Imperial College London

Machine Perfusion for Assessing and Optimizing Kidney and Pancreas Allografts

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Declaration of Originality

I Karim Ezzat Hamaoui 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 Information derived from the published and unpublished work of others has been acknowledged in the text and a list of references is given in the bibliography.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: The predominate issue in transplantation today is the inadequate supply of suitable organs for an increasing number of patients on the transplant waiting lists. In efforts to address this demand there has been an increasing use of expanded-criteria and donation-after-cardiac death donor kidneys and pancreases, however these organs are at higher risk for reperfusion injury. Advances in organ preservation thus need to focus on techniques to assess and optimise organ viability prior to transplantation.

Methods: This research focuses on organ viability assessment during preservation using rapid sampling micro-dialysis (rsMD); preconditioning organs with novel endothelial localising anti-coagulants to prevent micro-vascular thrombotic complications; development of both novel hypothermic machine perfusion (HMP) solutions to minimise reperfusion injury, and HMP strategies that offer superior preservation of organ integrity compared to static cold storage (SCS). Using HMP and normothermic-reperfusion models of porcine and human kidney and pancreatic grafts, this project has investigated strategies addressing these themes.

Results: rsMD can successfully provide detailed real-time information on tissue and organ viability during both SCS and HMP. Pre-conditioning grafts with novel localising anticoagulant proteins has been successful in ameliorating disturbances in macro and micro-vascular perfusion and graft microcvascular thrombosis, processes which play key roles in reperfusion injury and organ dysfunction. Separately the application of a novel adenosine/lidocaine based preservation solution in renal HMP preservation can potentially ameliorate reperfusion injury seen using conventional solutions. Post-ischaemic HMP reconditioning has been investigated in the context of recovering organs with an extreme period of SCS which may be a potential option to expand donor organ pools. Finally models of pancreatic HMP have been successfully established, opening the potential for organ viability assessment and optimization.

Conclusion: This research has been successful in its overall objective to develop novel translational strategies that have high potential for clinical implementation. In doing so the goal would be to enable an expansion of the pool of acceptable donor organs by improving the methods used to determine and optimise their viability, specifically more effective preservation techniques and by addressing specific post-operative complications through graft preconditioning.

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Abbreviations

AGE	advanced glycation end products
AKI	acute kidney injury
AL	Adenocaine
APC	antigen presenting cell
ARF	acute renal failure
AT	anti-thrombin
ATF6	activating transcription factor 6
ATP	adenosine tri-phosphate
BM	basement membrane
BMI	body mass index
CAM	cell adhesion molecule
CBF	capillary blood flow
CCL2	chemokine ligand 2
CCL5	chemokine ligand 5
CCL5	chemokine ligand 5
CD	cluster of differentiation
CIT	cold ischaemia time
CRP	c-reactive protein
CV	Cardiovascular
CVA	cerebrovascular accident
DAMP	damage associated molecular pattern
DBD	donation after brain death
DCCT	diabetes control and complications trial
DCD	donation after cardiac death
DGF	delayed graft function
DTI	direct thrombin inhibitor
DVT	deep vein thrombosis
ECD	expanded criteria donation
eNOS	endothelial nitric oxide synthase
EPCR	endothelial protein C receptor
ER	endoplastic reticulum
ERK1/2	extracellular signal-regulated kinases
FCD	functional capillary density
FDA	food and drug agency
FDP	fibrinogen degradation products
GFR	glomeruli filtration rate
GST	glutathione-S-trasferase
HbA1C	glycated haemoglobin
HBP	heparin binding proteins
Hchole	hypercholesterolaemia

HEPES	hydroxyethyl-piperazine-ethane-sulphonic acid
HES	hydroxyethyl starch
HIT	heparin-induced thrombocytopaenia
HMGB1	high mobility group box protein
HMP	hypothermic machine perfusion
HRP	horse radish peroxidase
HSP	heat shock protein
HTK	histidine-tryptophane-ketoglutarate
HTN	hypertension
ICAM	intracellular adhesion molecule
iCOX	inducible cyclo-oxygenase
ICT	islet cell transplantation
IF	immediate function
IFN-y	Interferon gamma
IGL	Institute George Lopez
IL	interleukin
INF-g	interferon gamma
iNOS	inducible nitric-oxide synthase
IR	ischaemia reperfusion
KTA	kidney transplant alone
LDL	low density lipoprotein
LMWH	low molecular weight heparin
Lox	lactate oxidase
LPs	Lipopolysacchoride
mAB	monoclonal antibody
MAC	membrane attack complex
MAPK	mitogen activated protein kinases
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MPS	Machine Preservation Solution
NADH	nicotinamide adenine dinucleotide
NHRP	normothermic haemo-reperfusion
NHS	National Health Service
NMR	normothermic reperfusion
NO	nitric oxide
NOS	nitric oxide synthase
OPS	orthogonal polarisation spectral imaging
PAI	plasminogen activator inhibitor
PAK	pancreas after kidney
PARs	protease activated receptors
PDGF	platelet derived growth factor
PEG	polyethylene glycol

PERK	protein kinase-like ER kinase
PFI	perfusion flow index
PKC	protein kinase C
PTA	pancreas transplant alone
QALY	quality of life adjusted year
RBC	red blood cell
RBC	red blood cell
RCT	randomised control trial
ROS	reactive oxygen species
rsMD	rapid sampling microdialysis
SCD	standard criteria donation
SCS	static cold storage
SPK	simultaneous pancreas kidney
TF	tissue factor
TLR	toll like receptor
TM	thrombomodulin
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
UFH	unfractionated heparin
UPR	unfolded protein response
UW	University of Wisconsin solution
VCAM-1	vascular cell adhesion molecule
VLDL	very low density lipoprotein

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Chapter 1:

Introduction

1.1.1 Kidney Transplantation

Kidney transplantation is the optimal treatment for patients with end-stage renal failure [1] and provides the best clinical outcomes, optimal quality of life and cost savings when compared with other modalities of renal replacement therapy [2] .

In the UK by end 2012 54,824 adults were receiving renal replacement therapy, an absolute increase of 3.7% from 2011, and approximately a 75% increase in the number of patients since 2000, with the median patient age for the start of replacement therapy at 58 years. Furthermore the percentage of these patients aged greater than 70 years increased from 19.2% in 2000 to 24.9% in 2012 [3].

In patients on replacement therapy the 1 year survival rate is 79%, with 5 year survival rates depending on patient age; 58% if aged 18-34, and 12% if greater than 75 years [4] . Comparatively the most recent 1 and 5 year patient survival rates for deceased kidney transplant recipients in the UK are over 95% and 88% respectively.

In parallel with the improvement in transplant results over the years to current levels demand has increased, with continuing emergence of new indications and decreasing contra-indications for receiving a transplant.

Despite the demonstrated advantages of transplantation, the full potential of these benefits to patients cannot be obtained due to the severe shortage of donated kidneys [5]. Indeed the largest problem facing the transplant community is the critical shortage of donor organs.

In the UK in 2014 2142 deceased donor, and 1243 living donor kidney transplants were performed. With 5881 patients registered on the national kidney transplant waiting list the number of patients have declined for the fifth year running (see Figure 1). This is due to patients leaving the transplant list, while the number of people joining the list is relatively stable. Despite this decline the number of patients registered has risen approximately 8% since 2005 and 70% since 2000 [6,7].

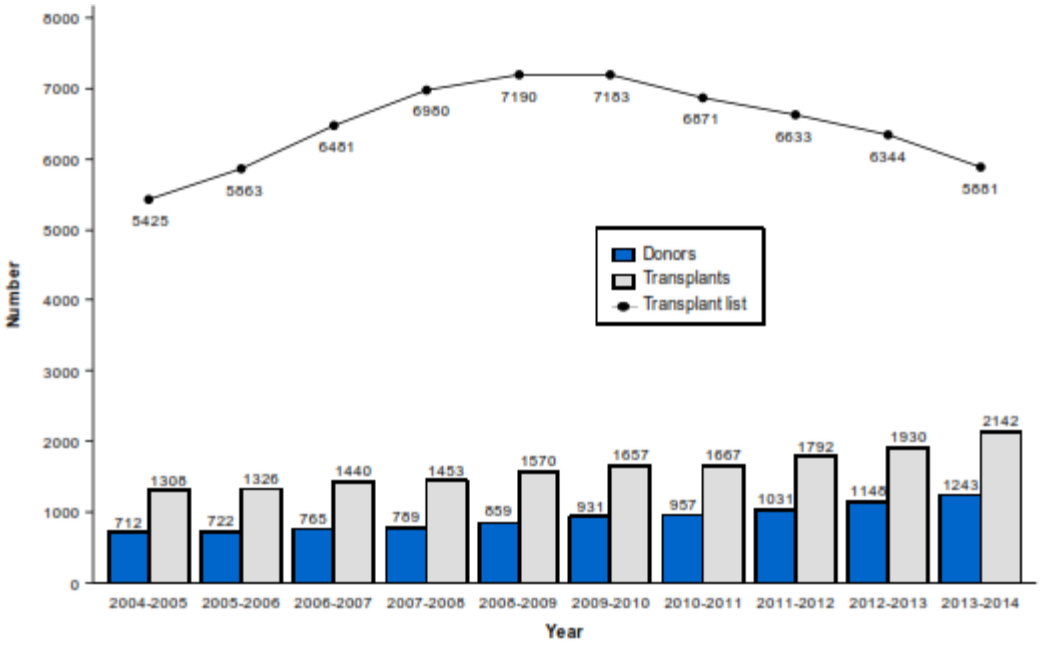


Figure 1 Deceased donor (DCD and DBD) kidney programme in the UK, April 2004 - March 2014, Number of donors, transplants and patients on the active transplant list at 31 March 2014.

Deceased Donor Kidney Transplantation

To meet this demand the organ supply pool has been expanded by a number of creative strategies. The most successful of which is the living donor donation scheme. The number of living donor kidney transplants has increased from 338 in 1999 to 1243 in 2014 – a threefold increase, and now accounts for 34% of kidneys transplants. This increase however significant does not match the need of potential recipients.

An alternative to living donor kidney transplantation is the use of allografts from deceased donors - either donation after brain death (DBD) or donation after cardiac death (DCD). At present deceased donors comprise the largest fraction of donated organs in the UK, with 2142 transplants occurring in 2013-14.

DBD describes a donor who has had primary brain death where cardiac circulation and respiration are intact or maintained by medical measures. DCD donors are grouped via the Maastricht Classification which has 5 categories[8]. These categories are further defined by being controlled or uncontrolled. Categories I, II and V are designated uncontrolled and categories III and IV are controlled

Category 1: Death has occurred outside of the hospital. For these individuals to be potential donors, the moment of sudden death needs to have been witnessed, the time that it occurred documented, and 'resuscitation' continued after death.

Category 2: Unsuccessful resuscitation where CPR is started outside of hospital following a collapse. Death is confirmed on admission to hospital.

Category 3: Patients on intensive care units with non-survivable injuries but where brain stem death criteria are not fulfilled who have treatment withdrawn and are awaiting cardiac arrest.

Category 4: Cardiac arrest in a brain stem dead individual.

Category 5: Unexpected cardiac arrest in a hospitalised setting.

Overall the number of DCD donors in the UK has increased the past decade compared to DBD donation which has remained relatively stable (see Figure 2).

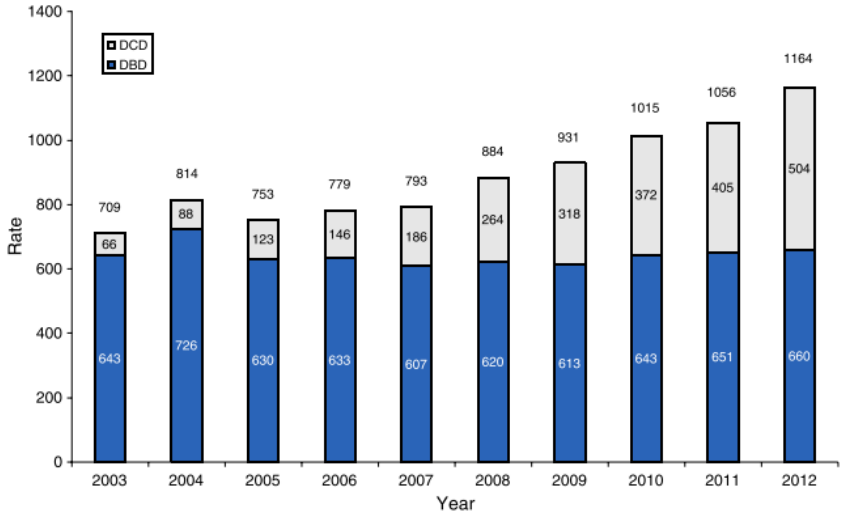


Figure 2 Number of deceased donors in the UK, January 1, 2003 to December 31, 2012. Overall the number of DCD donors has increased the past decade while DBD donation has remained stable [9].

The number of transplants arising from DBD donors has however decreased the past 14 years, with 1157 transplants performed in 2014 (vs. 1359 in 2001) . Conversely there has been an increase in the number of transplants (784 in 2014 vs. 56 in 2001) from DCD donors, led by successful initiatives to pursue potential donors more aggressively and accept a broader range of organs for transplantation to address the increasing numbers of patients on the waiting list [9] (see Figure 3).

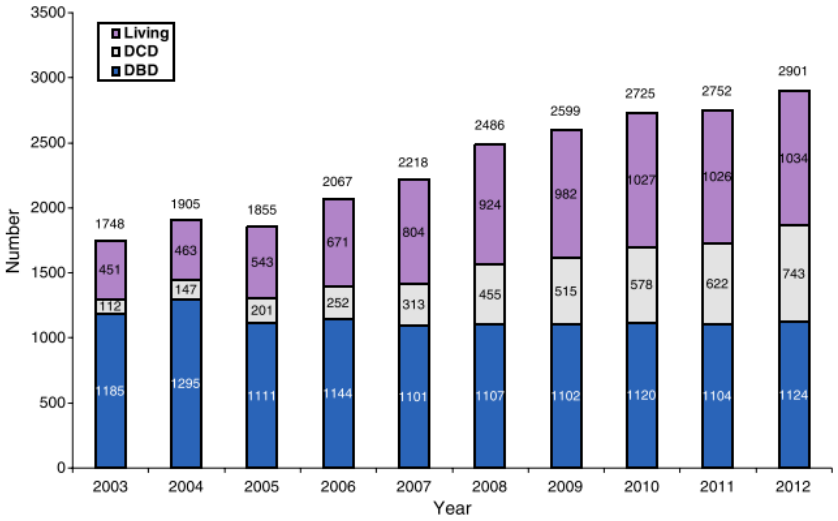


Figure 3 Number of kidney transplants in the UK, January 1, 2003 to December 31, 2012. The largest increasing donor sub-group is the DCD cohort[9].

The pattern of demand for kidney transplants outstripping the suitable supply of organs is repeated, with only some specific regional variations, throughout the world. Efforts to expand the various donor pools are prevalent in most countries [10], and at present it is realized the largest potential growth in recruitment of donors is from the DCD group. Of note a more aggressive pursuit of kidneys from DCD donors has the capability to increase the number of organ donors by 2.5-4 times and thus potentially eliminate waiting lists completely [11,12] .

1.1.2 Pancreas Transplantation

The Extent of the Problem

Diabetes mellitus is one of the fastest growing chronic diseases in the world with an estimated 171 million people, or 2.8% of the world's population, suffering from diabetes in 2000[13]. In the United Kingdom more than 1.8 million people (or 3.5% of the population) are diabetic, with a projected

increase to 2.6 million by 2030. Diabetes is a relentless disease affecting multiple organ systems insidiously[14] causing blindness, renal disease, impotence, neuropathy, foot ulcers, amputations, and heart disease; which not only shortens life expectancy but adversely affects quality of life in the process.

In the UK diabetes costs the National Health Service (NHS) between an estimated £1.3[15] and £2.7 billion per year, with a mean expenditure of £1600 on each diagnosed diabetic, and with treatment for diabetes and associated complications accounting for approximately 9% of NHS hospital budgets[16]. With the expected increased prevalence of diabetes over the next two decades the cost of treatment will rise steeply.

Defining the Problem

Diabetes mellitus is a condition that results from the failure of pancreatic beta cells to appropriately produce insulin. From a patho-physiological point of view, two predominant types of diabetes mellitus exist and must be differentiated[17]. Diabetes mellitus type I or juvenile-onset diabetes mellitus results from a total or near total selective T-cell mediated auto-immune destruction of the insulin-producing beta cells within the islets of Langerhans in the pancreas. It has a peak onset in adolescence, hence the name, but may occur at any age and is believed to result from a combination and interaction of both environmental and genetic factors. Autoimmune destruction of beta cells has usually been progressing for a number of months or years, with symptoms emerging only once less than 20% of the total beta cell mass remains[18]. Patients hence become dependent lifelong on exogenously administered insulin immediately at diagnosis.

The second form known as Type 2 diabetes, or non-insulin dependent diabetes mellitus, involves a complex metabolic syndrome characterised by peripheral insulin resistance[18]. In Type II diabetes, genetic predisposition for initial systemic resistance to endogenous insulin progresses slowly for a period up to 10 years before Beta cell failure supervenes and diabetes is diagnosed. Because the pancreatic beta cells retain their ability to produce insulin initial pharmacological treatment includes insulin secretagogues and insulin sensitizers, with insulin replacement reserved only for late phases of the disease.

Treatment Strategies for Diabetes

Under physiological conditions, blood glucose levels are kept within a very narrow range by a sensitive feedback mechanism controlling the secretion of insulin [18]. In the case of Type I diabetes slight under or over replacement of insulin compared to what is physiologically required will result in

a disproportionate change in glucose and fatty acid metabolism in liver and adipose tissues [14]. From a theoretical point of view it is clear exogenous insulin therapy cannot mimic the minute by minute physiological variations of insulin secretion in vivo necessary to maintain the same degree of blood glucose control[19].

Minimising hyperglycaemia has however been recognised as an important goal in the management of both types of diabetes and tight control of blood glucose levels is beneficial in preventing progression of subsequent secondary diabetic complications. The Diabetes Control and Complications Trial[20] (DCCT) showed tight blood glucose control depicted by target Glycated haemoglobin (HbA1C) levels of less than 7% in Type 1 Diabetics lowered the incidence of diabetic retinopathic, neuropathic, and nephropathic lesions by 47%, 60%, and 39% respectively. One important issued noted however in the trial was that the cost of achieving such tight control, and thus a reduction in secondary complications was a three-fold increase in the number of severe hypoglycaemic episodes that occurred. Thus it is apparent that no true physiological control of blood glucose levels can be accurately achieved with interval exogenous insulin administration, and even welcomed tight control carries additional risks that cannot be ignored.

In order to artificially control blood glucose levels to a similar physiological degree as performed by Beta cells a closed loop system must be used whereby insulin levels are adjusted on a moment-to-moment basis according to requirement[18]. There are two methods of achieving this continuous control. One method would be to utilise an insulin pump attached to a continuously monitoring blood glucose sensor[18]. At present external pumps are commonly used with manual control in clinical practice. However truly automated devices are troubled by the requirement of an accurate and continously monitoring sensor which has not been perfected neither has the ideal target tissue for monitoring, e.g. skin, subcutaneous fat. Despite technological progress in these designs over the past few decades, concerns of safety and reliability for planned implantable pumps means this truly automated approach is still several years away from clinical application. Thus the alternative and currently only available strategy to provide an effective closed loop system in Type 1 insulin dependent diabetics is beta cell transplantation in the form of a whole vascularised pancreatic transplant, or islet cell transplantation (ICT).

United Kingdom Pancreas Transplantation Trends

Pancreatic transplantation can broadly be considered in terms of whole pancreas and ICT. Interest in ICT has increased in recent years predominately arising from the development of the Edmonton

Protocol, which has given rise to clinical trials involving cadaveric donor pancreata whereby the islets are infused via the portal vein. These result in persistent insulin secretion, but have the disadvantage that top-up from anti-hyperglycaemic medications is still required [21–23]. ICT has shown both reduced frequency of hypoglycaemic episodes [20] and reduced microvascular complications [24–26]. It also improves quality of life and patient survival [27–29]. Persistent insulin independence however has not been achieved through ICT and this remains a great drawback. Additionally, ICT often requires multiple transplants to attain enough beta cell mass unlike whole transplantation which only requires one. Despite a number patients continuing to require exogenous insulin hypoglycaemic awareness with fewer hypoglycaemic episodes, thought to be due to the initial restoration of glycaemic control, the glucagon and epinephrine response, and overall resetting of anti-hypoglycaemic controls, which then persists after islet graft failure[30].

However evidence that ICT is a potentially viable treatment has led to advocates supporting introduction of such clinical programs and indeed the NHS now fund ICT particularly for patients with reduced hypoglycaemia awareness or those taking immunosuppressive drugs because of a previous kidney transplant[31].

Whole pancreas transplant however remains predominate definitive treatment for Type 1 diabetes. In the United Kingdom the number of patients registered on the active transplant list for a simultaneous-pancreas-kidney (SPK), pancreas-after-kidney (PAK) or pancreas-transplant-alone (PTA) has increased by more than 6 fold over the past 14 years, from 45 patients in 1998-99 to 270 patients in 2013-14 (see Figure 4). The number of pancreatic donors and transplants has also increased steadily since 1998-99 from 30 donors, resulting in 24 transplants, to 456 donors and 246 transplants in 2013-14 [7]. The majority (76%) were SPK transplants, while 11% were pancreas only transplants (PTA/PAK) and 13% were islet transplants. Between 2012-13 and 2013-14 the number of DBD pancreas donors increased by 1% to 365, while transplants from donors after brain death increased by 6% to 203, similarly the number of DCD pancreas donors decreased by 1% to 91, while transplants from donors after circulatory death increased by 5% to 43[7].

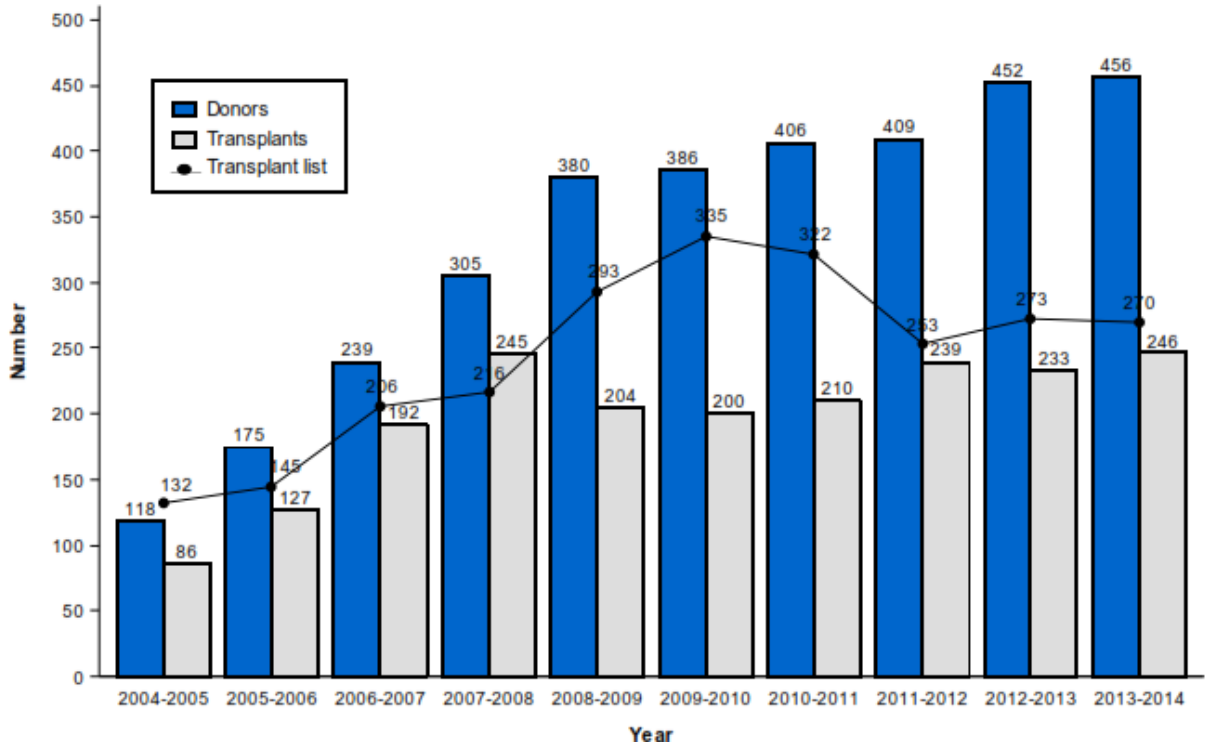


Figure 4 Deceased pancreas and islet programme in the UK, 1 April 2004 - 31 March 2014, Number of donors, transplants and patients on the active transplant list at 31 March. [7]

As can be seen in Figure 4 despite the availability of greater numbers of deceased donors than the number of patients awaiting a pancreas transplant fewer transplants are performed than needed. This had improved in recent years with demand stabilizing over the past 3 years but one reason for the continued disparity is that the proportion of pancreases retrieved but that subsequently cannot be used for transplantation is greater than in kidney, liver or heart transplantation, and as such there is a vast underutilization of pancreatic donors. Such pancreases however are not completely discarded and are generally used in approved national research projects.

Pancreatic Transplantation Effects on Secondary Diabetic Complications

As mentioned the DCCT[20] study demonstrated the beneficial effects of tight glycaemic control on development and progression of secondary diabetic complications. With the success of pancreatic transplantation it would be reasonable to assume that similar ameliorations in diabetic lesions would be observed in pancreatic transplant recipients. Patients demonstrate almost immediate freedom from insulin and a return to normoglycaemia after transplantation, with HbA1c levels normalising after several months[32].

Diabetic Nephropathy

In SPK and PKA the goal of the procedure is to treat uraemia with the renal allograft, and prevent recurrence of diabetic lesions by achieving euglycaemic control. In PTA the aim is stabilization or regression of established diabetic lesions, specifically nephropathy[33]. Diabetic lesions in recently transplanted kidneys in SPK at 1 year are absent compared to kidney transplant alone (KTA), and at 10 years there is regression of characteristic diabetic lesions in autologous kidneys in PTA [34]. However pancreas transplantation can have deleterious effects on renal function – with chronic dehydration (bladder drainage) and calcineurin-inhibitor exposure (immune suppression) implicated in most cases[33]. Thus overall though the damage from continuous hyperglycaemia is removed post pancreatic transplantation, the added requirement for immune suppression may lead to progressive long term renal insufficiency.

Diabetic Retinopathy and Neuropathy

Most patients who undergo pancreatic transplantation have a plethora of secondary diabetic complications already, and most have active retinopathic lesions, or have been treated for such lesions with photo-laser therapy. Studies are inconclusive but resolution of hyperglycaemic states in SPK and PTA recipients may limit the progression of already established lesions, and reduce the need for laser therapy [35]. Most benefit may however only be observable in patients with early lesions, as damage may be irreparable in more advanced stages of retinopathy [33]. In addition after SPK patients experience improvement in symptoms of autonomic dysfunction (gastric emptying)[36], and improved sensory and motor neuropathies as measured by conduction studies [37].

Quality of Life after Pancreatic Transplantation

While regulation of glycaemic control with pancreas transplantation can provide some amelioration of secondary diabetic complications, the procedure's effect on patient quality of life may be the most important and beneficial outcome[33]. Recipients of pancreas transplants enjoyed improvements in their quality of life when compared to patients that received a kidney allograft alone [18]. Consistent findings in quality of life assessments in pancreas transplantation is improved perception of diabetes specific issues such as satisfaction with diet flexibility, health management with the patient relieved to be free from dietary restrictions, frequent blood glucose monitoring, and insulin therapy [18]. In addition to feeling better about their lives, pancreas transplantation

recipients (specifically SPK) can expect to live an addition 10 to 15 years longer compared to diabetics on a transplant waiting list and recipients of KTA respectively [38].

1.2 The Impact of DCD and ECD Organ Transplantation

The most prevalent deceased donor cohort is the DBD group. DBD describes a donor with primary brain death where circulation and respiration are intact or maintained by medical intervention. Conversely a DCD donor refers to the situation where the criteria from brain death is not met, but where survival is unexpected, and where cardiac death occurs before organ procurement. Cardiac death can be either unexpected and uncontrolled (uncontrolled DCD) or deliberate and controlled (controlled DCD) whereby supportive intervention is systematically withdrawn after consent from the family [8].

Use of DCD donors is not without limitations. Unlike DBD donors, DCD donors are subject to lengthier warm ischaemic injury (10-45mins) during the time between cardiac arrest and initiation of cold preservation flush and organ retrieval [39]. Despite this, outcomes for DBD and DCD kidney transplants are similar in terms of both allograft and recipient survival, but with DCD kidneys at higher risk of primary non-function (PNF), DGF 20-80% vs. 20-30% for DBD [40], necessitating further dialysis, with reports also suggesting an increased risk of acute rejection and poorer long term function [41].

To add to these differences a DBD or DCD donor can also be a standard criteria donor (SCD) or an expanded criteria donor (ECD) depending on whether the appropriate standard or expanded criteria are met, potentially increasing the risk of complications, specifically with older DCD donor organs negatively impacted by DGF [42].

Thus one of the major consequences of expansion of donor pools with DCD/ECD cohorts is the increased risk of PNF and prolonged DGF due to donor organ damage, and their associations on immediate and long-term outcomes. It is the immediate and longer term complications associated with PNF and DGF in these organs that have tempered the acceptance and widespread use of kidneys from these donor pools.

1.2.1 Expanded Criteria Donors

In addition parallel to the expansion of certain donor pools, the demographic and health profiles of the majority of donors has changed, with steadily older donors with co-morbidities increasingly common place [43], so called marginal, expanded (or extended) criteria donors (see Figure 5). 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% [9].

Strategies to expand the donor pools have thus been limited by the comparative outcomes of grafts from these older ECD and DCD donors to SCD and DBD groups respectively.

SCD refers to donors <50 years of age with no history of hypertension (HTN) or diabetes and where donation occurs after brain death (DBD). While ECD donors are those who are >60 years of age or aged 50-59years with two of either 1) Cause of death cerebrovascular accident (CVA); 2) a history of HTN; or 3) terminal serum creatinine >133 micromoles/litre.[44]

ECD kidneys through their inherent description are associated with declining renal function, a reduced nephron reserve and decreased graft viability, with their transplant predisposing to a 77% increased risk of graft failure [45] and an average graft survival expectancy of 5.1 years, compared to 10 years for a younger non-ECD kidney [46]. 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.

Transplant however of such inherently higher risk kidneys is not without benefit, with multiple studies demonstrating a definitive survival advantage to receipt of an ECD kidney vs. remaining on dialysis and waiting for an SCD kidney [47].

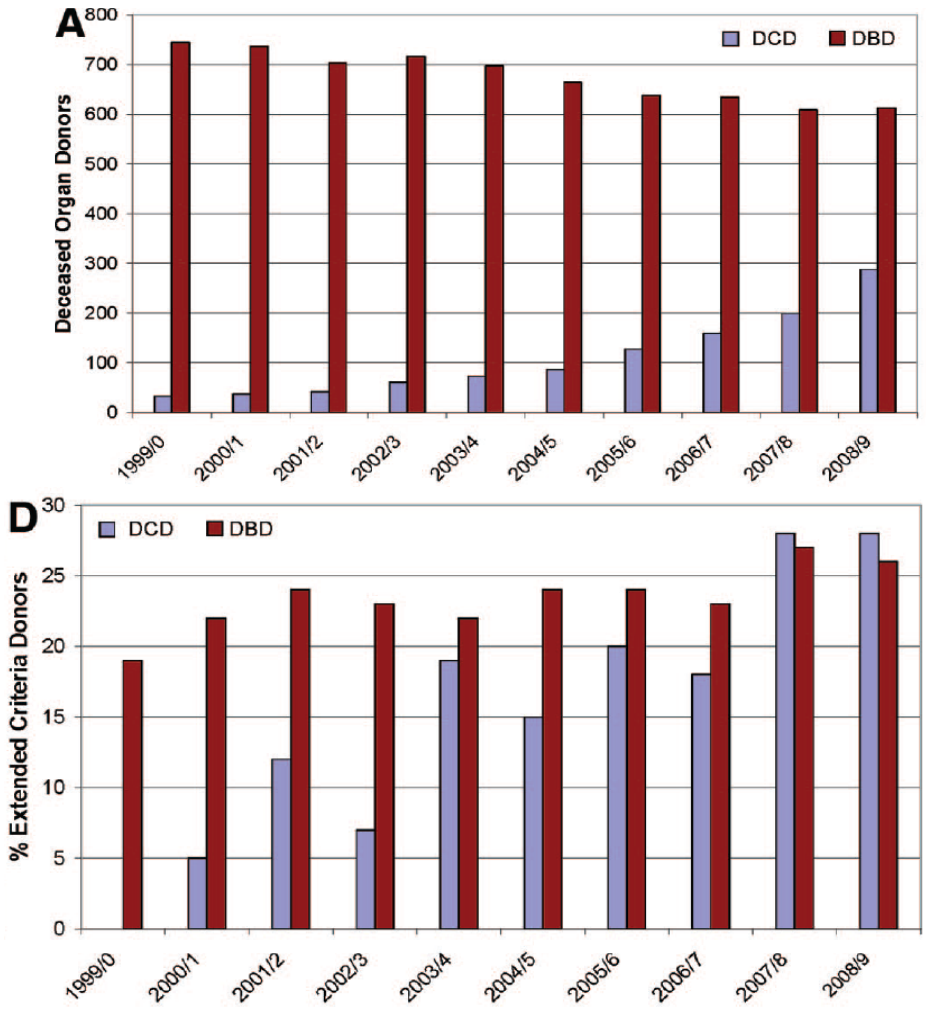


Figure 5 Donor trends April 1, 1999 to March 31, 2009. (A) Total number of deceased organ donors and (D) Expanded criteria donors, highlighting the increase in both DCD and in particular ECD/DCD donors in the UK. From Summers et al.[48].

1.2.2 Delayed Graft Function

DGF itself is can be defined as the need for more than one episode of dialysis in the immediate post-operative period [49] indicating a temporary failure of the transplanted kidney to resume adequate function. Approximately 20%-80 of kidney transplant recipients experience some degree of DGF in the first week depending on a number of variables, with type of donor allograft a predominant factor. This is supported by the acceptance that DGF is rarely experienced in living donor transplantation suggesting that donor factors themselves significantly influence DGF. The five key

contributing variables are related to donor tissue quality, donor brain death and related stress, preservation characteristics, immune factors and recipient variables [50].

Damage sustained by the organ during the transplantation process causes an acute halting of organ function in the postoperative period and this DGF deleteriously leads to a more complex post-operative period for the patient. The presence of DGF increases the likelihood of acute rejection (probably by unmasking hidden donor foreign antigens to the recipient) in the early period post-transplant [50]. It is this increased immunogenicity that is likely to contribute to instances of interstitial nephritis and tubular atrophy, and negatively affect long-term graft and patient survival. DGF increases the risk of graft loss by 41%, and acute rejection by 38%, with worse graft function at 3.5years compared to patients without DGF [41]. Deservedly DGF is associated with prolonged periods of hospitalization, higher treatment costs and adverse effects on transplant patients' general rehabilitation [51,52].

DGF is thus the clinical end result of predominantly ischaemic stress and injury sustained during organ retrieval and preservation, with the damage caused by this insult then accentuated during the reperfusion period, so called ischaemia-reperfusion (IR) injury [41], leading to a spectrum of irreversible damage the effect of which causes impaired long term organ function.

1.3 Ischaemia Reperfusion Injury

Ischaemia is defined as the cessation of arterial blood flow that subsequently deprives tissue of oxygen. There are various stages of the transplantation process where kidneys are exposed to ischaemic stress and these can be classified according to the temperature of the local environment – warm or cold. During retrieval warm ischaemic injury is sustained in the time between donor cardiac arrest and cold perfusion of preservation solution in-situ. This period is known as the primary warm ischaemia time or WIT. This is followed by further cold ischaemic damage (also known as cold ischaemia time or CIT) that ensues after flushing and storage of the allograft with cold preservation solution, and then transport to the recipient centre.

In DBD donors warm ischaemia can be minimised where access to abdominal vasculature can be achieved prior to withdrawal of support, allowing quicker institution of cold perfusion after cardiac arrest has occurred. While in DCD donation time between cardiac arrest and the start of cold perfusion is variable but demonstrated to be significantly longer.

The main goal of organ preservation is the maintenance of the functional, biochemical and morphological integrity of the graft during retrieval from the donor and transport to recipient: from harvest to revascularisation [53] .

During the period of implantation between removal of the organ from cold preservation and reperfusion there is a second period of warm ischaemia (known as secondary warm ischaemia). Upon revascularisation there is a further period of unavoidable damage due to the sudden influx of oxygenated warm blood into an organ that has undergone cold ischaemic and then warm ischaemic stress for a number of hours – known as reperfusion injury (see Figure 6). This was first demonstrated experimentally in a canine model of IR injury by Jenning et al. in the 1960, when they reported an unexpected acceleration of cellular necrosis and tissue damage after inducing transient ischemia and subsequent reperfusion, compared to permanent ischaemia [54].

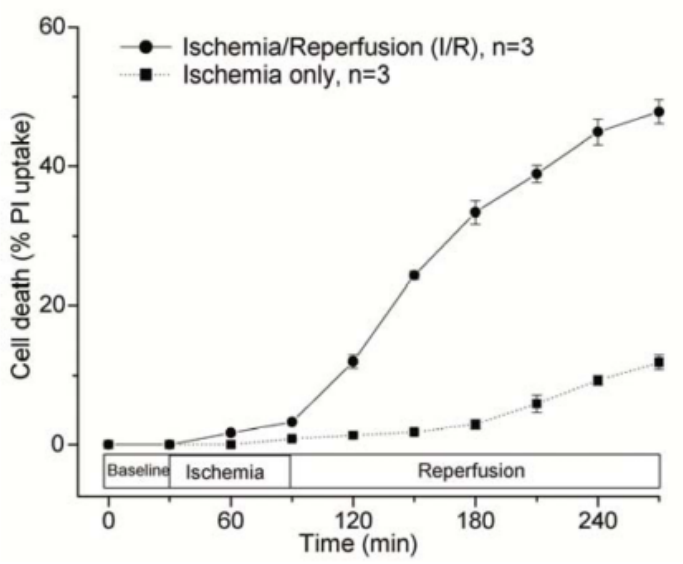


Figure 6 Cell death during IR injury - specifically either ischemia and subsequent reperfusion or prolonged ischemia, highlighting the problem and damage endured during reperfusion. Reprinted from Vanden Hoek et al. [55].

The theoretical premise of IR injury is that in any given tissue exposed to ischaemic stress a number of cells will suffer irreparable damage, that ultimately leads to cell death. A larger percentage of cells in this tissue will sustain damage but still remain viable, with the potential for self-repair and return to normal function (see Figure 7). As the former sub-group of cells will definitively undergo cell death, the degree and severity of IR injury remains with the number of cells in the 2nd subgroup that are salvageable and can actually remain viable after reperfusion [56]. Thus the aim of preservation strategies is to optimise the potential for these cells to be rescued, and improve function of the organ after transplantation. The cellular damage that occurs is based directly on the lack and then return of oxygen, the development of cellular oedema and intracellular acidosis, calcium overload, and the generation of reactive free radicals [57].

The following sections will describe and discuss the pathological entities involved in IR injury. The dominant discussion will relate to renal IR injury however the same mechanisms are involved in IR injury to all organs.

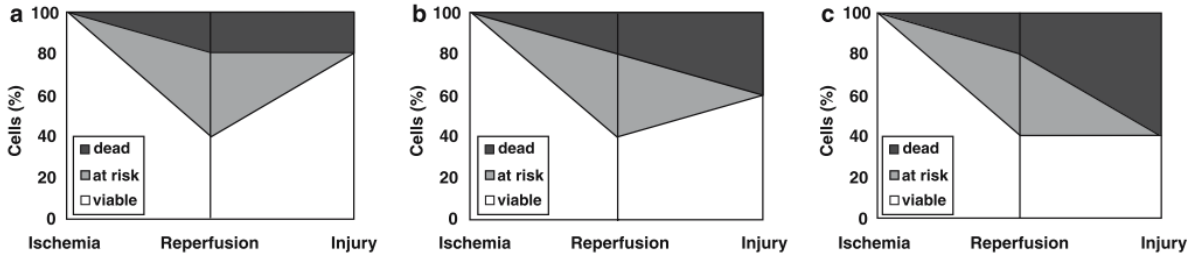


Figure 7 The concept of ischemia-reperfusion injury, where the fate of cells at risk (grey) determinates the full extent of tissue injury with either a complete recovery (a), a partial recovery (b) or full reperfusion induced injury (c). From Dorweiler et al [58].

1.3.1 Types of Ischaemic and Reperfusion Injury

In kidneys the susceptible target of the initial warm ischemic insult are the proximal tubular cells. These morphologically and functionally specialised cells have high metabolic requirements to facilitate transport of ions, water, and macromolecules across several cell membrane layers by highly selective transport systems[59]. These cells however can tolerate short exposures to warm ischaemia, but not without exhibiting alterations in trans-epithelial potentials, cell-cell junctions, with increased leakage and slowing of the active transport mechanisms[60] . Longer periods of cold ischaemia can be similarly tolerated [57]. It is the combined insult of warm, then cold ischaemia and finally reperfusion upon transplantation that causes damage and functional deficits (see Figure 8).

Cold ischaemic injury starts during the retrieval procedure when the kidney is flushed with cold preservation solution. Cold preservation is based on the principle of suppression of cellular metabolism through hypothermia. According to van't Hoff's rule tissue cooling from normothermic temperatures to 0°C decreases the rate of metabolic activity 12 times, with 10-12% of metabolism remaining at 4°C [61,62] . Metabolism is slowed but not halted and hypothermia itself is detrimental to tissue, and mitochondrial swelling and rounding, extra- and intra-cellular oedema, and margination of chromatin in the nucleus has been seen in kidney proximal tubules [63]. Thus though cellular damage occurs during cold ischaemia, warm ischaemic stress is more detrimental.

With warm revascularisation and the return of oxygen, reperfusion injury as the effecter phase of ischaemic injury ensues [59]. This is the final event that can cause profound injury with marked cell death in the transplanted organ. Thus while ischaemic injury is associated with the lack of oxygen, reperfusion injury is initiated by the return of oxygen.

Tubular cells are the key cell type whose dysfunction is predominately related to the functional delay in a kidney after transplant. It is endothelial cells (ECs) lining the lumen of blood vessels that are the first cells to sustain warm reperfusion damage [64]. Hypothermia followed by re-warming (37˚C) induces a pronounced apoptosis in ECs and renal interstitial cells, the extent of which depends on the duration of the cold incubation [65]. The damage that occurs is facilitated by several of the processes that started during cold preservation. Once normothermic blood flow is re-established cellular metabolism is re-initiated and cell repair mechanisms occur in parallel with apoptosis, autophagy, and necrosis; the final degree of damage sustained dependent on the balance between cell death and regeneration[57]. These processes involve elements of direct cell damage mediated through activation of innate immune processes that target both endothelial and renal interstitial and tubular cells. However IR injury to endothelial cells also results in microvascular dysfunction which is widely described in key organs systems[57] and is a serious problem as it potentiates tissue damage and limits recovery of function.

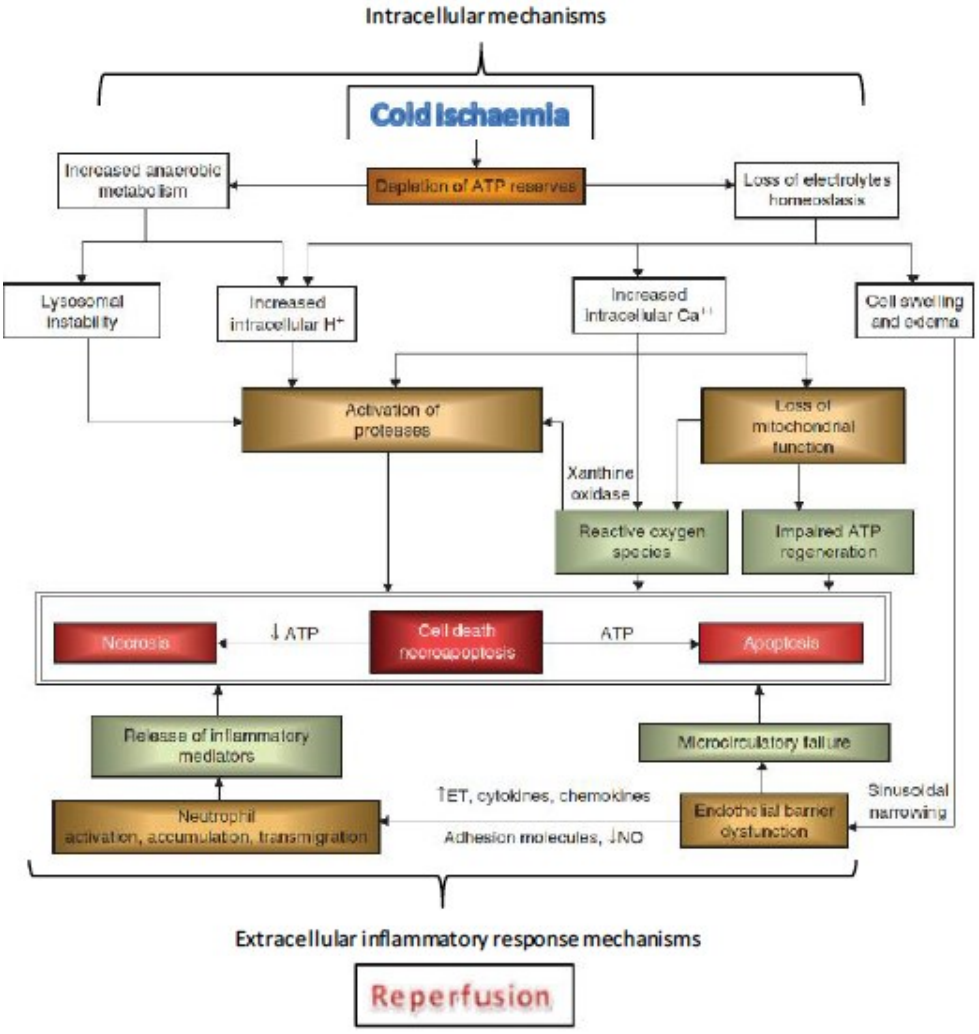


Figure 8 A schematic representation of illustrating the connection between mechanisms prevalent during ischaemia and reperfusion injury. Cold SCS induces ATP depletion resulting in the loss of cellular electrolyte balance with increases in anaerobic metabolism. This promotes the accumulation of cellular calcium causing lysosomal instability. There is an inhibition of mitochondrial oxidative phosphorylation decreasing ATP synthesis and increasing activation of cellular proteases. Electrolyte disturbances results in cellular oedema of both intrinsic cells of the tissue and endothelial cells. Endothelial cells undergo 'activation' with induction of cellular adhesion molecules, production of inflammatory modulators (cytokines and chemokines), and an increased vascular permeability all of which contribute to neutrophilic accumulation and transendothelial migration into the tissue upon reperfusion. Infiltrating neutrophils and leucocytes cause parenchymal damage by local production of reactive oxygen species (ROS), while activated endothelial cells and organ specific cells produce ROS through disrupted mitochondrial oxidative phosphorylation and xanthine oxidase. The degree of accumulated of cellular damage results in either recovery, or cell destruction through necrosis or apoptosis depending on availability of ATP. Adapted from Zaouali et al. [66].

1.3.2 Cellular Pathogenesis of IR Injury

Apoptosis vs. Necrosis

In necrosis cell integrity is loss, with release of cytosolic contents into the extracellular environment evoking an inflammatory response. The degree of necrosis is dependent on the severity and duration of ischaemia. Apoptosis however is a highly coordinated and programmed process, results in cellular degradation and confinement in a series of contained apoptotic cell bodies, without arousing an inflammatory reaction, but that requires adenosine tri-phosphate (ATP) to take place [58]. The contribution of each to tissue damage during IR is based on the phase of IR - during ischaemia with a lack of oxygen and thus minimal ATP generation, necrosis prevails. Upon reperfusion and the influx of oxygen cells that had started apoptotic cascade, triggered by ischaemic insult, are able to proceed. Reperfusion thus paradoxically produces damage through the provision of oxygen and ATP generation enabling apoptotic processes to complete. Experimental comparisons of IR and ichaemia has shown that cell death during prolonged ischaemia was 17% compared to 73% in reperfused tissue [67]. Reperfusion itself also triggers an local inflammatory cell reaction and this phase of injury is characterised by micro-vascular dysfunction and poor tissue perfusion – aptly named the capillary 'no re-flow' phenomenon [68].

Deprivation of oxygen

It is generally accepted that within minutes of the deprivation of oxygen there is switch from aerobic to anaerobic glycolytic metabolism through the activation of anaerobic genes as oxygen debt rises; the rate of change dependent on the basal metabolic demands of the tissue. Anaerobic metabolism of glucose however, produces an insufficient supply of ATP, 2 molecules vs. 38 molecules under normal conditions. To compensate for this discrepancy glucose consumption is increased (by up to 12 fold in some estimates) [69], resulting in a progressive net depletion of energy substrates, and subsequent starvation of cells, reflected by lower glycogen granules observed in ischaemic tissue [57].

Normally nicotinamide adenine dinucleotide (NADH) is generated from the reduction of NAD+ through the metabolism of glucose to pyruvate and processes within the Kreb's cycle. In anaerobic conditions NAD+ can only be regenerated by pyruvate's fermentation to lactic acid. As ischaemia progresses acid build up causes a progressive excess of free hydrogen ions, lowering cytoplasmic pH, a uniform observation in ischaemic tissues [70] [71] , which correlates well with the degree of tissue injury [72] [73] . Moreover hydrogen ion expulsion from the cell via H+ATPase pump and Na/H+

exchanger is lost as the intracellular ionic disturbance worsens [53] . The excess acid inhibits key glycolytic enzymes to stem further acid production. In adequately perfused tissue lactate acid is washed out into the circulation, and glucose metabolism continues, but in an ischaemic setting a lack of blood flow brings glycolysis to a halt. As glycolysis slows ATP production ceases. After 40 minutes of warm ischaemia all ATP is lost [74]. Any ATP available is metabolised to adenosine di-phosphate (ADP) and degraded to osmostically active inorganic phosphates, adenosine, inosine, and hydrogen ion depriving cells of substrates essential to generate further ATP, and exacerbating both osmotic flows and cytoplasmic acidity. It is these small molecules in parallel with the accumulation of toxic glycolytic products and intermediaries, including lactate, creatine, NADH, and free radicals, that cause the patho-histological sequelae characteristic of ischaemic injury.

Cellular Oedema

ATP is the required energy unit for the majority of cell functions. A lack of ATP causes inhibition of ATP dependent Na+/K+ ATPase and impairs a cell's ability to maintain membrane polarity and cell excitability, due to unchallenged trafficking of Na and K ions into and out of the cell. As Na accumulates it draws with it excess water causing cellular swelling. Furthermore, accumulation of glycolytic intermediaries as anaerobic metabolism slows acts to accelerate the increase in the osmotic load within the cell, attracting more water through simple diffusion, and exacerbating the ischaemic oedema [57] .

Cellular oedema results in disruption to cell membranes including those of the endoplasmic reticulum (ER), mitochondrion, and golgi apparatus [75]. An increase in cell volume results in activation of membrane stretch receptors, with a dissipation of membrane semi-conductance, both of outer cell membranes and those of the ER, mitochondrion, and golgi apparatus. A key use of preservation solutions is to limit cellular oedema and these biochemical and intracellular sequale (see Section 1.7.1.3 Formulation of Organ Preservation Solutions).

ER and Mitochondrial Stress

The ER is responsible for synthesis, maturation, and trafficking of a wide range of proteins, the biochemical reactions for which require a delicate environment that is based on pH, ionic concentrations, and re-dox potentials, and if disturbed causes ER functionality to be lost [76]. Several factors can cause ER associated stress and dysfunction in ischaemic tissue – namely hypoxia, energy substrate depletion, and an imbalance in ER calcium distribution [77]. The key response by

the ER to external stress is the unfolded protein response (UPR) the primary purpose of which is to enable adaptation to the changing environment and several pathways have been elucidated to be active in this protective process and involve ER localised transmembrane proteins specifically: Insitol-requiring enzyme-1, double-stranded RNA-activated protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6) [78]. These proteins are core adaptive UPR signal transducers and when activated promote expression of genes encoding for ER chaperones molecules, induction of mis-folded ER degradation, and anti-oxidant enzymes. If however experience excessive or prolong stress the UPR may fail to resolve the environmental changes and restore ER functionality. At this point the UPR proceeds to activate apoptosis – designated as the maladaptive UPR. The same pathways initially promoting the adapative response switch and mediate apoptosis. The transcription factor CHOP is activated downstream of PERK and ATF6 and induces pro-apoptopic proteins, while down regulating anti-apoptotic Bcl-2 family protein members. Insitol-requiring enzyme-1 through interaction with adaptor factor tumour necrosis factor receptor-associated factor-2 activates ER membrane associated caspases, key cellular initiators of programmed cell death. Finally dysfunction in calcium homeostasis with a cellular re-distribution and build up within the mitochondrial matrix causes a disruption of inner membrane polarity and generation of free radicals, which augment pro-apoptotic pathways.[78]

Mitochondrial dysfunction alone however is one of the critical events in ischaemic stress as it is the initiator of both apoptotic and necrotic cascades leading to cell death. Mitochondrion membranes facilitate oxidative pathways and stretching and unfolding causes changes in distances between membrane components resulting in a loss of coordination of oxidative phosphorylation. Once mitochondrial damage ensues if ATP is available, as is the case upon revascularisation with newly available oxygen, cytochrome c amongst other proapoptopic mediators [79] is released from the mitochondrial membrane causing the activation of initiator pro-caspases 8 and 9 [80,81]. This starts a cascade that in turn activates downstream caspase 3 [82] initiating programmed cell death [83]. ER associated calcium redistribution to the mitochondria augments pro-apoptopic mediatior activation. Caspase 3 is considered to be the key apoptotic molecule responsible for cleaving various proteins, disabling cell structure, and inhibiting repair processes [84,85]. The caspase family of proteins have substrates in plasma membranes, lysosomes, mitochondria, and nuclei [57] . When ATP is unavailable necrosis ensues. Histologically mitochondrial densities and the loss of cristae integrity heralds an irreversible phase of cellular injury [86] .

Calcium Re-distribution

During ischaemia there is an intracellular build-up of calcium ions, a result of both redistribution from the ischaemically stressed ER due to the UPR [79] , and reverse action of the Na/Ca exchanger [87–89] . Calcium pyrophosphate and uric acid is formed as a result and these 'danger signal' molecules bind intracellular proteins to form inflammasome complexes which stimulate the production of both pro-inflammatory mediators known collectively as cytokines [90,91]. Calcium also activates cytoplasmic proteases and phosphoplipases namely the caspases and calpains [92], which in severe intracellular acidosis augment the action of pro-apoptotic proteins released from the damaged mitochondria [93] .

Calcium ions are sequestered in the mitochondrial matrix to offset the ionic potential of excess intracellular sodium and maintain mitochondrial membrane potential [94]. Mitochondrial uptake is regulated by phospholipase C-delta-1, whose expression is up-regulated in cold ischaemia [95]. Calcium overload causes damage to the organelle and as key phosphorylative enzymes (complexes I,III,IV) are inhibited ATP depletion accelerates [96] . The influx of extracellular calcium that occurs during reperfusion facilitates pro-apoptotic protein (cytochrome c) release via the mitochondria permeability transition pore, which is normally closed, but which opens under high calcium loads [57]. Prolonged opening of the mitochondria permeability transition pore differentiates between reversible and irreversible cellular injury [96,97] . The unregulated calcium entry in the cell also activates cell membrane bound enzymes specifically phospholipase A2 which leads to formation and extracellular release of arachidonic acid metabolites (leukotriene B4, thromboxane A2) starting a pro-inflammatory cascade which perpetuates by recruitment and activation of neighbouring parenchymal, endothelial, and immune cells.

Free Radicals and Reactive oxygen species

Reactive oxygen species (ROS) play an important role in ischaemia induced cell damage [98,99]. ROS are generated from the metabolism of oxygen and biologically important species include the superoxide (O_2-), hydroxyl (OH-) and peroxynitrite (ONOO-) anions [100]. Under physiological conditions 95% of cellular oxygen is metabolised in the mitochondria at the end of the electron transport chain by tetravalent reduction with the direct formation of water (H_2O), and generation of ATP[101]; the remaining 5% are reduced in a series of univalent pathways with the formation of free radical intermediaries, such as O_2- and hydrogen peroxide which forms OH- anions [102]. These species play a role in normal intra- and inter-cellular communication, and are quickly reduced by the anti-oxidant enzymes superoxide dimustase, catalase, and glutathione peroxidise to water.

In ischaemia oxidative phosphorylation for the purpose of ATP generation is halted, however molecular oxygen can still be present within the mitochondria, and dysfunction of respiratory chain cytochromes during this period causes excess formation of O_2- anions from this residual oxygen[103]. Additional O_2-, hydrogen peroxide and OH- anions are formed via the still active alternative univalent pathways. These free radicals are highly reactive due to the free and unpaired valence electrons and mediate mitochondrial injury, as well as causing damage to intracellular lipids, nucleic acids and proteins[104] - so called 'oxidative stress'. It has been shown that the mitochondrial permeability transition is induced by ROS and in turn triggers mitochondrial ROS generation[105].

Furthermore under these conditions physiologically non-ROS producing enzymes (xanthine dehydrogenase, XO) can undergo conformational changes (induced by high calcium levels [106]) to isoenzymes capable of forming ROS[107].

1.3.3 Ischaemic Reperfusion Injury and the Kidney

1.3.3.1 Tubular Dysfunction

Ischaemic injury to proximal tubular epithelial cells leads to both structural and functional changes in their cell membranes. During IR activation of cytoplasmic calpains by increasing calcium levels leads to digestion and severing of cytoskeletal actin filaments which disrupts the structure and stability of epithelial cell membranes[108,109] and leads to functional changes in and obstruction of the tubular apparatus.

This instability and in concert with local cytokine effects disrupts apical tight junction integrity. ZO-1, ZO-2[110] and cingulin tight junction proteins are dephosphorylated, forming insoluble complexes with cytoskeletal proteins such as fodrin[111]. These new complexes alter the permeability and polarity of the epithelial membranes and result in back leak of glomerular filtrate into the interstitium with ensuing oedema[112]. At the same time Na+/K+ ATPase membrane channel proteins re-localise to the apical plasma membrane. This reduces Na transport out of the proximal tubules, increasing Na+ delivery to the distal tubular region. This delivery can activate tubuloglomerular feedback at the macula densa and induce afferent arteriolar constriction (through Renin and Angiotensin) which further negatively impacts glomerular filtration. These membrane

disturbances can help explain the observed increase in fractional Na excretion in transplant patients during the initial post operative period.[113]

Zona adheren junctions are also affected during IR injury. A lack of ATP results in a breakdown of the cadheren-actin cytoskeleton interface that makes up zona adheren junction[109] . E-cadherin, and alpha/beta catenin (proteins linking cadherin and actin) become phosphorylated by activated cytoplasmic kinases and dissociate from the plasma membrane[114–116]. Inflammatory cytokines may induce similar junctional changes acting on B-catenin through a wnt/wingless signal transcriptional pathway which regulates proliferative, and apoptotic gene expression[117], suggesting that disruption in adheren junctional complexes may start during ischaemia but are perpetuated during reperfusion by inflammatory mediators.

At the point of reperfusion depending on the degree of ischaemic injury epithelial cells can be shed into the tubular lumen. Epithelial cell exfoliation is mediated by disruption in cell-matrix adhesion specifically and primarily by B1 integrin proteins which anchor cells to the basement membrane. In a similar mechanism to protein re-localisation seen in zona adheren junctions integrins re-distribute to apical membranes which has two effects: 1) mediating detachment of the epithelial cell into the tubule lumen, and 2) facilitating through the new apical integrins interaction and possible adhesion with other shed cells - potentially worsening tubular obstruction[117]. Despite convincing experimental evidence in-vivo analysis has not yet confirmed these findings[113], and the true mechanism may be a complex interaction between other proteins of the integrin family, and the local effect of pro-inflammatory cytokines such as tumour necrosis factor (TNF), interleukin (IL)-1 and interferon gamma (IFN-γ).

1.3.3.2 Inflammatory Role of Tubule Cells

Tubular epithelial cells contribute to the inflammatory process by secreting both cytokines (TNF-α, IL-6, IL-1), chemokines (monocyte chemoattractant protein 1 [MCP-1], IL-8, CCL5 [chemokine ligand 5], fractalkines), and transforming growth factor beta (TGF-β) in response to IR injury [113]. Tubule cells then interact with infiltrative immune cells that perpetuate the initial inflammatory response provoked by reperfusion and endothelial activation[118].

Furthermore chemokines induce migration and adhesion of leucocytes to epithelial membranes facilitating direct macrophage induced tubular injury.

Proximal tubular epithelial cells also express the CD40 receptor for CD154 a ligand expressly found on T-cells. Tubular interaction with T-cells and binding of this ligand-receptor complex activates c-Jun N-terminal kinases and mitogen activated protein kinase (MAPK) pathways which stimulate IL-9 and MCP-1 production by these cells[119]. In parallel CD40 induces CCL5 production by epithelial cells, actions of which are amplified by T-cell (Th2 subset) secretion of IL-4 and IL-13[120].

The expression of toll-like receptor (TLR) 2 and 4 on epithelial cells after ischaemic injury[121] serves as another potential activator in the interstitium of both the innate immune response and specifically complement - exacerbating pro-inflammatory cascades and identifying tubular cells as a target for direct cell mediated damage. Tubule cell expression of C5a complement receptors increases after IR injury[122]. C5a is a key immunomodulator and chemoattractant recruiting and activating macrophages, neutrophils and T-cells, and binding of C5a generated during IR exacerbates the inflammatory infiltrate into the interstium. Blockade with anti-C5a monoclonal antibody (mAB) limits neutrophil and macrophage invasion and affords protection from renal dysfunction in mice[122].

1.3.3.3 Inflammatory Cells in Ischaemia Reperfusion Injury

Macrophages

Macrophages are active in both innate and adaptive immunity and are actively involved in the pathogenesis of IR injury. During IR macrophages become activated (through stimulation by chemokines such as MCP-1) exhibiting potent phagocytic activity and secreting key cytokines (IL-1, IL-6, IL-8, IL-12 and TNF-α) [123]. Within hours of reperfusion macrophages are adherent to renal endothelial of the vasa recta in the post capillary venules of the outer medulla[124]. This influx and infiltration enhances inflammatory cascades with subsequent recruitment of more macrophages and neutrophils and induction of apoptosis in the allograft. Macrophage activity has been implicated in the progressive development of renal fibrosis post IR injury[125] - illustrating a further potentially chronic role of these cell types rather than just in an acute phase of IR. In contrast phenotypic switching of macrophages from the M1 (pro-inflammatory) to M2 (anti-inflammatory) cell type may promote renal repair after ischaemia[126,127] . Macrophages have a key role in IR pathogenesis and subsequent tissue repair the nature of which however is still being investigated and is undoubtedly a complex interplay with other inflammatory cells and species.

Neutrophils

Neutrophils are key players in the innate immune response and active in phagocytosis, producing reactive oxygen and nitrogenous species, and antimicrobial peptides. The role of neutropils in IR injury has been widely established but their involvement in IR mediated renal damage is considered to be less than in other tissue types such as cardiac and skeletal muscle[128]. The exact role of neutrophils in IR mediated renal damage is still ill-defined, and despite discrepancies in the extent of their involvement in the IR process it is likely that one mechanism of injury is through direct damage by plugging renal microvasculature with release of reactive species and proteases [129].

Lymphocytes

CD4 T cells have been implicated as important mediators in the initial phase of injury during renal IR. CD4 knockout mice were protected from IR injury, and this damage was restored by adoptive CD4 transfers, while in contrast CD8 knockouts showed no effect on IR injury [130]. More recently distinct CD4 cell subsets including: CD4+, ygT, and Tregs, have been explicitly linked to IR pathogenesis with Th1 (IFN-γ) and Th17 cells (IL-17) promoting immune activation and damage, and Tregs limiting inflammation and contributing to tissue repair[131]. The functional variants of T-cell subpopulations reflect their differences in tissue-specific T cell/innate immune cell infiltrates and kinetics in IR injury, meaning that different T-cells will promote or inhibit damage at different temporal stages of IR mediated injury[132].

Natural Killer cells

Natural Killer (NK) cells are cytotoxic lymphocytes that lack T-cell or B-cell membrane receptors. They are potent components of the innate immune response and can target and kill infected cells directly as well as produce cytokines including IFN-γ and TNF-α. The known role of NK cells in acute kidney IR injury is still limited but due to their important cytotoxic, secretory, and stimulatory roles NK cells are predicted to be important in inducing IR renal injury. The exact contribution of NK cells to renal IR injury is still not well defined. NK cells can directly kill tubular epithelial cells after IR, and NK cell depletion in wild-type C57BL/6 mice has a protective effect, with the adoptive transfer of NK cells to Rag2−/− c −/− mice, which naturally lack NK, natural killer T , T, and B cells, worsening injury [133].

1.3.3.4 Inflammatory Mediators in Ischaemia Reperfusion Injury

Cytokines

Cytokines are low-molecular-weight regulatory glycoproteins released from various cell types and are key inflammatory mediators [134] involved in every aspect of immunity and inflammation, including: innate immunity, antigen presentation, haemopoetic cell differentiation, cellular recruitment and inflammation, and adhesion molecule expression [135]. IR injury is an inflammatory reaction that is potentiated by cytokines secreted by endothelial and tubular cells of the renal allograft as well as infiltrating immune cells of the recipient.

These cytokines act within the allograft to increase 1) expression of cellular adhesion molecules, 2) vascular permeability in the allograft, 3) recruitment and activation of endothelial and inflammatory cells into pro-inflammatory states, 4) activation of the complement system as well as expression of TLRs, 5) generation of ROS, and 6) activation of aracadonic acid derivatives (prostaglandins and leukotrienes) which serve to amplify cytokine release [135]. Key cytokines identified in IR injury include tumour necrosis factor, interleukin-1, and interleukin-6.

Tumour necrosis factor

TNF exists as several specific proteins - TNF-α and β - produced by immune cells (mononuclear phagocytes, neutrophils, activated lymphocytes, natural killer cells) and also endothelial and mast cells[136]. During IR injury TNF stimulates endothelial cells to express a variety of cell adhesion molecules; intracellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1) and E-selectin, while also inducing vascular and endothelial permeability which enables egress of inflammatory cells into the renal parenchyma[137]. TNF is a potent neutrophil activator as well as a chemotactic agent, mediating cell adherence but also infiltration, degranulation, and development of the respiratory burst and formation of ROS.[135]

Interleukin 1 (IL-1)

IL-1 is primarily produced by mononuclear phagocytes, however during periods of cellular stress such as IR injury endothelial cells have been shown to secrete IL-1. IL-1 is a key activator of T-lymphocytes and up-regulates IL-2 and IL-2 Receptor production and expression (which induces clonal T-cell proliferation) [138]. It is also a stimulator of hepatic acute phase protein production (C-reactive protein [CRP], complement) and shares similar effects as TNF - promoting endothelial expression of ICAM-1, VCAM-1 and E-selectin [136].

Interleukin 6 (IL-6)

IL-6 is the key inducer of hepatic acute phase proteins, and mediates T-cell activation, growth, and differentiation, as well as B-cell differentiation into plasma cells [135]. Its synthesis, capable from mononuclear phagocytes as well as T- and B-cells, endothelial cells, fibroblasts, hypatocytes and haemopoetic cells, is induced by both TNF and IL-1 [138].

Chemokines

Chemokines are a subgroup of cytokines whose primary effect is chemotaxis and leukocyte activation [134]. Chemokines activity have been documented in a number of reperfusion injury models in brain, heart, liver and kidney injury [139] . During IR injury chemokine induction is stimulated via a variety of mechanisms including ROS stress [140] [141] , allied pro-inflammatory cytokines (TNF-α, IL-1) [142], complement activation [143], TLR receptor activation and the NFk-B transcription pathway [139] .

A key chemokine is IL-8 which plays a predominate role in neutrophil recruitment, has been shown to be upregulated in clinical kidney transplantation of cadaveric allografts, with concentrations correlated to the degree of cold ischaemic injury [144] , and inhibition of the IL-8 receptor mitigating IR injury in murine renal transplant models [145].

Another chemokine of note is MCP-1. MCP-1 is a potent chemotactic protein acting on monocytes, T cells, and NK cells. It is implicated in monocytic infiltrative diseases, including IR injury in cardiac [146], cerebral ischaemia [147] , and in particular in renal ischaemia where mice deficient in the MCP-1 receptor demonstrated decreased tubular injury and monocytic infiltrates during acute kidney injury (AKI) [148] .

Other prominent chemokines include CCL5 (regulation upon activation, normal T cell expressed and secreted) and Fractalkine/CX3CL1 [149]. These are key players in general IR inflammation, and are likely active in renal IR injury. Fractalkin/CX3CL1 in particular is expressed on injured endothelial cells and is a potent chemotactic and adhesion molecule for macrophages with their expression up-regulated in post-ischaemic kidneys, and receptor inhibition shown to confer a degree of protection from macrophage infiltration [150] . Despite recent suggestions the exact role of these molecules have yet to be elucidated in renal IR.

1.3.4 Transcription Factor NFk-B

NFk-B is a family of transcription factors that integrates a complex network of extracellular signalling pathways, resulting in the transcriptional regulation of a large number of genes related to inflammation, immunity, apoptosis, cell proliferation, and differentiation.

NFk-B by binding to discrete DNA sequences known as B elements in promoter and enhancer elements of target genes can induce or repress gene expression. NFk-B activation through a number of stimuli results in nuclear translocation and can proceed either through a series of classical or alternative, and hybrid pathways.

The complete repertoire of target genes encompasses key inflammatory molecules; cytokines (Il-1,2,6 and IL-2 receptors), chemokines (IL-8, IL-10, MCP-1, CCL5), colony stimulating factors, TNF-α/β and INF-β; cell membrane surface molecules (T-cell receptor B chains, B2-microglobin, CAMs [selectins, ICAM, VCAM, ELAM], and major histocompatibility complex [MHC] Class I and II); inflammatory enzymes (iNOS, iCOX, PLA); acute phase proteins (Tissue factor, metalloproteinases); and allied transcription factors that act to regulate, mediate, or promote inflammatory gene transcription [such as c-rel, c-myc, IFR, IkBa].[151]

Depending on the pathway involved expression of specific gene subsets may be induced or repressed. Transcription of a number of key inflammatory mediators is guaranteed by short stimulations of NF-B regardless of stimulus concentration. These include negative regulators of NF-B activity (IkBa ,IkBe, and A20) and pro-inflammatory cytokines (IL-6, IL-8, MCP-1, and IP10). Some genes however are transcribed only with prolonged NF-B activation, such as cell surface receptors, adhesion molecules and signal adapters, and key chemokines such as CCL5.

There are numerous stimuli relevant to NFk-B activation in kidneys and include inflammatory cytokines (IL-1/2, TNF-α, IFN-γ) and growth factors, pathogen and cellular damage-associated molecular patterns, TLRs and nucleotide-binding oligomerization domain receptors (NOD like receptors), metabolic (high glucose, advanced glycation end products [AGEs]) or genotoxic stress, vasoactive and endothelial related agents (endothelin, angiotensin II, thrombin), cell membrane activation proteins (CAMs, fibronectin), immune mediators, proteinuria, and mechanical stretch.[152]

Renal NFk-B activation in the setting of reperfusion injury regulates neutrophil, macrophage, lymphocyte, and dendritic cell biology, as well as endothelial cell activation, and has been

documented in both in vivo and in vitro models in intrinsic glomerular cells such as podocytes and mesangial, tubular, and endothelial cells during renal IR injury. [153]

1.3.5 Complement

The complement system plays an important part in innate immune response processes. It is composed of a group of serum and cell membrane bound proteins whose interplay with each other and other molecules of innate and adaptive immune systems means it has diverse roles in inflammation, and IR injury [154] .

Three different complement pathways can be distinguished: the alternative pathway, the classical pathway, and the MB-lectin pathway and all three are active in IR injury to variable degrees depending on the models and organs involved.

After activation of any of the three pathways there is a merged formation of a the complement component C3 and enzyme C5 convertase and ultimately the formation of the membrane attack complex (MAC) formed by a C5b-C9 complement complex [154], with the split products of C3, C4, and C5 formed in the pathway capable of immuno-modulating functions [155] [156] .

The predominate pathways responsible in renal IR injury appear to be the alternative [157] and MB-lectin pathways [158] with the role of classical pathway in the inflammatory milieu occurring during and after renal ischaemia [154] only recently emerging as an important mechanism.

Select inhibition of the alternative pathway affords protection from ischemic injury in kidney models [157]. In murine models of AKI , C3 production from tubular epithelial cells is stimulated and the endogenous complement inhibitor Crry expressed on the tubular basolateral surface is depressed after IR [159]. While C3a has been demonstrated to play a crucial role in chemokine production by tubular epithelial cells after IR, supporting the role of the alternative pathway after IR injury [160], with small interfering RNA (siRNA) gene slicing targeting C3 and caspase 3 improving renal function and affording structural protection in a murine transplant model [161].

The MAC and C5a also contribute to ischemic AKI with C5a receptor antagonists demonstrating protective effects against IR injury [162]. In addition MAC deposition on tubular epithelial cells is known to stimulate production of key inflammatory cytokines - TNF-α and IL-6 [163].

1.3.6 Toll like receptors

TLRs are a family of 13 trans-membrane prototype pattern recognition receptors and are key components of the innate immune response primarily due to their ability to recognise pathogen related molecules. Recognition and engagement of exogenous molecules by TLRs induces generation of cytokine and chemokines, upregulating co-stimulatory molecules, enhancing antigen processing by antigen presenting cells, and guiding T-cell induction and activation, that altogether initiate and amplify the innate immune response[91].

Signal transduction and activation of innate responses by TLRs is via several pro-inflammatory kinases and the NF-κB pathways, that lead to generation of pro-inflammatory cytokines [134]. TLRs have also been shown to recognise endogenous cellular molecules derived during cellular injury (damage-associated molecular patterns [DAMPs]) - such as products from degradation of macro-molecules, proteolytic cascades, and intracellular components of ruptured cells, such as hyaluronan, fibronectin, as well as heat shock proteins (HSPs), high-mobility group box protein-1 (HMGB1) and host DNA [164]. Though predominately present on immune related antigen presenting cells TLRs are now known to be expressed on a variety of non-immune functioning cells. In particular TLR2 and TLR4 are constitutively expressed on renal tubular epithelial cell membranes and cells of the Bowman capsule [165], which recognise to HSP-60 and HSP-70, and the expression of which is up-regulated during renal ischaemia and reperfusion injury [121].

More recently transgenic knockout of TLR3 in mice was shown to be protective in renal IR with less renal damage biomarker production Neutrophil gelatinase-associated lipocalin, lower cumulative kidney damage, and improved renal blood perfusion [166].

Experimentally loss of function TLR4 gene mutations are associated with improved kidney graft function post operatively, with TLR4 expression upregulated in cadaveric vs. living donor kidneys [121], while in clinical liver transplantation the TLR4 genotype enhanced sterile inflammation and was linked to poor graft survival [167].

TLR signally by HMGB1 has been shown to accelerate the inflammatory response to IR injury in a positive feedback loop instigated by TLR mediated ROS release [121]. TLR activation and signally thus not only impacts immediate damage during IR, but persistent activation through these pathways impacts recipient allo-responses, graft rejection, and chronic allograft injury. The role of TLR is acute injury has been widely described, and though definitively involved in chronic responses the exact mechanism played in chronic injury is less understood.

1.3.7 Nitric Oxide

Nitric Oxide (NO), or endothelium relaxing factor is a labile free radical produced in varying degrees through the conversion of the amino acid L-arginine to L-citrulline, catalysed by nitric oxide synthase (NOS). It plays a role in both normal and abnormal tissue function and contributes to the pathogenesis of IR injury[99] . There are three isoformic variants of NOS - nNOS1 and eNOS are constitutive, calcium/calmodulin dependent, and primarily in neuronal and endothelial cells respectively, while iNOS is calcium independent, inducible and involved predominately in inflammation[99] . All three variants are found in the kidney.

The extent of NO's role as a protective or deleterious entity in inflammation is dependent on the site, cellular origin, quantity and rate of production, and precipitating factor for its generation. Constitutive NOS is regarded to be associated with cytoprotective formation of NO, while iNOS expressed predominately after hypoxic or cytokine exposure is associated with deleterious generation of NO that is actively involved in the progression of IR injury and in kidneys, acute renal failure (ARF). Ultimately the degree of damage is dependent on balance and shift between NO and ROS formation and their ultimate metabolism during ischaemia and subsequent re-perfusion

1.3.7.1 Cytotoxic NO

Cytotoxic properties of NO are related to its interaction with other ROS produced during periods of ischaemia and hypoxia, and subsequent reperfusion. NO can react with O_2- anions to form ONOO-. Depletion of the NOS cofactor BH4 during ichaemia may also trigger changes in NOS function, switching from NO to O_2- formation.

1.3.7.2 Cytoprotective NO

More recently NO has been shown to be a potentially cytoprotective molecule in IR injury, with several key properties[168].

NO acts as an anti-oxidant free radical scavenger capable of reacting with O_2- and reducing superoxide mediated tissue damage[169] [170]. This reaction produces the highly toxic ONOO-, the reactivity of which however is determined by the NO:02 -ratio with excess NO reducing ONOO-elicited activity[171]. NO is also able to inhibit mitochondrial respiration, and reduce ROS formation during reperfusion by competitively binding to cytochrome-C oxidase[172]. During ichaemia binding is more pronounced due to the lack of O_2, mitigating a degree of preconditioning, and during

reperfusion ischaemia is slowly abated by progressive competition for binding by oxygen, preventing sudden resumption in electron transport chain activity, and less ROS formation.

NO can mediate inflammatory processes specifically preventing inflammatory neutrophilic infiltration, and cytokine production[173]. TNF-α is a key cytokine involved in accumulation of neutrophils and production of allied cytokines, as well as inducing pro-inflammatory gene expression via the NF-KB pathway[174]. Direct NO administration in murine models of renal and pulmonary IR injury using NO-donors (molsidomine [170], nitroprusside [175]) has been shown to reduce IR damage and apoptosis via a significant reduction in TNF-α. While liver preconditioning through NO leads to a reduction in IL-1 release, with protection against IR injury[176].

NO also influences the expression of a number of cell adhesion molecules (CAMS) which play a key role in the cellular inflammatory aspect of IR injury. During and after IR injury CAM are upregulated on vasculature endothelial cells - with selectins mediating the initial tethering of neutropils to activated endothelial an important step in IR pathogenesis. L-selectin is constitutively specific for leucocytes while P and E- selectin are expressed on endothelial cells in response to stimuli - such as ROS, and cytokines (IL-1, TNF-α)[58]. IR injury has been shown to decrease intracellular NO levels and increase P-selectin expression[177]. Delivery of NO by molecular donors in both animal and human in-vitro models decreases leukocyte endothelial attachment, rolling, and infiltration, and inhibits the expression of P-selectin[177] , E-selectin, VCAM-1 and ICAM-1, achieved possibly by inhibition of TNF-α [137] and blockade of NFk-B.

NO can exhibit both pro-apoptotic and anti-apoptotic effects [178]. NO can reduce the amount of apoptosis induced by TNF-α, ROS, and glucose deprivation, while conversely high concentrations of intracellular NO can induce necrosis and apoptosis [99] . The mechanisms by which NO mediates these opposite effects are only slowly being elucidated, and may differ between cell types. Possible mechanisms include through a diminished response of the intracellular g-protein messenger enzyme guanylyl cyclase to NO [178] , down regulation of the proapoptotic gene p53 [179], direct dose-dependent inhibition of apoptotic caspases [180], and even the induction of HSP70.

In parallel to down regulating CAM expression NO has been shown to regulate the chemokine response. NO acts by modulating the production[142] of the potent neutrophil chemoattractants macrophage inflammatory proteins (MIP-1 and 2) that are expressed during IR injury[181]. The mechanisms for many of investigated protective effects of NO likely lie with modulating the effects or actions of transcription factors such as NFk-B and AP-1, and the MAPKs.

MAPKs are signal transducers modulating gene expression and protein production involved in cell proliferation, differentiation, survival, and apoptosis. NO has been shown to affect activation of several species: extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases, p38 kinases, guanylyl cyclase and cGK, and through one of these pathways NO exhibits its beneficial IR protective effects .

Transcription factors are protein complexes that control the transcription of DNA, with NFk-B involved in cellular responses to stressful stimuli, cytokines, ROS, bacterial or viral pathogens. Genes regulated by NFk-B include those for iNOS, TNF-α, IL-6 and IL-1 - all key species in the pathogenesis of IR injury. NO may inhibit NFk-B activity by a number of mechanisms: scavenging of ROS (O_2-), interrupting NFk-B DNA binding, and stabilising NFk-B precursors (IkBa) .

1.4 The Coagulation Cascade and Ischaemia Reperfusion Injury

1.4.1 Physiology of Coagulation

The coagulation system is composed of both cellular and macromolecular protein elements. Cellular components include platelets, inflammatory cells, and in few numbers RBCs and vascular micro-particles), while the proteins are a series of coagulatory and anti-coagulatory enzymes and co-factors [182].

Once coagulation is activated - through trauma or other stimuli - cells interact with the macro-molecular coagulation proteins initiating a series of enzymatic cascades that lead to the formation of a fibrin meshwork. Enzymatic proteases exist in two predominant forms - as a pre-cursor form known as a zymogen and an activated form.

Historic models divided the coagulation cascade into 3 components [183] - the intrinsic, extrinsic and common pathways. The intrinsic relies on exposed collagen in damaged vessels to activate Factors XII, XI, IX and VIII. The extrinsic on stabilisation of endothelial bound Tissue Factor (TF, an transmembrane glycoprotein) which then complexes with the zymogen Factor VII to form the activated serine protease TF-FVII complex (TF-FVIIa). In both instances the end products of each activated Factor VIII (FVIIIa) and TF-FVII converge on the common pathway by activating Factor X which cleaves prothrombin (FII) into thrombin (FIIa). Thrombin in turn acts on fibrinogen to generate fibrin which is then cross linked to other fibrin molecules in an insoluble fibrin meshwork.

The most recent model of coagulation is based on TF being the key trigger of coagulation [183] with limited requirement for contact activation of the intrinsic pathway, the TF-VIIa complex able to activate Factor IX directly ('the josso pathway'), and thrombin able to activate Factor XI [184]. The TF extracellular domain binds and increases the activity of FVII/VIIa. Thus TF initiates the pathway for extrinsic coagulation, playing a critical role in thrombin generation, and thrombus formation. Because TF-VIIa can also activate FIX directly, to form a complex with FIXa and FX, an essential amplification loop re-generating FXa is initiated [185]. This is potentiated in the presence of thrombin activated platelets.

Coagulation reactions do not take place in the liquid plasma phase of the circulation, but in the solid phase occurring on cell membranes or the surface of developing thrombi. The primary step in endothelial cells acquiring a thombogenic phenotype is upregulation of bound TF.

In a normal state there is a constant generation of TF and activation of small quantities of the coagulation proteases, with fibrin formation. However to prevent uncontrolled fibrin formation and thrombosis a tightly controlled endogenous anti-coagulant system exists, predominant in the circulation and at the endothelial surface. During episodes of stimuli endogenous anti-coagulant mechanisms are inactivated and overwhelmed by the pro-thrombotic stimulus.

There are three endogenous anti-coagulant systems: anti-thrombin III (AT), tissue factor pathway inhibitor (TFPI), and the protein C system (see Figure 9) . Evidence of their importance in maintaining vascular fluidity and preventing unintentional thrombosis is derived from analysis of congenital and experimental, deficiencies or functional defects of ATIII [186] , TFPI [187], and Protein C[187] that increases thrombotic risk.

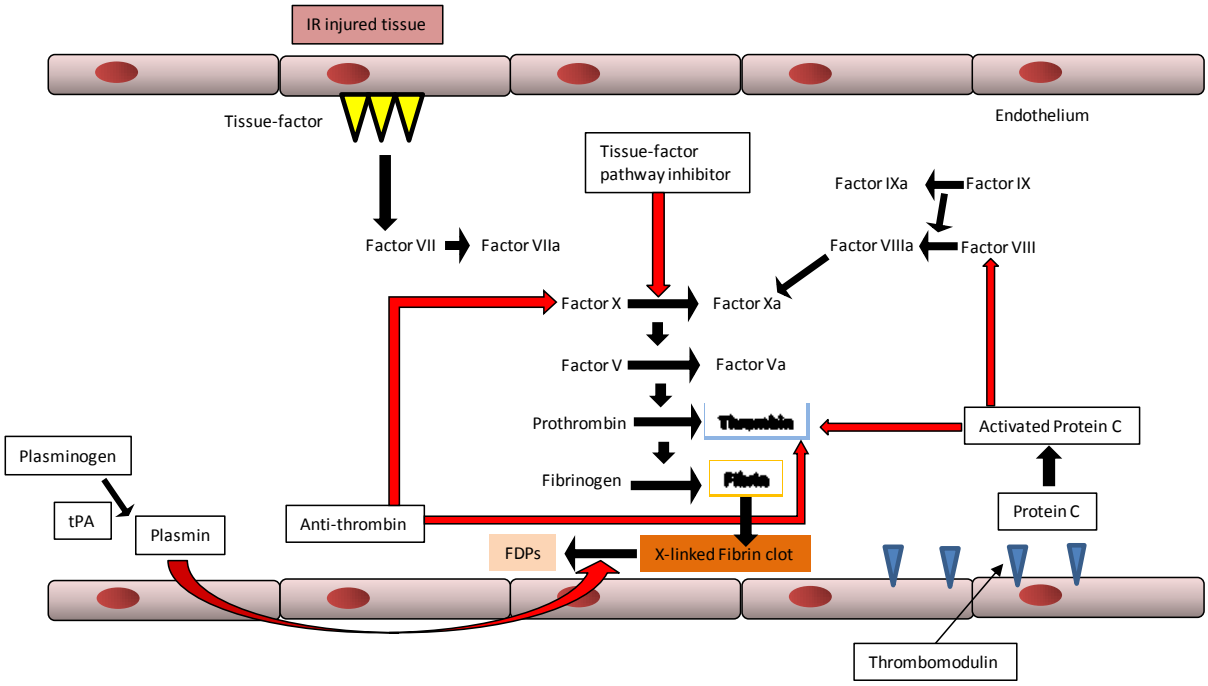


Figure 9 Overview of the coagulation network and various endogenous inhibitors. The process begins with tissue factor contacting factor VII/VIIa in blood after IR injury or during inflammation. Thrombin is generated by a series of feedback and amplification steps resulting in generation of fibrin polymers with fibrin deposition (not shown). The three main regulators of coagulation are anti-thrombin, tissue- factor pathway inhibitor, activated protein C / thrombomodulin. Fibrinolysis is facilitated by plasmin formed from the cleavage of plasminogen by tissue plasminogen activator (t-PA).

1.4.1.1 Anti-thrombin

AT is a potent proteinase inhibitor, inactivating the majority of serine proteases in particular thrombin and FIVa and FXa. Inhibition is catalysed by heparan and dermatan sulphate which are glycoaminoglycans present within the glycocalyx of the endothelial cell membrane. These co-factors potentiate by a factor of 100 the activity of ATIII [188,189].

1.4.1.2 TFPI

TFPI is produced by endothelial cells. It binds to minute quantities of generated FXa then proceeds to inhibit the TF-FVIIa complex on the endothelial surface [190,191].

1.4.1.3 Protein C System

Protein C is a vitamin K dependent protein produced by the liver. It is a circulating molecule activated by it binding to the thrombin-thrombomodulin complex on the endothelial surface. Thrombomodulin (TM) is a 100-kDa transmembrane protein produced by endothelial cells in either a bound or soluble circulating form. Once bound to TM protein C associates with protein S (a cofactor), and the endothelial protein C receptor (EPCR) which serve to accelerate protein C activation 20-fold[192]. Bound to its cofactors Protein C suppresses FV and FVIII, while independently protein C directly inhibits FXa [193].

1.4.1.4 Endogenous Anti-fibrinolytic Systems

Once fibrin is generated by the action of thrombin on fibrinogen there are intrinsic fibrinolytic mechanisms in action to prevent widespread coagulation and thrombosis. The primary system is through the protease tissue plasminogen activator (t-PA). t-PA is released by endothelial cells and transforms plasminogen on the cell surface or the clot itself into plasmin. Plasmin degrades fibrin with the release of fibrin degradation products (D-dimers). t-PA itself is solely produced in the micro-circulation. In the kidney a specific plasminogen activator is evident u-PA - expressed during remodelling in tissue repair and angiogenesis, as well limiting vascular homeostasis during localised infection [194,195]. Fibrinolysis is controlled by alpha-2 antiplasmin (a plasmin inhibitor) and plasminogen activator inhibitor 1-3(PAI 1-3). PAI-1 also mediates inflammatory responses through the induction of leucocytes (neutrophils and lymphocytes), activation of neutrophils via TLR-4 and clearance of apoptotic cells and debris [196].

1.4.2 Coagulatory and Inflammatory Cross Talk During Ischaemia Reperfusion Injury

There is much cross talk between inflammatory mediators and coagulatory proteases. Coagulation proteases produced as a direct consequence of the inflammation accompanying innate immune responses activated during IR injury continuously refuel the coagulation-inflammation cycle perpetuating and strengthening the inflammatory reaction. It is evident that unlike during coagulation initiated through the intrinsic pathway and direct acute vessel injury inflammation induced coagulation is not balanced by natural anti-coagulatory and endogenous fibrinolytic systems. However, coagulatory enzymes can also modulate key processes in inflammation including leucocyte activation, cytokine release, CAM expression, and NFk-B signalling. Mechanisms in this relationship have been elucidated in in-vitro and in-vivo models of endotoxic and IR based inflammation.

1.4.2.1 TF and TFPI

The key initiator of inflammation induced coagulation is TF[185] with TFPI a potent endogenous inhibitor. TF is not constitutively expressed on endothelial cells, present on cells not in direct contact with blood. Histologically, TF is present in adventitial layers of vessels, creating a 'TF envelope' that can be initiate coagulation quickly after disruption to the endothelial[197]. Inflammation induced coagulation can be initiated through TF up regulation or theoretically TFPI inhibition.

During IR injury cytokine activated monocytes and macrophages expressing TF are recruited into allograft. Leucocyte-endothelial adherence enables immune cell derived TF forming complexes with FVIIa, as well as transfer of TF at the endothelial surface, activating the coagulation cascade[198,199].

Despite mononuclear cells considered to be responsible for substantive TF expression in the vasculature recent findings have demonstrated endothelial expression as potentially key step in IR induced activation of coagulation. Endothelial have been shown to express TF through a variety of mechanisms.

In-vitro models have shown TF expression can be induced by a variety of stimuli [182,197,200] including thrombin, inflammatory cytokines and chemokines (IL-6, IL-1, TNF-α, IFN, MCP-1), a result of CAM expression and receptor binding (ICAM, P-selectin), soluble and deposited complement

(C5a), AGEs, oxidised low density lipoproteins (LDLs) as well as hypoxia and ROS [201,202]. TF expression can also be inhibited by enhanced eNOS generation, specifically in endotoxin and cytokine induced expression in vitro [203]. Expression is mediated by a number of intracellular kinase signalling pathways (e.g. protein kinase C [PKC], and MAPKs) and also NFk-B [197]. In-vitro TNF-α induces TF expression on cultured endothelial cells, and blockade of thromboxane receptors inhibits cytokine induced expression by limiting activity of PKC and NADPH oxidase, with decreased ROS generation [204,205]. TLR2 and TLR4 activation can induce TF expression in macrophages and endothelial cells respectively via a JAK2-STAT3, and the NFk-B signalling pathways [206,207]. The complexity of interactions between inflammatory and coagulatory cascades as well as the nature of in-vitro experiments has proven difficult in translating in-vitro TF expression profiles using some in-vivo models [208].

In-vivo cardiac IR models demonstrate endothelial TF expression mediated by ROS and reduced by treatment with ROS scavengers, and directly by TF mAb [209,210]. However similar models have suggested that TF expression may in fact predominate in cardiomyocytes, with endothelial expression limited [211,212], pointing to TF solely as an initiator (regardless of localization) and thrombin as the dominant effector in infarct size. TF-VIIa inhibition in murine cardiac IR reduces activation of NFk-B and IL-6 expression with TLR4 down regulation a potential mechanism [213].

In contrast renal models have firmly implicated up regulated endothelial TF expression during IR injury as key to mediated damaged [214–216]. TFs role in renal IR is abrogated with TF antisense oligonudeotides [217], recombinant TFPI[218], direct thrombin inhibitors, and in protease activated receptor-1 (PAR-1) or TF deficient mice [215]. Furthermore extended CIT (24 v 6 hours) is associated with up regulation of endothelial TF expression in murine renal transplantation, and induces a procoagulant state with greater loss of endothelial integrity and tubular necrosis[219]. Early inflammation in renal allografts appears not to have an immunogenic source[220] but rather IR mediated and activation of coagulation cascades through TF likely plays an important part in this process. Similar findings have been shown in hepatic models of IR injury [221–224].

Studies in clinical transplantation are limited by comparison. Studies available indicate a chronic pro-coagulant state, with renal transplant recipients demonstrating increased TF plasma activity compared to healthy controls [225]. In contrast immunohistology has localised of TF in biopsies from normal and transplanted kidneys[226]. In normal kidneys TF localised to glomerular epithelium and basements membranes only. Donor kidneys showed TF reactivity in renal capsule, and adventitia of large arteries but not on endothelial cells. Some pre-implantation biopsies even demonstrated

depleted glomerular TF. Furthermore long-term allografts with progressive dysfunction and those with advanced rejection in contrast to plasma activity exhibited diminished TF expression in glomerular epithelium and basement membranes.

CRP is marker of inflammation. It is also an inducer of TF expression in vitro and in vivo and promotes a prothrombotic and inflammatory phenotype [227–230]. The kidney allograft is known to locally produce CRP in glomeruli and cortical tubules[231] during episodes of acute rejection, and increased levels of CRP perioperatively and on follow up are associated with poorer graft survival [232,233]and the development of metabolic syndrome[234]. Experimentally in murine renal IR models CRP exacerbates reperfusion injury by upregulating macrophage activation and FcγR expression[235] and impairing tubular epithelial regeneration[236]. CRPs role in inflammatory reactions in the kidney has multiple dimensions. Studies to date have yet to fully elucidate the acute role for CRP in the immediate IR setting but TF involvement is a plausible component.

Despite TFPI being the main inhibitor of the TF-VIIa complex and thus thrombin formation the complete role of TFPI during inflammation related coagulation has not been fully characterised. Clinical studies show that TFPI levels are not decreased in inflammatory endotoxaemia[237]. This may not be representative however as the majority of TFPI is endothelial bound with only 10-20% in the circulation bound to lipoprotein[238]. Recombinant TFPI treatment however blocks inflammatory thrombin generation and necrosis [218] as well as reducing microcirculatory 'no-reflow' dysfunction (capillary malperfusion upon revascularisation) [239] in renal and cardiac IR models, and reduces mortality in severe endotoxaemia via IL-6 [185]. Available evidence does not point to depletion or dysfunction of TFPI during inflammatory states, such as endotoxaemia. It may be that overwhelming inflammatory stimuli overcome the endogenous activity of TFPI, and thus pharmacological treatment increasing TFPI levels above physiological levels may enable activity to counter thrombin generation.

1.4.2.2 APC

There is a down regulation of APC activity during inflammation. In endotoxaemia APC zymogens levels are decreased due to consumption from the amplified generation of thrombin and fibrin, impaired synthesis due to the acute phase response, and degradation from neutrophilic elastases [185,240]. In IR injury during transplantation the influence from degradation likely predominates, related to the activation, adherence and infiltration of neutrophils and macrophages. Release of TNF-α, which in turn stimulates IL-6 production, and IL-1 down regulates endothelial TM expression

via NFk-B, disrupting a key step in APC functionality [241,242]. Cofactor protein S is found bound in plasma to complement C4b (60%) and also in a free soluble form. During IR cytokine release, ROS, and TLR activation stimulates the acute phase response with increased complement production and activation reducing the free:bound ratio of protein S, rendering a relative deficiency for APC [243,244]. Finally the EPCR receptor is downregulated in endotoxaemic stress, and experimental receptor blockade in animal endotoxaemia models results in severe thrombosis[245].

In addition to anti-coagulatory properties APC exerts anti-inflammatory effects. This functionality is mediated in part by PAR1-4 which are the predominate G-protein receptors mediating cell signalling by coagulation proteases, but also by binding to EPCR receptors[246]. The anti-inflammatory properties have been demonstrated in endotoxaemic, renal, and hepatic IR models [247]: APC reduces leucocyte-endothelial adhesion and activation through down regulating cytokine (IL-6, TNF-α) and chemokine (MCP-1) production [248–252]; down regulates ICAM, ECAM and E-selectin mobilization from Weible Paladi bodies to the endothelium[253]; and ameliorates mitochondrial oxidative stress[254]. The APC-EPCR complex preserves endothelial barrier integrity by inducing membrane sphingosine 1-phosphate receptor activation and signalling (S1P)[255], and inhibiting endothelial cell apoptosis [256,257]. EPCR, through the similarity of its homology with major histocompatibility complex receptor CD1 may also play a direct role in mediating inflammation, and experimental transgenic expression of EPCR in mice enhances APC levels protecting against endotoxaemia[257].

Loss of TM from the endothelium also contributes to the pro-inflammatory phenotype during IR. TM reduces MAPK and NFk-B signalling[258]. In addition the TM-thrombin complex inhibits thrombin-induced endothelial activation[259], activating thrombin-activatable fibrinolysis inhibitor (TAFI), a procarboxypeptidase B which is a potent C5a inhibitor contributing to a reduction in microvascular dysfunction[260].

Shear stress stimulates TM transcription and endothelial expression via krupple like factor 2, and in a transplant IR setting, SCS where by pulsatile and pressure stimulation of the vasculature is absent, upon reperfusion the endothelium is compromised and less able to tolerate the inflammatory influx [261–263].

1.4.3 Thrombin - The Key Coagulatory Protease

Thrombin has a central role in the coagulation cascade whose generation once started is accelerated by a series of feedback amplification loops resulting in fibrin deposition at the site of endothelial injury. The haemostatic actions of thrombin are depicted in Figure **9**. Thrombin is a multifunctional molecule also possesses non-haemostatic properties and in particular is involved in inflammation[264–266]. Thrombin induces cytokine (e.g. IL-6 and IL-8) and chemokine (MCP-1) production in a number of cell types including endothelial cells [267,268] , mural cells, epithelial cells, adipocytes, and leucocytes[269]. Thrombin can induce endothelial expression of adhesion molecules (P-selectin, E-selectin and ICAM)[270], promotes release of growth factors (such as platelet derived growth factor [PDGF]) and chemokines (PAF)[271] promoting endothelial-leucocyte-platelet interactions and adhesion[272], as well as production of platelet-activating factor [273], endothelin [274], von Willebrand factor [275], and plasminogen activator and inhibitor [276]. Thrombin directly activates complement (C3 and C5) and modulates innate immune responses by altering secretion and expression of cytokines and receptors in antigen-presenting cells[277–279]. Thrombin also increases endothelial permeability, a feature of inflammation, indirectly by stimulating histamine and prostacycline[280] release from platelets and endothelial cells, and directly through the S1P signalling pathway, that in contrast when stimulated by APC/EPCR actually reduces barrier failure [279,281,282]. Thrombin influences tissue repair and potentiates the effects of VEGF, and clot-bound thrombin, which is protected from AT inactivation, contributing to angiogenesis [265]. The PAR receptors form the crucial molecular link between coagulation and inflammation and the majority of extra-haematostatic effects of thrombin are mediated by PARs.

1.4.3.1 Protease Activated Receptors

The extra-haemostatic pro-inflammatory cellular effects of coagulation proteases, such as thrombin and FX, as well as the anti-inflammatory effects of the APC pathway are mediated by PAR signalling[283].

Four types of PAR (PAR 1–4) have been described as part of the G-protein-coupled family of receptors each with a transmembrane domain [284]. PAR receptors are found on various cell types including mononuclear cells, endothelial cells, platelets, fibroblasts, and smooth muscle cells[285] and are illustrated in Figure 10.

PAR activation by coagulation proteases is a unique 'self' mechanism the extracellular PAR receptor domain is cleaved by the protease, revealing a previously cryptic neo-aminoterminus and ligand for

the same receptor, initiating signal via the transmembrane domain. The proteolytic cleavage of extracellular PAR domains by thrombin or factor Xa activates of a canonical G-protein mediated pathway with downstream signalling that triggers multiple transcription-regulated, and cell-specific events[286].

The specific effect of PAR activation is dependent on the tissue in question, and there is a difference in expression between humans and mice[287] rendering direct translation of potential interventions complicated.

PAR-1, PAR-3 and PAR-4 are thrombin receptors, PAR-2 is activated by TF/FVIIa, TF/FVIIa/Xa, while FXa and the APC/EPCR complex can also induce signalling via both PAR-1 and PAR-2 [288]. PAR 2 can also be activated by alternative proteases such as trypsin and mast cell tryptase[284]. Thrombin-activated PAR-1 has also been shown to transactivate PAR-2 by donating the tethered ligand forming a heterodimer and inducing signalling [289].

Stimulation of PARs on endothelial cells leads to induction of pro-inflammatory mediators - IL-6, IL-8, TGF-β, MCP-1, PDGF, and CAM (ICAM-1, P-selectin). PAR-1 on monocytes and macrophages activates cells and enhances leucocyte-endothelial-platelet adhesion with production of IL-1, TNF-α, IL-6, MCP-1 and IL-10, downregulating IL-12 release; while PAR-2 enhances IL-1, IL-6, and IL-8 release[290].

PAR-1 has an interesting dual role being the primary thrombin receptor but also a receptor for the anti-coagulant (and anti-inflammatory) APC/EPCR complex. Thrombin however is a 100 fold more potent PAR-1 activator than APC/EPCR, with levels of APC required to match thrombin activation higher than those found endogenously in-vivo. Thrombin is the primary PAR-1 agonist on endothelial cells thus the predominant effects of PAR activation are pro-inflammatory[291].

However while PAR-1 thrombin activation induces a pro-inflammatory enhancing response, activation by APC/EPCR results in an anti-inflammatory response that preserves endothelial integrity. These contrasting effects are mediated by differential coupling of activated PAR-1 to different S1P proteins. APC/EPCR activation results in a PAR-1/S1P-R1 association preserving endothelial integrity, while thrombin activation in PAR-1/S1P-R3 causing barrier disruption and endothelial permeability[282].

PAR-2 shares use of intracellular adaptor proteins with TLR4 in activating NFk-B during inflammation potentially modulating responses[292]. Transactivation of PAR-2 by PAR-1 ligands is also implicated in the anti-inflammatory effects of PAR-1 (via APC) [289].

PAR-3 acts as co-factor for PAR-1, with receptor dimerization regulating intracellular signalling in endothelial barrier failure[293]. Both PAR-3 and PAR-4 may accelerate TF induced thrombin generation on vascular smooth muscle surfaces[294], while PAR-4 is implicated directly in cardiac IR injury [295]and endothelial activation[296], and in platelet aggregation [297,298].

Despite animal models demonstrating benefit of APC, TM, and TFPI, in reducing inflammatory responses in endotoxaemic and IR models [237,299–307], this has not been translated into clinical practice. The most recent randomised control trial (RCT) for APC use in severe sepsis (PROWESS-SHOCK Trial) demonstrated no survival benefit in patients, with data supported by a Cochrane systemic review [308]; neither have trials involving recombinant TFPI [300], or TM [309]. PAR signalling in endotoxaemic murine models has demonstrated that single PAR deficiency does not protect against inflammation, but that multiple simultaneous inhibition is required to down regulate inflammatory responses [310].

There maintains an inherent difficulty in translating animal model based IR treatments to clinical practice due to differences between murine and human expression and function of coagulation proteases and receptors, as well as the complex interaction between coagulation and inflammation[311]. In clinical practice these interactions are further confounded by multiple interlacing patient co-morbidities and pathologies, as well as differing geno- and pheno-types, and concurrent pharmacological treatments and medications. The temporal differences in PAR activation, differences in expression and dose-related responses of the wide range of proteases, and interaction between PARs themselves serves to highlight the complexities in these pathways and is one further reason why beneficial interventions in experimental models targeting PARs (e.g. APC) may have not been successful in clinical practice[312].

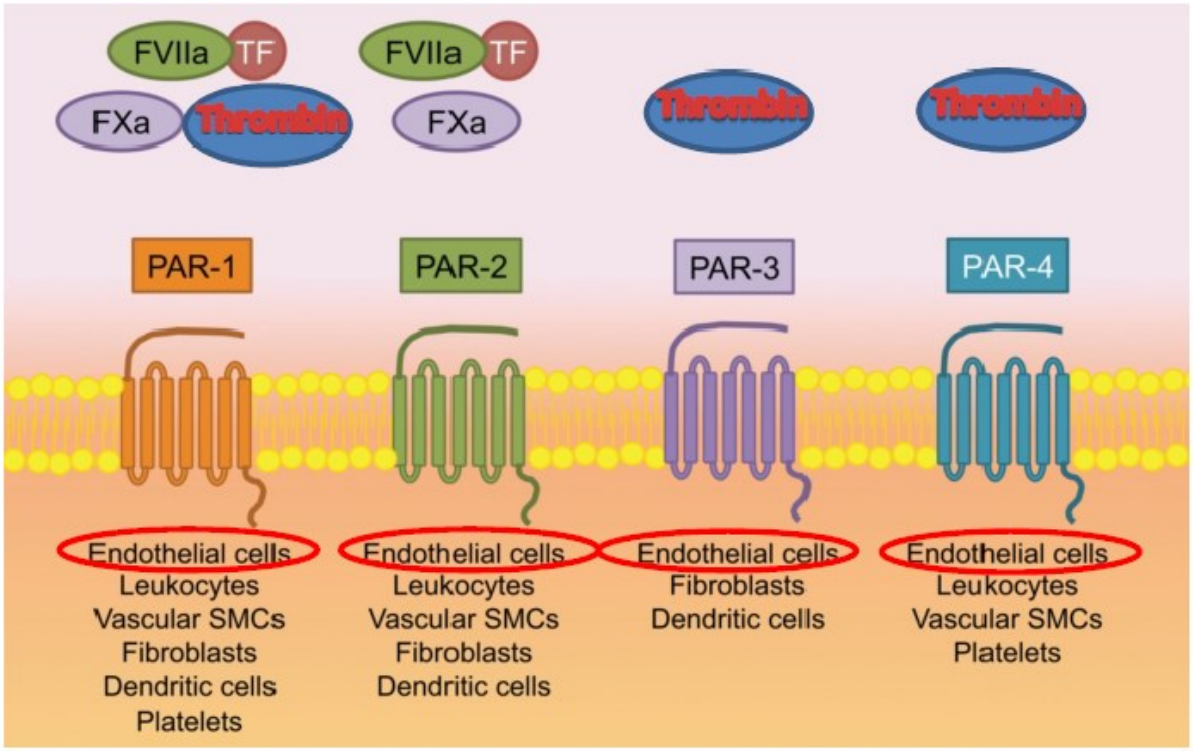


Figure 10 Diagrammatic representation of PAR receptors, their various activators, and the predominant cell type of their expression. In particular thrombin is a key activator of PAR 1,3, and 4, which are all expressed on endothelial cells, demonstrating thrombin wide non-coagulatory potential. Adapted from Esmon et al. [313].

1.4.3.2 Anti-thrombin

AT is the primary inhibitor of thrombin and functions as a general protease inhibitor. Heparan sulfates are endogenous glycosaminoglycans that promote AT function at the endothelium. When heparan sulphates binds to AT it induces a conformational change that increases the rate of thrombin inactivation by a 100 fold. Thrombin has several functional binding regions for binding sodium, an active site, and 2 exosites (exosite I and II). Sodium binding modulates thrombin function in a prothrombotic or anti-coagulant fashion - with absence of sodium favouring thrombin binding to Protein C. The active site is highly negatively charged and substrate access to it is restricted by presence of 'insertion loops' of thrombin peptides. Exosite I is an important electropositive binding site responsible for binding fibrinogen, TM and involved in recognising thrombin substrates directly (fibrinogen, PAR1, FV and FVIII) or indirectly APC and FXIII) [314]. Exosite II is located on the opposite to site I and binds highly sulphated polysacchardes such as heparan sulphate, as well as for TM, and is the principle site for thrombin-AT and thrombin-protein C interactions [264].

During inflammation AT levels are reduced by similar mechanisms as for APC. However inflammatory cytokines also reduce synthesis of glycosaminoglycans within the glycocalyx - heparan sulphate being one of them, resulting in less effective natural inhibition of thrombin by AT [283,315].

In a small animal translational model of liver IR injury AT administration lowered plasma myeloperoxidase and leucocyte infiltration, with less organ damage and liberation of hepatic enzymes[316]. AT treatment improved the hepatic blood flow and capillary perfusion - which is disrupted during IR due to leucocyte-endothelial interactions, the generation of thrombin and fibrin(ogen) which potentiates inflammatory responses, and obstruction of microvessels through endothelial barrier failure and oedema. As well as inhibiting thrombin, and thus potentially reducing thrombin induced PAR signalling, AT induces prostacyclin release from endothelial cells [317]; a potent vasodilator and inhibitor of leucocyte migration and endothelial permeability. Intravital microscopy has shown AT treatment to reduce rolling and adherence to the endothelial during experimental mesenteric IR[318], while also limiting systemic coagulatory and inflammatory responses[301].

AT has also been used with success in transplantation models of IR injury. In experimental murine cardiac transplantation using an allogenic rejection model AT induced indefinite graft survival[319]; while in xenogenic rejection (which is extremely proinflammatory, prothrombotic and prone to hyperacute rejection) AT prolonged graft survival albeit in-conjunction with a complement receptor inhibitor[319]. Furthermore in a pig-to-primate xenogenic renal transplant model AT treatment increased graft survival and doubled time to graft failure[320]. Early results with AT treatment in combined human kidney-pancreas transplantation, and pancreas re-transplantation are promising with less renal and pancreatic IR injury, decreased rates of pancreas allograft thrombosis and pancreatitis, and better graft function [321][322]. Despite these early successes AT use in clinical trials for severe sepsis, as a modulator of systemic inflammation, proved less beneficial in terms of patient survival[323].

1.4.3.3 Fibrin and Fibrinogen

Fibrinogen is a 340kDa glycoprotein produced in the liver and is an acute phase protein. In normal physiology plasma concentrations range from 2-4 g/L. During inflammatory conditions this level can increase several fold [324]. It is composed of two sets of 3 polypeptide chains (Aa, Bb, and g). Coagulation occurs when thrombin acts on fibrinogen to form fibrin where thrombin cleaves at the N-terminals of Aa and Bb chains releasing fibrinopeptides A and B. This cleavage exposes

polymerization sites on the activated fibrin molecule. Fibrin monomers polymerize at these site by bind to complementary sites on other molecules forming larger 'protofibrils'. Thrombin activated FXIIIa stabilises the fibrin clot formation catalysing isopeptide bonds between g chains in adjacent molecules.

Fibrinolysis of fibrin clots is dependent on plasmin. Its zymogen plasminogen is activated by t-PA or uPA and the activated plasmin cleaves fibrin at various sites releasing X and Y fragments, D-Dimers, D and E fragments, Bβ15-42, and smaller a chain remanents [325–327].

Various experimental models have been used to investigate the role of fibrin(ogen) during inflammatory responses - including endotoxaemia, IR, and chronic inflammatory diseases. Fibrinogen and/or fibrin [fibrin(ogen)] were first demonstrated to have a pro-inflammatory potential in more than five decades ago. In-vivo and in-vitro studies showed fibrin(ogen) inducing leucocyte migration [328–330] and modulating inflammatory actions of both leucocytes, ECs, and platlets by increasing cytokine and chemokine responses [331].

In-vitro exposure to fibrin activates ECs with upregulation IL-8 mRNA transcription and cytokine release, a potent chemotactic and inducer of leucocyte activation and migration. Similarly exposing mononuclear cells to fibrinogen induces an inflammatory phenotype [332] with increased formation of ROS[333], as well as cytokine (TNFa, Il-1, IL-6) [334,335], chemokine (MCP-1, MIP-1 and 2), and matrix metalloproteinase-9 expression [332,336].

Inflammatory effects of fibrin(ogen) are mediated by a variety of receptors, but intra cellular signalling is propagated by key inflammatory kinases (MAPK, PKC) and transcription factors (NFκB)[332]. In-vitro TLR signalling (TLR-2 and 4) is associated with fibrin(ogen) induced cytokine [337] and chemokine expression [336] in murine models. This supports the concept of fibrin(ogen) as an acute phase protein acting as a DAMP mediating pro-inflammatory responses through innate immune signalling mechanisms.

Fibrin(ogen) binds the leucocyte CD11b/CD18 integrin receptor (also known as Mac-1 or complement receptor 3) [338,339] inducing and mediating ROS production, cytokine expression, and leucocyte migration. CD11b/CD18 was previously identified as a complement receptor further demonstrating the close interaction and complex interplay between coagulation proteases and mediators of inflammation. Fibrinogen binding to CD11b/CD18 has also been shown to be important in the pathogenesis of chronic inflammatory conditions such as skeletal muscle injury, multiple

sclerosis, rheumatoid arthritis , colitis, and colitis associated cancers[324], further highlighting the spectrum of involvement of coagulation proteins in inflammatory states.

Fibrin(ogen) also mediates ligand–receptor interactions with leukocytes via by acting on endothelial membrane receptors. Fibrin(ogen) binds ICAM-1 on endothelial cells, augmented by VE-cadherin, acting as a leucocyte bridge enhancing endothelial-leucocyte adhesion [340,341]. Through ICAM-1 fibrinogen also induces local vasoconstriction with increased production of endothelin-1[342], though this may be balanced by signalling through other membrane integrins [324]. Fibrin(ogen) signalling through both receptors affects endothelial barrier integrity and permeability by altering tight junction protein occludin and ZO-1 and 2 expression bound to cellular actin[343–345].

Most recently the VLDL receptor has been identified as a novel receptor for fibrin modulating trans-endothelial leucocyte migration[346].

Fibrinogen can also mediate platelet activation and aggregation by binding to aIIBb3 integrin on platlets while P-selectin the key endothelial-platelet CAM requires the presence of fibrinogen to maintain continued platelet expression [324].

Fibrinogen like protein 2 (fgl2) is a membrane bound protein that exhibits prothrombinase activity (directly converting prothrombin into thrombin), resulting in fibrin deposition, microvascular thrombosis and tissue inflammation. Fgl2 expression on endothelial cells has been shown to be upregulated by TNF-α in murine cardiac IR models, and mediates formation of fibrin rich microvascular thrombi, contributing to ischaemic tissue injury and the no-reflow phenomenon [347,348]. Thus fgl2 provides a further potential mechanism of microvascular dysfunction relating to coagulation activation in I/R conditions.

Despite evidence supporting fibrin(ogen)'s role in endothelial leucocyte adhesion and infiltration in-vivo studies have shown that leucocytes and platelets do not accumulate in fibrin clots [312,341]. This however alludes more that fibrin(ogen)ic inflammatory effects may not predominate during an overwhelming stimulus for coagulation, but during inflammation may have a key underappreciated non-haemostatic role to act and augment inflammatory responses. On the other hand an overwhelming inflammatory response, such as during IR injury, can activate the coagulation system, with the deposition of fibrin, and in the inflammatory milieu propagate obstruction of microvascular vessels.

Inhibition at specific points of the coagulation cascade has been shown to ameliorate IR injury a number of small animal models [237,299–307] , and this can be in part related to less activation of PAR receptors and potentially less generation of fibrin(ogen) and related degradation products which have inflammatory effects.

In murine crescentic glomerulonephritis direct thrombin inhibitors protected kidneys better against inflammation than did PAR-1 deficiency, and though thrombin inhibition also decreases PAR-4 activation these findings suggest involvement of fibrin(ogen) or its derived products[349]. Furthermore TF induced inflammation was abrogated by fibrinogen depletion with ancrod, with similar effects to use of a direct thrombin inhibitor[350]. Fibrinogen deficient murine models of myocardial and cerebral ischaemia demonstrated smaller sized infarcts and reduced mortality [351,352]. However only recently it was suggested an absolute lack of fibrinogen may be as detrimental as the increased levels seen during IR. A partial reduction of fibrinogen, seen in heterozygous mice models though can improve renal function and overall outcome in a murine model of renal IR injury [353].

1.4.3.4 Fibrin(ogen) Fragments in Experimental Models

During fibrin formation, and then fibrin clot breakdown by the action of plasmin various degradation products are produced - designated fibrinogen degradation products (FDP). Thrombin cleaves fibrinogen into fibrin releasing fibrinopeptides A and B. Conflicting evidence exists as to the inflammatory potential of these fragments. Determination of any isolated effect however is further complicated by co-existent thrombin activity. While both have been suggested to function as chemoattractants [354–356], recently it was shown that proinflammatory effects may differ between the two peptides, with B identified to be the primary inflammatory agent [327,357]. In complete contrast a different study determined both to have anti-inflammatory effects capable of reducing extravascular induced inflammation[358]. In both studies thrombin impact was abolished. The full role of these fibrinogen fragments remains controversial.

1.4.3.5 D-Dimers

After formation of activated fibrin monomers a stable clot forms by cross-linking between protofobrils. Through the action of plasmin fibrin clots are degraded releasing D-dimers and other FDPs. Studies have demonstrated a variety of pro-inflammatory properties for D-dimers. In-vitro exposure of human promomocytic leukemic cells, and peripheral monocytes to D-dimers induces expression of IL-1 (a and b), uPA, TF, PAI-2 [359]; and IL-1, IL6 and PAI respectively [360,361]. Some reports have suggested that the inflammatory potential for D-dimers on monocytic cells is minimal [362] or even anti-inflammatory [363].

Levels of D-dimers in plasma however are used as a marker of stabilized cross-linked fibrin formation, indicating the activation of coagulation and subsequent fibrinolysis. Determination of the D-dimers levels as a diagnostic parameter is useful in the detection of thromboembolic complications, where elevated levels are used in the diagnosis of deep vein thrombosis and pulmonary embolism, and higher levels of D-dimer are associated with increased rates of multi-organ dysfunction and patient mortality in severe sepsis and with disseminated intravascular coagulopathy [364,365]. Higher rates of D-dimers point to not only greater coagulation activity but also a co-existing inflammatory response mediated by the FDPs, fibrinogen, thrombin and other key proteases.

Various other fragments are generated during fibrin(ogen) cleavage and fibrinolysis - such as fibrin fragment E (FnE), fibrinogen fragment E (FgnE), Bβ15–42, and Bβ43–63.

FnE and FgnE have been shown to differentially influence angiogenesis (Fne promoting angiogenic and FgnE as an anti-angiogeneic), as well promote IL-6 release from macrophages through binding CD11c. FnE in particular is associated with N-terminal disulfide knot II (NDSKII) binding of VE-cadherin and this interaction is thought to be involved in mediating monocytic and neutrophilic migration[341]. Little in all is known on the full inflammatory potential of these fragments.

The Bβ43–63 peptide was only recently identified as a cleavage fragment of fibrin(ogen) but has been shown to exhibit anti-angiogenic and anti-tumour effects in-vitro.

Bβ15–42 is fragment of the N-terminal of the β chain generated during fibrinolysis by the cleavage of cross linked fibrin by plasmin, and whose sequence is independent from fibrinopeptide B. Its role in inflammation has been investigated by various groups worldwide. Early studies suggested the isolated Bβ15–42 fragment is a chemoattractant for neutrophils and fibroblasts [354,357], and

capable of inducing IL-8 expression in-vitro in a human oral squamous carcinoma cell line, determined using mAb against Bβ15–42 and a synthetic peptide sequence mimicking fibrin exposure [366]. More recently however Bβ15–42 has been demonstrated to be protective against inflammation especially in IR based injury.

Bβ15–42 protects against IR in-vivo in both murine and porcine myocardial ischaemia[367–369] and murine cardiac transplant models[370]. Bβ15–42 reduced infarct size and necrosis, NDSKII induced leucocyte recruitment competing for binding to VE-cadherin[367], as well as improved cardiac function[370]. A follow up phase IIa clinical trial investigating Bβ15–42 treatment for myocardial infarction at reperfusion reported smaller necrotic core zones on MRI early after cardiac events, but with similar longer term outcomes [371].

Functionally Bβ15–42 is also able to ameliorate single [372] and polymicrobial [373] endotoxaemia and lung transplant[374] induced endothelial barrier failure in murine models, by signalling through the Rho-kinase, inhibiting activation, and preserving VE-cadherin stability, maintaining endothelial integrity.

In a large animal porcine model of haemorrhagic shock Bβ15–42 protected animals against myocardial, pulmonary hepatic, and intestinal IR injury; with higher pulmonary and cardiac functional indices, better tissue perfusion and oxygenation, lower levels of cytokine (IL-6) production and neutrophil infiltration [375].

In mouse models of hepatic[376] and renal[377] IR injury Bβ15–42 treatment preserved histological integrity, reduced cytokine expression, maintained endothelial barrier function, and improved blood velocity and organ perfusion.

Bβ15–42 fragment's protective effects however require the presence of fibrinogen, with protection abrogated by fibrinogen depletion[367], suggesting that Bβ15–42 may be part of an endogenous feedback mechanism to regulate the pro-inflammatory effects of coagulatory molecules at both specific sites of inflammation and systemically. This study supports similar findings in renal IR that suggest complete fibrinogen depletion may be harmful[353]. Evidence proves Bβ15–42 is a promising therapeutic agent, however the complete mechanism of action, and in what clinical pathologies it would be most beneficial still requires investigation.

1.4.4 Exogenous Anti-coagulants in Ischaemia Reperfusion Injury

A number of exogenous anticoagulants have demonstrated promising results in the reduction of IR injury and inflammatory states. These include heparin and heparin like compounds, direct thrombin inhibitors (DTI), and FXa inhibitors (see Figure 11).

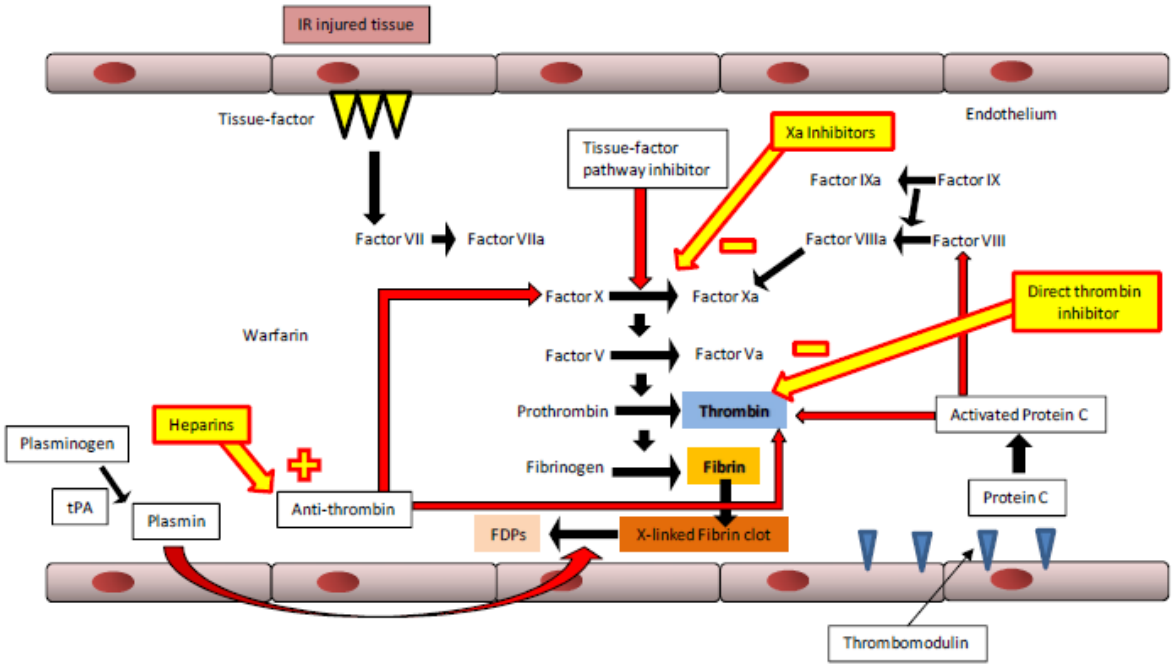


Figure 11 Overview of the coagulation network various endogenous inhibitors and exogenous inhibitors. Reprint of Figure 11 demonstrating the mechanism and location of action of heparins, DTIs and FXa inhibitors.

1.4.4.1 Heparin

Exogenous heparin is similar in structure and nature to endogenous heparan sulphate and is part of the same glycosaminoglycan family[378]. Heparin also termed unfractionated heparin (UFH) has a mean molecular weight of 15,000 Da with the structure determined by repeating sequences of uronic acid and amino sugar containing disaccharides. It is a highly sulphated polysaccharide composed of and D-glucose-amine and hexuronic acid residues bound by glycosidic linkages[265].

Similar to endogenous highly sulphated polysaccharides heparin binds AT via a unique pentasaccharide sequence[379]. Binding to AT induces a conformational change that increases thrombin inactivation by a factor of a 100 fold. The circulating thrombin complex is irreversibly composed of a ternary with AT bound to the thrombin active site while heparin (bound to AT) and

the exosite II[264]. Heparin clearance is predominately through binding of endothelial and macrophage heparin receptors with a smaller percentage cleared by the kidneys [265].

Low molecular weight heparin (LMWH) has a mean molecular weight of 5000 Da and is composed of less than 18 saccharide units in length, it is formed from by partial hydrolysis or enzymatic degradation of UFH[379]. Due to its smaller size then standard UFH LMWH is less able to bind both thrombin exosite II and AT (requiring a molecule > 18 saccharide units), and is thus a greater inhibitor for FXa[380]. Advantages over UFH are a longer half-life and predictable dose responses reducing the need for frequent monitoring.

Because it can be synthetically produced It is one of the most important anti-coagulants in clinical practice today and is used in prevention and treatment of arterial and venous thrombosis , including limb, pulmonary and associated with myocardial infarction [381]. In addition it is used in extracorporeal circuits and haemodialysis to prevent thrombosis.

Despite being used clinically for its anticoagulant properties evidence has suggested that heparin possesses anti-inflammatory properties. The close interaction between coagulation and inflammation, its effects on endogenous anti-coagulants that also exhibit anti-inflammatory properties logically support this. Various exogenous anticoagulants have been analysed in I/R injury models to reveal their protective properties.

In particular the use of heparin in myocardial IR has been extensively studied[382].

In a canine model heparin and an N-acetylheparin analogue administrated prior to reperfusion both reduced the size of infarcts and improved coronary endothelial vasodilatory function[383,384]. In isolated cardiac organ reperfusion experiment heparin and N-acetylheparin reduced the infarct size and improved myocardial function[385].

While low molecular weight heparin infusion in an isolated reperfusion rabbit heart model with damage mediated by complement (human plasma) reduced tissue injury and preserved discordant organ function[386], and in a related murine model reduced occurrence of arrhythmias.

Heparin and a non-anticoagulant heparin derivative (O-desulfated heparin) both equally preserved coronary endothelial vasodilatory function, reduced inflammatory cell infiltration, and reduced infarct size in a canine IR model[387]. The anti-inflammatory properties of both agents were attributed to inhibition of NFk-B transcription factor activation.

The anti-inflammatory potential of heparin have also been demonstrated in clinical settings [388–390]. Use of heparin-bonded circuits reduced incidence of myocardial infarction, with less requirements for ionotropic support, and less post-operative complications after cardiopulmonary bypass. While use of a higher dose of systemic heparin demonstrated a non-significant but observable trend of lower systemic cytokines (IL-6 and TNF-α) compared to standard doses.

The anti-coagulatory mechanisms of heparin are well established. There are several potential mechanisms for anti-inflammatory effects however that have been investigated.

Heparinoids (UFH, LWMH, and some derivatives) exhibit non-specific protein binding and more than 100 different heparin binding proteins (HBP) have been identified[391]. HBPs are diverse in origin and function and can be involved in coagulation and fibrinolysis, a variety of growth factors, involved in lipid metabolism, be extracellular matrix proteins, and in mediation of immune responses [392](including acute phase proteins[393] and complement[394]). Heparin binding of HBPs can modulate the structure and function of the HBP in question and thus heparin has a wide spectrum of potential effects based on the heterogeneity of HBPs.

Endogenous heparinoids such as heparan sulphate in the glycocalyx of endothelial cells bind cytokines and chemokines (e.g. INF-γ) stabilising them, protecting them from proteolytic degradation, and localising them to sites of inflammation within the vasculature, where in the proximity of their receptors binding is enhanced during inflammation[395]. Because heparinoids can bind inflammatory cytokines/chemokines , exogenously administered soluble heparin (UHF or LMWH) can attenuate inflammatory responses by binding these mediators, and neutralising them by preventing binding to their receptors.

Heparinoids can also interfere with endothelial-platelet-leucocyte interactions, and inhibit leucocyte infiltration in tissues. In vitro UFH and LMWH inhibited P- and L- selectin binding of their receptor ligands, as well as inhibiting thrombin and PAF induced leucocyte-endothelial P-selectin adhesion[396,397], by directly binding to P-selecting on activated endothelial cells[398]. UFH and LMWH can also bind CD11b/CD18 receptors on neutrophils, inhibiting the receptor binding with fibrinogen, complement or FX and limiting inflammation and neutrophil migration [399].

Once bound at the glycocalyx and cell membrane heparin can be internalised into the cytosolic compartment of endothelial cells[398], vascular smooth muscle cells [400], hepatocytes[401], and cardiac myocytes[402]. Based on its electro-chemical structure, heparin once within the cell can bind and interfere with NFk-B transcription factor translocation in activated endothelial cells,

lymphocytes, and monocytes, limiting inflammatory gene activation and transcription of pro-inflammatory mediators[387]. In a murine cardiac IR model heparin directly inhibited TNF-α expression, and by inhibiting P-selectin mediated leucocyte-endothelial adhesion limited inflammatory cell infiltration and further generation of inflammatory cytokines[403]. Finally in-vitro heparin modulated cytochrome c (a key component of the mitochondrial electron transfer chain), limiting its reactivity by 200 fold, potentially inhibiting cytochrome c activation of the caspase cascade and thus inflammatory apoptosis[404].

UFH has traditionally been used because of its relatively low cost, ability for simple monitoring and effective reversibility with the antidote protamine. Despite experiment evidence for potential benefit the use of heparin as an anti-inflammatory agent been largely limited by the its anti-coagulant effects and the risk of haemorrhage, as well as the persistent risk of heparin resistance and heparin-induced thrombocytopenia (HIT).

Heparin like molecules, and associated glycoaminoglycans, like pentosan poly sulphate have also been investigated in animal models with a limited degree of success to reduce reperfusion injury and expression of genes associated with IR injury[405,406]. These effects however have not been translated into clinical trials, though pentosan with limited anticoagulant ability may have benefit as risk of bleeding complications in post-operative patients would be less than with conventional heparins.

1.4.4.2 Direct Thrombin Inhibitors

The most widely studied and clinical exploited DTI is hirudin extracted from the parapharyngeal glands of the medicinal leech Hirudo medicinalis, and was the first anticoagulant used in humans in 1905. Hirudin is a polypeptide of 65 amino acids, stabilised by three disulphide bridges, with a molecular mass of 7000 Da and is one of the most potent and natural DTI[314]. In current medicine hirudins today are produced as recombinant proteins by genetically modified yeasts.

Effective thrombin inhibition by AT requires the catalytic effect of heparin bound to the AT-Thrombin complex. In contrast hirudin inhibits thrombin directly and independently of any other co-factors. Hirudin forms non-covalent and irreversible 1:1 complexes with thrombin, binding to exosites I and II [264]. Unlike heparin, hirudin inhibits both free and clot-bound thrombin molecules, as well as thrombin bound to fibrin(ogen) degradation products[407,408]. Heparin-AT complexes on

the other hand are relatively ineffective in accessing bound thrombin. Hirudin also exhibits no interaction with circulating plasma proteins, and so shows a more stable and predictable dose-response compared to heparin[409]. The lack of plasma protein binding also renders the potential for immunologic resistance and HIT like syndromes low[410]. Renal clearance accounts for 90% of the systemic clearance of hirudins, and t1/2 lengthens with renal dysfunction.

The recombinant hirudin lepirudin was the first DTI approved for clinical use in Europe (European Medical Evaluation Agency) in 1997, and the US (U.S Food and Drug Administration) in 1998 as a treatment of HIT that is complicated by co-existing thrombosis[410]. Since another recombinant hirudin, desirudin gained approval for use in thrombosis prophylaxis after major surgery. There are a number of recombinant hirudins and hirudin analogues in clinical and experimental use.

Hirudins such as lepirudin, bivalirudin (Hirulog) and desirudin have been used in various trials for acute coronary syndrome in myocardial infarction and subsequent angioplasty [411–414] and unstable angina [415]; for the prophylaxis and treatment of DVT in high risk patients[416,417]; and in lower extremity bypass [418]. Hirudins have also been assessed for anticoagulation in scenarios involving artificial surfaces, such as haemodialysis circuits[419] and complicated high risk cardiac bypass surgery[420,421], and extracorporeal membrane oxygenation[422].

Because DTIs are able to mitigate thrombin in nonbound and clot-bound forms hirudins have been found to be promising in various animal IR models (e.g. lung, heart or brain) [423–425] by reducing the proinflammatory effects of thrombin [426–428].

Bivalirudin attenuated the inflammatory effects of thrombin in a carrageenan-induced rat paw oedema model [429]. Since bivalirudin binds to both the catalytic site and the anion-binding exosites (I and II) of thrombin [430,431], it blocks thrombin effects mediated both by PAR cleavage and activation as well as by the non-catalytic activities of the mitogenic and chemotactic peptide domains[196,432].

By binding to the thrombin active and preventing PAR-1 from cleavage [426][332], hirudins thereby diminished thrombin signalling [433] of CAM expression[434], and secretion of cytokines and chemokines; VEGF[435], IL-6 [436], IL-8 and MCP-1 [437].

In in-vitro endotoxaemia models hirudin analogs (lepirudin) effectively ameliorated endotoxin induced platelet activation [310,438,439]. While melagatran a hirudin analogue and a thrombin active site inhibitor diminished platelet P- selectin expression [426,440].

1.4.4.3 Direct FXa Inhibition

FXa can induce expression of pro-inflammatory chemokines and cytokines (TNF-α, IL-6, IL-8 and MCP-1) as well as CAM (P-selectin) through PAR-1 and PAR-2 signalling [200,441]. Several known endogenous and exogenous synthetic anti-coagulants can inhibit FXa, such as LMWH or APC. Direct FXa inhibitors, e.g. rivaroxban, have been investigated in treatment of acute coronary syndrome, with experimental data suggesting a non-anticoagulant anti-inflammatory profile, with rivaroxban associated with a significant increase in endothelial TM. In-vitro rivaroxban down regulated expression of IL-6, TNF-α and MCP-1 in thrombin activated endothelial cells, to the same extent as select PAR-1 inhibition[428].

The upstream position of FX in the coagulation cascade means that FXa inhibition prevents thrombin generation, but could also allow functions of existing thrombin to continue, such as activating protein C, thus maintaining an endogenous anti-coagulant and anti-inflammatory mechanism active. Combined with recent preliminary data supporting FXa inhibition as both potentially inflammation modulating as well anti-coagulant has led to suggestions that direct FXa inhibitors may be superior than DTIs[313]. However FXa inhibition in context of alternative inflammatory conditions (such as endotoxaemia, or transplantation IR injury) and interactions with other pathological processes need to elucidated before any further translational use is feasible.

1.5 The Microcirculation in Organ Transplantation

In transplantation the endothelium is the first site where inflammatory cells in the recipients' oxygen rich warm blood encounter and interact with the donor tissue - and is thus the site of the first challenge with the initiation of IR related cascade of inflammatory and coagulatory events. It is now recognised that microvascular dysfunction is an early and rate-determining effect of the IR injury to endothelial cells and then their interaction with and promotion of the inflammatory responses. In the immediate peri-transplantation setting the endothelium is where the post-operative function of the allograft is determined.

1.5.1 Physiology of the Endothelium

The endothelium is composed of a single layer of cells that line the entire vascular system. Approximately 10 trillion (10-13) endothelial cells form a 0.2 um think haemo-compatible barrier with a surface area estimated to be between 3000-6000m2[442], forming an almost 1 kg organ[443]. They are flat shaped cells juxtaposed in a mosaic fashion so their long axis is orientated to the direction of blood flow. They are attached to the basal membrane which is high in content of collagen and glycoproteins and thus acts as a barrier between blood and the extra-cellular matrix.

The endothelial itself is not a passive component of the vasculature, it is dynamic and able to respond to stimuli and stress emanating from the circulation. Microscopically cultured ECs have a cobblestone appearance, but if exposed to shear stress in a perfusion chamber similar to that in the circulation cells re-orient and align themselves along the axis of stress [444,445] and this is seen in-vivo in cardiopulmonary bypass patients where endothelial of saphenous grafts elongate and re-organise themselves to the new direction of the arterial flow [446,447]. The actual concept of the endothelium as an organ stems from these observations and recognition of the various consequences and diseases that are secondary to endothelial injury and dysfunction - including diabetes, atherosclerosis, HTN, sepsis and inflammatory syndromes.

Endothelial cells are the gatekeeper and primary interface between blood and tissues, and in addition to a general role relevant to controlling extravasation of fluid, solutes, hormones, macromolecules, haemostatic and inflammatory cells, have a specific role in the maintenance of blood fluidity by regulating thrombosis, thrombolysis, platelet adherence, vascular tone and blood flow[448]. They are dynamic with both metabolic and synthetic functions, exerting autocrine, paracrine, and endocrine influence on smooth muscle, platelets, and peripheral inflammatory cells.

Endothelial cells are phenotypically variant in different vascular trees relating to the specific tissue and organ and related to the perfusion dynamics and shear stresses that predominate in and differentiate between the larger arteries and veins, and micro-circulatory arterioles, venules and capillaries. Arterioles are the thinner, smaller branches of arteries, and are the principal controllers of resistance in the microcirculation. Venules act as larger lower pressure reservoirs through which the circulation is directed back to the heart. In conjunction with smooth musde cells the endothelium controls microcirculatory perfusion through local vasoregulation mediated by direct action and through the response to locally generated and systemic vasoactive agents - such as NO, endothelin, prostacyclin, and adrenaline.

Capillary beds lie between arterioles and venules and are the smallest of vessels. With luminal walls consisting of only a single layer of endothelial cells, thus shortening the distance for diffusion between blood and tissue they are the site of exchange of molecules between the circulation and tissue - making an effective capillary circulation a necessary prerequisite of tissue perfusion and organ function[315]. In fact the majority of endothelial cells - 600g of the 1kg organ - are micro-vascular capillary endothelial cells[443] - where the exchange of nutrients, hormones, and immune cells between tissues and the circulation is dominant.

1.5.2 The Endothelium in Ischaemia Reperfusion Injury

1.5.2.1 Endothelial Injury

Endothelial dysfunction is a pathogenic early consequence of IR injury. ECs are the first cells of the allograft to experience the effects of reperfusion with warmed oxygenated blood after ischaemia. The dysfunction seen is caused by the multiple pathological sequelae started during ischaemia and then accentuated during reperfusion - specifically: ROS mediated damage, ion homeostasis and osmotic stress leading to cellular swelling, and membrane dysregulation and activation, production of pro-inflammatory mediators, and apoptosis [449].

Prolonged hypoxia has deleterious effects on cellular metabolism as described in Section 1.32. These cellular changes occur in endothelial cells and at the same time to adjacent renal interstitial tubular causing both cell types to enter an activated state. There is an upregulated Secretion of pro-inflammatory cytokines is induced and up-regulated by induction of pro-inflammatory transcription factors (such as NFk-B), while there is an increased expression of endothelial cell adhesion molecules. This promotes recruitment, activation and infiltration into the renal parenchyma of

recipient immune cells (macrophages, lymphocytes, neutropils). This inflammatory infiltrate serves to complement the local inflammatory processes by allied secretion of cytokines, destruction of apoptotic cells, and further recruitment of inflammatory cells. In parallel activation of the complement system and generation of ROS by tubule, endothelial and recruited inflammatory cells promotes the sustained activation of these inflammatory cascades.

IR related endothelial injury is associated with a multitude of features: altered cell membrane potentials, changes in cell membrane fluidity, disturbances of ionic intra- and intercellular flux, cellular swelling, altered cytoskeletal organization, concerted loss of energy stores, diminished production of important bioactive constitutive anti-inflammatory species (NO, prostacyclin), and production of inducible pro-inflammatory agents (endothelin, thromboxane A2) [449,450]. During ischaemia inflammatory genes for CAMs and cytokines are induced, while genes for cNOS and TM are inhibited[451]. These plethora of cellular responses to the initial ischaemic injury are then amplified during reperfusion.

Morphologically cells are swollen, with loss of pinocytitic vesicles, detachment from the basement membrane (BM) with adherence of activated leucocytes to endothelial surfaces [449]. The reperfusion phase is characterised by specific micro vascular events. Lymphocytes, macrophages, and granulocytes migrate to the allograft and through increased expression of adhesion molecules – in particular ICAM-1, VCAM-1, P-selectin and various other integrins– producing an infiltrate that results in dissolution of the endothelial cell lining, worsening interstitial oedema, and causing deeper parenchymal injury from ROS and infiltrating cells [91].

There is an increase in cytokine and chemokine production – locally form ECs and from the infiltrating macrophages and leukocytes. Complement, leukotrienes, platelet activating factors, thromboxane A2, TNF-A, IL-1, and endothelins are central to potentiating the inflammatory infiltrate by up-regulating endothelial cell and leukocyte adhesion molecules[220,452] .

In the initial stages of reperfusion hyperaemia prevails, this gives way to a progressive and longer phase of diminishing flow, characterised by aggregation of leukocytes and platelets and activation of coagulation cascades – the no reflow phenomenon[453]. There are two distinct types of no-reflow: “structural no-reflow", characterized by an irreversible damage to endothelial and supporting cells; and where there is a disturbance of the microcirculation caused by vasoconstriction and micro-thrombi and -emboli - “functional no-reflow"[315].

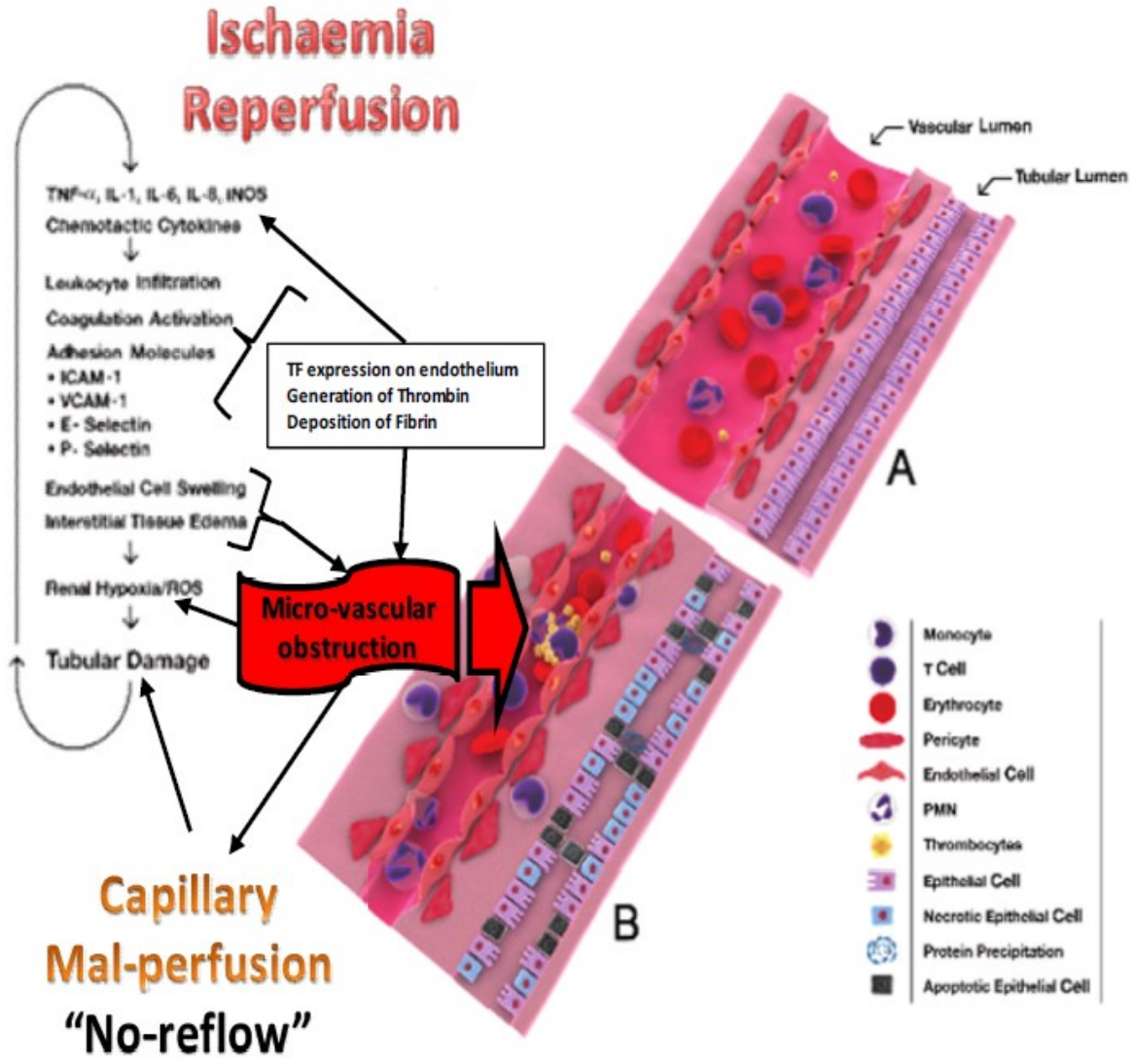


Figure 12 Microvascular injury and tubular damage before (A) and after (B) renal IR injury. Highlighting interface between coagulation, inflammation, and development of microvascular dysfunction with microcirculatory failure and 'no reflow'. These processes are amplified by continued activation of coagulation cascades and generation of thrombin and fibrin, which potentiate inflammatory responses, further restricting perfusion, with ongoing relative hypoxia and ROS generation. The final clinical entity encountered is delayed organ function. Adapted from Legrand et al. [454].

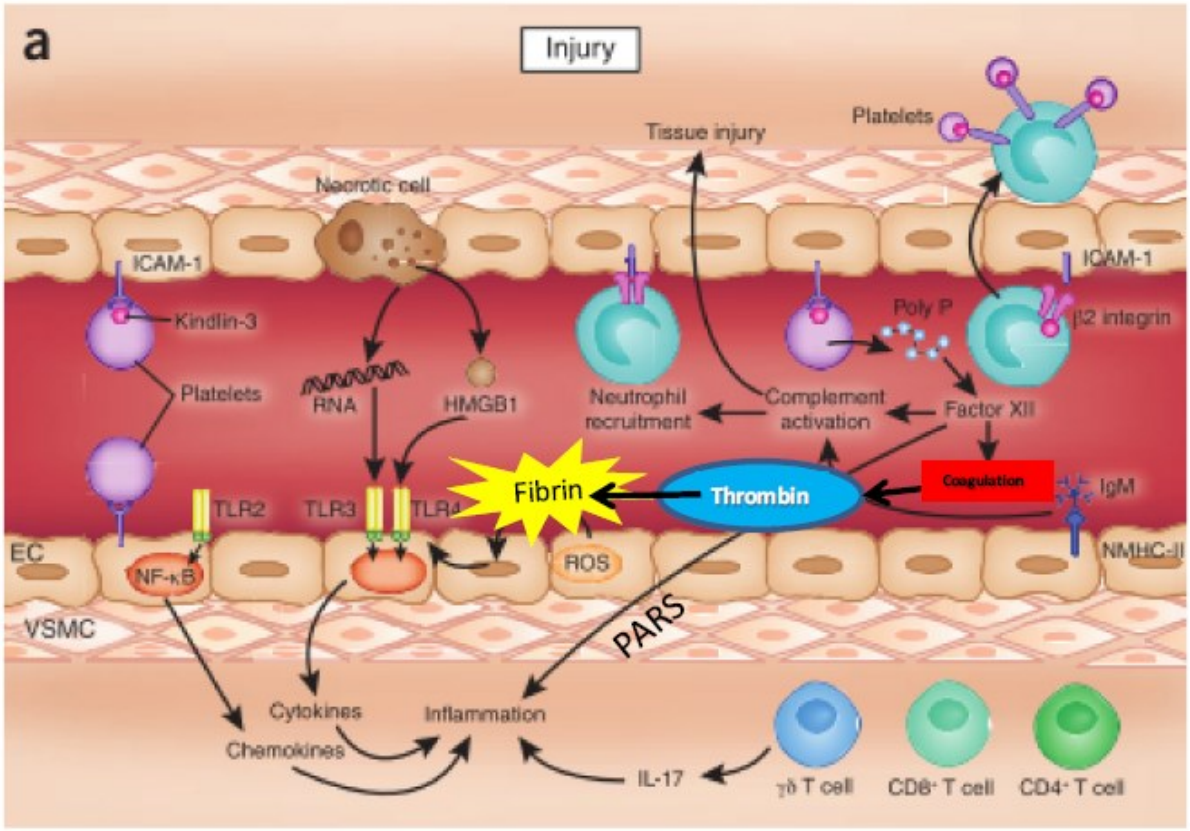


Figure 13 Overview of cellular and inflammatory cascade involvement in IR injury. (a) IR injury results in activation of the innate immune system, with hypoxia during the ischemia precipitating TLR-dependent stabilization of transcription factor NF-kB. NF-kB activation stimulates expression of pro-inflammatory genes for inflammatory mediators and cell adhesion molecules. TLR-4 expression is increased by ROS generated from interstitial as well as endothelial cells, and is activated by endogenous ligands and damage associated molecular pattern molecules such as HMGB1. TLR-3 is similarly activated. During reperfusion, increased cell adhesion molecule expression and platelet-leucocyte-endothelial interactions facilitates an inflammatory infiltration into the tissue. Platelets further activate coagulation factors leading to generation of thrombin and subsequent fibrin formation contributing to the development of not just a proinflammatory and but also procoagulant micro-environment within the vascular bed of organ. Thrombin further potentiates pro-inflammatory responses via PAR receptors. Diagram adapted from Eltzschig et al. [455].

1.5.2.2 Endothelial Activation

An important feature of endothelial cells is that they can enter an 'activated' state that results in the transcription of new genes and affording cells a new range of cell membrane receptors and paracrine abilities. Activation allows the endothelium to modulate local inflammatory cascades, interact with and direct action of peripheral leucocytes, and shift the local interstitial and endovascular environment into a pro-coagulant state. Stimulus to activation ranges from local trauma to vessels and endothelium directly, inflammatory cytokines (TNF-α, INF-γ) and triggers of the innate and acquired immune responses, stressful metabolic disturbances such as hypoxia and ROS. In particular hypoxia induced molecular changes (via NFk-B pathways) in endothelial cells include up regulation of pro-inflammatory genes, and signally pathways that potentiate the IR inflammatory response[456]. Following activation endothelial cells assume an inflammatory phenotype that is characterised by accelerated ROS production, further release of cytokines (e.g. IL-8) and allied inflammatory mediators (platelet activating factor [PAF-1]), increase expression of CAMs binding leucocytes and platelets, as well as promoting complement activation and inactivation of protective eNOS and NO[457,458] .

1.5.2.3 Leucocyte-Platelet-Endothelial Interactions During Ischaemia Reperfusion

Leukocyte infiltration into post-ischemic tissue involves the well-established process of margination, rolling, adherence and migration of leukocytes through the endothelial barrier.

Leucocyte-endothelial interactions are mediated by increased CAM expression - predominately P- and L-selectin [459] (which are rapidly increased soon after the start of IR injury), ICAM [460], and E-selectin [461]. P-selectin is responsible for the initial tethering of polymorphonuclear cells to endothelial walls [462], while L-selectin expressed on leucocytes mediates rolling [463]. ICAM-1 and 2 on endothelial cells binding to leucocyte function associated antigen-1 (or CD11/18) secures leucocyte endothelial attachment and activates the inflammatory cells before migration [464].

ROS generation via XO in both endothelial cells and activated leucocytes perpetuates the inflammatory process by stimulating the expression of further CAM[465] (via transcription factor activation such as NFk-B), continued production of cytokines and chemokines [466], and increased venular permeability. In addition diminished expression of cNOS and production of NO, has been implicated facilitating these inflammatory processes [467,468] with experimental evidence to support this through NO inhibitor and NO donor administration in animal models reproducing IR microvasculature features, and reducing IR damage respectively [469,470]. With the adherence and

activation of leucocytes on endothelial membranes further cytokines are generated including platelet activating factor 1 (PAF-1), and there is an increased generation of ROS in particular by XO[471] and NADPH oxidase [458].

The recruitment of leucocytes into the post ischaemic microcirculation is accompanied by the activation and accumulation of platelets, in particularly the venular microcirculation. In the kidney [472] (as well as the brain [473] and liver [474]) platelets can be seen to accumulate early by 30-45mins after reperfusion. The temporal relationship between leucocytes and platelets adherence in IR has lead to proposals of an interdependence for recruitment in inflammatory tissues. During IR injury 25% of platelets adhere directly to the endothelium, with the remaining binding to leucocytes, in particular neutrophils attached to the endothelium [475].

1.5.2.4 Endothelial Permeability

During renal ischaemic injury increases in peri-tubular capillary permeability have been documented [476]by two distinct mechanisms; increased 1) para-cellular or 2) transcellular movement of molecules and fluid. While paracellular transport has been the widely described the role of transcellular transport during IR injury has only recently been thought to be an important element.

Cellular junctions evident in endothelial cells are similar in nature to tubular epithelial cells but are less spatially and structurally organised. Cadherin adheren junctions are present between endothelial cells throughout the vasculature, while tight-junctions are can be prominent such as in the blood brain barrier or sparse and simple enabling a more leaky barrier interface such as in venules or capillaries of the renal vascular [448]. The disparity in cell-junctions in endothelial cells in different vascular beds, and from other barrier formations (e.g. tubular epithelial cells) suggests an important functional role in mediating permeability [477,478] .

Unfortunately the majority of evidence for endothelial-endothelial cell interactions during IR injury has come from in-vitro models. It is important to realise that in-vitro responses of cultured immortalised endothelial cell lines may not reflect those actually present in-vivo, in particularly as there is altered expression of key cell markers compared to cells studied in-vivo [479].

During and after ischaemia ATP depletion and free radical ROS damage are suggested to be the primary causes of increased paracellular permeability. Studies in mouse models have attributed increased permeability to re-distribution of occludin proteins from the ZO-1 antigen in tight junctions, along with internalization of vascular endothelium cadherin [480]. In parallel lack of ATP

and ROS can alter normal actin cytoskeletal structure in endothelial cells in particular F-actin, with surface breakdown, aggregation and dispersal of F-actin within the cytoplasm [481]. This break down renders the already leaky 'barrier' less resistant to fluid and macromolecular shifts from the circulation. Finally ROS can also increase basal actin stress fibres in endothelial cells and trigger endothelial contraction increasing paracellular permeability, which is thought to be mediated by a complex interaction of Rho family GTPases, tyrosine kinases, PKC, and cAMP dependent protein kinases[482] suggesting numerous other stimuli to these changes still to be deciphered.

1.5.2.5 Contribution of Leucocytes and Platelets to IR Endothelial Injury

The contribution of ROS to increases in endothelial barrier permeability are important. However the source of these ROS must be distinguished. The barrier dysfunction caused by IR is the effect of both ischaemic damage to endothelial cells as well as damage during reperfusion with the influx of oxygen, inflammatory cells, and production of cytokines. The major sources of ROS (superoxide and hydrogen peroxide) during reperfusion is activated leucocytes, followed by endothelial and interstitial renal cells via NADPH oxidase, uncoupled eNOS, and XO.

Despite the acceptance that leucocyte adherence and accumulation is linked to increased in endothelial barrier dysfunction during IR recent studies have pointed to complex interactions between leucocytes, platelets, endothelial derived cytokines, and ROS at various spatial and temporal points during reperfusion to be responsible as opposed to a simple single event of leucocyte/endothelial interaction and adherence [483].

None the less neutrophil accumulation has been shown to be a direct cause of IR induced barrier failure rather than a consequence. A strong correlation exists between the degree of IR induced permeability and the extent of the neutrophil adherence and infiltration through the venular endothelium into tissues[484] [470], with experimental neutropaenia[485] and immune blockage of CAM's[486] (P-selectin, ICAM-1, CD11/CD18) diminishing barrier failure during IR.

Despite the interdependence between platelet and neutrophil endothelial adherence the exact role of platelets in mediating endothelial barrier permeability is still not defined, with studies describing a range of involvement from no effect of platelets in inducing barrier failure[487] to even a potentially protective effect[488].

1.5.2.6 Histological Findings of Renal Specific Vascular and Tubular Damage During Ischaemia Reperfusion Injury

Despite the biochemical and cellular effects of IR on endothelial cells, the histopathological changes seen in IR injury (in human and animal models) and more specifically renal IR are subtle and limited to the arteriolar smooth muscle [489] [490]. Definitive evidence of endothelium injury and changes are not routinely seen in renal biopsies in transplant recipients, and when present abnormalities are mild [40,449,491,492]. There are potential sampling biases in human kidneys (cortex sampled vs. medulla) and difficulties in detecting endothelia injury in animal models that may explain this phenomena.

Evidence of ischaemic endothelial failure in other vascular beds however demonstrates that renal endothelial injury has a plausible and potentially important role in renal dysfunction. Coronary and cerebral IR reveals separation of endothelial tight junctions, endothelial cell detachment from the basement membrane and blebbing, and even endothelial necrosis [493,494] .

In contrast examining IR renal tubular injury studies have definitively found higher levels of brush border injury, tubular necrosis, cell shedding, tubular dilation, and interstitial inflammation in kidneys with delayed graft function compared to immediate functioning allografts [40,492,495] , while other groups have found no distinct differences in these kidneys [496,497] .

Considering the evidence the histological findings of both tubular and even endothelial injury do not correlate with the degree of dysfunction seen in kidneys with delayed graft function. Thus the effects of IR may not be easily detectable on histology as they are functional rather than persistent morphological effects, especially early in the post operative period.

1.5.3 Cardiovascular Risk Factors for Endothelial Dysfunction

Cardiovascular disease risk factors (HTN, hypercholesterolaemia [HChole] & obesity, and diabetes) are an interrelated group of diseases whose pathogeneses are manifested in the systemic vasculature, with the major cellular target the endothelium. These diseases are known to negatively influence outcomes in affected transplant recipients. The emergence of the expanded donor criteria defining a potentially viable, but with higher risk of graft dysfunction, subset of the donor pool has meant the impact of these co-morbidities amongst donors is gaining precedence. Separately and in-concert they affect oxidative stress and ROS production, impaired vasomotor function, leucocyte-endothelial interaction, endothelial barrier dysfunction, as well as the recruitment of platelets and potentiate a pro-thrombotic environment[498], see Figure 14.

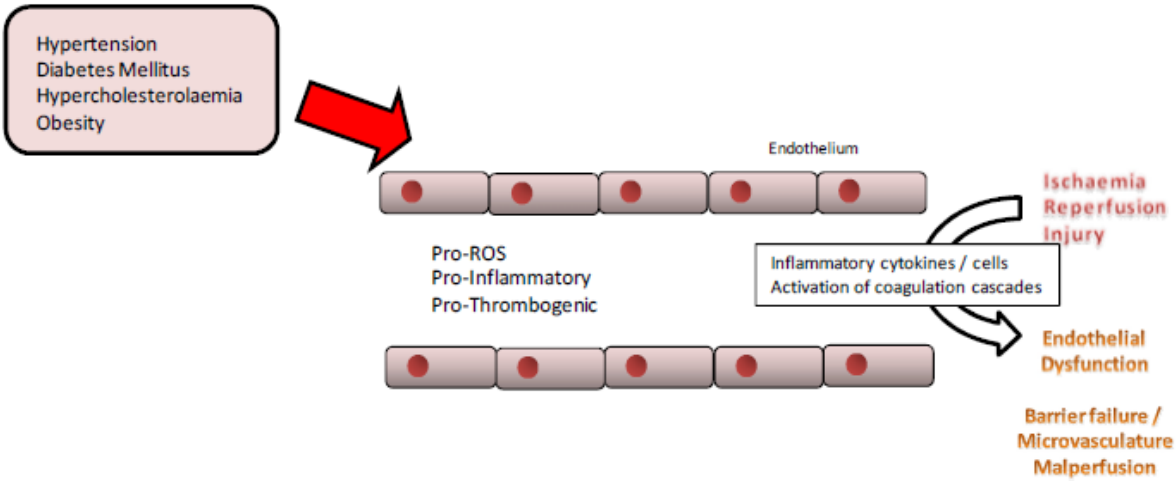


Figure 14 The role cardiovascular disease risk factors in IR induced endothelial injury and barrier failure. Hypertension, diabetes mellitus, hypercholesterolaemia, and obesity all induce a pro-oxidative, pro-inflammatory, and pro-thrombotic environment. These effects are exacerbated by interactions of inflammatory cells and coagulation proteases with the endothelia, resulting in barrier failure and poor perfusion during IR injury [499].

1.5.3.1 Hypertension

Animal models of HTN (such as spontaneously HTN rats, chronic angiotensin II infusion, and salt retaining models) demonstrate an increase in ROS production and oxidative stress. Endothelial and leucocyte NADPH oxidase is implicated in the production of superoxide in HTN enhanced ROS. Despite HTN affecting only the arterial system in terms of pressure, increased ROS is seen in both arterioles and venules. Superoxide as an inhibitor of dilating NO, or a promoter of endothelin production, as well as links to activation of angiotensin II receptors, and TNF-α from leucocytes are potential patho-mechanisms [500–503].

The majority of complications associated with HTN are related to endothelial activation or vasomotor dysfunction. Vasomotor dysfunction has been primarily attributed to reduced NO bioactivity; with endothelin and angiotensin II receptor activation (possibly from increased ROS) and angiotensin 1 receptor mediated T-cell activation with release of TNF-α (which induces a pro-oxidant and inflammatory state) as suggested causes. [501,504]. Endothelin alone stimulates cell proliferation, upregulating gene expression of collagenases, prostaglandin endperoxidase synthase and PDGF, and these is cross talk between endothelin, NO and prostacyclin in the control of vasomotor processes [505]. These same mechanisms are also associated with increases in endothelial permeability, through angiotensin II receptor activation, ROS generation, and activation of stress transcription factors (NFk-B and hypoxia inducible factor 1) and production of vascular endothelial growth factor and prostaglandins [506–508]

HTN also enhances leucocyte endothelial cell adhesion - via increased expression of CAMs. Angiotensin 1 receptor activation with ROS formation via NFk-B and induction of VCAM and P-selectin are key to these observations [509–511] .

The combined effects of HTN mediated cellular changes on endothelial integrity and function means that HTN itself is associated with degenerative and rarefactive remodelling with a loss of up to 50% of the micro-vasculature in some tissue beds [498].

Finally HTN in patients is associated with abnormalities in clotting factors, platelet activation, and fibrinolysis posing an increased risk of thrombosis. This may be related to endothelial production of platelet stimulants (endothelin) as well as reduced generation of inhibitors (prostacyclin, NO), with implications of an angiotensin II mediated pathway. [512–514]

1.5.3.2 Hypercholesterolaemia

HChole is also associated with accelerated ROS generation [500] - the primary source being NADPH oxidase and xanthine oxidase from endothelial and leucocyte cells, mediated by T-cell derived INF-γ. [515]. HChole induced ROS is further implicated in dysfunctional endothelium dependent vasodilatation (NO inactivation) [515–517], and barrier function [518,519] (through endothelin VEGF and ROS). In both cases lipid lower statins normalises endothelium permeability and vasodilatation [516,518] though the effects may be mediated by anti-inflammatory properties rather than lipid lowering actions (such as NFk-B inhibition)[152]. HChole is also associated with an impaired angiogenic response to VEGF and related growth mediators - thought to be partly mediated through ROS inactivation of NO, and increased expression of endostatin [520,521]. Finally HChole and oxidised LDL appears to enhance platelet responsiveness and thrombosis, and promote leucocyte/endothelial adhesion. There are 2 proposed mechanisms: T-cell derived INF-γ and ROS upregulate CAM expression (CD40/40L) for leucocytes, with adherent leucocytes forming leucocyte-platelet (P-selectin CAM expression) and leucocyte-platelet endothelial aggregates that can activate coagulation cascades via tissue factor induction on the endothelium; and reduced NO bioavailability (possibly through inactivation by ROS or oxidised LDL) [517,522,523]. Obesity with a high body mass index (BMI) is usually associated with HTN, HChole and diabetes. The effect of a large quantity of adipose tissue on endothelial function is likely mediated by circulating adipose derived hormones (adipokine, adiponectin, leptin). Obesity is associated with increased sensitivity to activation of endothelial cells from further stimuli, as well as platelet hyper-reactivity, and increased thrombogenesis [524,525] .

1.5.3.3 Diabetes

Hyperglycaemia is a major stimulus for ROS generation, with impairment of intrinsic anti-oxidant defences. ROS formation is primarily mitochondrial and enhanced through glucose and NADPH oxidase, which are linked to AGEs and PKC C activation [526]. NADPH oxidase and angiotensin I receptor activation is upregulated [527]. Oxidative stress in diabetes is implicated in impaired endothelial vasomotor function via several mechanisms including: endothelin, AGEs, leukotriene and prostaglandin synthesis, and NO inactivation [528].

Together hyperglycaemia and diabetes induced ROS have been linked to increases in leucocyte/endothelial adhesion and endothelial permeability - clinically evident as retinopathy and nephropathy of diabetes. P-selectin and receptors for AGE (RAGE) on endothelial cells bind leucocyte B2 integrins[529]. Reduction of circulating glucose (insulin), anti-oxidant scavengers or a direct PKC

inhibitor have all been shown to blunt these hyperglycaemia induced responses. [530,531]. Leucocyte interactions with the endothelial, the generation of inflammatory cytokines and growth factors (VEGF), the excess ROS and hyperglycaemia evident in diabetes, and signalling pathways involving peroxisome proliferator-activated receptor g [PPARg] and PKC are all implicated in the hyperpermeability seen in diabetics [532,533]. These same process are also responsible for the anti-angiogenic effects of diabetes in retinopathy[520] and may be active in wound healing and repair in other tissues.

Finally diabetes is associated with a hypercoaguable and pro-thrombotic state[534]. Systemically this is seen by elevated levels of clotting factors (VII, VIII, XII) [535], increased numbers of activated circulating platelets, and a down regulation of intrinsic fibrinolytic processes [536]. There is an increased expression of P-selectin, with enhanced thromboxane production[537]. In parallel AGE activation of endothelial and monocytic tissue factor increases the production of thrombin [538], the key molecule needed for the formation of fibrin .

1.5.3.4 Effect of Ischaemia Reperfusion on At Risk Endothelial in Transplantation

Cardiovascular risk factors render tissue more vulnerable and more at risk in IR injury. These risk factors induce a low grade inflammatory response - both systemically and in specific vascular beds - which induces endothelial cells into an activated state and more sensitive to secondary stresses. In both cases CV risk factors and IR injury promote a pro-oxidative, pro-inflammatory, and pro-thrombotic phenotype - which manifests as more deleterious injury and organ dysfunction (see Figure 15). In transplantation this is further complicated by the potential immune response provoked by the transplant itself, and trauma during surgery. These CV factors have been likened to a 'double edged sword' where by an organ from an ECD donor is initially compromised, and then more liable to yield more damage during IR and transplantation, with organ dysfunction. This is somewhat confirmed in renal transplants with glomerular, tubular, parenchymal and vascular abnormalities seen on pre-implantation biopsies predictive for delayed graft function and graft failure[40].

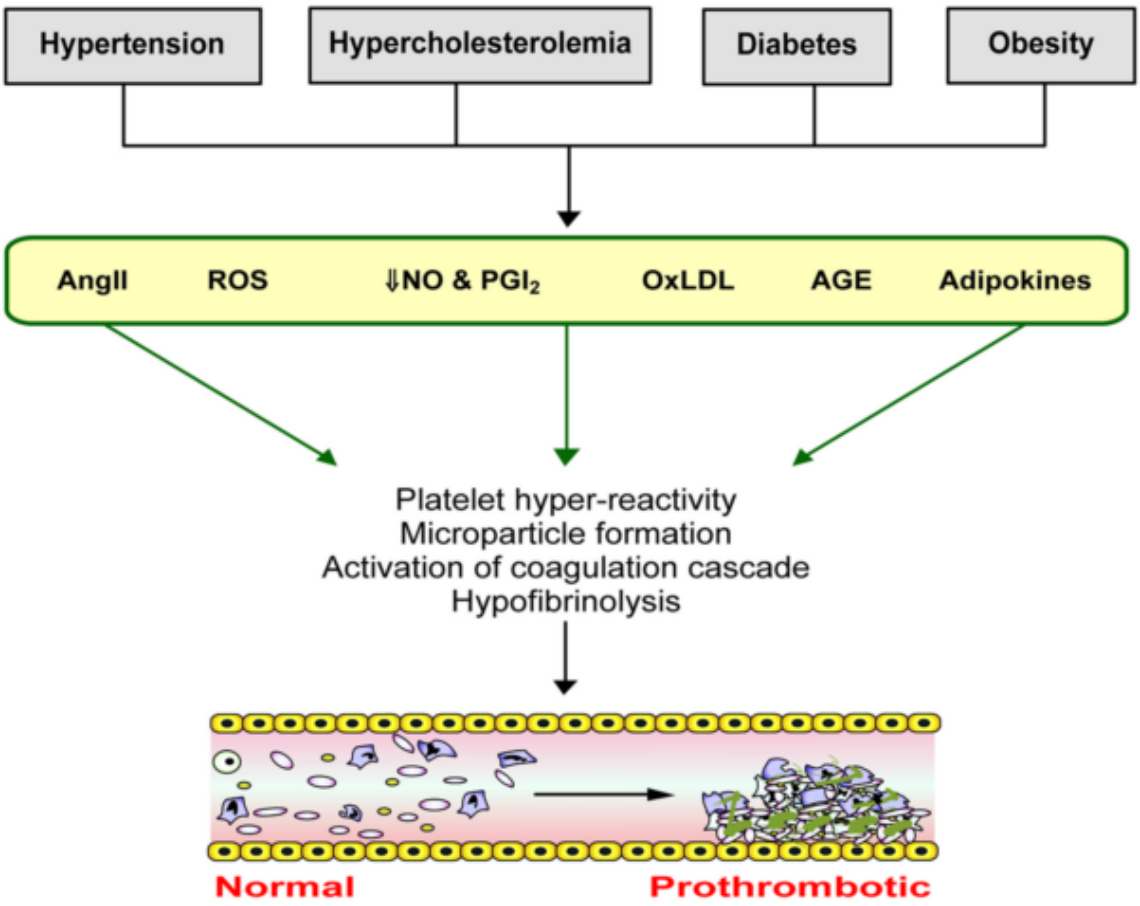


Figure 15 Cardiovascular disease risk factors induce a prothrombotic phenotype in the vasculature. During IR injury this already 'inflamed' at risk endothelium predisposes to a more potentiated inflammatory response. AngII = angiotensin II, oxLDL = oxidized low density lipoprotein, AGE = advanced glycation end-products, ROS = reactive oxygen species, NO = nitric oxide, PGI2 = prostacyclin. Adapted from Granger et al. [498].

1.5.4 Role of Tissue Perfusion in IR Injury and Transplantation

1.5.4.1 Physiology of Renal Microcirculation

The primary subunit of the kidney is the nephron whose function is to facilitate filtration and resorption of solutes and metabolites from the circulation, with the formation of concentration urine for excretion[539]. Each human kidney has approximately 1.3 million nephrons. The kidneys accounts for 2% of total body weight but receive 25% of total cardiac output, this high blood flow is needed for effective filtration[540].

A single nephron is composed of a glomerulus where filtration occurs through the bowman's capsule (fed by an afferent and drained by an efferent arteriole), the proximal and distal tubules (primary resorption of solutes), the loop of henle (resorption of water), and the collecting duct. Resorption of solutes and water from the filtrate into peri-tubular capillaries and vasa recta is achieved using active and passive transport. The components of nephrons are spread across the kidney parenchyma, with the cortex consisting primarily of glomeruli, tubules and proximal aspects of the collecting duct; and the medulla S3 segments of the proximal tubule, look of Henle and distal collecting duct. The medulla has a high metabolic activity from reabsorption of sodium by the think ascending limb of the loop of Henle. Due to the requirements of a strict osmotic gradient to facilitate water reabsorption blood flow is significantly lower in the medulla compared to the cortical regions, with the medulla receiving only 10% of renal blood flow [541]. This regional difference will play a major role in ischaemic insults and recovery of kidney function in clinical practice.

1.5.4.2 Alterations of Tissue Perfusion

Decreased renal blood flow is of critical importance in initiating and then potentiating ischaemic kidney injury and ARF. After acute ischaemic kidney injury a substantially reduced total blood flow of between 40-50% is evident upon reperfusion in both animal and human models, with regional deficits in tissue flow likely playing an important role in extending ischaemic injury into the reperfusion phase [542–545]. Following ischaemic injury the reduced blood flow significantly contributes to a deteriorating GFR. Animal models of ARF have pointed to persistent perfusion deficits in the outer medulla, rather than the cortex or inner medulla, as the prominent regions affected [546–548]. As oxygen tension under normal physiological conditions decreases between the

outer cortex to the inner medulla[549], combined with the high regional metabolic activity the impact of reduced blood flow to this cortico-medullary region is potentially magnified.

In the majority of animal ARF models, the S3 proximal tubule segment (outer medulla) and not the thick ascending limb of the loop of Henle that is most susceptible to ischemic injury [550–552]. This is likely due to the IR related reductions in medullary blood flow and oxygen delivery while at the same time cortical blood flow is restored to near physiological levels. Furthermore S3 segments have a limited capacity to undergo anaerobic metabolism compared to the thick ascending limb of the loop of Henle. Though compared to the proximal tubule, there are fewer histological changes in the thick ascending limb, IR upregulates expression of inflammatory genes, inducing cytokine secretion [540]. Cytokines from the thick ascending limb and the S3 act as effectors of positive feedback enhancing inflammation and potentiating vascular obstruction, via a number of proposed mechanisms.

Rat and canine models of AKI and ARF have demonstrated reduced post-glomerular flow rates and perfusion in peritubular capillaries [553] and the outer medulla using morphomotry and carbon injections [554], radio nucleotide imaging [543], contrast enhanced MRI [555–558], with an increased total renal vascular resistance[559,560] and vascular congestion on histological analysis [561,562] during reperfusion. Using laser Doppler flowmetry a mouse model of ARF demonstrated reduced total renal blood flow by 45%, and GFR by 68% during reperfusion, with N-acetyl-cysteine pre-treatment ameliorating these deficits - indicating NO and antioxidants as potential modifiers in the pathomechanism [563]. In a large animal pre-clinical isolated porcine kidney reperfusion model, after 24h CIT organs demonstrated decreased flow rates, GFR, and tubular sodium reabsorptive ability [564]. The rat transplant model supports AKI investigations and further shows extended CIT correlated to the degree of medullary vascular congestion, most prominent in the outer stripe, with red cell trapping and vessel obstruction using radiolabel erythrocytes [544].

Clinically kidney transplant recipients with DGF demonstrated lower GFR, lower blood flow and higher resistance indices (Doppler flow) [565], with higher transcapillary pressure differentials compared to functioning allografts [566] - implicating afferent arteriolar constriction as a cause of reduced perfusion and dysfunction. Similarly assessing intra-operative renal perfusion using an IR camera the rate of re-warming time was correlated with return of renal function post-operatively, with greater parenchymal perfusion in LD compared to DCD/DBD donor organs [567]. Recently intraoperative spectrometry of DCD donor kidneys 30 minutes into reperfusion revealed allografts with DGF exhibited impaired cortical haemoglobin saturations, with capillary congestion and lower

microcirculatory perfusion [568] - suggesting that despite macroscopic reperfusion areas of the kidney parenchyma may still endure hypoxic stress. Total cortical parenchymal flow is also lower in allografts experiencing DGF vs. immediate function [569]. In parallel a high resistance index on Doppler US is also predictive of acute tubular necrosis and acute rejection in transplanted renal allografts [570,571].

Examining the cortical kidney microcirculation using orthogonal polarisation spectral (OPS) imaging peri-operatively in renal transplant recipients at 5 and 30 minutes demonstrated a 'no reflow' pattern with heterogeneous perfusion, oscillating flow and scattered microthrombi in peritubular capillaries[572]. The investigators noted a correlation between cortical capillary blood flow and creatinine clearance, suggesting microcirculatory perfusion may be useful in predicting IR induced draft dysfunction. Similar findings were seen using OPS clinically in pancreas[573,574] and liver transplantation[575,576], with poor capillary perfusion and microcirculatory dysfunction correlated to graft dysfunction, demonstrating the spectrum of impact IR microcirculatory dysfunction has in the global field of organ transplantation.

The causes of altered renal perfusion are not completely understood. There may be an imbalance in production and activity of endogenous regulators of vasodilatation and vasoconstriction - such as NO and endothelin. This is partly supported by studies demonstrating improvement in IR injury with pre-treatment by vasoconstrictor antagonists [170,577,578].

Congestion in peri-tubular capillaries and outer medulla may be due to aggregation of leucocytes and platelets [129,579] combined with vasoconstrictive elements, and development of pro-inflammatory and pro-thrombotic cascades. Congestion has been shown to shunt blood flow from the outer medulla potentiating hypoxic and ischaemic stress during reperfusion [580–582].

IR damage to endothelial cells with resultant swelling and vessel permeability with interstitial oedema can also contribute to malperfusion through directly compressing peritubular capillaries causing vessel obstruction [553]. Interstitial oedema may also contribute to haemoconcentration, increasing the potential for platelet/leucocyte/endothelial interactions. This is particularly important as these interactions in the IR environment will potentiate further endothelial barrier failure [583,584].

Lastly activation of the clotting cascade by IR effects - ROS, hypoxia, and the subsequent activation of complement and inflammatory cells may negatively affect rheological properties of the blood. Over all three components of Virchow's triad seem to be met with an activated and damaged

endothelium, congestion of flow through the microcirculation, and activation of coagulatory proteases. The final factor is likely the inflammatory process that occurs in parallel - and it is now widely accepted that there is critical and continuous cross talk between the inflammation and coagulation, especially in IR injury[585].

The mechanisms of altered perfusion may also be divided by the separate units of the vascular bed they relate to and specific changes seen in arterioles, capillaries, and venules all contribute to poorer tissue perfusion.

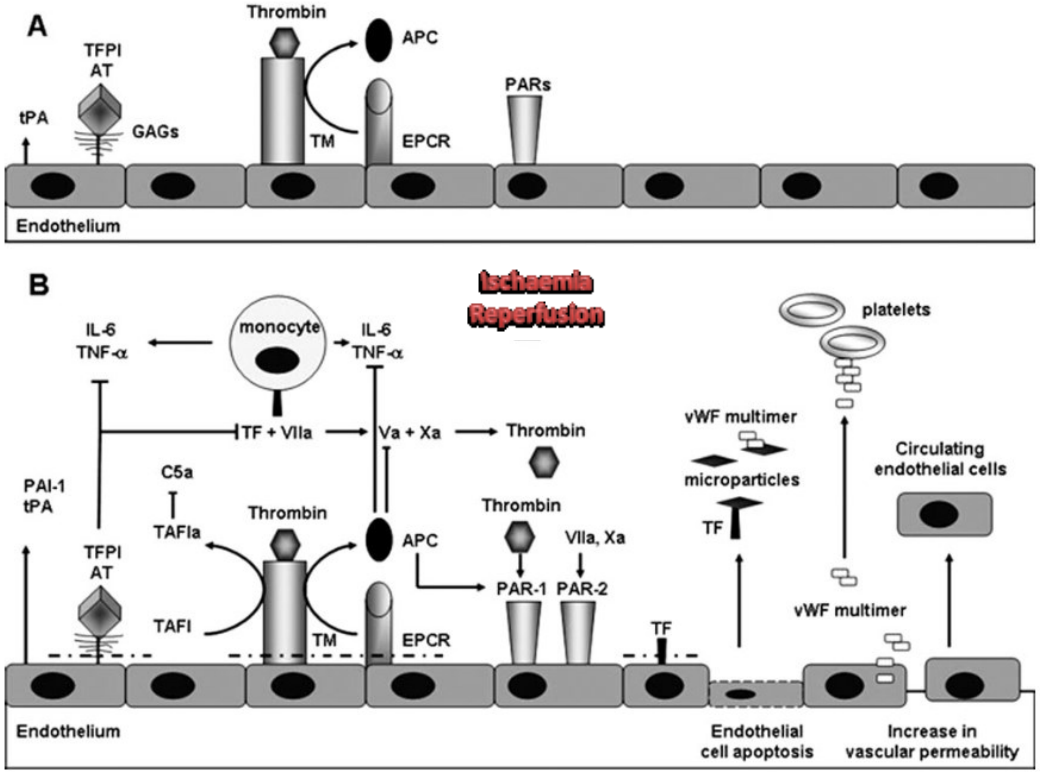


Figure 16 The key role of the endothelium in normal physiological situations and in inflammation during IR injury. (A) Normally the endothelium prevents coagulation by: expressing thrombomodulin (TM) and the endothelial PC receptor (EPCR) supporting generation of activated PC by thrombin, expressing TF pathway inhibitor (TFPI) and antithrombin (AT), and secreting tissue-type plasminogen activator (t-PA) promoting fibrinolysis. (B) During IR there is a local and systemic activation of inflammation which leads a an increase in the release of pro-inflammatory cytokines, with activation and subsequent dysfunction of the endothelium. With activation there is a release of metalloprotease enzymes, apoptosis of some endothelial cells, detachment of others, and a general loss of barrier function, leading to interstitial tissue oedema. Induction of TF on circulating inflammatory cells and graft endothelial cells, release of von Willebrand factor, and subsequent platelet adhesion and aggregation, promotes a pro-coagulant environment within the graft. There is a decrease in glycosaminoglycans production, and endogenous anticoagulant proteins (TFPI, AT, EPCR, and TM) are cleaved from the endothelium surface transforming the endothelial into a pro-coagulant phenotype.

Furthermore circulating and locally produced APC and AT are consumed. Platelet activating inhibitor (PAI-1) levels increase and overwhelm t-PA, limiting fibrinolysis and shifting to further fibrin formation and deposition. Thrombin-activatable fibrinolysis inhibitor (TAFI) activity is reduced leading to enhanced complement activation and action of C3a and C5a activity. PAR receptors are further activated by thrombin, TF-FVIIa complexes and FXa, as well as APC modulating cytokine release. Adapted from Schouten et al. [240]

1.5.4.3 Arterioles

IR causes a progressive endothelium dependent vasoconstriction of arterioles. During ischaemia there is an inducible production of potent vasoconstrictors such as endothelin-1, endothelial angiotensin II, sympathetics, adenosine, thromboxane A2, and leukotrienes that act to counter constitutive and inducible endogenous vasodilators such as NO, acetylcholine, bradykinin, and prostaglandin E2[586]. This effect is then accentuated after reperfusion, by excess ROS production from endothelial cells and the recruitment and adherence of leucocytes[449,587] . Whether such vasoconstriction arises with certainty due to increased presence of endothelial derived vasoconstrictors, or a deficiency in effect of endogenous dilators, such as NO is still being investigated[468,588,589]. Evidence for this vasoconstriction is found in spectral Doppler wave forms during the initial hours and days after transplantation in kidneys with DGF that show higher restrictive indices compared to immediate functioning grafts [571,590,591].

1.5.4.4 Capillaries

Capillaries are responsible for the exchange of nutrients, hormones, oxygen, CO2, and metabolites between the circulation and the organ in question[208]. Key beds in the kidney include the glomerulus, and the peri-tubular capillaries. Dysfunction in these beds is likely key to the poor functional status of kidneys affects by IR clinically experiencing DGF and reduced glomerular filtration rate (GFR). IR manifests with an increase in fluid infiltration into the interstitium (oedema) a result of increased endothelial barrier permeability, rather than higher transcapillary hydraulic pressure [92,592] . Diminished NO levels and leucocyte-endothelial synergy have been implicated in this process [587]. This mechanism may also be active in diminishing effective GFR[566]. In parallel with increased fluid filtration IR is associated with perfusion deficits and decreased capillary perfusion density. This appears to stem from obstruction of capillaries by stiffer, activated leukocytes and platelets combined with swollen and partially detached endothelial cells narrowing capillary lumens [593].

1.5.4.5 Venules

Venules are the primary site of endothelial dysfunction and the ensuing inflammatory response in IR related microvascular injury. The predominate features involve leucocytes: leucocyte-endothelial adhesion and transendothelial infiltration, platelet-leucocyte aggregation, with extravasation of albumin and fluid into the interstitium due to increased barrier permeability; and increased ROS formation[594–596] .

1.5.5 Derangement of Coagulation in Ischaemia Reperfusion Injury

The recent developments seen in the understanding of thrombin physiology, and the interlacing interactions of coagulation proteases and inflammatory mediators has presented an opportunity for pharmacologic intervention using novel anti-coagulants in a variety of processes and conditions that have traditionally not been treated by such means in the past. In particular interventions using anti-coagulants targeting inflammation in IR injury in organ transplantation has the most potential for impact because at present IR mediated injury in marginal allografts and the subsequent delayed function and complications that arise and impact graft and patient survival represents one of the major challenges in transplantation today.

In transplantation settings of IR injury, activation of coagulation cascades and their interactions with recipient (and potentially donor) leucocytes and platelets in the allograft microvasculature leads to the generation of and deposition of intravascular fibrin(ogen), propagation of which may cause occlusion of the microvasculature and obstruct capillary perfusion [597].

The localisation of coagulation to areas of infectious inflammation functions to isolate regions within in a kidney and contain the pathogenic spread [312]. In a similar manner during IR, on-going coagulation reducing perfusion through the micro-circulation would feedback and potentiate the progressive thrombosis and ischaemia in the affect regions. Hypoxia during reperfusion will augment the pro-inflammatory and pro-coagulant phenotype of endothelial, resulting in amplification of fibrin deposition, worsening micro-coagulopathy and inflammation further diminishing microvascular perfusion. In parallel the inflammatory processes occurring during IR and the generation of pro-inflammatory cytokines from endothelial cells, immune cells and cells of the organ amplify and ensure continued activation of coagulation proteases. There is also inhibition of intrinsic mechanisms of fibrinolysis and natural anticoagulation (which are also anti-inflammatory) ensuring continued inflammation. Despite evidence of intravascular fibrin(ogen) deposition during IR this

does not lead to overt organ damage, instead it produces a transient inflammatory milieu with-in the allograft, affecting normal tissue function [598]. This transient inflammation results clinically as delayed graft function which can last from several hours and days to weeks.

1.5.6 Coagulatory Disturbances in Kidney Transplantation

IR injury is the exacerbation of ischaemic tissue damage upon reestablishment of the circulation and influx of oxygen and cellular blood components. It is an unavoidable process in organ transplantation, and the degree of injury sustained a major contributor to allograft function, and both patient and allograft graft survival.

The patho-physiology of IR injury is complex and not fully understood. Animal and a number of human studies have shown that ROS [99], components of the innate immune system such as complement [154], as well as leucocytes and platelets [128] contribute to I/R injury. The interplay between components of the inflammatory response and coagulation cascade has been highlighted as potential key player, previously underappreciated in IR pathogenesis [599][266].

Overt renal allograft thrombosis as an entity is a serious complication in renal transplantation. Arterial thrombosis occurs in 0.2-7.5%, and venous thrombosis in 0.1-8.2% of renal allografts[600]. There is a heterogeneity in allograft thrombosis rates in the literature, for which there are several possible reasons: classification and diagnosis of thrombosis differs between institutions and countries (clinical, histological, after allograft removal, radiological), the majority of reports are retrospective, and differentiation is frequently not made between arterial or venous thrombosis. A brief review of the literature demonstrates this (Table 1).

None the less it is a detrimental complication that is variably implicated by various analyses in 2-7% [601,602], 17-35% [603,604], and as high as 44% of all cases of early graft loss in adults [605]. Furthermore the most recent analysis of paediatric kidney recipients since 1995 (North American Paediatric Renal Transplant Cooperative Study [NAPRTC]) suggests despite improved immunosuppression in the current era of transplantation 2.7% of all paediatric allografts are lost to thrombosis, with thrombosis accounting for 35% of all lost allografts in the 1st year, and 18% of all graft losses [606,607].

1.5.6.1 Causes and Risk Factors

Numerous retrospective analyses of graft outcomes and complications has suggested a number of risk factors and donor / recipient pathologies associated with allograft thrombosis[600,602,604,608–616]. Donor factors include: age(<6 or >60), cold ischaemia time >24 hours, vessel atheroma, deceased donor, history of stroke, use of a right kidney. Recipient factors include: Recipient age <5–6 or >50, Peritoneal dialysis, diabetes mellitus, atherosclerosis of renal or iliac vessels, previous history of thrombosis, technical/surgical problems, use of iliac graft anastomosis in young donors, hemodynamic instability, thrombophilia, use of artificial grafts, no use of thromboprophylaxis, acute allograft rejection, and delayed graft function.

An important aspect of these risk associations is that no one study has identified all of them, and specific risks are not seen in every study analysis. Thus the interactions between risk factors, in terms of cumulative or even exponential risk are not known.

1.5.6.2 Current management of kidney allograft thrombosis

Diagnosis of Post-transplant graft thrombosis is based on a mixture of evidence of failing function, clinical findings, and radiological imaging including ultrasound scan (USS), magnetic resonance imaging (MRI), and biopsy[600]. Venous thrombosis is usually accompanied by a swollen, tender allograft and haematuria, while arterial thrombosis may only present with sudden anuria and by pain over the graft region. In an arterial/venous thrombosis picture symptoms may be mixed and sparse. Clinically it can be difficult to distinguish graft thrombosis from acute rejection, as thrombosis frequently occurs in rejection episodes. Partial thrombosis may also present as delayed graft function, until the process progresses to complete thrombosis where the full clinical picture is more recognisable[617,618]. However delayed function can also mask graft thrombosis, where thrombotic events normally occur within the first 10 post days postoperatively, with 93% of cases occurring by day 7[601,619], and DGF may have been evident since transplantation. Doppler USS is reliable technique to detect renal artery or vein thrombosis, and can help to distinguish between episodes of acute rejection, thrombosis and delayed function [591,620–623].

Successful salvage of allografts from thrombosis is reported in the literature using injection of anti-fibrinolytic agents, endovascular aspiration, and surgical thrombectomy [624–626]. In the majority of cases graft rescue is disappointing and generally allograft thrombosis causes irreversible loss of function and necessitates surgical graftectomy.

Apart from addressing the risk factors associated with allograft thrombosis, by either carefully scrutinizing donors or optimising the recipient post transplantation the only available preventative measure that is widely available is prophylactic anti-coagulation. There has been no national, or international census on suggested practice, neither have there been any high quality trials assessing the benefit of such treatment on thrombosis prevention and graft loss[627]. None the less several studies and analyses have been made examining the effectiveness and safety of routine anti-coagulation in kidney transplant recipients, particularly in high risk cohorts such as patients with diagnosed thrombophilia. The common repertoire of prophylactic anticoagulants used include both oral and intravenous medications; aspirin, unfractionated heparin (UFH), and low molecular weight heparin (LMWH). Debate and evidence remains to address whether such anticoagulation is effective in preventing thrombosis, which anti-coagulant is most effective in different donor/recipient cohorts, and if the risk of bleeding is a permissible and acceptable risk. A summary of the studies addressing the issue of anti-coagulation in kidney transplantation is presented in Table 2.

Author	Nationality	Year	n	Recipient age	Donor (% DBD/ DCD)	% RAT + RVT	% RAT	% RVT	Study design
Jordan [628]	Canada, Toronto	1970-1980	341	13-67	84	4.4% (15)	3.5% (12)	0.9% (3)	Consecutive c.s.
Penny [601]	Australia, ANZDATA	1980-1992	6153	N/A	N/A	2.2% (134)	1.1% (70)	1.0% (64)	Case-control
Murphy [629]	Ireland, Belfast	1989-1992	202	adults	N/A	4.5% (9)	1.0% (2)	3.5% (7)	Retrospective c.r.
Benedetti [630]	USA, Minnesota	1985-1993	998	16-74	51	1.4% (14)	0.4% (4)	1.0% (10)	Retrospective c.r.
Bakir [631]	Holland, Groningen	1986-1994	558	14-71	100	6.1% (34)	2.0% (11)	3.4% (19)	Consecutive c.s.
Ojo [604]	USA, UNOS	1990-1996	84513	>18	N/A	0.9% (751)	N/A	N/A	Case-control
Perez [619]	Spain, A Coruña	1988-1997	827	44	100	5.7% (47)	2.3% (19)	3.4% (28)	Retrospective c.r.
Englesbe [632]	USA, Michigan	1993-1997	714	all	43	1.82% (13)	0.8% (6)	1.0% (7)	Consecutive c.s.
Samhan [633]	Kuwait, Hawaly	1993-1998	151	all	24	3.3% (5)	2.6% (4)	0.7% (1)	N/A
Osman [615]	Egypt, Mansoura	1976-1999	1200	all	0	0.5% (12)	0.4% (5)	0.1% (1)	Consecutive c.s.
Mazzucchi [634]	Brazil, Sao Paulo	1995-1999	356	18-70	64	1.4% (5)	0.3% (1)	1.12% (4)	Retrospective c.r.
Parada [635]	Portugal, Coimbra	1980-2001	1000	41	98	0.9% (9)	0.6% (6)	0.3% (3)	Prospective
Orlic [636]	Croatia, Rijka	1971-2002	725	N/A	53	1.1% (8)	0.6% (4)	0.6% (4)	N/A
Stechman [637]	UK, Oxford	1997-2002	401	27-75	78	1.0% (4)	0.75% (3)	0.25% (1)	Prospective
Hernández [617]	Spain, Tenerife	1996-2004	870	18-76	100	4.8% (42)	3% (26)	1.8% (16)	Retrospective c.r.
Sanni [614]	UK, Newcastle	1990-2005	1308	N/A	93	2.8% (36)	N/A	N/A	Retrospective c.r.
Dimitroulis [638]	Greece, Athens	1980-2005	1367	N/A	44	2.3% (31)	2% (27)	0.3% (7)	Consecutive
Salehipour [639]	Iran, Shiraz	1988-2006	1500	Apr-70	20	1.1% (16)	0.6% (9)	0.5% (7)	Retrospective c.r.
Głyda [640]	Poland, Juraszów	1995-2005	327	46.5	100	0	0	0	Retrospective c.s

Table 1 Summary of studies of kidney transplant outcomes detailing rates of kidney allograft thrombosis. RAT: renal artery thrombosis, RVT: renal vein thrombosis. c.s: case series. c.r: case review. N/A: not addressed.

Author	Nationality	Year	n	Recipient age	Donor (%DBD/ DCD)	% RAT + RVT	% RAT	% RVT	Study design
Amézquita [613]	Spain, Madrid	1990-2006	772	N/A	100	5.5% (42)	N/A	N/A	Case-control
Sharma [610]	USA, Richmond VA	2000-2006	401	adults	72	0.49% (2)	N/A	N/A	Retrospective c.r.
Kamali [641]	Iran, Tehran	1998-2007	718	21-58	0	N/A	3.1% (22)	N/A	Retrospective c.r.
Haddiya [642]	Rabat, Morocco	1998-2008	67	38-50	0	4.5% (3)	N/A	N/A	Retrospective c.r.
Phelan [605]	Ireland, Dublin	1990-2009	2381	>18	100	0.2% (7)	0.75% (11)	1.2% (30)	Retrospective c.r.
Luna [612]	Badajoz, Spain	1992-2009	577	adults	100	6% (35)	N/A	N/A	Retrospective c.r.
Zilinska [643]	Slovakia, Bratislava	2008-2009	103	adults	N/A	N/A	3.9% (4)	2.9% (3)	Retrospective c.r.
Bessede [644]	France, Paris	1971-2010	3129	adults	93	N/A	1.9% (61)	1% (31)	Retrospective c.r.
Eufrásio [645]	Portugal, Coimbra	1980-2010	2000	43 +/- 13	96	0.1% (2)	1% (20)	0.4% (8)	Retrospective c.r.
Srivastava [646]	India, Uttar Pradesh	1989-2010	1945	6-56	0	0	0.46% (9)	0.15% (2)	Retrospective c.r.
Ghadiani [647]	Iran, Tehran	1994-2010	385	35 +/- 14	2	N/A	9% (35)	3% (12)	Retrospective c.r.
Aktas [648]	Turkey, Ankara	1975-2011	1843	3-66	24	N/A	0.2% (5)	0.1% (2)	Retrospective c.r.
Sagban [649]	Dusseldorf, Germany	1997-2012	338	44 +/- 13	0	0	1.2% (4)	1.2% (4)	Retrospective c.r.

Table 1 continued. Summary of studies of kidney transplant outcomes detailing rates of kidney allograft thrombosis. RAT: renal artery thrombosis , RVT: renal vein thrombosis. c.s: case series. c.r: case review. N/A: not addressed.

Author	Nationality	Year	Study Design	Cohort Details	Group	Type of Treatment	n
Esfand-iar [650]	Iran, Tehran	2007-2008	Prospective RCT	Paediatric kidney transplantation	Control Anti-coag Group	No anticoagulation Heparin /Aspirin	63 24
Osman [651]	Egypt, Mansoura	2003-2005	Prospective RCT	Living donor kidney transplantation	Control Anti-coag Group 1 Anti-coag Group 2	No anticoagulation LMWH (Post Op 1/52) UFH (Post Op 1/52)	25 25 25
Eng [652]	Kentucky, USA	2005-2009	Retrospective c.s	Adult kidney transplantation	Control Anti-coag Group	No anticoagulation Aspirin / UFH / LMWH / LMWH+Warfarin; Post Op	213 28
Pawlicki [611]	Poland, Katowice	2006-2008	Retrospective c.s	Adult kidney transplantation	Control Anti-coag Group	No anticoagulation Heparin (2/7) + LMWH (5/7); Post Op	48 11
Murashi-ma [653]	USA, Philadelphia	2003-2007	Retrospective c.s	Adult kidney transplantation in recipients at high risk for VTE (e.g. Thrombophilia)	Control Anti-coag Group	No anticoagulation Anti-coagulation (50% Thrombophilia)	32 16
Kusyk [654]	Australia, Camperdown	1998-2003	Retrospective c.s	Adult kidney transplantation	Control Anti-coag Group	No anticoagulation UFH ± LMWH; Post Op	310 16
Mathis [655]	USA, New Jersey	1998-2002	Retrospective c.s	Adult kidney transplantation	Control Anti-coag Group	No anticoagulation Heparin; Post Op	697 28

Table 2. Summary of studies investigating the merits of anti-coagulation in kidney transplantation. Ages are represented as either mean ± SD, a range, or a description of the cohort age. ASA: aspirin. RCT: randomised controlled trial. c.s: case series. UFH: unfractionated heparin. LMWH: low molecular weight heparin. VTE: venous thrombo embolism.

Author	Group	Recipient Age	Donor Age	Donor DD %	RAT + RVT %	RAT %	RVT %	Bleeding	
								% Conservative Management	% Requiring Reoperation
Esfandi-ar [650]	Control	10.4	18.2	0	7.90	0	0	0	0
	Anti-coag Group	9.6	16	0	0	0	0	0	0
Osman [651]	Control	29.4 ±8	35.3±10	0	0	0	0	32	0
	Anti-coag Group 1	28.3±8	35.9±11	0	0	0	0	44	4
	Anti-coag Group 2	26+/-6	33±9	0	0	0	0	48	0
Eng [652]	Control	Adults	N/A	N/A	0	0	0	27	1.7
	Anti-coag Group	31-53	N/A	N/A	0	0	0	32 (21.1% of ASA, 71.4% of UFH/LMWH)	14 (5.2% of ASA, 42.9% of UFH/ LMWH)
Pawliki [611]	Control	46 ±11	45 ±12	100	0	0	0	12.5	4.1
	Anti-coag Group	46 ±11	45 ±12	100	0	0	2	57.9 (63% of UFH, 50% of LMWH)	9
Murash ima [653]	Control	N/A	N/A	N/A	18.8	NA	NA	6.3	NA
	Anti-coag Group	N/A	N/A	N/A	6.3	NA	NA	31.3	NA
Kusyk [654]	Control	Adult	N/A	N/A	0	0	0.97	1.3	2.2
	Anti-coag Group	Adult	N/A	N/A	0	0	0	25	37
Mathis [655]	Control	Adult	N/A	N/A	0	0	0	0	0
	Anti-coag Group	Adult	N/A	N/A	7.1	0	0	14	50

Table 2 continued. Summary of studies investigating the merits of anti-coagulation in kidney transplantation. Conservative management included transfusion of blood products. Bleeding rates are described as: the percentage of patients bleeding requiring intervention with percentage of those bleeding on specific anti-coagulants in brackets. DD: deceased donor, RVT: renal vein thrombosis, RAT: renal artery thrombosis. N/A: not addressed. UFH: unfractionated heparin. LMWH: low molecular weight heparin. VTE: venous thrombo embolism.

The lack of sufficient controlled and randomised trials of suitable power limits any attempt at producing a protocol based on high quality evidence. Most protocols in use by transplant centres is based on anecdotal evidence and individual unit experience.

Recently a survey of anticoagulation practices of 35 transplant centres in France revealed a high degree of variability in patient management[627]. The survey addressed the management of 4 hypothetical situations necessitating decisions on patient anticoagulation in the peri-operative period based on the units standard practice, dealing with issues of thrombophilia, history of thrombosis, concurrent anti-coagulation, atrial fibrillation, diabetes, and advancing age. Overall there was a large diversity in anti-coagulation practice, with no common management strategy, and this likely reflects the complexity of anti-coagulation in renal transplant recipients in the absence of good evidence from the literature, and national/regional guidance.

Bleeding complications in patients cannot be overlooked and in what studies are available (Table 2) document bleeding rates ranging from as low as 1% to as high as 57% in patients on post-operative anti-coagulation and higher in those on UFH, with similar high rates requiring reoperation[611,650–655].

Use of heparins, especially LMWH, in chronic renal impairment is problematic due to the variability and unpredictability in dose response due to insufficient renal clearance. Renal transplant patients in general are also susceptible to such problems, with a greater risk of bleeding with UFH, than non transplant recipients[656]. Thus use of UFH in the peri-operative period may be cautioned as ideal target coagulation parameters to prevent thrombosis but limit bleeding still need be determined. Furthermore despite heparin possessing anti-inflammatory properties, which may be beneficial in during transplantation these effects are only observable at higher doses than those deems clinically safe[657].

Emerging acceptance of the complex overlap and interface between coagulation cascades and inflammatory responses during IR injury means that the episodes of thrombosis clinically detectable are possibly the result of a progressive and exaggerated IR response. The initial IR injury that initiates coagulation and intravascular fibrin deposition in the microvasculature, could overwhelm the recipient innate anti-coagulatory and fibrinolytic processes. This process through interactions with platelets and leukocytes, and potentiated by cytokines and activation of the graft endothelial into an inflamed and pro-coagulant phenotype, can lead to extension and acceleration of thrombosis into the larger vessels -arterial, venous or both - with subsequent allograft thrombosis.

Eventual graft thrombosis does not frequently occur - as evidence by the relatively low incidence of thrombosis in the literature, instead it is likely that the clinical entity encountered is DGF.

During human transplantation there is a demonstrated malperfusion of the renal micro-circulation (cortical and medullary regions)[658] with subsequent effect on renal function, highlighted by greater and discontinuous perfusion deficits in kidneys with DGF[568,569,659,660] and also subsequent graft failure. Furthermore damage to peritubular endothelial cells is associated with DGF, and though damage is initiated by IR responses, it may be potentiated in areas with poor perfusion[661].

Renal transplant recipients demonstrate higher activity of AT, Protein C, and Protein S, with higher levels of D-Dimers in the post-operative period suggesting increased thrombotic processes in these patients[662,663]. These finding correlate with other studies that show significant release of inflammatory cytokines (IL-6 and IL-8), as well as prothrombin fragment 1+2 (marker of coagulation activation) in cadaveric kidney recipients within 30 minutes of implantation demonstrating the overlap between inflammatory processes and coagulation events in mediating IR injury[664].

A similar study demonstrated that higher levels of D-Dimers are released within several minutes into the recipient circulation in kidneys that subsequently exhibit DGF compared to immediate functioning kidneys[618]. Release however of pro-thrombin fragment 1+2 did not follow this trend, suggesting to the authors that resident fibrin deposits in the graft (from retrieval and preservation) may have been the cause of the D-Dimer release. Prevalence of remnant micro-thrombi and fibrin in static cold stored kidneys is documented. However this does not negate the key role rapid activation of coagulation by the transplanted kidney may have, leading to the generation of fibrin and clot formation damaging the allograft and resulting in delayed function[665]. Ex-vivo flush, for example with streptokinase (a plasminogen activator) of kidney allografts with biopsy proven intraglomerular micro-vascular thrombosis prior to transplantation salvaged kidneys and allowed transplantation[666]. Despite this intervention 25% of salvaged kidneys demonstrated DGF.

Renal transplant recipients have higher levels of t-PA and lower PAI activity compared to healthy controls, suggesting ongoing activation of coagulation proteases necessitating continued and up regulated activation of the fibrinolytic system[667]. Furthermore PAI activity was higher in recipients with chronic allograft rejection compared to those with stable renal function - indicating that increased activation of coagulation (and in these patients less fibrinolysis) - is not limited to the early IR insult but can persist within chronic inflammatory reactions in these patients. Peritubular capillaritis, as well as peritubular thrombosis are both seen in renal transplant allograft biopsies of

kidneys with chronic rejection, again indicating the wider spectrum and involvement of activation of coagulation cascades and thrombosis in transplant pathology, from perioperative IR injury to ongoing inflammation[668,669].

Stasis of blood within an allograft during the WIT induces coagulation activation with thrombin generation, and the activation of platlets with leukocyte-platelet-endothelial complexes formed in the glomerular and peritubular microcirculation [670,671]. These processes converge on reperfusion with fibrin deposition, the development of microthrombi, occlusion of allograft microvasculature. Impaired perfusion, and further hypoxia enhances endothelial and leukocyte activation, which amplifies activation of coagulation cascades (via TF expression) with more thrombin generation, fibrin(ogen) deposition, and allograft invasion by leucocytes, which is independent of allogenicity [220]. In summary effects of IR induced inflammation on haemostasis are mediated by increased production of pro-inflammatory cytokines, which potently activate coagulatory cascades while seriously inhibiting both fibrinolysis and natural anticoagulatory mechanisms.

Recently DTIs have been trialled in experimental porcine kidney auto-transplant models with successful outcomes. Melagatran is a low molecular weight specific thrombin inhibitor with action on free and bound thrombin (half-life of 1.7 to 2.5 hr). Melagatran administration (to the preservation solution) during SCS preservation, as well as after transplantation to the recipient animal reduced the pro-inflammatory activity of circulating immune cells, as well as renal injury (reduced P-selectin and MCP-1), and inflammatory infiltrates. Animal and graft survival, with good function and no DGF, was doubled from 4 to 9 days with melagatran treatment compared to controls[672]. In a similar porcine DCD auto transplant model melagatran also improved short and longer term graft survival, with reduced DGF peri-operatively, with less interstitial fibrosis and good function at 3 months [673,674].

1.6 Ischaemia Reperfusion Injury and the Pancreas

The cellular mechanisms of renal IR injury, in particular those related to endothelial cell stress and damage also play a dominant role in pancreatic reperfusion induced damage during transplantation. Pancreatitis and graft thrombosis are the two primary entities that arise from such IR mediated injury.

1.6.1 The Aetiology of Early Pancreatic Graft Thrombosis

Though reports indicate that recruitment of marginal donors may be of benefit to expanding certain donor pools, with patient and graft survival rates comparable to ideal donors in some cases; older age, higher BMIs, and increased organ ischaemia time (as in DCD) are identified risk factors for post-operative graft thrombosis [675].

Furthermore procurement and preservation injury, reperfusion injury upon implantation, and inherent hypercoagulability of recipients are increasing seen to play a dominant role in graft thrombogenesis [676]. Considering the possible factors it would seem that Virchow's triad is met in most pancreatic transplantation operations; with endothelial damage and activation secondary to haemodynamic instability prior to retrieval, the stasis of residual blood within the pancreatic graft during procurement and preservation (even with through washout), and the recipient's hypercoagulable diabetic state [677]. It is to this effect that vascular thrombosis has been coined 'the Achilles heel' of pancreatic transplantation [676,678].

Rates of pancreatic graft thrombosis are variable but venous vs. arterial thrombosis predominates, and reports in the literature suggest an occurrence in 3.1-17% of grafts [679–683]. It is the leading non-immunological cause of pancreatic graft failure [678,684,685]. Thrombosis can be classified as either completely or partially occlusive. Complete occlusion, with-out sufficient collateral vessels would result in a swollen, painful, and failing graft that would require removal if interventions to salvage the allograft proved unsuccessful. Partially occlusive thrombosis can be harder to detect, as clinical signs may not be as obvious, with the only indicator DGF. Thus partially occlusive thrombosis is likely underreported and occurs more frequently than complete occlusion [684]. It is unknown what percentage of pancreatic DGF involves or is due to partial thrombosis of large or even smaller vessels.

The pathogenesis of graft thrombosis is inherently related to the degree of IR injury each organ sustains in retrieval and transplantation [321], and pancreatic tissue is extremely vulnerable to

ischaemic stress. IR by definition describes damage sustained during the period of ischaemia after retrieval and then the further damage that is inflicted once perfusion is re-established when the organ is transplanted to the recipient. A key finding in pancreatic IR related deterioration in graft microcirculation is decreased capillary perfusion, with increased macromolecular permeability and leucocyte adhesiveness in post-capillary venules [686]. Orthogonal polarisation spectral imaging analysis of the pancreatic microcirculation during clinical pancreatic transplantation demonstrated that poor post-operative endocrine and exocrine function correlated with microcirculatory dysfunction and impairment of microvascular perfusion in the acute phase post transplantation[573].

This leucocyte-endothelial interaction and inflammatory process heralds platelet activation and aggregation with generation of subsequent ROS, and the release of further pro-inflammatory cytokines, growth factors, and cytotoxic proteases, which further disrupts graft perfusion through platelet stagnation in non-perfused vessels and possibly accumulation in pancreatic venules [687]. Histologically pancreatic IR injury is characterised by interstitial and interlobular oedema, vacuolization, granulocyte infiltration, scattered necrosis[688] and apoptotic cell death[689].

The degree of parenchymal micro-circulation disruption and ischaemic tissue injury caused by re-perfusion in IR has been correlated with the severity of post operative oedematous and necrotising graft pancreatitis [686,690]. Pancreatitis itself is thus one manifestation of graft micro-circulatory disruption, and the subsequent hypoxic tissue damage and inflammatory process that prevails in the reperfusion setting. Inherent interactions between pro-inflammatory and pro-coagulant cells, cytokines, chemokines, and adhesion molecules, and key components of the coagulation cascade (e.g. fibrin) thus play a decisive role in post-ischaemic pancreatic injury [687].

The interface between inflammation and coagulation has been described in previous sections, and is understandably complex[290]. Venous thrombosis for instance is now understood to be an early consistent and sensitive marker of anti-body mediated humoral rejection in heart transplants, and several small animal studies of acute humoral xenograft rejection illustrate the point by demonstrating a complete inhibition of rejection with the use of anti-thrombin agents[691]. Coagulatory proteases are thus thought to be an early inflammatory stimulus in anti-graft responses, and parallels results from sepsis animal studies that show coagulatory proteases actually amplify LPS induced inflammatory responses [692].

Thus the inflammatory process evident in pancreatic grafts after IR injury is likely one stimulatory factor for the activation of coagulation cascades, with down regulation of fibrinolysis and a decrease

in anti-thrombin III (a potent endogenous anti-coagulant) that has been demonstrated in pancreatic grafts during reperfusion. This points what appears to be to the development of a local hypercoaguable state in the pancreatic vascular bed, and is probably a contributing aspect to the development of graft thrombosis[693].

In addition to both IR injury and early humoral rejection several other possible patho-physiological causes and risk factors for graft thrombosis have been identified. Post operative analysis of pancreas recipients has revealed consistently higher platelet counts than diabetic controls[694]. In situations where endothelial damage is prevalent (such as IR injury in pancreatic grafts) a concurrent thrombocytocis may potentiate micro-circulatory dysfunction – so called 'sticky platelet syndrome' – through increased production of regulatory chemokines and adhesion molecules – such as von Willebrand factor and fibronectin, inducing vasoconstriction (thromboxane A2), and in conjunction with tissue factor activate the coagulation system and promote thrombosis and vascular occlusion within the graft[695].

Inherited thrombophilia in transplant organ recipients is an important factor in the post-operative graft thrombosis. Major inherited thrombophilic disorders include factor V leiden, protein C deficiency, protein S deficiency, prothrombin gene mutations, and anti-thrombin deficiency. Retrospective reports state that up to 18% of patients with pancreatic graft thrombosis are thrombophilic[696]. Considering the inflammatory and possible pro-coagulant local process occurring in the pancreatic vasculature, an inherited pro-coagulant disorder in the recipient would most likely exacerbate the thrombotic tendency in the post-operative period.

Peritoneal dialysis has been identified as an independent risk factor for renal graft thrombosis – though the patho-physiological cause is still elusive. This risk could also be conferred to diabetic patients on dialysis who receive a pancreatic graft as well as a renal allograft[697].

Furthermore the identified cardiovascular risk factors discussed in Section 1.53 affect both donor allograft endothelial as well as the recipient inflammatory and coagulatory profiles, rendering the pancreas more prone to microvascular dysfunction, thrombosis, and graft failure after transplantation.

1.6.2 Current Management of Early Pancreatic Graft Thrombosis

Various surgical and endovascular procedures have been described to salvage pancreatic grafts suspected of developing thrombosis[698–700]. Such developments are necessary to reduce graft

loss and morbidity in affected patients, however preventative measures that would reduce the incidence of thrombosis are important considerations.

The lack of evidence in the form of clinical trials, as well as the increasing difficulties in designing such trials to determine best management have led Units in the UK in Oxford [676], Newcastle and Manchester, as well as Europe [678] to incorporate locally devised systemic anti-coagulation protocols post-operatively in efforts to reduce the incidence of early graft thrombosis, but with no consensus on the therapeutics employed.

Despite the widespread, albeit heterogeneous use of systemic anticoagulation, rates of thrombosis are still high[684]. Furthermore systemic anticoagulation introduces the additional risk of bleeding into a post operative patient [700,701] with recent reports quoting a 4-8% incidence of major bleeding[679,702].

Similar to the observed benefits in other models of tissue IR injury and transplantation various systemic anti-coagulants have been shown to confer a protective benefit in IR pancreatitis such as Anti-thrombin III, and potentially IR related microvascular dysfunction and thrombosis [690].

The beneficial mechanism of action of heparin in pancreatitis however may only partly be due to its anti-coagulatory actions, but also due to its innate ability to inactivate plasma and tissue proteases, as well as slowing leucocyte recruitment and endothelial adhesion. Thus it may provide a two-prong anti-coagulatory and also anti-inflammatory benefit[703].

Effective prevention of microvascular graft thrombosis would subdue one of the most troublesome complication in the early period post-transplantation, and ultimately improve graft survival of pancreases retrieved from 'ideal' as well as marginal donors, and allow substantive expansion of the donor pool.

1.7 Organ Preservation in Transplantation

Organ transplantation is one of the key advances that has been made in the field of medicine in the last 50 years and for thousands of patients around the world with end-stage organ failure it remains the only effective therapy available. The supply of organs has always been an important issue in transplantation, in particular regarding moral & ethical, societal, logistical, and medical dimensions to the use of living and cadaveric donors. The impact of these issues on the quality and efficacy of treatments is of extreme importance to the overall multi-disciplinary approach in use by countries with successful organ transplant systems in place.

The key to ensuring optimal quality of organs lies in the period between retrieval and implantation namely organ preservation where efforts are made to preserve the organ ex-vivo against mounting ischaemic, and then reperfusion, damage initiated upon re-vascularisation.

As described in Section 1.3 the theoretical premise of IR injury is that in any given tissue exposed to ischaemic stress a number of cells will suffer irreparable damage, that ultimately leads to cell death. A larger percentage of cells in this tissue will sustain damage but still remain viable, with the potential for self-repair and return to normal function. As the former sub-group of cells will definitively undergo cell death, the degree and severity of IR injury remains with the number of cells in the 2nd subgroup that are salvageable and can actually remain viable after reperfusion [56]. Thus the aim of preservation strategies is to optimise the potential for these cells to be rescued, and improve function of the organ after transplantation. The cellular damage that occurs is based directly on the lack and then return of oxygen, the development of cellular oedema and intracellular acidosis, calcium overload, and the generation of reactive free radicals [57].

The key to effective organ preservation is addressing the various pathological processes occurring during IR with the aim to minimise the effects of these process on cells and tissues in the organ so it may function as effectively as possible after transplantation[704].

Organ preservation solutions are designed to minimise this damage and attempt to address the various biochemical and histological processes that occur during this ischaemic interval. Currently there are two predominate methods of cold preservation: Static cold storage (SCS) and hypothermic

machine perfusion (HMP). HMP generates a controlled continuous or pulsatile re-circulating flow of preservation solution through the vasculature of kidney at 0–4°C.

1.7.1 Static Cold Storage

Current techniques for 'static cold storage' incorporate the use of cold preservative solutions to minimize cellular injury and decrease metabolism until the allograft is implanted in the intended recipient.

At transplantation reperfusion with the recipient's blood initiates further cellular damage termed IR injury [96]. SCS starts with a rapid vascular washout to facilitate cooling, removal of blood components, and equilibration of the preservative solution with the surrounding tissue. SCS relies on the effects of hypothermia alone, supplemented special preservation solutions aimed at modulating the progressive cellular and molecular changes evident during ischaemia and then reperfusion [705].

There are limits to the beneficial effects of hypothermic preservation on organ damage. Metabolism can only be slowed by a factor of 1.5-2 for every 10^{o}C drop in temperature [704,706]. Metabolism is not arrested fully however, and recent evidence indicates that during retrieval cooling is not sufficiently quick and inevitable ischaemic injury occurs[96]. Once cooled to hypothermic temperatures cold ischemia damage supervenes. Briefly disruption of cytoskeletal structure and mitochondrial integrity reduces the formation of ATP, and ATP dependent ion pumps fail, resulting in cellular oedema and tissue damage from sodium and calcium influx. The continuing low levels of metabolism result in accumulation of toxic metabolites, with formation of ROS and free radicals once reperfusion is re-established at transplantation. With reperfusion of the allograft follows influx and activation of leucocytes, alterations in endothelial cell structure, with endothelial entering a pro-inflammatory phenotype, cytokine release from interstitial, endothelial, and immune cells, potentiates inflammatory processes with subsequent activation of innate immune responses and the coagulation cascade leading to microvasculature injury, perfusion failure, and prolongation of IR damage leading clinically to delayed function [96].

Endeavours to produce preservative solutions that intervene in as many of the changes as possible to effectively limit the cellular and metabolic derangements identified in cold ischaemia and reperfusion, have led to the development of various solutions over the years.

1.7.1.2 Cold storage preservation solutions in relation to the development of clinical organ transplantation worldwide

With the arrival of clinical transplantation by the 1960s, there was a resurgent interest in the knowledge gleamed from anatomists and physiologists concerning the techniques and theories for keeping organs viable and functioning whilst outside the body [707–712].

Artificial perfusion circuits were developed with then novel automated pumps to study the physiology and function of organs[710,713,714]. These experiments attempted to use whole blood as well as synthetic blood substitutes that were predominately crystalloid based, to both demonstrate organ function and determine the potential for preservation. It was at this time that hypothermia emerged as a potential tool in these experiments for optimising preservation through its ability to alter metabolic activity[712], with the potential benefit for clinical transplantation increasingly realised[715].

Sir Roy Calne's team at Westminster Hospital in London led one of the first investigations into the merits of cooling kidneys prior to transplantation. Using cooled heparinised blood vascular flushing was shown to render more effective mass cooling of tissue than simple surface cooling[716]. Though this novel yet practical method of cold preservation would emerge superior within a few decades, the use of blood as the perfusate would not, instead acellular flush and preservation solutions would predominate the field of organ preservation.

One of the first preservation solutions developed was in 1969 by GM Collins and co-workers at UCLA in the US. This pioneering solution was acellular and designed to replicate the intracellular electrolyte balance of mammalian cells, attempting to prevent the cellular consequences of ischaemia (warm and cold) as they were understood at the time [717]. A number of versions of these solutions were developed aptly named Collins solutions, with the most widely used a version modified for use in the Euro-Transplant network in Europe in 1976[718]. This solution termed 'Euro-collins' solution was subsequently used in transplant centres worldwide for nearly two decades.

The advent of the Collins solutions allowed renal preservation to be extended to 24-36 hours; long enough for tissue matching and sharing/transport of organs between centres. This allowed the rapid expansion of transplantation as a clinical therapy, in particular for kidney transplantation, but also

for other organs such as the liver, pancreas, heart, and lung. Flexible techniques for organ procurement were designed which allowed all donor organs to be safely procured with minimal damage by being cooled in situ all the while quickly and simultaneously removed in a bloodless field, and further dissected and packaged ('benching') on a back table[719].

The initial achievements made in clinical transplantation through the use of Collins solutions were sufficient for clinical needs at the time. However despite the success with Collins solutions in the kidney, preserving the heart, lung, liver, and pancreas were not as successful. At the time in 1970s however preservation of these organs was not a priority. The biggest problems facing transplantation were related to immunogenic rejection, and it was not until the introduction of cyclosporine for immunosuppression that extrarenal organ transplantation became an effective treatment of patients with cardiac, lung, hepatic failure, or diabetes. At this time there was a renewed interest in the field of organ preservation, in particular for extra-renal organs and research into improving solutions for preservation dominated. [720]

These efforts culminated with development of the University of Wisconsin (UW) solution by Belzer and colleagues[721]. UW solution then dominated clinical abdominal organ preservation for almost two decades before a series of novel solutions emerged as viable and accepted alternatives. Despite other solutions available UW remains as the gold standard solution for abdominal organ transplantation today[722].

Tolerated periods of cold ischaemia vary depending on the organ in question; as little as 6h-8h for the heart and lung respectively, 12-15 hours for the liver, and 24 hours for the kidney, with extended CIT a risk factor for DGF post transplantation. Since the fixture of UW as the dominant preservation solution in abdominal organ preservation a number of alternatives emerged for SCS organ preservation including: Marshall's citrate, Bretschneider's (Custodiol) Solution, Celsior, Kyoto University Solution, and Institute George Lopez-1 solution.

1.7.1.3 Formulation of Organ Preservation Solutions

Loss of Energy Substrates

Because hypothermia does not abolish metabolic activity the solution should contain substrates necessary for ATP production. High-energy amino acids (e.g. glutamic acid) are able to restore ATP

dependent enzyme processes. By augmenting solutions with the ATP precursor adenosine, ATP synthesis can resume after reperfusion and replenish the pool of high-energy phosphates[705].

Cell Swelling

Preservations solution must limit transmembrane osmotic flows that arise due to inhibition of Na/K+ ATPase, and K+ / Na+ flux in and out of the cell. By mimicking the intracellular environment (high K+, lower Na+) this flux gradient can theoretically be abolished. However high K+ concentration solutions (so called 'intracellular solutions') also cause vasoconstriction. The specific effect of such vasoconstriction is not known but may alter microvasculature perfusion dynamics during preservation if dynamic machine perfusion is utilized, and even in the initial period during reperfusion further potentially exacerbating reperfusion injury and 'no-reflow'. Alternatively extracellular solutions can be used, with a low K+ and high Na+ content, that generates external osmotic pressure counterbalancing the cytoplasmic colloidal pressure[723]. A low potassium solution would be suggested to cause potassium efflux from cells preservation solutions have been described utilising either of these principles with no benefit of one over the other demonstrated[705].

To further limit transmembrane flux impermeants can be used such as mannitol (182 Da), raffinose (504 Da), glucose (180 Da), lactobionic acid (357 Da), and gluconate (195 Da)]. These molecules are large enough to leave capillary vessels and enter the interstitial spaces but either too large or too charged to enter the cell. They exert sufficient osmotic pressure within the extracellular space such as to counter the physiological intracellular driving force and limit cellular oedema. However the impermeant content must be close to the intracellular concentration of osmotically active molecules in the 110 – 140 mM range[53].

During flushing and washout at retrieval there is a rapid equilibration between the solution and the interstitium with diffusion of solution contents into the extravascular space. A colloidal agent must thus also be used to limit interstitial oedema, and a number have been extensively investigated e.g. albumin (70 KDa), hydroxyethyl starch (HES, 250 KDa), polyethylene glycol (PEG, 20 – 35 KDa), and dextran 40 (40 KDa)[53].

Finally Ca2+ homeostasis should be maintained in several ways[724]. The ideal preservation solution should thus theoretically have a low Ca2+, can have a variable Na+ content with carefully adjusted K+ and magnesium (Mg2+) concentrations.

Intracellular Acidosis

During the hypoxia of warm and cold ischaemia anaerobic glycolysis prevails with formation of lactic acid and H+ ions. Furthermore hydrolysis of ATP yields further H+. A lack of ATP renders extrusion by the H+/ATPase and Na+/H+ exchanger inadequate and there is a progressive build up of H+ and development of intracellular acidosis[725]. Intracellular acidosis causes damage to cytoplasmic contents including mitochondrial membranes, cytochromic enzymes of the electron transfer chain, and components of the ER. Sufficient damage will result in necrosis or apoptosis once reperfusion is established. Buffers within the preservation solution can help to minimise the variations in pH that develop during ischaemia. Theoretically during reperfusion and availability of oxygen excess intracellular H+ can then be removed by endogenous mechanisms. Various buffers can be used including bicarbonates, phosphate, citrate, hydroxyethyl- piperazine-ethane sulphonic acid (HEPES), histidine, and tryptophan, but must be able to diffuse into the intracellular compartment, and function at low temperatures during hypothermic preservation[726,727].

Oxidative Stress

Finally the generation of ROS during ischaemia and on subsequent reperfusion is a key initiator and cause of cell damage during IR. During reperfusion cells endogenous anti-oxidant mechanisms are enabled in efforts to limit ROS mediated damage - such as superoxide dismutase, catalase, glutathione peroxidase and heme oxygenase-1 [728]. Extracellular availability of anti-oxidant species is limited by contrast and ROS scavengers in the preservation solution, such as allopurinol and reduced glutathione are theoretically beneficial[729].

1.7.1.4 Static cold storage preservation solutions used in clinical practice

There are several common themes to the various preservation solutions developed [730] in particular related to ameliorating sequelae that occur during IR injury (Table 3).

Collins

Collins was one of the first preservation solutions developed and was modelled on intracellular electrolyte levels with a high potassium and glucose as the impermeant[731]. Though this novel solution enabling successful preservation of the kidney, heart, liver and lung, the associated maximal

storage time was shortened. This was attributed to glucose aggravating anaerobic glycolysis. Modifications to Collins solution emerged - replacing glucose with mannitol or sucrose, which enable longer CIT. The final most widely used variant was the Euro-Collins modification in Europe and was in large scale use up until the late 1980s[732,733].

Marshall's citrate

Marshall's citrate solution was designed as an alternative to Collins solution with modifications to improve buffering and cellular oedema. It is a hyperkalaemic crystaloid solution with high levels of Na+ and Mg2+, and offered improved intracellular buffering with citrate. A variation using HEPES instead of citrate exists[734], but citrate theoretically also chelates calcium, preventing intracellular build-up. Mannitol is used as the impermeant to limit cellular swelling, and with Marshall citrate preservation of kidneys for 24-40h can be safely achieved[735]. Unfortunately this protection could not be afforded to the liver and so limited its application with multi-organ abdominal retrieval.

University of Wisconsin solution

Developed by Belzer and Southard UW solution is the gold standard for abdominal organ transplantation today. The primary strategy employed by UW can be summarized as: 1) Prevention of oedema (raffinose, lactobionate); 2) Supplementation with ATP precursors (adenosine); and 3) antioxidants and ROS defence (allopurinol and reduced glutathione)[720].

Inclusion of HES in UW is contentious. It was added to improve oncotic and colloidal support. However studies have shown HES can form red blood cell (RBC) aggregates during flushing, and at hypothermic temperatures the viscosity limiting effective washout[736].

Despite these potential flaws transplantation centres in the 1990s in the US and Europe demonstrated improved DGF rates in kidney transplants with UW. This was followed suit by improvements in liver transplantation, with safe preservation times extended up to 24h. Since then numerous comparative studies have shown UW to be superior over other solutions for pancreas, heart, kidney and lung preservation[736].

Bretschneider's (Custodiol) Solution

Histidine-tryptophane-ketoglutarate (HTK) otherwise known as Custodiol or Bretschneider's solution was first developed as a cardioplegia for use during cardiac bypass surgery in 1970s by H.J.

Bretschneider at the University of Gottingham in Germany[737]. The solution had three main components - histidine (as a buffering agent), and the amino acids tryptophane (a membrane stabilizer) and ketoglutarate (a substrate for anaerobic glycolysis), with mannitol as an osmotic agent. The potential benefits linked to development and use of HTK lies in the extracellular electrolyte composition wit low concentrations of Na+ , K+ , and Mg2+, with hypokalaemia perhaps limited vasospasm during flush and retrieval; furthermore because HTK had a low viscosity it was considered to be more effective than UW during organ flushing, which contained HES and which causes RBC aggregates[738]. However due to the low viscosity larger volumes at lower flow rates through the organ of HTK were needed to achieve complete tissue equilibration. Early studies demonstrated HTK to be equally effective as UW solution in kidney and liver preservation [739,740]. However later studies suggested that HTK was inferior in DCD liver preservation when extreme CITs of longer than 8> were encountered[741]. Despite critical papers HTK use with standard CITs in kidney and liver transplantation is considered safe and as effective as UW solution. Furthermore lower preservation costs per donor case with HTK meant it has become increasingly popular over last 20 years[742].

Recently modifications to HTK have been reported in efforts to enhance the protective capacity of the solution, newly named Custodiol-N [743]. Changes include addition of the amino acids glycine and alanine (to inhibit hypoxic cell injury), the iron chelator deferoxamine, and a new membrane-permeable hydroxamic acid derivative LK 614 to inhibit ROS injury. Furthermore histidine has been substituted with the derivative N-acetyl-L-histidine (due to the theoretical risk of toxic side effects in combination with oxygen), L-arginine to aid NO production, and aspartate for resumption of the TCA Kreb's cycle during reperfusion.

Celsior Solution

Celsior is recently developed solution extracellular type solution with a high Na+ concentration. It was initially developed for cardiac allograft preservation and sought to combine benefits of various components of UW solution (lactobionate and mannitol - osmotic agents) and from HTK (histidine - buffering agent). Glutathione is added before use as ROS scavenger. The use of solution has been extended from cardiac preservation and has demonstrated successful preservation in the lungs, pancreas, and kidney [744–746].

Kyoto University Solution

A new preservation solution has recently been developed at the Kyoto University in Japan. The theoretical basis of this solution is the importance of saccharides and electrolyte balance during hypothermic preservation in limiting cellular oedema and the solution contains trehalose, an alpha linked disaccharide[747]. The osmotic strength of the solution is further augmented by gluconate and HES. There are two primary variants with an intracellular (low Na+, high K+) and extracellular (high Na+, low K+) electrolytic composition.

Further improvements to Kyoto solution can be made by addition of ROS scavengers (N-acetylcysteine), secondary messenger compounds dibutyryl cAMP and even a NO donor (nitroglycerin) and such modified Kyoto solutions have demonstrated superior preservation in experimental lung and liver transplantation compared to UW solution [748,749].

IGL-1

The Institute Georges Lopez (IGL) in France has developed IGL-1, a preservation solution in composition identical to UW solution with exception to the substitution of the colloid HES for PEG-35 [750], which alleviates potential stimulus of RBC aggregation and adds potential benefits of PEG.

In large animal porcine renal transplantation models IGL-1 preserved kidneys demonstrated reduced MHC type II, cellular apoptosis and interstitial fibrosis, and superior GFR and renal function at 15 days post op compared to UW solution[751,752]. In a non-randomised prospective multicentre trial IGL-1 preservation of deceased donor kidneys (20% DCD) compared to UW solution showed a trend of better renal function in the initial 2 weeks with similar rates of DGF, and patient survival at 12 months follow-up, demonstrating equality to UW solution [753].

A series of pilot studies successful in liver preservation for orthotropic liver transplantation, with better survival, and histology demonstrating less necrosis and better hepatic microcirculation after reperfusion[754,755]. Most recently a porcine pancreas transplant model demonstrated equal outcomes and effectives of IGL-1 for preservation as compared to UW solution [756].

The use of PEG as substitute colloid for organ preservation is based on a number of observations that suggest PEGs (with low molecular masses) may confer a protective benefit in transplantation settings [757]. Firstly, unlike HES it does not form aggregates with RBCs during flush and washout

within the allograft. However the exact mechanism of the protective actions of PEG have not yet been fully elucidated but PEG is thought to act by stabilisation of the lipid monolayer in cell membranes. In part by 1) dehydrating action to reduce lipid molecular motion and cause denser packing of membrane lipids, decreasing fluidity; 2) at higher surface pressures PEG induces compression of glycerophospholipids. Temperature and pH of the local environment both influence PEG binding and it may be that at hypothermic temperatures that most beneficial effects on membrane stabilisation can be seen. However the act of binding to cell membranes and conferring relative stability may also have an indirect effect coined 'immunocamouflage' [757]. Binding of PEG to cell membranes creates organised layers of 'structured water' which act as a 'bumper' preventing approach, interaction, and fixation of immune leucocytes during reperfusion[758]. Limiting leucocyte endothelial interactions is beneficial, and can limit subsequent proliferation of the IR inflammatory response in the allograft and ameliorate subsequent IR damage, as such injury has a negative impact on graft outcome.

Aqix RS-I

Aqix RS-1 (Aqix Ltd, UK) is a recently developed SCS preservation solution that is undergoing experimental studies[759]. It based on a non-phosphate-buffered solution, with organic buffers, amino acids and metabolic substrates and possesses the characteristics of normal physiological interstitial fluid. Aligning Aqix composition to interstitial fluid theoretically enables the solution to maintain the ionised status of cell membranes, as well as function of enzymatic and receptor components. At 286mOsmol/L the osmolarity is similar to serum, the ionic content equal to that of can isotonic solution, and ionic conductivity comparable to human serum at 12.6 mS cm^{-1}. The combination of bicarbonate and Good's BES asits buffer allows pH to be maintained between 7.2 and 7.46, over a large temperature range of 4-37˚C, demonstrating the flexibility of the solution and the potential use in transplantation in moving from hypothermia to normothermia.

Initially designed for neurophysiological studies, its ability to preserve tissue in hypothermic, and normothermic conditions has lead to interest in its use in organ preservation. Predinical porcine studies in kidney and liver preservation demonstrate improvements in organ function compared to conventional solutions at both hypothermic[760] and normothermic [759,761] preservation temperatures.

	Preservation Solutions										
	Collins	Euro Collins	Belzer UW	HTK	Celsior	PBS	HOC	Custodiol-N	IGL-1	Belzer-MPS	Polysol
Sodium	10	9.3	25.0	15.0	100	120	80	16.0	120	100	136.0
Potassium	115	115.1	125.0	10.0	15	-	80	10.0	30	25.0	5.0
Calcium	-	-	-	0.015	0.25	-	-	0.02	-	0.5	9.0
Magnesium	30	4.7	5.0	4.0	13.0	-	35	8.0	5	5.0	14.0
Chloride	15	15.0	-	51.03	41.5	-	-	30.04	20	1.0	-
Phosphate	50	57.6	25	-	-	60		-	25	25	0.8
Sulfate	30	4.7	5.0	-	-	-	40	-	5	-	4.0
Bicarbonate		9.3		-	-	-	-	-	-	-	25.0
Glucose	25	1.9		-	-	-	-	-	-	10.0	16.0
Gluconate	-	-	-	-	-	-	-	-		85	95
Glutamic acid	-	-	-		20.0	-	-	-	-	-	-
Raffinose	-	-	30.0	-	-	-	-	-	30	-	-
Lactobionate	-	-	100	-	80	-	-	-	100	-	-
HES (g/L)	-	-	50	-	-	-	-	-	-	50	-
Citrate	-	-	-	-	-	-	55	-	-	-	-
Histidine	-	-	-	180.0	30.0	-	-	124.0	-	-	-
Adenosine	-	-	5.0	-	-	-	-	-	5		5.0
a-ketoglutarate	-	-	-	1.0	-	-	-	2.0	-	-	-
Adenine	-	-	-	-	-	-	-	-	-	5.0	5
Pyruvate	-	-	-	-	-	-	-	-	-	-	0.2
Allopurinol	-	-	1.0	-	-	-	-	-	1	1.0	1.0
Glutathione	-	-	3.0	-	3.0	-	-	-	3	3	3.0
Mannitol	-	32	-	30.0	60.0	-	185	30.0	-	30	-
Tryptophane	-	-	-	2.0	-	-	-	2.0	-	-	-
Ribose	-	-	-	-	-	-	-	-	-	0.5	-
HEPES	-	-	-	-	-	-	-	-	-	10.0	-
PEG-35	-	-	-	-	-	-	-	-	0.03	-	8
Amino acids	-	-	-	-	-	-	-	-	-	-	11.0
Phenol red	-	-	-	-	-	-	-	-	-	-	0.07
N-Acetylhistidine	-	-	-	-	-	-	-	57.0	-	-	-
Sucrose	-	-	-	-	-	140	-	33.0	-	-	-
Aspartate	-	-	-	-	-	-	-	5.0	-	-	-
Glycine	-	-	-	-	-	-	-	10.0	-	-	-
Alanine	-	-	-	-	-	-	-	5.0	-	-	-
Arginine	-	-	-	-	-	-	-	3.0	-	-	-
Deferoxamine	-	-	-	-	-	-	-	25.0	-	-	-
LK 614	-	-	-	-	-	-	-	7.5	-	-	-
pH	-	7	7.4	7.2	7.3	7.1	7.1	-	7.4	7.4	7.4
Osmolality (mOsm/kg)	-	375	320	310	320	310	400	-	320	-	320
Relative price (US$)	-	275	200	181	228	-	-	-	184	200	-

Table 3 Composition of common preservation solutions. PBS: Phosphate Buffered Sucrose. Units of solutes are in mmol/L unless stated otherwise. Data is collated from several sources[62,751,762–767].

1.7.1.5 Comparison of Solutions for Cold Storage in Kidney and Pancreas Transplantation

Kidney

UW solution remains the most widely used preservation solution for kidney transplantation worldwide (See Figure 17). In the UK 2012 Guidance to the National Organ Retrieval Service recommended UW solution use for all in-situ perfusion during DCD organ retrieval, especially in multi-abdominal organ retrieval (liver, kidney, pancreas or liver and kidney), while for kidney alone Marshall's solution is an alternative to UW. In DBD donors apart from multi-organ kidney, pancreas, and liver retrieval where UW is preferred, Marshall's Citrate or UW solution is recommended. For back table perfusion and SCS packing UW is preferred for the liver and pancreas, while Marshall's or UW solution can be used for the kidney [768]. In the UK Marshall's citrate is the most commonly used preservation solution, largely on account of its relatively low cost [769].

Despite the widespread use of UW solution worldwide in organ and especially kidney preservation from DCD donors, there is conflicting evidence for on benefits of UW compared to other SCS preservations solutions.

Recently a meta-analysis of 15 prospective trials including 3584 kidney transplants systematically examined effects of various preservation solutions on DCD kidney outcome - specifically DGF[770]. Euro-collins was associated with a higher risk of DGF than UW and HTK in two RCTs each respectively. There was an equal risk of DGF when using UW compared with Celsior (3 RCTs) and HTK (2 RCTs). Only two RCTS compared UW to HTK (no meta-analysis possible) both reported similar rates of DGF. Unfortunately there was limited data regarding other comparisons and outcomes to form any further conclusions but the data does suggest there is no difference in the incidence of DGF with the use of the three dominant solutions Celsior, HTK and UW.

A number of retrospective trials have also examined outcomes with UW preserved kidneys compared to HTK. A recent single centre retrospective review reported that equivalent patient survival, allograft function, rejection , and graft survival at 2 years compared to UW. HTK use however conferred a higher risk of early (<6 months) allograft loss, attributed to PNF, and exclusively in ECD allografts. Suggesting HTK may not be suitable for use with ECD donors[771]. Similarly analysis of the UNOS database for DBD/DCD kidney transplants showed no increased risk for DGF, but an increased risk of death-censored graft survival for allografts preserved with HTK (n=5728)

versus UW (n=15898)[741]. Based on this recent data the routine use of HTK in abdominal organ retrieval, especially from ECD donors, has come into question.

A recent single centre retrospective review of DBD/DCD kidney transplants from France comparing UW (n=610) to Celsior (n=201) preservation found similar allograft function at 1 month and at 1 year, without a difference in 5y survival[772]. With similar results from another retrospective analysis, with no difference in DGF or rejection, nor graft and patient survival at 3 years [746].

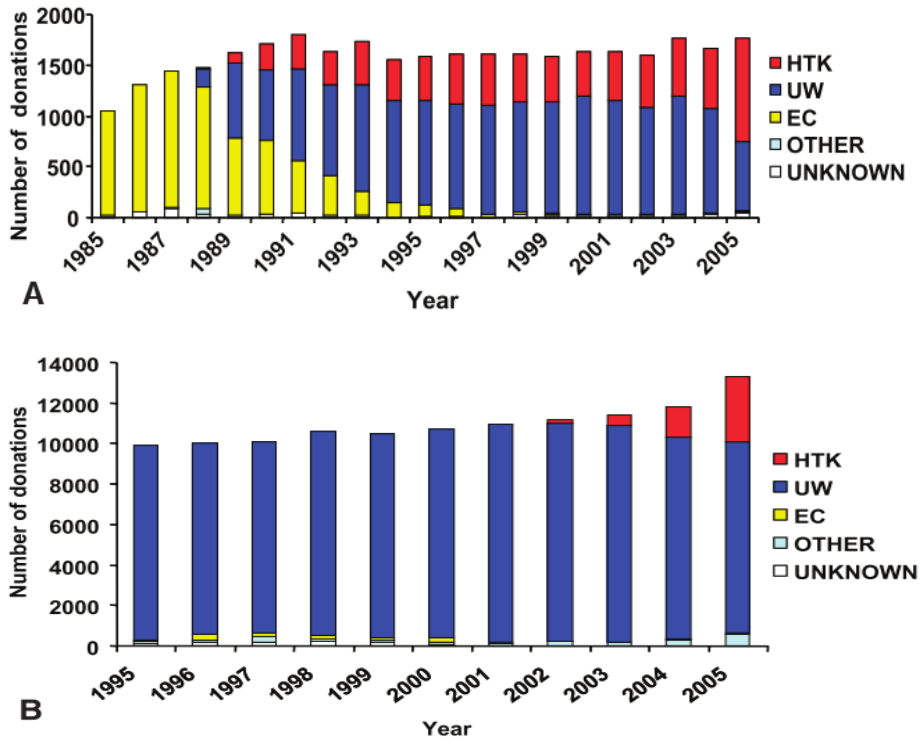


Figure 17 Cold storage solutions in use in the Eurotransplant Region and the US. (A) Cold storage solutions in Eurotransplant region in deceased donors from 1985–2005. (B) Cold storage solution in the United States in deceased donors from 1995–2005. Adapted from Maathuis et al. [736].

Pancreas

Belzer UW is the most commonly used SCS preservation solution in the world for pancreas preservation[682]. It is also the only solution recommended for pancreas retrieval in the UK[768]. Potential alternatives to UW are Celsior and HTK, and several investigators have examined their potential use in clinical preservation.

The largest published retrospective review comparison of HTK (n=258) vs. UW (n=50) use for pancreas preservation from the University of Indiana reported equivalent outcomes in terms of graft function, survival, and patient survival with each solution[773]. Similarly the only RCT comparing UW (n=27) to HTK (n=41) for pancreas preservation recently reported similar graft and patient outcomes[774].

Analysis of registry data however from UNOS of all pancreas transplants performed in the US between 2004 and 2008, of allografts preserved with HTK (n = 5728) vs. UW (n = 15 898) indicated that despite no effect on DGF, there was an increased risk of death-censored graft loss, predominately for loss after 12 months[741]. This registry data is supported by a recent retrospective analysis that highlighted an increased risk of pancreas allograft DGF, and post-operative complications (e.g. graft thrombosis) in HTK preserved allografts compared to UW, but with no impact on graft or patient survival[775]. This registry data must however be considered with caution regarding thrombotic complications because as mentioned anti-coagulation is not uniform in practice. A further retrospective comparison in 2 centres in Michigan reported similar graft and patient survival, with similar allograft function at 90 days, but with more acute rejection within the first 180 days with HTK use(25% vs. 9.8%) vs. UW. [776] . The worrisome reports from these analyses, despite other evidence indicating an equivalency of HTK to UW are likely to prompt surgeons to remain cautious of HTK and prefer UW.

Equivalent outcomes for UW and Celsior in pancreas transplantation have been reported by two RCTs[777,778]. Both were small studies however and a power calculation was not conducted to determine if patient numbers were sufficient. A further comparative trial demonstrated similar equivalency, however the where significant differences in characteristics between groups such as recipient age and BMI, and surgical technique employed that could have confounded and biased results[779]. With more use of DCD donor organs with lengthier WITs, and ECD donors of increasing age, the effect of co-morbidities and ischaemic damage on these organs is increased with the

potential for inferior function and survival after transplantation. Considering the dominance of SCS preservation methods the past 4 decades, and the many transplant programmes facilitated by this effective form of preservation, the challenge of maintaining allograft viability in the face of these increasing numbers of ECD and DCD organs that are being used worldwide to expand donor pools has most likely reached the limits of basic SCS preservation.

Despite the emergence of numerous new SCS preservation solutions designed to address mechanisms of this injury, SCS at the most effective level only slows the extent of ischaemic damage. To improve organ viability a more dynamic and active form of preservation is needed to both fulfil the metabolic needs of the organs, as well as intervene and prevent damage during reperfusion. HMP of organs is a preservation modality that can potentially address the needs of such as strategy, and many groups have moved towards machine perfusion revisiting its possibilities in clinical practice.

1.7.2 Machine Perfusion Organ Preservation

The growing need for kidney transplantation stems from it being the optimal treatment for patients with end-stage renal failure[780] and providing the best clinical outcomes, optimal quality of life and cost savings when compared with other modalities of renal replacement therapy[2]. In the UK in 2005 approximately 40000 adults were receiving renal replacement therapy – a 28% increase in the number of patients since 2000, with median patient age for the start of replacement therapy 65 years. In patients on replacement therapy the 1 year survival rate was 79%, with 5 year survival rates depending on patient age; 58% if aged 18-34, and 12% if greater than 75 years[4]. Comparatively the most recent 1 and 5 year patient survival rates for kidney transplant recipients in the UK stood at 96% and 83% respectively. Despite the demonstrated advantages of transplantation, the full potential of these benefits cannot be obtained due to the severe shortage of donated kidneys[781]. Recent strategies to improve access to organs suitable for transplantation include increasing utilization of kidneys from deceased donor cohorts previously considered unsuitable for transplantation, and development of newer methods of preservation superior to SCS, specifically HMP as a tool for organ viability assessment and optimization.

1.7.2.1 The Need for Improved Preservation

Efforts underway to expand the potential donor pool have included use of 'Expanded Criteria' (ECD) or 'marginal' kidney donors, as well as increasing use of kidneys from non-heart beating donors (NHBD – now donation after cardiac death, DCD). According to the United Network for Organ Sharing (UNOS) marginal or ECD kidneys are from donors aged above 60 years of age, or above 50 years of age with two or more of: HTN, history of CVA, or terminal creatinine levels greater than 133 micromoles/litre. NHBD or DCD donors are classified by the Maastricht criteria. The majority of kidneys from DCD donors are from Type II (cardiac arrest with unsuccessful resuscitation) and III (expected cardiac arrest / withdrawal of treatment) Maastricht categories. DCD kidneys thus may experience prolonged periods of warm ischaemia, and this manifests as higher rates of post-operative graft dysfunction[782], specifically delayed (DGF) >80%, and primary (PNF) >9%, non-function in transplant recipients of Type II category kidneys; compared to 23% DGF in cadaveric heart beating donors[783]. Similarly kidneys from marginal and ECD donors are presumed to be of compromised quality and more susceptible to ischaemic injury, and when used in clinical transplantation are also associated with increased rates of both DGF and PNF when compared to

non-marginal donor kidneys[784]. DGF itself necessitates continued dialysis, and is associated with a then increased risk of acute rejection and poorer long term outcome [736].

Almost 100,000 patients are registered on the United States organ transplant waiting list awaiting a suitable kidney[785]. In the UK the number of patients awaiting a kidney transplant has risen by 16% since 2001 with 5881 patients currently on the national kidney transplant waiting list [6], with 2804 new registrations, and 2142 deceased donor kidney transplant performed in 2013-2014. The majority of patients wait approximately 3 years to receive a kidney. Of a total of 1243 deceased kidney donors, 521 were DCD donors, and 821 transplants resulted from such donors; an annual increase of 10%, and an increase of 558% over the last decade. The DCD donor cohort is the largest growing group of donors. In comparison the number of DBD kidney transplants is stable having risen only 2% to 1321 transplants in 2013 since 2004. Overall only 83% of DCD kidneys retrieved were transplanted, compared to 87% of DBD kidneys.

Furthermore deceased donors profiles are changing with more donors trending to be classified as ECD being older (37% >60 years of age vs. 14% in 2003), with higher a BMI (24% > 30 BMI, vs. 16% in 2003), and subsequent more co-morbidities that will affect transplant outcome[9].

With the use of ECD and DCD donors to meet the transplant demand, strategies are needed to optimize the viability of donated kidneys and ensure they function to the best of their ability. Optimising organ viability begins with choice of preservation method. Currently there are two dominant methods for preservation – SCS and HMP.

As discussed in previous sections SCS involves flushing the retrieved organ with a preservative solution - commonly Marshall's hypertonic citrate (Soltran, Baxter Healthcare) or Belzer UW solution (Viaspan, Bristol Myers Squibb), and then packing on ice for transport. SCS preservation relies on the suppression of cellular metabolism via hypothermia as the organ is flushed free of blood, and replaced by the infused cold preservative solution. Most centres in the UK use SCS to preserve kidneys for transplantation. This method of preservation however was developed in an era of younger donors with higher quality organs[736] and with the recent trend of diminishing availability of heart beating donors, and increasing reliance on marginal and DCD organs, the limitations of using SCS for effective preservation is thought by many in the field to have been reached.

At present a kidney allograft may be preserved with SCS and retain viability up to 24 hours in clinical scenarios and theoretically 120 hours for research purposes (see Table 4).

	Clinical (hours)	Experimental (hours)	Optimal SCS Solution
Kidney	24	120	<24 h: UW, HTK, PBS >24 h: UW
Pancreas	12	96	UW, HTK
Liver	12	48	UW, HTK

Table 4 Current status of clinical and experimental cold storage preservation of abdominal grafts. Adapted from 'tHart et al. [786].

Increasing cold ischaemia time is associated with delayed graft function, and post operative complications in liver, cardiac, pancreatic, and renal allografts[787] at transplantation. As mentioned with current initiatives to expand donor pools, marginal organs will be increasingly more prone to ischaemic injury, and preservation techniques developed for use with 'ideal' grafts may be inadequate for these more susceptible organs. This is invariable with the case of recipients of kidney grafts preserved with conventional cold storage from DCD donors who experienced rates of primary non-function, and delayed graft function 2-4x as high as recipients from DBD donors[788]. HMP as a method of preservation that may provide additional benefit in such marginal allografts [789].

1.7.2.2 Historical Development of Machine Perfusion

One of the first attempts at isolated organ perfusion was by Langendorf in 1895 when he devised a simple non-circulating organ perfusion apparatus consisting of a medium reservoir and a siphon tube connected to the organ, with the perfusate driven by gravity.

Development of perfusion techniques as a method to preserve organs outside the body to make organ transplantation a potential reality really only began after the introduction of the first successful technique for vascular anastomosis by Alexis Carrel in the early 1900s. By the 1930s Carrel in collaboration with famed aviator and innovator Lindbergh after 10 years of work successfully designed one of the first ever sterile all glass pulsating perfusion apparatus [790] to extracorporeally perfuse a whole organ (Figure 18). This innovation gained both Carrel and Lindbergh international fame and paved the way for Carrel being awarded the Nobel prize for Physiology and Medicine in

1912. Research into organ perfusion and preservation techniques was halted with the advent of world war II and it was only in the 1960's that interest in organ perfusion resumed.

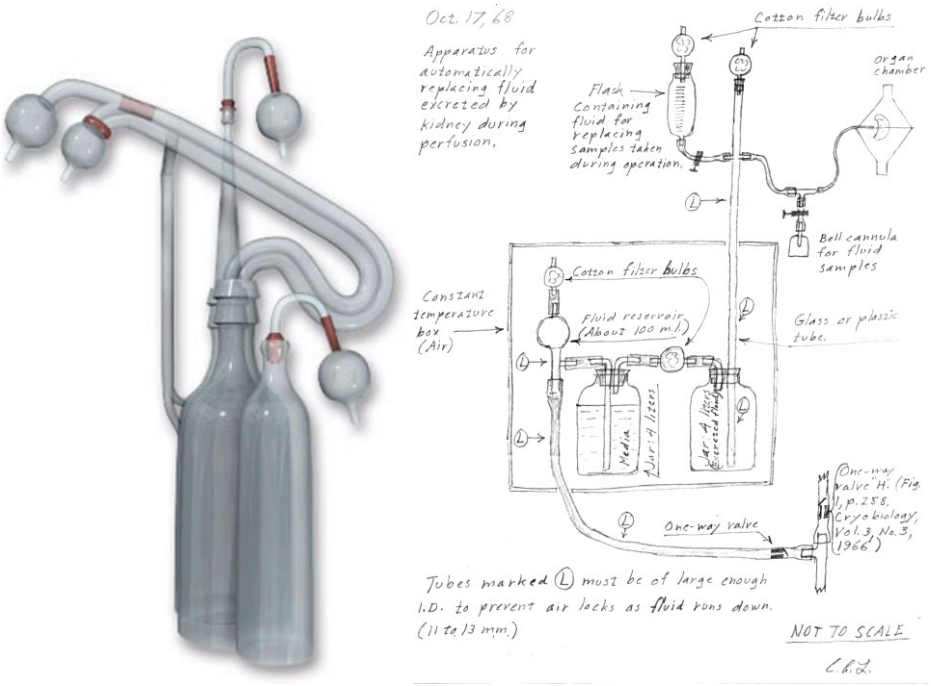


Figure 18 Model of a Lindbergh glass perfusion pump (left) and schematic of Lindbergh's perfusion apparatus dated 1966. Adapted from Cowan et al. [791]

In the early 1960s a group at the Naval Medical Research Institute were able to recreate the perfusion apparatus Carrel had developed 30 years previously, and successfully publish notable articles on mammalian ex-vivo perfusion[790]. Finally Carrel and Lindburgh's original work gained widespread recognition at the Tissue Culture Association meeting in Miami in 1964. That same year Lillehei and his associates published they had successfully preserved in-vitro for 24hours a canine kidney which functioned normally on re-implantation in the original animal[790].

By 1965 Belzer and Najarian had started a kidney transplant program for living and decreased donors in San Francisco. It was here that Belzer began what would be the most important development in research into machine organ perfusion. In contrast to the glass Lindbergh perfusion apparatus which incorporated a gas-driven and gravity based pump system for perfusion, the Belzer apparatus incorporated two mechanical pumps. Use of this relatively novel technology made the apparatus much larger than Lindbergh's pump, at the size of motorcycle, but which still remained relatively transportable, with use of a specially modified truck (Figure 19). Canine experiments of renal transplantation using HMP preservation for 72 hours showed promising results and two years

later the first human cadaveric kidney transplant preserved by HMP for 17 hours was performed. The graft functioned immediately and HMP became a distinct reality. Several more successful transplants followed in the next few years and by the early 1970s Belzer unsuccessfully applied HMP to livers and hearts.[790].



Figure 19 Photograph of the Belzer Perfusion Machine. Adapted from Belzer et al. [792].

By the mid 1970s the development of an effective SCS solution by Collins that successfully met the clinical requirements for renal transplantation saw SCS, rather than HMP, rapidly adopted by most clinical transplant centres around the world [18,790].

With the development of improvement of liver and pancreas transplantation in the 1980s after introduction of newer immunosuppressive agents Belzer once more attempted to expand HMPs use in clinical transplantation. Initial forays into pancreatic perfusion were promising but technical difficulties left further work abandoned, and HMP remained a clinical entity only for renal transplantation for the next 20 years [18,790].

1.7.2.3 Machine Perfusion in the UK

Though HMP continued to be used in isolated centres in the UK, US and Europe, over the past two decades, due to technical, logistical, and economics costs of instituting and running HMP systems SCS – which seemed to be the cost effective method to provide sufficient preservation – was the dominate method of organ preservation the world over [789]. The potential benefits of HMP over SCS have however led to a recent resurgence of interest in machine organ preservation and its use in clinical practice (Figure 20). The most recent data suggests that 25-35% of all kidneys in the US undergo HMP[793], with and up to 67% of DCD donor kidneys [10].

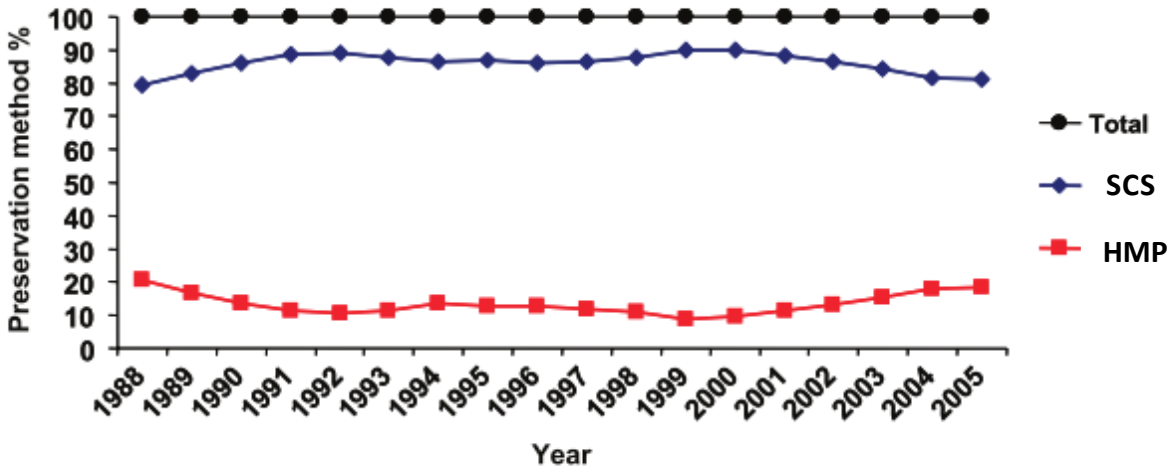


Figure 20 Use of HMP and SCS in the United States between 1988 and 2005. Chart is based on the Organ Procurement and Transplantation Network data; October 2006. Recent data from 2009 suggests HMP use has increased to 36%. Adapted from Maathuis et al. [736]

Currently in the UK out of 21 kidney transplant centres in England and Wales 8 centres report use of machine perfusion for kidney preservation in clinical practice, and according to the BTS between 2000-2007 2% of all kidneys from deceased donors underwent HMP, rising to 20% of kidneys from the DCD subset[4]. Comparatively in the US the use of HMP increased substantially and by 2008 data from the Organ Procurement and Transplantation Network showed 20% of all kidneys in the US underwent HMP, including 65% of DCD (non-ECD) kidneys, and 50% of all ECD kidneys[794]. The renewed interest in HMP lies in the merit for the potential 'rescuing' of marginal, and DCD kidneys that are increasingly being used in clinical practice[795]. HMP provides a viable conduit for organ viability assessment and modulation, in a subset of the donor pool (DCD and ECD donors) that is likely to increase in size over the ensuing years as the waiting list demand for organs increases. The ability to evaluate these organs, and select organs for transplantation based on viability could significantly increase the number of available organs in the donor pool.

In clinical practice a kidney would be retrieved, flushed with cold storage solution, and either immediately attached to and perfused on a portable perfusion device, or transported using SCS back to the base hospital for perfusion on bench-top perfusion machine. In both settings multiple parameters related to perfusate flow through the kidney can be measured. These perfusion dynamics include – 1) pulse pump rate, 2) perfusate temperature, 3) perfusion pressure (systolic, diastolic and mean pressures), 4) real-time flow and 5) vascular resistance. In addition to the

capability of monitoring perfusion dynamics, the circulating perfusate can be sampled for levels of damage and injury biomarkers. In both instances perfusion dynamics and biochemical perfusate analysis have been described in the assessment of organ viability and suitability for organ transplantation.

1.7.2.4 Theoretical Basis of Hypothermic Machine Perfusion and Graft Re-conditioning

HMP of an organ generates a controlled re-circulating flow of preservative solution at hypothermic temperatures in the 0–4°C range[796]. This continuous flow permits perfusion of the organ promoting a washout of blood and equilibration with the perfusate medium; delivering oxygen and nutrients and removing toxic metabolites. In addition it allows for the possibility of real-time viability assessment, provision of metabolic support during perfusion, and the potential for addition of pharmacologic agents to the circulating perfusate that can potentially improve perfusion parameters and organ viability[797]. The benefits of HMP are summarised schematically in Figure 21. Importantly retrospective reports and evidence of lower rates of DGF in machine pumped kidneys, have been augmented by the recent publication of an large international prospective RCT, confirming a definitive and significant benefit on DGF and both 1 year and 3 year survival rates in machine perfused kidneys, especially so in ECD kidneys, compared to SCS [798].

HMP is based on the concept of preserving the organ in a 'better environment' [799]. Similar to SCS HMP slows down metabolism, reducing oxygen requirements and ATP depletion, by circulating a cold preservative solution through the organ via pulsatile or non-pulsatile flow, theoretically providing nutrients, and facilitating washout out of toxic metabolites and free radicals.

The mechanisms of HMP improving allograft viability are based on the concepts of hypothermic reconditioning[800]. Evidence suggests that HMP acts by preserving allograft energy metabolites, acting to suppress programmed cell death and instead promote autophagy, and limiting endothelial activation that upon reperfusion would potentiate a greater inflammatory response. [801]

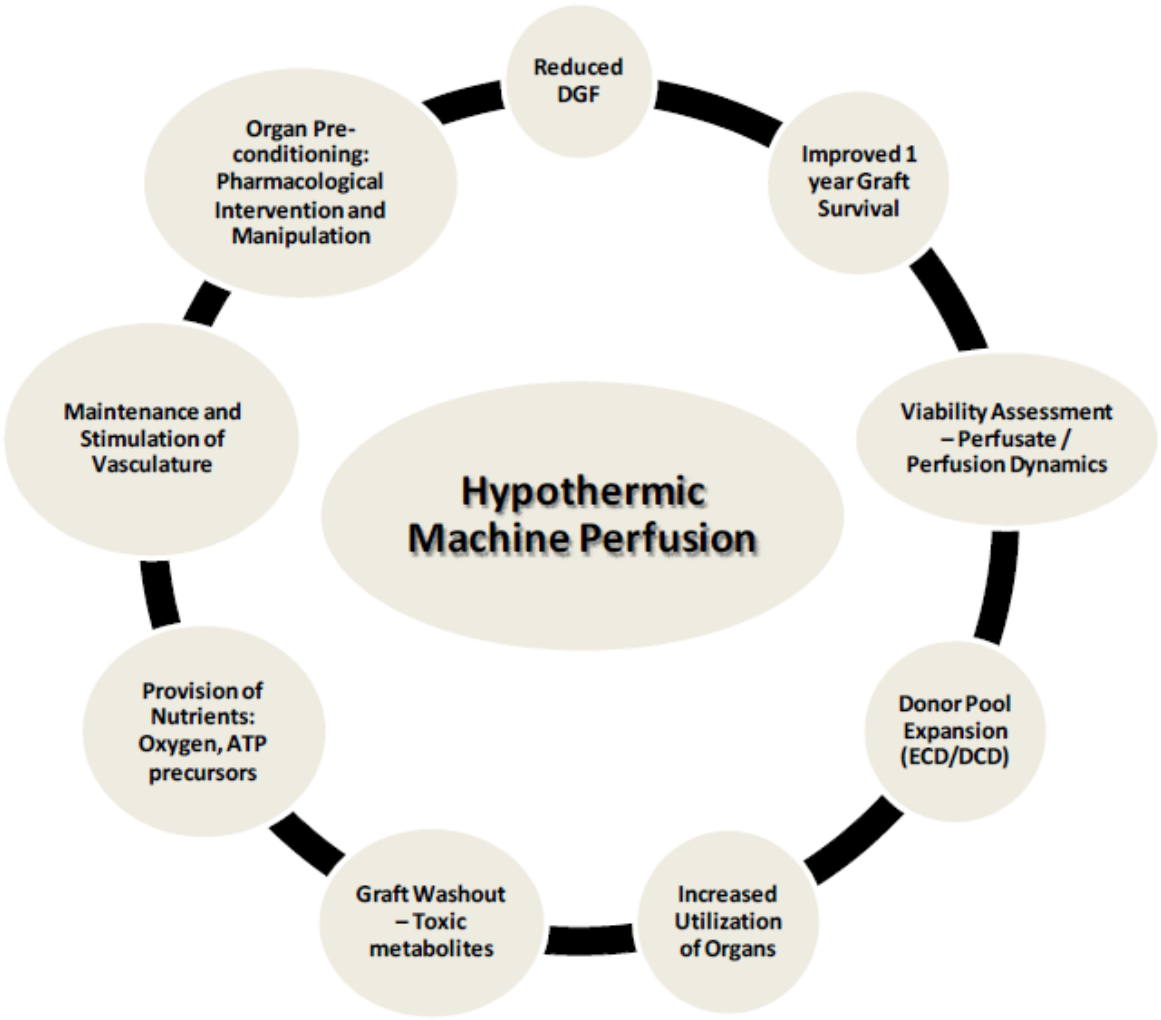


Figure 21 Schematic summary of the important benefits of ex-vivo HMP for renal preservation prior to transplantation. Benefits can be divided into physiological and theoretical vs. clinical [802].

HMP and Oxygen

An important aspect of HMP is perfusate oxygenation[803,804]. The 1st generation of commercially available renal perfusion machines (RM3 from Waters Medical Systems, Lifeport from Organ Recovery System) do not have an inbuilt hollow-fibre in-line oxygenator within the perfusion circuit. Hollow-fibre in-line oxygenators are used in cardiopulmonary bypass machines and are an effective method of perfusate oxygenation. Both machines depend on atmospheric pressure to oxygenate the perfusate, the RM3 unit by passively sweeping room air (21% O_2) over the semi-permeable

membrane within the cassette at about 2–4 L/min, while the LifePort has no active or passive method of perfusate oxygenation and instead relies directly on atmospheric pressure.

From commercial literature the 2nd generation of renal perfusion machines by these companies have been designed with the ability to oxygenate the perfusate. These apparatuses have only recently gained FDA and CE approval, and as of yet no reports have been published on their use clinically or in research settings.

In a recent study comparing the two 1st generation machines, the RM3 and Lifeport, researchers performed fluid gas analysis on the perfusate during 24 hours HMP of canine kidneys [805]. Oxygenation was by atmospheric pressure with both machines, and in both cases such application provided a perfusate PO_2 between 22-24 kPa (175-180 mmHg). At hypothermic temperatures (4-7°C) metabolism is sufficiently suppressed that such oxygenation would theoretically be suitable for the remaining active energetic processes [722,730].

Oxygenation during HMP is not a new idea when three decades ago oxygenated perfusate during 48 hours of HMP was shown to have restored 50% of the ATP:ADP ratio in ischaemically damaged rabbit kidneys[806,807]. Furthermore a number of studies have reported that with non-oxygenated HMP ROS mediated damage (measured by lipid peroxidation) is elevated despite better graft function compared to SCS preservation, suggesting hypoxic injury during HMP may be implicated[808–810].

Several studies using porcine auto-transplantation models have demonstrated approximately 20 hours of actively oxygenated HMP to be superior to SCS, with improved cortical microcirculatory perfusion, less proximal tubular damage, less ROS mediated damage, less cytokine expression, and better allograft function after transplantation[811,812]. Buchs et al.[813] demonstrated using magnetic resonance imaging that oxygenated HMP restored ATP levels in ischaemically damaged porcine DCD kidneys (30 minutes WIT), but with no benefit in non-damaged kidneys. There are conflicting reports however where 21 hours of oxygenated vs. non-oxygenated HMP in a porcine kidney autotransplantation model demonstrated no benefit to active oxygenation, with equity of functional outcomes[814].

Further evidence of the benefit of hypothermic oxygenation however is found from liver studies where spectroscopy demonstrated re-oxygenation of cytochrome oxidases in SCS preserved rat

livers after 2 hours of oxygenated HMP[815]. In these livers mitochondrial dysfunction upon reperfusion was avoided, which is central to restoring normal physiological tissue function, and maintaining tissue homeostasis.

Hypothermic oxygenation after SCS also preserved mitochondrial and cytosolic HSP70 during isolated rat liver perfusion, with early resumption of aerobic metabolism and exocrine function [816]. Because mitochondrial damage is critically linked to mechanisms of programmed cell death, ameliorating mitochondrial injury through hypothermic oxygenation can avoid progression to cellular apoptosis and potentially necrosis, and instead initiate autophagy and tissue repair[817,818].

Promoting effective autophagy, permitting apoptosis, and limiting cell necrosis through hypothermic oxygenation may also limit activation of innate immune responses upon reperfusion. Release of DAMPs by necrotic cells provides ligands for innate response receptors. The high mobility group box B1 (HMGB-1) protein once released during reperfusion is recognised by toll like receptors (TLR-4) both on immune cells and also on the vascular endothelium, promoting a pro-inflammatory and pro-thrombotic phenotype within the allograft during reperfusion. In a porcine autotransplant model hypothermic oxygenation reduced levels of such DAMPs (HMGB-1), as well as TLR-4 expression, and improved allograft performance[812]. In a similar model active oxygenation during HMP improved both short term and long term (3 months) allograft function, with less fibrosis and chronic inflammation compared to non-oxygenated HMP pumped kidneys[819]

Notwithstanding it must be remembered data from the large multi-centre Eurotransplant RCT has also established the limited but significant superiority of non-oxygenated HMP over SCS in clinical kidney transplantation[820]. At present no trials have been performed investigating the efficacy of oxygenated HMP in clinical practice.

The Endothelium

The endothelium has a key role in IR injury. Newer research points to a vasoprotective and endothelial mediated effect of HMP. HMP of porcine livers reduced endothelial von Willebrand factor (which is upregulated in endothelial activation and IR injury), and platelet aggregation after transplantation. Analysis of perfusate from kidney HMP compared to the 'flush' solution from SCS stored kidneys demonstrated that after HMP there was Providing a potential mechanism for HMP to

permit a reduction in leukocyte activation and a decrease in IR activated inflammation during reperfusion[821].

It is now known that endothelial gene expression is strongly influenced by vascular shear stress[822]. During organ retrieval and SCS the cessation of vasculature flow promotes a pro-inflammatory and pro-coagulatory phenotype, which could worsen IR responses after transplantation[263]. Flow mediated endothelial dysfunction is linked to decreased expression of the mechanosensitive transcription factor Kruppel-like-factor 2 (KLF-2), which normally suppresses inflammatory signals and cytokine production[261]. HMP has been shown to restore levels of KLF-2 compared to SCS during reperfusion[823].

1.7.2.5 Clinical Benefits of Hypothermic Machine Perfusion vs. Static Cold Storage

The benefits of HMP compared to SCS in kidney preservation can be seen in both experimental and clinical studies and include improved rates of delayed graft function and patient survival. Not all studies however link HMP use with decreases in rates of DGF and improved patient survival. Several recent studies examining this comparison deserve to be mentioned.

In 2003 a meta-analysis by Wight et al. of published literature comparing SCS to HMP between 1971 and 2001, the paper confidently concluded that use of HMP is associated with a 20% risk reduction for DGF compared to standard SCS (even though the studies included were small and heterogenic).

In 2005 Schold et al. examined the use of HMP on ~85000 kidney transplant recipients between 1994 and 2003 through the Scientific Registry of Transplant Recipients in the US, and reported that HMP use led to a significantly decreased incidence of DGF compared to SCS (19.6% vs. 26.6%), but without a significant effect on long term survival[824].

In 2006 Matsuoka et al reported analysis on 4618 ECD kidneys registered with the United Network for Organ Sharing in the US between 2000-2003. Use of HMP in management of 912 ECD kidneys reduced the incidence of DGF (26%) compared to 3706 ECD kidneys (37%) managed with standard SCS. Despite kidneys allocated to the HMP group having a greater number of risk factors negatively affecting viability a similar 3 year patient survival rate to was evident between HMP and SCS kidneys. The group also concluded that the decrease in DGF may have a cost-effective benefit to offset the increased cost of a HMP program by decreasing need for dialysis post-operatively.

Not all retrospective studies however linked HMP with improvements in DGF and patient outcomes. In 2007 Opelz et al. in their retrospective analysis of 91674 deceased donors transplants from the Collaborative Transplant Study database looked at the effects of kidney preservation methods and times. They concluded that deceased donor kidneys should be transplanted in less than 18h, but that less cold ischaemia times had no effect on graft function, and suggested that HMP was not superior to SCS in terms of graft survival. In sharp contrast Stratta et al. published their retrospective analysis of 141 ECD kidney transplantation comparing HMP to SCS, and reported that despite longer cold ischaemia times (~24hrs for HMP vs. ~19hrs for SCS), recipients of ECD kidneys managed with HMP had similar survival and functional outcomes as recipients of ECD kidneys undergoing SCS – but that rates of DGF were markedly less in the HMP group (11% vs. 37% for SCS kidneys)[825].

Also in 2007, the Oxford Transplant Unit published a retrospective record review[826] of 36 Maastricht category III and IV DCD donor kidneys comparing HMP preservation to conventional SCS. With a cold ischaemia time of ~15hrs they noted immediate renal function in 72% of HMP pumped kidneys, compared to 11.8% of kidneys undergoing SCS, and a significantly shorter period of hospitalization (8 days in the HMP group vs. 14 days for the SCS group) for the machine perfused kidney recipients, supporting other reports on the potential cost-effectiveness of HMP. A more specific retrospective study published by the same group in 2008 compared clinical outcomes of 30 recipients receiving controlled DCD kidneys (Maastricht III only) managed with HMP compared to 30 recipients of controlled DCD kidneys preserved by SCS. Results re-enforce their previous findings, demonstrating a significantly reduced rate of DGF (53.3% vs. 86.6%), shorter hospital stays (10 days vs. 14 days), and better short term graft function at 1, 6, and 12 months post-operatively in the controlled DCD HMP group than in the SCS group[827].

More recently the first international multi-centre RCT in the Euro-transplant region comparing HMP vs. SCS in kidney transplantation was published by Moers et al. in 2009[828]. This RCT randomly assigned a kidney from 336 consecutive deceased (DBD and DCD) kidney donors to either SCS or HMP. Recipients were followed up for 1 year and showed a significant risk reduction for DGF in the HMP group (20.8% HMP vs. 26.5% SCS), with an improvement in 1 year patient survival for HMP preserved kidneys (94% vs. 90%), and 3 year (91% vs. 87%) vs. SCS kidneys. The graft survival advantage at 3 years in HMP kidneys was most pronounced for kidneys re-covered from ECD donors (86% vs. 76%), with DGF having a profound effect on graft survival in DBD kidneys, but which was improved by HMP preservation compared to SCS[798].

Further subgroup analysis examining kidneys from only DCD donors (n=164) demonstrated further benefit with reduced incidence of DGF (and of 4 days shorter duration) at 53.7% vs. 69.5% in HMP kidneys vs. SCS, and improved 1 year survival in recipients of HMP DCD kidneys[820]. HMP kidneys also had greater eGFR up to 1 month after transplantation, while 1-year graft and patient survival was similar (93.9% vs. 95.1%) compared to SCS preserved DCD kidneys, but superior in HMP kidneys at 3 years (91% vs. 86%)[798]. [829]

Machine perfusion reduced the incidence of DGF (adjusted odds ratio: 0.43; 95% confidence interval 0.20–0.89; P = 0.025). DGF was 4 days shorter in recipients of machine-perfused kidneys 53.7% (adjusted odds ratio: 0.43; 95% confidence interval 0.20–0.89; P = 0.025). DGF was 4 days shorter in recipients of machine-perfused kidneys (P = 0.082). Machine-perfused kidneys had a higher creatinine clearance up to 1 month after transplantation (P = 0.027). One-year graft and patient (P = 0.082). Machine-perfused kidneys had a higher creatinine clearance up to 1 month after transplantation (P = 0.027). One-year graft and patient survival was similar in both groups (93.9% vs. 95.1%). up to 1 month after transplantation (P = 0.027). One-year graft and patient survival was similar in both groups (93.9% vs. 95.1%).

In the Euro-Transplant Senior Programme of kidneys from donors >65 years are preferentially transplanted into patients >65 years of age[830]. HMP significantly reduced the incidence of PNF (3.5% HMP vs. 12.9% SCS), and improved 1-year graft survival (89% HMP vs. 81% SCS), but did not influence DGF which (29.4% HMP vs. 34.1% SCS). Incidentally CIT was short averaging 10.75 hours between both groups, and HMP benefits on DGF may require longer ischaemic exposure to be realised. None the less when DGF had occurred, 1-year graft survival was significantly better after HMP compared with SCS (84% vs. 48%)

In contrast to the results from Euro-Transplant a RCT conducted by surgeons at Cambridge University, UK in DCD kidney transplantation demonstrated no benefit to HMP, with similar rates of DGF (57.8% HMP vs. 55.6% SCS), and 12 month graft function between SCS and HMP cohorts[831]. There were several fundamental differences between these two studies. In the UK study immunosuppression and use of UW for SCS / flushing was standardized, while in the Eurotransplant study participants had a choice of immunosuppression and between UW or HTK for flush and SCS (this reflects the nature of the Eurotransplant study which included cross border collaborations between a number of European countries and centres). A further key difference is timing of HMP,

where kidneys were only perfused once transported back (by SCS) to the central transplant centre from a local/regional hospital where the retrieval occurred. In the Euro-Transplant study kidneys were placed immediately on a machine perfusion device after retrieval, and it use of HMP throughout cold preservation may be necessary to achieve benefit. However several large animal studies have reported conflicting results for HMP re-conditioning after SCS, and the exact nature and mechanism of HMP benefit after a period of SCS as compared to HMP immediately after retrieval still need to be elucidated[823,832].

A retrospective review of high risk uncontrolled DCD (Maastricht categories 1 and 2) donor kidney transplantation (n=27) in Lyon, France reported superior 3 year graft survival in HMP preserved uncontrolled DCD recipients compared to ECD (n=30) donor kidney recipients (100% vs. 82% respectively), without PNF in either cohort[492]. DGF however was more prevalent in DCD vs. ECD kidneys (81% vs. 28% respectively). Other outcomes (GFR and histology on protocol biopsy) were similar between the DCD and ECD groups at 3 years. Despite higher DGF rates with uncontrolled DCD allografts, 3 year function and survival was similar to ECD allografts suggesting that DGF does not have the same impact in DCD compared to ECD organs. These results could also be explained by the careful selection of the DCD donors - without past medical history (non-ECD DCD), and use of HMP, but suggestions that the cytokine surge in DBD may in fact contribute to outcomes after DGF have made[833].

Jochmans et al. reviewed the outcomes of DCD kidney transplantation (n=287, 94% controlled DCD) in Belgium over the 10 year period 2000-2009 [834]. Fifty-three percent of kidneys underwent HMP. PNF occurred in 1% and DGF in 31% of recipients. Multivariate analysis revealed SCS (vs. HMP), CIT, and use of HTK solution (vs. UW) were independent risk factors for the development of DGF. Interestingly analysis of delayed vs. immediate HMP revealed no univariate risk for DGF. Five-year patient and death-censored graft survival were 93% and 95% for Belgium vs. DCD kidney transplant recipients in the Netherlands.

Recently O'Callaghan et al conducted a meta-analysis examining outcome benefits of HMP vs. SCS[835] in kidney transplantation. The analysis included 7 RCTs (1475 kidneys) and 11 non-RCTS (728 kidneys) and determined the risk of DGF was less with HMP use vs. SCS, with no effect on PNF, and no detectable impact on longer term graft or patient survival. Bathini et al. and colleagues performed a systematic review on the effect of HMP preservation vs. SCS specifically in DCD kidney transplantation[836]. They included 9 studies with overlap of 5 prospective studies with O'Callaghan

et al. and the remaining retrospective (including an analysis of UNOS registry data) or cohort based studies. HMP pumped kidneys from DCD donors experienced decreased DGF compared to SCS preserved DCD kidneys, with a trend toward improved allograft survival at 1-year in HMP kidneys. Both groups contest that due to the poor heterogeneity in studies (e.g. definition of DGF, immunosuppression regimes) and a general lack of sufficient power in available studies, and relatively short follow-up to-date means further study is required to assess the overall impact of HMP on graft function and outcome.

A more recent analysis of data from the Scientific Registry of Transplant Recipients in the US included outcome data from all SCD (n=71,192), ECD (n=15,122), and DCD (n=8,395) donor kidney recipients between 2000 and 2011[837]. The risk of DGF was found to be lower with HMP. Stratified by the type of donor and CIT, the adjusted odds of DGF was lower with HMP across all CITs in SCD, for CIT>6 hours in ECD, and for 6>CIT<24 hours in DCD kidney allografts. Length of CIT is known to be a risk factor for DGF, irrespective of storage modality, however HMP has been shown to reduce this effect. A recent RCT from University of Miami group exploring effect of differing HMP durations in 66 pairs of donor kidneys reported that longer HMP associated CIT time with HMP was beneficial vs. shorter HMP CIT time [838]. Longer HMP time kidneys had reduced rates of biopsy proven acute rejection at 12 months, with no effect on organ function (DGF, PNF), nor on graft and patient survival compared to short HMP time. This supports the premise that preservation strategies should target reducing exposure to CIT but also utilise HMP to limit the impact of any CIT on graft function.

A single centre observational, retrospective review of 88 ECD kidney transplants from Barcelona analysed the influence of HMP (n=37) vs. SCS (n=51) on graft outcomes[839]. Univariate analysis revealed improved eGFR at 48 hours post operation (11±8 vs.16±14 ml/min/ m^2; P=0.021) and at discharge (29±13 vs. 42±19 ml/min/m^2; P=<0.0001), with reduced DGF (7±7.6 vs. 3.2±2.7; P=0.036), as indicated by requirement for dialysis, and shorter hospital stay (2.43±1.2 vs. 1.76±1days; P=0.006) in HMP preserved kidneys. While multivariate analysis confirmed a reduced rate of DGF (35.1% vs. 47.1%; P=0.03, OR=0.34) and need for dialysis (P=0.005, OR=0.65) and also revealed a higher 1-year graft survival (95% vs. 84%; P=0.025, HR=1.915) in HMP vs. SCS preserved kidneys.

1.7.2.6 Hypothermic Machine Perfusion and Allograft Viability Assessment

Machine Perfusion Dynamics

Machine perfusion improves immediate graft function, mediates improved survival rates, and enhances preservation of the microcirculatory integrity of the preserved organ. It allows for extended preservation, reversal of damage sustained through warm ischaemia time, and the potential for graft viability assessment through monitoring of perfusion dynamics and biochemical perfusate analysis. At present renal flow rate and vascular resistance are the only two accepted parameters used as indicators of kidney viability. Approximately 15% of all HMP preserved kidneys in the USA are discarded, predominately because of a high renal resistance [840], however relatively little information is definitively known about the true predictive value of RI and other perfusion characteristics on transplantation outcome or their use for selecting or discarding kidneys for transplantation.

Several groups have published extensively on the topic of viability assessment using HMP – notably the Newcastle group by Talbot et al., in Japan by Matsuno et al., Guarrera et al. in the US, Kosieraszki et al in Poland, and Ploeig et al. in the Netherlands, however many additional centres have published their experiences with HMP viability assessment.

The Newcastle clinical viability protocol has evolved over the past decade and sets criteria for HMP preservation and assessment of DCD donor kidneys using a unique perfusion apparatus with hypothermic Belzer Machine Preservation Solution (MPS) as the perfusate. Perfusion time is approximately 4 hours, with perfusion pressure set at 50-55mm Hg. Viable organs are required to exhibit a Perfusion Flow Index (PFI – an index relating flow to pressure) of 0.4/ml/min/100g/mmHg or greater, with flow rates >25ml/min/100g of tissue, and a graft weight increase less than 25% post machine perfusion[841]. In addition levels of glutathione-S-transferase (GST, an indicator of tubular damage and propensity for re-perfusion injury) in perfusate are required to be less than 200IU/100g of kidney.

In their experience use of HMP for DCD kidneys and these viability criteria increased utilization of DCD kidneys, and over a 24 month period increased graft survival rates from 45.5% (graft survival of Type II Maastricht donor kidneys using SCS, n=10) to 88.4% (n=28), and though DGF rates were still high[842], renal function and long term graft (and patient) survival was comparable to recipients of heart beating donor kidneys[782] . A modified protocol (desired perfusate GST levels changed to be

<100IU/100g of tissue) successfully identified poorly functioning kidneys unsuitable for single transplantation, but were instead utilized for dual kidney transplantation [843], and early functional outcomes in dual transplant recipients (n=23) at 3 and 12 months were similar to patients receiving a single kidney (n=115). This study demonstrates the potential use of HMP viability assessment in identifying kidneys suited for dual rather than single transplantation, minimizing non-use of DCD kidney allografts.

In Japan a perfusion system known as LPS-II is used for cadaveric kidney preservation using cryoprecipitated AB positive plasma. Perfusion pressures are set to between 30-50mmHg, with perfusate circulated at 8-10oC, and pH maintained between7.4-7.6. Kidneys are selected for transplantation based on a minimum flow rate of 0.4 ml/min/g (40/ml/min/100g), and a concurrent decrease in perfusion pressure. Though subject to similar cold ischaemia times (~10hrs), kidneys with higher flow rates >90/ml/min/100g equated to increased rates of immediate function, and a decreased duration of ATN post-operatively, no incidence of PNF, compared to kidneys with flow rates of 40-65ml/min/100g which exhibited 25.7% PNF, and only 8.6% immediate function [844]. In another study using the LPS II apparatus kidneys perfusing with flow rates of 110ml/min/100g and an equivalent resistance index of 0.55 were successfully transplanted without incidence of DGF post-operatively[845]. Recently Matsuno et al. used the LPS-II perfusion apparatus and these perfusion criteria to successfully transplant 17 uncontrolled DCD kidneys with high initial donor creatinine levels (>3.0mg/dl), without incidence of PNF but with instances of severe DGF [846].

In Poland, Kosieraszki et al. performed a retrospective analysis of their 6-year experience with HMP for kidney preservation (n=234) to determine the value of each measured perfusion and perfusate parameter in predicting DGF. Kidneys were perfused for ~30hrs. No single parameter successfully predicted DGF, but flow <45/ml/min and RI of >0.44, and perfusate lactate and LDH levels (indicators of cell death) >1/75mmol/L and > 709U/L respectively were associated with a higher incidence of delayed function[847]. Kwiatkowski et al. presented long term results of renal transplant patients who had received a HMP managed kidney (n=227) vs. patients with kidneys preserved with SCS (n=188). In this non-randomised analysis kidneys stored by HMP had improved 5y graft survival, and less incidence of returning to dialysis, despite longer CITs [848].

In the US Polyak et al. reviewed their experience with 650 preserved kidneys in their institution between 1993 and 1999 (402 HMP vs. 248 SCS)[849]. HMP time was ~25hrs, systolic HMP pressure was ~ 60mmHg, kidneys with flow rates < 50ml/min/100g and RIs > 0.85 mmHg/ml/min/100g were

discarded, with idea flow rates >100ml/min/100g and RI <0.4 mmHg/ml/min/100g. They showed that HMP reduces rates of DGF compared to SCS (9% HMP vs. 24% SCS), especially so in ECD donor kidneys (14% HMP-ECD vs. 37% SCS-ECD). In addition HMP mediated a 10% increase in 1 year graft survival in ECD donor kidney recipients compared to SCS. Guarrera et al. from the same institution published data that suggested even kidneys with poor HMP characteristics (flow <80 ml/min/100g and RI >0.4 mmHg/ml/min/100g) can still be transplanted with adequate outcomes, but that such perfusion data should take into account other donor variables[850]. This supports material from Mozes et al. who reviewed perfusion dynamics of discarded kidneys. HMP perfusion criteria at 4 hours are most frequently used at his institution with decisions regarding acceptance for transplant. He concluded that kidneys perfusing with RI <0.6 mmHg/ml should not be discarded based solely on suboptimal perfusion parameters. Kidneys with RI of 0.4-0.6 demonstrated similar outcomes as transplanted kidneys with RI <0.4. Refining perfusion criteria they decreased the discard rate of HMP kidneys in their region from 20% in 2002 to 12% and 3% in 2003 and 2004 respectively[851].

Sellers et al. also in US, compared outcomes of 568 HMP vs. 268 SCS managed kidneys between 1990 and 1995. HMP time was ~20-24 hrs. Overall graft survival was similar for both groups – despite worse donor and recipient characteristics in the HMP group. Kidneys with >40 minutes of WIT undergoing HMP had higher survival rates vs. SCS kidneys with >40mins WIT. DGF rates were higher after SCS (20.2%) vs. HMP (8.8%)[852].

In Spain, Suarez et al. use a Waters RM3 Perfusion Device to pump ECD donor kidneys using a modified Belzer MPS solution at 4oC – with added mannitol, bicarbonate, and vasodilators – at a perfusion pressure of 40-45mmHg, for ~17hrs[853]. RI, in conjunction with renal biopsy, was the criteria to determine transplant suitability, and kidneys with RI > 0.4 were not used for transplantation. In a small study use of these criteria increased utilization of ECD kidneys otherwise considered unsuitable for transplantation, with no incidence of PNF post-operatively. Continuing their work Peri et al. from the same institution in Spain recently published a report detailing their experience with ECD donor kidneys and HMP between 2004 and 2007[854]. From 292 ECD donors (206 brain death, 86 DCD / Maastricht 2) one kidney underwent SCS and one HMP. Kidneys were discarded if RI > 0.4 mmHg/ml/min. Discarded kidneys had lower flow rates compared to transplanted kidneys (56 vs. 78.8 ml/min). Length of hospital stay was lower in the DCD HMP group (13 days) vs. DCD CD group (35 days) and though HMP kidneys experienced longer CIT than SCS controls they showed no significant differences in renal function.

Similarly in Russia, Reznik et al. describe a decreasing RI, with an increasing renal flow rate, as a reliable predictor of viability in uncontrolled DCD kidneys (in conjunction with adequate biopsy histology). In a small study comparing SCS (n=17) with HMP (n=21) DCD kidneys displaying immediate function post-transplantation reached a suitable RI after 1-3hrs of HMP, while kidneys that experienced DGF required 6-12hrs of HMP to reach acceptable levels, prompting the conclusion that up to 10hrs of HMP may be required for ischaemically damaged DCD kidneys to be recovered for transplant[855].

Patel et al. performed several retrospective reviews of approximately 268 kidneys that underwent HMP and transplantation at the University at Buffalo (SUNY), New York. HMP kidneys demonstrated improved perfusion dynamics over time, with average flow reached by 2 hours[856]. Kidneys with initial poor HMP dynamics but with early improvement by 2 hours were less likely to be discarded, but demonstrated higher incidence of DGF compared to kidney exhibiting ideal perfusion from start of HMP. Further analysis from the same group observed higher rates of DGF (75%) with HMP kidneys perfused at higher pressures (> 23mmHg, n=24) vs. lower pressures (<23 mmHg, n=49), with overall higher resistances in high pressure perfused kidneys, but comparable flow rates[857]. Graft survival at 1 year was similar. Perfusion pressures can be increased to support lower flow rates but may not be beneficial, use of the PFI calculation as advocated by Talbot et al. may minimize perfusion at higher pressures which may be detrimental to organ function[858]. They also correlated perfusion dynamics during HMP with results of pre-implantation biopsies - classified as Normal (n=60) or Abnormal Histology (n=57, glomerulosclerosis or arteriosclerosis >10% in sampled glomeruli or arterial lumens/intrarenal arteries respectively) [859]. Allografts with abnormal pre-implantation biopsies showed significantly lower flow and higher resistances. Pathology did not predict DGF, but 1-year graft survival (96.2% vs. 80%) and eGFR (58 vs. 48 mL/min) was lower in kidneys with abnormal histology, though these metrics did not reach significance. Despite abnormal pathological findings kidneys improving flow parameters may allow transplantation of allografts previously considered for discard with acceptable results.

Analysis from the Euro-Transplant HMP RCT of 336 DCD and DBD kidneys high RI at the end of HMP was an independent risk factor of DGF (odds ratio [OR] 38.1; 95% CI 1.56–934; P=0.026) however the predictive value of RI on DGF was poor (AURoC 0.58)[860]. RI was also found to be an independent risk factor for 1-year graft failure (hazard ratio 12.33 [1.11–136.85]; p=0.004).

A retrospective analysis of 440 transplanted DCD kidneys preserved by HMP was conducted at the University Medical Centre in Maastricht, Netherlands[861]. RI at the start of machine perfusion was significantly and independently associated with both PNF (OR 2.040, 95% CI 1.362–3.056; p = 0.001), and DGF (OR 2.345, 95% CI 1.110–4.955; p = 0.025). However the predictive quality was moderate (0.609, 95% CI 0.538–0.681), and there as not association between RI and graft nor patient survival.

A retrospective analysis of 115 deceased donor kidney transplants (85% DBD) from Romania correlated using linear regression HMP Resistive index to Doppler US assessment of renal vascular resistance at 1 day post transplantation(R=0.813, R^2=0 .661, adjusted R^2=0.657, F(1.80)=156.34, p<0.0001, Std. Error of estimate: 0.04166)[862]. This important study demonstrates the prognostic use of the HMP resistance in predicting allograft perfusion post transplantation. Poor renal vascular resistance post transplantation is associated with DGF and poor graft survival, possibly due to detrimental effects of IR injury on renal microvascular perfusion. Thus HMP may confer benefits on post implantation function by better preserving the microvasculature during cold preservation and facilitating better vascular perfusion once transplanted.

Renal Machine Perfusion Perfusate biomarkers

Research has focused on the use of machine perfusate biomarkers for pretransplant in vitro assessment of kidney viability[863–868] . Evidence remains controversial and based largely on animal models. Biomarkers most likely will be incorporated in clinical viability scores, rather than be used in isolation. Some of the more-established work has focused on GST, lactate dehydrogenase (LDH), N-acetyl-β-D-glucosaminidase (NAG), alanine aminopeptidase, aspartate aminotransferase, and heart-type fatty acid binding protein[869].

Glutathione S-transferase

GST is found in the kidneys and is involved in detoxifying metabolites and conjugating glutathione [863–865]. The 2 subtypes, α-GST and π-GST, found in proximal and distal tubules are released during ischemia. Proximal tubules, being metabolically more active, are more vulnerable to hypoxia, making α-GST a more-sensitive marker[864], and a potential tool for pretransplant assessment of DCD kidney viability. Quantification of total GST is technically easier and often is used as a surrogate marker of α-GST levels[870,871] .

Initial reports on GST use in organ viability assessment were promising. During HMP, α-GST levels correlate with warm ischemia time; with prolonged ischemia being associated with higher levels[866,867]. Similarly, levels are lower in functioning, compared to non-functioning, grafts from DCD donors[866,867]. Further research into functioning DCD kidneys demonstrated that GST levels are significantly higher at the end of HMP in kidneys with DGF compared with kidneys with immediate function[870]. Moers et al., were the first to show from Eurotransplant data that GST levels, although related to warm ischemia time also represent an independent risk factor, but with poor predictive value for DGF [870,872]. A systematic review of 23 studies from the US reported that GST, among other biomarkers is significantly associated with DGF[873]. However recently it was suggested that only the π-GST subtype is independently associated with DGF[874].

Other research has shown isolated cases of high GST levels occurring in functioning DCD grafts, showing that GST levels cannot necessarily be considered in isolation [869]. Similarly, based on their experience with a case of immediate graft non-function in a kidney with normal GST but low flow rates, Balupuri et al also suggested other factors should be considered in addition to GST levels [875]. They postulated that GST may originate from capillary beds, and that low levels of GST may be related to small capillary mal-perfusion, not necessarily reflecting ischemic damage, and recommended that a flow rate of at least 50 mL/min/100 g kidney is required for GST levels to be

clinically relevant. The Newcastle clinical viability protocol recommends a GST content at 4 hours of less than 200 IU/L/100 g of renal mass[876].

Lactate dehydrogenase

LDH is a nonspecific marker of cellular injury. It was hoped that its level in kidneys undergoing HMP would reflect the extent of ischemic injury [869]. However, research by Daemen et al did not confirm a significant correlation between LDH and warm ischemia time or graft outcome[867]. In a group of DCD functioning grafts, this biomarker was found to be a good discriminator of immediate versus DGF, but not independently predictive of graft failure within the first year post-transplant[870]. LDH use as a biomarker may be of value in the context of other determinants of graft function, but it is unlikely to have independent predictive value[872].

N-acetyl-β-D-glucosaminidase

NAG is a lysosomal enzyme. There is limited work looking at NAG levels in renal perfusate of HMP grafts and their correlation with ischemic damage [869]. Research by Moers et al. showed that like LDH, NAG levels constituted a risk factor for DGF but have no independent predictive value of graft failure in the first year post-transplant[870].

Alanine aminopeptidase

Alanine aminopeptidase is a peptidase located in renal cells and involved in cell regulation. Like GST, it is excreted in the urine secondary to renal tubular damage and has been investigated as a pretransplant marker of posttransplant renal viability [869]. In DCD kidneys alanine did demonstrate a correlation of alanine aminopeptidase with DGF, but not PNF, and but with a poor predicative value [870,877].

Aspartate aminotransferase

Aspartate aminotransferase is an enzyme seen in hepatic and renal parenchymal cells that has been studied in the context of HMP. Most research has focused on its use in liver transplant, but there is also evidence for its applicability in renal transplants [869]. Higher levels are associated with an increased risk of delayed, as opposed to immediate, graft function after transplantation but with a poor predictive value[870].

Heart-type fatty acid binding protein

Heart-type fatty acid binding protein (h-FABP) is found mainly in the heart, and in smaller amounts, in the small intestine, the skeletal musde, and the distal tubules of the kidneys[868] and is involved in the uptake of fatty acid from the cytosol into the mitochondria. It is an independent risk factor for DGF, but with poor predictive value[870].

1.7.2.7 Machine Preservation solutions

University of Wisconsin Solution

The most widely used perfusate for HMP is a formulation of the SCS Belzer UW solution, where lactobionate is replaced by gluconate, a suggestion from Dr. F. O. Belzer and Dr. J. H. Southard who first developed UW solution at University of Wisconsin[878]. This HMP variant of UW solution is known as Belzer MPS.

There are several further differences between SCS UW solution and Belzer MPS the predominant being an inversion of the sodium/potassium electrolyte content - with Belzer MPS representing a more extracellular environment electrolytes. Furthermore Belzer MPS contains glucose as a substrate for gluconeogenesis, the addition of mannitol as a further osmotic agent, and omission of adenosine.

Despite Belzer MPS being used as the dominant HMP perfusate Belzer UW was initially designed as a HMP perfusate by Belzer and colleagues[722]. Experimental evidence suggests however that Belzer UW is superior to Belzer MPS when used for HMP of kidneys from ECD donors, whereas Belzer MPS is better for HMP of kidneys from uninjured SC donors[879].

Belzer MPS is marketed and distributed with the two most commercially available organ perfusion systems by Organ Recovery Systems (ORS, Des Plaines, Illinois, Model LifePort) and Waters Medical Systems (WMS, Rochester, Minnesota, Model RM3).

Belzer-MPS thus continues to be the predominant HMP perfusion solution, and despite numerous studies having attempted to alter the composition of MPS to improve its preservation performance, it has to-date remained commercially unchanged. Together Belzer UW used for SCS preservation of abdominal organs, and Belzer MPS used for clinical HMP of kidneys are considered the gold standard for organ preservation today and used by most centres worldwide.

However with increased understanding of the base molecular changes and mechanisms underlying IR injury in transplantation several groups have attempted to develop new solutions to address the increasing use of ECD and DCD donor organs which are more susceptible to IR injury, and which may not be effectively addressed by the components of the traditional UW based preservation solutions.

Polysol

Polysol is a preservation solution developed in the Netherlands at the Academic Medical Centre in Amsterdam, for use in both SCS and HMP preservation [767]. It is based on tissue culture media, is both a low viscosity and extracellular type solution, with an iso-osmolar osmolality (320mOsm/L), and polyethylstarch as the main colloid. Raffinose, trehalose and gluconic acid are the saccharides impermeants used to resist cellular oedema, while glutathione, ascorbic acid, selenium and alpha-tocopherol are added as free radical scavengers.

Polysol has been assessed in a porcine kidney auto-transplantation model, and was used for 20 hours of both SCS or HMP of kidneys, with favourable outcomes (improved initial flushing of allografts, and both better microcirculatory perfusion and renal function after transplant) compared to controls undergoing SCS with HTK or UW solution alone [880–882].

Polysol has also been evaluated in experimental porcine and murine liver HMP and transplantation, with limited success compared to SCS [766,883,884].

Vasosol

Guarrera and colleagues at Columbia University in New York have developed a novel solution based on Belzer MPS with additional components targeted to ameliorate specific mechanisms of IR injury. This solution was designated Vasosol. Rationale of addition of α-ketoglutarate, L-arginine, N-acetylcysteine, nitroglycerin, and prostanglandin E1 was to provide a supply of energy substrates, improve microcirculation, and support endogenous antioxidant systems. Initial experiments utilizing a canine kidney auto-transplant model with Vasosol for SCS preservation demonstrated better graft function compared to kidneys preserved with UW solution [885]. In the US a randomised prospective clinical trial of 162 machine perfused kidneys compared Vasosol HMP with conventional HMP using Belzer MPS demonstrated improved early graft and shorter hospital stay in recipients of Vasosol perfused kidneys [886]. This paper was retracted shortly after publication due to concerns regarding logistical conduct of the study between the multiple centres, however the data and conclusions drawn are still of worthy note. Vasosol has also been used in liver HMP preservation in particular the first clinical study of liver HMP that was published in 2010 by Guarrera et al where HMP with Vasosol improved patient recovery and lower incidences of biliary complications [887]. Allografts were from DBD donors, and HMP via dual perfusion of hepatic artery and portal vein.

Lifor

Lifor was developed as a normothermic machine perfusion solution. It contains cellular nutrients and a non-protein oxygen carrier that works at temperatures higher than 4°C. It has been used to machine perfuse porcine kidneys nomorthermically vs. HMP with Belzer MPS, with improved HMP flow dynamics using Lifor suggestive of better preservation [888,889]. Lifor has also been evaluated in porcine liver machine perfusion investigating differing perfusion temperatures between 4-21°C [890].

Custodiol-N

Recently Custodiol-N (the modified HTK solution) has been supplemented with dextran to allow kidney HMP. Using a ex-vivo porcine kidney machine perfusion and isolated reperfusion model modified Custodiol-N was compared to Belzer MPS [743]. Kidneys undergoing 20 hours of HMP with the modified Custodiol-N demonstrated higher renal blood flows, oxygen consumption and creatinine clearances compared to Belzer MPS perfused kidneys during isolated haemoreperfusion. This preliminary work suggests that there is further potential for the expansion of newer preservation solutions that may provide superior preservation and function after reperfusion compared to the current gold standard Belzer MPS.

1.7.2.8 Economic Benefits of Machine Perfusion

Improved outcomes in recipients of kidneys managed by HMP compared to SCS has been shown to result in a decreased length of hospitalization and need for dialysis. Any cost additional cost of HMP compared to SCS could potentially be absorbed by savings as a result of the improved outcomes.

Two cost-effectiveness studies have been conducted by UK groups comparing HMP and SCS, one by NICE[4] and one by the National Institute for Health Research[891]. Both analyses however used only preliminary data from two ongoing RCT comparing HMP to SCS, one still on-going[847] and one now complete[892] . Both were largely inconclusive with comparable data supporting both SCS and HMP. The NICE technology appraisal guidance is due for review August 2010.

In an analysis by Wszola et al. from Poland comparing HMP to SCS they concluded that as well as of clinical benefit – increased long term graft survival (mean F/U at ~7 years) and decreased need for post-operative dialysis and incidence of return to dialysis – HMP costs were equalized to SCS costs by 16 months post transplantation[893].

In the recent RCT by Moers et al. cost-effectiveness analysis suggested that any additional costs of HMP are compensated for by the improved clinical outcomes, especially as the reduced incidence of DGF, PNF, and graft failure means a subsequent reduced need for dialysis[894]. This new data supports historical 1997 data from Johns Hopkins in Baltimore, US that demonstrated the likelihood of requiring dialysis after renal transplantation is 2.33 times higher if SCS is used compared to HMP in kidneys from donors >50 years of age, with a potential net saving of $14700 realized through use of the paradoxically more expensive HMP preservation method [852] .

More recent economical analysis from the Euro-Transplant RCT combined the short-term data from the study with a Markov model with a 10-year time horizon[895]. They included cost of hospital stay, dialysis, and complications in their analysis and concluded that HMP reduced the risk of DGF and graft failure at lower costs than conventional SCS preservation. The analysis revealed cost savings of $86 750 per life-year gained in favour of HMP, with an incremental cost-utility ratio of minus $496 223 per quality-adjusted life-year (QALY) gained after transplantation, demonstrating gain to life-years and QALYs while at the same time reducing costs.

HMP in Practice

There are several commercially available machine perfusion devices – and these are in use in various centres worldwide, however many institutions have developed custom perfusion apparatuses. The perfusate is commonly Belzer MPS, a modified UW solution specific for HMP, with some units preferring to add specific pharmacological agents to the perfusate to improve kidney perfusion dynamics. Initial perfusion pressures in HMP are variable – with advocates for both higher (>55 mmHg systolic) and lower (<40 mmHg systolic) settings[896]. Most units that utilize HMP as a method of assessment of organ viability in kidney transplantation utilize a parameter which is a derivative of flow through the renal allograft – whether it be PFI, RI, or actual flow rates. The duration of HMP is heterogenic amongst centres – varying from several hours to over 24 hours. Biomarkers of cellular damage in the perfusate are measured by some groups – e.g. GST, LDH, Lactate – to provide additional viability information. Parameters based on HMP are considered in conjunction with other screening tools for selection of suitable organs for transplantation – WIT, CIT, donor age and medical history, biopsy result, and macroscopic appearance of the organ – in deciding to use or discard a retrieved organ.

1.7.2.9 Machine Perfusion Devices

There are currently two commercially widely available perfusion devices designed for kidney perfusion. They are the Waters Medical System 'RM3 Renal Perfusion System', and the Organ Recovery System 'Lifeport' (See Figure 22). 2nd generation machines from both manufacturers are also being rolled out into the commercial arena. The Lifeport is portable compact perfusion apparatus, while the 1st generation RM3 is a bench-top based perfusion system, and the 2nd generation device is transportable. The most recent large RCT comparing SCS to HMP in the Eurotransplant region utilized the Lifeport device[892] . This device provided HMP in transit from the site of organ retrieval to the base hospital where the potential operation would occur. In the UK of 21 kidney transplant centres 8 are reported to use the Lifeport for HMP preservation.



Figure 22 Organ Perfusion Machines. 1st generation Waters Medical RM3 Renal Perfusion System with dual organ cassette for perfusion of two kidneys (top left) and 2nd generation waves machine (top right), Organ Recovery Systems 1st generation Lifeport device (bottom right), and 2nd generation Lifport device (bottom right).

Several studies have been conducted to compare the effects of both these machines in HMP of kidney allografts. In a retrospective report of 5 years of kidney transplantation (n=774, ECD and DCD donors) at their institution Guarrera et al. [897] found that for similar periods of CIT DGF rates were lower in kidneys pumped by the RM3 (31%, 90/378) vs. the Lifeport (41%, 162/396). In addition

kidneys managed with the RM3 had better graft function at 1 year, and better 1 year graft and patient survival, compared to recipients of kidneys managed with the Lifeport. In a second smaller study by Kazimi et al. 89 kidney transplant records (2% DCD) were retrospectively analysed [898]. Their main finding was hospitalization was significantly longer for recipients of kidneys managed with HMP on a Lifeport (15 days) vs. RM3 unit (9 days).

Recently an RCT reported on comparison of the Waters Medical RM3 vs. the ORS Lifeport for HMP preservation of 50 deceased donor kidneys (48% ECD) prior to transplantation [899]. DGF was similar using both HMP devices (32%). However patients with DGF required an average of 4.66 haemodialysis sessions when using the RM3 vs. 2.65 with the Lifeport($P = 0.005$). Overall 1-year graft survival was 80% vs. 96% in the RM3 and Lifeport groups, and 66% vs. 94% for ECD kidneys respectively. Finally interstitial fibrosis and tubular atrophy on biopsy was more common in the RM3 preserved allografts 45% vs. 0% ($P = 0.03$) in Lifeport pumped kidneys, but creatinine levels were similar between the groups. An important note in this study is that pressures were maintained at 45mmHg in the RM3 perfused kidneys vs. 30mmHg with the Lifeport. Lower pressures have been reported to be superior to higher pressures during HMP and thus this small but key difference introduces a large potential bias and confounding factor in interpreting these results [900]. Despite the reasons stated for choosing a higher pressure in the RM3 group being that sufficiently higher pressures were required to achieve equal flows compared to perfusion with the Lifeport, the data presented shows that flow rates were not equal between groups (higher in the RM3 kidneys) as were RI indices. In such as comparison between two modalities it would have been more useful to maintain one target perfusion variable similar between the two modalities (flow rate or pressure). In this case this study not only compares two preservation devices but also two perfusion pressures and so in both cases neither group would act as a good control for each other.

Results from this recent RCT are in stark contrast to those from an experimental canine animal model of DCD kidney allograft preservation and autotransplantation where the RM3 and the Lifeport were compared [805]. Kidneys underwent 45mins of WIT then 24 hours of HMP using the RM3 (n=8) and the Lifeport (n=8), followed by auto-transplantation and observation for 7 days. RM3 kidneys were perfused at a systolic pressure of 45mmHg, Lifeport at 30mmHg. RI in kidneys on both devices was similar for the 24 hours of perfusion, and histology indicated equivalency of preservation injury. Serial creatinine over 7 days was similar between RM3 and Lifeport preserved kidneys, however a separate subgroup with 8 additional animal demonstrated pulsatile flow is superior to non-pulsatile flow with lower creatinine levels after transplantation. Despite RM3 perfusion operating at a higher

temperature (2 vs. 5 ˚C) renal function and preservation injury was similar. This study utilized similar perfusion targets as the Wszola et al. RCT, but did not report any differences in early renal function. It may be that differences would have been evident after 7 days in this study and on longer assessment of allograft survival but this was not the remit of the protocol. There have been no experimental or clinical reports to date of results with use of the 2nd generation Waters Medical Systems or Organ Recovery Systems devices. Furthermore the debate between pulsatile vs non-pulsatile or continuous perfusion has not been resolved and with further studies needed to determine the optimal parameters for effective HMP.

1.8.1 Rapid Sampling Microdialysis

Currently, besides flow rate and vascular resistance during HMP, there are no other entirely accepted indicators of kidney viability [841,842,846–848,850,851,853–855,901–903] and 12-18% percent of kidneys are discarded due to concerns about their suitability for transplantation [6]. Attempts have been made to determine renal biochemical profiles through the analysis of renal artery effluent, however, its use is limited and there is no reported consensus on its role in predicting graft outcome [873]. An assessment of both renal tissue viability and *ex vivo* function during preservation would facilitate a greater level of discrimination in identifying those organs which are at higher risk of delayed function and potentially non-function and would enable a more accurate selection of kidneys which are suitable for transplantation.

Microdialysis is a minimally invasive technique that allows the interstitial composition of a target tissue to be sampled, followed by *ex vivo* analysis, providing an accurate method of monitoring tissue biochemistry and metabolism over time. A fine tubular dual lumen microdialysis probe, with a semi-permeable bio-compatible membrane tip is introduced into the target tissue of interest. A physiological isotonic solution is pumped through the inlet port of the probe at a low, constant, and controlled rate and past the semi-permeable membrane tip where it equilibrates with the surrounding extracellular tissue fluid. Relatively low molecular weight compounds easily diffuse into or out of the lumen of the catheter down their concentration gradients while 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 the molecule of interest. A schematic representation of this process is demonstrated in Figure 23.

Tissue ischaemia occurs when local oxygen delivery fails to meet cellular metabolic needs. As oxygen deficiency progresses, through flow-dependent oxygen consumption, anaerobic metabolism ensues. This causes subsequent changes in the concentration of metabolites involved in the glycolytic pathway. Microdialysis techniques are capable of sampling and measuring these glycolytic substrates specifically lactate, as a potential marker of tissue ischaemia.

The use of conventional off-line microdialysis for *in vivo* tissue viability assessment has been validated in a variety of research and clinical scenarios [904], including monitoring of cerebral ischaemia [905–909], bowel ischaemia [910–912], free flap surgery [913–915], and post-operatively in kidney [916,917] and liver [918–921] transplantation. Microdialysis techniques can be translated

easily from an experimental to a clinical setting due to the availability of FDA-approved and CE marked probes [904]. There are limited reports of the use of microdialysis as a tool for organ viability assessment during SCS [916,922,923] or HMP [924] and may be due to the theoretically limited metabolism occurring in this period compared to normothermic temperatures. However, the clear need for greater tissue assessment compels us to propose that microdialysis could be used to effectively quantify and investigate the development of ischaemic injury and stress during this period.

The Department of Bioengineering at Imperial College London has developed a novel online rapid sampling microdialysis (rsMD) analyser capable of measuring the concentration of markers of tissue ischaemia at 30 second intervals. To date no such system has been used to directly characterise and compare the effects of SCS and HMP on renal biochemical activity and the ischaemic injury sustained during preservation in real time. If capable of accurately monitoring tissue ischaemia during organ preservation, rsMD may have a promising future role in graft viability assessment during the preservation period.

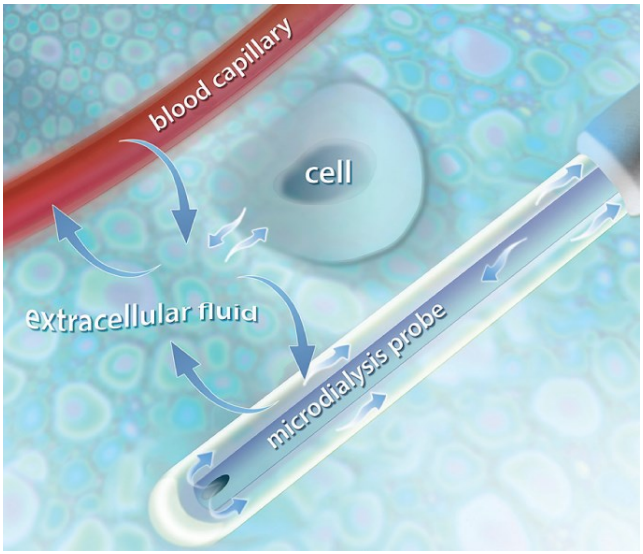


Figure 23 Schematic representation of a microdialysis catheter. Fluid isotonic to the target tissue extracellular fluid is pumped through the microdialysis catheter at a known rate. Molecules at high concentration in the tissue equilibrate across the semi-permeable microdialysis membrane and can be analysed in the collected perfusate (the microdiasylate). Courtesy of CMA Microdialysis AB from http://www.microdialysis.com/.

1.8.2 Addressive Therapeutics

1.8.2.1 Theory of Targeting

Therapeutic targeting simplistically describes selectively delivering a pharmacological agent to the site of disease, and increasing the therapeutic ratio of that agent at that specific site relative to non diseased areas.

The holy grail of targeted therapeutics is the smart 'Magic Bullet' concept, where by an exogenous molecular agent selectively seeks out, finds and binds exclusively and irreversibly to specific 'targeted' entity in a biological system producing a therapeutic effect [925]. The actual desired effect need not be mediated by this interaction directly but potentially indirectly through associated molecules at the target site. In practice though without unrestricted access to the target molecule, knowledge of the amount of molecule to be targeted, and considering an infinite association constant for the components, designing a 'Magic Bullet' is not feasible.

The closest practical intervention is distributive-retention targeting - exemplified by clinical use of mAB. But this is an en-mass targeted approach with the actual proportion of antibodies that bind the target agent usually low, with little useful effect; as they are distributed to all extracellular locations in the biological system and catabolised by processes distant to the target site - leaving a low proportion of the agent to bind the target.

Addressive targeting is an interesting approach that can likened to that employed by leucocytes. Here differential and temporal expression of CAMs guides and directs inflammatory cell trafficking to sites of vascular or tissue damage and mediates a local rather than a systemic response[925]. It is characterised by several molecular interactions (acting in sequence or simultaneously) with low affinity to identify a site where an agent will aggregate and concentrate mediating a localised effect.

Addressive targeting has recently been applied in organ transplantation, with the design and clinical testing of a complement inhibitor molecule designed with endothelial binding functionality[926,927]. Based on the highly effective human complement receptor 1 (CR1) the construct designated as APT070 (or Mirococept) consists of the first 3 consensus domains of CR1 and a membrane-targeting synthetic peptide, which facilitates binding of the molecule to the phospholipid bi-layer of the endothelial cell surface and protection against complement activation. Activation of the complement system has been shown to be involved in pathogenesis of tissue damage in IR injury and a potential therapeutic target[928]. Mirococept has been evaluated in animal models of limb-ischaemia, and renal and cardiac transplantation to ameliorate both IR injury

and IR injury and rejection [929], and is currently undergoing MRC funded Phase 2 trials (EMPIRIKAL) in the UK in kidney transplantation with DGF the primary end-point.

The use of membrane-targeting synthetic peptides in organ transplantation introduces the concept of 'organ painting'. Organ painting simply put is organ pre-conditioning or pre-treatment prior to transplantation with therapeutic agents that (through membrane-targeting peptides) are able to localise to the membranes of endothelial cells of the organ vasculature, 'painting' the organ from the 'inside'. Such compounds can be termed 'cytotopic' *(From the Greek: kytos + topos*)[930].

1.8.2.2 Membrane-targeting synthetic peptides

Natural Mechanisms

Approaches to design therapeutic agents for addressive therapies have utilised several aspects of natural mechanisms of protein and cell membrane interactions. Several intracellular proteins have been shown to undergo reversible binding to inner leaflets of cell membranes, such as ras and Src[925]. The mechanism of reversible binding is based on a two site interaction between the protein and the cell membrane.

Such a protein would have undergone post-translational modification, with the attachment of hydrophobic group (normally myristoyl or palmitoyl) to the effector amino acid sequence of the protein. The hydrophobic group inserts in the hydrophobic interior of the lipid membrane bi-layer, and is then anchored by an electrostatic interaction between positively charged amino or guanidino side-chains in the effector amino acid, and the negatively charged phospholipid heads of the membrane. This anchoring provides sufficient energy to enable stable binding and attachment. Dissociation is through phosphorylation of series residues near to, or contained within, the main effector amino acid sequence, which reduces the overall positive charge of the protein, weakening the electrostatic attraction component of the interaction that enabled stable binding, with subsequent de-attachment. Once released intracellular phosphatases dephosphorylate the serine sequences, resetting the mechanism. [931]

Theoretical Approach to Designing Therapeutic Addressives

Smith et al. at theorised that the additive binding concept could be retained, with the switch elements discarded, in a system of addressive targeting coined a 'sequential membrane addressin' (SMA) to design a moiety that could be produced for attachment to an 'effector' protein and allow targeting of that protein to specific biological sites[925].

Simplistically each SMA could be regarded as a molecular post code and several SMAs could be combined to produce a targeted address, where each consecutive element in the moiety specifies for a more specific sequence. e.g. SMA-1 acts as postal are code (all cell membranes), SMA2 as the region within the a town (acidic phospholipid membranes) etc. (1).

SMAs have been designed using the myristoyl- electrostatic switch (MES) concept, but other mechanisms that interact with outer cell membrane components are feasible e.g. using sLex carbohydrates to target sites on P- and E- selectin CAM receptors, or antibody fragments to bind cells modified by dansyl hapten[932]. The CR1 inhibitor Mirococept was designed using the MES concept for the SMA.

Mirococept (APT070)

Mirococept is a derivative of the anti-complement molecule CR1 (or CD35) conjugated to a MES based membrane localising peptide[925,933]. The CR1 component comprises the 'head' of the molecule, and the three sequences of CR1 are based on the findings that these initial 3 sequences of SCR at the N-terminal effect the relevant biologic activities of CR1[934]. The membrane localising 'tail' component is myristoyl (SMA1) and the SMA2 is a positive peptide (Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp). The tail is conjugated to the CR1 head via an activated disulphide thiol bridge ([S-2thiopyri- dyl]-Cys-carboxamide). The structure of Mirococept is shown in Figure 24.

The membrane localising tail sequence has been demonstrated to successfully adhere to both murine and porcine endothelial cells in the vasculature of murine and porcine kidneys (see Figure 25), and porcine hearts, in models of transplantation and IR injury [933,935,936], as well as endothelial cells in human kidneys[936] (see Figure 26).

Localization of Mirococept is rapidly complete within several minutes and the dissociation half-life averages several hours, with experiments using radiolabelled tails suggesting that modified (localising) CR1 is retained 5–10 times longer than the unmodified CR1 form [935].

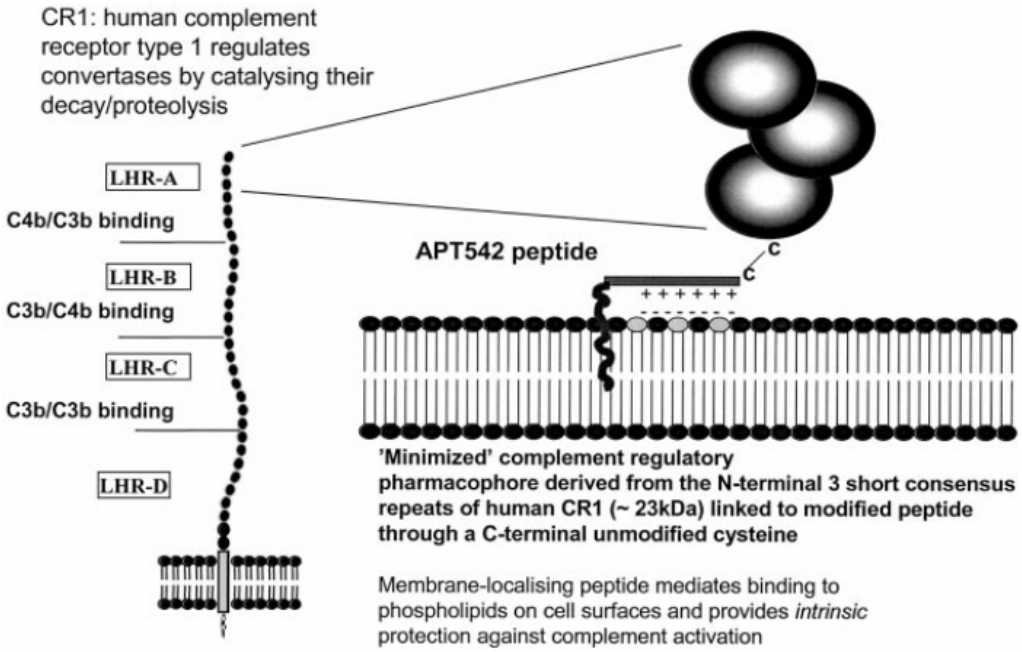


Figure 24 Outline structure of Mirococept [925].

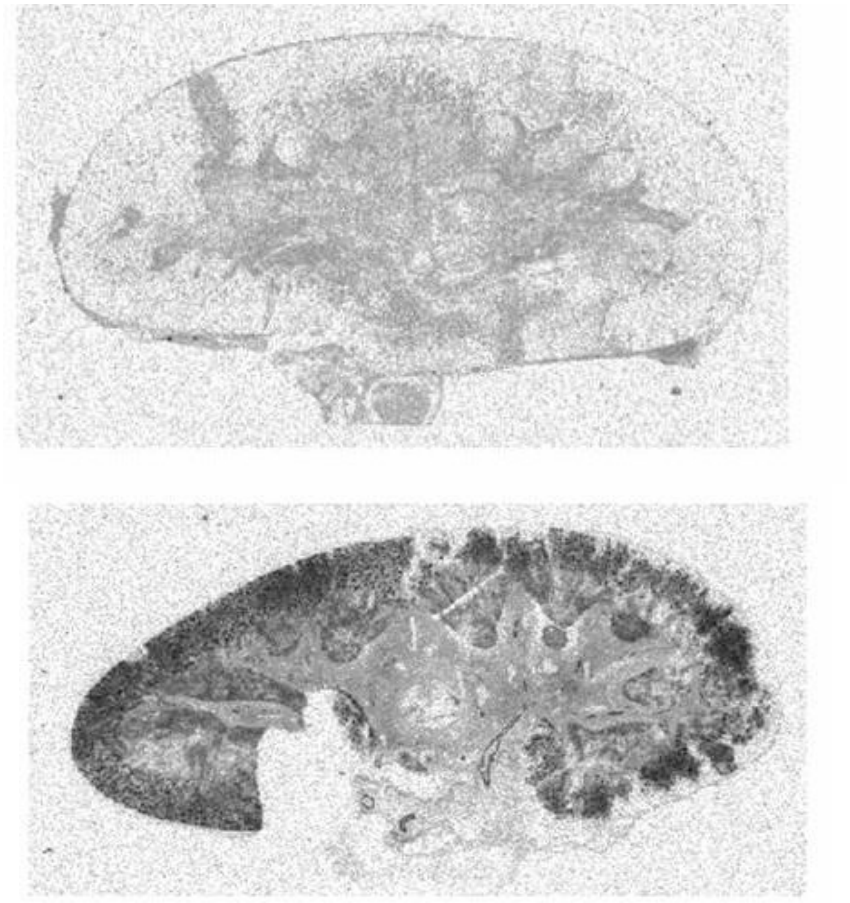


Figure 25 Demonstration of non-transgenic addressive targeting of myristoyl tethering agents using whole-organ autoradiography using ^{35}S or ^{14}C labelled anti-complement proteins. Top: porcine kidney perfused with the radio labelled CR3 anti-complement component of Mirococept (without a conjugated tail) demonstrating only 10% retention. Bottom: porcine kidney perfused with radiolabelled Mirococept (intact protein) with 90% retention. Provided by Dr Richard Smith, King's College London.

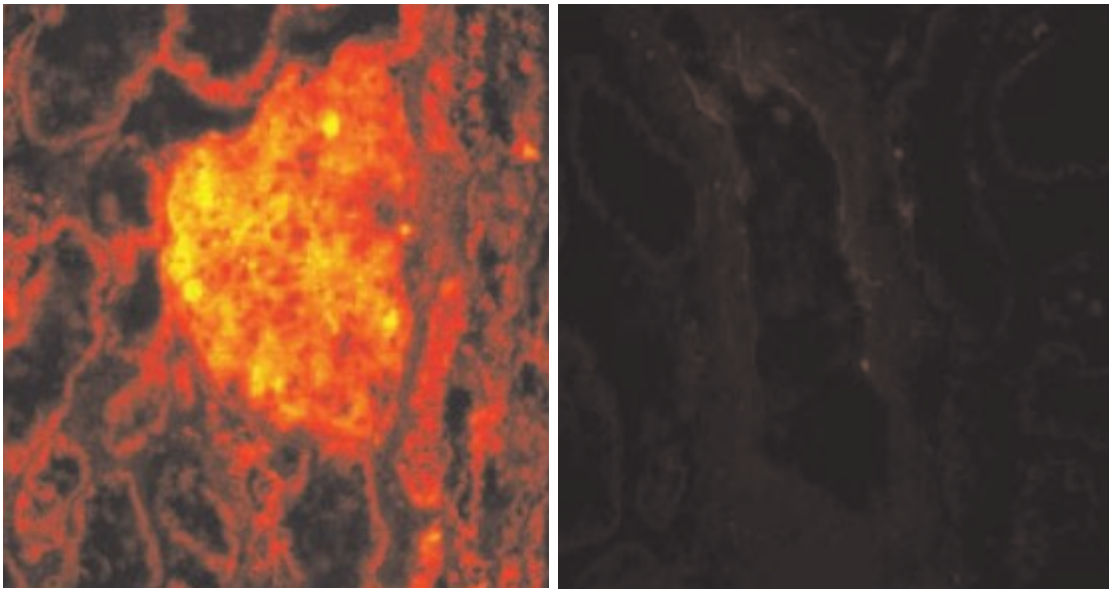


Figure 26 Photomicrograph demonstrating binding of Mirococept within a human kidney. Left: Immuno-histochemistry of human kidneys perfused with preservation solution containing Mirococept showed that the agent bound to glomeruli and tubules. Right: human kidney perfused without Mirococept showed no staining [936].

1.8.2.3 Use of Targeted Anti-coagulants in Organ Transplantation

The interplay between coagulation cascades and inflammatory responses during IR injury and especially during organ transplantation has been demonstrated in numerous experimental and clinical models. TF expression [214–216], thrombin generation [264–266], and fibrin deposition [331] is known to be actively up regulated during IR responses, and exogenous treatment with TFPI[239], APC [247], TM [937], AT [307,690], and direct (and indirect) thrombin inhibitors[672–674], have all shown variable degrees of benefit in reducing injury and organ function during IR injury.

Several of these anti-coagulant (and potentially anti-inflammatory) proteins contain heparin-binding domains, which lead to the interactions with glycosaminoglycans like heparan sulfate. The concentrating presence of these proteins on the glycocalyx ensures maintenance of a quiescent rather than an activated state of the endothelium. During IR however the endothelial surface and glycocalyx is injured with loss of endogenous anti-coagulant proteins, and activation of endothelial cells that promotes both a pro-coagulant and a pro-inflammatory phenotype and response.

Despite efficacy in experimental models TFPI, APC[938], and TM[309] have not shown clinical correlates. DTIs have shown benefit in IR during experimental organ transplantation [672–674], and

clinically use of DTIs during cardiopulmonary bypass and acute coronary syndrome [411–414] ameliorates cardiac injury.

Use in transplantation is less certain. Heparin has been used extensively in prevention of thrombosis in kidney and pancreas allografts [627,684,939], and is known to possess anti-inflammatory properties, but at clinically safe doses only anti-coagulant rather than anti-inflammatory effects dominate. Even at clinical doses in the prevention of allograft thrombosis (which maybe evidence of progression of IR related coagulation with extension from the micro-circulation into the macro-vasculature) risk of bleeding is high, and prevention of thrombosis not guaranteed.

An ideal approach would be an addressive anti-coagulant which localises to within the allograft exerting an anti-coagulatory (and thus potentially anti-inflammatory) effect locally at the site of the IR response rather than systemically. This would be potentially more effective and omit the need for systemic anti-coagulation which confers a serious risk of bleeding to patients.

Thrombalexin (TLN; PTL004) is one such novel cytotopic agent. It is a sister molecule to Mirococept, where the anti-complement CR1 head has been replaced with a Hirudo medicinalis based anti-coagulant moiety. The anti-coagulant head is a derivative of the DTI bivalirudin (Hirulog), designated HLL. Similar to the relationship between CR1 and SCR, HLL is a shorter peptide than Hirulog and comprised of the active peptide sequences mediating anti-thrombin activity. The overall structure and potential function of TLN is illustrated in Figure 27

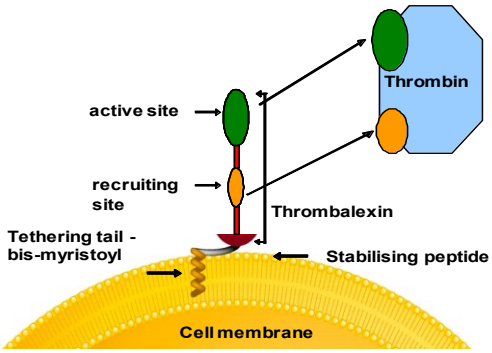


Figure 27 The cytotopic anti-thrombin molecule: Thrombalexin. TLN has direct inhibitor of thrombin enzyme activity via the anti-coagulant HLL peptide that is related to Hirulog (Bivalirudin). The cell membrane tethering tail is bis-myristoyl based. Provided by Dr Richard Smith, King's College London.

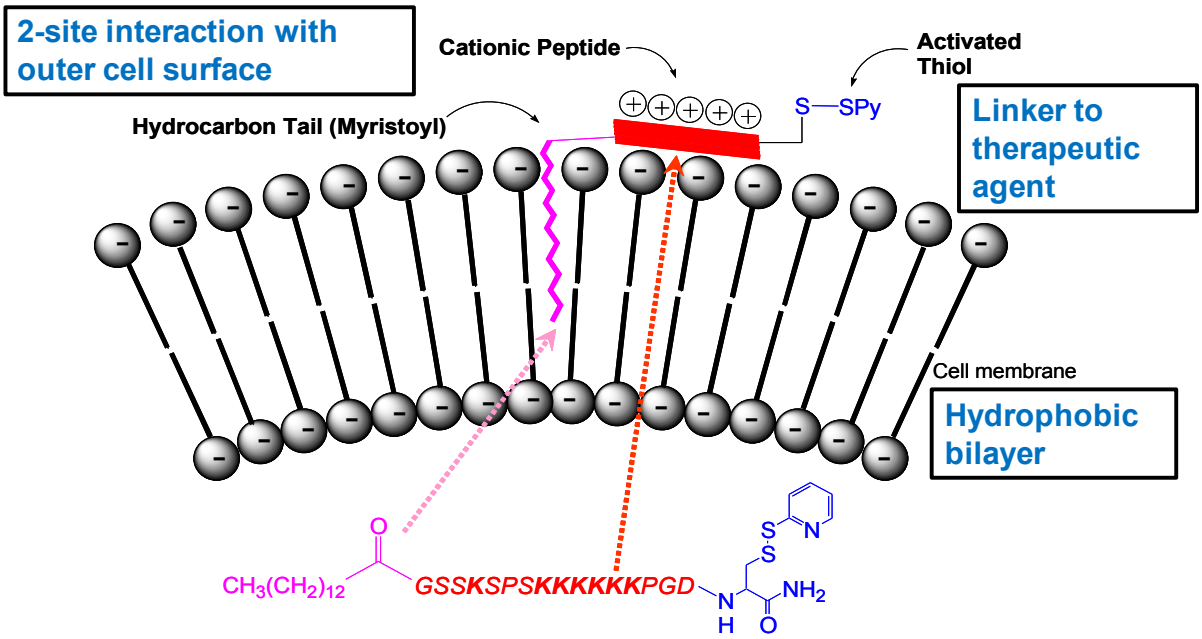


Figure 28 Schematic of the three primary addressive components of the Thrombalexin: 1) Based on the MES comprising a bis-myristoylated peptide tail; 2) with a positive peptide stabilizer conjugated to the HLL peptide by; 3) a disulphide bridge (Gly-Ser-Ser-Lys-Ser-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-Pro-Gly-Asp-S-2thiopyridyl-Cys-carboxamide). Provided by Dr Richard Smith, King's College London.

The membrane localising tail is similar to Mirococept and based on the MES comprising a bis-myristoylated peptide; with a positive peptide stabilizer conjugated to the HLL peptide by a disulphide bridge. TLN has demonstrated the ability to incorporate into outer cell membranes of endothelial cells and thus confer a cell-associated anti-coagulant activity that is stable to cell washing cycles. The three main components of TLN are illustrated in Figure 28.

Due to the nature and size of the hydrophobic anchor internalization of the TLN conjugate is discouraged once it is membrane bound, and is thus able to maintain presentation of the functional anti-coagulant moiety within the intravascular space. Despite structural differences TLN has similar localising characteristics as Mirococept, with demonstrated affinity for porcine endothelial in-vitro, and murine endothelial in-vivo [940].

In the development of TLN several variants of the HLL localising compound were designed. One such early variant designated PTL006, comprises the HLL peptide head with the localising tail based on a pegylated phospholipid (glycophosphatidyl inositol [GPI]) anchor. PTL006 has been shown to localise to glomeruli in rat kidneys (see Figure 29) but comparative analysis has determined that TLN

(PTL004) binds more strongly once attached to cell membranes with slower cell-cell transfer than PTL006 [941].

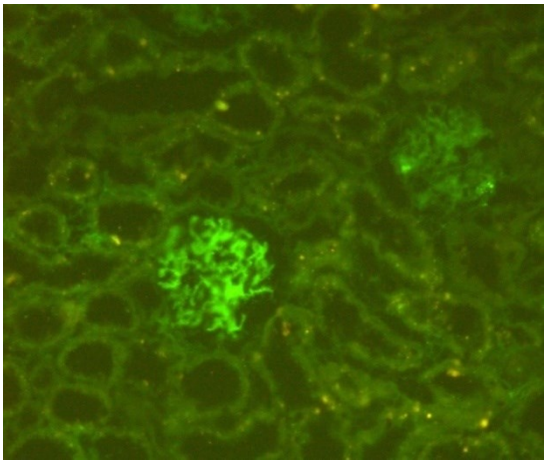


Figure 29 Fluorescent (FAM) labelled PTL006 perfused through a rat kidney demonstrating localisation to glomeruli and peritubular vessels. Provided by Dr Richard Smith, King's College London. [941].

The anti-coagulant HLL moiety has been shown to possess similar functional anti-thrombin properties as unmodified Hirulog. Expression of HLL in-vitro on porcine endothelial cells by transfection of these cells with DNA constructs encoding for the Hirulog-like molecule demonstrated an inhibition of clotting on exposure of these cells to human plasma compared to control group cells [942,943]. Though the model in this study is xenogenic, and immunogenic factors are an important consideration, the demonstration that the hirudin proteins inhibited thrombus formation is evidence of effective in-vitro functionality in context of pro-thrombotic stimuli.

In a murine model of lipopolysaccharide (LPS) induced sepsis, transgenic expression of this hirudin based anti-coagulant protein on endothelial cell walls inhibited the widespread intravascular thrombosis and consumptive coagulopathy commonly associated with severe endotoxaemia [944].

More recently in a skin graft presensitized recipient rat renal allograft transplant model (SRAT) TLN pre-treatment (via flushing) of allografts during cold storage and prior to transplantation improves graft function and extends graft survival to 9 days vs. 2 days for untreated controls[940]. In the same model PTL006 demonstrated similar survival benefits[945]. Thus considering the original description of the anti-coagulant fusion proteins to be used in this study – the hirudin element has been proven to inhibit thrombosis in two different animal models.

1.8.3 Organ Preservation Solutions

The increasing use of ECD and DCD organs to expand donor pools and meet the requirements of the growing list of patients awaiting a kidney allograft has led to a resurgence of novel methods to optimise marginal organ viability. HMP has been shown to confer benefit in kidney allograft preservation compared to SCS [946]. Developments in the understanding of the base molecular changes and mechanisms underlying IR injury in transplantation has prompted interest in developing new preservation solutions that may more effectively ameliorate IR injury in marginal allografts.

Adenocaine (AL) solution (Hibernation Therapeutics Global Ltd, Kilquade, Ireland) is a novel normokalemic non-depolarising preservation solution initially developed for use as a cardioplegia and for adult cardiac allograft preservation.

AL solution is based on the theoretical benefits of using a non-depolarising membrane approach in-conjunction with the two key constituents adenosine and lidocaine in providing superior allograft preservation and ameliorating IR injury. Experimental and early clinical evaluation of AL solution in cardiac surgery and transplantation indicates superior organ preservation and function post reperfusion.

Adenocaine has been shown to exert protective and beneficial effects on cardiac function after periods 6-8 hours of SCS using a murine ex-vivo cardiac reperfusion model with better functional recovery of coronary and aortic flow rates vs. allografts stored in conventional solutions such as Celsior [947–949]. AL appeared to afford cardioprotection by preserving myocardial high-energy phosphates, with down regulation of the heart balanced by the expense of a high acid-load during ischemia, and then recovery of myocardial pH during reperfusion[950], which enable better function on reperfusion and during re-animation[951].

The polarizing concept of arrest and protection using Adenocaine is currently being used in a number of US centres as all blood microplegia[952], but there is only one case reported of AL microplegia used in a high-risk adult patient undergoing cardiac surgery[953]. Furthermore Adenocaine exerts an anti-inflammatory effects on in-vitro isolated porcine leucocytes, inhibiting ROS generation, CAM expression, and leucocyte-endothelial adhesion and cell infiltration[954]. There is potential for application of AL preservation solution to other organs, in particular kidney preservation prior to transplantation. Adenocaine Machine Preservation Solution (AL-MPS) is a variant of Adenocaine specifically modified in-house to facilitate HMP of abdominal organs designed to target specific elements of renal physiology in an effort to improve organ preservation. AL-MPS is composed of a

modified KHB base with 4mM adenosine; 0.5mM lidocaine; 0.26mM calcium; 16mM Magnesium; and 4% bovine serum albumin (BSA) and a comparison with other preservation solutions can be seen in

1.8.3.1 Adenosine Physiology

Adenosine is produced by all mammalian cells and regulates a wide spectrum of physiological activities[955]. It regulates renal renin production, the glomerular filtration rate (GFR) and renal vascular tone, and is also a key regulator of tubular-glomerular feedback [955,956]. Tissue adenosine levels are increased during states of negative energy balance where the hydrolytic rate of ATP is greater than that of ATP synthesis, such as in conditions of ischaemia and tissue hypoxia during pathological insults to the kidney[955,957].

Extracellular adenosine arises from enzymatic hydrolysis of ATP or ADP with high levels (>5mmol/L) during ischaemic tissue injury[958]. ATP and ADP may be released into the interstitium via granular release from platelets, inflammatory cells, and even endothelial cells. ATP and ADP are hydrolysed by ectopyrase (or CD39) in AMP, which is the degraded by enzyme ecto-5'-nucleotidase (CD73) into adenosine[959–961].

Extracellular ATP deficits from breakdown into adenosine are recognised damage signals promoting further tissue injury and destruction[962–964]. Thus CD73 may serve as both a remover of deleterious ATP and generation of cytoprotective adenosine during ischaemic tissue injury[965]. Conversely adenosine is thus also a precursor and substrate for ATP production.

Adenosine effects are mediated by a series of G-protein coupled purinergic adenosine receptors (AR) that are either high affinity receptors (10-100nM) and include A1AR, A2AAR, and A3AR receptors, or low affinity (>1μM) such as A2BAR where such levels are frequently only encountered in pathological conditions[955,966,967].

Adenosine has an important role in modulating inflammatory injury highlighted by the fact that its receptors are highly expressed on the endothelium and immune cells both critically involved in IR injury. Renal AR expression however is heterogeneous (see Figure 30), temporal and dependent on the type of insult - whether ischaemia, hypoxic, inflammatory or potentially a composite as in transplant related IR injury[965].

1.8.3.2 Adenosine in the Kidney

A1 Adenosine Receptors are expressed in most parts of the kidney, especially in distal afferent arterioles, proximal tubules, collecting ducts, and papillary epithelia [966], see Figure 30. These receptors regulate vascular tone, TGF and renin release - and stimulation with exogenous adenosine results via a PKC and ERK/MAPK signalling [968] in renal arteriolar vasoconstriction, with a decline in GFR, and an increase in NaCl, HCO3-, phosphate and fluid resorption [956].

A1AR receptors are believed to be a key player in modulating renal IR injury. However both A1AR activation and blockade can be protective depending on the nature of the pathological insult to the kidney e.g. nephrotoxic vs. ischaemic injury [965].

During ischaemic insults A1AR stimulation reduces GFR and afferent cortical blood flow - suggesting in fact that ischaemic effects may be mediated by these same receptors [955]. The decrease in GFR, renin release, sympathetic outflow and fluid transport/reabsorption associated with A1AR stimulation however would theoretically reduce the filtration work load of the allograft and with it renal oxygen demand and thus consumption, which is a potentially beneficial feedback effect in the setting of ischemic renal injury [965].

Furthermore though adenosine is a renal vasoconstrictor through interaction with afferent arteriolar A1AR receptors it is only so when not affecting the bulk of renal A2AR receptors at the same time [969,970]. In-vivo activation of the more prevalent A2AR receptors (found predominately in glomerular epithelium and adjacent vasculature) produces an overall persistent vasodilatation in kidney allografts, with reduced vascular resistance, despite a relative and short lasting vasoconstriction in afferent arterioles [971–973].

The A2AR receptors facilitate increased medullary blood flow and oxygenation, while lowering medullar transport activity [955] through NO generation - with A2AAR agonists improving medullary flow and oxygenation after IR injury [970,971]. Thus adenosine functions as a link between renal energy demand and supply through its endogenous vaso-constricting and -dilatory effects.

These receptors are also known for their immuno-regulating ability however renal protection against IR injury has been shown to be independent of macrophage activation. [974]

A2AAR receptor effects are mediated by Gs protein stimulation of adenylate cyclase and protein kinase-A and results in a cAMP Response-Element Binding-mediated cytoprotection [969,975,976].

A2BAR receptors are expressed primarily on the renal vasculature [977,978] and function through the same signalling pathway as A2AAR receptors, producing similar effects. The protection afforded by A2BAR receptor subset is thus targeted to the endothelial and in a limited extent the tubular epithelial, compared to A2AAR receptors that also modulate infiltrating immune cell activity [979].

The distribution and mechanism of the A3 Adenosine receptor sub-type in the kidney is still not clear. Neither is the role A3AR plays in IR based tissue injury with contrasting reports in the literature suggesting activation or suppression differentially modulates cellular damage depending on the mechanism of injury [980,981].

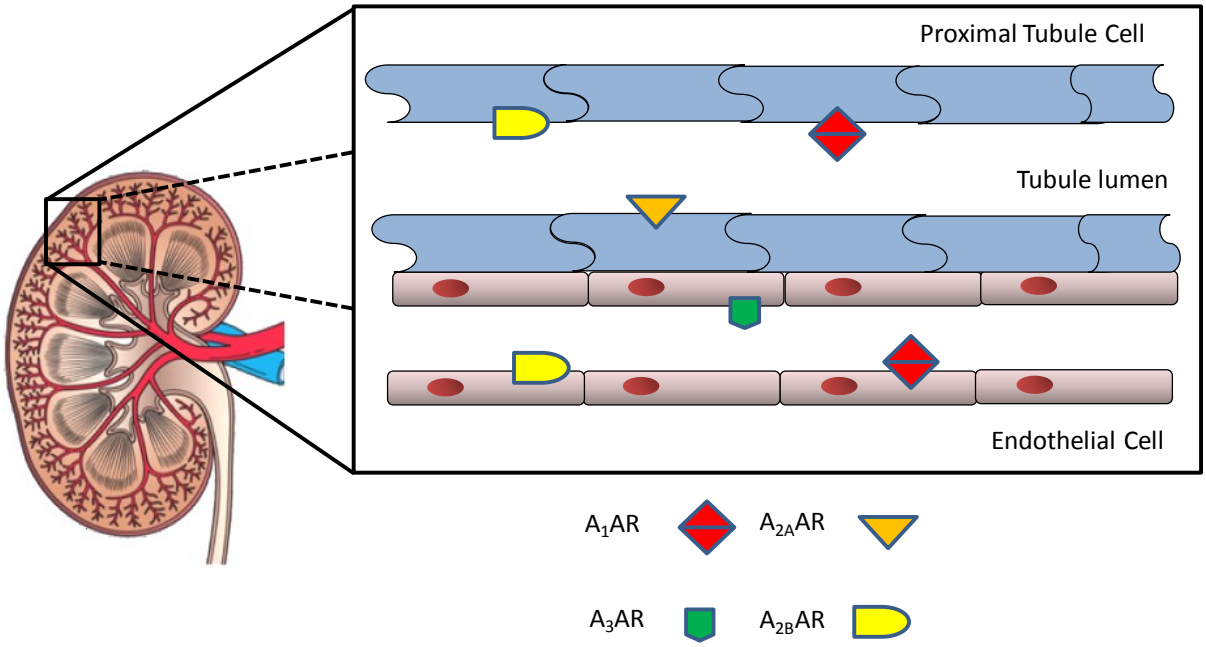


Figure 30 Adenosine receptors (ARs) in the kidney mediating cytoprotection. A1AR: endothelial and tubular activation produces cytoprotection. A2AAR: found on leukocytes (e.g., neutrophils and lymphocytes) and tubular cells afford IR protection by reducing inflammation. A2BAR: renal tubular and endothelial expression may also facilitate renal protection. A3AR: expressed on many renal cell types but importantly on endothelial cells, and signalling appears variable and dependent on the type of injury but may be detrimental to kidney function during ischemia [982].

1.8.3.3 Lidocaine

Lidocaine is a local anaesthetic agent that acts through inhibition of voltage sensitive fast opening sodium channels. It is also used as an anti-arrhythmic drug, [983] and, prior to the use of amiodarone was drug of choice during cardiac resuscitation[984,985]. Its effects on sodium channels also means it posses negative chronotropic and inotropic effects, and acts as a vasodilator [986].

Independent of its mechanism of action via sodium channel inhibition lidocaine has also been shown to exert anti-histimainic and anti-inflammatory effects. The anti-inflammatory mechanisms of lidocaine have yet to be elucidated. Possible mechanisms include inhibition of: 1) leucocyte endothelial interaction [987]; 2) the release inflammatory cytokines and chemokines [988]; 3) the production of superoxide anion ROS by XO[989,990]; 4) ROS damage by scavenging hydroxyl free radical species [991]; and 5) sodium/calcium exchange and thus reducing cellular calcium accumulation during ischemia [992–994].

Studies have shown that continuous infusion of lidocaine protects against myocardial IR injury in the in-vitro rat heart [995] and in the in-vivo rabbit [996], cat [997], pig [998], mice [999] and canine heart reducing infarct size[1000], and also against cerebral IR injury limiting the degree of apoptosis[1001].

Mechanisms of action of lidocaine for limiting IR injury may be related to the 5 proposed anti-inflammatory effects suggested, however recent evidence suggests lidocaine may limit tissue injury by alternative uncharacterised anti-apoptotic pathway[999].

These mechanisms may not be mutually exclusive but related, for example increased cytosolic Ca during IR is a potential co-factor for formation of XO, and thus inhibition of sodium/calcium exchange and limiting calcium accumulation by lidocaine may serve to decreased ROS production[1002], while the same mechanism may also help to preserve mitochondrial bioenergetics, reduce deleterious mitochondrial calcium loading during reperfusion, and exhibit better preservation o ATP [1003].

Recently lidocaine has been shown to inhibit IL-1, IL-6, and IL-8 production and ICAM-1 expression on activated endothelial cells in-vitro [987,1004], and both blocks TNFα production[988] via NFk-B, and TNFα signalling by attenuating the PI-3K subunit p85's recruitment to TNF receptor, and limiting leucocyte-endothelial adhesion, endothelial barrier permeability, and eNOS activity[1005]. Lidocaine has also been shown to reduce P-selectin expression on activated platelets in-vitro, and thus potentially may produce a similar effect on endothelial cells[1006].

In contrast, the effects of lidocaine on renal function are controversial. Lidocaine pre-treatment of renal donors has been previously reported in studies to enhance renal excretory function and reduce the incidence of delayed graft function in cadaveric transplantation [1007–1009]. While other reports suggest worsening of renal function after IR injury with local anaesthetic intervention [1010].

Perhaps one of the most suggestive reports is of an ex-vivo reperfusion model using canine kidneys where low dose lidocaine (80.5µmol/L) addition to Euro Collins SCS solution had a significant cytoprotective effect on allograft function evident after 48 hours of SCS [1011]. Reperfusion dynamics demonstrated superior viability in-terms of higher GFR and renal flow rates, lower urinary LDH, with evidence of less lipid peroxidation, and histologically reduced tubular necrosis, dilatation, interstitial oedema and cell detachment.

Parallel to this however lidocaine has been suggested to impair repair of the ureteric epithelial layer and increase the risk of urological complications by decreasing urothelial cell viability, with the effect concentration (lowest: 9mM, highest 31mM) and time dependant [1012].

1.8.3.4 Electrolytes

Magnesium is the second most prevalent intracellular cation after potassium and is a co-factor in hundreds of enzymatic reactions for energy metabolism and nucleic acid synthesis. Its actions have been likened to a physiological antagonist of endogenous calcium, and is involved in hormone receptor binding; gating calcium channels, with effects on transmembrane ion flux; muscle-fibre contraction;; the control of vasomotor tone; cardiac excitability; and both neuronal activity and neuro- transmitter release[1013]. Only 1% of magnesium is present in blood and approximately one fifth of total magnesium bound intracellularly to ATP within soft tissue, with ATP depletion during ischaemia releasing bound magnesium, increasing free levels[1014,1015]. Due to its natural calcium antagonist, and anti-inflammatory properties it has historically been an adjunct in surgical cardioplegia since the 1970s [1016] protecting against I/R related cytoplasmic calcium overload[1017], with magnesium treatment during myocardial IR also reducing infarct size by augmenting an adenosine mediated pathway[1018]. The inclusion of magnesium in AL is based upon its effects on membrane stabilisation, facilitating ATP production, and observed anti-inflammatory properties in IR settings.

From a scientific standpoint, there are at several areas of concern with potassium concentrations of 10 mmol/L and above in conventional preservation solutions. Such high potassium levels may promote ionic imbalances, with cellular sodium and calcium loading leading to interstitial cell and endothelial cell dysfunction and microvascular injury [951,1019]. Vascular endothelial cells can become depolarised and activated, entering into a pro-inflammatory and thrombotic state with increased permeability [1020]. Finally potassium is a potent vasoconstrictor which during preservation and subsequent reperfusion can promote a higher vascular resistance and vessel

spasm, impairing effective flushing and inducing mal-perfusion [951,1021]. AL seeks to avoid these potential effects, with a normokalemic potassium content.

AL as a composite of adenosine, lidocaine, and magnesium has been demonstrated in cardiac models to afford better protection than conventional preservation solutions possessing membrane voltage stabilisation, vasodilatory, anti-inflammatory and anti-coagulant properties, as well as been shown to preserve high-energy phosphates during ischaemia[950,954,1022–1024]. With the increasing use of ECD and DCD donor organs being used to expand donor pools interventions to optimise kidney viability are in much clinical need, and the cytoprotective properties of AL may prove beneficial in kidney transplantation.

	Preservation Solutions										
	Collins	EC	Belzer UW	HTK	Celsior	HOC	Custodiol-N	IGL-1	Belzer-MPS	Polysol	Al-MPS
Sodium	10	9.3	25.0	15.0	100	80	16.0	120	100	136.0	141
Potassium	115	115.1	125	10.0	15	80	10.0	30	25.0	5.0	5.9
Calcium	-	-	-	0.015	0.25	-	0.02	-	0.5	9.0	0.224
Magnesium	30	4.7	5.0	4.0	13.0	35	8.0	5	5.0	14.0	16
Chloride	15	15.0	-	51	41.5	-	30.04	20	1.0	-	6.3
Gluconate	-	-	-	-	-	-	-		85	10.0	-
Phosphate	50	57.6	25	-	-		-	25	25	0.8	1.2
Sulfate	30	4.7	5.0	-	-	40	-	5	-	4.0	16
Bicarbonate		9.3		-	-	-	-	-	-	25.0	25
Glucose	25	1.9		-	-	-	-	-	10.0	16.0	6.7
Glutamic acid	-	-	-		20.0	-	-	-	-	-	-
Raffinose	-	-	30.0	-	-	-	-	30	-	-	-
Lactobionate	-	-	100	-	80	-	-	100	-	-	-
HES (g/L)	-	-	50	-	-	-	-	-	50	-	-
Citrate	-	-	-	-	-	55	-	-	-	-	-
Histidine	-	-	-	180.	30.0	-	124.0	-	-	-	-
Adenosine	-	-	5.0	-	-	-	-	5		5.0	4
a-ketoglutarate	-	-	-	1.0	-	-	2.0	-	-	-	-
Adenine	-	-	-	-	-	-	-	-	5.0	5	-
Pyruvate	-	-	-	-	-	-	-	-	-	0.2	-
Allopurinol	-	-	1.0	-	-	-	-	1	1.0	1.0	-
Glutathione	-	-	3.0	-	3.0	-	-	3	3	3.0	-
Mannitol	-	32	-	30.0	60.0	185	30.0	-	30	-	-
Tryptophane	-	-	-	2.0	-	-	2.0	-	-	-	-
Ribose	-	-	-	-	-	-	-	-	0.5	-	-
HEPES	-	-	-	-	-	-	-	-	10.0	-	-
PEG-35	-	-	-	-	-	-	-	0.03	-	20	-
Amino acids	-	-	-	-	-	-	-	-	-	11.0	-
Phenol red	-	-	-	-	-	-	-	-	-	0.07	-
N-A-H	-	-	-	-	-	-	57.0	-	-	-	-
Sucrose	-	-	-	-	-	-	33.0	-	-	-	-
Aspartate	-	-	-	-	-	-	5.0	-	-	-	-
Glycine	-	-	-	-	-	-	10.0	-	-	-	-
Alanine	-	-	-	-	-	-	5.0	-	-	-	-
Arginine	-	-	-	-	-	-	3.0	-	-	-	-
Deferoxamine	-	-	-	-	-	-	25.0	-	-	-	-
BSA (g/L)	-	-	-	-	-	-	-	-	-	-	40
Lidocaine	-	-	-	-	-	-	-	-	-	-	0.58
LK 614	-	-	-	-	-	-	7.5	-	-	-	-
pH	-	7	7.4	7.2	7.3	7.1	-	7.4	7.4	-	7.4
Osmolality (mOsm/kg)	-	375	320	310	320	-	-	320	-	-	NA

Table 5 Composition of AL-MPS compared to common preservation solutions. N-A-H: N-Acetylhistidine. Units of solutes are in mmol/L unless stated otherwise. Data is collated from several sources[62,751,762–767].

1.8.4 Post-Ischaemic Organ Reconditioning

Despite the growing evidence for benefits of HMP in allograft preservation implementation of HMP protocols similar to those described in the Eurotransplant Trial is logistically complex and resource intensive. The necessity of this technology being timely available at the site of organ retrieval for transport to the recipient centre, as well as continuous monitoring during preservation and transport, has limited uptake of HMP for routine organ preservation, where SCS may actually be sufficient for the majority of harvested organs. Use of HMP likely lies in preservation of ECD and DCD allografts for reconditioning and assessing viability. Interest has focussed on post-ischaemic hypothermic reconditioning as a technique that may allow benefits of HMP to be realized in allografts after a period of SCS.

Koetting et al. [800] first demonstrated the benefit of 90 min of hypothermic reconditioning, by oxygenated HMP compared to non-oxygenated HMP to recover organs after prolonged (18 hours) SCS ischemic injury. Oxygenation during HMP produced substantial 3-fold higher clearances of creatinine and urea, and less apoptotic caspase-3 activity during organ viability assessment on an isolated reperfusion circuit. The authors concluded that continuous oxygenation during HMP effectively ameliorated energetic breakdown and deterioration of mitochondrial redox balance. The same group in another study using a porcine renal autotransplantation model demonstrated that a short period (2 hours) of reconditioning after prolonged (21 hours) SCS proved as effective as continuous HMP for the whole storage time [812]. Active oxygenation was provided in both continuous HMP and 2 hours of post-ischaemic reconditioning HMP, and equally improved urea and creatinine clearance, as well as tubular function, with lower expression of TLR-4, lower HMGB-1 release. A short period of HMP even after extended SCS can also normalise expression of KLF-2 reducing the pro-inflammatory endothelial phenotype [823]. Furthermore a period post-ischaemic reconditioning of even 1 hour duration is as effective as 4 hours, after 18 hours of SCS, with improved creatinine clearance, tubular reabsorption, and renal flow on an isolated reperfusion circuit [1025]. Furthermore the use of pulsatile flow in HMP appears to be the important factor in HMP vs. non-pulsatile flow to ensure benefits of HMP are realized compared to SCS [801,823].

Other reports offer conflicting evidence. Using isolated porcine DCD kidney reperfusion model a short (4 hour) period of HMP after prolonged (18 hours) SCS was found to confer no advantage over simple SCS preservation, compared to 18 hours of continuous HMP [810]. In this study the Lifeport machine was used which offers no capacity for active or passive oxygenation and relies solely on

atmospheric pressure[805]. Furthermore ROS mediated lipid peroxidation damage was higher in HMP than SCS kidneys, further implicating a lack of oxygenation during HMP as an important factor in these results compared to studies with oxygenation.

Concerns for the use of oxygenation during HMP are predominately related to the potential formation of excess ROS from a high concentration of oxygen. This has supported the use of free radical scavengers in HMP perfusate, such as superoxide dimutase. Evidence of ROS or other damaging effects of oxygenation are lacking in kidney preservation and transplantation models. Liver studies have suggested however that high (>95%) concentrations of oxygen increase ROS generation during reperfusion, and so lower concentrations (21%) have been advocated[1026]. More recent reports however are inconsistent, with one group observing less oxidative injury and better function during reperfusion when 100% oxygen was used for rat liver HMP compared to 21% or with non-oxygenated HMP[1027].

The overwhelming body of evidence points to HMP - oxygenated or non-oxygenated- as superior to SCS preservation. The development of HMP is being directed to towards reconditioning and optimising viability of less viable DCD and ECD donor allografts with the intent to promote their immediate function, and increase longer term survival, allowing an expansion of the donor pool with these donor cohorts. At present the full capacity of HMP to recondition an organ is unknown, but research investigating the limits to which HMP can be effectively used will allow a better application of HMP in clinical practice.

1.8.5 Pancreatic Hypothermic Machine Perfusion Preservation

1.8.5.1 Historical development of pancreatic machine perfusion

The preservation method of choice for both ICT and whole pancreas transplantation is currently SCS. Despite the definite benefits of transplantation, the shortage of organs is a predominant factor in limiting ICT and whole pancreas transplantation. This, combined with the increased use of ECD, has led several groups to attempt to utilise the success of HMP with kidneys, in the maintenance and assessment of the quality of pancreatic islet cell and pancreatic grafts [869].

Use of HMP confers a number of advantages already demonstrated in kidney preservation. In particular the potential for viability testing, and more so the possibility to pharmacologically intervene and optimise pancreatic tissue viability has been the key driver behind recent research.

Despite this interest pancreatic HMP remains a mostly unexplored option. Historically in the decades where Belzer was developing renal HMP experimental attempts were made to apply HMP to the pancreas, with some limited success compared to that enjoyed in the kidney.

There were several notable attempts reported including the Minneapolis group [1028,1029] under Najarian and Sutherland, and Brynger et al.[1030] in the 1970s and early 1980s, and Kenmichi [1031] in Japan in 1992. Using a canine auto-transplant model Najarian's group demonstrated that using a variety of low perfusion pressures (5-25mmHg), 24 hours of hypothermic perfusion of pancreases with a plasma perfusate, was successful in preserving pancreases with good function upon transplantation[1028]. Brynger et al. [1030] successfully perfused segmental canine pancreas allografts for 24 hours on a Gambro machine at high pressures (50mmHg) with an albumin containing electrolyte solution, and despite oedematous change of 135-215% 6 of 9 grafts functioned after transplantation. Further canine work however comparing pancreatic HMP preservation to SCS however demonstrated pancreatic HMP to be a technically complex procedure that was inferior to SCS with poor graft survival in those perfused organs, related to post-HMP oedema (see Figure 31) [1029].

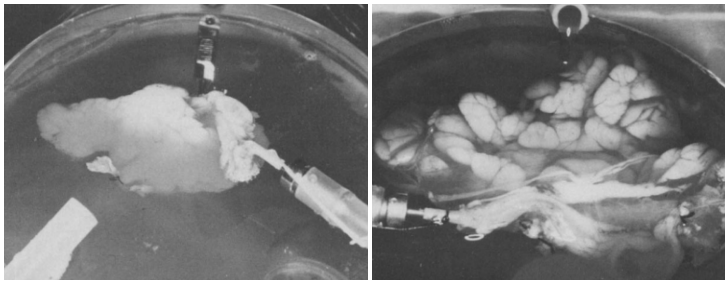


Figure 31 Early attempts with a segmental canine pancreatic allograft undergoing HMP; initially (left) and after 48 hours displaying severe oedema (right) [1029].

The complexity involved in such perfusion meant that pancreatic HMP remained a non-viable clinical entity for the next few decades with limited experimental reports exploring various aspects of using HMP for preservation or viability assessment. Kenmochi et al. in 1992 for example used perfusion dynamics from a short 1 hour period of HMP to predict graft viability upon transplantation of warm ischaemically damaged allografts[1031]. They concluded that flow metrics obtained during HMP could be a potential predictor of reperfusion injury in compromised allografts. Most recently our group at Imperial College London has experimented with HMP of segmental porcine pancreatic grafts with a promising results[1032].

There were several attempts at normothermic pancreatic haemoperfusion both as methods of preservation and viability assessment that were trialled in large animal models in the 1970s-90s by Eckhauser el al. in Michigan, USA[1033], and Saito in Tottori, Japan[1034] (see Figure 32), Barthel et al. in Gottingen, Germany[1035], and Lykoudis et al. in Greece[1036].

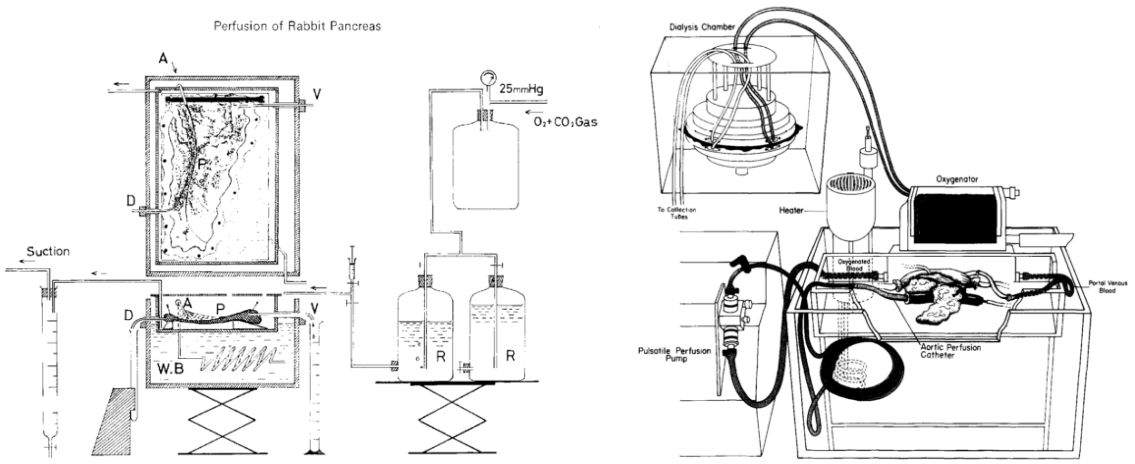


Figure 32 Historical attempts at pancreatic perfusion. Left: schematic diagram of the perfusion apparatus used by Saito et al. for normothermic perfusion of the rabbit pancreas set in a chamber [1034] (A, arterial cannula (the superior mesenteric artery); D, pancreatic duct cannula; P, pancreas; R, perfusion fluid reservoir; V, portal vein cannula; W.B, water-bath). Right: Modified perfusion apparatus using inline dialysis unit by Eckhauser et al. [1033].

However despite experimental reports application of these strategies to clinical practice were not attempted predominately due to technical difficulty in achieving successful perfusion, and because of the relative simplify that SCS offered.

With current pressure to expand donor pools and address the growing list of patients awaiting a pancreas there has been an increasing use of ECD and DCD donor organs. Unfortunately such organs carry with them an increased risk of graft delayed function and graft failure. Once again interest has rekindled in strategies to apply the merits of HMP in these organs optimising pancreatic preservation and potentially allowing viability assessment.

It can be assumed that because renal HMP preceded other forms of dynamic organ preservation attempts to apply HMP to other organs have routinely been based on modifying renal protocols. There are however a number of reasons why such renal protocols cannot be directly translated to the pancreas first identified by the Minnesota Group [1028].

The main physiological difference with the pancreas is its low flow and pressure environment. This means that machine perfusion has the capacity to damage the fragile vascular endothelium leading to platelet activation and thrombosis on graft reperfusion [1037–1039].

1.8.5.2 HMP in Islet Cell Transplantation

Recently there has also been interest in HMP to facilitate preservation and liberation of pancreatic islets for ICT [869]. In several reports Taylor et al. [1040,1041] used a 24 hour HMP porcine model. They found the highest insulin content was in islets harvested from machine perfused pancreata. Islet yield was 1.6-1.8 times greater than the fresh group and 2-3 times greater than cold storage group.

One of the previously reported disadvantages of machine perfusion was organ oedema, thought to lead to bleeding and necrosis[795]. This group found that though they observed moderate oedema, there was no associated loss of function. They further suggested that the oedema appeared to aid in enzymatic digestion, producing a greater yield and purity of islets compared with pancreas subjected to 24 h of SCS [795]. It was noted however that islets were oedematous, suggesting a degree of ischaemic injury and thus that HMP for islet isolation may not be solely beneficial.

In a small (n=4) human model, Leeser et al. [1042] used 13 hour low-flow machine perfusion compared with two groups of cold storage of <8 and >8 h. They found that machine perfusion may be beneficial in pancreata with prolonged cold ischaemic time, which would normally be less likely to meet criteria, by maintaining islet yield, viability and function. They reported islet yields from machine perfused pancreata of 3435 IEQ/g, compared with 5134 IEQ/g and 2640 IEQ/g respectively for those cold stored for <8 and >8 h. Mean islet viability after perfusion was 86% (vs. 74% and 74% for the <8 and >8 h groups) and insulin secretion index was 6.4 (vs. 1.9 and 1.8 for the <8 and >8 h groups).

1.8.5.3 Key issues in the development of pancreatic perfusion models

1) Animal model

In development of experimental translational models one of the key considerations for large animal models is the species utilized. Historically canine models have been used extensively, however the porcine model provides a consistent and reliable source of tissue[1043]. In both instances despite similarities to the human pancreases definitive anatomical differences are present.

2) Type of pancreatic graft

Types of pancreatic graft that can be retrieved can be classified as segmental[1044] or whole[1035] for ex-vivo perfusion and include or not include the duodenum. Choice will depend on the degree of translational applicability desired, with a whole pancreatic graft including a duodenal segment most akin to what would be encountered in a clinical setting. Never the less a whole porcine pancreatic graft has a more complex and fragile anatomy compared to the human pancreas, but is one of the best approximations available in a non-primate species.

3) Perfusion Solution

Clinically Belzer MPS is the gold standard for HMP. Despite the development of several new experimental solutions none have reached clinical practice, have been used primarily in kidney, and a lesser extent in liver models. The Minnesota group used a plasma based perfusate supplemented with albumin to enhance the osmotic potential of the solution and limit oedema[1028]. Alternatives to Belzer MPS include the SCS variant Belzer UW solution. Belzer UW was initially developed as HMP solution for kidneys[722], and has proved beneficial in DCD donor allografts experimentally[879]. Karcz et al. used Belzer UW in the initial development of pancreatic HMP models at Imperial College London. Any solution employed would likely require supplementation with osmotic agents to help minimise allograft oedema.

4) Perfusion Protocols

The inherent differences between the kidney and the pancreas necessitates lower perfusion pressures, and flow expectations. The kidney as a whole is a high-flow filter, while the pancreas is both a glucose sensor and reservoir of potent digestive enzymes. Excessive barotrauma can damage the pancreas and result both in oedema and potentially graft pancreatitis upon reperfusion and transplantation[1028]. Despite this understanding optimal perfusion pressures in hypothermic, and

even normothermic instances have not been established. However because recent evidence suggests that perfusion pressures <40mmHg during renal HMP are optimal [882], it is probable that even lower pressures would be needed for pancreatic HMP.

5) Viability Assessment

HMP has been used a tool for viability prediction using perfusion dynamics and perfusate analysis for biomarkers. However suitably predictive markers have not been found[873]. Physiological differences between the kidney and pancreas suggest that some markers used in renal assessment may be useful in the pancreas but that independent and pancreas specific indicators exist. With ideal perfusion pressures lower in the pancreas expected flow dynamics will be less than in renal HMP and thus viability criteria for the kidney[876] will not be suitable. Calculated PFI (ml/min/100g/mmHg) developed by the Newcastle group in the UK may however provide a useful metric as it incorporates both flow rates[1031] and systolic perfusion pressures used but also accounts for the weight of the pancreatic allograft.

Assessment of tissue metabolism using rsMD would provide insight into both tissue ischaemic injury, and also changes in tissue metabolism during preservation. Assessing organ viability in an experiment addressing the modality of hypothermic preservation can also be done after preservation during reperfusion. The gold standard would require an auto-transplant model, however an isolated reperfusion model would provide an indication of immediate organ viability and function[722]. Microdialysis, if incorporated during normothermic reperfusion can provide additional real-time information on reperfusion injury. Furthermore functional pancreatic viability can be assessed during reperfusion by glucose stimulation and monitoring insulin release, as well as pancreatic exocrine secretions.

Finally an important aspect of any large animal organ preservation and perfusion work is developing protocols and a strategy that could be applied to human allografts in clinical practice.

Chapter 2:

Basic Methodology

2.1.1 Hypothermic Machine Organ Perfusion System

2.1.1.1 The RM3 Hypothermic Perfusion Machine

The Waters Medical Systems (Rochester, MN, USA) RM3 organ perfusion machine was initially designed as a two-part kidney preservation system which includes (1) a control unit (the RM3) for the pulsatile perfusion and monitoring of one or two kidneys and (2) a sterile, disposable, single-use cassette designed to circulate the perfusate through the kidney(s). The following details of the RM3 device and its operation have been extracted and adapted from the RM3 Device Manual [1045].

The RM3 machine provides a simple and efficient method for hypothermic pulsatile preservation for one or two organs by maintaining pressure, flow and temperature with the self contained 'control' or 'base' unit monitoring perfusion parameters and controlling cassette function. The cassette is a circulation system consisting of a central chamber to house the organ, venous and arterial reservoirs, a bubble trap, oxygenator, heat exchanger, and a pulsatile pump head. Pressure, flow and perfusate temperature are under control of the RM3 base unit, and free for alteration and evaluation by the operator. A schematic diagram of the base unit and cassette can be seen in Figure 33.

2.1.1.2 The RM3 Control Unit

The RM3 control unit regulates the pulsatile perfusion of the perfusate, and in real-time monitors perfusion pressure, perfusate flow and perfusate temperature .

Readings for monitored parameters are displayed on the central display screen as isolated values or in a graphical representation of trend over time. During normal operation the largest section will display the real-time analog waveform, the menu or the trend graphs. A second section will display the numerical information which includes systolic, mean and diastolic pressures, flow from both the right and left kidneys, temperature of the perfusate, renal resistance of the right and left kidneys and the pump pulse rate. The third section displays the identification of the menu keys located on the front panel overlay. A photograph of the display screen can be seen in Figure 34.

The RM3 control unit has variable stroke volume control, allowing the operator to mechanically adjust the occlusion of the pulsatile pump arm. By turning the stroke volume knob clockwise, the volume of perfusate from the pump head to the kidneys is increasing along with a corresponding increase in pressure.

In addition the RM3 contains an alarm system which will signal an audio as well as a visual alarm for user selected alarm conditions.

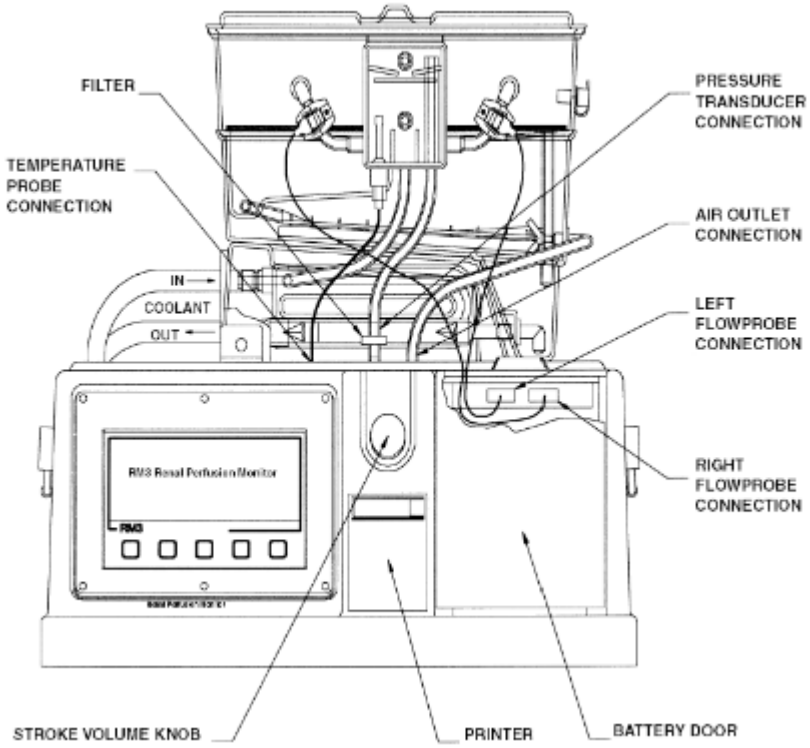


Figure 33 Schematic of RM3 base unit and organ cassette. Adapted from RM3 Manual [1045].



Figure 34 Photograph of the RM3 base unit display with perfusion readings on the right side of the screen.

2.1.1.3 The Cassette

The MOX DCM-100 cassette is a gravity flow system circulating perfusate through the attached kidneys. The perfusate is pumped from the arterial reservoir, through the heat exchanger to the bubble trap where it is delivered to the cannulated kidney(s). The perfusate returns to the arterial reservoir by gravity through the right and left venous reservoirs and the membrane oxygenator. The cassette is easily mounted on the RM3 by the mounting brackets on the left front and right rear connectors on the cassette and allows positioning of the pulsatile pump head into the pump arms of the RM3. A photograph of a cassette in operation can be seen in Figure 35.

A pressure transducer line, an inlet tubing to the ambient air pump, temperature probe cable and flow transducers are then attached to the RM3 base unit. To complete the installation, the heat exchange lines from the ice water bath or the refrigeration unit are attached to the cassette. For the most efficient operation, the inlet connection is located on the top and the outflow connection on the bottom. A temperature probe located in the bubble trap will control a circulation pump located in the ice water bath and thermostatically maintain the temperature of the perfusate. This temperature was correlated with the temperature of the organ being perfused using a separate temperature monitor in the tissue to ensure effective cold preservation.

The bubble trap separates any trapped air bubbles from the perfusate to prevent air bubbles from reaching the kidneys. It also provides mounting positions for the perfusate temperature probe and the pressure transducer line. Since the bubble trap is operated at the perfusion pressure, it is critical to insure that there are no air leaks in the pressure system. It is recommended by the manufacturers that all tubing connectors are tie-strapped for airtight connections.

Sample ports are located on the bubble trap. The syringe access pressure port allows the operator to regulate the proper perfusate level at all times. By inserting a needle and syringe into the pressure port and either infusing or withdrawing air, the perfusate level can be moved up or down. The sample port below the perfusate level indicator line can be used to infuse products or remove a sample of the perfusate. A persistently rising fluid level indicates a leak in the pressure system and the perfusate level should be at the indicator line at all times for accurate pressure measurements.

Perfusate migrating down the pressure transducer line will damage the transducer and so a hydrophobic filter is used during operation of the RM3.

The pulse pump arm of the RM3 control unit and the pump head in the cassette provide pulsatile flow. The pump head includes a pair of occlusive valves placed in series and is alternately compressed and released by the cam-driven arm of the pump arm. The pump head must be placed in the pump arm assembly with the holding brackets positioned directly at the base of each valve in the pump head. During initial priming of the cassette, some air may be trapped in the pulsatile pump head. To remove this air, the discharge end of the cassette (the nearest the coolant source) should be raised from the top of the RM3 and held at an upward angle while manually squeezing the pump head.

The RM3 control unit has a variable stroke volume control, allowing the operator to manually adjust the occlusion of the pulsatile pump arm. By turning the stroke volume knob on the base unit the flow through the pump head is altered; e.g. turned clockwise the volume of perfusate from the pump head to the kidneys is increased along with a corresponding increase in pressure.

The flow 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, flow from the left, into the right reservoir. These reservoirs are calibrated in 20ml increments. The flow rate is measured by timing the flow

from each kidney into the venous reservoir. The graduated marking on the venous reservoirs of the cassette are provided to permit direct measurement of the perfusate.

The perfusate in the cassette can be cooled by either the ice water bath in the RM3 or by means of an auxiliary refrigeration system.

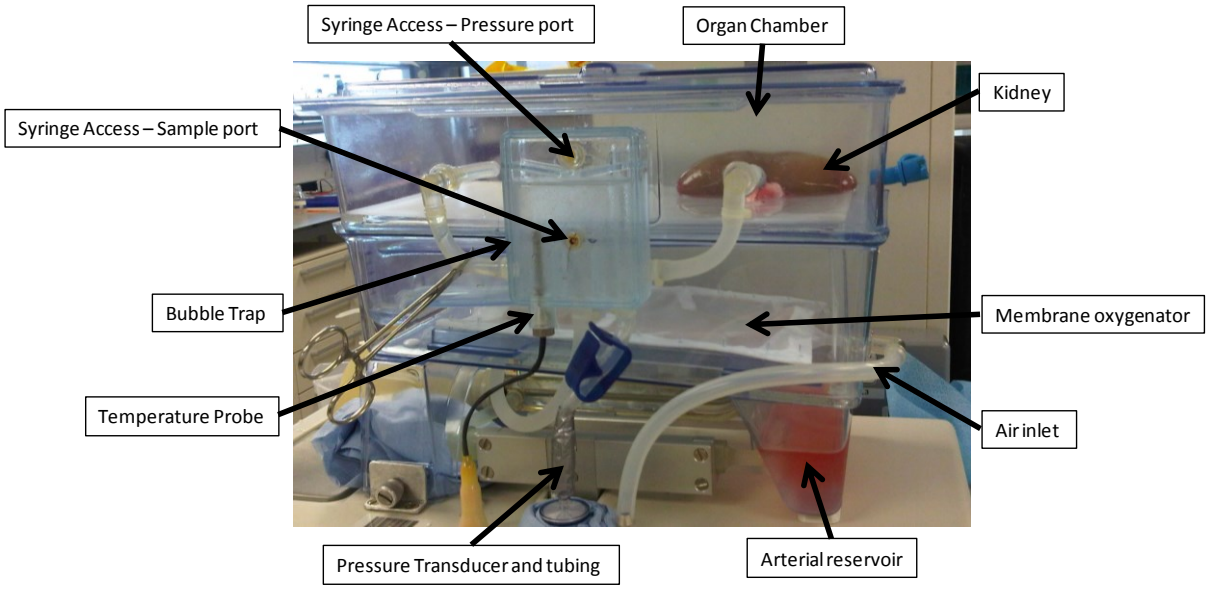


Figure 35 Photograph of organ cassette in use with a porcine kidney. Key components of the cassette are labelled.

2.1.1.4 Measuring flow with the RM3

The RM3 uses two methods of measuring flow. The first method uses real-time flows measured with Transonic ultrasonic clamp-on flow probes using the ultrasound signal method to insure precision measurements on each inlet tube from the bubble trap into the organ chamber. In this manner, sterile perfusate does not come in contact with the flow probe.

The second method for measuring flow is the Direct Timer method. This method measures the filling time on the volume increments on the venous reservoir and automatically places the value in the appropriate display area. The direct timer method is utilised if there is a primary malfunction with the real-time flow probes during an experiment.

How the Flow probes Work

The Transonics flow probes in the RM3 use the ultrasound to measure the transit time ultrasound between to transducers to accurately measure perfusate flow. The flow probes are pre-calibrated for use with the RM3 and perfusate. The left and right flow probes are attached to the RM3 directly to the flowmeter board located inside the battery door. The flow probes are clamped onto outflow arterial outflow tubing from the bubble trap (see Figure 36).

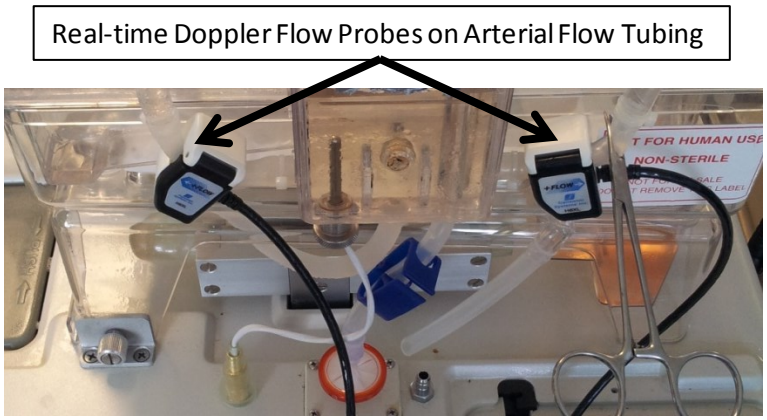


Figure 36 Photograph of real-time flow probes on arterial tubing.

Direct Timer Method

'L Flow Timer' (left flow timer) and 'R Flow Timer' (right flow timer) in the SYSTEM MENU measures the rate of flow from each kidney using the cassette's venous reservoirs. The markings in the venous reservoirs are in 20ml increments, ranging from 0 to 80ml. When selecting the manual timer method, the RM3 Flow Timer is switched from Auto (using the flow probes and real-time flow) to Man (manual). Once the left or right timer is selected, the LEVEL key will determine the volume of perfusate measured. The levels that can be selected are 20ml, 40 ml, 60 ml and 80 ml, which correspond to the volume of perfusate collected in the venous reservoir. The flow is measured by occluding the tube exiting from the respective section of the venous reservoir chamber. The right kidney chamber will flow into the left venous reservoir and the left kidney chamber into the right venous reservoir.

For Example (taken from RM3 Manual):

To measure the right flow:

1. From the MAIN MENU select the SYSTEM MENU.
2. In the SYSTEM MENU select R Flow Timer (right flow timer).
3. Adjust the timer for level 20ml.
4. Occlude the tube exiting the left venous reservoir to the oxygenator.
5. When the perfusate reaches the 0 mark, press the START key. This starts a digital counter on the display.
6. When the perfusate reaches the 40ml mark in the reservoir, press the STOP key.

The RM3 will compute the flow and display flow readings on the digital display, and calculate flow resistance. These values will remain displayed until a new flow volume is calculated, at any time, using the Direct Timer method.

2.1.1.5 Pressure Measurements

A replaceable Honeywell transducer measures pressure. Systolic, mean and diastolic pressures are displayed digitally as well as in analog waveform. Pressures are continuously updated for digital display and in real-time for the analog display. The fluid level in the bubble trap should be at the perfusate level mark to insure accurate pressure measurements. This can be accomplished using a 30ml syringe with a 22 gauge needle or smaller in the top injection port to adjust the fluid level in the bubble trap. Using Pressure and Flow measurements the Resistance to flow is calculated and displayed by the RM3 base unit.

2.1.1.6 The Circulation (CIRC) Pump and Reservoir

The circulator pump, is a submersible pump located in the 5L coolant reservoir. It circulates approximately 3.5 litres per minute through the cassette's heat exchanger to cool the perfusate. The electrical connector is located inside the reservoir in the upper front section. Control of hypothermic cooling time depends on the ambient conditions. The use of a cassette cover will help maintain temperature for a longer period of time.

2.1.1.7 The Air Pump

The air pump will circulate approximately 500 ml/min of ambient air (21% O_2) over the oxygenator membrane in the cassette. It is operated from the SYSTEM MENU with a port connected at the top of the RM3 cabinet to the right of the pressure port. The air pump allows more air to circulate across the membrane.

2.1.1.8 The 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).

2.1.1.9 RM3 Operational Checklist

Before each experiment the RM3 machine was primed and made ready for attachment of the pancreatic graft to the perfusion circuit. Furthermore after a graft is removed, and subsequently reattached to the organ perfusion cassette the flow probes and pressure readings are zeroed before continuing perfusion.

1) Attaching the perfusion cassette to the RM3 Machine via the top mounted screw clamps
2) Adding 1.5L of water and ice to the coolant reservoir.
3) Connecting the cassette gas inlet tubing to the RM3 machine.
4) Connecting the temperature probe to the cassette and RM3 machine.
5) Attaching the Transonic flow probes to the arterial tubing.
6) Attaching the coolant tubing to the cassette.
7) Priming the cassette with the desired volume of perfusate (in this case UW solution).
8) De-bubbling 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.

2.1.2 Normothermic Reperfusion

2.1.2.1 Modification of the RM3 Perfusion Apparatus

The RM3 unit was modified to facilitate normothermic reperfusion of grafts for viability assessment (see Figure 37). To perfuse organs normothermically a water heater (Grant Instruments FH16D Flow Heater) was externally attached to the circulation pump and the cassette via the cassette's heat exchanger ports. Where two RM3 units were being used the cassettes were attached in series. The external water heater maintained the temperature of the water between 35-41˚C to warm the perfusate to a target temperature of between 37±2˚C (human grafts) or 38±2˚C (porcine grafts) depending on the experimental protocol.

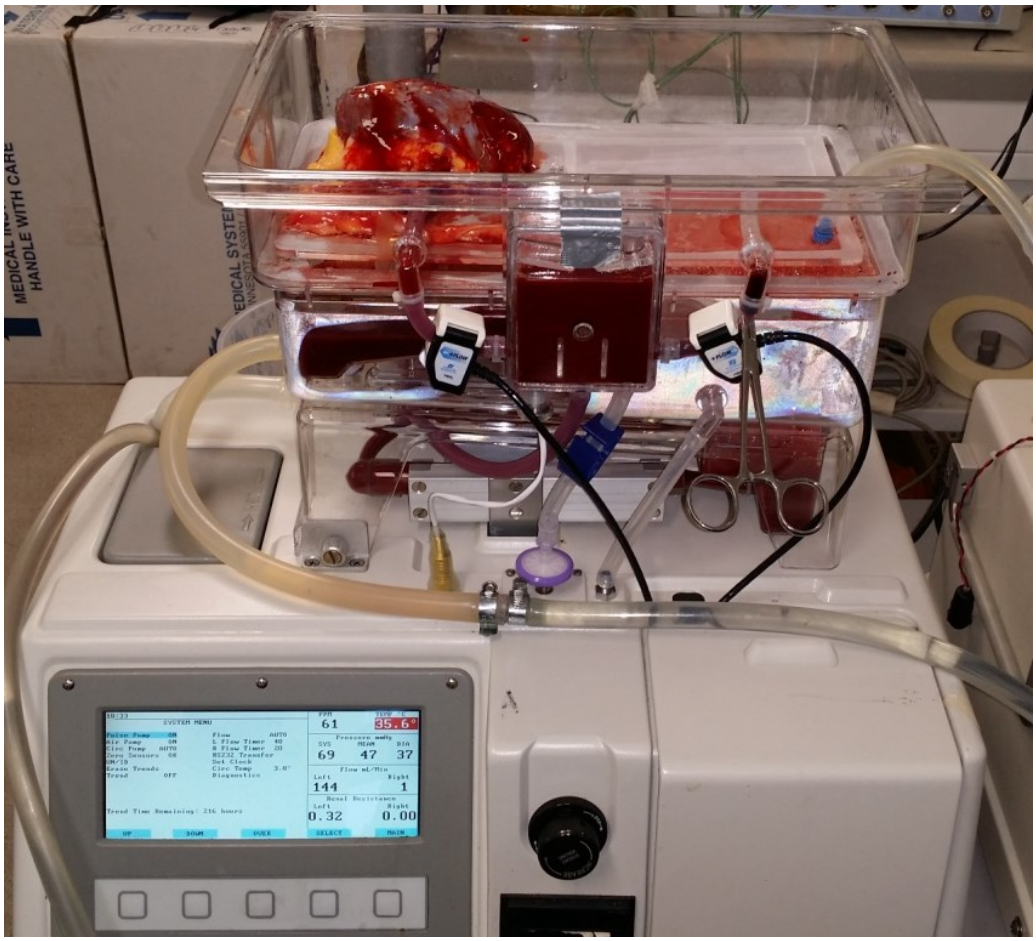


Figure 37 Photograph of kidney undergoing normothermic haemoreperfusion. Provision of oxygen via hollow probe into the arterial reservoir, and normothermia maintained by use of an external heated re-circulator.

2.1.2.2 Provision of Oxygen

Oxygen was administered by inserting a thin hollow probe through which an oxygen / carbon dioxide mixture (95% O_2, 5% CO_2) was passed into the arterial reservoir to allow effective oxygenation of the perfusate (see Figure 37). Carbon dioxide was included in the mixture to supplement acid buffering within the perfusate during reperfusion.

2.1.3 Retrieval of Grafts

2.1.3.1 Porcine Grafts

Porcine kidney and pancreas grafts for this project were obtained from pigs at a local slaughterhouse Cheale Meats Ltd, Brentwood Essex. Pigs were white crossed large pigs (mixed breed) weighing between 70-120kg.

There were two methods used for retrieval of porcine organs targeting a shorter (15 minutes) and longer (45 minutes) WIT.

Shorter (15 minutes) ischaemia time

Pigs were sacrifice by stunning, and then exsanguination. After death the internal thoracic and abdominal organs were removed and supplied for dissection and retrieval of the kidneys and/or pancreas and associated vasculature. Time between death and flushing was aimed to be maintained at 15 minutes. During this time organs were dissected and made ready for flushing and cold storage transport to the laboratory. After approximately 15 minutes organs were flushed with 0.5-1L of organ preservation solution (UW, Soltran or Adenocaine Solution depending on the project protocol) at a pressure of 100cm H_2O. The period until flushing in pancreatic retrieval was longer due to the time needed to accurately dissect the organ and ligate key vessels, ice was placed near the organs to facilitate cooling during dissection in this case until flushing. Organs were flushed until effluent via the venous drainage was clear, and then packed in the same flush preservation solution on ice and transported back to the laboratory. Transport time was approximately 4 hours.

Longer (45 minutes) ischaemia time

Pigs were sacrifice by stunning, and then exsanguination as part of the commercial processing line at the slaughterhouse. The time between death and provision of the organs was approximately 25-30 minutes, and included a 10 minute period in a 65˚C water bath.

After this period the internal thoracic and abdominal organs were removed and supplied for dissection and retrieval of the kidneys and/or pancreas and associated vasculature. A rapid dissection was conducted to minimise further warm ischaemia time (5-10 minutes) and cold flush initiated. During flushing dissection and back benching of organs continued and the organs made ready for cold storage transport to the laboratory. Organs were flushed with 0.5-1L of organ preservation solution (UW, Soltran or Adenocaine depending on the project protocol being used) at a pressure of 100cm H_2O. Organs were flushed until effluent via the venous drainage was clear. Transport time was approximately 4 hours.

Autologous Blood Collection

If blood was required as part of the project protocol autologous porcine blood (1L) was collected from the animal before death in a container with 10,000 IU of heparin (Multiparin®; CP Pharmaceuticals, Wrexham, UK) to prevent extracorporeal thrombosis. This blood was then placed in a cold storage bag for transport back to the laboratory.

2.1.3.2 Graft Anatomy

Porcine Kidney

Porcine renal anatomy and size is similar to that of the human kidney. During dissection the renal capsule was dissected and partially removed. The renal artery and vein were isolated and benched to ensure no leaks were evident, and the ureter preserved. Flushing was via the renal artery and out flow via the renal vein (see Figure 38). Once at laboratory further dissection of the graft, and preparation for machine perfusion was performed. All bench work was undertaken with the graft placed on an ice/UW mixture. Aberrant and earlier missed vessels were located and tied. The kidney

graft was weighed using electronic scales. The renal artery was cannulated with a perfusion cannula used for attachment to the RM3 arterial tubing. The vein and ureter were left to drain freely.

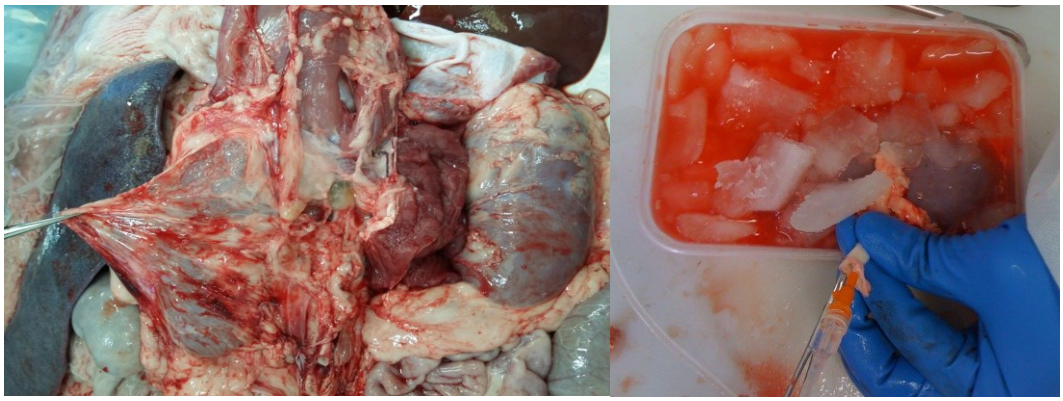


Figure 38 Porcine kidneys. Prior to benching (left) and being flushed (right).

Porcine Pancreas

The porcine pancreas model has historically been used for experimental pancreas transplantation due to the anatomical and immunological similarities with humans [18]. The similar size & function of the porcine pancreas to the human type make swine amenable for research using equipment designed for human sized organs and tissues – in this case the RM3 Perfusion system. Porcine pancreatic anatomy though similar to human pancreatic anatomy has several distinct differences in morphology, as well as arterial and venous vasculature [1043].

Lobe structure

The porcine pancreas is a retroperitoneal structure ventral to the 1st, 2nd, and 3rd lumbar vertebrae. It weighs approximately 25-200g (depending on the size of the animal) and is triradiate in shape [1043]. It is composed of three lobes, with irregular margins and a nodular surface. The splenic lobe (corresponding to the tail and body in the human pancreas) is situated posteriorly and is attached to the spleen and the stomach. The duodenal lobe corresponding to the head of the pancreas) is located adjacent to the duodenum while the connecting lobe (corresponding to the uncinate process) is an extension of the pancreas which is attached to the anterior aspect of the portal vein. In addition there is a bridge of pancreatic tissue serving as an anatomical connection between the splenic and connecting lobes [1043]. Anatomy of the porcine pancreas can be seen in Figure 39.

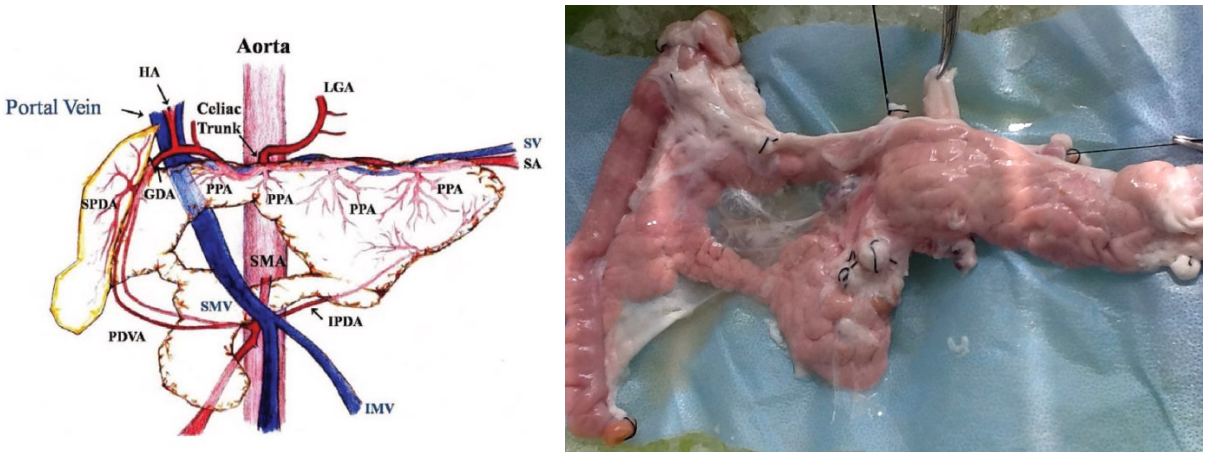


Figure 39 The porcine pancreas. Depiction of porcine pancreatic anatomy including arterial and venous network (left) adapted from Ferrer et al. [1043]. Photograph of benched whole porcine pancreatic graft (right).

Arterial Supply

The blood supply of the pancreas is provided by the celiac trunk and superior mesenteric arteries [1046]. The DL of the pancreas receives its blood supply from the superior pancreaticoduodenal artery, a branch of the gastroduodenal artery. The SL is supplied by the splenic artery; and the inferior CL and bridge of tissue supplied by the pancreaticoduodenal artery branch of the SMA.

Venous Drainage

The portal vein receives blood from stomach, pancreas, intestine, and spleen. On its way from the root of the mesentery to the liver, the portal vein penetrates the pancreas at an acute angle so that it lies caudally on the ventral surface and rostrally on the dorsal surface of the pancreas passing through the ring of pancreatic tissue [1043]. The portal vein has two branches that drain into it, the splenic vein and the superior mesenteric vein. The splenic vein drains the body and tail of the pancreas, is usually embedded within pancreatic tissue. The superior mesenteric vein passes through the portal ring receiving the inferior pancreaticoduodenal vein – draining the duodenal lobe. The inferior mesenteric vein usually flows into the superior mesenteric vein – a distinct anatomical variant compared to human pancreatic venous drainage [1043]. The gastroduodenal vein empties into the superior mesenteric vein immediately before its junction with the splenic vein, and receives drainage from the superior pancreaticoduodenal vein that also anastomoses with the inferior pancreaticoduodenal vein.

Ductal System and Histology

The pancreatic duct is composed of two separate ducts draining the body and tail – anatomising to form the common pancreatic duct prior to the pancreatic sphincter, though several anatomic variations exist [1043]. Histologically pancreatic islet cells are relatively indistinct, but functionally similar to humans.

Pancreas Dissection and Benching

Upon provision of the internal thoracic and intra-abdominal organs the aorta was immediately identified and clamped distal to the superior mesenteric artery origin. Lumbar vessels were clipped and the aorta cut 5-10 cm proximal to the origin of the celiac trunk. After the prescribed warm ischaemia time the proximal aorta was then cannulated and cold preservation solution infused into the pancreas via the aorta and through the celiac trunk and superior mesenteric artery. At this point ice was placed in the vicinity of pancreas to facilitate rapid cooling. The pancreas was then dissected out en block and placed on a tray of ice and preservation solution. The distal splenic artery and vein, the left gastric artery and vein, the distal gastroduodenal artery and vein, the hepatic artery and vein, components of the superior mesenteric artery (distal to branches supplying the pancreas), as well as lumbar arteries from the dorsal aortic wall and the aorta itself distal to the superior mesenteric artery origin, were then tied with silk ligatures and washout continued with cold preservation solution until clear solution emerged from the portal vein. Any leaks from aberrant arterial vessels were identified, clipped and tied with appropriate size silk ligatures.
The final graft composition was based on the extent of dissection and two methodologies were employed to produce a segmental porcine pancreatic graft or a whole organ.

Segmental Allograft

The final graft was composed of the splenic and duodenal lobes of the pancreas – supplied by the celiac trunk +/- the superior mesenteric artery segments of the aorta. Venous drainage was composed of the splenic, gastroduodenal, and superior mesenteric veins draining into the portal vein. The small bowel and attached duodenal pancreatic tissue, and tissue composing the lower the half of the pancreatic 'portal ring' surrounding the portal vein was carefully isolated and removed. No further attempt was made to manipulate the pancreatic ductal system. The graft was then placed in an organ transfer bag with approximately 200ml of preservation solution and the bag placed on ice for transport back to the laboratory. A photograph of a segmental porcine pancreatic graft can be seen in Figure 40.

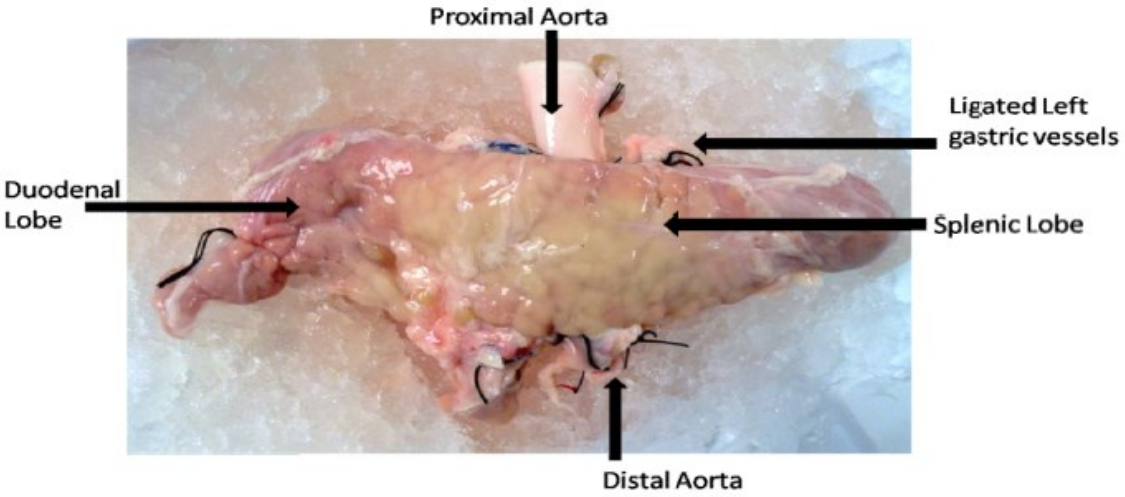


Figure 40 Benched segmental porcine pancreatic graft.

Whole Organ Graft

The whole organ allograft comprised the entire porcine pancreas - involving the splenic, duodenal, connecting lobe and tissue surrounding the portal vein (see Figure 39). A section of small bowel from just distal to the gastric pylorus up to the equivalent of D3 was preserved. The aorta was tied distal to the origin of the SMA to preserve the arterial supply to the inferior portions of the pancreas. The splenic vessels were tied at the splenic hilum. The vessels and ducts to/from the liver were tied at the portahepatis.

Pancreatic Bench Work

Once returned to the laboratory further dissection of the graft, and preparation for machine perfusion was performed. All bench work was undertaken with the graft placed on an ice/UW mixture. Aberrant and earlier missed vessels were located and tied. The pancreas graft was weighed using electronic scales. The proximal aortic segment was cannulated with a perfusion cannula used for attachment to the RM3 arterial tubing. The portal and mesenteric venous vessels were left open ended for venous effluent drainage. In whole organ grafts an enterotomy was performed and a foley (14-F) catheter inserted and secured by purse string suture into the small bowel to collect pancreatic and small bowel secretions. A benched porcine pancreatic graft can be seen in Figure 41.

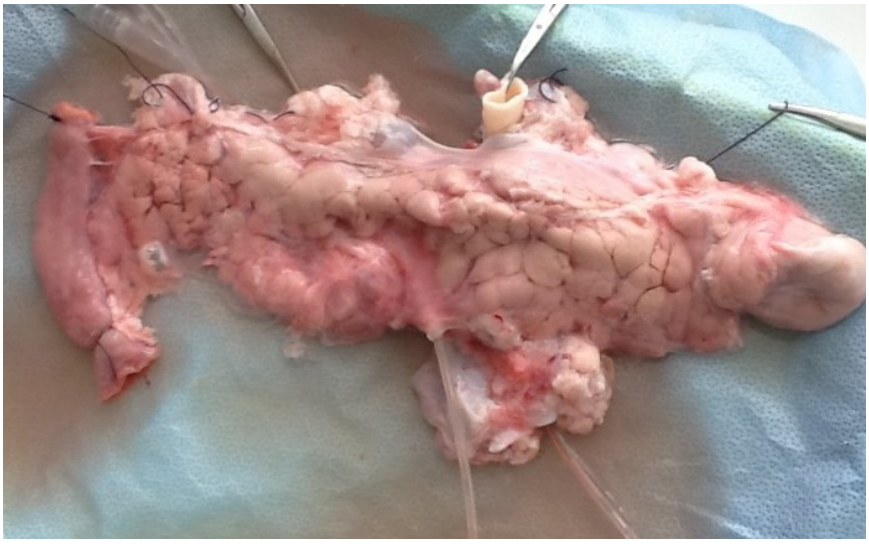


Figure 41 Benched whole porcine pancreatic graft.

2.1.3.3 Attachment of the Benched Grafts to the Perfusion Cassette

Only one organ was run on the RM3 at any one time. Once the RM3 machine has been prepared and the cassette primed the opposite arterial tubing from the bubble trap was clamped shut. To attach the organ the circuit flow was reduced (using the Stroke Volume knob on the RM3 base unit) and the cannulated arterial segment of the dissected graft was slowly filled with perfusate and de-bubbled. The cannulated artery was then connected to the unclamped arterial tubing of the cassette and the flow then altered using the stroke volume knob to reach the desired initial flow and/or pressure readings.

Chapter 3:

Application of Rapid Sampling Microdialysis in Organ Viability Assessment During Preservation: A Comparison of SCS vs. HMP

Application of Rapid Sampling Microdialysis in Organ Viability Assessment During Preservation: A Comparison of SCS vs. HMP

3.1 Objectives

Microdialysis is a minimally invasive technique that allows the interstitial composition of a target tissue to be sampled, followed by ex vivo analysis, providing an accurate method of monitoring tissue biochemistry and metabolism over time.

Imperial College London has successfully developed a novel online rsMD analyser capable of measuring the concentration of markers of tissue ischaemia at 30 second intervals. To date no such system has been used to directly characterise and compare the effects of SCS and HMP on renal biochemical activity and the ischaemic injury sustained during preservation in real time. If capable of accurately monitoring tissue ischaemia during organ preservation, rsMD may have a promising future role in graft viability assessment.

Objectives were two-fold: 1) to assess the feasibility of using rsMD to monitor changes in glycolytic metabolites in the renal cortex and medulla during SCS and HMP in a porcine model; 2) to compare the effects of SCS and HMP on renal cellular metabolism and ischemia during the preservation period.

3.2 Methods

An experimental porcine DCD model was employed to determine the feasibility of using the rsMD system for tissue viability assessment during *ex vivo* hypothermic preservation. This was conducted in collaboration with Prof Boutelle's team in the Department of Bioengineering at Imperial College London who designed and built the in-house rsMD system.

3.2.1 Organ Procurement

Twenty-two DCD (Maastricht Type 2 equivalent) kidneys were retrieved from 11 adult pigs (70-100 kg), terminated at a local abattoir by stunning and exsanguination. Organs were retrieved by laparotomy and rapid en-bloc dissection of both kidneys. as per the shorter ischaemia protocol detailed in Section 3.1.3.1. After a median ischaemia time (WIT) of 15 minutes (range: 11-20 minutes) organs were cold flushed with University of Wisconsin (UW) solution (Viaspan, Bristol Myers Squibb, UK) at low pressure (100 cm H_20), until the venous effluent was clear. Organs were then stored in the flushing solution and transported on ice to the laboratory.

3.2.2 Organ Preservation

Kidneys underwent either a further 24 hours of SCS or 10 hours of HMP. Ten kidneys underwent 24 hours of SCS (n=10, mean temperature 3.5 ^{o}C) in the original flushing solution, while twelve underwent 10 hours of HMP (n=10, mean temperature 7.8 ^{o}C) on a Waters Medical Systems RM3 perfusion machine. Mean cold ischaemia time (CIT) prior to start of in-lab preservation (24 hours of SCS or 10 hours of HMP) and microdialysis monitoring was 254 minutes (range: 160-380 minutes). Target systolic perfusion pressure was 40 mmHg during the first 4 hours of HMP, which was adjusted hourly. After 4 hours no further changes to the perfusion system were made. Perfusion dynamics were monitored throughout.

UW solution (Viaspan) was used as the HMP perfusate in these experiments. This solution was originally developed by Belzer and colleagues as a HMP solution, and despite experimental work that has shown it is as effective as the machine preservation solution (MPS) variant [1047], at present only the Belzer MPS variant is FDA approved for clinical HMP use. The same solution was chosen for both preservation modalities to observe and determine differences in renal tissue biochemistry between SCS and HMP, and not influenced by differences in perfusate composition.

Following SCS or HMP preservation support was stopped abruptly and each kidney was allowed to warm passively for 2 hours to detect changes in lactate concentrations as tissue temperature increased and ischaemic cellular injury accumulated. This was a massive challenge for the kidneys, to test their reaction to the rewarming injury and the potential affect of each preservation method on the "reaction" of the kidney to the challenge. The protocol for this experiment is summarised in Figure 42.

HMP kidneys experienced approximately 14 hours of total CIT (~4 hours SCS for transport and 10 hours for HMP), while SCS kidneys underwent 28 hours of total CIT (~4 hours for transport and 24 hours of SCS). These time periods were chosen to mimic extreme clinic settings.

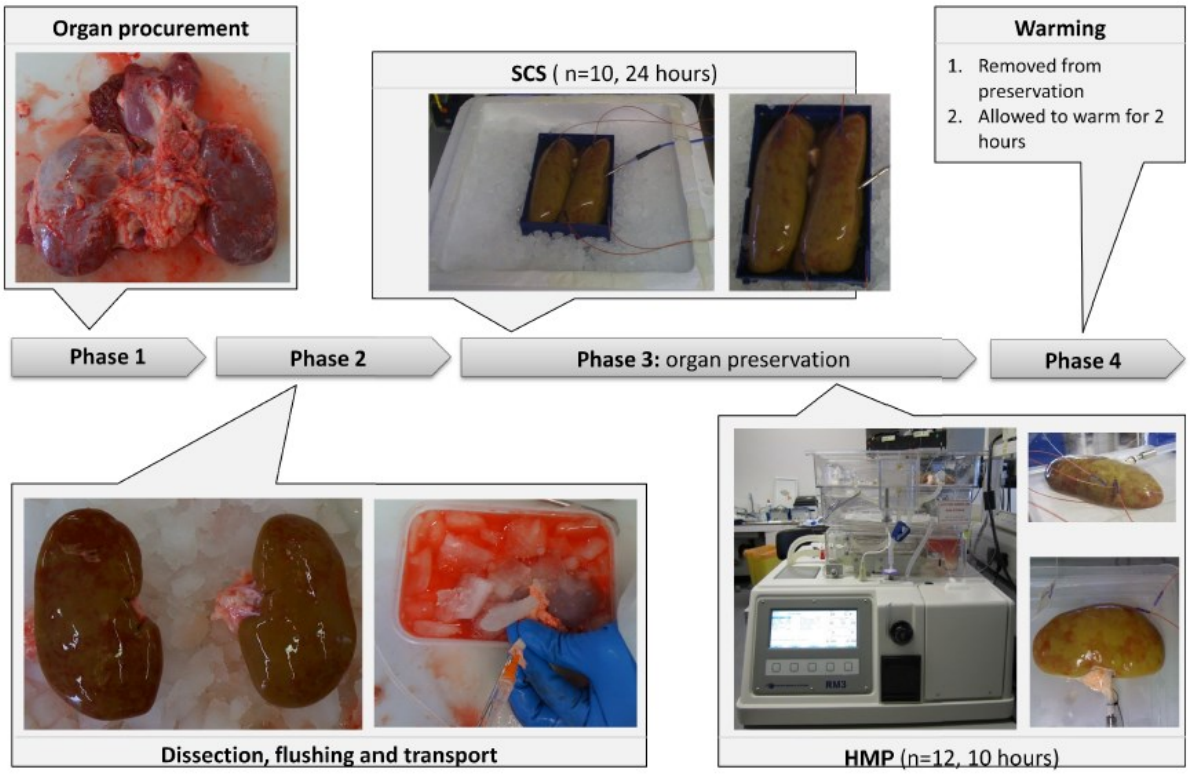


Figure 42 Diagram of the experimental protocol detailing the sequential phases.

3.2.3 Rapid Sampling Microdialysis

RsMD assessment of tissue metabolism was conducted in collaboration with the Department of Bioengineering, Imperial College London using an custom made system.

Two microdialysis probes (MAB11.35.4, Microbiotech, Stockholm, Sweden) were inserted into each kidney, one tunnelled superficially into the parenchyma of the lateral cortex and one placed deeper perpendicularly into the medulla (see Figure 43).

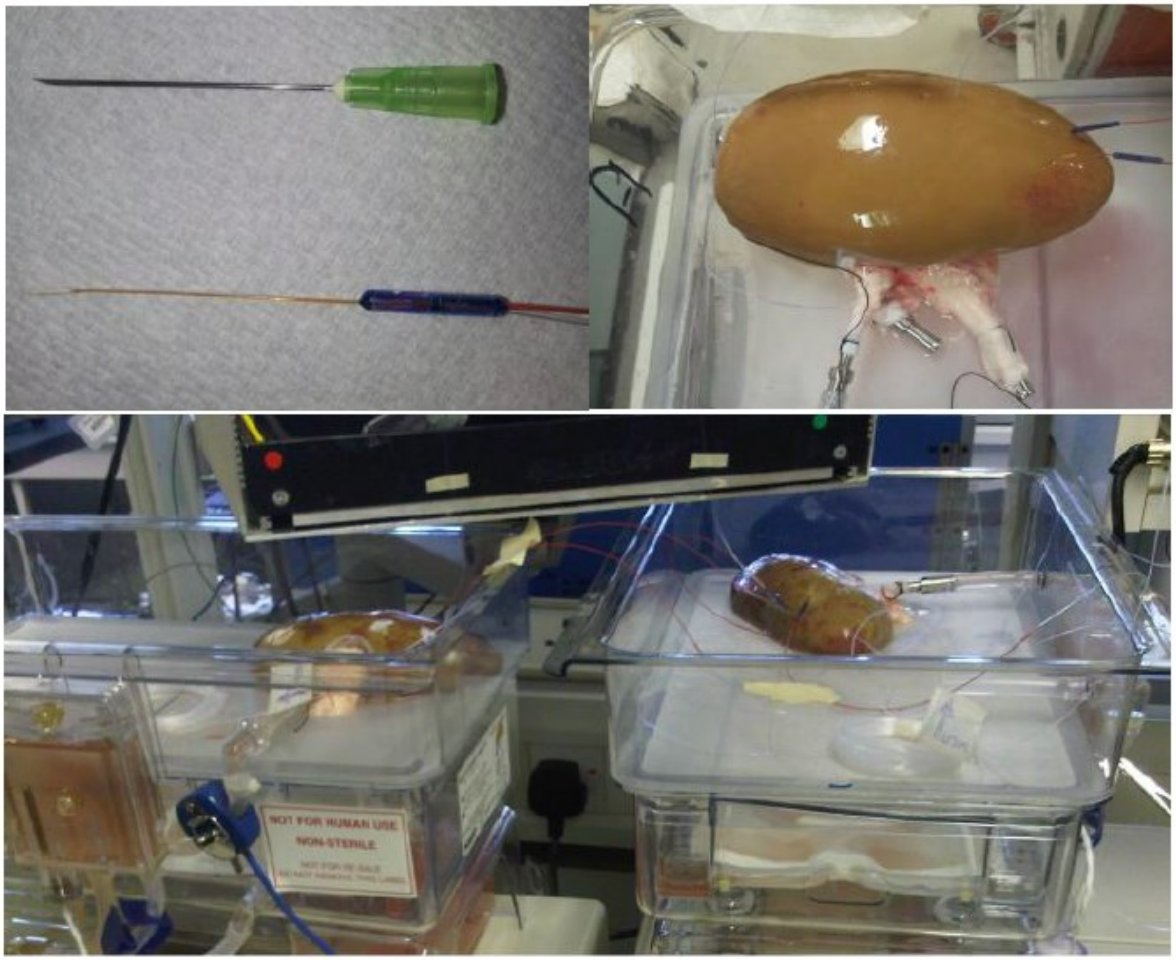


Figure 43 Microdialysis monitoring in porcine kidneys. Comparison of size of a microdialysis probe to a green hypodermic needle (top left), and a kidney undergoing monitoring with probes in cortex and medullary regions (top right). Two kidneys undergoing HMP and microdialysis (bottom).

A fine tubular dual lumen microdialysis probe, with a semi-permeable membrane tip is introduced into the tissue of interest. A physiological isotonic solution is pumped through the probe inlet tubing at a constant flow rate, past the semi-permeable membrane tip, creating a concentration gradient across the membrane. Low molecular weight compounds diffuse across the membrane, down their concentration gradients, while 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.

Cortical probes were analysed in real-time; for the medulla, the liquid stream was collected in storage tubing and 'played back' afterwards to recreate the real time. In addition perfusate samples taken during 10 hours of HMP (0, 3, 6, 10 hours) were analysed for levels of lactate and glucose by using the rsMD system.

Extensive changes were made to the rsMD system currently used to monitor brain injury [907], bowel ischemia [912] and free flap surgery [915], to make it suitable for monitoring a pair of kidneys (see Figure 44). The probe inlet tubing was 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; slower flow rates would have resulted in an increased recovery but would have increased the lag time. The outlet tubing of the probe was connected to the 200 nanolitres (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) pumped 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 was connected to two separate sample loops of the valve, either side of the analysis loop, allowing two kidneys to be monitored simultaneously. Every 30 seconds a 200 nL dialysis sample was automatically injected into the analysis flow stream, alternating between each kidney. On some occasions the valve switching frequency was reduced to every 150 seconds. 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 K_m 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 Macbook Pro portable computer (Apple Computers, Cupertino, CA).

The recorded current peaks were separated in Matlab (R2011b, MathWorks, US), to produce results for each kidney individually, and were converted into concentrations based on calibrations (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 microdialysis probe and the analysis system.

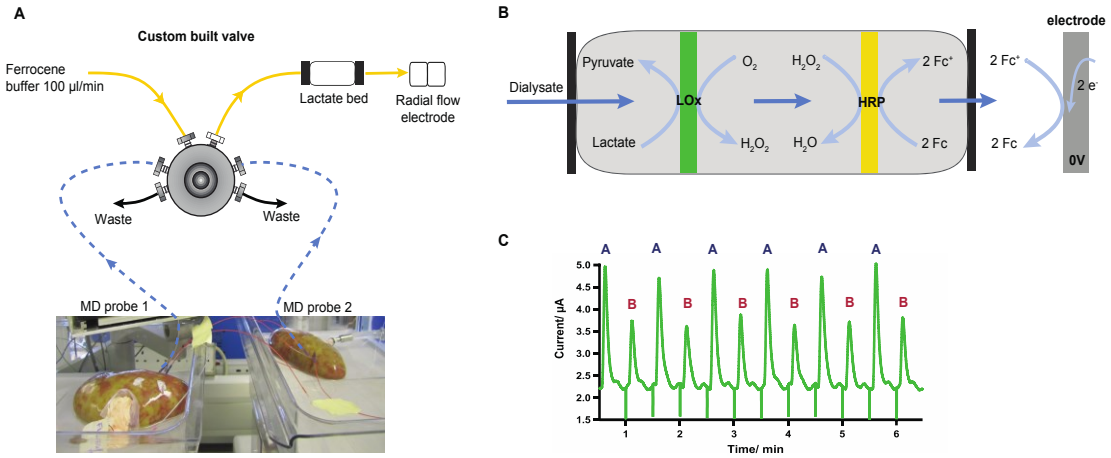


Figure 44 A. A microdialysis probe is inserted into the cortex of each kidney. The probe outlets are connected to the flow injection valve of the rsMD system, either side of the analysis loop, using low-volume FEP tubing, represented here by dotted blue lines. Every 30 s a 200 nL dialysate sample is injected into the analysis flow stream, alternating between each kidney, providing real-time data from two kidneys simultaneously. B. The dialysate sample flows through an enzyme reactor which contains two consecutive membranes, the first loaded with lactate oxidase and the second with horseradish peroxidase to a glassy carbon electrode held at 0 V. The reaction is mediated by ferrocene monocarboxylate. C. The current is recorded as peaks, alternating between each kidney. The peak height is proportional to the lactate concentration in the dialysate sample. Abbreviations: rsMD: rapid sampling microdialysis, nl: nanolitre, V: volt.

3.2.4 Storage and delayed analysis of on-line microdialysis

The second microdialysis probe was placed into the medulla of each kidney. The probe inlet tubing was connected to a syringe pump and perfused with T1 solution at a flow rate of 1 µL/min. The outlet of the probe was connected to a length of coiled storage tubing (fine bore polyethylene tubing, 0.4 mm inner diameter, 12 m for SCS experiments and 6 m for HMP) primed with T1 solution. This lower flow rate was chosen to increase the probe recovery and to reduce the length of storage tubing required. Usually this would increase the time delay between the probe and the assay however this was not an issue in this case as the samples were analysed later. At the end of the

kidney monitoring experiment, during which one probe for each kidney was assayed online, the stored dialysate from the second probe was then pumped into the on-line assay, effectively 'playing back' the online liquid stream collected during the experiment. To do this the end of the coiled tubing that had been connected to the probe was detached and connected to a syringe pump and perfused with T1 solution at a flow rate of 2 µL/min, while the other end of the coiled tubings were connected to two separate sample loops of the valve, either side of the analysis loop, as before, allowing the data to be analysed with temporal resolution. The dialysate was analysed as described above for the online assay. Differences in flow rate and delay were accounted for to compare these results to the online results (see Figure 45).

In an experiment to compare online and offline methods, two probes were placed in the same region of the medulla; one probe was analysed online and one offline, as described above. The levels obtained by the two methods were coincident. The mean difference between the two probes was 24.7 ± 62.5 µM, compared to a mean difference of 269 ± 66.7 µM comparing two probes in two different regions (the cortex and medulla), both analysed using the online method (data not shown).

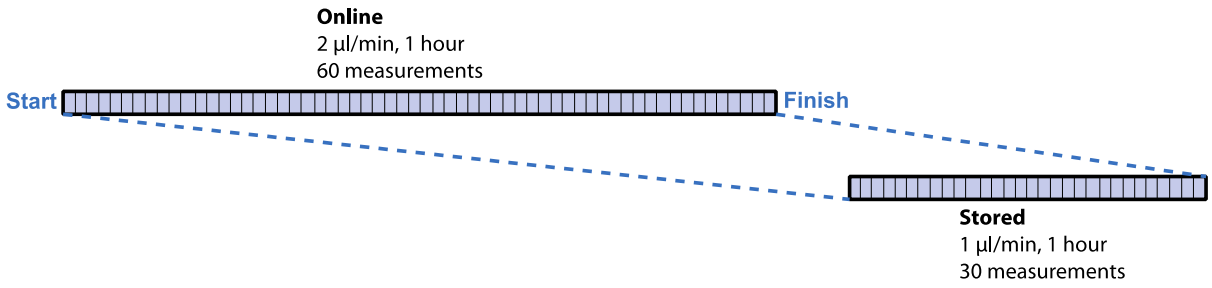


Figure 45 Schematic comparison of the online and stored analysis methods. Online analysis was carried out at 2 µL/min, giving 60 measurements in 1 hour. Stored samples were collected at 1 µL/min and analysed at 2 µL/min, giving 30 measurements in 1 hour. Abbreviations: µL: microlitre.

3.2.5 Perfusate analysis

Perfusate samples were taken at the start of HMP and then at 3, 6 and 10 hours. The samples were stored at -80 °C and were analysed at a later date. The rsMD system was re-configured and was set up in the same way as the system used to monitor brain injury patients. Each sample was manually injected into the system until 6 peaks were obtained for each of glucose and lactate.

3.2.6 Statistical Analysis

For intra-preservation group comparisons, tests were done using the Wilcoxon Signed Rank Test (two-tail), while the Mann Whitney U-Test (two-tail), was used for inter-preservation group comparisons. Significance was taken to be $p<0.05$. Perfusion dynamics are represented as means +/- SD. Lactate levels are represented as median and interquartile range, and graphically using box plots showing the median and interquartile range, with whiskers showing the 10^{th} and 90^{th} percentiles. For the initial 40 minutes of warming, the data was compared to an assumed linear increase, using area under the curve analysis. A one-sample t-test (two-tail) was used to test whether the data was significantly different from a linear increase.

3.3 Results

3.3.1 HMP Dynamics

Ten kidneys underwent 24 hours of SCS and a further twelve underwent 10 hours of HMP. Ten kidneys underwent 24 hours of SCS and a further twelve underwent 10 hours of HMP. Perfusion parameters were similar between HMP kidneys with systolic pressures of 36 ± 8.5 mmHg, flow rates of 27.7 ± 6.1 ml/min/100g, PFIs of 0.75 ± 0.27 ml/min/100g/mmHg, and resistances of 0.39 ±0.16 mmHg/ml/min by the end of perfusion, with a trend of improving perfusion metrics and based on clinical protocols, were considered indicative of kidneys suitable for transplantation [841] (Figure 46).

3.3.2 Microdialysis

Overall medullary lactate levels were greater than cortical levels throughout preservation and passive warming. RsMD data from two kidneys that underwent HMP was excluded from analysis due to technical problems.

3.3.2.1 Preservation

SCS – Cortex and Medulla

SCS cortical lactate levels declined from 114 (84-164) to 65 (41-118) µM (p=0.002, n=10) between the 15-30 minutes interval and the 1-1.5 hour interval. Levels remained low and stable for the remainder of the 24 hours (63 [60-151] µM at 23.5-24 hour interval, Figure 47a). In contrast, however, medullary lactate levels were stable and consistent throughout monitoring (Figure 49), with significantly greater levels throughout the preservation period in all cases and at all time intervals compared to the cortex (median cortical levels at 15-30 minutes, 1-1.5 hours, 9.5-10 hours and 23.5-24 hours: 114 (84-164) µM, 65 (41-118) µM, 68 (53-102) µM, 63 (60-151) µM, compared to medullary levels: 600 (480-826) µM p=0.004, 478 (421-712) µM (p=0.002), 474 (361-568) µM p=0.002, 374 (343-624) µM p=0.004 respectively, n=10).

HMP – Cortex and Medulla

During HMP, cortical lactate levels were stable for the initial 1.5 hours of perfusion (98 [76-104] µM at 15-30 minutes and 124 [76-179] µM at 1-1.5 hours, p=0.322, n=10). Between the 1-1.5 hours and 9.5-10 hour intervals, median levels increased to 230 (201-291) µM (p=0.002, n=10, Figure 47b).

Similar to the SCS data, HMP medullary lactate levels were greater than cortical levels (median levels at 15-30 minutes, 1-1.5 hours, and 9.5-10 hours: 98 [76-104] µM, 124 [76-179] µM, 230 [201-291] µM in the cortex compared to 425 [149-645] µM p=0.027, 315 [167-447] µM p=0.009, 551 [369-877] µM p=0.004 in the medulla, n=10, Figure 49).

SCS vs. HMP

There were no significant differences in cortical lactate levels during the first 1.5 hours of SCS and HMP preservation. By the 9.5-10 hour interval, the median cortical lactate concentration in HMP kidneys was significantly higher than in those preserved by SCS (HMP 230 [201-291] µM vs. SCS 68 [53-102] µM, p<0.001, n=10, Figure 48). In the medulla, there was no significant difference in lactate levels between the two methods during the entire preservation period (n=10, Figure 48).

3.3.2.2 Warming

Cortical lactate profiles for SCS kidneys undergoing 2 hours of passive warming showed a significant increase in lactate, detected between the end of 24 hours (65 [59-169] µM) and after 1 hour of warming (121 [103-216] µM, p=0.004, n=9), with no further increase by 2 hours (133 [104-225] µM, p=0.652). Maximum lactate levels were detected within the first hour of warming (median 11 [9-15] minutes after warming onset, Figure 50), which then decreased to stable levels, although still higher than pre-warming levels, within 1 hour.

Similarly there was a significant increase in cortical lactate levels between the end of HMP (241 [206-276] µM) and after 2 hours of warming (470 [320-586] µM, p=002, n=10). In contrast to SCS, however, the time to maximum detected lactate in HMP kidneys was significantly longer (median 120 [112-120] minutes after warming onset, p<0.001 vs. time to peak in SCS group, Figure 50).

Overall there was a significant increase in cortical lactate after warming in both groups, with the increase in the HMP group significantly greater than the SCS group (SCS median increase 56 [49-62] µM vs. HMP 143 [88-353] µM p=0.006, Figure 51).

Medullary data indicated a significant increase in lactate during 2 hours of warming in both groups (median values before and after warming, SCS: 381 [359-652] µM to 667 [617-738] µM, p=0.008, n=9; HMP: 517 [375-850] µM to 926 [738-1137] µMp=0.002, n=10) with a similar increase in both groups (median values SCS: 248 [93-357] µM, HMP: 241 [168-297] µM, p=0.968,Figure 51). Similarly, the median time to maximum detected medullary lactate levels was 120 [100-120] minutes in SCS and 98 [90-120] minutes in HMP kidneys (p=0.357).

Specific area under the curve analysis of the lactate profiles during the initial 40 minutes of warming (Figure 52) demonstrated no significant difference compared to a linear increase model in both cortical and medullary regions of HMP kidneys. Cortical lactate levels in SCS kidneys however, demonstrated a significant difference, compared to a linear increase, in a positive direction, corresponding to the lactate spike seen in this preservation group, while medullary levels also showed a significant difference from a linear increase but in a negative direction, indicating that levels decreased during this period, before increasing.

The mean increase in temperature in both SCS and HMP kidneys during 2 hours of warming was similar in both groups (median values: SCS 13.2 [11.6-13.3] °C vs. HMP 11.1 [10.1-11.4] °C, p=0.625). The final median temperature after 2 hours of warming in the SCS group was 17.1 (15.7-17.2) °C and 17.9 (17.5-18.7) °C in the HMP group (p=0.312).

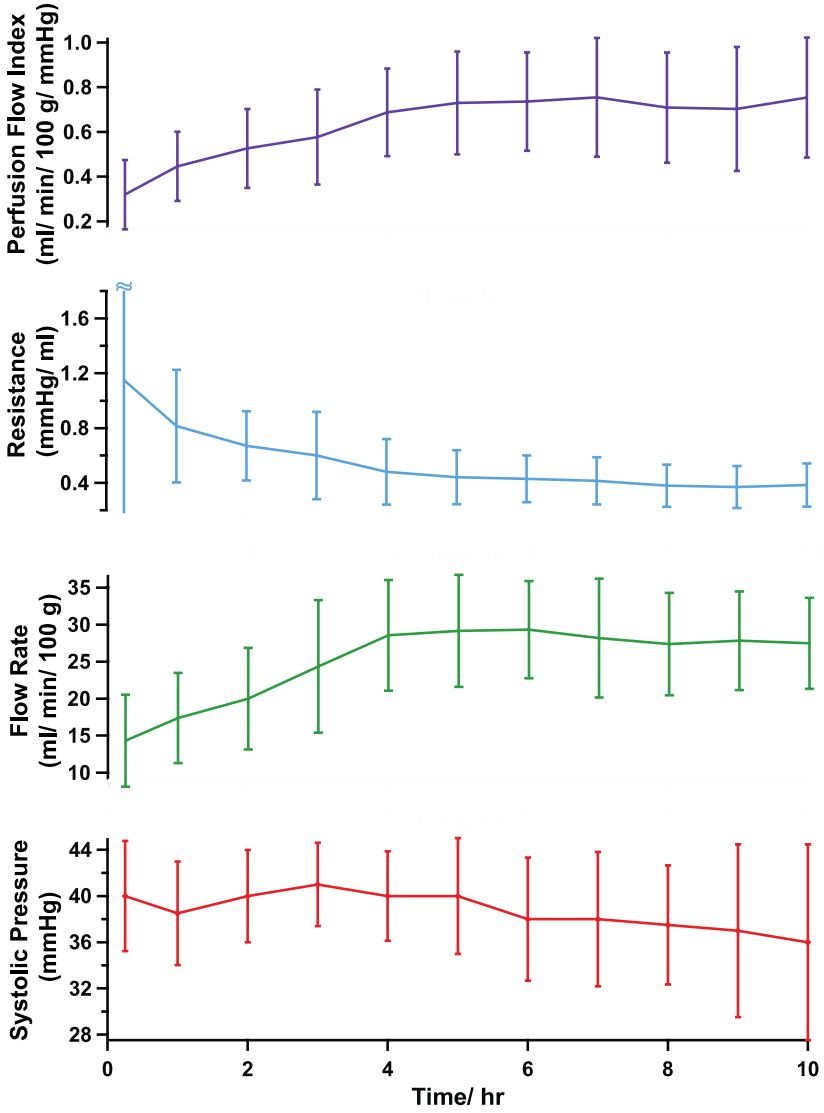


Figure 46 Perfusion dynamics over 10 hours of HMP for 12 kidneys. Perfusion characteristics were indicative of organ viability, with improving flow rates and perfusion flow indices. (Mean +/- standard deviation). Target systolic perfusion pressure was 40 mmHg during the first 4 hours of HMP, which was adjusted hourly. After 4 hours no further changes to the perfusion system were made. Abbreviations: HMP: hypothermic machine perfusion.

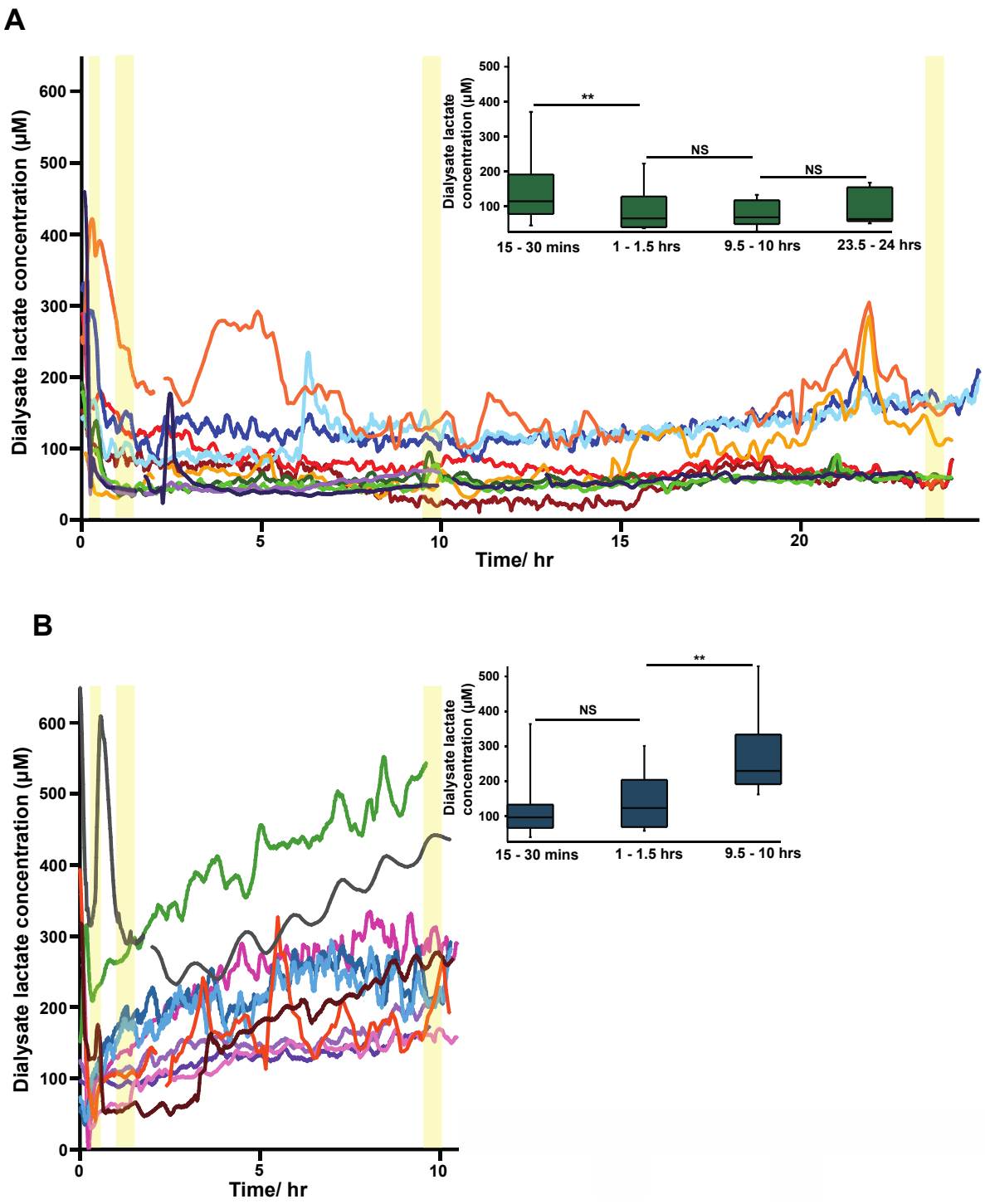


Figure 47 Dialysate cortical lactate profiles for kidneys undergoing A. 24 hours of cold storage (n=10). B. 10 hours of HMP (n=10). Each line represents real-time data of an individual kidney with a point every minute (5 minutes in a few cases - 2 SCS, 2 HMP, for section from 2 hours to end) smoothed with a Savitsky-Golay 21-point filter to minimise the effect of any anomalous results. Inset: Median lactate concentrations of SCS kidneys at 4 time intervals (15-30 minutes, 1-1.5 hours, 9.5-10 hours, and 23.5-24 hours) during 24 hours of preservation and of HMP kidneys at 3 time intervals (15-30 minutes, 1-1.5 hours and 9.5-10 hours) during 10 hours of preservation. Shaded regions indicate the data window averaged to give the data presented in

the box plots. Boxplots show median levels and interquartile range. Whiskers indicate 10th and 90th percentiles. Significance: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, NS = not significant. For SCS, p=0.002 for 15-30 minutes vs. 1-1.5 hours, p=0.770 for 1-1.5 hours vs. 9.5-10 hours and p=0.098 for 9.5-10 hours vs. 23.5-24 hours. For HMP, p=0.322 for 15-30 minutes vs. 1-1.5 hours and p=0.002 for 1-1.5 hours vs. 9.5-10 hours. Abbreviations: µM: micromolar.

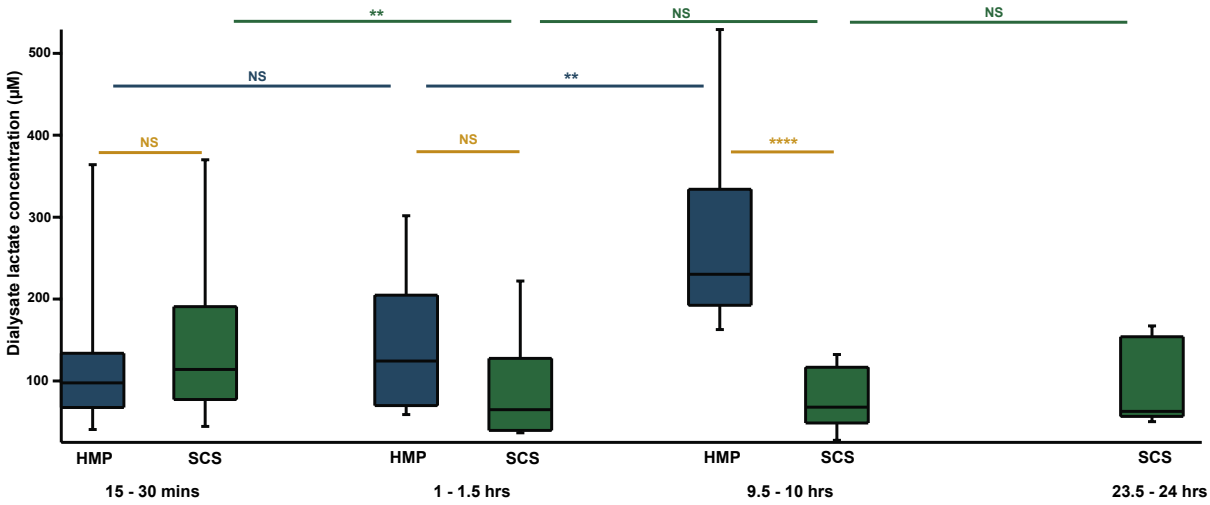


Figure 48 Median dialysate cortical lactate levels in SCS and HMP kidneys during 24 hours of SCS (n=10) and 10 hours of HMP (n=10). Lactate levels were similar during the first 1.5 hours of preservation, median values at 1-1.5 hours: HMP 124 (76-179) µM vs. SCS 65 (41-118) µM. At the 9.5-10 hours interval lactate levels were significantly higher in HMP kidneys vs. SCS kidneys (230 [201-291] µM vs. 68 [53-102] µM, p<0.001). Boxplots show median levels and interquartile range. Whiskers indicate 10th and 90th percentiles. Significance: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, NS = not significant. For SCS, p=0.002 for 15-30 minutes vs. 1-1.5 hours, p=0.770 for 1-1.5 hours vs. 9.5-10 hours and p=0.098 for 9.5-10 hours vs. 23.5-24 hours. For HMP, p=0.322 for 15-30 minutes vs. 1-1.5 hours and p=0.002 for 1-1.5 hours vs. 9.5-10 hours. For SCS vs. HMP, p=0.393 at 15-30 minutes, p=0.075 at 1-1.5 hours and p<0.001 at 9.5-10 hours. Abbreviations: µM: micromolar.

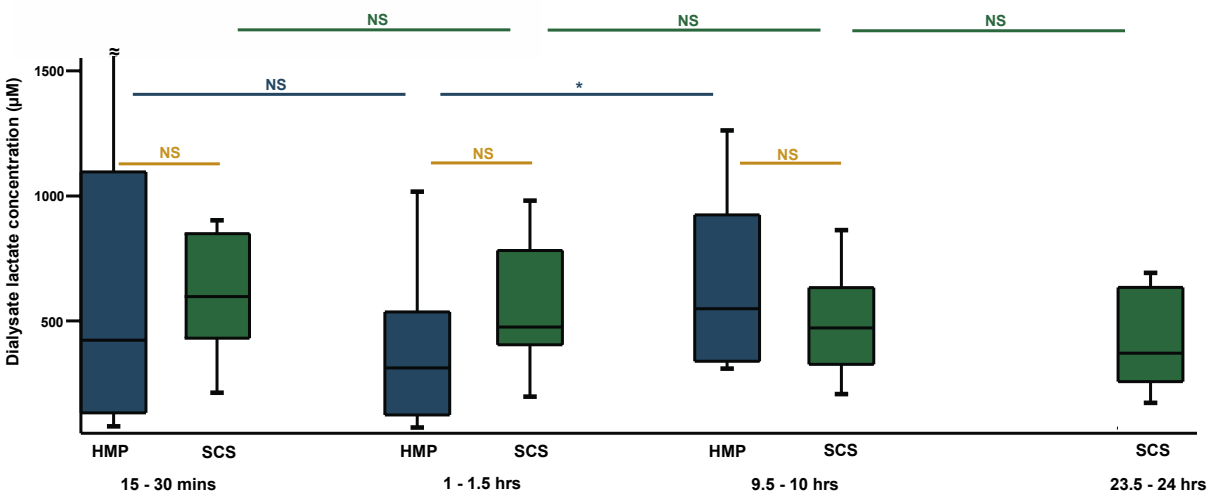


Figure 49 Median dialysate medullary lactate levels in SCS and HMP kidneys during 24 hours of SCS (n=10) and 10 hours of HMP (n=10). For kidneys stored using SCS, lactate levels remained stable from onset (478 [421-713] µM, 474 [361-568] µM and 374 [343-624] µM at 1.5 hours, 10 hours, and 24 hours respectively). During HMP, lactate levels remained stable for the initial 1.5 hours, and then increased between the 1-1.5 hours and 9.5-10 hours intervals (315 [167-447] µM at 1.5 hours to 551 [369-877] µM at 10 hours). Comparing SCS to HMP, lactate levels were similar throughout preservation. Boxplots show median levels and interquartile range. Whiskers indicate 10^{th} and 90^{th} percentiles. Significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, NS = not significant. For SCS, p=0.193 for 15-10 minutes vs. 1-1.5 hours, p=0.625 for 1-1.5 hours vs. 9.5-10 hours and p=0.359 for 9.5-10 hours vs. 23.5-24 hours. For HMP, p=0.105 for 15-30 minutes vs. 1-1.5 hours and p=0.014 for 1-1.5 hours vs. 9.5-10 hours. For SCS vs. HMP, p= 0.315 at 15-30 minutes, p=0.089 at 1-1.5 hours and p=0.353 at 9.5-10 hours. Abbreviations: µM: micromolar.

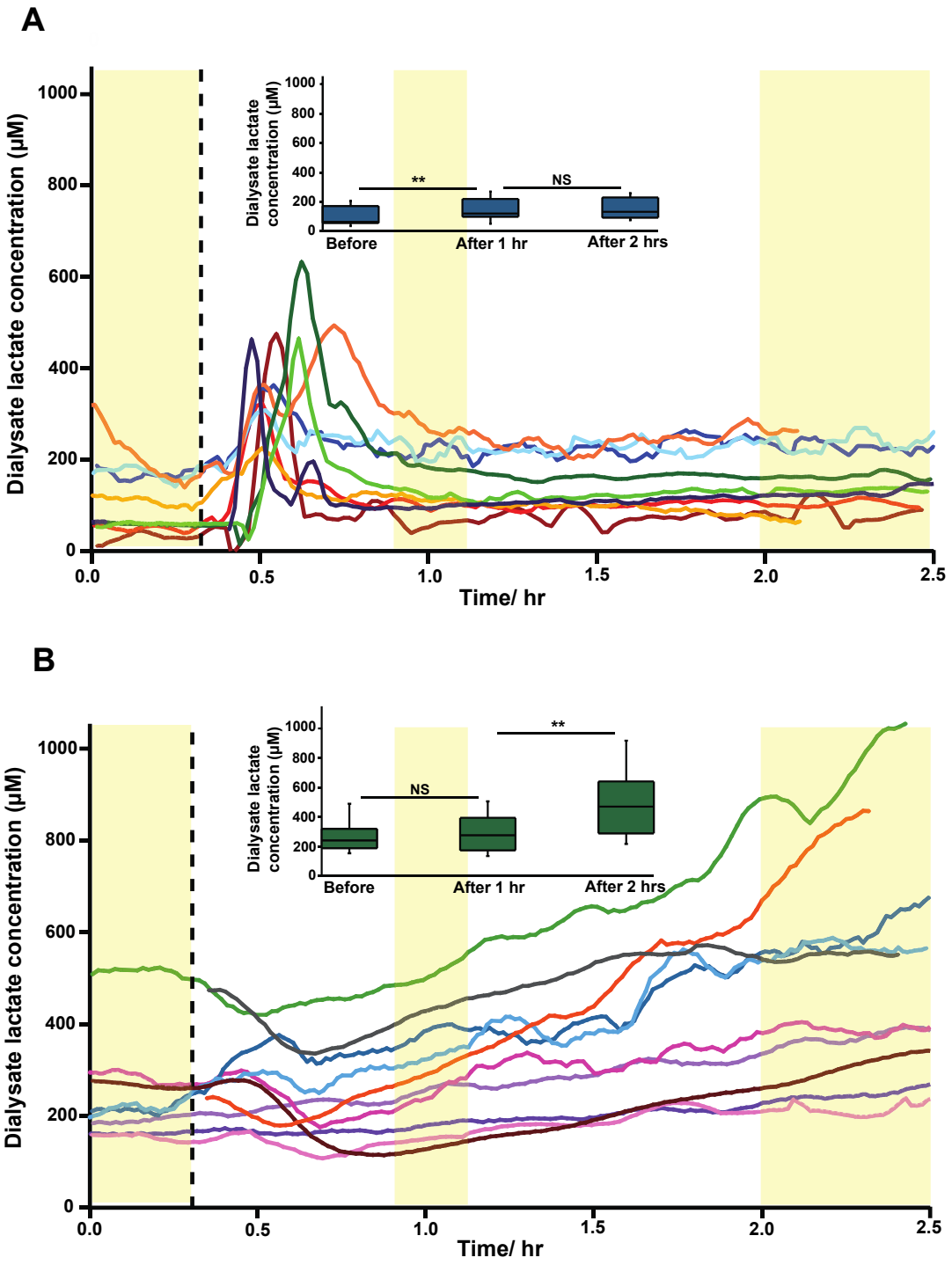


Figure 50 Dialysate cortical lactate profiles for kidneys undergoing 2 hours of passive warming to ambient temperature after preservation. Each line represents real-time data of an individual kidney with a point every minute, smoothed with a Savitsky-Golay 21-point filter to minimise the effect of any anomalous results. A. 24 hours of SCS (n=9), B. 10 hours of HMP (n=10). Inset: median readings for lactate concentrations at 3 time intervals (30 minutes before warming, after 1 hour warming, after 2 hours warming) during 2 hours of passive warming. Shaded regions indicate the data window averaged to give the data presented in the box plots. Boxplots show median levels and interquartile range. Whiskers indicate 10th and 90th percentiles. Significance: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, NS = not significant. For SCS,

p=0.004 for before vs. after 1 hour warming and p=0.652 for 1 hour vs. 2 hours after warming. For HMP, p=0.275 for before vs. after 1 hour warming and p=0.002 for 1 hour vs. 2 hours after warming. Abbreviations: µL: microlitre.

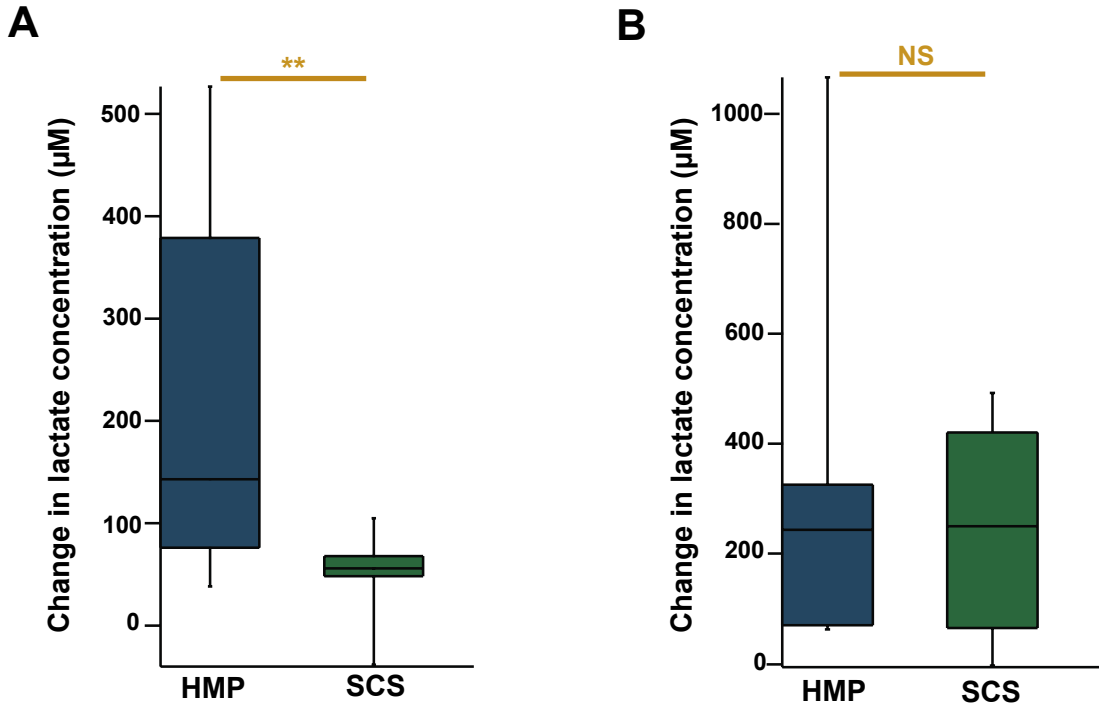


Figure 51 A. Comparison of cortical lactate increase in the two groups. The increase in the HMP group is significantly greater than the SCS group (median values: HMP 143 [88-352] µM vs. SCS 56 [49-62] µM, p=0.006). B. Comparison of medullary lactate increase, which is similar between both groups (median values: HMP 241 [168-297] µM, vs. SCS 248 [93-357] µM, p=0.968). Boxplots show median levels and interquartile range. Whiskers indicate 10th and 90th percentiles. Significance: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, NS = not significant. Abbreviations: µM: micromolar.

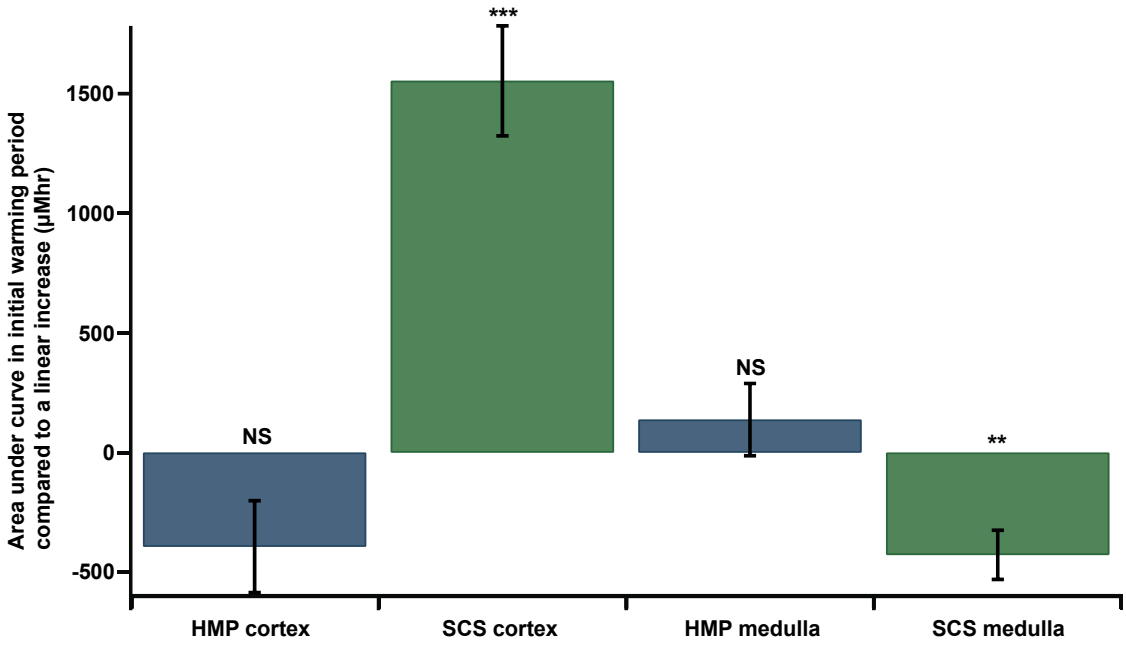


Figure 52 Area under curve analysis of lactate profiles during warming, compared to a linear increase model during the initial 40 minutes of warming. No significant changes in lactate levels in HMP kidneys during this period were detected. Cortical lactate levels in SCS kidneys demonstrated a significant increase, corresponding to the lactate spike seen in this preservation group, while medullary levels decreased slightly during this period. Significance: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, NS = not significant. HMP cortex p=0.071, SCS cortex p<0.001, HMP medulla p=0.387, SCS medulla p=0.004. Abbreviations: µM: micromolar.

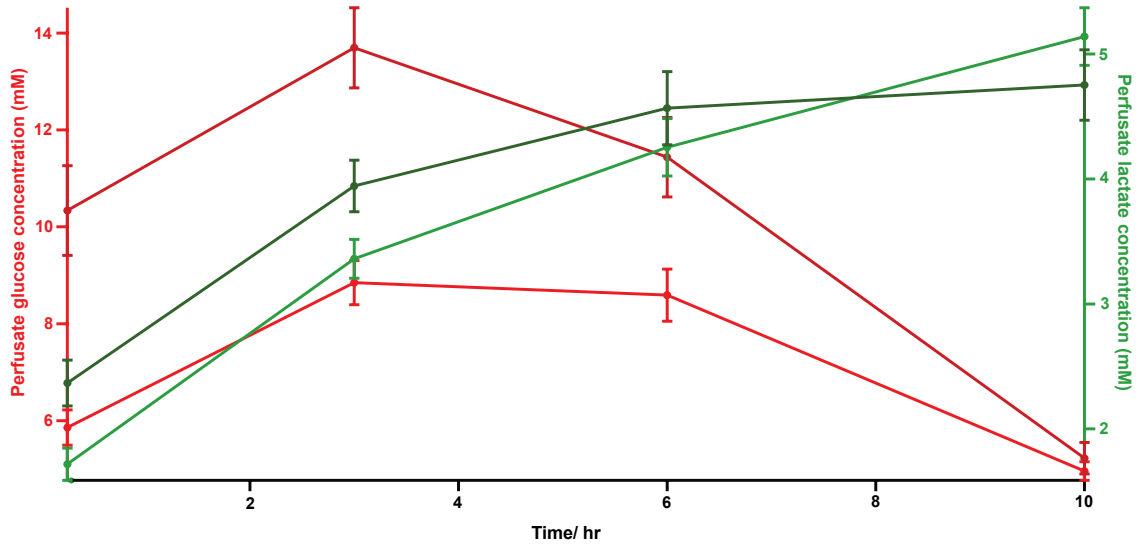


Figure 53 Perfusate lactate (green) and glucose (red) concentrations during 10 hours of HMP for two kidneys. The darker and lighter colours indicate the two kidneys. Lactate increases during HMP, while glucose peaks after several hours and declines until the end of perfusion. Abbreviations: mM: millimolar.

3.4 Discussion

We have used rsMD to identify the differing effects on renal energy metabolism brought about by SCS and HMP, both during hypothermic preservation and subsequent warming. Both modalities show contrasting effects, and these differences may help elucidate the reported clinical superiority of HMP, that is observed to afford metabolic resilience on the renal parenchyma to warm ischaemia after initial cold preservation.

Associations between DGF and increased risk of graft loss [41], acute rejection [50], longer hospital stay and high costs [51] mean there is an emerging need for better viability testing prior to transplantation to identify and potentially reduce the risk of poor outcome, especially with the increasing use of organs from ECD and DCD donors. The period of preservation between retrieval and transplant provides a unique opportunity to assess organ viability, particularly when preservation utilises HMP [841].

Lactate, as an end-product of anaerobic glycolysis, formed by the conversion of pyruvate by lactate dehydrogenase, is a key marker of tissue hypoxia, ischaemia and cell stress, which is potentially reversible.

3.4.1 Lactate and The Kidney

The physiology of lactate in the kidney, however, is complex [1048]. Under normal physiological conditions the kidney plays a key role in removing lactate from the systemic circulation by uptake and metabolism, as well as in using lactate as a metabolite for gluconeogenesis [1049,1050]. The fate of lactate in the kidney differs between the cortex and medulla. The medulla is the primary site for local production of lactate from glucose, while the cortex uses lactate as both an oxidative energy substrate, and to form glucose for local and systemic use. Such an intricate recycling system is likely to be a way of providing the medulla and cortex with energy for their complex tasks [1051].

Use of perfusate lactate as a biomarker of ischaemic tissue injury in HMP preserved kidneys has been investigated and associations between higher levels of perfusate lactate and risk of DGF have been found [847,1052]. Similarly perfusate lactate dehydrogenase (the enzyme responsible for the inter-conversion between lactate and pyruvate) has been evaluated in a number of studies and shown to be associated with primary and delayed function, as well as with graft failure [870]. Lower perfusate lactate in HMP has been taken as an indicator of better organ viability, as judged

by improved microvascular perfusion and post-transplant function [1052,1053]. Measuring perfusate lactate, however, provides no indication as to the anatomical source in the perfused organ and thus is a sensitive but not site-specific indicator of tissue metabolism. RsMD allows quantification of local levels of lactate within the interstitium of the renal parenchyma – both the cortex and the medulla - the two principle and distinct metabolic regions of the kidney.

3.4.2 Cortical Metabolic Profiles During SCS and HMP

It was initially theorised that tissue lactate in HMP preserved kidneys would be lower than in SCS, as a result of the washout effect in HMP, with levels in SCS preservation predicted to rise progressively as lactate accumulation occurs during cold ischaemia - interestingly the reverse was found. Cortical lactate remained consistently low and stable for the duration of SCS, while levels in HMP kidneys increased progressively. The stable, low lactate levels seen during 24 hours of SCS have not been reported previously [916,923].

HMP differs from SCS in two key respects. Firstly, in HMP there is a sustained pulsatile perfusion, which allows buffering of intra- and extra-cellular acidosis, and removal of metabolic products. Secondly, SCS kidneys were typically stored at a lower temperature (4.6 °C lower on average). The protocols for this study were designed to mimic clinical practice as closely as possible to ensure its translational potential of the data generated. Clinically, the temperature at which HMP is performed is between 4-8 ^{o}C, while SCS temperatures are lower at 2-4 ^{o}C[1054,1055]. In our study, these differences were maintained. Hence, in SCS, local glycolytic activity may have been limited due to: 1) a finite local supply of glucose and ATP precursors. 2) the inhibitory effect of acidosis on glycolytic enzymes and therefore glycolysis rates. 3) the lower temperatures during storage, slowing cellular function and therefore reducing both the demand for energy and energy metabolism rates. During HMP, oxygen delivery via oxygen dissolved in the UW solution is much lower than with haemoperfusion, but even after 18 hours of perfusion remains above 100mmHg, however, tissue VO_2 is also reduced to the extent that the delivery would be theoretically sufficient for basal metabolic requirements [722].

Lactate metabolism is however complex in the kidney. Further explanation for the increasing lactate levels seen during HMP is that direct perfusion provides the possibility for the removal of local metabolic products from one part of the kidney for redistribution to other parts of the kidney, as the perfusate is recycled. This may re-establish local transport and redistribution of

glucose and lactate between the cortex and medulla, which would facilitate low levels of energy and glucose production in the cortex and further glycolysis in the medulla.

Analysis of the perfusate demonstrates that perfusate lactate levels increase over the duration of HMP, while glucose levels increase in the first 3 hours of perfusion and then decrease over the remaining preservation period. Figure 53 shows data from perfusate analysis of two HMP kidneys. The increasing levels of glucose in the perfusate suggest that local production of glucose through cortical gluconeogenesis is occurring [1056,1057] as UW solution does not inherently contain glucose. This glucose can be transported to the medulla by the perfusion medium, enabling further glycolysis to occur, leading to the increasing lactate levels seen in both the tissue and the perfusate.

This explanation is supported by the higher lactate levels seen in the medulla, compared to the cortex. The ability of HMP to re-establish glucose-lactate recycling, as well as to deliver low levels of oxygen and nutrients in the perfusate, may be sufficient to facilitate resumption of inherent physiological function to a certain degree. In contrast, SCS stored organs receive no perfusion, which may explain the difference in metabolic profiles seen between these two modalities.

3.4.3 Cortical Metabolic Profiles During Warming after SCS and HMP

The 2 hours of warming after cold preservation was designed to test the reaction of the kidney parenchyma to an extreme challenge and the effect to this reaction of SCS vs. HMP. During warming, increasing lactate levels likely represent further, more deleterious cell damage and death, as the type of ischaemia changes from hypothermic to sub-normothermic. The initial spike in lactate levels in SCS, observed in the first 30 minutes of warming, was likely a result of a surge in the limited metabolic activity and in anaerobic glycolysis, with the sudden increase in temperature. The subsequent decrease to lower stable levels representing remaining surviving cortical cells with minimal anaerobic potential. HMP profiles, however, do not show any initial surge in cortical lactate. Instead they show a gradual increase over the 2 hours, potentially due to an increased basal anaerobic glycolytic ability - as without continued machine circulation any contribution from medullary sources is negligible. The progressive cortical lactate increase in HMP kidneys may thus represent similar cell damage as in SCS kidneys but protracted over a longer time interval, possibly indicating a better “ability” of HMP kidneys to tolerate the warming insult.

3.4.4 Metabolic Profiles in the Medulla

The medulla can be considered to be a physiologically distinct organ to the cortex with respect to function and metabolic activity [549,1056,1057]. A similar trend was seen in medullary lactate profiles during preservation by both HMP and SCS, with no observable differences between the two modalities. Furthermore similarities in SCS and HMP kidneys' medullary profiles during warming suggest any real effect of HMP may dominate in cortical regions of the kidneys.

3.4.5 Microdialysis Studies of Renal Metabolism

Investigating energy metabolism in renal SCS, Keller et al. [916] used conventional microdialysis (off-line with samples analysed every 30 minutes) in a porcine autotransplant model and showed an initial decrease in cortical lactate during a relatively short CIT of approximately 2 hours. The decrease was expected as microdialysis monitoring commenced prior to retrieval and flushing, and represents decreased metabolism secondary to flushing and cold storage.

In their later study [923] using longer CIT (24 hours), in an *ex vivo* porcine renal preservation model, they showed an overall increasing trend in cortical lactate levels during 24 hours of SCS, in stark contrast to our findings. Furthermore in the same experiment kidneys undergoing 24 hours of warming (at 37^{o}C - without any preceding hypothermia) lactate levels peaked after several hours and remained high. In contrast in our experiments warming lactate profiles (in SCS kidneys) demonstrated a rapid peak (within 15-30mins) followed by a quick decline to a stable nadir. In our protocol warm ischaemia exposure was at a lower temperature (21^{o}C) and post a period of hypothermic preservation so a direct comparison cannot be made accurately. However given the different protocols, differences in lactate profiles could be attributed to the period of SCS in our experiments.

Microdialysis monitoring during *in situ* clamping of renal arteries to instigate ischaemia [1058], vascular thrombosis [917], and DGF [1059] in porcine models demonstrates a build up of cortical lactate and depletion of glucose. This is an expected phenomenon in ischaemic settings, which can be explained by the switch to anaerobic production of lactate, with glucose consumption.

Fonouni et al. [922] recently investigated differing CIT duration on renal energy metabolism during anastomosis and reperfusion in a porcine autotransplant model. They observed a similar lactate rise and peak in the initial period during the anastomosis, after 24 hours of SCS, followed by a decline until vascular reperfusion. These findings correlate with our data, however due to the superior temporal resolution of our rsMD system we were more accurately able to determine the duration and level of the warming peaks. In the same study, kidneys with a shorter SCS CIT of 6

hours showed a progressive increase in lactate in the same period, and interestingly this profile resembles the warming profile for HMP kidneys in our study. It is possible to surmise that these less compromised (6 hours SCS) kidneys are of a similar viability as the HMP kidneys in our study possibly due to the effect of HMP preservation on the renal parenchyma.

In a novel study Baicu et al. [1060] combined microdialysis with renal HMP preservation to determine the efficacy of perfusate supplementation, in restoring depleted energy intermediaries, but failed to quantify perfusate or tissue lactate levels. The authors concluded that measuring interstitial chemical changes by microdialysis may provide a better means of assessing organ viability than by renal effluent analysis alone.

3.4.6 Limitations of this Study

There are some inherent limitations in this work. UW was used as both the SCS solution and HMP perfusate in these experiments. Despite UW solution being initially designed by Belzer and Southard to be a HMP perfusate, the UW Belzer MPS variant is the predominate perfusate used in conventional renal HMP. The predominate difference between UW and Belzer MPS is the electrochemical composition with an inversion of the sodium / potassium content; UW being an intra-cellular solution and Belzer MPS extra-cellular [705]. The theoretical benefits of using one solution is that differences in their composition should not affect any observed differences between preservation modalities. A limitation is that the use of one solution in the protocol does not truly replicate clinical practice, however recent experimental evidence from a canine kidney auto-transplant model suggests equity in effect and outcomes in kidney HMP preservation using either solution - UW or UW Belzer MPS[1047].

The Waters Medical Systems RM3 organ perfusion unit was used in these experiments with a normal hypothermic perfusion temperature between 4-8^{o}C. As indicated in Section 1.7.2.9 MACHINE PERFUSION DEVICES the Organ Recovery Systems Lifeport immerses the organ in cold preservation solution in addition to perfusion thus maintaining organ temperature lower than with the RM3. The rsMD lactate profiles observed in these experiments are specific to kidney HMP using the RM3 and may be different with other devices.

Comparatively different cold ischaemia times were used in SCS and HMP. There were several reasons. Twenty-four hours of SCS was chosen in this experimental setting to represent an elongation of the normally accepted duration of SCS in clinical practice [705]. The ten hours of HMP was chosen to match clinical scenarios with the rational that; the benefits of HMP are likely

to be experienced after 4-5 hours of HMP [1061] with minimal benefit after 10 hours; the average time for HMP in the UK is approximately 10 hours [831]. Furthermore more than 10 hours of HMP in the current model was not feasible due to logistical constraints. Such differences in cold preservation time however would potentially only impact rsMD data from the 2 hours post preservation warming period and not that during hypothermic preservation, in particular the 1st 10 hours. Acknowledging this limitation an additional experiment using two kidneys was performed comparing 10 vs. 24 hours of SCS on subsequent warming lactate profiles to investigate the effect of the different cold storage time. In these kidneys a similar warming profile (with a lactate surge within 15-30 minutes followed by a decline to a stable nadir) was seen for both durations of CIT (data not shown).

rsMD monitoring was started only once the organs were back at the laboratory, and so there is no data for the critical time period that includes animal donor death, recovery warm ischaemia, organ flushing, and the first few hours of cold storage transportation.

The 2 hour post preservation warming period was included to test the viability of the kidneys post preservation. This period was chosen to exert a challenge on the kidneys in an experimental setting and may only partly reflect the clinical period of anastomosis formation. This is because the 'anastomosis' takes on average less than an hour, and many transplant surgeons attempt to limit the temperature increase by applying ice-slush or cold external rinse.

Finally in these experiments it was not possible to observe tissue lactate concentrations after the period of ambient warming, which would require either a porcine auto-transplant model or use of *ex vivo* isolated reperfusion to simulate the initial period of after reperfusion after revascularisation.

3.4.7 rsMD in Context and Future Work

Continuous online microdialysis has provided an important insight into the chemical processes active during the *ex vivo* phase of a donor kidney during SCS and HMP, offering, for the first time, a possible biochemical explanation regarding the protective role of HMP for kidneys. Further studies would need to be conducted to confirm this. Determining the potential of this technology in assessing allografts prior to transplantation requires an assessment of organ function in grafts with variable degrees of initial ischaemic injury, to correlate lactate profiles with functional outcome. This was not feasible using the current model. However the ability to monitor real-time

cellular metabolism in an organ prior to transplantation has the potential to provide unique and vital information on graft function post-transplantation.

The next step is to use rsMD in a porcine kidney auto-transplant, or an *ex vivo* isolated reperfusion model, and to monitor multiple chemical markers of anaerobic metabolism (e.g. glucose, pyruvate, citrate and adenosine tri-phosphate) and kidney function in real-time. Furthermore rsMD monitoring will enable assessment of organ viability in experimental model testing strategies to improve preservation or ameliorate reperfusion related tissue damage.

Chapter 4:

Addressive Targeting of Anti-thrombin Agents to Graft Vasculature during HMP: Preconditioning Organs to Ameliorate Post Transplant Micro-vascular Thrombosis

Addressive Targeting of Anti-thrombin Agents to Graft Vasculature during HMP: Preconditioning Organs to Ameliorate Post Transplant Micro-vascular Thrombosis

4.1 Objectives

Combining the use of addressive anti-thrombin agents with the emergence of HMP as an increasingly effective tool in organ preservation allows a strategy to potentially pre-condition the graft endothelial and vasculature prior to transplantation. Our hypothesis is pre-treating a renal graft with Thrombalexin using HMP as a delivery tool will enable effective vasculature tethering, forming an anticoagulant 'coat' that would potentially diminish pro-coagulant processes by inhibiting thrombin activity, and improve organ perfusion.

Ex-vivo organ perfusion models have been developed for investigating novel interventions that can ameliorate organ injury and improve viability. These models incorporate ex-vivo organ reperfusion models of porcine kidneys, as well as a direct pre-clinical model using human kidney grafts not suitable for clinical transplantation and subsequently donated for research. These organ perfusion models were used in a series of experiments investigating the feasibility and effect of using HMP for pre-conditioning kidney grafts with Thrombalexin.

4.2.1 Experimental Groups

The aim of these experiments was to evaluate the potential of ex-vivo organ pre-treatment with Thrombalexin in ameliorating microvascular thrombosis and macro and micro vascular perfusion deficits using a normothermic ex-vivo renal haemo-reperfusion (NHRP) circuit with minimally anti-coagulated autologous whole porcine blood. Four experiments were conducted. Experiment 1 tested an early variant of TLN (designated 'PTL006'), while Experiments 2-4 tested the current TLN construct. Experiment 4 involved use of human kidney grafts allocated for use in this research. Endothelial tethering of these proteins in porcine and human kidneys was evaluated using a specifically modified fluorescent labelled 'tail'. Details of the experimental groups and the protocols used is summarised in Figure 54.

<table>
<tr><th>Organ Procurement</th><th colspan="3">Hypothermic Perfusion</th><th>Normothermic Reperfusion</th></tr>
<tr><td>Graft retrieval, flush, and SCS transport ➔</td><td>HMP Stabilization: 4 hours ➔</td><td colspan="2">HMP Organ Pre-conditioning with TLN/PTL006: 1.5 hours (Washout-1,Treatment, Washout-2) ➔</td><td>NHRP Challenge: 6 hours</td></tr>
<tr><td rowspan="5">UW Solution or Marshall's Citrate</td><td rowspan="5">Based on clinical protocols [841]</td><td colspan="2">Experimental Porcine Groups</td><td></td></tr>
<tr><td><u>Experiment 1:</u>
CG1: n=7, control (Unmodified perfusate) Vs PTG1:n=7, TLN (PTL006) (2.1µM in perfusate)
<u>Experiment 2:</u>
CG2: n=8, control (Unmodified perfusate) Vs PTG2: n=8, TLN (2.1 µM in perfusate)
<u>Experiment 3:</u>
TCG3: n=4, Tail-only control (4.2µM Tail-only Proteins in perfusate) Vs PTG3: n=4, TLN (4.2µM in perfusate)</td><td>NHRP challenge with porcine (autologous) blood</td></tr>
<tr><td colspan="2">Experimental Pre-clinical Group</td><td></td></tr>
<tr><td colspan="2"><u>Experiment 4: Human kidneys</u>
CG4: n=1, control (Unmodified perfusate) Vs PTG4: n=1, TLN (4.2µM in perfusate)</td><td>Porcine blood - pro-coagulant xenogenic stimulus</td></tr>
</table>

Figure 54 Schematic summary of experimental protocols for TLN testing in porcine and human renal grafts. CG: Control Group, PTG: Protein Test Group, UW: University of Wisconsin Solution.

4.2.2 Organ Retrieval and Hypothermic Machine Perfusion

Kidneys were retrieved from landrace pigs terminated at a local abattoir by stunning and exsanguination. This model of sacrifice was akin to substantial head injury and also DCD as animals were only accessed once cardiac death had occurred. Organs were retrieved as per the shorter ischaemia protocol detailed in Section 3.1.3.1. Access was achieved through a midline incision, Kidneys were retrieved via en-block dissection, flushed with preservation solution (500-1000ml at 100cm H_20) and packed on ice for transport to the laboratory. Paired kidneys were matched between treatment and control groups. All kidneys were perfused initially on a Waters Medical Systems RM3 device for 4 hours of HMP stabilization with preservation solution (500ml) according to clinical protocols [841] with a target systolic pressure of 40mmHg. Kidney weight pre- and post- HMP was recorded. Perfusion dynamics were recorded throughout.

Approval was granted for research use of human kidney grafts initially retrieved but subsequently not used for transplantation by a National Research Ethics Committee (IRAS code: 84202) and NHS Blood and Transplant (the national regulator of organ transplantation in the United Kingdom). One pair organs were used in this study as part of a direct pre-clinical model in experiment 4, and underwent identical perfusion protocols as porcine kidneys.

4.2.3 Cytotopic Peptides

The TLN molecule is a conjugate peptide of the direct thrombin inhibitor [D-Phe-L-Pro-L-Arg-L-Pro-Gly-Gly-Gly-L-Asp-Gly-L-Asp-L-Phe-L-Glu-L-Glu-L-Ile–L-Pro-L-Glu-L-Glu-L-Tyr-L-Leu-Gly-Gly-L-Cys-amide] and the cytotopic tail APT3146 [(N-alpha, N-epsilon *Bis*-myristoyl Lys-Ser-Ser-Lys-Seryl-Pro-Lys-Lys-Asp-Asp-Lys-Lys-Pro-Gly-Asp-(S-2-pyridyldithio) Cys] [1062].

The peptide had a mass of 4649 Daltons as determined by time-of-flight mass spectrometry and was prepared by reaction of the two components in aqueous solution followed by further purification [1063].

A fluorescently labelled-version, lacking anticoagulant activity (FAM-PTL), was used to study localisation of TLN within the kidney.

The component peptides were prepared using solid phase synthesis by Cambridge Research Biochemicals, Billingham, UK and the conjugate also synthesized at scale by Almac Sciences, Craigavon, UK.

4.2.4 TLN Organ Pre-Treatment

After HMP stabilization kidneys underwent preconditioning with TLN. This consisted of three 30 minutes perfusion episodes:

1) Washout-1: 30 minutes HMP with 300mls of fresh perfusate to limit the amount of remaining donor blood in the organ and perfusate from the initial 4 hours of HMP ;

2) Treatment: perfusion with 400ml of TLN spiked perfusate (Protein Test Groups 1-4; PTG1-4); or either un-modified perfusate (Control Groups 1, 2 and 4; CG1, CG2, CG4) or perfusate with Tail-only proteins (Tail Control Group 4, TCG4); and

3) Washout-2: 30 minutes HMP with 300ml of fresh perfusate to facilitate removal of non-adherent TLN from the organ vasculature. A diagram summarising this protocol can been seen in Figure 55.

The same flow driven perfusion parameters during HMP were used. HTK was used as a treatment medium in Experiment 1, and lab made glutathione-free University of Wisconsin solution in Experiments 2-4 (as glutathione content of UW solution has theoretical deleterious effects on TLN activity). Between each perfusion episode kidneys were removed and kept on ice in UW solution.

4.2.5 Normothermic Haemo-reperfusion

Kidneys underwent 6 hours of normothermic reperfusion using minimally anti-coagulated whole blood (WB) perfusate (10,000U/L Heparin); 600mls, 3:1 ratio WB:0.9% saline. RM3 units were setup to facilitate normothermic perfusion (see Figure 56). Once the perfusate was 39˚C kidneys were connected to the RM3 arterial tubing. Flow driven perfusion setting were maintained initially as per during HMP and then set to between 50-70mmHg and maintained equal between kidney pairs.

Human grafts were perfused with porcine whole blood to increase the likelihood of a pro-coagulopathic reaction.

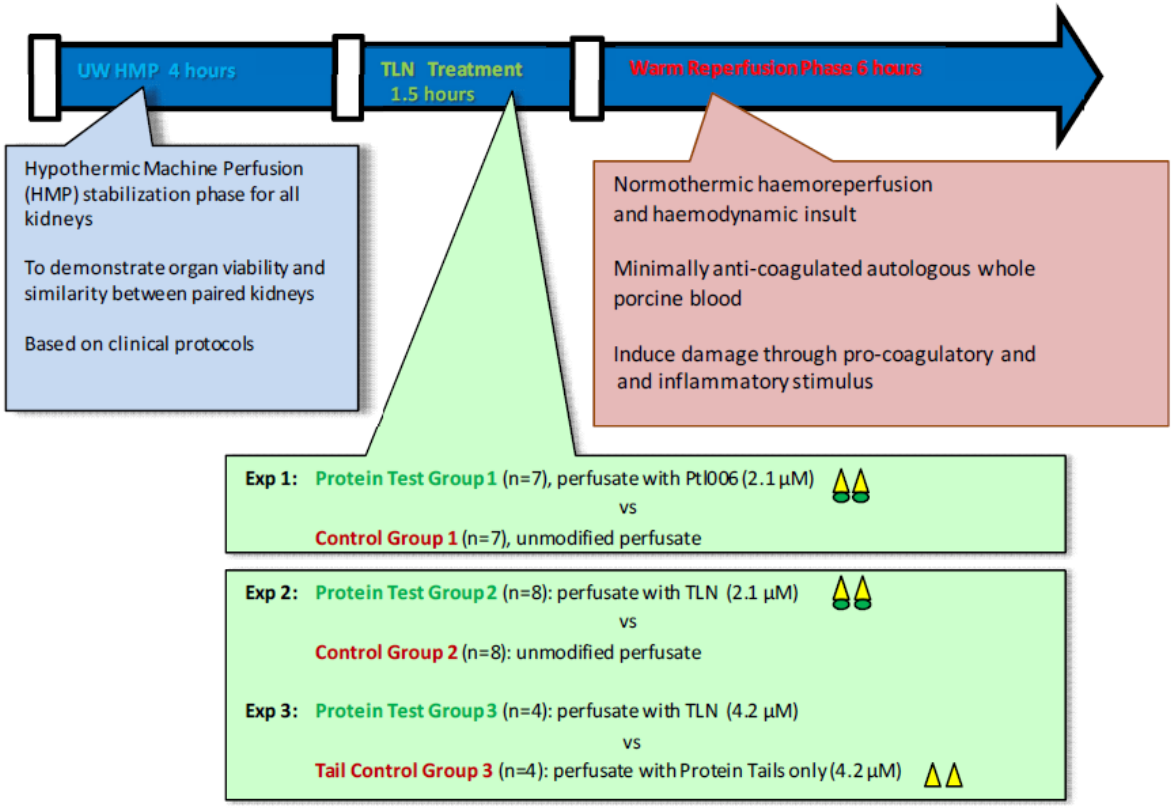


Figure 55 Diagram of TLN testing protocol. Three distinct phases: HMP, TLN treatment and Normothermic reperfusion.

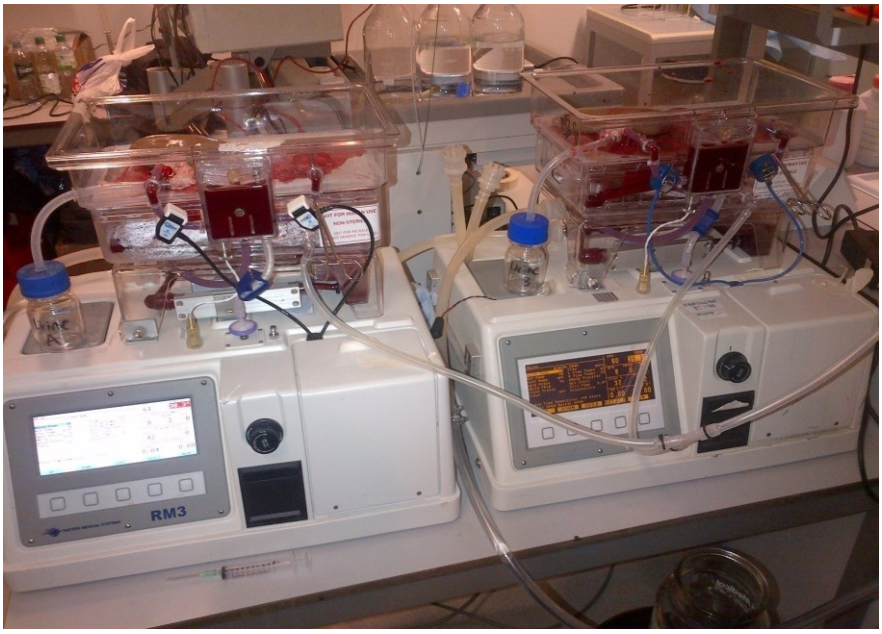


Figure 56 Photograph of normothermic haemoreperfusion reperfusion circuits using modified Waters Medical RM3 perfusion device.

4.2.6 Orthogonal Polarization Spectral Imaging

OPS imaging technology was invented by Cytometrics, Inc. (Philadelphia, PA, USA) while developing a videomicroscope to take high contrast images of blood in the microcirculation using reflected light [1064]. The principle of OPS is based on cross-polarisation of reflected light. As can be seen in Figure 57 light is linearly polarized in one plane by an emitter and projected through a beam splitter onto the target tissue. The majority of the reflected light retains its polarization and thus is unable to pass through to the analyser (orthogonal polarizer) and form the image. However light that penetrates the tissue deeper undergoes multiple scattering events and becomes depolarized. This depolarised 'scattered' light is reflected back which passes through the analyser and back-illuminates the absorbing foreground material. A wavelength of 548nm (green light) is used for the emitted light which is the optimal wavelength for absorption by oxy- and deoxy-hemoglobin in RBCs, enabling blood vessels of the microcirculation to be visualized.

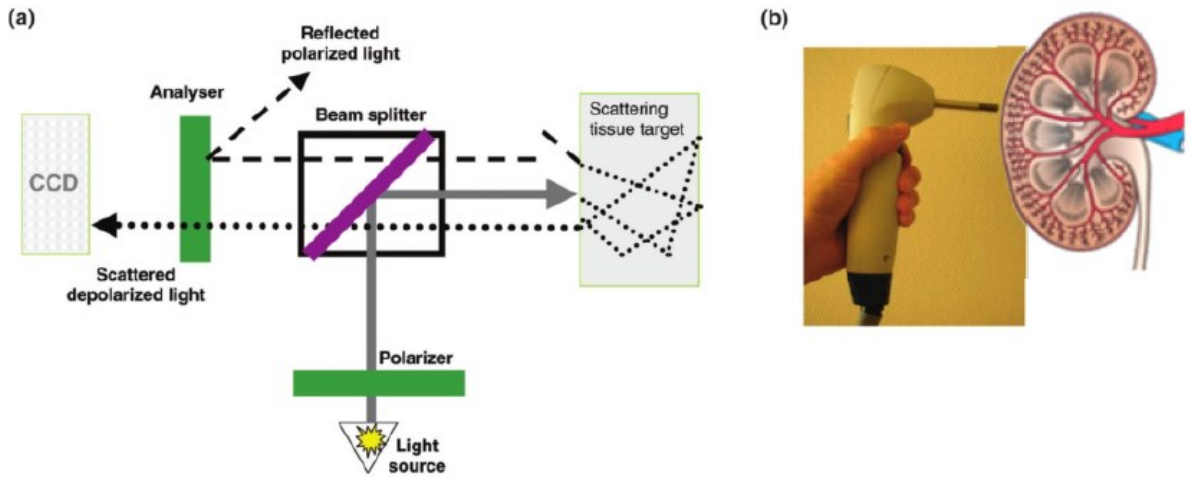


Figure 57 Schematic of the orthogonal polarization spectral imaging apparatus (a) built into a simple hand-held device that can be used to assess the renal microcirculation (b). Adapted from Spronk et al. [1065].

Studies of the human microcirculation using OPS imaging in various pathological states have shown a wide spectrum of different clinical applications with evident impact on the diagnosis, treatment or assessment of prognosis.

OPS has been validated in various pathologies in animal models [1066,575,1067,1068,574] and also partly in humans [1069,1070]. OPS has also been used to investigate changes in microcirculation in clinical transplantation settings specifically the kidney [572], pancreas [573] and liver [576,1071]. Assessment of the microcirculation demonstrated dysfunctional perfusion resembling 'no reflow' with hypoperfusion in kidney, pancreas and liver allografts that experienced delayed function [572,573,1071].

Imaging of the renal microcirculation in this experiment was assessed using a hand held OPS imaging system (Cytoscan TM, Cytometrics Inc., Philadelphia, Pa., USA), whereby the kidney capsule was removed and probed applied to the kidney. Images were recorded for ≥2 areas at 6 hours of NHRP in 4 pairs of kidneys. Linearly polarized light at a wavelength of 548 nm (absorption spectrum of haemoglobin[Hg]) is able to penetrate tissue up to a depth of approximately 200–500 µm [1072]. Erythrocytes (containing Hg) are visualized as dark areas, while the surrounding tissue lacking Hg appears lighter in contrast.

Analysis and quantification of microdynamic parameters was performed offline by frame-to-frame analysis of the recorded images using a computer-assisted image analysis system (CapiScope,

KKTechnology, United Kingdom). Analysis included a quantitative assessment of capillary diameters (D), functional capillary density (FCD) and RBC velocity (RBCv). In each sample diagnostic fields (region of interest) each measuring 782x782 µm (0.611 mm^2) of interest that were isolated and analysed. FCD was defined as the ratio of area of RBC perfused capillaries per area of observation (mm^2), directly reflecting the state of nutritive tissue perfusion[1073]. vRBC (µm/s) was measured in the midpoint of ten capillaries that crossed a designated grid within the ROI by means of frame-to-frame analysis (Modification of De Backer's score)[1074]. Individual volumetric capillary blood flow (CBF; pl/s) was calculated from RBCv and D metrics according to the formula vCBF = ∏(D/2) x vRBC . To evaluate changes in overall capillary perfusion, a perfusion index (PI) was calculated from using the FCD and CBF metrics (PI = FCD x CBF, pl/s/mm^2). The degree of homogeneity (heterogeneity index, HI) in CBF was assessed by expressing the standard deviation (SD) relative to the difference between the maximum and minimum of the CBF (obtained from all observations from 1 experiment at the time point).

4.2.7 Rapid Sampling Microdialysis

RsMD was instituted in these experiments in line with the methodology described in Section 3.2.3 Rapid Sampling Microdialysis. In brief microdialysis probes (MAB11.35.4, Microbiotech, Stockholm, Sweden) were inserted superficially into the parenchyma of the lateral cortex at the start of NRHP. These were connected to an in-house built rapid-sampling microdialysis analyser. Every 30 seconds a 200 nL dialysis sample was automatically injected into the analysis flow stream, alternating between each kidney. The stream accelerates the dialysate through an enzyme reactor containing LOx and 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 Macbook Pro portable computer (Apple Computers, Cupertino, CA). Lactate levels were analysed in real-time to reveal information on interstitial lactate concentration during the 6 hours of reperfusion, as an indication of tissue viability and metabolism.

4.2.8 Clotting Assays

Perfusate samples were taken during the pre-conditioning Treatment and Washout-2 phases. Samples were analysed using a simple clotting assay using citrated plasma and calcium chloride with time to visual formation of fibrin in glass test tubes at normothermic temperatures reflecting relative concentrations of TLN in perfusate samples. Blood perfusate samples were taken at 0 minutes, 20 minutes, 1 hour, 3 hour and 6 hours of NHRP and collected into citrate blood collection tubes. Tubes were centrifuged at 3000rpm for 4 minutes, and the plasma stored at -80oC. Plasma was analysed for levels of fibrin degradation products (INNOVANCE® D-Dimer Siemens Healthcare Diagnostics), fibrinogen and Prothrombin Factor 1+2 (Quadratech Diagnositics Ltd, UK) using commercially available kits.

4.2.9 Histology

Wedge biopsies were taken for histopathological analysis after 6 hours of NHRP and fixed in 10% formaldehyde or snap frozen with liquid nitrogen and stored at -80°C. Tissue blocks were processed in paraffin wax blocks by routine, automated, procedures. Sections were cut at 4 µm and stained with 1) hematoxylin and eosin (H&E) to assess morphology), 2) periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin to assess tubular brush border.

Immuno-histochemistry was conducted on frozen sections. Sections were incubated with primary antibody to fibrin(ogen) (No. 350, American Diagnostica Inc) that reacts with the human fibrin(ogen) Beta -chain only, reacting with fragments X & Y, but not D & E, (therefore with fibrin monomer) for 1 hour at 37°C, washed, and then incubated with a biotinylated secondary antibody for 1 hour at 37°C. After the sections were washed, the streptavidin– horseradish peroxidase conjugate was applied, and the sections were stained with diaminobenzidine. For each run, a negative control was processed by deleting the primary antibody and applying an isotype-specific control antibody (Zymed).

Samples were evaluated for morphology and overall structure using light microscopy. Variables specifically assessed were endothelial detachment, presence of tubular debris/tubular intratubular

cell detachment, tubular dilatation, and peritubular vessel congestion. Slides were blinded for the assessor and variables scored using a scale of 0 - 4 according to the degree of changes seen in each field of view at 10x magnification, where: 0 denoted normal cells with normal no abnormalities; 1: changes affecting 25% of the sample; 2: changes affecting 25-50%; 3: changes affecting 50-25%; and 4: changes affecting >75% of the sample. Peritubular congestion was assessed in slides from paired kidneys separately and comparatively ranked with slide demonstrating more congestion graded 1, the other 0. Three fields of view were analysed in each slide for each of these changes.

4.2.10 TLN Adherence

Two porcine kidneys underwent the complete experimental protocol but were treated with a TLN-tail derivatised at the N-terminus with a FAM fluorescent derivative (FAM-PTL) to histologically demonstrate tethering and adherence to the porcine kidney microvasculature. One human graft underwent the HMP and Pre-conditioning phases of the experimental protocol, and was treated with FAM-PTL to demonstrate similar tethering and microvasculature localisation in a human graft.

4.2.11 Statistics

Values are represented as mean and standard deviation. Continuous variables were plotted as levels versus time curves for each kidney and the mean area under the curve calculated. Continuous variables from paired contra-lateral kidneys were evaluated using the paired students T-test or non-parametric Wilcoxon signed rank test as appropriate. Repeated measures were analysed for differences using ANOVA for repeated with measures with Bonferroni correction for post-hoc tests as required. Data and statistical analysis was performed using Microsoft® Excel Software (Reading, UK). A p value of ≤ 0.05 was considered significant.

4.3 Results

4.3.1 Kidney Characteristics

A total of thirty-eight porcine kidneys and two human kidney grafts were utilised in this project. Porcine kidneys experienced a retrieval WIT of 15 minutes (Range: 10-20), CIT until perfusion of 298 minutes (Range: 219-480), and had a mean graft weight of 233g (Range:107-330). Table 6 summarizes porcine kidney characteristics in the experimental groups.

Kidney Characteristics	Warm Ischaemia Time (minutes)		Cold Ischaemia Time Before HMP (minutes)		Weight Pre-HMP (g)	
Overall for All Kidneys (n=36)	15 (10-20)		298 (219-480)		233 (107-330)	
Experiment 1	CG1	PTG1	CG1	PTG1	CG1	PTG1
	13.6 (2.4)	12.8 (2.6)	345 (80.6)	348 (80.9)	175.1 (229-116)	185.7 (258-107)
Experiment 2	CG2	PTG2	CG2	PTG2	CG2	PTG2
	16 (2)	16 (2)	334 (76)	334 (76)	253 (188-330)	237 (197-315)
Experiment 3	TCG3	PTG3	TCG3	PTG3	TCG3	PTG3
	15.5 (0.6)	15.5 (0.6)	247 (16)	247 (16)	257 (249-295)	251 (240-290)

Table 6. Porcine kidney characteristics.

4.3.2 Hypothermic Machine Perfusion Stabilization

Perfusion characteristics and weight gain in kidneys during initial HMP stabilization were not significantly different between the Protein Treated Groups (PTG1,PTG2,PTG3) and the Controls Groups (CG1, CG2, TCG3), and are summarised in Table 7 and Table 8.

HMP Stabilization Perfusion Dynamics	Systolic Pressure (mmHg)		Perfusate Flow Rate (ml/min/100g)		Perfusion Flow Index (ml/min/100g/mmHg)	
Experiment 1	CG1	PTG1	CG1	PTG1	CG1	PTG1
	46.2 ± 9.4	43.5 ± 6.3	21.4 ± 6.7	23.4 ± 8.2	0.55 ± 0.22	0.52 ± 0.17
Experiment 2	CG2	PTG2	CG2	PTG2	CG2	PTG2
	38.7 ± 3.8	38.3 ± 3.8	16.2 ± 6.2	16 ± 5.8	0.42 ± 0.08	0.39 ± 0.12
Experiment 3	TCG3	PTG3	TCG3	PTG3	TCG3	PTG3
	36.8 ± 2.7	36.8 ± 1	12.7 ± 3.8	13.6 ± 4.2	0.38 ± 0.08	0.37 ± 0.13

Table 7. HMP perfusion dynamics in Treatment and Control Groups in Experiments 1-3

	Percent Weight Change Post HMP (%)		Percent Weight Change Post NHRP (%)	
Experiment 1	CG1	PTG1	CG1	PTG1
	42.5 ± 12.6	42.8 ± 12.9	10.9 ± 7.8	11.9 ± 9.4
Experiment 2	CG2	PTG2	CG2	PTG2
	20.6 ± 7.8	21.8 ± 7.2	9.8 ± 7.9	14.2 ± 14.8
Experiment 3	TCG3	PTG3	TCG3	PTG3
	14.8 ± 5.5	14 ± 5.5	19 ± 10	27.8 ± 4.4

Table 8. Percent weight change during HMP and NHRP

4.3.3 TLN Delivery and Adherence

4.3.3.1 Perfusate Analysis

Perfusate samples taken during organ preconditioning from TLN treated kidneys (PTG1, PTG2 and PTG3 Groups) were assessed via a clotting assay. Results are expressed as a multiple of the clotting time for a control sample of unmodified perfusate, and are shown in Figure 58. A higher clotting ratio indicates a greater concentration of TLN present in the sample. Experiment 1 demonstrated a significant decrease in the clotting time ratio between 0 minutes of TLN treatment (2.19 ± 0.17) and 15 minutes (1.89 ± 0.12, p=0.001) which remained stable at the end of treatment (1.92 ±0.11). Furthermore clotting times did not significantly increase between the start and end of the washout phases (1.15 ± 0.04 vs. 1.26 ± 0.1). Experiment 2 and 3 also demonstrated a similar trend with decreasing clotting time ratios during TLN treatment, but with these differences not reach

significance. In Experiment 2 there was also significant increase in clotting time ratios between the start and end of the washout phase, with clotting time ratios in the washout samples were significantly lower than those at 30 minutes of TLN treatment. While in Experiment 3 clotting time ratios did not increase during the washout phase, and remained stable.

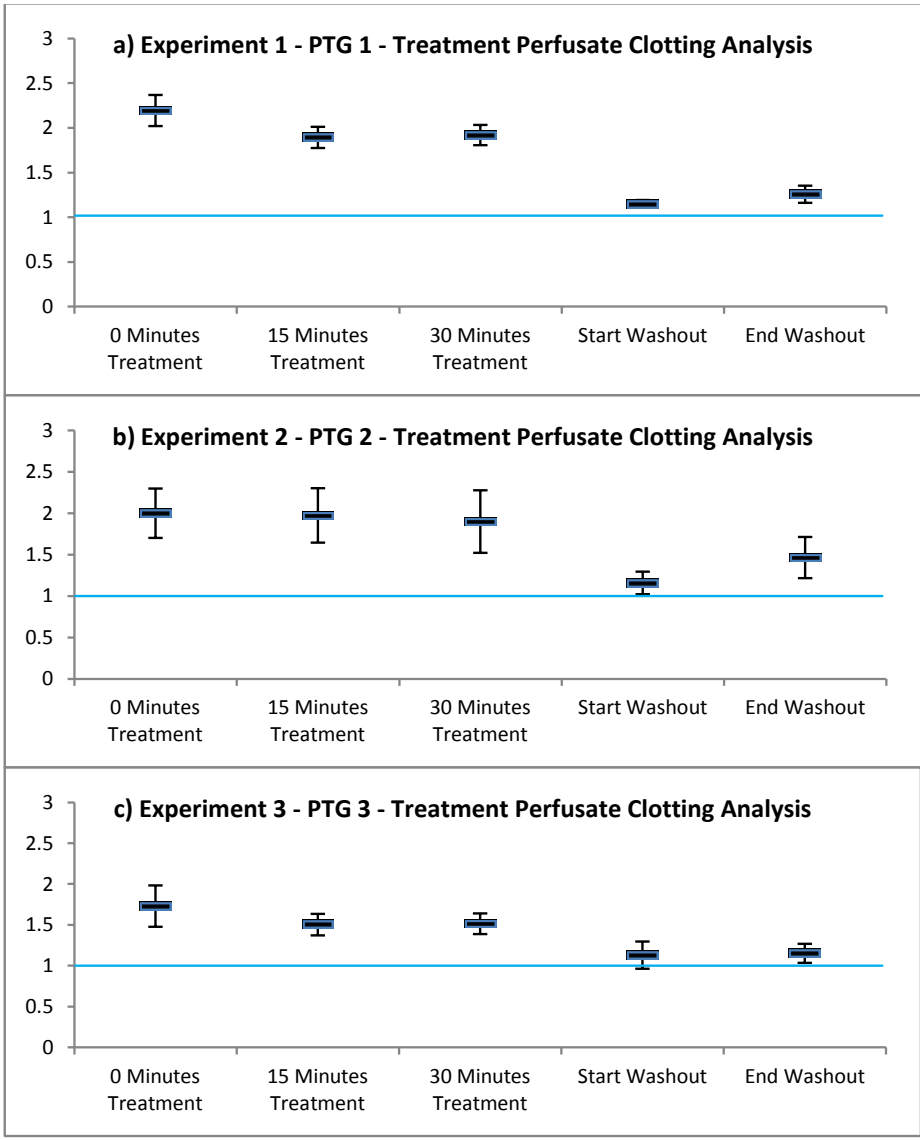


Figure 58 Perfusate Clotting Analysis. Clotting times are expressed as multiple of the clotting time for a control sample of unaltered perfusate (represented by the blue line). The larger the value the greater the concentration of TLN. present. Experiment 1: ANOVA p=0.001, 0 mins (2.19 ± 0.17) vs. 15 mins (1.89 ± 0.12) p=0.001, 30mins (1.92 ±0.11) vs. start washout (1.15 ± 0.04)p<0.001, start vs. end of washout (1.26 ± 0.1) NS, 30mins vs. end of washout p<0.001. Experiment 2: ANOVA p=0.001, Clotting time ratios trended lower during treatment: 0 mins (2.0 ± 0.09), 15 mins (1.97 ± 0.11), 30mins (1.89 ±0.14), p>0.05 for all treatment comparison. Start of washout (1.15 ± 0.02) vs. end of washout (1.46 ± 0.06) p=0.001 and 30mins vs. both start of washout and end of washout p=0.001, p=0.002 respectively. Experiment 3: ANOVA p=0.004, Clotting time ratios trended lower during treatment: 0 mins (1.72 ± 0.06), 15 mins (1.5 ± 0.02), 30mins (1.51 ±0.02), p>0.05 for all treatment comparisons. Start of washout (1.12 ± 0.03) vs. end of washout (1.15 ± 0.01) p=0.7 and 30mins vs. both start of washout and end of washout p=0.06, p=0.08 respectively.

4.2.3.2 Fluorescent Labelled PTL

Treatment with FAM-PTL histologically demonstrated tethering and adherence to the porcine kidney microvasculature after treatment, with continued adherence after 6 hours of NHRP challenge (Figure 59).

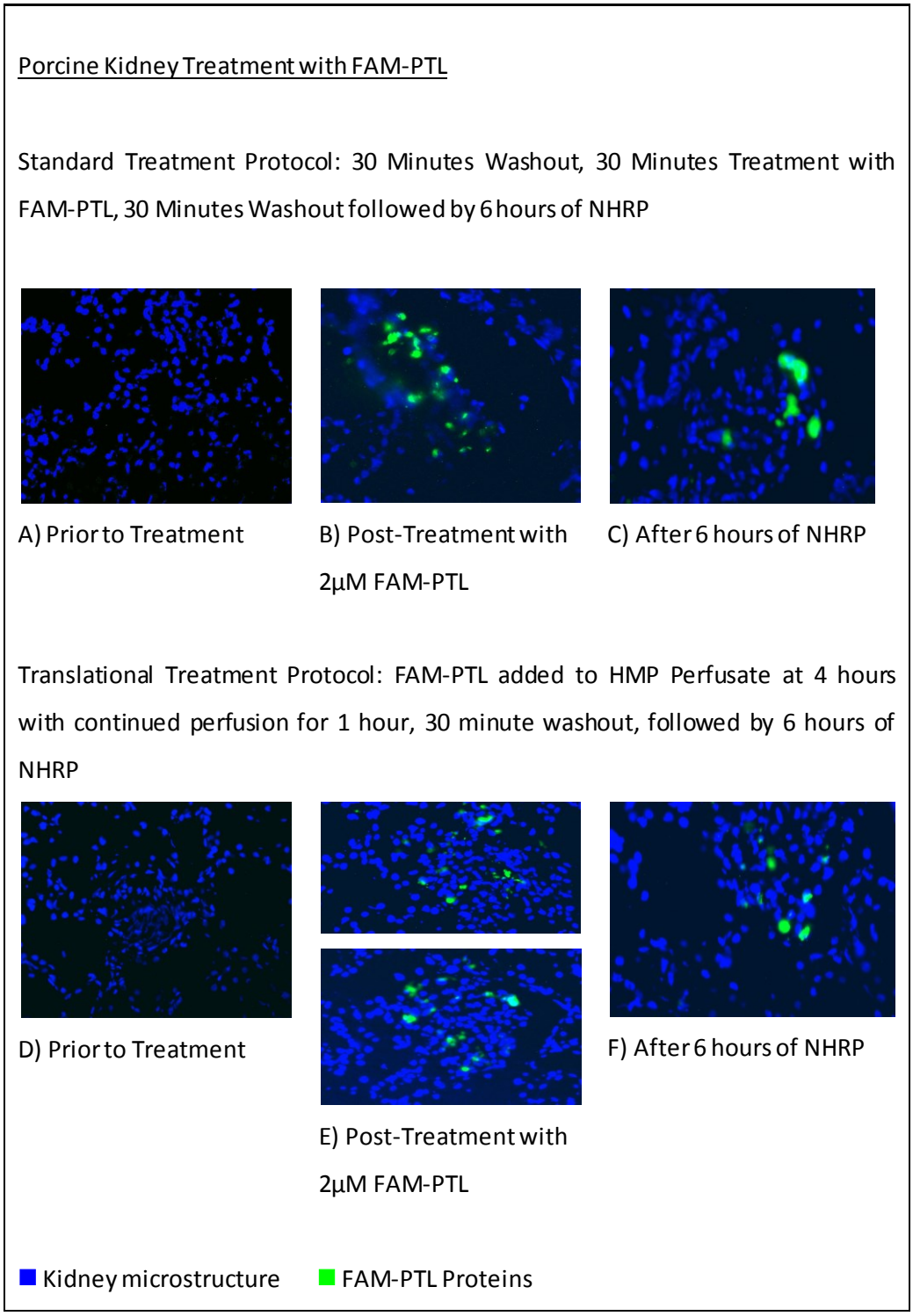


Figure 59 Histology of biopsies under UV light taken from a porcine kidney during experimental treatment with fluorescent labelled (FAM-PTL) tail molecules. Prior to treatment (A); after

treatment demonstrating presence of the proteins adherent to the kidney microvasculature (B); and with continued adherence after 6 hours of normothermic reperfusion with blood (C). A 2nd porcine kidney underwent a slightly modified treatment protocol that was designed to as clinically translational as possible, where by the proteins are simply added to the perfusate during HMP, rather than requiring removal of the kidney and perfusion with ' protein spiked' solution. No proteins were present adherent to the microvasculature prior to injection of the FAM-PTL into circulating HMP perfusate (D); with FAM-PTL present 1 hour after addition (E), and also after subsequent 6 hours of normothermic reperfusion (F).

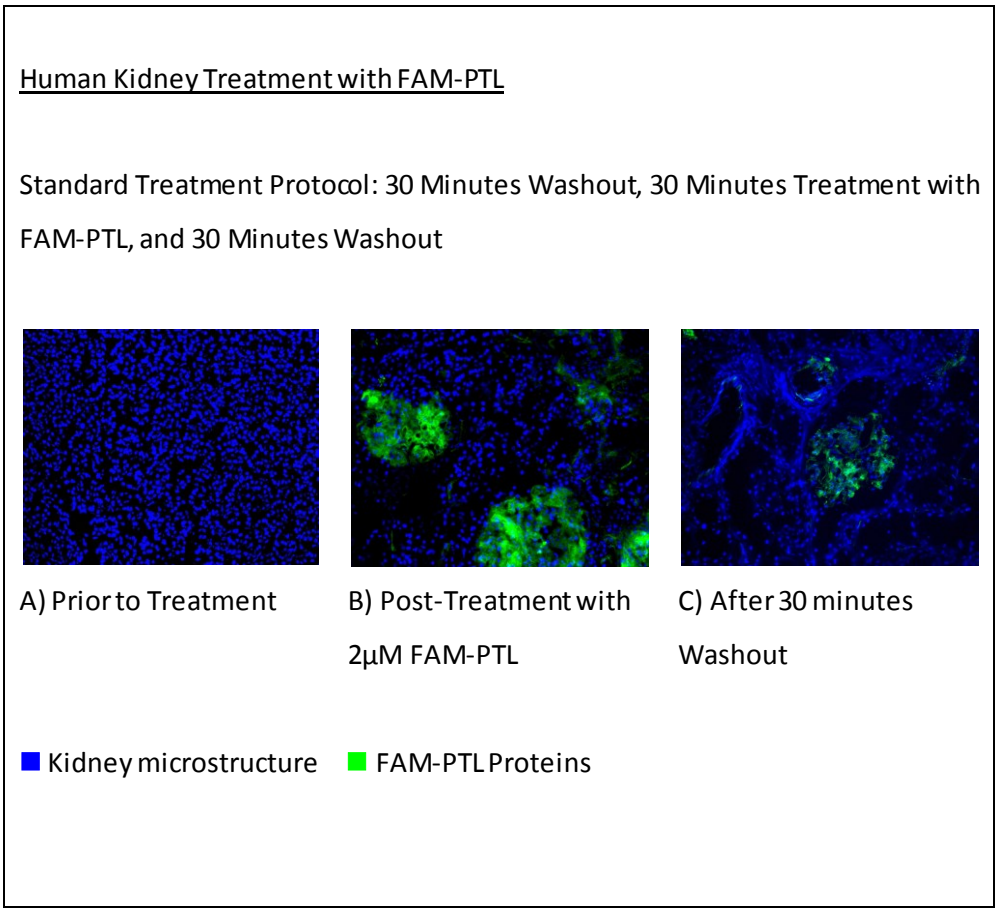


Figure 60 Histology of biopsies under UV light taken from a human kidney as part of experiment 5. Prior to treatment with fluorescent labelled (FAM-PTL) tail molecules (A); after treatment with FAM-PTL demonstrating presence of the proteins adherent to the kidney microvasculature (B); and with continued adherence after the further 30 minutes of Washout-2 perfusion (C).

4.3.4 Normothermic Haemoreperfusion

4.3.4.1 Vascular Perfusion Dynamics

Protein Treated kidney groups demonstrated superior blood flow and PFI compared to the Control Group kidneys (Figure 61 for a summary of NHRP perfusion data). Renal blood flow rates were on average 26.4%, 33.7%, and 45% higher, and PFIs 28.9%, 32.1%, and 44.4% greater in protein treated PTG1, PTG2 and PTG3 kidneys compared to controls. Weight gain was not different between Treated and Non-treated groups (Table 8). Systolic perfusion pressures were similar between groups in each experiment during NHRP: CG1: 70.7 ± 17.9 mmHg vs. PTG1: 71.7 ± 9.2 mmHg; CG2: 48 ± 18.5 mmHg vs. PTG2: 49.5 ± 18.27 mmHg; TCG3: 50.5 ± 3.5 mmHg vs. PTG3: 49.5 ± 0.63 mmHg; p=0.349, p=0.316, p=0.777 respectively.

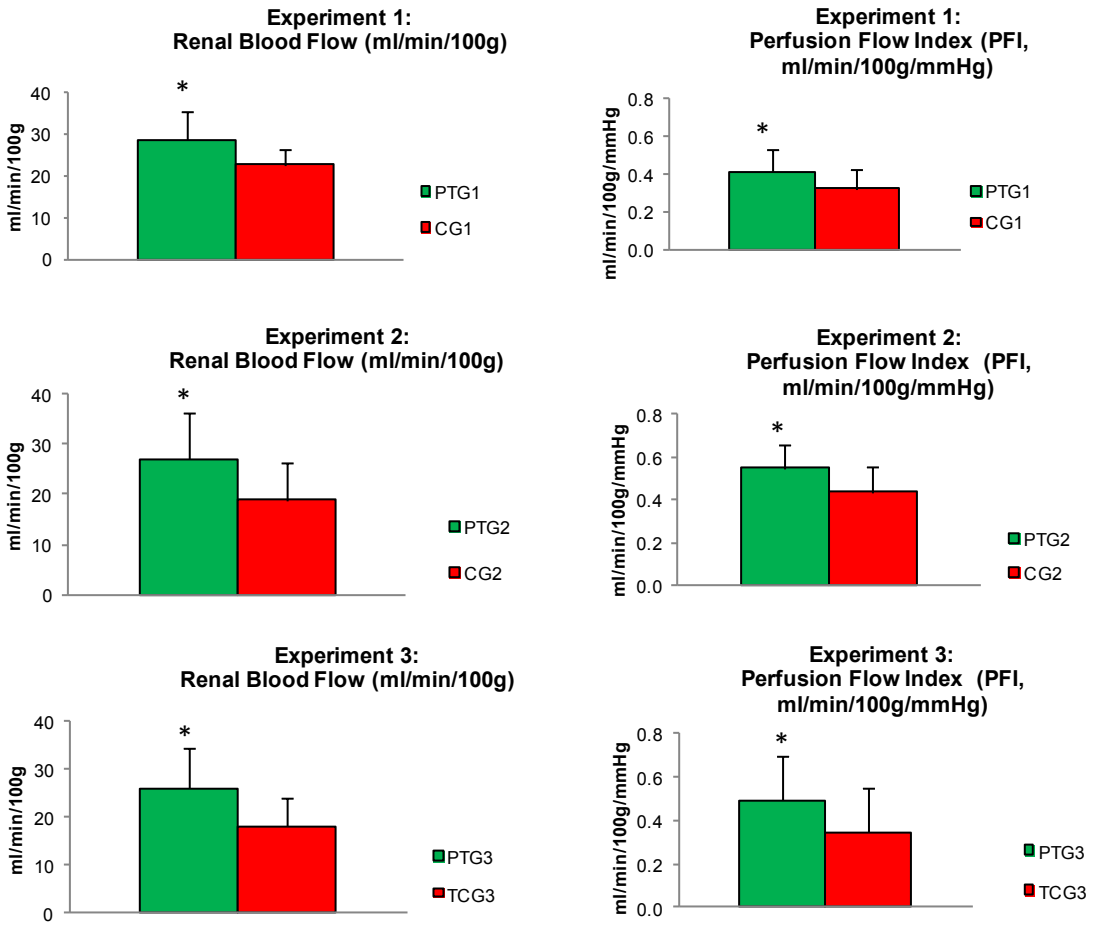


Figure 61. NHRP renal perfusion dynamics in experiments 1-3. Protein Test Group kidneys demonstrated higher renal blood flow rates (PTG1: 30.6 ± 6.8, PTG2: 26.7 ± 9.9, PTG3: 25.9 ± 8.6 ml/min/100g) than control kidneys (CG1: 23.1 ± 3.7, CG2: 20.4 ± 7.7, TCG3: 17.9 ± 6.2 ml/min/100g; p=0.02, p=0.022, p=0.009 respectively), as well as higher perfusion flow indices

PTG1: 0.43 ± 0.12, PTG2: 0.53 ± 0.23, PTG3: 0.49 ± 0.16 ml/min/100g/mmHg) vs. control kidneys (CG1: 0.32 ± 0.1, CG2: 0.43 ± 0.23,TCG3: 0.34 ± 0.12 ml/min/100g/mmHg; p=0.0494, p=0.031, p=0.009 respectively) during six hours of NHRP challenge. Two-tailed paired t-test. *=significant difference vs. corresponding control group.

4.3.4.2 Orthogonal Polarisation Spectral Imaging

In Experiment 2 OPS data was recorded and analysed for 4 pairs of kidneys after 6 hours NHRP to assess the microdynamics of the kidney microvasculature (Figure 62). FCD was similar between treated and non treated kidneys however TLN treated kidneys had 44% larger diameter capillaries, 50% faster RBCv, 3.5 times greater CBFs and PIs compared to the non-treated control kidneys (Figure 63).

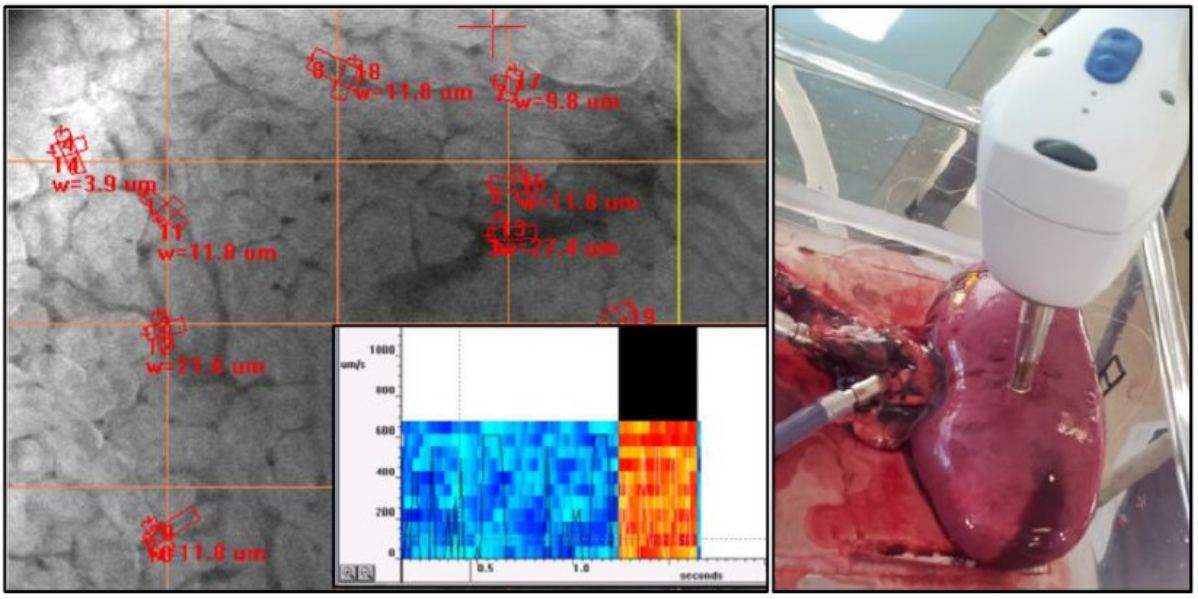


Figure 62 OPS Imaging: Example of post-capture frame analysis (LEFT) and a kidney undergoing OPS assessment (RIGHT).

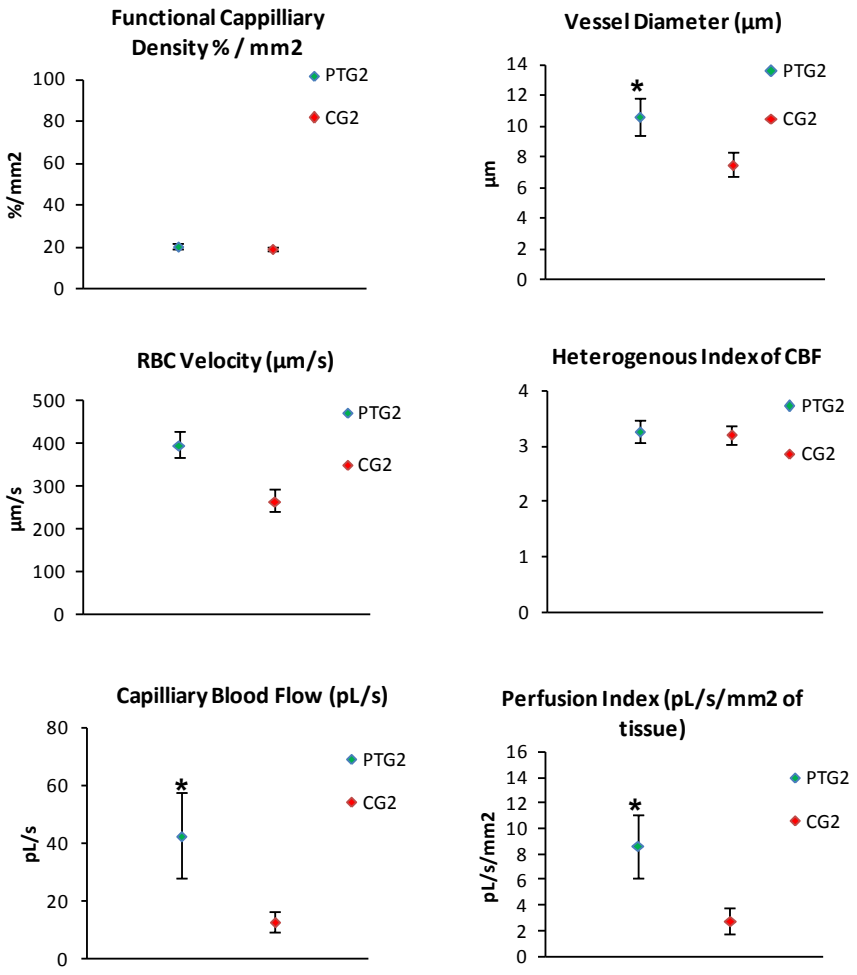


Figure 63. OPS microcirculatory perfusion dynamics in kidneys at 6 hours of NHRP. Functional capillary density (FCD) was similar between treated and non treated kidneys (CG2: 19.3 ± 3.1 % vs. PTG2: 20.5 ± 2.0 %, p=0.4). TLN treated kidneys recorded vessel diameters were larger (CG2: 7.44 ± 0.81 µM vs. PTG2: 10.77 ± 1.23 µM, p=0.052), and RBC velocity (CG2: 272.1 ± 26.3 µM/s vs. PTG2: 403.2 ± 31.2 µM/s, p=0.011), calculated CBF (CG2: 13.2 ± 3.65 µM3/s vs. PTG2: 41.9 ± 14.5 µM3/s, p=0.032), and the PI (CG2: 2.77 ± 0.98 pl/s/mm^2 vs. PTG2: 9.05 ± 2.48 pl/s/mm^2, p=0.021) higher compared to control kidneys. The HI calculation revealed similar levels of homogenous perfusion within the areas sampled within each kidney (CG2: 3.21 ± 0.18 AU vs. PTG2: 3.27 ± 0.21 AU, p=0.79). Two-tailed paired t-test. *=significant difference vs. control group.

4.3.4.3 Rapid Sampling Microdialysis

Data of cortical micro-dialysis levels were recorded for four pairs of kidneys during Experiment 2 during the HMP stabilization phase, and NHRP. Data from two pairs during HMP and one during NRHP were excluded due to background signal interference in two, and technical problems in one,

during recording that prohibited accurate analysis. During HMP cortical microdialysis lactate concentrations were similar between the non-treated control and treated kidneys (CG2: 0.128 ± 0.006 mM/min vs. PTG2: 0.137 ± 0.04 mM/min, p=0.78). In NHRP cortical lactate levels were higher in non-treated vs. treated kidneys (CG2: 3.28 ± 1.04 mM/min vs. PTG2: 1.67 ± 0.28 mM/min, p=0.072), with the difference approaching significance (Figure 64).

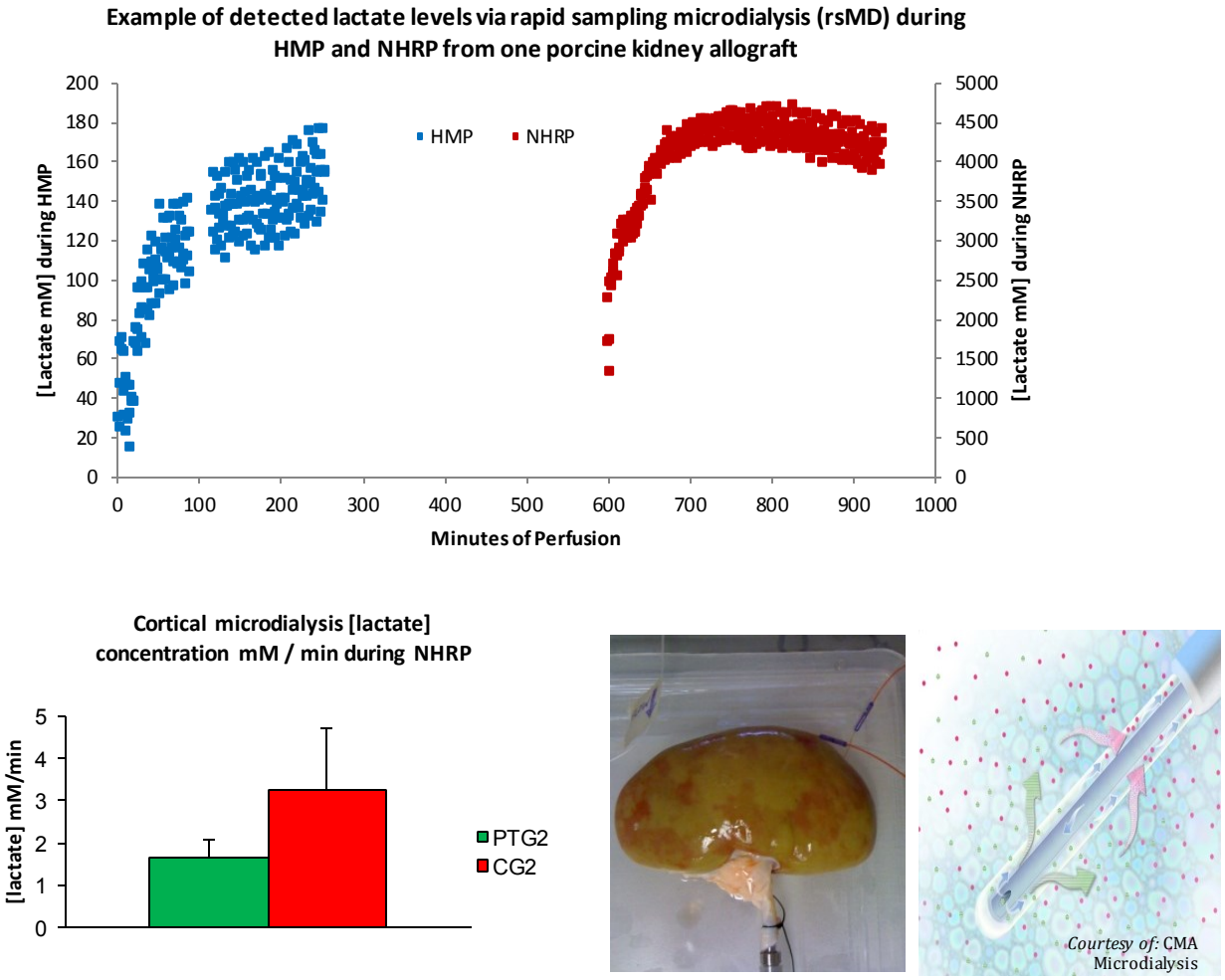


Figure 64 rsMD: Scatter diagram of rsMD readings measured from one kidney during HMP and NHRP (Top). NHRP cortical lactate concentrations trended higher in non-treated vs. treated kidneys during (CG2: 3.28 ± 1.04 mM/min vs. PTG2: 1.67 ± 0.28 mM/min, p=0.072, bottom left). Photograph of kidney undergoing microdialysis monitoring by insertion of probes into cortex and diagram of microdialysis catheter tip (bottom right).

4.3.4.4 Haemoreperfusion Clotting Analysis

Clotting data is summarised in Table 9. Higher levels of D-Dimers (average AUC) were observed over the 6 hours of perfusion in the Experiment 1 control group, CG1(14.5 ± 7.9 mg/L) vs. the treated kidneys, PTG1 (9.5 ± 5.1 mg/L, p=0.051) with this difference at significance. No other differences were observed in clotting parameters between treated and non-treated kidneys in the Experiments 2 and 3.

NHRP Perfusate Clotting Analysis	D-dimers (mg/L)		Fibrinogen (µg/L)		Prothrombin Fragment 1+2 (mg/L)	
Experiment 1	CG1*	PTG1	CG1	PTG1	CG1	PTG1
	14.5 ± 7.9	9.5 ± 5.1	0.385 ± 0.06	0.373 ± 0.21	0.006 ± 0.003	0.007 ± 0.002
Experiment 2	CG2	PTG2	CG2	PTG2	CG2	PTG2
	5.63 ± 2.2	11.2 ± 11.06	1.08 ± 0.19	1.2 ± 0.24	0.005 ± 0.001	0.009 ± 0.002
Experiment 3	TCG3	PTG3	TCG3	PTG3	TCG3	PTG3
	6.18 ± 9.6	6.4 ± 7	0.78 ± 0.07	0.79 ± 0.33	0.002 ± 0.001	0.001 ± 0.002

Table 9. NHRP blood perfusate clotting analysis for Experiments 1-3. * Higher levels of D-dimers were observed in control CG1 (14.5 ± 7.9 mg/L) vs. the treated kidneys, PTG1 (9.5 ± 5.1 mg/L, p=0.051). Two-tailed paired t-test.

4.3.4.5 Histology

Light microscopy histological assessment of biopsy samples between Control (CG1-2, TCG3) and Treatment (PTG1-3) Groups showed no significant differences in endothelial detachment, tubular debris/epithelial detachment, tubular Dilatation, and peritubular congestion, all p-values > 0.5 (Table 10, Figure 65).

	Endothelial detachment		Tubular Debris		Tubular Dilatation		Peritubular Congestion	
Experiment 1	CG1	PTG1	CG1	PTG1	CG1	PTG1	CG1	PTG1
	1.4 ±0.24	1.27 ±0.19	4 ± 0	4 ± 0	4 ± 0	4 ± 0	0.6 ± 0.24	0.4 ± 0.24
Experiment 2	CG2	PTG2	CG2	PTG2	CG2	PTG2	CG2	PTG2
	1.08 ±0.08	1.08 ±0.08	3.5 ± 0.5	3.5 ± 0.5	3.5 ± 0.5	3.5 ± 0.5	0.6 ± 0.24	0.5 ± 0.29
Experiment 3	TCG3	PTG3	TCG3	PTG3	TCG3	PTG3	TCG3	PTG3
	1.13 ±0.13	1 ± 0	3 ± 0.58	3 ± 0.58	3.25 ± 0.25	3 ± 0.24	0.5 ± 0.29	0.5 ± 0.29

Table 10. Histology scores of biopsies taken after 6 hours of NHRP assessing endothelial detachment, tubular debris/epithelial detachment, tubular Dilatation, and peritubular congestion. No significant differences were seen between treated and non-treated kidneys, with similar degrees of reperfusion induced vascular and tubular damage and micro-vascular congestion. Two-tailed Wilcoxon signed rank test.

Immuno-histochemistry for fibrin(ogen) was conducted in biopsies from kidneys in CG1 and PTG1 groups and demonstrated a generalised fibrin(ogen) deposition in interstitial tissues and peritubular capillaries. There was a generalised deposition of fibrin in all biopsies examined with no difference between treated and non-treated kidney grafts (see Figure 66).

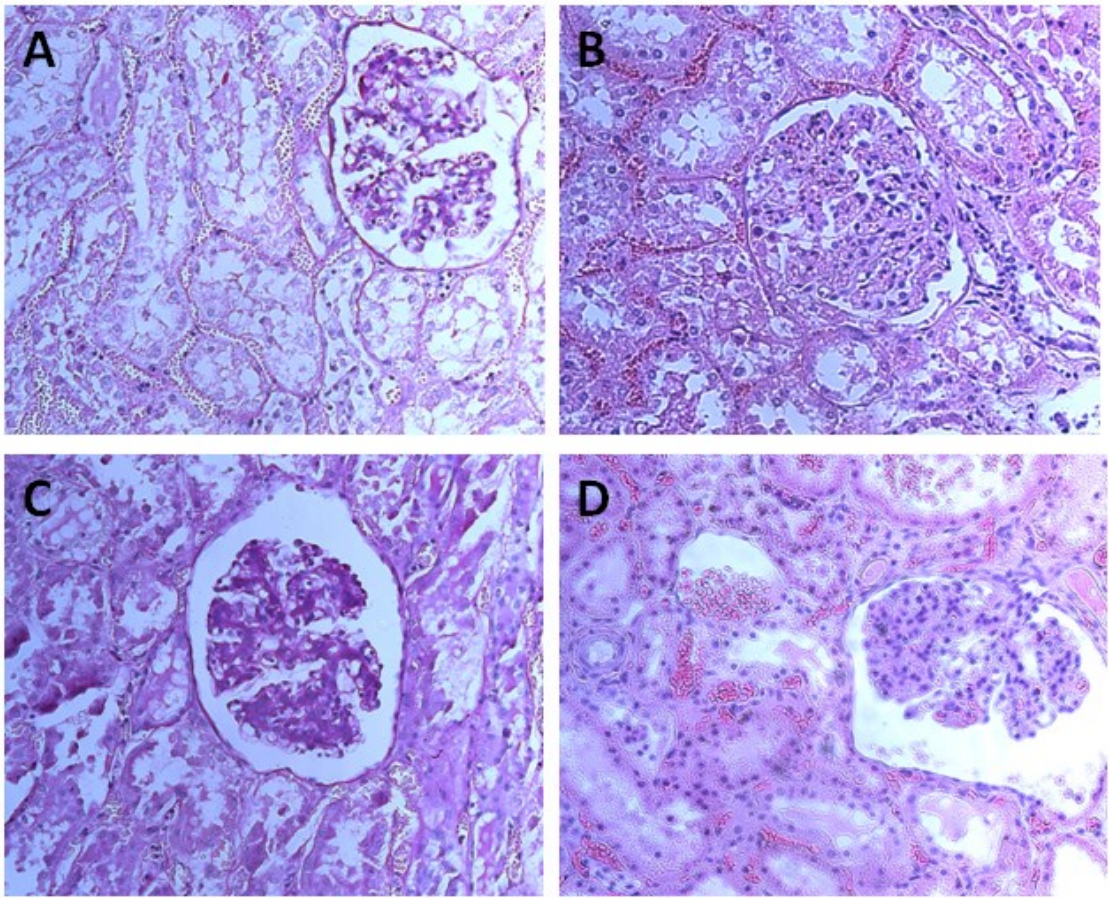


Figure 65 Photomicrographs of light microscopy of Protein Treated vs. Control kidneys after 6 hours of NHRP. H&E staining, x20. A: PTG1 kidney, B: CG1 kidney, C: PTG2 kidney, D: CG2 kidney. Showing similar degrees of tubular debris, dilatation, and peritubular congestion.

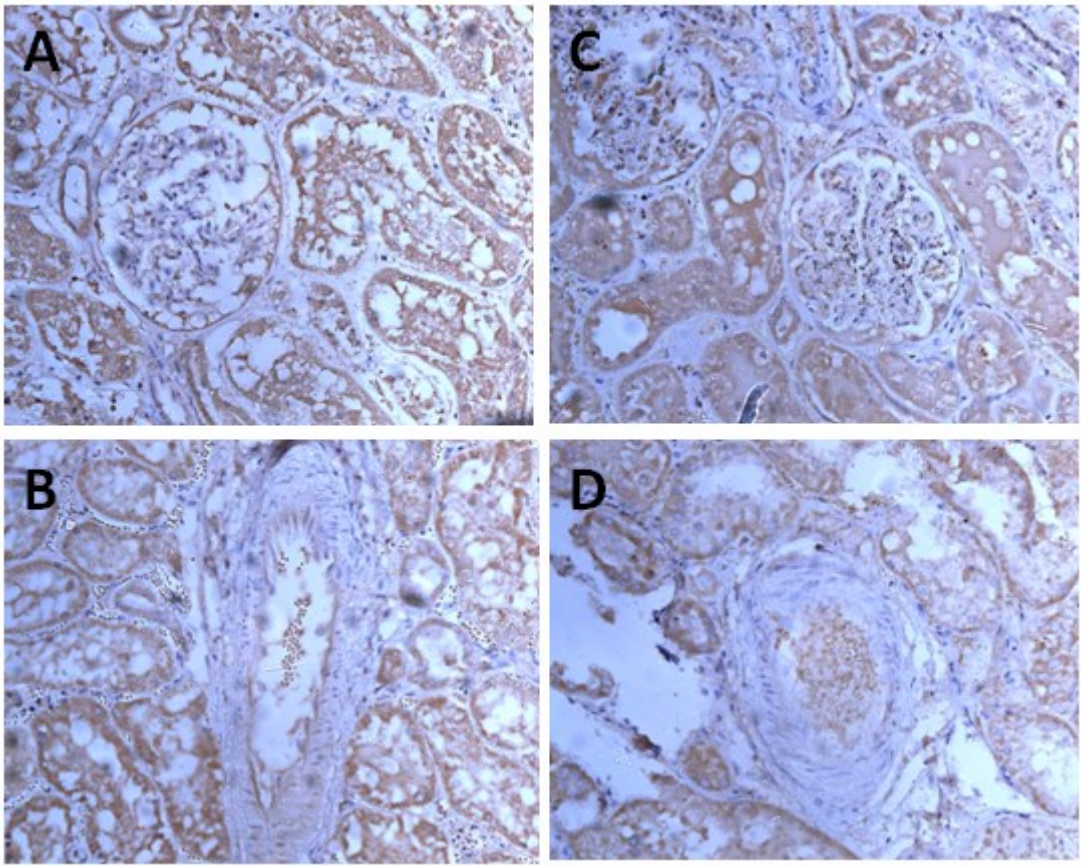


Figure 66 Immuno-histochemistry for fibrin(ogen) in Protein Treated and Control kidneys after 6 hours of NHRP. A and B: Protein treated (PTG1) kidney, C and D: Control (CG1) kidney. There is generalised intravascular and interstitial fibrin deposition to a similar degree in both groups of kidneys.

4.3.4.6 Pre-clinical Experimentation with Human Kidney Grafts

A pair of DCD kidney grafts were used in a pre-clinical model in Experiment 4. CIT was 78 hours (4680 minutes) until start of the experiment. Table 11 summarizes kidney characteristics, perfusion dynamics, perfusate clotting results and microdialysis cortical lactate levels in these kidneys. During HMP stabilization perfusion characteristics were superior in the CG4 control kidney vs. the PTG4 kidney.

Kidney Characteristics	Warm Ischaemia Time (minutes)		Cold Ischaemia Time Before HMP (minutes)		Weight Pre-HMP (g)	
Experiment 4	CG4	PTG4	CG4	PTG4	CG4	PTG4
	NA	NA	78 hours	78 hours	210	230
HMP Stabilization Perfusion Dynamics	Systolic Pressure (mmHg)		Flow Rate (ml/min/100g)		Perfusion Flow Index (ml/min/100g/mmHg)	
Experiment 4	CG4	PTG4	CG4	PTG4	CG4	PTG4
	37.8	49.5	18.2	10.2	0.48	0.2
Weight Changes	Percent Weight Change Post HMP (%)		Percent Weight Change Post NHRP (%)			
Experiment 4	CG4	PTG4	CG4	PTG4		
	14.3	15.6	1.2	-0.8		
RSMD [Lactate]	HMP [Lactate] mM		NHRP [Lactate] mM			
Experiment 4	CG4	PTG4	CG4	PTG4		
	0.17	0.28	6.997	4.442		
Perfusate Analysis	Ddimers (mg/L)		Fibrinogen (µg/L)		Pro-thrombin Fragment 1+2 (mg/L)	
Experiment 4	CG4	PTG4	CG4	PTG4	CG4	PTG4
	4.74	1.89	3.7	1.2	NA	NA

Table 11. Human kidney graft characteristics, HMP dynamics, weight changes, rsMD levels during NHRP, and NHRP blood perfusate clotting data.

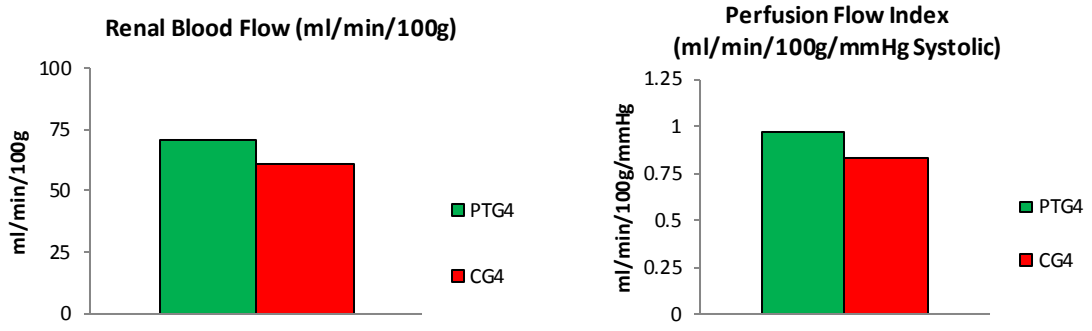


Figure 67 NHRP perfusion dynamics in a treated and non-treated human kidney pair. Renal blood flow: PTG4: 70.8 ml/min/100g vs. CG4: 60.7 ml/min/100g, PFI: PTG4: 0.97 ml/min/100g/mmHg vs. CG4: 0.83 ml/min/100g/mmHg.

Despite initially more viable perfusion dynamics HMP in the CG4 control kidney during the NHRP the opposite was observed. Systolic perfusion pressures were similar (CG4: 73.2 mmHg vs. PTG4: 72.9 mmHg) but TLN treated kidneys demonstrated superior perfusion with 16.6% higher renal blood flow and 16.8% higher perfusion indices as seen in Figure 67. Micro-dialysis lactate concentrations were lower in the control kidney during HMP prior to treatment but were subsequently 36% lower in the treated PTG4 kidney during NRHP. D-Dimer and fibrinogen levels were 2.5-3x higher over the 6 hours of perfusion in the control CG4 group. There was insufficient sample for PF1.2 testing. Percent weight change was not different after HMP stabilization nor after the NHRP challenge between the non-treated and treated kidney. Light microscopy demonstrated a similar degree of reperfusion injury in the treated and non-treated graft (see Figure 68). A human DCD kidney was also treated with FAM-PTL. FAM-PTL was observed in biopsies after the 30-minutes of treatment, after then still present after the washout phase (Figure 60).

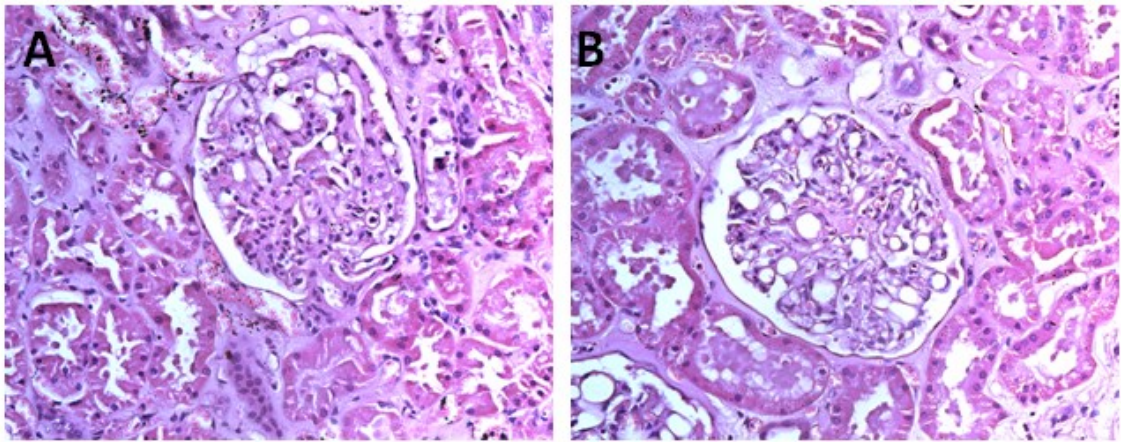


Figure 68 Photomicrographs of light microscopy of Protein Treated vs. Control human kidneys after 6 hours of NHRP. H&E staining, x20. A: PTG4 kidney, B: CG4 kidney. Showing similar degrees of tubular debris, dilatation, and peritubular congestion.

4.4 Discussion

ECD and DCD donor kidneys are more susceptible to microcirculatory failure during reperfusion, with increased risk of subsequent DGF. The increasing use of these organs to expand the donor pool means any approach that could ameliorate micro-vascular dysfunction and improve organ perfusion at the reperfusion stage is in high clinical demand.

Thrombalexin and its variants are a novel series of membrane localising 'cytotopic' anti-coagulant constructs that have proven efficacy in improving graft function in murine kidney transplantation models[940]. This is the first study of preconditioning large animal organs with addressive cytotopic anti-coagulant agents using HMP as a delivery vehicle, and further assessing the benefits of this pre-treatment using a NHRP challenge. Using a series of translational porcine ex-vivo renal perfusion and haemo-reperfusion models we demonstrated HMP can be used to deliver TLN into the vasculature of a kidney graft, and facilitates cytotopic tethering and adherence; improving macro and micro-vascular organ tissue perfusion, with biochemical evidence of less thrombophilia during NHRP challenge. These significant advances in pre-conditioning the graft, in particular the microvasculature endothelium, are derived from the TLN cytotopic agent, that localises to endothelial cells and presents a Hirulog based direct thrombin inhibitor moiety within the intravascular space, conferring local thrombin inhibition and both anti-coagulant, and potentially anti-inflammatory effects. Inhibition of thrombin reduces fibrin(ogen) activation and cross linked fibrin generation, and in parallel decreased inflammatory responses mediated by PAR receptor activation.

IR injury sustained during organ transplantation is a complex interplay between endogenous coagulative and inflammatory processes, with endothelial and tissue damage characterised by microcirculatory failure and the 'no re-flow' phenomenon [348,1075]. The clinical effect of this injury is DGF, which increases the risk of later immunologic rejection [1076]. Improving microvasculature perfusion improves organ function.

With the increasing use of ECD and DCD organs to address the growing need of patients on transplant waiting lists, the impact of IR injury is seen by the higher rates of DGF in these more susceptible organs and reluctance of their use by surgeons [1077,1078]. However use of the DCD/ECD cohort could expand the donor pool by 40% [11]. Interventions that can improve organ viability, and improve function in the perioperative period during which IR injury is most dominant are the key to better utilization of these donor organs [12].

HMP is recommended for use in ECD and DCD donor allograft preservation and clinically such preserved organs encounter less DGF after transplantation [891,946,1079]. HMP is also used as a tool for allograft viability assessment using a variety of perfusion dynamic calculations and perfusate biomarkers to aid identification of potentially unsuitable allografts [1061,1080–1082]. As of yet HMP is has not been widely utilized in organ preconditioning using pharmacological agents. Several reports have described augmenting traditional HMP preservation solutions with agents to reduce IR injury (e.g. N-acetylcysteine, L-arginine, nitroglycerin, prostaglandin E1, paparavine) and improve perfusion dynamics [1083–1087]. Use of HMP to delivery pharmacological agents to pre-condition allografts against specific injury after transplantation has not been widely attempted. HMP however would be an ideal tool to deliver addressive therapeutics, and precondition the organ vasculature before transplantation.

4.4.1 TLN Delivery

HMP successfully delivered TLN into the kidney vasculature, with tethering and adherence. The decrease in clotting time ratios using the simple plasma-CaCl based clotting assay in samples from Experiment 1 taken as treatment progressed demonstrates the removal of TLN (specifically the PTL006 early variant) from the perfusate and sequestration onto the vasculature, while at the same time demonstrating functionality of the HLL anti-coagulant moieties. Similar trends were seen Experiment 2 and 3 demonstrating removal of TLN from the perfusate however the significance was limited. In Experiment 2 and 3 TLN was used to treat kidneys as opposed to the earlier TLN variant PTL006 in Experiment 1, with the primary difference between the two molecules the mechanism of the tethering peptide. Experiment 3 utilised a higher concentration of TLN vs. Experiment 2, despite this increase in amount of TLN clotting times were similar in both Experiment 2 and 3. The manufacturing process for TLN used in these experiments is laboratory based compared to pharmaceutical quality production, thus the potential for inconsistencies in batch qualities exist despite through care. Furthermore Experiment 3 only utilised 4 kidneys in each arm limiting statistical potential.

Cytotopic protein adherence in these experiments however was then confirmed using the FAM-PTL analogue. This qualitatively demonstrated the presence of the TLN after treatment, with continued adherence to the kidney microvasculature and ultra-structure after 6 hours of NHRP challenge when using the perfusion protocols in these experiments. Use of a directly translational protocol to deliver FAM-PTL in porcine organs demonstrated similar adherence, further emphasising the potential for translational application of this large animal ex-vivo organ perfusion work.

4.4.2 NHRP Challenge

Isolated organ reperfusion circuits using autologous blood have developed as ex-vivo methods to experimentally assess organ viability in large animal models without the need for live auto-transplantation [1088]. Any extra-corporeal organ haemoperfusion work requires anti-coagulation to inhibit clotting during blood procurement, storage and also development of clots within the perfusion circuit and graft being perfused. In general heparin or sodium citrate is used to ensure adequate ant-coagulation, with these agents used alone or in combination; some studies using high doses of heparin alone (15,000-25,000U/L)[1089,1090]; lower doses of heparin (5,000-10,000U/L) but with added citrate (18-50ml/U)[1088]; while others also advocate in addition a continuous heparin infusion (3,000U/L/hr) into the circuit[722].

The normothermic haemoreperfusion phase of the protocol used minimally heparinised (10,000U/L) autologous whole porcine blood. The aim of the NHRP phase of the protocol was to induce a haemodynamic and pro-coagulant insult and damage the kidney to determine the effect of the presence of the TLN proteins tethered to the kidney endothelium. The dose of heparin was chosen to ensure adequate retrieval and storage of the blood until the NHRP phase, but balanced so as to not completely abolish any coagulatory potential. This was successful as demonstrated by the detection of D-Dimers levels during reperfusion, indicating fibrin deposition and subsequent fibrinolysis; with histological evidence of tubular and endothelial damage, and a generalised and diffuse pattern of fibrin(ogen) deposition, which is characteristic of IR injury [312].

4.4.3 Normothermic Macrovascular Perfusion Dynamics

These experiments confirmed that TLN anchored to endothelium can also inhibit activation of clotting. Both macro-vascular and micro-vascular perfusion metrics (renal blood flow, PFI) were persistently superior in TLN treated kidneys compared to the non-treated and Tail-only protein treated controls. Macro-vascular PFI is a useful calculation as it incorporate both renal flow rates and systolic pressure but corrects for differences in kidney mass. In these experiments observed renal blood flow rates and PFIs were between 25-45% superior in the TLN treated kidneys vs. controls. Relative flow rates during ex-vivo NHRP can be considered indicators of relative organ viability [1091,1092].

Ameliorating the microcirculatory perfusion dysfunction that is seen in renal IR injury in both experimental and clinical settings has been shown to be important and beneficial in improving initial graft function after transplantation, and limiting DGF [249,317,563,1093,1094].

4.4.4 Haemoperfusate Clotting Activity

Immediately after transplantation there is systemic activation of the coagulatory system [665]. The interlace between coagulation and inflammation renders the ischaemically damaged endothelium vulnerable to reperfusion mediated damage, and subsequent microcirculatory failure. This provokes further ischaemia and tissue injury manifested as DGF, with a higher risk of immunologic rejection [1076].

TLN is a DTI and anchored to endothelium can mitigate local thrombin action. Because thrombin has both coagulatory and inflammatory effects (via PAR-1-4) thrombin inhibition can ameliorate both these sequelae [672,314].

Direct thrombin inhibitors reduce IR injury during systemic administration in various animal IR models (e.g. kidney, lung, heart and brain) [423–425,1095] by reducing the pro-inflammatory effects of thrombin [426–428]. Recently in a porcine model of kidney auto-transplantation intervention with a DTI (melagatran) by either adding it to the UW during SCS for 24 hours, or in conjunction with systemic administration to the animal prior to nephrectomy improved allograft function, and survival after subsequent re-transplantation [672].

4.4.5 Normothermic Microvascular Perfusion Dynamics

In our study OPS of the microcirculation demonstrated better perfusion in TLN treated kidneys during the NHRP challenge. The improved calculated microvascular PI (pl/s/mm2) in these kidneys stemmed from larger diameter cortical capillaries, and faster blood flow through these vessels.

The presence of larger calibre vessels can be due to relative vessel dilation [1096,1097] - secondary to potentially less thrombin action at the endothelial cell surface, and less generation of local vasoconstrictors[274,1098] (e.g. endothelin) or alternatively more release of nitric oxide (NO). However the TLN HLL anti-coagulant moiety is an analogue of Hirulog, and Hirulog has actually be shown to inhibit NO bioavailability in endothelial cells [1099,1100].

Alternatively the larger capillaries could be due to less fibrin formation and aggregation of cellular blood components at the endothelial surfaces, rendering better transit through vessels. OPS microcirculatory analysis routinely underestimates vessel diameter due to presence of undetectable endothelial-leucocyte interactions at the glycocalyx that narrow the vessel lumen reducing the

diameter of the channel available for the passage of blood[572]. The larger observed diameter in these vessels may relate to a relative inhibition of dynamic leucocyte aggregation at the endothelial due to less thrombin mediated inflammatory activation via PAR receptors, and diminished fibrin generation. Fibrin it-self is known to be a potent pro-inflammatory mediator[1101].

These changes correlated with reduced levels of clotting activity during NHRP challenge in TLN treated kidneys, with lower DDimer levels indicating less fibrinolysis and thus indirectly less intravascular fibrin formation. The lack of statistical difference in DDimer levels in the 2nd and 3rd experiments points to 1) the traumatic nature of the animal sacrifice used in this uncontrolled DCD model (where a cytokine surge from stunning may render blood more pro-coagulant); and 2) DDimers levels may be a useful indicator of the effects of TLN treatment in a closed haemo-reperfusion circuit, but may not be the best marker as they are an indirect measure of thrombin activity.

Faster RBC velocity could be attributed to the relatively lower intravascular clotting activity and less obstruction to flow in capillaries. Maintaining equal systolic perfusion pressures between control and TLN treated kidneys during NHRP dictates that any observed differences in renal perfusion blood flow dynamics (blood flow rates, PFI) are due to inherent differences in the kidney vasculature and effect of the presence of the addressive anti-coagulant proteins. The similar changes in weight in kidneys during HMP and NHRP further indicates that perfusion differences between treated and non-treated grafts are not due to tissue oedema limiting blood flow.

4.4.6 Rapid sampling Microdialysis

The effect of improved perfusion seen in treated kidneys is further demonstrated by the trend of lower concentrations of detected rsMD cortical lactate. Lactate as the end product of anaerobic glycolysis is one of several surrogate markers of tissue ischaemia, and tissue lactate measured by microdialysis has been demonstrated to be raised in allograft hypoperfusion associated with ischaemia and thrombosis [917,1059].

During reperfusion the oxygen debt is not immediately remediated, and mitochondrial damage limits effective ATP production and so anaerobic pathways remain active. The lower levels of tissue lactate detected via rsMD in TLN treated kidneys in this model likely reflects the better overall tissue perfusion, both in relative oxygen delivery, and potentially as well as washout of toxic metabolites[1102,1103]. The higher variability seen in the control group reflects the variation in graft damage sustained during sacrifice and retrieval, and is an inherent component of a

uncontrolled DCD model and may be a reason for the lack of statistical significance. Furthermore rsMD analysis was available only for 4 pairs of kidneys due to technical difficulties with the rsMD system itself. Finally localisation of the probes to the cortex was done visually without image guided confirmation introducing the slight possibility of placement of part of the rsMD probe in an alternate region of the kidney parenchyma - such as the medulla (which has different metabolic profiles to the cortex [1104]).

4.4.7 Addressive Anti-coagulant Therapy

There have been several reports of attempts to use addressive-type targeting of anti-coagulants in experimental models of systemic IR injury (sepsis) as well as xenotransplantation where coagulation is a dominant pathology. These data complement those obtained in other model systems illustrating the benefits of localised anticoagulation.

Early work by colleagues Chen et al. in developing the HLL moiety of TLN generated transgenic mice expressing hirudin and TFPI tethered to the endothelium by fragments of CD4 and P-selectin [1105]. During LPS induced sepsis in these transgenic mice expression of hirudin or TFPI inhibited widespread intravascular thrombosis and consumptive coagulopathy associated with severe endotoxaemia, but with limited risk of bleeding. Intravascular thrombosis and coagulation is an identified reliable marker of early acute humoral rejection, and a xenograft model is extremely pro-thrombogenic with widespread fibrin deposition[312]. In a series of experiments these colleagues used hearts from these transgenic mice and transplanted them into rats in a xenograft model, with both endothelial TFPI and hirudin expressing allografts surviving for more than 100 days (vs. 3 days in controls) and rendered completely resistant to acute humoral xeno-rejection[1106]. These reports demonstrate the feasibility and benefit of targeting anticoagulants to graft endothelial cells and inhibiting coagulation, especially in so using an extremely pro-thrombotic model of acute humoral rejection, but also highlighting the potential for use of such therapy in modulating IR related coagulation events.

Ding et al. synthesized a TM based fusion protein by linking the extracellular domain of mouse TM to an antibody for PECAM-1 [937]. TM is a potent endogenous anti-coagulant normally present on the vasculature endothelium, but presence and activity is reduced during IR mediated endothelial injury. The TM fusion proteins localised to inflamed PECAM-1 expressing endothelial in the pulmonary vasculature in a murine lung IR model and inhibited fibrin deposition and leucocyte infiltration, suppressing NFκB activation, preserving pulmonary function and blood oxygenation, without

haemorrhage. Similarly Crikis et al. reported that using transgenic mice expressing human endothelial TM, cardiac graft survival in a model of acute vascular rejection was longer than wild type mice[1107]. In the same study these transgenic mice demonstrated a reduced inflammatory response in stasis-induced thrombosis and a mouse-to-rat xenograft model, with reduced HMGB1 levels in LPS induced endoxtaemia. Thus targeting anti-coagulants to the endothelium mediates both anti-coagulatory and anti-inflammatory effects in several models of IR injury, however transgenic therapeutics are complex and with limited scope for current application to clinical practice.

Targeting and delivering localising thrombin inhibitors into an organ prior to transplantation is simple, and potentially safer in terms of anti-coagulant side-effects and more effective than systemic treatment of the recipient where there is no guarantee of success [611,650–655]. Furthermore the use of localised DTIs introduces the potential for anti-inflammatory effects of these agents in modulating IR responses at the site of injury (the graft vasculature), with DTIs having recently demonstrated benefit on organ function in an experimental porcine renal transplant model [672].

4.4.8 Pre-clinical Human Kidney Model

Use of appropriate pre-clinical models is a pre-requisite to the development of protocols for clinical introduction. The data obtained from TLN pre-conditioned human kidneys correlated with our findings in the porcine models. These experiments utilized porcine blood during the NHRP challenge phase, thereby presenting a pro-coagulant and pro-inflammatory xenogenic stimulus to the organs. In this context xenogenic models are useful because of the prominent coagulopathy that develops [1092,1108] as evidenced by the progressive increase in fibrin degradation products during NHRP in the control kidneys.

Histological evidence demonstrated HMP can successfully deliver TLN into human grafts with tethering to the renal microvasculature. During normothermic reperfusion the superior perfusion with less intravascular coagulation, and lower cortical lactate in the treated kidney correlated with the porcine models.

The pre-clinical model using human kidneys not used for research utilized porcine blood during the NHRP challenge and presented a pro-coagulant and pro-inflammatory xenogenic stimulus to the organs. This discordant model is different to the conventional ex-vivo xenogenic perfusion models frequently described in the literature [1092,1108]. The conventional approach entails perfusing a porcine graft with human blood, where apart from the potential ABO blood group incompatibility

(pigs have only two blood groups A and O), Gal carbohydrate residues on porcine endothelium readily and aggressively cross-react with preformed antibodies in human blood, instigating acute humeral xenograft rejection[1109]. The model described in these experiments involves human endothelium interacting with porcine whole blood. Apart from basic incompatibilities between blood groups porcine blood has not been shown to contain high concentrations of antibodies to other endothelial glycocalyx components [1110,1111]. The human-to-pig reaction in our model is thus theoretically less pro-coagulant compared to a pig-to-human xeno-reaction, but none-the-less means it is more pro-coagulant and pro-inflammatory than would be with use of cross matched whole human blood. Importantly, experimentally it is a suitable test of effect of the presence of the TLN in the pre-treated graft.

4.4.9 Mechanism of Effect

The definitive mechanism of TLN in this model still requires further elucidation. The likely effect of TLN is through inhibiting thrombin action and the associated coagulatory, and potentially inflammatory sequelae associated with normal thrombin function. However the localisation of TLN to the endothelium also introduces the possibility of interaction with the glycocalyx and other membrane associated proteins. Cytotopic painting of the endothelium with TLN can be considered a potential example of 'immunocamouflage' where by the cell membrane surface is modified by non-immunologic molecules creating a barrier[1112]. This camouflage could then prevent direct interactions between plasma and cellular components of the blood and the endothelium, limiting IR inflammation and coagulation. Thus there are several limitations to our work that require mentioning. The aim of this study was on establishing efficacy in a large animal pre-clinical model of using HMP to deliver TLN into renal grafts, pre-conditioning the endothelial, and observe the subsequent effects on macrovascular and micro vascular tissue perfusion. This study did not look at the endothelial membrane and glycocalyx interactions with TLN, nor on endothelial activation though it is likely that localisation of these proteins to the endothelial in an IR setting modulates activity of these cells during reperfusion. Further work is being directed to investigate the interactions at the endothelial surface.

The other caveat to be applied to interpreting this work is that the histological outcomes did not reveal remarkable differences in the treated grafts, and this is likely due to the experimental time frame employed. Despite use of isolated organ reperfusion circuits as well established initial alternatives to in-vivo experimentation the relatively short duration of ex-vivo reperfusion is not a sufficient timescale for development of histological changes that can be seen in-vivo during

transplantation [1113] with the predominant effects of reperfusion in this model on cellular and molecular processes within the graft. Extending experimentation and reperfusion time to 24 hours and longer would allow further development of histological sequale. Ongoing research would thus ideally would involve large animal porcine auto-transplantation and incorporate aspects of the ex-vivo experiments including OPS during the initial 4-6 hours, and rsMD during preservation and then reperfusion and during the 1st 24-48 hours post operatively. Such experiments would provide further translational evidence for a clinical and functional effect of the changes in circulation and organ perfusion seen in these ex-vivo experiments. However this type of experiment was beyond the scope of the initial objectives of these experiments.

4.4.10 Organ Pre-Treatment in Context and Future Work

The addressive approach suggest here using HMP to deliver TLN is novel and ideal. An allograft would theoretically undergo HMP for several hours as part of a transplant centre's clinical protocol, then TLN would be added to the perfusate, preconditioning the endothelial with tethering of the proteins. The allograft would ultimately be transplanted with the thrombin inhibiting anti-coagulants localised to the allograft vasculature, limiting the need for systemic anti-coagulation (with associated bleeding risks) and be potentially more effective. The DTI effects of the TLN would inhibit both the pro-coagulant and pro-inflammatory actions of thrombin - ameliorating IR responses, and improve post-operative allograft perfusion and theoretically function. For the first time the feasibility has successfully been demonstrated of pre-conditioning kidney grafts with addressive thrombin inhibiting therapeutics using HMP, in ex-vivo porcine and pre-clinical human kidney perfusion models, and that the presence of these agents improves organ perfusion. There is a high potential for application of this addressive technique to clinical practice, not solely for kidney transplantation, but for also for other organs, for example pre-conditioning marginal pancreatic allografts which are very susceptible to reperfusion injury and allograft thrombosis.

Chapter 5:

Development of Adenocaine as a Novel Hypothermic Machine Perfusion Preservation Solution

5.1 Objectives

Adenocaine as a composite of adenosine, lidocaine, and magnesium has been demonstrated in cardiac models to afford better protection than conventional preservation solutions possessing membrane voltage stabilisation, vasodilatory, anti-inflammatory and anti-coagulant properties, as well as been shown to preserve high-energy phosphates during ischaemia (5,9,77–79). With the increasing use of ECD and DCD donor organs being used to expand donor pools interventions to optimise kidney viability are in much clinical need, and the cytoprotective properties of Adenocaine may prove beneficial in kidney transplantation.

Adenocaine Machine Preservation Solution (AL-MPS) is a variant of Adenocaine specifically modified to facilitate HMP of abdominal organs. The objective of this study was test the benefits of AL-MPS compared to University of Wisconsin (UW) solution (Belzer MPS, the gold standard machine preservation solution) for HMP kidney preservation using a series of large animal ex-vivo porcine kidney perfusion and reperfusion models.

5.2 Methods

5.2.1 Experimental Protocol

Twenty porcine kidneys were used in this study. In the laboratory one kidney of each pair underwent HMP with either AL-MPS (AL, n=10) or UW (Belzer MPS) (UW, n=10) solution for 10 hours on a Waters Medical Systems RM3 perfusion machine (TM). Kidneys then underwent viability assessment for 2 hours using an oxygenated normothermic reperfusion (NMR) circuit with Kreb Henseleit Buffer (KHB) solution. The protocol for this experiment is summarised in Figure 69. Ten hours of HMP was chosen as a suitably duration period to that used in recent clinical trials comparing SCS to HMP (ref). Longer cold perfusion overnight (i.e. 24 hours) was not logistically possible with current RM3 perfusion systems.

5.2.2 Preparation of Solutions

AL-MPS was prepared in- laboratory and refrigerated for 24 hours before each experiment. The AL-MPS solution was composed of a KHB base with 4mM adenosine; 0.5mM lidocaine, 0.26mM calcium, 16 mM Magnesium, and 4% BSA. Commercially available UW solution was used as the control perfusate.

5.2.3 Graft Retrieval

Porcine kidneys were obtained from adult landrace pigs after sacrifice at a local abattoir. Local guidelines were adhered to. Organs were retrieved as per the longer ischaemia protocol detailed in Section 3.1.3.1. Kidneys were flushed at a pressure of 100cmH_20 with 500ml of either AL-MPS or UW depending on the assigned experimental group. At the laboratory grafts were further benched for HMP and the renal artery cannulated for perfusion.

5.2.4 Hypothermic Machine Perfusion

Kidneys were perfused hypothermically (5-7˚C) on two commercially available Waters Medical RM3 perfusion units for 10 hours. 1L of preservation solution was used in either circuit. Perfusion was based on clinical protocols[1114] with a target systolic perfusion pressure of 40mmHg by 15 minutes of perfusion. Volume driven flow was then adjusted at 1, 2 and 3 hours of HMP to maintain a target systolic pressure of 40-45mmHg, and then left to take its course.

5.2.5 Reperfusion Circuit

The first four pairs of kidneys in these experiments only underwent HMP. In the following six pairs a normothermic reperfusion assessment of viability was introduced after the initial period of cold HMP. After 10 hours of HMP 6 kidney pairs were then connected to a NMR circuit to assess organ viability. Reperfusion with KHB was performed after a 20 minute re-warming period at room temperature to mimic the implantation of the kidney into the recipient. The Waters Medical RM3 units were modified to facilitate normothermic reperfusion by in line incorporation of an automated temperature controlled water pump (Grant) with the target temperature set to 39oC. The circuit was primed with 1L of KHB (BRAND) supplemented with 2g glucose, 1.8mg H_2CO_3, 10g/L (1%) BSA, spiked with 1000mmol/L creatinine, and oxygenated by bubbling a mixture of 95% O_2 / 5% CO_2 gas mixture through RM3 cassette arterial reservoir. The ureter was cannulated to allow collection of urine. Urine volume was replaced with fresh KHB. Reperfusion was maintained for 2 hours. Initially systolic perfusion pressure was set to 40 mmHg and gradually increased to 50 mmHg within the first 10 minutes and then maintained between 45-50mmHg.

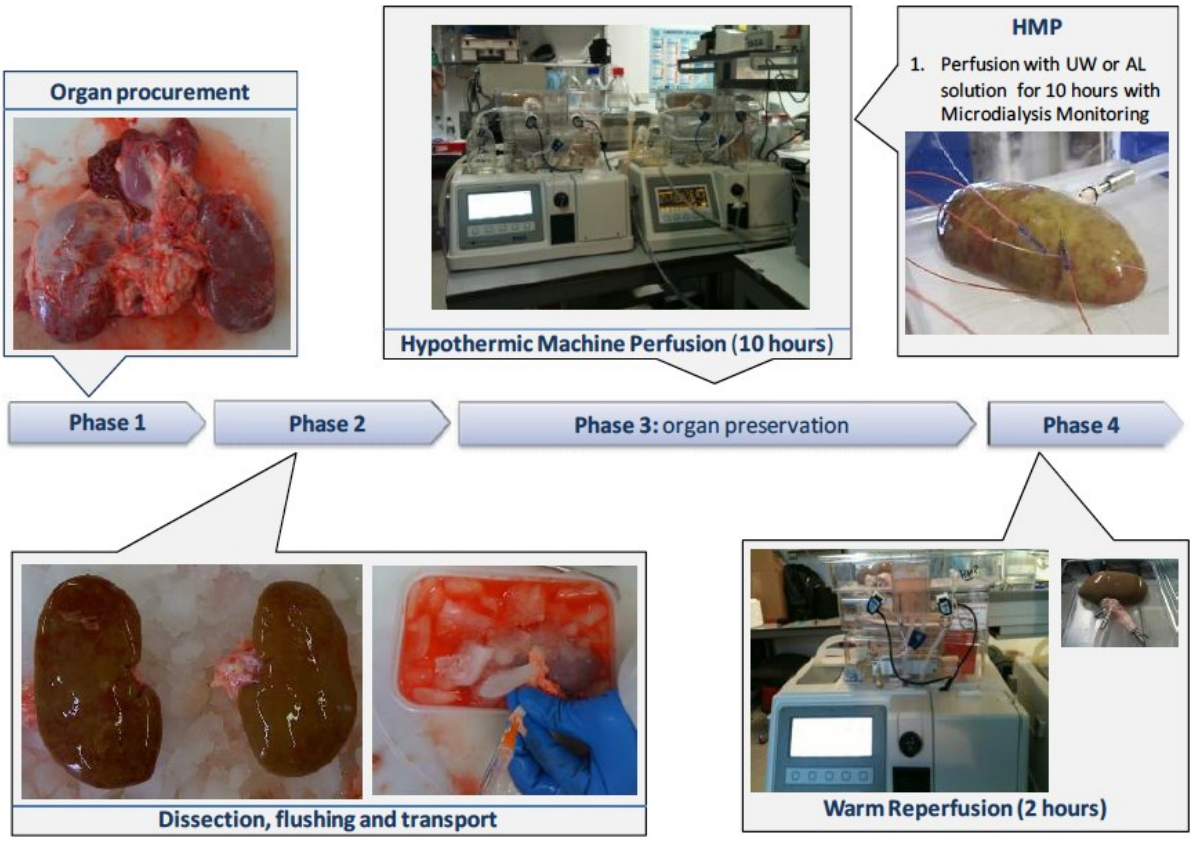


Figure 69 Diagram of experimental protocol for testing of novel AL solution vs. UW solution during HMP.

Outcome Measures

Real-time perfusion dynamics (perfusion pressures, flow rates, and resistances) were recorded throughout HMP and reperfusion. Kidneys were weighed pre-HMP, post-HMP, and post-Reperfusion, to quantify oedematous change. During reperfusion urine output was collected and measured. Perfusate was iso-volumetrically replaced with the amount of urine lost, using KHB with 1% BSA. Urine samples were collected at 1 and 2 hours, and reperfusate samples collected at 5mins, 1hour and 2 hours of reperfusion. All reperfusate and urine samples were centrifuged for 3 min at 3500rpm and the supernatant stored at -80 degrees for later analysis. Samples were analysed for electrolytes, creatinine, lactate dehydrogenase (LDH). Blood gas analysis was performed real-time on arterial and venous reperfusate samples at 30mins, 1 hour and 2 hours of reperfusion and allowed calculation of oxygen consumption.

5.2.6 Rapid sampling microdialysis

RsMD was instituted in these experiments in line with the methodology described in Project 1. In brief microdialysis probes (MAB11.35.4, Microbiotech, Stockholm, Sweden) were inserted superficially into the parenchyma of the lateral cortex at the start of HMP. These were connect to an in-house built rapid-sampling microdialysis analyser. Every 30 seconds a 200 nL dialysis sample was automatically injected into the analysis flow stream, alternating between each kidney. The stream accelerates the dialysate through an enzyme reactor containing LOx and 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 Macbook Pro portable computer (Apple Computers, Cupertino, CA). Lactate levels were analysed in real-time to reveal information on interstitial lactate concentration during the 10 hours of HMP, and then 2 hours of normothermic reperfusion.

5.2.7 Histological Analysis

Needle core biopsies were taken pre-HMP, post-HMP, and wedge biopsies after 2 hours of normothermic reperfusion, fixed in 4% formalin, dehydrated, and embedded in paraffin wax. 4 µm sections were cut and stained with hematoxylin and eosin, (periodic acid-Schiff) for light microscopy evaluation.

Tubular dilation, epithelial flattening, epithelial shredding, tubular debris, vacuolation, condensed tubular nuclei, and glomerular shrinkage were each scored over three fields using a semi-quantitative scale (0 normal; 1 mild (0-25% affected); 2 moderate (25-50% affected); 3 severe (>50% affected).

5.2.8 Analytical methods

During HMP and reperfusion, recorded flow rates were adjusted for kidney weight to calculate flow rate per 100g (ml/min/100g). PFI (ml/min/100g/mmHg) values which incorporate flow rate and pressure per 100g of tissue were calculated for each kidney.

During reperfusion creatinine clearance was calculated to determine GFR per gram of tissue as (urinary creatinine × urinary volume/reperfusate creatinine). Fractional excretion of sodium was calculated as (urinary sodium × urinary flow)/(GFR × reperfusate sodium)×100)/g. Urinary LDH/GFR ratio per gram of tissue was similarly calculated. Oxygen consumption was calculated as (difference between arterial and venous partial pressure of oxygen)×flow rate/100g of tissue.

5.2.9 Statistical Methods

Values are represented as mean and standard deviation. Continuous variables were plotted as levels versus time curves for each kidney and the mean area under the curve calculated. Continuous variables from paired contra-lateral kidneys were evaluated using the paired students T-test or non-parametric Wilcoxon signed rank test when distribution assumptions were not met. Histology scores were compared using the Wilcoxon signed rank test. Data and statistical analysis was performed using Microsoft® Excel Software (Reading, UK). All tests were 2-tailed and a p value of ≤ 0.05 was considered significant.

5.3 Results

5.3.1 Adaptation of AL Cardioplegia for kidney Machine Perfusion

A pilot experiment utilized un-modified AL as the HMP perfusate for perfusion of a single porcine kidney (WIT:15 minutes, SCS CIT 135 minutes). Perfusion failure occurred after 2 hours due to massive oedema (77% weight gain), and poor perfusion dynamics: perfusion pressure >100mmHg, flow rate: 3ml/min/100g, resistance: 8.8 mmHg/ml. The perfusate composition was adjusted with addition of 4% BSA to prevent oedema, and facilitate machine perfusion, this final solution was designated AL-MPS.

5.3.2 Kidney Characteristics

Ten pairs of porcine kidneys were used in these experiments (AL group n=10, UW group n=10). Kidney characteristics were similar between experimental groups. WIT was identical for both AL and UW kidneys (WIT: 34.6 ±14.5 minutes) while SCS CIT in AL kidneys was 270.4 ±22.5 minutes, and 264 ±18.8 minutes in UW kidneys, p=0.356. Average kidney weight was 308.4g±107.5 in AL group, and 316g ±109.8 in UW group (p=0.816).

5.3.3 Hypothermic Machine Perfusion

Perfusion dynamics during HMP are shown in Figure 70. After the initial 3 hour stabilization phase perfusion where systolic pressures were maintained at 40mmHg pressures remained stable in AL perfused kidneys (Systolic pressure during 3-10h of AL HMP: 39.6 ±4.1mmHg) but decreased in UW kidneys (UW=35.7 ± 4.6 mmHg, p=0.022). HMP perfusate flow rates in AL kidneys were 20% higher compared to UW kidneys (Flow rates: AL=21.5 ±7.7; UW=17.9 ± 7.7 ml/min/100g, p=0.011). The PFI during the initial stabilization phase was 25% greater in AL kidneys vs. UW kidneys (0-3h of HMP: AL=0.50± 0.2, UW=0.40 ± 0.17; p=0.033), but equalised during the remainder of HMP (3-10h of HMP: AL=0.55 ± 0.2, UW=0.54 ± 0.23 ml/min/100g/mmHg; p=0.817) with an increase in PFI in UW group to the levels seen with AL. Resistance was not different during HMP (AL=0.59 ±0.29, UW=0.63 ±0.27 mmHg/ml/min; p=0.462).

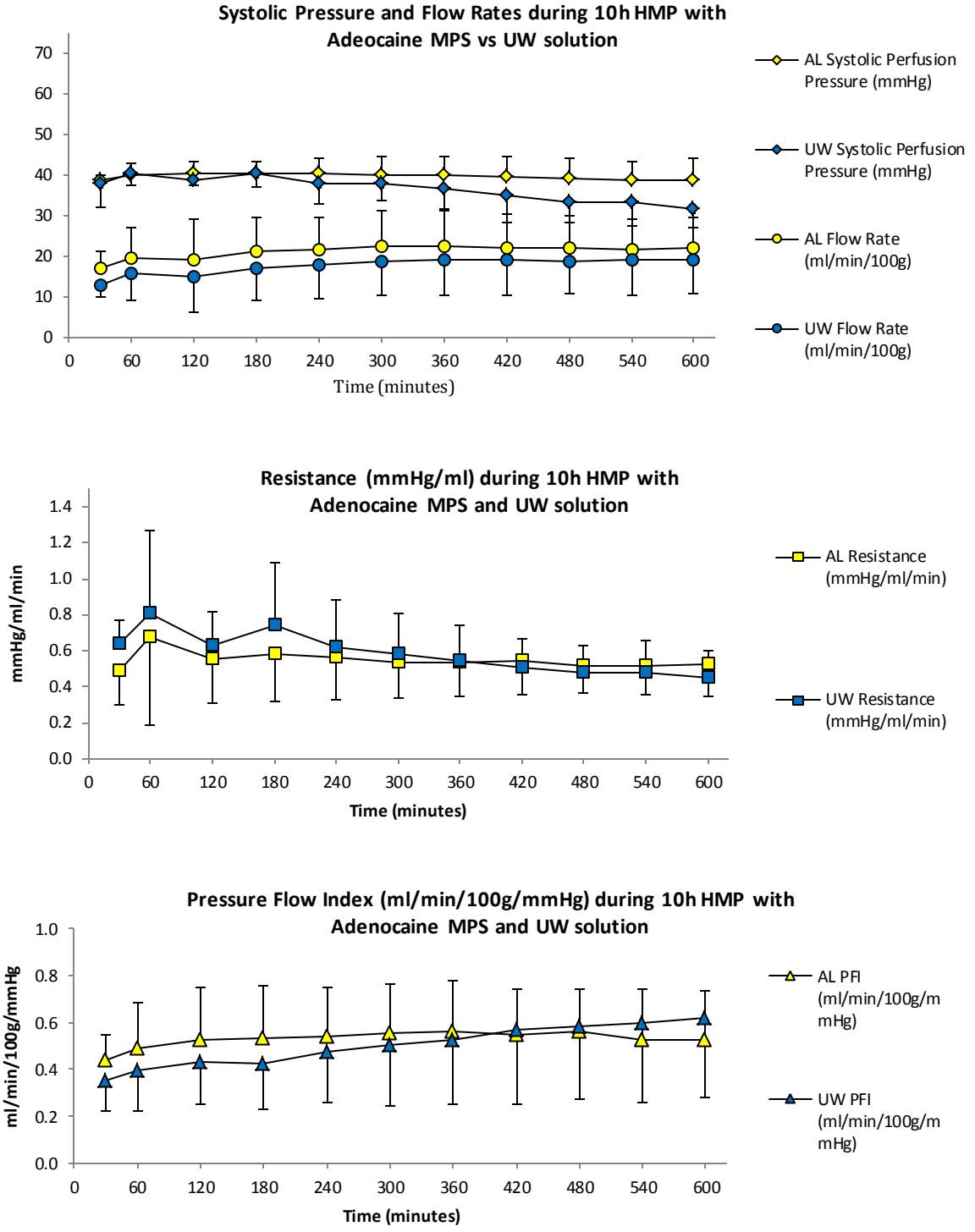


Figure 70 Perfusion dynamics during HMP with AL and UW solutions for 5 hours. Systolic perfusion pressure and flow rates (top), resistance (middle) and perfusion flow index (bottom).

5.3.4 Normothermic Reperfusion

5.3.4.1 Reperfusion Dynamics

After 10 hours of HMP kidneys underwent viability assessment on a isolated normothermic reperfusion circuit. Reperfusion dynamics were similar in both AL and UW perfused kidneys (Systolic pressure: AL=46.1±3.2, UW=46.2±2.2 mmHg, p=0.856; Reperfusion flow rate: AL=44.7±10.4, UW=50.5±17.4 ml/min/100g, p=0.319; Resistance: AL=0.21±0.04, UW=0.17±0.11 mmHg/ml/min, p=0.305; PFI: AL=0.97±0.23, UW=1.1±0.37 ml/min/100g/mmHg, p=0.317). Reperfusion dynamics are depicted in Figure 71 and Figure 72.

5.3.4.2 Weight Change

There was a 38.8±8.2% significant increase in graft weight after AL HMP vs. 18.9 ±8.6% with UW HMP (p=0.002). While weight increase after RP trended lower in the AL group (9.7 ±7.9%) vs. the UW group (21.9 ±10.7%, p=0.113). Weight changes are represented in Figure 73.

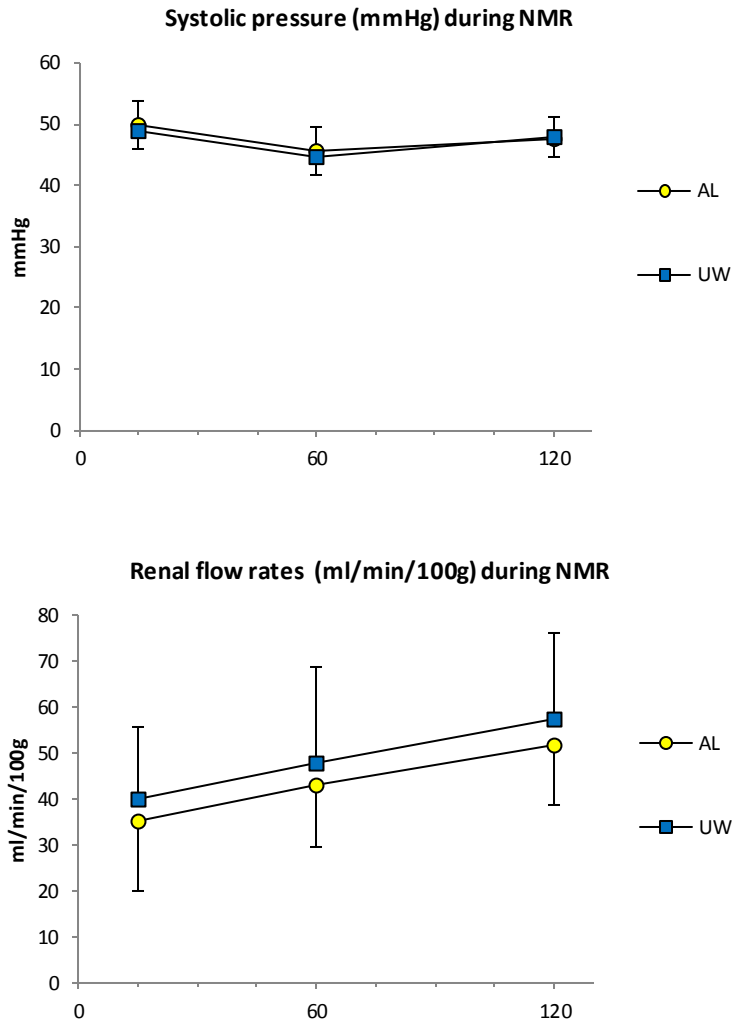


Figure 71 Reperfusion Dynamics in AL and UW kidneys. Systolic pressure (top) and renal flow rates (bottom). There were no differences in reperfusion dynamics between the two groups of kidneys.

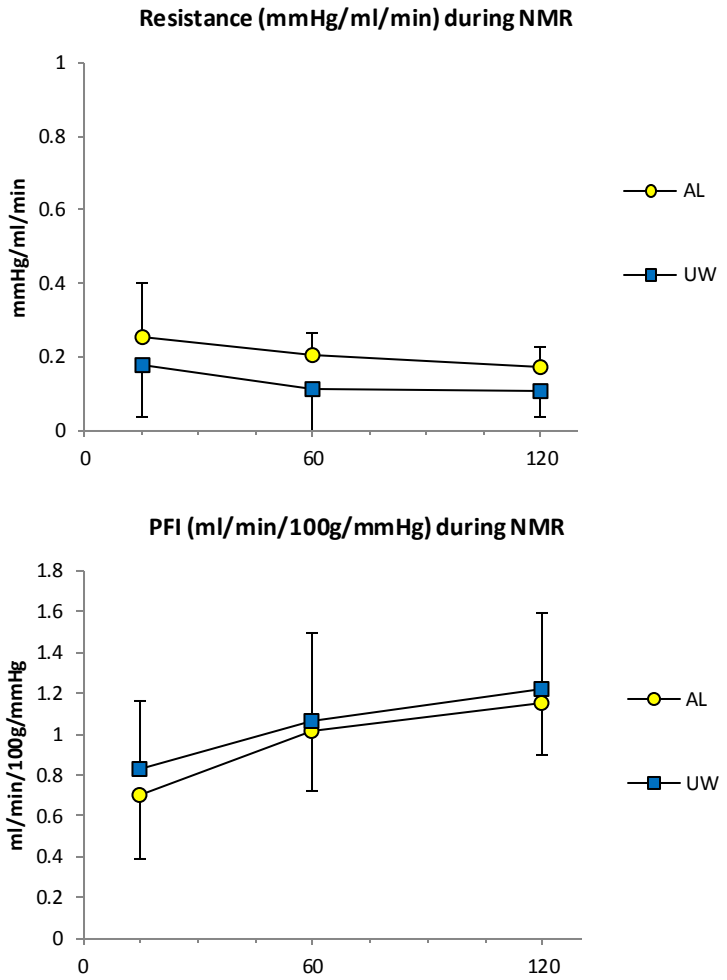


Figure 72 Reperfusion Dynamics in AL and UW kidneys. Resistance (top) and perfusion flow index (bottom). There were no differences in reperfusion dynamics between the two groups of kidneys.

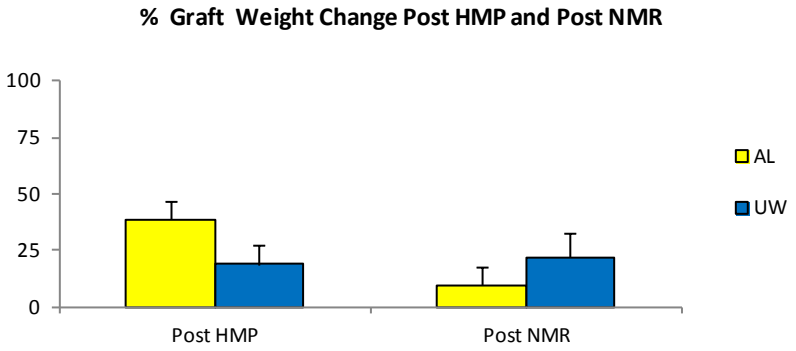


Figure 73 Percent weight change in AL and UW kidneys after HMP and NMR. There was a 38.8±8.2% significant increase in graft weight after AL HMP vs. 18.9 ±8.6% with UW HMP (p=0.002). While weight increase after RP trended lower in the AL group (9.7 ±7.9%) vs. the UW group (21.9 ±10.7%, p=0.113).

5.3.4.3 Urine output/GFR

Urine output trended higher at 60 and 120 minutes of RP in UW perfused kidneys (Urine output 60mins: AL= 0.79±0.67, UW=1.26±1.3 ml/hour/g, p=0.41; Urine output 120 minutes: AL= 1.10±0.74, UW=1.25±1.51 ml/hour/g, p=0.748). Despite significantly lower reperfusate creatinine concentrations at the end of RP in AL perfused kidneys (AL=413.2 ± 103, UW=512.9±75.5 mmol/L, p=0.026) overall creatinine clearance was not different between the preservation solutions (AL= 0.016 ± 0.01, UW = 0.018 ± 0.01 ml/min/g, p=0.73). Fractional sodium excretion per gram of tissue was not different (AL=0.3±0.18, UW=0.27±0.11 AU, p=0.748), and assessment of tubular damage via urinary LDH/GFR ratio indicated similar levels of ischaemic injury (AL=0.29±0.4, UW=0.28±0.09 IU/ml/min/g). Functional assessment of graft viability is depicted in Figure 74.

5.3.4.4 Oxygen consumption

Overall oxygen consumption was comparable between AL and UW perfused kidneys during reperfusion (AL=3.46±2.5, UW=3.09±1.7 kPa/ml/min/g, p=0.58) and is shown in Figure 74. Furthermore the buffering capacity of AL kidneys was superior to UW kidneys with preservation of higher levels of reperfusate bicarbonate during 2h of reperfusion (AL=17.1±3.3, UW=12.1±4.3 mmol/L, p=0.02, see Figure 74).

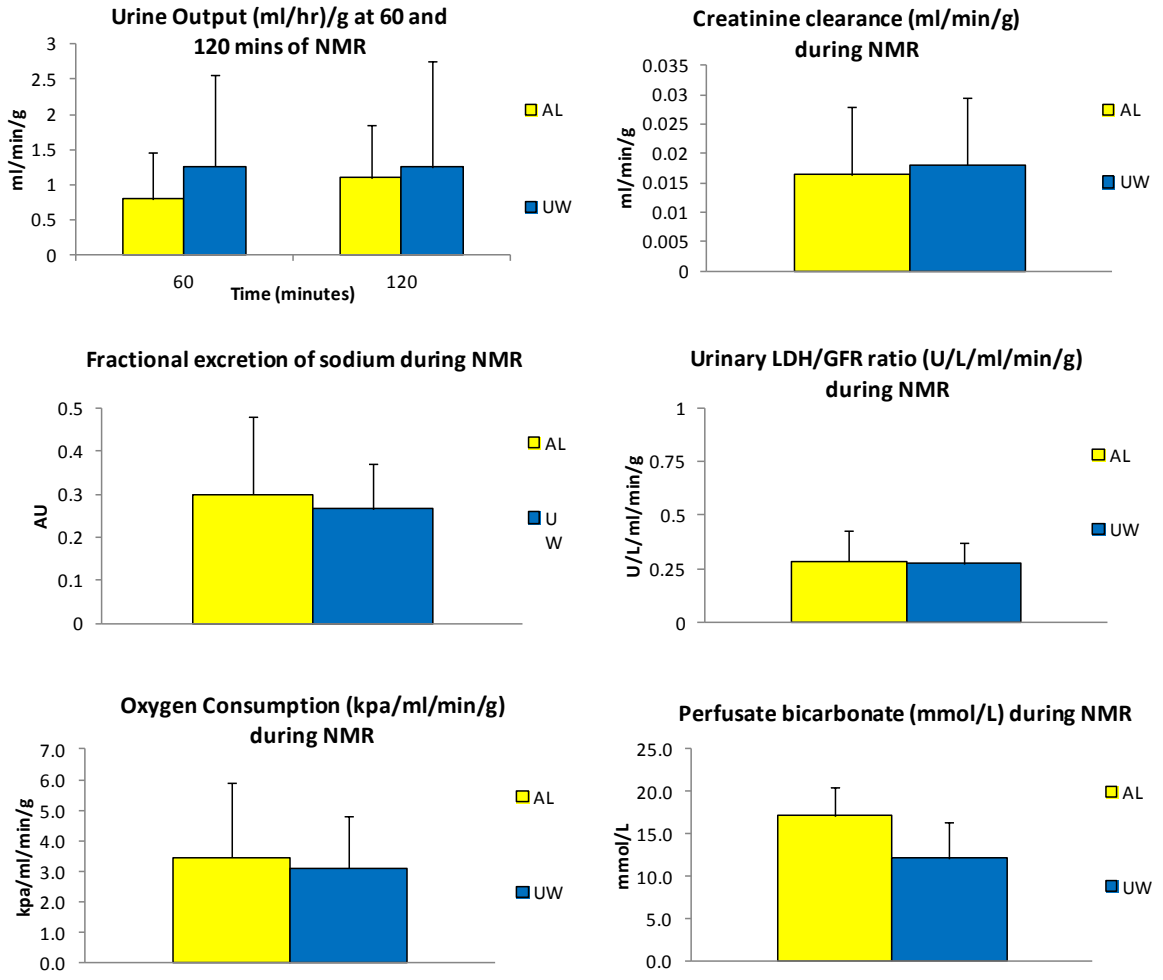


Figure 74 Functional and biochemical assessment of AL and HMP graft viability during NMR. Urine output, creatinine clearance, fractional excretion of sodium, urinary LDH/GFR ratio, oxygen consumption and perfusate bicarbonate levels.

5.3.4.5 Rapid sampling microdialysis

Cortical tissue lactate concentrations were quantified using rsMD in kidneys during both 10 hours of HMP and 2 hours of reperfusion (Figure 75). During HMP cortical lactate levels were comparable between AL and UW perfused kidneys (AL= 0.154 ±0.096, UW= 0.144±0.038 mM/min, p=0.851). During reperfusion cortical lactate levels trended lower in AL kidneys vs. UW kidneys (AL= 0.66±0.306, UW= 0.887±0.534 mM/min, p=0.33). Lactate levels were also quantified in the reperfusate using blood gas analysis and were significantly lower in the AL vs. UW group (AL=3.1±0.4, UW=4.1±1.0 mmol/L, p=0.04) during reperfusion.

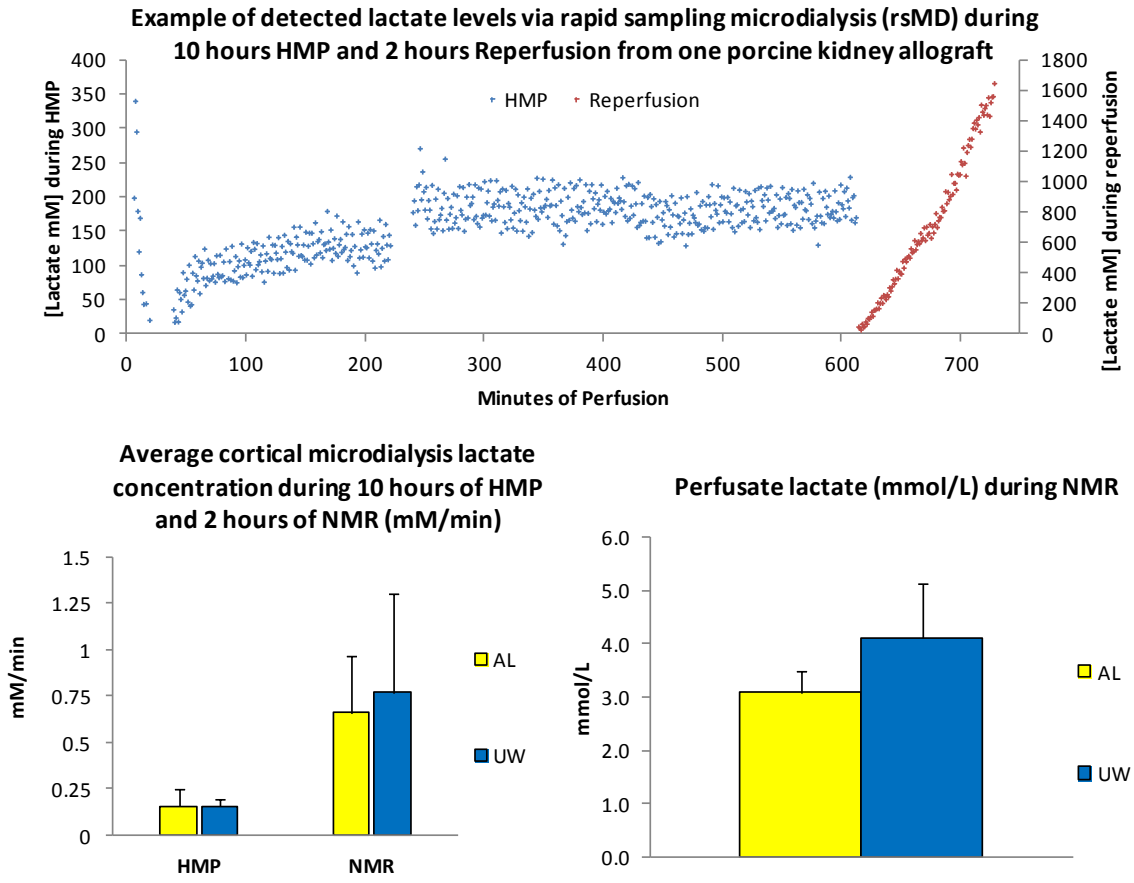


Figure 75 RsMD for tissue lactate during HMP and NMR in AL and UW kidneys. Example scatter diagram of rsMD readings measured from one kidney during HMP and NMR (Top). Average cortical tissue lactate during HMP and NMR in AL and UW kidneys (bottom left). Perfusate lactate concentrations during NMR (bottom right).

5.3.4.6 Histology

There were no significant differences in core biopsies between AL and UW kidneys in pre-HMP and Post-HMP samples. After NMR all kidneys exhibited evidence of reperfusion induced tubular damage with a significantly greater degree of tubular dilation in UW vs. AL kidneys (p=0.003), see Figure 76. There were no other significant differences (see Table 12).

Parameter	Tubular Debris		Tubular Dilation		Nuclear Fragmentation	
Group	AL	UW	AL	UW	AL	UW
Score	2.4 ± 0.5	2.6 ± 0.5	2.1 ± 0.6*	3.1 ± 0.7	1.2 ± 0.5	1.2 ± 0.2

Table 12 Histology scores of biopsies taken after 2 hours of NMR assessing tubular debris, tubular Dilatation, and nuclear fragmentation. There was a significantly greater degree of tubular dilation in UW vs. AL kidneys (p=0.003). There were no other significant differences.

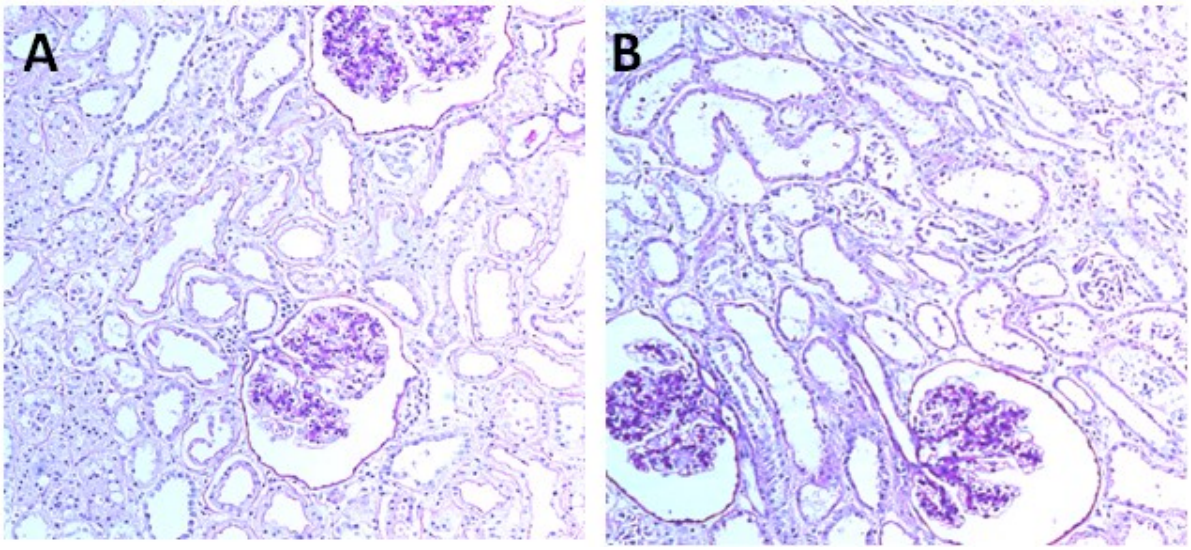


Figure 76 Photomicrographs of light microscopy of AL vs. UW perfused kidneys after 2 hours of NMR. H&E staining, x20. A: AL kidney, B: UW kidney. Showing greater degree of tubular dilatation in UW kidneys.

5.4 Discussion

Increasing use of DCD and ECD has led to a resurgence of methods to improve allograft viability and function. Interest has focussed on the further potential of HMP which has shown benefit compared to SCS in terms of graft survival and DGF[946].

The preservation solution used in clinical practice for renal HMP was first developed 40 years ago, and is the gold standard solution, but may not be the most effective solution for marginal allografts. Ischemia reperfusion injury is believed to contribute to graft function dysfunction and new methods of protection are required to expand with the increasing use of marginal kidneys secondary to organ shortages [1115]. Adenocaine MPS (AL-MPS) is a HMP solution based on the cardioplegia Adenocaine containing adenosine and lidocaine as the primary effector agents in the solution.

The present study was the first using a AL-MPS solution [1116] in a DCD porcine kidney HMP model. We report that Al-MPS solution showed significantly improved flow rates and flow indicies compared to UW during a period of 10 hours of HMP, and significantly lower perfusate lactate levels during 2 hours warm reperfusion. In all other areas, modified AL solution showed equivalency compared to UW in hypothermic perfusion dynamics, normothermic functional viability assessment, and histological injury.

5.4.1 Potential Benefits of AL-MPS in Renal HMP

In addition both adenosine and lidocaine have been demonstrated to exert protective effects on kidney physiology against IR injury. Through A1 adenosine receptors adenosine has been shown to lower renal oxygen consumption by reducing GFR through selective afferent arteriolar vasoconstriction, thus decreasing filtration workload during IR injury while cellular energetics are reconstituted and repaired[965]. This effect may be a mechanism of 'acute renal success' where in an ischaemic setting reduced formation of ultrafiltrate will decrease tubular workload and thus oxygen consumption, and aid recovery from the initial ischaemic insult[1117]. Conversely via A2 receptors adenosine facilitates an increase in medullary blood flow and oxygenation while at the same time lowering medullar transport activity [955] through NO generation[970,971].

While lidocaine is a local anaesthetic agent, it is also used as an anti-arrhythmic drug [983] and has been used in cardiac resuscitation[984,985] possessing negative chronotropic, inotropic effects, and vasodilatory effects[986]. Independent of its inhibition of voltage sensitive fast opening sodium channels lidocaine has also been suggested to exert anti-inflammatory effects by 1) inhibiting

leucocyte endothelial interaction [987]; 2) inhibiting release inflammatory cytokines and chemokines from endothelial cells [988]; 3) inhibiting production of superoxide anion ROS by XO[989,990]; 4) reducing ROS damage by scavenging hydroxyl free radical species [991]; and 5) by inhibiting sodium/calcium exchange reducing cellular calcium accumulation during ischemia [992–994].

The inclusion of magnesium in AL is based upon its effects as a natural calcium antagonist on membrane stabilisation and preventing ischaemic cytoplasmic calcium overload[1017], as well as facilitating ATP production[1013], and observed anti-inflammatory properties in IR settings[1018].

At a concentration of 5.8mM the potassium content of AL-MPS is normokalaemic and less than that of UW (25mM). High potassium concentrations above 10mM promote ionic imbalances[951], depolarise endothelial cells rendering them into an activated pro-inflammatory state[1020], and cause vasoconstriction during preservation[951,1021].

5.4.2 Comparison of AL-MPS to UW Solution during HMP

In this experiment AL-MPS was compared to UW, the gold standard perfusate for machine organ perfusion. UW does not contain adenosine, nor lidocaine, however UW does contain adenine a component and pre-cursor of adenosine, included in UW as an energy metabolite precursor. Adenine has not been shown to exert pharmacological effects as adenosine.

Overall during HMP flow rates were higher during perfusion with AL-MPS, suggesting more effective perfusion. A mechanistic effect of lidocaine's vasodilatory properties may be a factor in these differences[1118]. However differences may further stem from the relative viscosity of UW compared to AL-MPS. UW contains hydroxy-ethyl-start a pentose starch that acts as the primary osmotic agent in UW and which renders it viscous. AL-MPS however relies on an albumin supplement at isotonic levels (4%) as an oncotic agent.

The resistance values calculated by the perfusion machine however showed no difference between HMP solutions, and this in part can be attributed to its calculation using the mean perfusion pressure. The systolic pressure as an indication of potential barotrauma in our experience is the preferred value to be monitored. We utilised the systolic pressure, the flow rate, and weight of individual kidneys to calculate the PFI as a measure of effective organ perfusion. The PFI is thus a more informative metric than the Resistance Index.

With AL-MPS PFI levels remained within viable parameters (>0.4)[876] for the duration of perfusion, and during initial perfusion (0-3 hours) was superior to perfusion with UW. It is suggested a

minimum perfusion time of 4 hours is required before perfusion dynamics have stabilised sufficiently to allow there use in predicting organ function after transplantation [1061]. With AL-MPS perfusion stabilises within the 1st 60 minutes, compared to 4 hours with UW potentially allowing earlier use of perfusion dynamics for viability assessment to guide and plan recipient management.

Higher interstitial oedema after HMP with AL-MPS reflects the absence of HES, a potent oncotic present in UW. Greater than 25% weight gain in renal allografts with HMP has been suggested to predicate to poorer graft outcome[1114]. It is interesting that increased tissue water was found in the isolated heart perfused with AL cardioplegia but this affect functional recovery, as AL was shown to be superior in left ventricular performance than high potassium solutions[1119]. Similarly the association is however is not absolute[659] and the reliance on individual parameters is less sensitive in assessing viability than with combined indices such as perfusion dynamics[832]. During reperfusion, for example, the interstitial oedema was lower in AL perfused kidneys compared the UW solution .

The normokalaemic nature of AL-MPS compared has been attributed as one of the key factors in the benefits seen in cardiac preservation and subsequent re-animation. However note must be considered in drawing parallels to kidney preservation as cardiac preservation CIT is shorter (up to 4 hours clinically) and may not be ideal in situations with longer CIT as a relatively low potassium content may result in intracellular potassium depletion. The potassium content of AL-MPS however is in line with newer preservation solutions such as Polysol which have shown promise in experimental models[880].

5.4.3 NMR Viability Assessment

Isolated kidney reperfusion models have been used to determine renal preservation injury in a number of settings particularly for comparing preservation solutions[581,751,761,1120]. Functional assessment of kidney grafts during reperfusion demonstrated similar viability profiles in AL-MPS and UW perfused kidneys. Adenosine, through A1 and A2 receptors modulates renal arteriolar glomerular and medullary blood flow, limiting glomerular filtrate formation and reducing oxygen demand. This effect was not seen in these experiments; urine output trended lower with AL-MPS but not significantly, with creatinine clearance, fractional sodium excretion, oxygen consumption, and LDH/GFR ratio comparable to UW. Adenosine receptor activation produces both and immediate and delayed receptor mediated effect [968], suggesting preservation with adenosine in the hypothermic state would mediate a degree of benefit during subsequent reperfusion. However AL-MPS contained

a 4mM adenosine concentration which may be an insufficient level to exert a protracted effect in an ex-vivo reperfusion model.

In addition to using the LDH/GFR ratio for assessing tubular damage as proposed by Kehrer et al. [1121], ischaemic injury was further characterised using rsMD for cortical tissue lactate. The real-time nature of the rsMD system ensured dynamic changes during HMP and reperfusion were characterised. This high resolution technique demonstrated non-significant differences between cortical lactate profiles in both preservation solutions.

The trend of lower lactate in modified AL pumped kidneys suggests potentially less ischaemic damage during reperfusion, correlating with the trend of increased O2 consumption in these kidneys.

An alternative explanation of these trends is suggested by the data presented in Chapter 3. and relates to the intrinsic lactate metabolism inherent in the renal cortex. In contrast to traditional thought higher lactate may actually represent more viable cells able to undergo anaerobic metabolism during reperfusion when the oxidative enzymes are still undergoing repair and oxygen consumption and aerobic metabolism is not efficient. In the same sense a lower lactate signifies less cells able to undergo metabolism in the cortex. The limitation in significance in this data pronounces further experimentation will be required to clarify these trends, which should then be correlated to functional differences during reperfusion.

AL-MPS kidneys demonstrated a resilience to development of acidosis during reperfusion, maintaining higher perfusate bicarbonate levels than UW kidneys. This effect may in part be mediated by A1 receptors within the proximal convoluted tubules, activation of which as well as modulating filtrate formation is known to increase tubular bicarbonate reabsorption [1122,1123].

5.4.4 Adenosine and Lidocaine in Preservation Solutions

Lidocaine has been evaluated as a cytoprotective supplement to conventional SCS solutions with a degree of success by Erkasap et al. They added 0.081mmol/L of lidocaine to Euro-Collins solution, and investigated the effect of this composite solution to unmodified Euro-Collins in a series of experiments using canine kidneys. The effectiveness of 48 hours of SCS preservation with these solutions was compared by assessing organ viability using an isolated normothermic reperfusion circuit. Reperfusate flow rates, GFR, urine output, and fractional sodium excretion levels were all

found to be significantly higher; with markers of tubular damage (urinary LDH/GFR ratio), and lipid peroxidation (malondialdehyde tissue levels) significantly lower, after Euro-Collins/lidocaine preservation. A potential mechanism for these observations is based on lidocaine's ROS scavenging properties[1002]. This will be active during HMP, and so potentially limit lipid peroxidation and cell damage during reperfusion; hence promote better functional metrics. Acknowledging Euro-Collins solution was not used in our study, despite using a 7-fold higher concentration of lidocaine in AL-MPS than Erkasap et al. used such differences in functional reperfusion dynamics were not seen. These differences may stem from the fact that in the AL-MPS experiments grafts came from a DCD animal model with WIT more than 30 minutes, and thus considerably longer ischaemic injury, compared to Erkasap et al. where canine kidneys were retrieved under anaesthesia with minimal reported WIT before flushing.

Lopez-Neblina et al. developed a SCS preservation solution, Human Biosystems (HBS) composed of macro molecules, high-energy substrates, precursors, and co-factors, with a mixture of anti-proteolytic amino acids, antioxidants, and anti-inflammatory compounds[1124]. HBS contained adenosine as an energy substrate, and lidocaine as a membrane stabilizer, exact concentrations of each not reported. They compared effectiveness of HBS to UW solution and HTK in renal SCS preservation using a murine kidney transplant model. HBS preserved kidneys better, with improved allograft function and survival at 7 days. The specific role of both adenosine and lidocaine in improving allograft preservation cannot be determined considering the presence of other substances active to limit IR injury. However this study further highlights the theoretical benefits of adenosine and lidocaine in abdominal organ preservation, and supports their potential use.

5.4.5 Limitations

This study had several limitations. Despite widespread use of isolated organ perfusion models, they provide only a limited, acute and early assessment of organ function after preservation. A more direct translational approach would entail use of a porcine auto-transplant model. An optimal middle ground is use of an isolated haemo-reperfusion circuit which provides a more physiological challenge to the organ, and which can be maintained for several hours; use of which clinically has been demonstrated to predict allograft function. DCD porcine kidneys were used with a length WIT but with only a relatively short (4 hours) CIT prior to the start of HMP, determination of the effects of modified AL HMP on kidney function and metabolism using grafts exposed to longer lengths of initial SCS would allow further elucidation on the potential for use of AL MPS.

Adenosine and lidocaine concentration levels used in AL-MPS were in-line with levels in cardioplegic Adenocaine and chosen as a starting reference point. Both these agents have dose related effects and so higher concentrations of each in AL-MPS require evaluation. The AL solution in modified-Krebs Henseleit and 16 mM $MgsO_4$ was refrigerated for 24 hours before the experiment and, while it was visually checked for precipitates, future studies should include a full quantitative particulate analysis prior to kidney perfusion.

During NMR standard methods of determining renal function and creatinine clearance were utilised, and included ml for ml replacement of fluid filtered by the graft (urine) with stock KHB solution to ensure an equal and constant volume of fluid in the perfusion circuit. Despite being widely used and described in the literature [1125] this methodology however introduces an unavoidable and potential inaccuracy in the calculation of creatinine clearance due to the fluid movements.

The PFI calculation utilised pre-HMP weight. With significant changes in weight during HMP a recalculation of PFI using dynamic weights as perfusion progresses would be ideal to demonstrate dynamic perfusion metrics. This was not logistically possible however to measure kidney weight every hour.

5.4.6 Future Development of AL-MPS

The University of Wisconsin solution (Belzer MPS) has been the gold standard HMP solution for the past 40 years. Clinical studies have shown HMP with UW vs. SCS is beneficial for graft function and patient survival, however this benefit is less dominant with marginal allografts. With the increasing demand for kidney transplantation, and more dependence on marginal allografts, novel solutions that can ameliorate IR injury are needed. In our study cold machine perfusion with AL-MPS was superior to UW for the initial 4 hours of HMP and may allow earlier viability assessment. Furthermore AL-MPS HMP preservation demonstrated equivalency to UW in functional organ assessment during normothermic reperfusion. Dose related effects of the adenosine and lidocaine may be reason differences in organ viability between AL-MPS and UW were not observed. AL-MPS is the initial attempt introducing a successful cardiac preservation solution into the setting of renal HMP, and our results are promising allowing further refinement of the AL-MPS composition.

CHAPTER 6:

CAN POST ISCHAEMIC RECONDITIONING BY HMP RECOVER KIDNEYS FROM EXTREME COLD ISCHAEMIA?

6.1 Objectives

Hypothermic preservation has formed the mainstay for organ maintenance in transplantation, with SCS constituting the commonest and simplest method. Prolonged static storage is associated with deleterious graft outcomes, especially in marginal allografts. HMP preservation however has been shown to be beneficial for marginal organs promoting a washout of blood and equilibration with the perfusate medium; delivering oxygen and nutrients and removing toxic metabolites.

The use HMP to evaluate the effect of extreme CIT on parameters measured during machine perfusion was investigated. Furthermore, it aimed to establish whether HMP is able to reverse the effect of prolonged CIT and restore renal function.

6.2 Methods

6.2.1 Experimental Protocol

Eight porcine kidneys were used in this study. After retrieval one kidney of each pair underwent SCS for either 4 hours (SCS4, n=4) or 52 hours (SCS52, n=4). All kidneys after SCS underwent 10 hours of HMP reconditioning on a Waters Medical Systems RM3 device with UW solution. Heterogeneous clinical HMP pump times have been reported in the literature with averages varying between 10[831] to 31[838] hours. Thus 10 hours of HMP was considered to be a reasonable starting point for reconditioning in this extreme CIT model to allow for stabilization of HMP parameters for viability interpretation. After HMP kidneys underwent viability assessment for 2 hours using an oxygenated NMR circuit with KHB solution. The protocol for this experiment is summarised in Figure 77.

6.2.2 Graft Retrieval

Porcine kidneys were obtained from adult landrace pigs after sacrifice at a local abattoir. Local guidelines were adhered to. Organs were retrieved as per the longer ischaemia protocol detailed in Section 3.1.3.1. Kidneys experienced 45 minutes of in-situ warm ischemia prior to retrieval, benching and flushing. Kidneys were flushed at a pressure of 100cmH_20 with 1L of Eurocollins solution (Soltran, Baxter, Compton, UK). At the laboratory grafts were further benched for HMP and the renal artery cannulated for perfusion.

6.2.3 Hypothermic Machine Perfusion

Kidneys were perfused hypothermically (5-7˚C) on two commercially available Waters Medical RM3 perfusion units for 10 hours. 1L of preservation solution was used in either circuit. Perfusion was based on clinical protocols[1114] with a target systolic perfusion pressure of 40mmHg by 15 minutes of perfusion. Volume driven flow was then adjusted at 1, 2 and 3 hours of HMP to maintain a target systolic pressure of 40-45mmHg, and then left to take its course.

6.2.4 Reperfusion Circuit

After 10 hours of HMP kidneys were connected to a NMR circuit to assess organ viability. Reperfusion with KHB was performed after a 20 minute re-warming period at room temperature to

mimic the implantation of the kidney into the recipient. The Waters Medical RM3 units were modified to facilitate normothermic reperfusion by in line incorporation of an automated temperature controlled water pump (Grant) with the target temperature set to 39°C. The circuit was primed with 1L of KHB supplemented with 2g glucose, 1.8mg H_2CO_3, 10g/L (1%) BSA and oxygenated by bubbling a mixture of 95% O_2 / 5% CO_2 gas mixture through RM3 cassette arterial reservoir. The ureter was cannulated to allow collection of urine. Urine volume was replaced with fresh KHB. Reperfusion was maintained for 2 hours. Initially systolic perfusion pressure was set to 40 mmHg and gradually increased to 55 mmHg within the first 10 minutes and then maintained between 55-60mmHg.

Outcome Measures

Real-time perfusion dynamics (perfusion pressures, flow rates, and resistances) were recorded throughout HMP and reperfusion. Kidneys were weighed pre-HMP, post-HMP, and post-Reperfusion, to quantify oedematous change. During reperfusion urine output was measured and iso-volumetrically replaced using KHB with 1% BSA. Blood gas analysis was performed real-time on arterial and venous reperfusate samples at 30mins, 1 hour and 2 hours of reperfusion and allowed calculation of oxygen consumption.

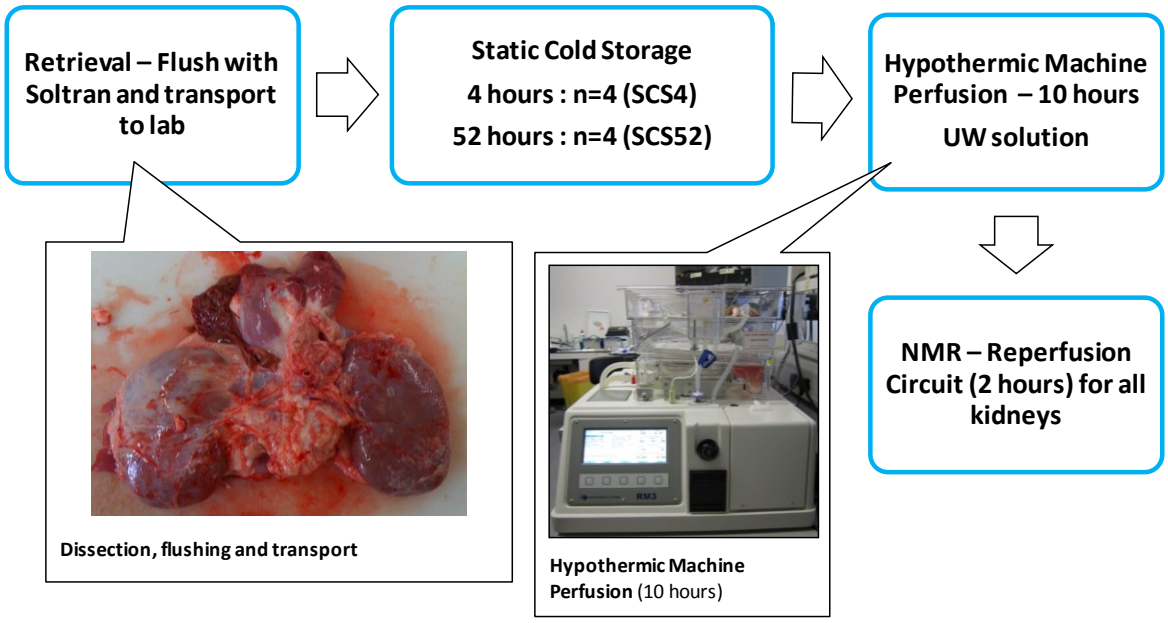


Figure 77 Diagram of experimental protocol investigating potential for hypothermic reconditioning after an lengthy cold storage period.

6.2.5 Analytical methods

During HMP and reperfusion, recorded flow rates were adjusted for kidney weight to calculate flow rate per 100g (ml/min/100g). PFI (ml/min/100g/mmHg) values which incorporate flow rate and pressure per 100g of tissue were calculated for each kidney. Oxygen consumption was calculated as (difference between arterial and venous partial pressure of oxygen) × flow rate/100g of tissue.

6.2.6 Rapid sampling microdialysis

RsMD was instituted in these experiments in line with the methodology described in Project 1. In brief microdialysis probes (MAB11.35.4, Microbiotech, Stockholm, Sweden) were inserted superficially into the parenchyma of the lateral cortex at the start of HMP. These were connect to an in-house built rapid-sampling microdialysis analyser. Every 30 seconds a 200 nL dialysis sample was automatically injected into the analysis flow stream, alternating between each kidney. The stream accelerates the dialysate through an enzyme reactor containing LOx and 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 Macbook Pro portable computer (Apple Computers, Cupertino, CA). Lactate levels were analysed in real-time to reveal information on interstitial lactate concentration during the 10 hours of HMP, and then 2 hours of normothermic reperfusion.

6.2.7 Histological Analysis

Needle core biopsies were taken pre-HMP, post-HMP, and wedge biopsies after 2 hours of normothermic reperfusion, fixed in 4% formalin, dehydrated, and embedded in paraffin wax. 4 µm sections were cut and stained with hematoxylin and eosin, (periodic acid-Schiff) for light microscopy evaluation. Tubular damage was scored for: tubular dilation, tubular debris, and nuclear fragmentation of tubular cells. were each scored over three fields using a semi-quantitative scale: 0 (normal); 1 (0-25% affected); 2 (25-50% affected); 3 (50%-75% affected) and 4 (>75% affected).

6.2.8 Statistical Methods

Values are represented as mean and standard deviation. Continuous variables were plotted as levels versus time curves for each kidney and the mean area under the curve calculated. Continuous variables from paired contra-lateral kidneys were evaluated using the paired students T-test or non-parametric Wilcoxon signed rank test when distribution assumptions were not met. Histology scores were compared using the Wilcoxon signed rank test. Data and statistical analysis was performed using Microsoft® Excel Software (Reading, UK). All tests were 2-tailed and a p value of ≤ 0.05 was considered significant.

6.3 Results

6.3.1 Hypothermic Machine Perfusion

6.3.1.1 Perfusion Dynamics

Perfusion dynamics during HMP are shown in Figure 78. There were no significant differences in overall perfusion dynamics during HMP between SCS4 and SCS52 kidneys. However the perfusion time necessary to reach viable perfusion parameters (measured resistance <0.5 mmHg/ml/min, and calculated PFI >0.4 ml/min/100g/mmHg) was significantly less in SCS52 (Resistance: 165±90 minutes, PFI: 120±85 minutes) than SCS4 kidneys (Resistance: 450±180 minutes, PFI: 330±180 minutes; p=0.008, p=0.027 respectively).

6.3.2 Normothermic Reperfusion

6.3.2.1 Reperfusion Dynamics

After 2 hours of NMR reperfusion dynamics were not significantly different between SCS4 and SCS52 kidneys (see Figure 79). (Systolic pressure: SCS4=55.7±1.3, SCS52=58.1±0.7 mmHg, p=0.080; Reperfusion flow rate: SCS4=69.6±19.64, SCS52=73.2±31.6 ml/min/100g, p=0.853; Resistance: SCS4=0.11±0.02, SCS52=0.19±0.14 mmHg/ml/min, p=0.305; PFI: SCS4=1.25±0.36, SCS52=1.26±0.53 ml/min/100g/mmHg, p=0.976).

6.3.2.2 Functional Assessment

Urine output during NMR was significantly higher in SCS4 kidneys (See Figure 80). (Urine output 60mins: SCS4= 0.71±0.21, SCS52=0.27±0.06 ml/hour/g, p=0.034; Urine output 120 minutes: SCS4= 1.03±0.19, SCS52=0.32±0.17 ml/hour/g, p<0.001). Total oxygen consumption during NMR was similar between kidneys (SCS4: 6.1 ± 5.6, SCS52: 5.7 ± 2.5 kPa/min/ml/g, p=0.894).

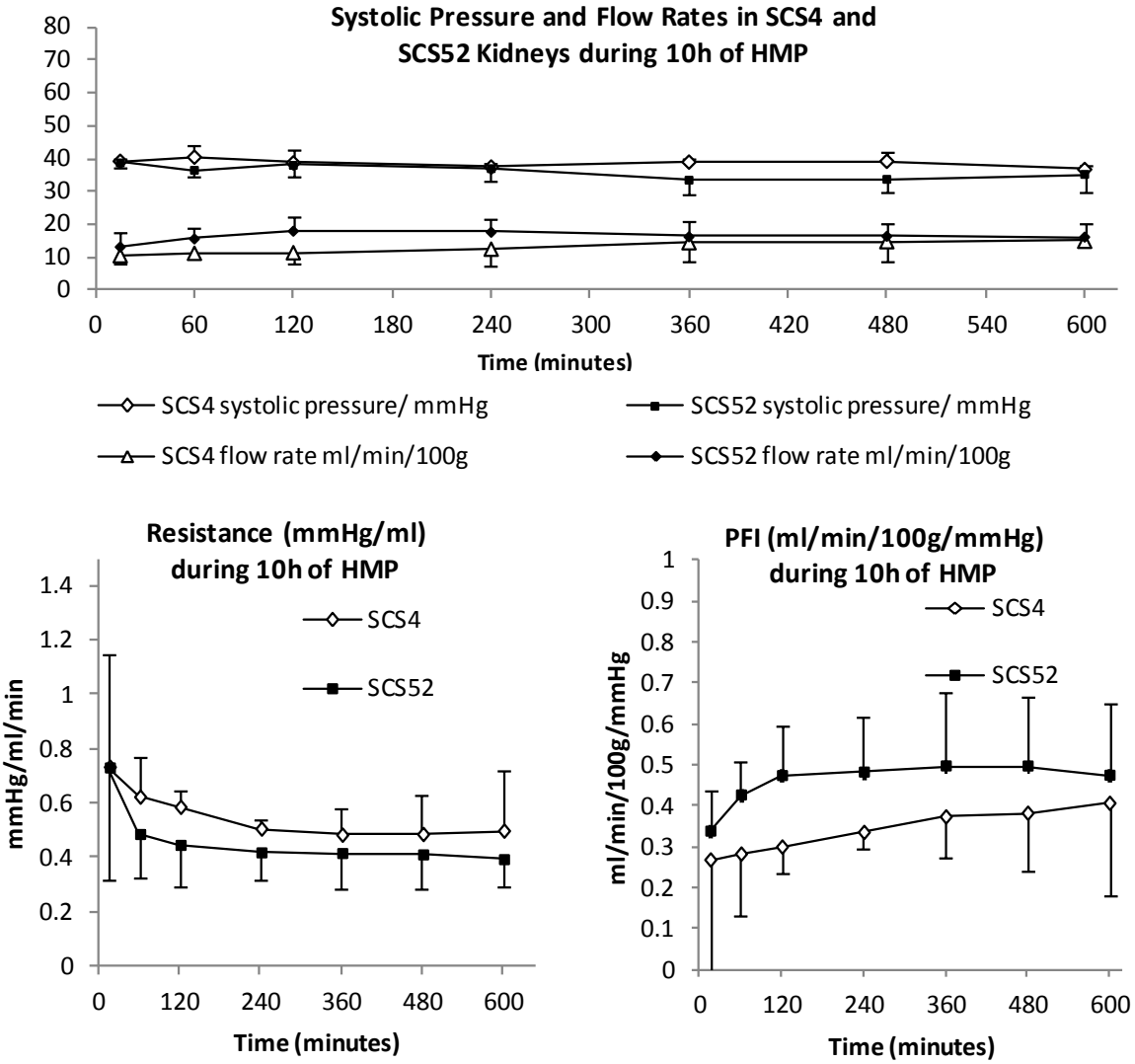


Figure 78 Hypothermic machine perfusion dynamics in SCS4 and SCS52 kidneys. There were no significant differences in HMP perfusion parameters between kidneys.

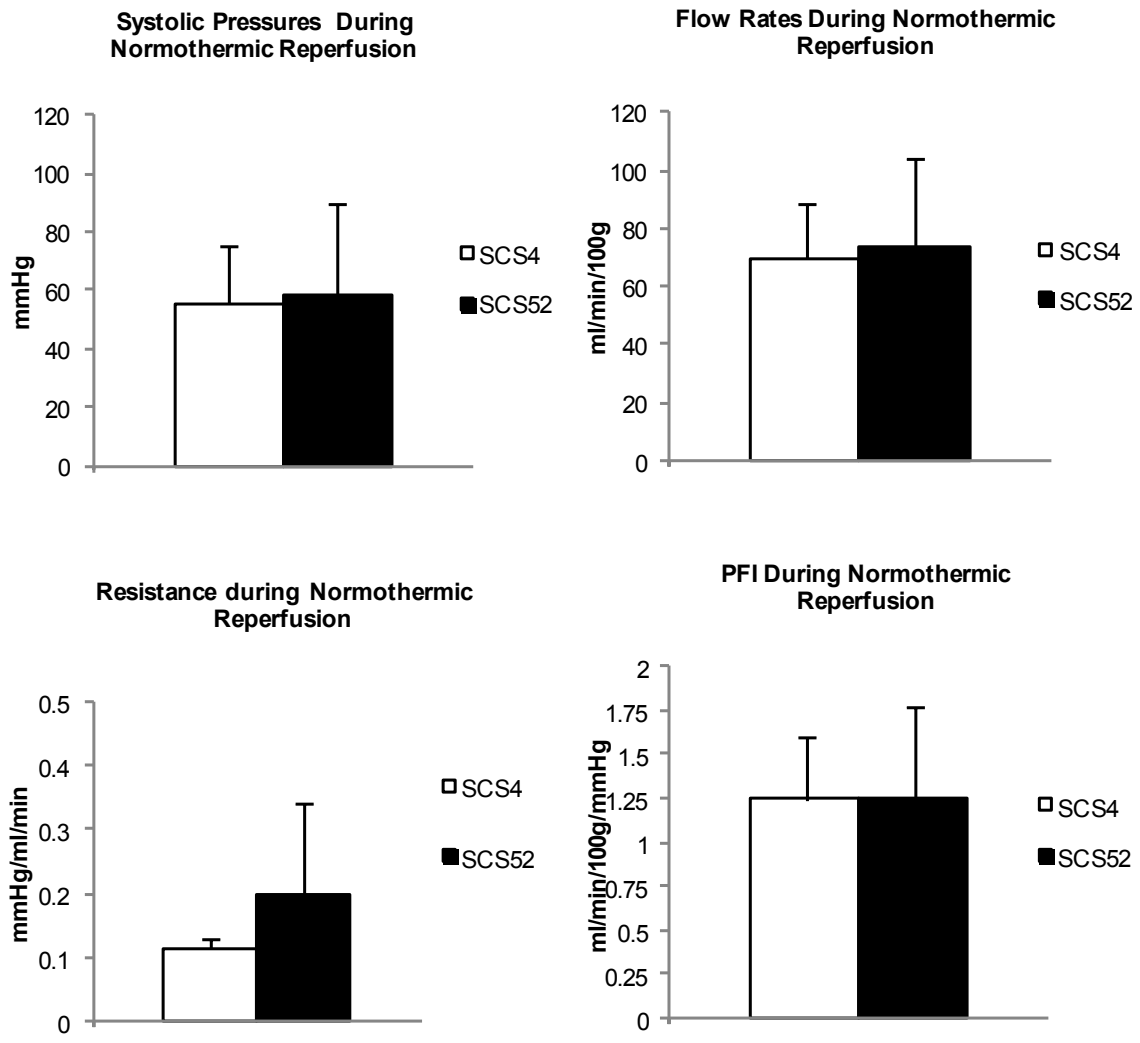


Figure 79 Normothermic reperfusion dynamics in SCS4 and SCS52 kidneys. There were no significant differences in NMR reperfusion parameters between kidneys.

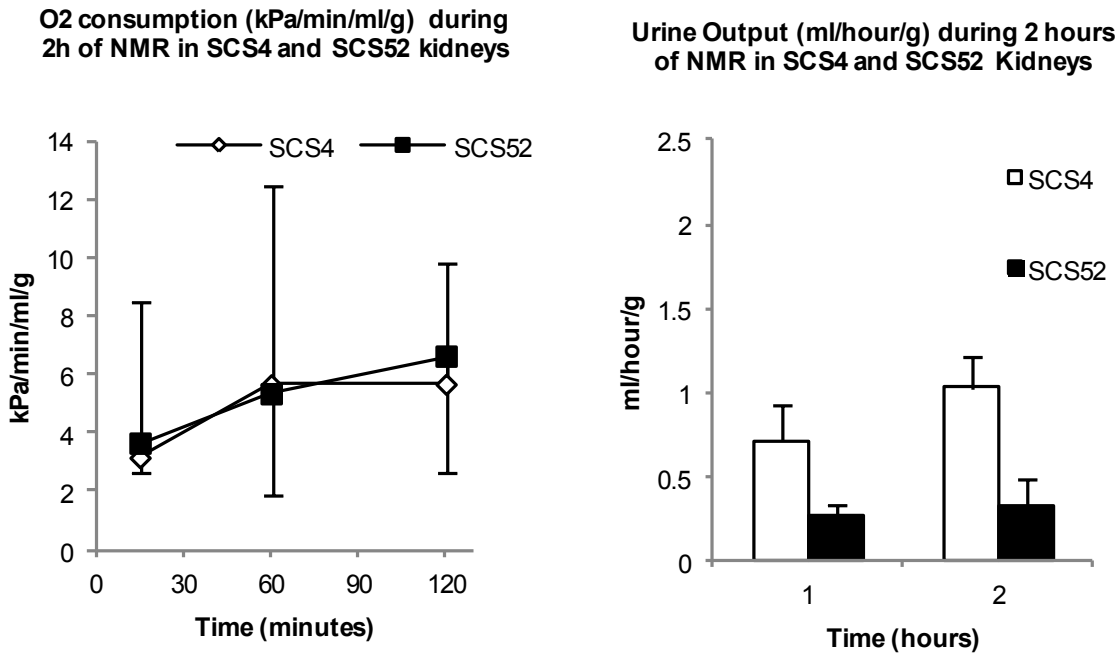


Figure 80 Functional assessment in SCS4 and SCS52 kidneys. Urine output during NMR was significantly higher in SCS4 kidneys. Urine output 60mins: SCS4= 0.71±0.21, SCS52=0.27±0.06 ml/hour/g, p=0.034; Urine output 120 minutes: SCS4= 1.03±0.19, SCS52=0.32±0.17 ml/hour/g, p<0.001). Total oxygen consumption during NMR was similar between SCS4 and SCS52 kidneys.

6.3.2.3 Rapid sampling microdialysis

Real time cortical tissue lactate concentrations were quantified using rsMD in kidneys during HMP (SCS4:n=4, SCS52:n=2) and NMR (SCS4:n=2, SCS52:n=2, Figure 81). During HMP cortical microdialysis lactate concentration trended higher between 1.5 and 10 hours in SCS4 and SCS52 kidneys, with overall lactate levels higher in SCS4 vs. SCS52 kidneys, though these differences were not significant (p=0.288, p=0.507, p=0.248 respectively).

During NMR detected lactate trended 2 fold higher in SCS4 kidneys (SCS4: 1.07 ±0.41 mM/min, SCS52: 0.50 ± 0.01 mM/min; p=0.285). and lactate increased between the start and end of NMR in both SCS4 (Start: 0.59 ± 0.23 mM ; End: 1.94 ± 1.04 mM, p=0.255) and SCS52 (Start: 0.2 ± 0.04 mM ; End: 1.06 ± 0.44 mM, p=0.241) kidneys by 3.3 and 5.4 times respectively (p=0.432). Cortical lactate correlated with perfusate lactate which also trended higher in SCS4 kidneys (SCS4: 3.7 ± 0.39, SCS52: 3.0 ± 0.69 mmol/L, p=0.055) with the difference bordering significance.

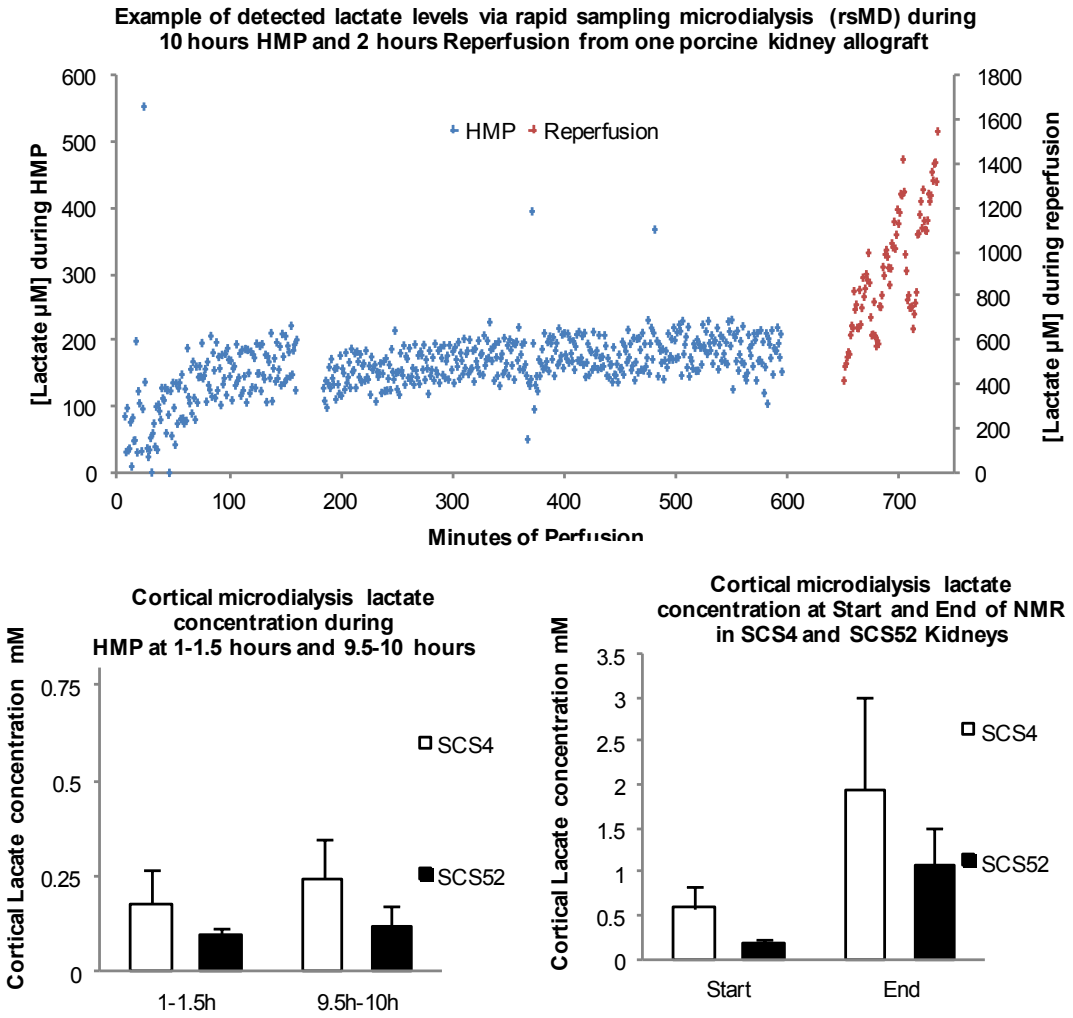


Figure 81 RsMD for tissue lactate during HMP and NMR in SCS4 and SCS52 kidneys. Example scatter diagram of rsMD readings measured from one kidney during HMP and NMR (Top). Average cortical tissue lactate during at 1.5 hours and 10 hours of HMP in SCS4 and SCS52 kidneys (bottom left). Average cortical tissue lactate during the NMR in SCS4 and SCS52 kidneys at start (1st 20mins) and end (last 20 minutes; bottom right).

6.3.2.4 Graft Weight Change

Kidney weight in the SCS4 group remained unchanged between flushing and the end of cold storage, while SCS52 kidneys lost weight (-17.1 %, p=0.012) after 52 hours of cold storage. Weight change after HMP (SCS4: 43.1 ±28.1%; SCS52: 46.3 ± 5.2%, p=0.826) and NMR (SCS4: 8.1 ±16.2%; SCS52: 13.4 ± 10.6%, p=0.092) was not different between both groups of kidneys (see Figure 82).

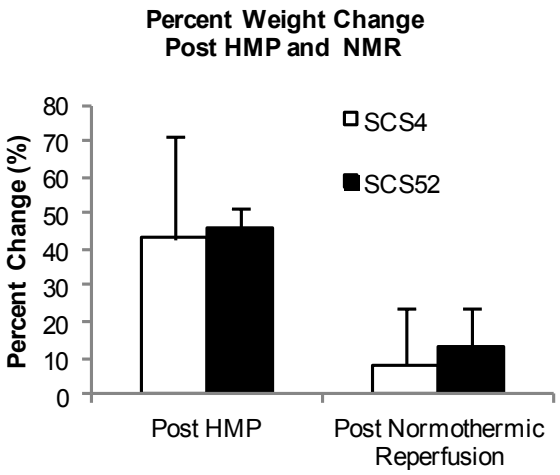


Figure 82 Percent weight change in SCS4 and SCS52 kidneys during HMP and NMR.

6.3.2.5 Histology

There were no significant differences in core biopsies between SCS4 and SCS52 kidneys in pre-HMP and Post-HMP samples. After NMR all kidneys exhibited evidence of reperfusion induced tubular damage as measured by tubular debris, tubular dilation, and fragmented nuclei, however there were no significant differences between SCS4 and SCS52 kidneys, see Table 13 and Figure 83.

Parameter	Tubular Debris		Tubular Dilation		Nuclear Fragmentation	
Group	SCS4	SCS52	SCS4	SCS52	SCS4	SCS52
Score	3.5 ± 0.58	4.0 ± 0	2.3 ± 0.5	2.7 ± 0.6	2.1 ± 0.2	2.3 ± 0.3

Table 13 Histology scores of biopsies taken after 2 hours of NMR assessing tubular debris, tubular Dilatation, and nuclear fragmentation. There was no significant difference in the degree of tubular damage in SCS4 and SCS52 kidneys.

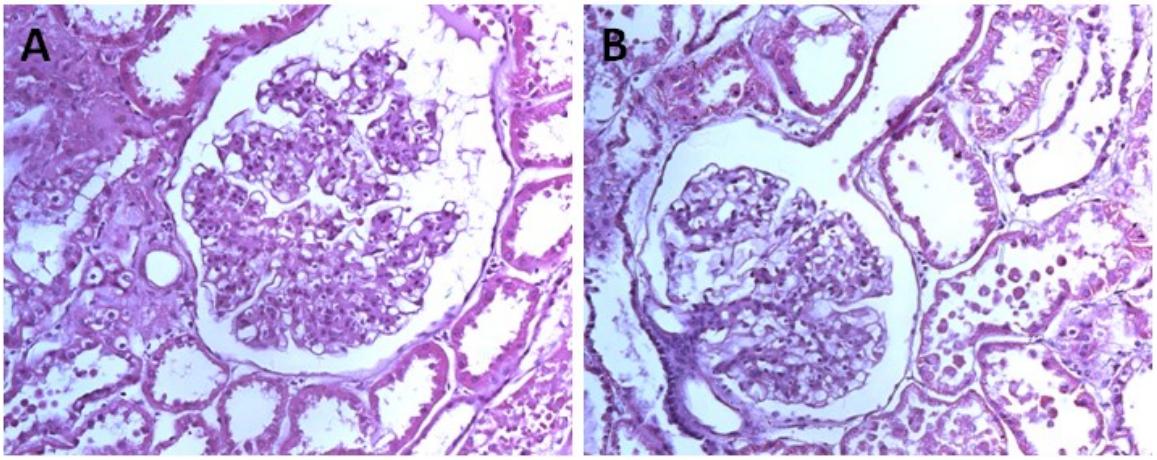


Figure 83 Photomicrographs of light microscopy of SCS4 vs. SCS52 kidneys after 2 hours of NMR. H&E staining, x20. A: SCS4 kidney, B: SCS52 kidney. Showing similar degrees of reperfusion injury.

6.4 Discussion

HMP reconditioning of kidneys after cold storage offers the possibility of re-vitalising allograft viability and measuring metrics for viability assessment, especially in 'marginal' organs[1126]. Characterising the potential for HMP reconditioning in DCD grafts exposed to long durations of cold storage is of particular interest for the successful expansion of the donor pool.

6.4.1 Effect of Long SCS on Post-Ischaemic HMP Dynamics

We have shown that HMP after an period of extreme SCS CIT in DCD porcine kidneys yields perfusion parameters indicative of suitable organ viability for transplantation[841], but with no demonstrated benefit on kidney function during isolated reperfusion.

Measured renal resistance and calculated PFI during HMP are used by clinicians as measures of renal viability[841] and resistance has been shown to be an independent risk factor for primary non-function[1127]. Perfusion dynamics are used by some centres to determine organ discard[856], though conservative advocates suggest such parameters to be used as only one of several factors in determining organ transplantability[1061,1127]. Furthermore resistance during HMP has been shown to correlate with the post-transplant reperfusion renal resistivity index[862].

Overall HMP perfusion dynamics were similar in kidneys previously exposed to relatively short SCS of 4 hours and extreme SCS of 52 hours. Furthermore if clinical HMP viability criteria[841] (Resistance and PFI levels) were applied SCS52 kidneys attainted levels of accepted viability significantly earlier during HMP compared to SCS4 kidneys with shorter cold ischaemic injury.

Lower HMP flow rates, high resistance, and low PFI values are associated with poorer graft outcomes. In this experiment the severely injured kidneys grafts perfused as well and achieved 'viability' quicker as those with limited injury. After extreme SCS there was a loss in kidney weight compared to weight recorded post flushing at retrieval. Similar weight loss is reported after extended SCS of kidney allografts by other groups[832,1128]. The weight reduction arises from recovery from 'flush' induced oedema at retrieval, suggested to stem from malfunction of the Na+K+ pump, cellular swelling, and dehydration of the renal parenchyma during SCS[1129]. The SCS4 kidneys with limited SCS time did not experience a reduction in mass. The reduced oedema in SCS52 kidneys facilitated better perfusion during HMP with less tissue osmotic pressure resisting pulsatile flow. The SCS4 kidneys required several more hours of HMP to achieve optimal perfusion, potentially explaining the equity between these kidneys in HMP dynamics. The benefit conferred on

SCS52 kidneys however was only evident until HMP equilibration in both SCS4 and SCS52 was achieved producing a similar degree of weight gain after HMP.

6.4.2 Effect of Post-Ischaemic HMP after Extreme Length of SCS on Viability

Despite SCS4 and SCS52 kidneys also perfusing with equity during NMR, and with comparable oxygen consumption, the SCS52 kidneys demonstrated significantly depressed renal function with lower urine production. Ten hours of HMP was not sufficient to ameliorate the level of extreme SCS ischaemic injury sustained by these kidneys, and oxygenation during HMP may be a potential factor.

Conventional HMP utilizes one of several commercially available perfusion machines. Two machines widely used are the Waters Medical Systems RM3 and the Organ Recovery Systems Lifeport. HMP in routine clinical practice at present does not incorporate active oxygenation of the perfusate. Recent experimental evidence has suggested that oxygenation may be potentially beneficial and restore cellular energy stores and improve allograft function during reperfusion [1130] though this may be limited to specific donor types[1131]. The RM3 unit used in our laboratory allows for passive oxygenation while the Lifeport provides no option for active or passive oxygenation. In our study passive oxygenation with room air on a RM3 was used during HMP as in clinical practice, this provides a perfusate oxygen pressure of approximately 24 kPa (180 mmHg) [805] which is theoretically sufficient for tissue needs at 4-7˚C [722,730] sustaining kidney metabolism. Active oxygenation delivering a higher partial pressure of oxygen may however afford further benefit in kidneys that have sustained severe ischaemic stress, such as those in the SCS52 group.

Furthermore pulsatility during HMP has been shown to mediate protective effects on the vasculature endothelial, with pulsatile perfusion limiting pro-inflammatory cytokine expression with reduced tissue expression of ICAM-1 [1132], and reduced TLR-4 mRNA levels[812]. It is now known that endothelial gene expression is strongly influenced by vascular shear stress [822]. During organ retrieval and SCS the cessation of vasculature flow promotes a pro-inflammatory and pro-coagulatory phenotype, which could worsen IR responses after transplantation [263]. Flow mediated endothelial dysfunction is linked to decreased expression of the mechanosensitive transcription factor Kruppel-like-factor 2 (KLF-2), which normally suppresses inflammatory signals and cytokine production[261]. HMP has been shown to restore levels of KLF-2 compared to SCS during reperfusion[823]. Because the NMR model utilized acellular KHB the full benefits of HMP mediated endothelial protection against IR injury may not have been fully appreciated without the added stress of the cellular and plasma components evident in a normothermic haemoreperfusion model.

rsMD observed a trend of higher cortical tissue lactate both during HMP and NMR in the SCS4 kidneys exposed to a shorter period of SCS. Lactate is the terminal product of anaerobic glycolysis; the primary source of ATP production during ischaemia[1133]. The kidney itself however is also an endogenous source of lactate during physiological aerobic conditions[1051]. Despite neither difference reaching significance an explanation for this trend may lie in SCS4 kidneys being intrinsically more viable due to the shorter SCS CIT. Cortical cells were able to undergo anaerobic glycolysis to either augment low grade aerobic energy production during passively oxygenated HMP, or simply maintain physiological lactate production.

This greater viability can be extrapolated to explain the higher lactate during NMR in the SCS4 kidneys, when lower levels would be expected vs. SCS52 kidneys, where full provision of oxygen would abort the need for anaerobic metabolism. In fact during IR a degree of anaerobic metabolism is necessary as the mitochondrial pathways required for aerobic ATP production are repaired[1134]. Though raised tissue lactate is indicative of allograft thrombosis and tissue ischaemia, this correlation is accurate in a transplantation[1059] or isolated haemoreperfusion model, where microcirculatory dysfunction can affect tissue perfusion and oxygen delivery. In this experiment with KHB such microcirculatory failure is absent, and augmented cortical lactate production suggests improved cortical cell viability.

However the sustained production of cortical lactate by the SCS52 kidneys during NMR in a comparable trend to the SCS4 kidneys further suggests a degree of viability in the renal parenchyma of these kidneys which was not expected. This may be related to the initial finding of reduced post-ischaemic graft weight before HMP in those kidneys. A longer CIT that allows greater equilibration between the solution and the organ parenchyma may facilitate better preservation and buffering, which then allows improved HMP perfusion due to less graft oedema. Thus though shorter lengths of CIT are invariably more desirable and less damaging than longer CITs, a longer CIT may confer a better SCS preservation effect from the solution. The longer term effect of this better equilibration however on renal function is unknown but the possibility that kidneys initially considered to be extremely marginal due to lengthy cold ischaemia may actually be useable after a period of post-ischaemic HMP deserves further thought and investigation.

6.4.3 Mechanisms of Effect of Post-Ischaemic HMP

Koetting et al.[800] demonstrated the benefit of 90 min of hypothermic reconditioning by oxygenated HMP compared to non-oxygenated HMP to recover organs after prolonged (18 hours)

SCS ischemic injury. No comparison was reported on HMP perfusion dynamics, but oxygenation produced substantial 3-fold higher clearances of creatinine and urea, and less apoptotic caspase-3 activity during organ viability assessment on an isolated reperfusion circuit. The authors concluded that continuous oxygenation during HMP effectively ameliorated energetic breakdown and deterioration of mitochondrial redox balance. The same group in another study using a porcine renal autotransplantation model demonstrated that a short period (2 hours) of reconditioning after prolonged (21 hours) SCS proved as effective as continuous HMP for the whole storage time [812]. Active oxygenation was provided in both continuous HMP and 2 hours of post-ischaemic reconditioning HMP, and equally improved urea and creatinine clearance, as well as tubular function, with lower expression of TLR-4, lower HMGB-1 release. A short period of HMP even after extended SCS can also normalise expression of KLF-2 reducing the pro-inflammatory endothelial phenotype[823]. Furthermore a period post-ischaemic reconditioning of even 1 hour duration is as effective as 4 hours, after 18 hours of SCS, with improved creatinine clearance, tubular reabsorption, and renal flow on an isolated reperfusion circuit[1025]. Additionally the use of pulsatile flow in HMP appears to be the important factor in HMP vs. non-pulsatile flow to ensure benefits of HMP are realized compared to SCS[801,823].

6.4.4 Limitations

There are several potential caveats to the interpretation of this study. The HMP dynamics and effects on HMP on kidney viability in the SCS4 group was considered in this model to reflect ideal kidneys with minimal CIT injury, and a suitable control for kidneys exposed to prolonged SCS prior to HMP, with the objective to determine the differences between kidneys exposed to different durations of CIT. The next evolution of these experiments will investigate the benefit of a period of HMP or not after exposure to extreme SCS CIT.

The significance of differences observed in the experimental groups may have been limited by the use of four pairs of kidneys for experimentation. Furthermore due to technical problems with the rsMD apparatus data was not available for all kidneys during both HMP and NMR (data available for 6 kidneys during HMP, and 4 during NMR).

During NMR renal urine output was measured however calculation of glomerular filtration by biochemical analysis of the perfusate and urine would provide a greater assessment of glomerular and tubular function, this resource however was not available during these experiments.

NMR using acellular KHB is useful for acute determination of renal function and viability, however isolated haemoreperfusion would provide a more physiological assessment and challenge in kidney grafts.

rsMD of tissue biomarkers concentrated on lactate as a marker of ischaemic injury, however detection of other glycolytic molecules such as glucose, pyruvate and ATP would provide a greater assessment of tissue metabolism. Finally HMP was only passively oxygenated as per clinical practice with the RM3 unit. There is however a potential benefit of actively oxygenated HMP after extreme SCS on renal viability which is another avenue of investigation.

6.4.5 Future Considerations

As post-ischaemic HMP reconditioning emerges as a viable technique for revitalising marginal allografts[1135] consideration must be made as to the length of SCS from which the allograft is being recovered. In our study porcine kidneys exposed to extreme CIT demonstrated HMP dynamics suggestive of viability with equity to minimally injured grafts. However this period of HMP reconditioning did not confer a functional advantage during isolated reperfusion. It will be important for future practitioners of this technique to be aware that graft weight loss during SCS, and hence resolution of flush induced oedema may affect perfusion dynamics during HMP. Nescessitating any subsequent viability assessment incorporating these parameters to be reconsidered. Furthermore despite the clinical success with conventional HMP, in its present form utilising passive oxygenation HMP may not be able to functionally recover grafts exposed to extreme SCS, suggesting that HMP interventions utilising active oxygenation of the perfusate may be better positioned to provide benefit in such organs.

Chapter 7:

Development of Pancreatic Machine Perfusion Models

7.1 Objectives

The objectives of this project were to develop a series of protocols for whole organ pancreas HMP as well as experimental normothermic reperfusion to allow assessment of organ viability and function. The overall goal of developing these models is to provide a superior more dynamic method of preservation, as a tool to allow assessment of pancreatic viability, and also a delivery mechanism for pharmacological intervention to optimise pancreatic integrity and viability. For example overall no strategy has yet been identified to be an effective measure in minimizing the incidence of early vascular thrombosis of a pancreatic graft[698]. An ideal option would be for a locally delivered addressive anti-coagulant which would curb graft thrombus formation without the worrisome risks associated with systemic anti-coagulation. This would also be a logical approach if indeed a local hypercoaguable state is induced in the pancreatic bed through IR injury, and an addressive anti-coagulant could be delivered using HMP.

In development of pancreatic perfusion models initially a comparison was made to determine a suitable pancreatic HMP perfusion pressure compared to a renal model of HMP using porcine grafts (Phase 1). This pressure was then used in a series of models to compare the effect of a period of post-ischaemic HMP after 24 hours of SCS to only SCS preservation in porcine pancreases, with viability determined using a normothermic reperfusion model (Phase 2). Finally the protocols developed in the porcine models were applied to a series of human pancreases to determine their applicability to clinical grafts (Phase 3). These human pancreases were grafts considered unsuitable for clinical transplantation and that had subsequently been donated for research through NHS Blood and Transplant.

7.2 Methods

7.2.1 Phase 1: Comparison of Renal and Pancreatic HMP Perfusion

Porcine pancreases and kidneys were retrieved from pigs using the shorter ischaemia protocol as described in Section 3.1.3.1. Briefly 9 kidneys were retrieved, flushed, placed on ice, and returned to the laboratory. There they underwent 5 hours of HMP on a Waters Medical Systems RM3 perfusion machine using University of Wisconsin (UW) solution as the circulating perfusate for 5 hours at 40mmHg (40 Systolic Group, 40SG, n=9). Seven porcine pancreases were similarly retrieved flushed with UW solution, placed on ice and transported to the laboratory. Four of these pancreases were based on a segmental pancreatic graft with perfusion via the celiac axis (splenic artery)through an aortic segment, outflow via the splenic and portal veins, and perfused on an RM3 with Belzer UW for 5 hours with a target systolic pressure of 30mmHg (30 Systolic Group, 30SG, n=4). The remaining three pancreases where based on whole organ grafts and similarly perfused on an RM3 for 5 hours with Belzer UW with a target perfusion pressure of <20mmHg (<20 Systolic Group, 20SG, n=3). Perfusion consisted of a priming phase (2h) followed by stable perfusion(3h). Perfusion dynamics, graft weight gain were recorded, and the PFI calculated for each organ. Belzer UW used for pancreatic HMP was modified by the addition of 60mmol/L of mannitol.

7.2.2 Phase 2: Short Post-ischaemic Pancreatic HMP vs. Pancreatic SCS

Porcine pancreases were retrieved from pigs using the shorter ischaemia protocol as described in Section 3.1.3.1. Briefly 6 porcine pancreases were retrieved, flushed, placed on ice, and returned to the laboratory. There pancreases underwent 24 hours of SCS. After SCS 3 pancreases (SCS group, n=3) underwent viability assessment on an isolated autologous NMR circuit. The remaining three pancreases (SCS-HMP group, n=3) underwent 5 hours of HMP at low pressure (<20mmHg) with Belzer UW (see Figure 85) and then underwent 2 hours of viability assessment via NMR. The protocol for this experiment is shown in Figure 84.

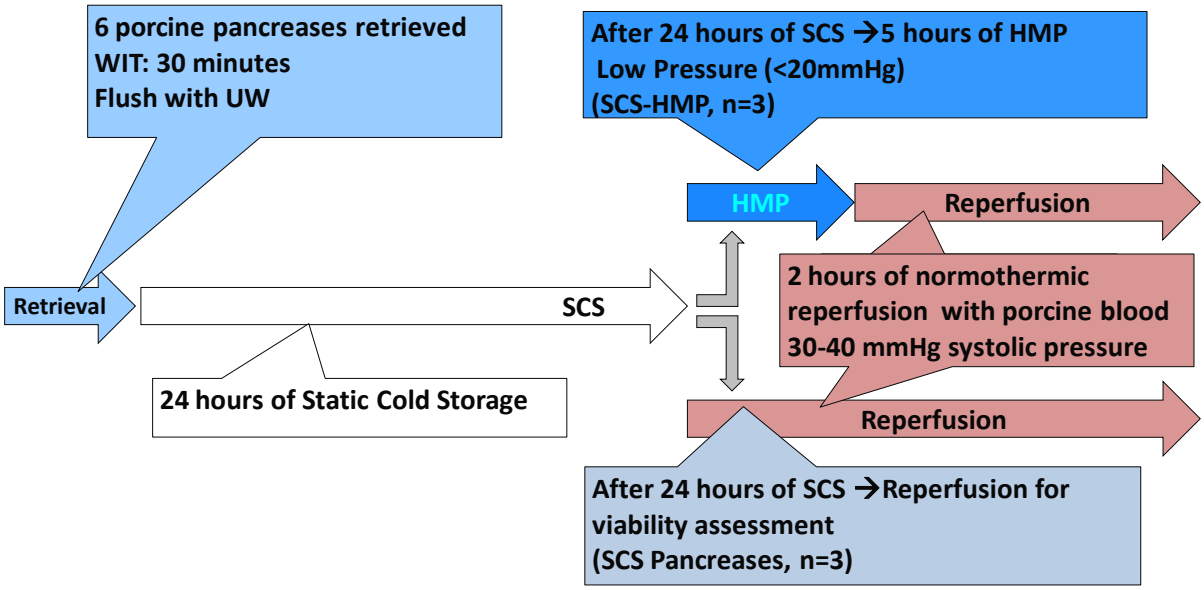


Figure 84 Diagram of experimental porcine pancreatic perfusion protocol comparing HMP to SCS. HMP consisted of a priming phase (2h) followed by stable perfusion(3h). NMR was conducted at a systolic pressure of 30mmHg. Perfusion dynamics, graft weight gain were recorded, and the PFI was calculated for each organ.

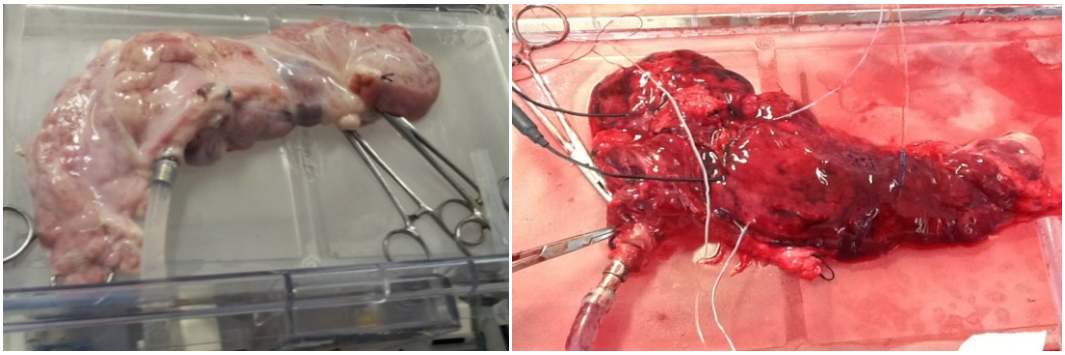


Figure 85 Whole porcine pancreatic graft undergoing HMP (left) and NMR (right).

Belzer UW used for pancreatic HMP was modified by the addition of 60mmol/L of mannitol. NMR perfusate involved a whole blood saline mixture (3:1), with 21mmol NaH_2CO_3, and oxygenated with a 95% O_2 / 5%CO_2 gas mixture.

Endocrine functionality was assessed by addition of 25mmol/l of glucose to the reperfusate at 30 minutes of NMR. Perfusate samples were collected during reperfusion, centrifuged at 3000rpm, and the supernatant stored at -80˚C. Samples were analysed at a later date for levels of glucose, insulin, c-peptide and amylase via the Chemical Pathology Department of Imperial College Healthcare NHS

Trust using routinely used assays. Exocrine functionality was assessed by cannulating the duodenum with a large bore foley catheter to collect pancreatic exocrine and bowel secretions.

Core biopsies were taken after SCS and HMP, and wedge biopsies after NMR. Biopsies were fixed in 4% formalin, dehydrated, and embedded in paraffin wax. 4 µm sections were cut and stained with hematoxylin and eosin for light microscopy evaluation. to assess for degree of reperfusion injury noting for degree of tissue necrosis and oedema.

7.2.3 Phase 3: Application of experimental pancreatic HMP protocols to human grafts

Human pancreases used in development of a direct pre-clinical model were obtained through NHS Blood and Transplant. These organs were considered to be unsuitable for clinical transplantation and that had subsequently been donated for research through NHS Blood and Transplant. Three human pancreatic grafts were used. Pancreases underwent a similar protocol consisting of 5h of HMP at low pressure (<20mmHg) and then 2h of NMR at 40mmHg. Perfusion was through a reconstructed Y-graft via the splenic and superior mesenteric arteries (see Figure 86). Perfusion dynamics, graft weight gain were recorded, and the PFI was calculated for each organ.



Figure 86 Human pancreatic graft undergoing HMP and NMR. Perfusion is via reconstructed Y graft. HMP (left) and NMR (right).

NMR utilised a KHB based solution as the reperfusate and included supplementation with 21mmol NaH_2CO_3, and oxygenated with a 95% O_2 5%CO_2 gas mixture.

Endocrine functionality was assessed by addition of 25mmol/l of glucose to the reperfusate at 30 minutes of NMR. Perfusate samples were collected during reperfusion, centrifuged at 3000rpm, and

the supernatant stored at -80˚C. Samples were analysed at a later date for levels of glucose, insulin, c-peptide and amylase via the Chemical Pathology Department of Imperial College Healthcare NHS Trust using routinely used assays. Exocrine functionality was assessed by cannulating the duodenum with a large bore foley catheter to collect pancreatic exocrine and bowel secretions.

Core biopsies were taken after SCS and HMP, and wedge biopsies after NMR. Biopsies were fixed in 4% formalin, dehydrated, and embedded in paraffin wax. 4 µm sections were cut and stained with hematoxylin and eosin for light microscopy evaluation to qualitatively assess for reperfusion injury noting for degree of tissue necrosis and oedema.

7.2.4 Rapid sampling microdialysis

The rsMD system was used to sample and quantify levels of tissue lactate in pancreases during both HMP and NMR this was instituted in line with the methodology described in Project 1. In brief microdialysis probes (MAB11.35.4, Microbiotech, Stockholm, Sweden) were inserted superficially into the ventral section of the pancreatic tail at the start of HMP. These were connect to an in-house built rapid-sampling microdialysis analyser. Every 30 seconds a 200 nL dialysis sample was automatically injected into the analysis flow stream, alternating between probes in two pancreases. The stream accelerates the dialysate through an enzyme reactor containing LOx and 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 Macbook Pro portable computer (Apple Computers, Cupertino, CA). Lactate levels were analysed in real-time to reveal information on interstitial lactate concentration after the 24 hours of SCS, during 5 hours of HMP, and then 2 hours of NMR.

7.2.5 Statistical Analysis

Values are represented as mean and standard deviation. Continuous variables were plotted as levels versus time curves for each organ and the mean area under the curve calculated. Where continuous variables were compared significance was assessed using a student's T-test or the non-parametric

equivalent when distribution assumptions were not met. In a comparison of more than 2 groups one way ANOVA with Bonferroni correction post-test was utilised. Data and statistical analysis was performed using Microsoft® Excel Software (Reading, UK). All tests were 2-tailed and a p value of ≤ 0.05 was considered significant.

7.3 Results

7.3.1 Examination of Kidney and Pancreas Machine Perfusion Dynamics

7.3.1.1 Organ Characteristics

40SG kidneys experienced 18.3 ±9.7 minutes of WIT, and 12.5 ±9.7 hours of CIT (range: 3.4-26 hours) prior to HMP. 30SG pancreases experienced 38.7 ±11.1 minutes of WIT, and 4.0 (±2.0) hours of CIT (range: 3-7 hours) prior to HMP, and all 20SG pancreases experienced equal WIT of 30 minutes and CIT of 26.2 ± 0.3 hours.

WIT in 40SG was significantly shorter than in 30SG pancreases (p=0.003), but not 20SG pancreases (p=0.082). All pancreases experienced statistically similar WITs (p=0.239). CIT was significantly longer in 20SG pancreases compared to 30SG pancreases (p=0.002) and 40SG kidneys (p=0.016), which also had similar CITs.

7.3.1.2 Hypothermic Machine Perfusion

Systolic pressures were different between all three perfusion groups (SG40: 40.5 ± 2.6 mmHg; SG30: 30.5 ±5.1 mmHg; SG20: 16.5 ± 5.4 mmHg; ANOVA $p<0.001$, $p<0.001$ for all comparisons).

Overall flow rates were similar in 40SG kidneys 32.6 ± 7.6 ml/min/100g and 30SG pancreases 26.5 ± 2.1 ml/min/100g, and lower in 20SG pancreases 12.9 ± 0.5 ml/min/100g compared to SG40 kidneys (p=0.002), but not SG30 pancreases.

Despite differing perfusion pressures and flow rate the PFI were similar between all three groups SG40: 0.81 ± 0.2 ml/min/100g/mmHg; SG30: 0.91 ± 0.21 ml/min/100g/mmHg; SG20: 0.83 ± 0.23 ml/min/100g/mmHg; ANOVA p=0.67) implying a similar ratio between pressure and flow dynamics. Perfusion dynamics are shown for all three groups in Figure 87, and average perfusion dynamics during HMP in Figure 88.

Weight gain was highest (95.2 ± 37.5%) in 30SG pancreases compared to SG40 kidneys (36.8 ± 13 %, p=0.002) and 20SG Pancreases (21.5 ± 8.7%, p=0.004), but not statistically different between SG40 kidneys and SG20 pancreases (p=0.410), see Figure 89.

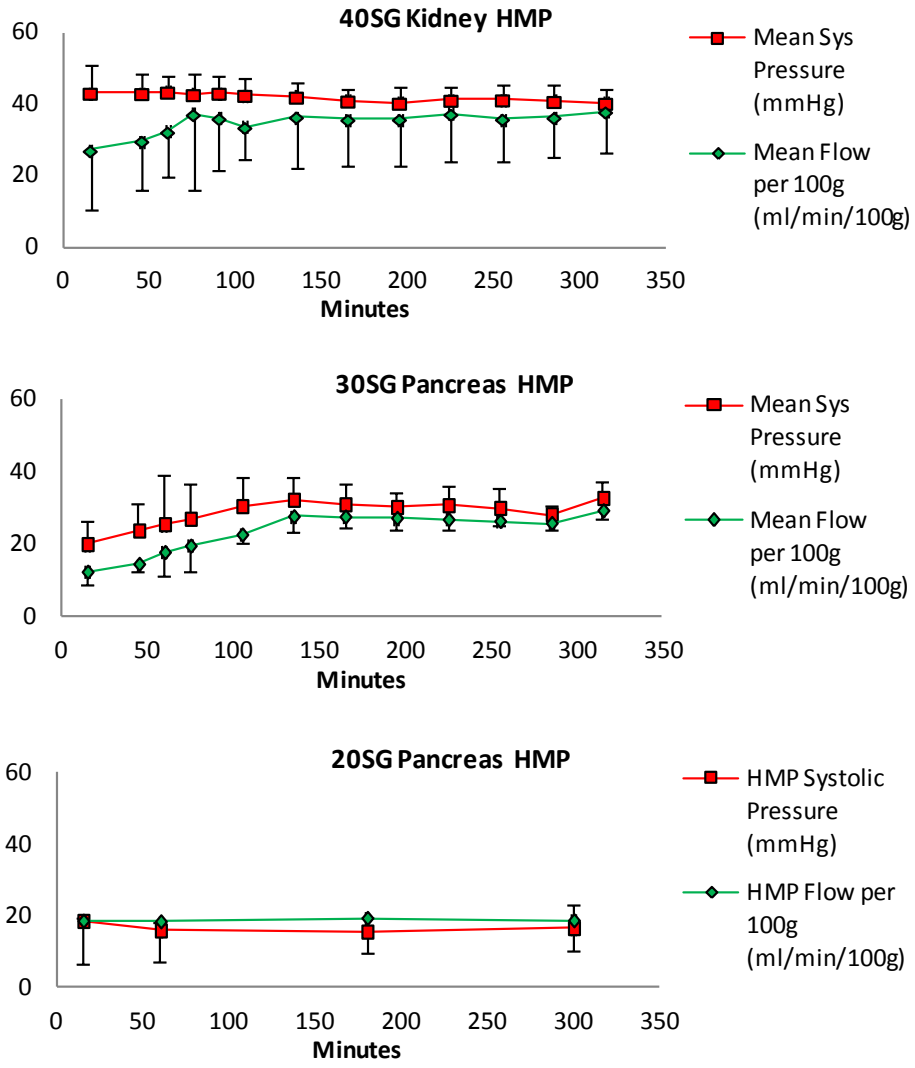
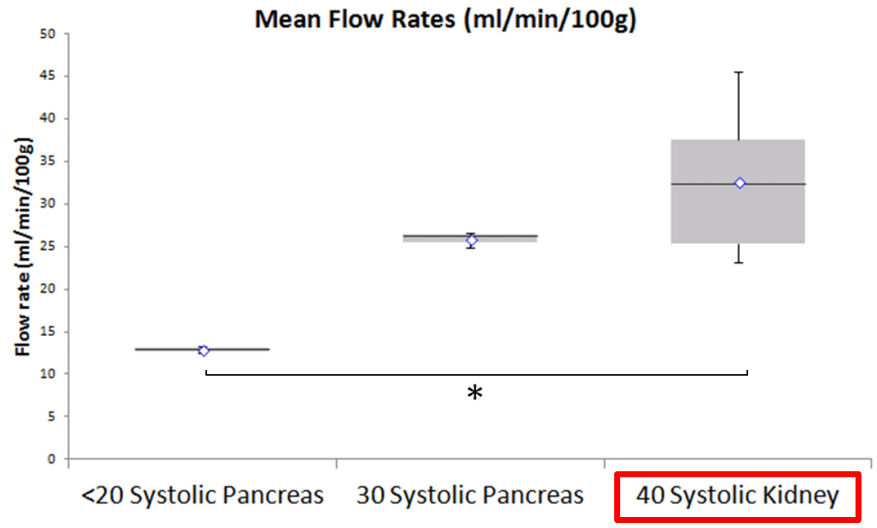


Figure 87 Perfusion dynamics comparing renal HMP to Pancreatic HMP. Kidneys were perfused at a higher pressure (Target: 40mmHg) compared to pancreases (30mmHg and 20mmHg). Flow rates and PFI were similar between all three perfusion groups.



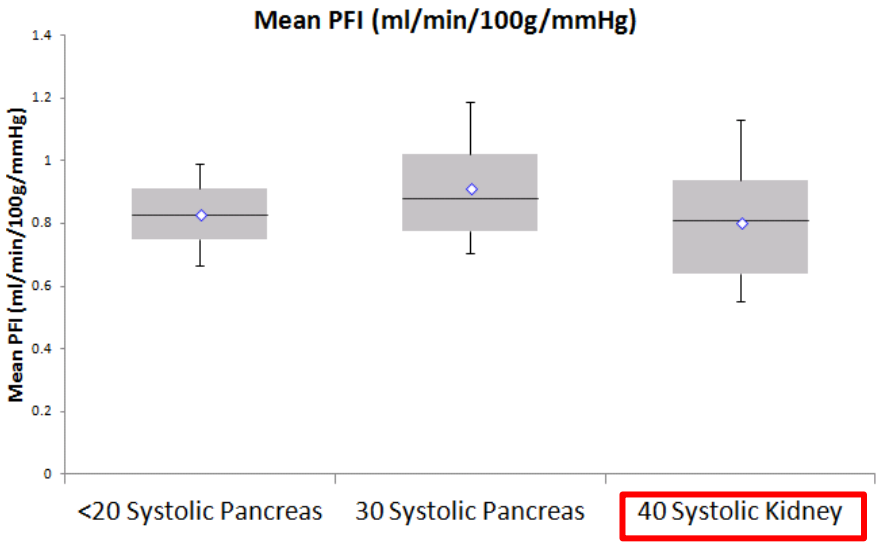


Figure 88 Average flow rates and PFI during HMP of kidneys perfused at 40 mmHg systolic pressure, and both pancreases perfused at 30mmHg systolic and 20mmHg systolic pressure. Flow rates were significantly less in the 20 Systolic Group pancreases vs. the 40 Systolic Group kidneys (p=0.002).

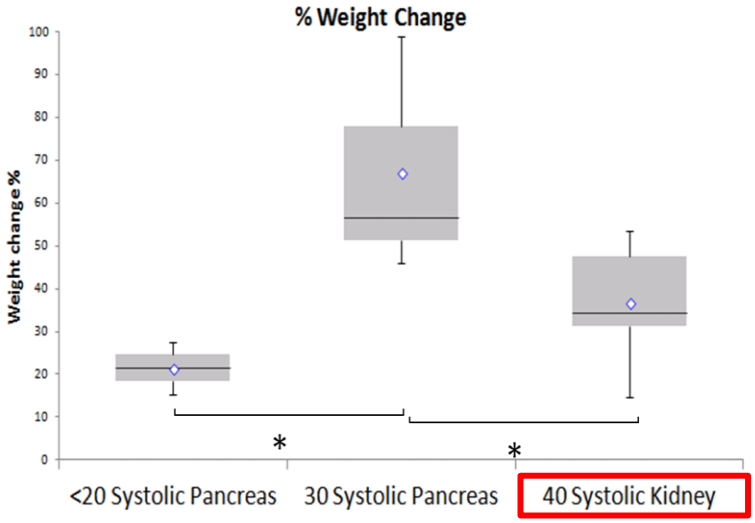


Figure 89 Percent weight change during HMP in kidneys and pancreases. 40 Systolic Group kidneys and 20 Systolic group pancreases, had significantly post HMP weight increases vs. 30 Systolic group pancreases (p=0.002, p=0.004 respectively).

WIT were the same (30 minutes) and CIT similar (26.8 ±0.35 hours vs. 26.2 ± 0.26 hours, p=0.23) in the SCS Group and SCS-HMP Group pancreases.

7.3.2.1 Hypothermic Machine Perfusion

During 5 hours of HMP at low pressure of 16.5 ± 3.5 mmHg, flow rates were stable at an average of 19.4 ± 10.1 ml/min/100g. Calculated PFI increased from 0.99 ± 0.61 ml/min/100g/mmHg after 15 minutes of perfusion to 1.35 ± 0.9 ml/min/100g/mmHg by 3 hours remaining stable until end of HMP (1.3 ± 0.83 ml/min/100g/mmHg at 5 hours). Machine measured resistance remained low throughout HMP at 0.15 ±0.04 mmHg/ml/min. Weight change after HMP was 19.4 ± 7.1 %. Perfusion dynamics and weight change are shown in Figure 90 and Figure 91 respectively.

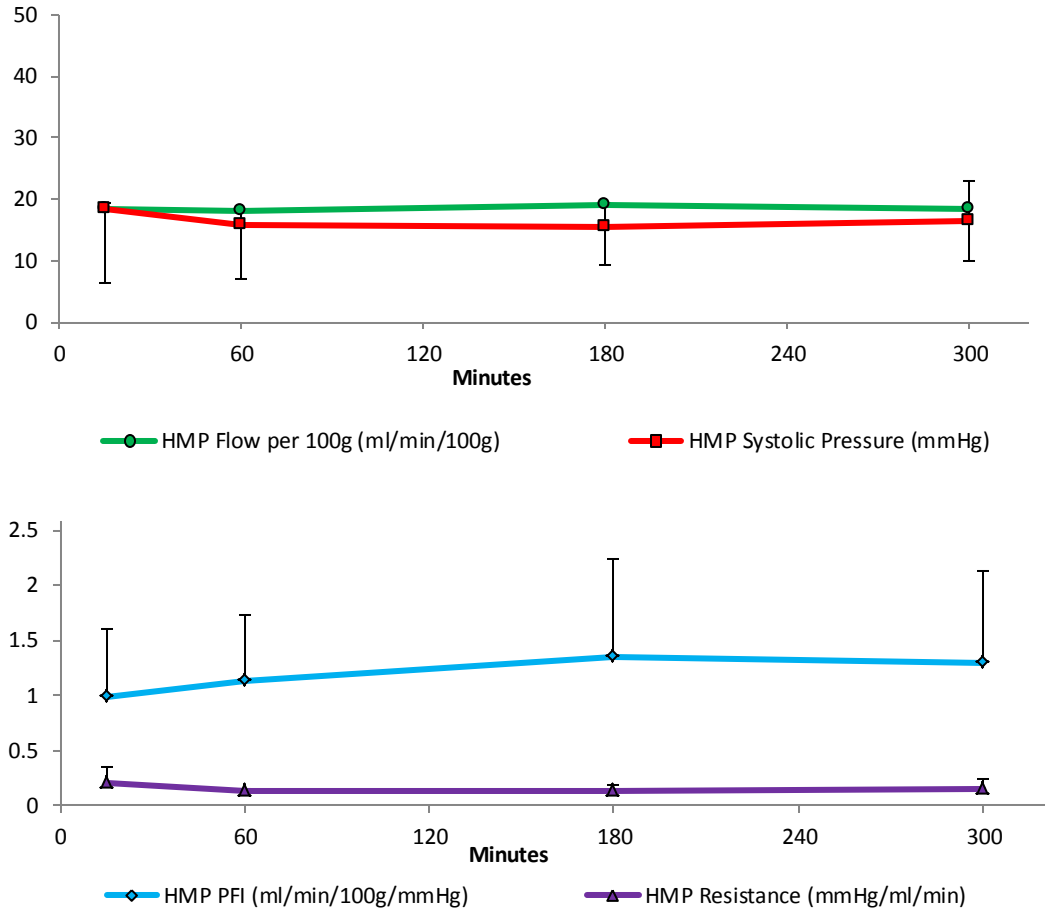


Figure 90 HMP perfusion dynamics in SCS-HMP pancreases. Stable perfusion was achieved at low pressures.

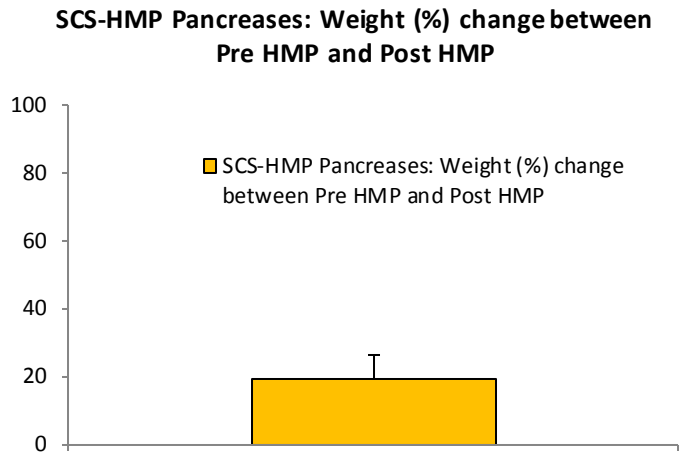


Figure 91 Weight change during HMP in SCS-HMP pancreases.

7.3.2.1 Normothermic Reperfusion

Reperfusion Dynamics

One SCS pancreas did not perfuse during NMR, with limited venous portal outflow and observable mass oedema. This experiment was stopped and data excluded from all analysis. Reperfusion dynamics in SCS and SCS-HMP pancreases shown in Figure 92 and Figure 93.

SCS pancreases were perfused at a higher pressure to SCS-HMP pancreases (SCS: 39.8 ±0.3 mmHg vs. SCS-HMP pancreases at 29.3 ± 2.1 mmHg, p=0.011).

Average flow rates and PFIs trended higher in SCS pancreases (Flow: 48.8 ± 12.6 ml/min/100g, PFI: 1.23 ± 0.33 ml/min/100g/mmHg) vs. SCS-HMP pancreases (Flow: 30.8 ± 23.7 ml/min/100g, PFI: 1.06 ± 0.85 ml/min/100g/mmHg), but these differences were not significant (Flow: p=0.35,PFI: p=0.776). Resistances also trended higher in SCS-HMP pancreases but not significantly (p=0.34).

PFI during reperfusion tended to increase by 12 ± 21.2% in SCS-HMP between 15 minutes and the end of NMR in SCS-HMP pancreases, but decrease by 32.5 ± 19.5% in the SCS only pancreases.

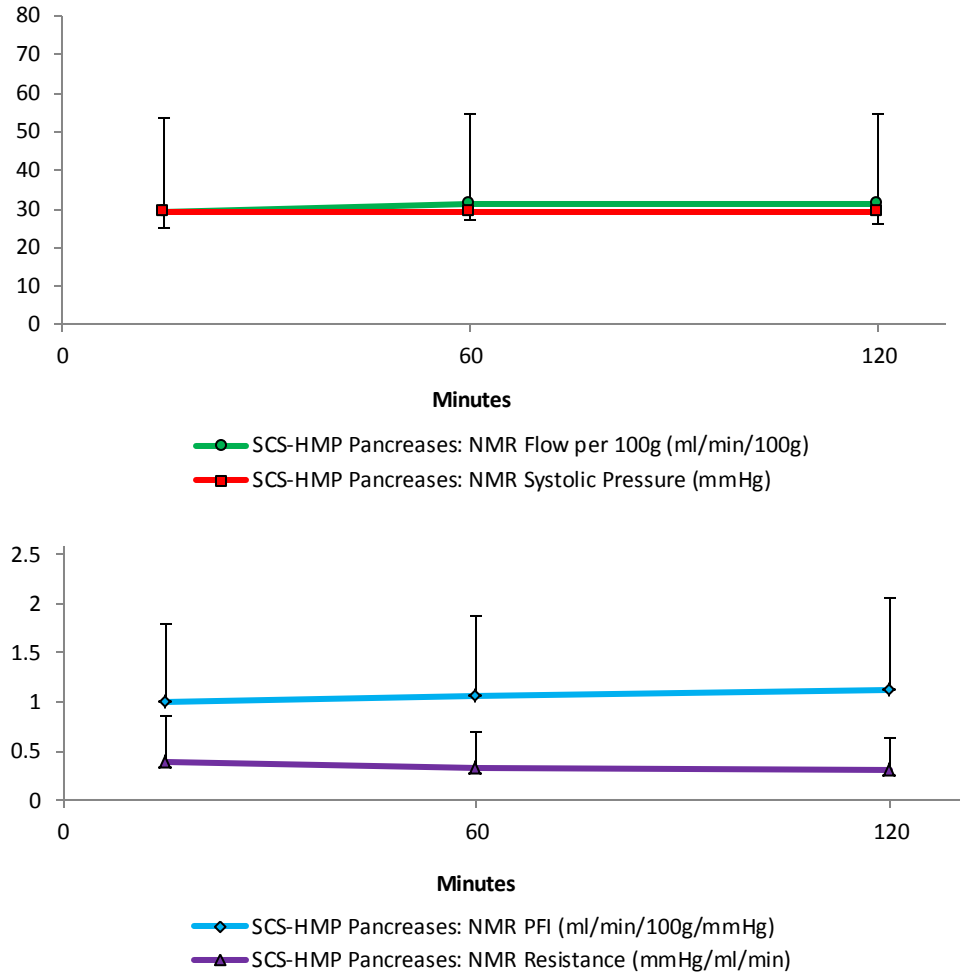


Figure 92 Normothermic reperfusion dynamics of SCS-HMP pancreases. Reperfusion demonstrated stable flow rates and PFI.

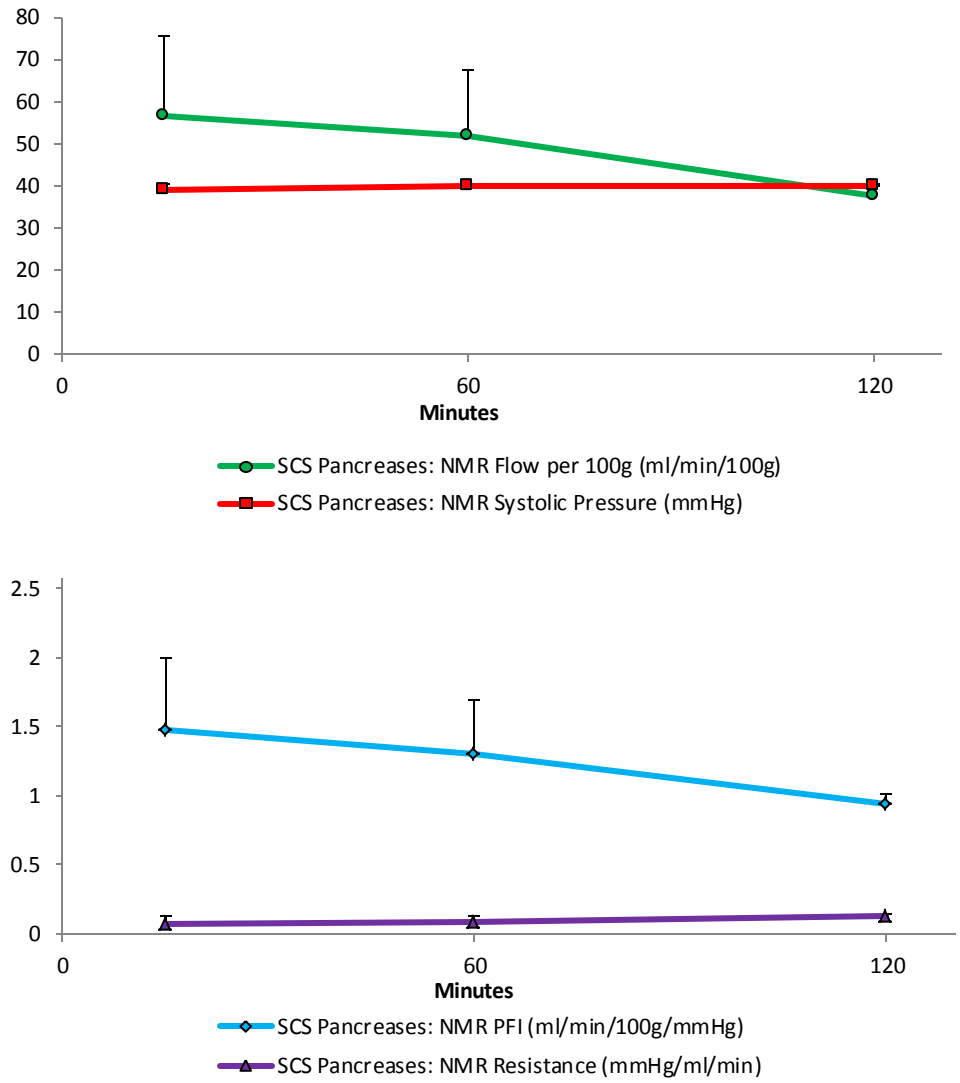


Figure 93 Normothermic reperfusion dynamics of SCS pancreases. Reperfusion demonstrated declining perfusion parameters during NMR.

Functional Assessment

After 30 minutes of NMR glucose was added to the NMR circuit to stimulate insulin secretion (see Figure 94). In both groups insulin was detected at 15 minutes, prior to active beta cell islet stimulation. Analysis indicated the presence of variable amount of glucose in the reperfusate blood (SCS Group: 4.7 ±0.4 mmol/L; SCS-HMP Group: 8.4 ± 2.4 mmol/L) prior to addition of 25mmol/L of glucose. Detected insulin levels in SCS-HMP pancreases remained stable during NMR (26.2 ± 28.9 uM/ml at 15 minutes, and 24.2 ± 23.5 uM/ml at 2 hours), while levels decreased from a peak at 15 minutes (17.3 ±15.4 uM/ml) to a trough at 2 hours (0.7 ± 0.7 uM/ml). Average insulin levels in SCS pancreases was 7.6 ± 3.5 uM/ml and in SCS-HMP pancreases 27.4 ± 25.7 uM/ml (p=0.373). Amylase levels were unable to be determined due to interference with the assay by haemolysis of

reperfusate blood samples. There were no exocrine of duodenal secretions from any porcine pancreases during reperfusion.

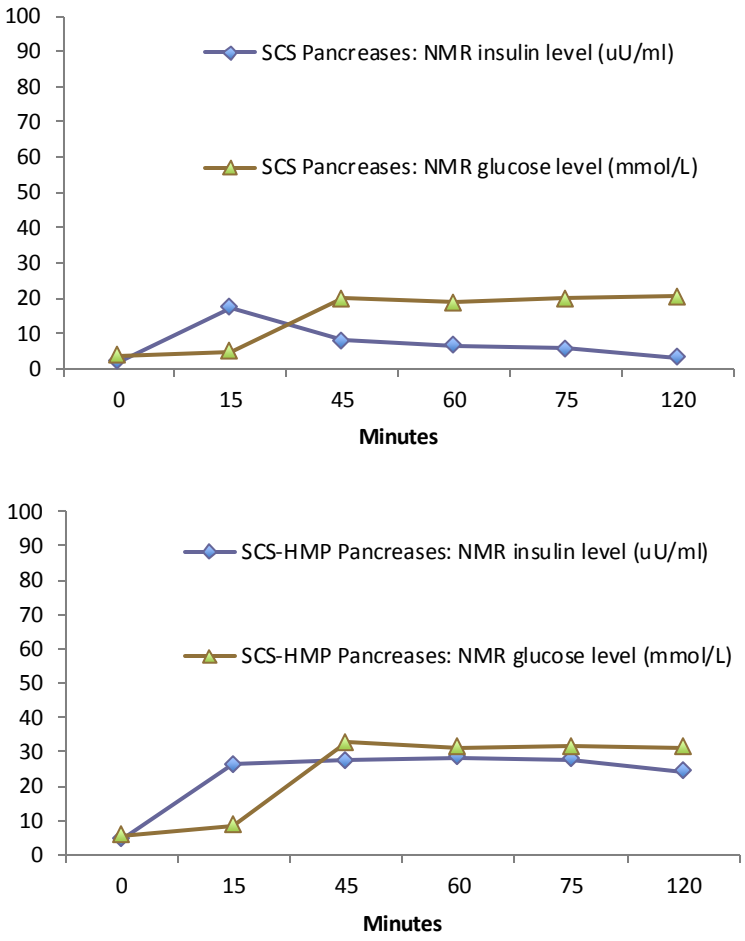


Figure 94 Insulin and Glucose levels during functional assessment of SCS and SCS-HMP pancreases.

Rapid sampling Microdialysis

During HMP pancreatic tissue lactate was detected via rsMD in all SCS-HMP pancreases. Detected lactate levels remained stable for the duration of HMP (1-1.5hours: 0.157 0.06 ± mM/min; 4.5-5 hours: 0.146 ± 0.08 mM/min, p=0.756). During NMR there was a progressive rise in tissue lactate in pancreases in both groups, with levels trending higher in SCS only pancreases (1.05 ± 0.51 mM/min) vs. SCS-HMP pancreases (0.71 ± 0.46 mM/min, p=0.44). rsMD lactate levels in pancreases during HMP and NMR are shown in Figure 95.

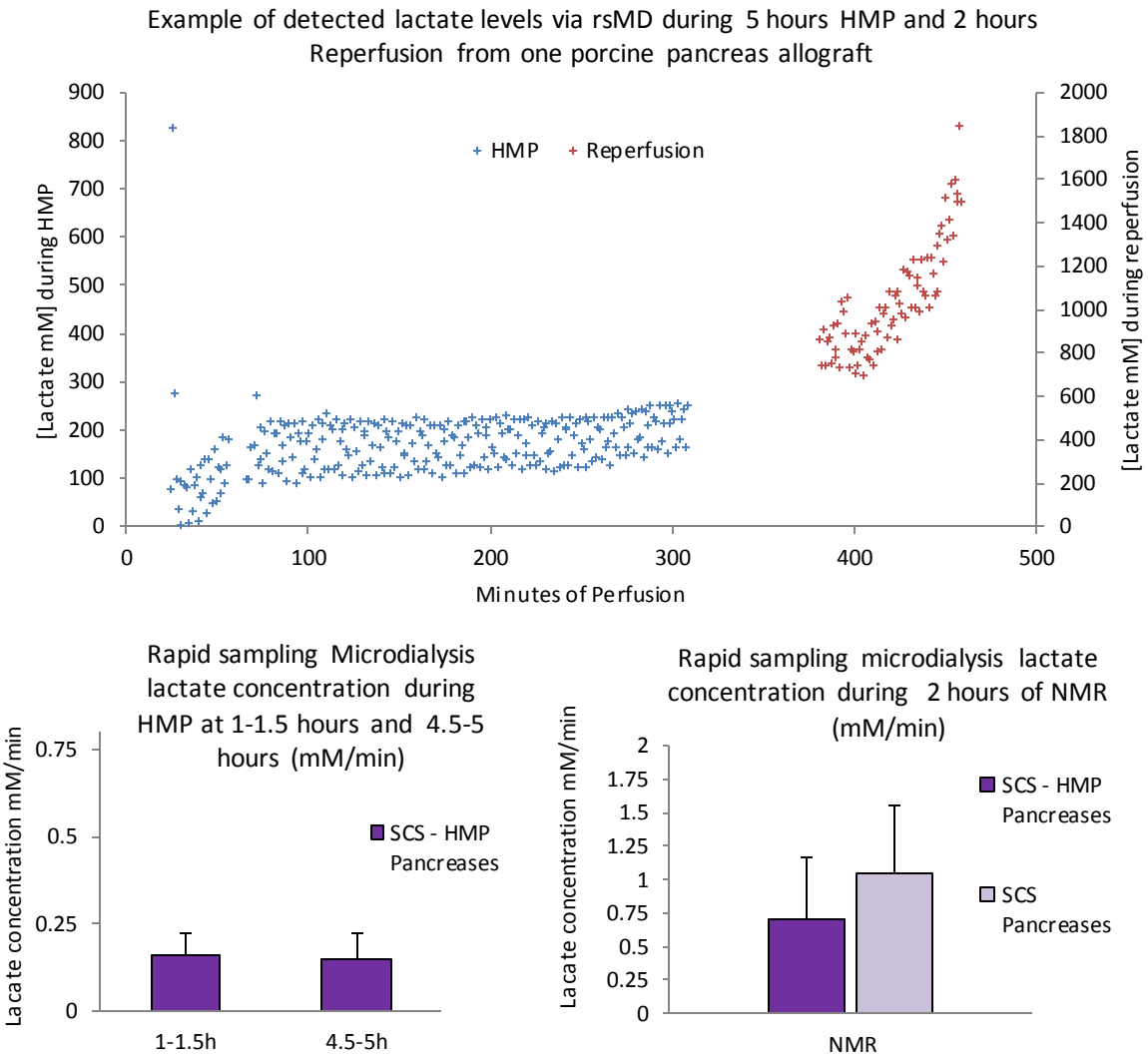


Figure 95 RsMD for tissue lactate during HMP and NMR in SCS-HMP pancreases, and during NMR in SCS pancreases. Example scatter diagram of rsMD readings measured from one SCS-HMP pancreas (top). Average tissue lactate during at 1.5 hours and 5 hours of HMP in SCS-HMP pancreases (bottom left). Average tissue lactate during NMR in SCS-HMP and SCS pancreases (bottom right).

Histology

Core biopsies were of limited histological value in determining post-SCS injury in both SCS and SCS-HMP groups. The majority of samples however demonstrated intact pancreatic cells with intact nuclei, considered normal and potentially indicative of viability. However a number of biopsies from both SCS and SCS-HMP pancreases demonstrated focal areas of necrosis and detachment of cells from the underlying connective tissue. After HMP there was no observable change compared to pre-HMP core specimens.

Wedge biopsies taken after reperfusion in both SCS and SCS-HMP pancreases demonstrated severe tissue injury, ranging from focal necrosis ranging encompassing 40% of specimens to 90% with extensive tissue disruption (see Figure 96). Despite these findings some areas of these specimens showed no evidence of necrosis, with intact acini and islet highlighting the variable and heterogeneous level of injury sustained.

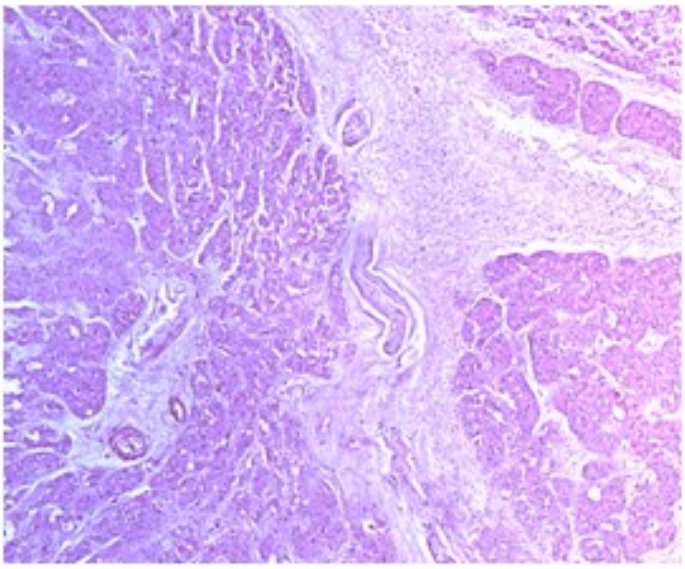


Figure 96 Photomicrographs of light microscopy of an SCS pancreas after 2 hours of NMR. H&E staining, x10. Demonstrating areas of severe necrosis but also areas of intact pancreas.

7.3.3 Application of Experimental Pancreatic HMP Protocols to Human Grafts:

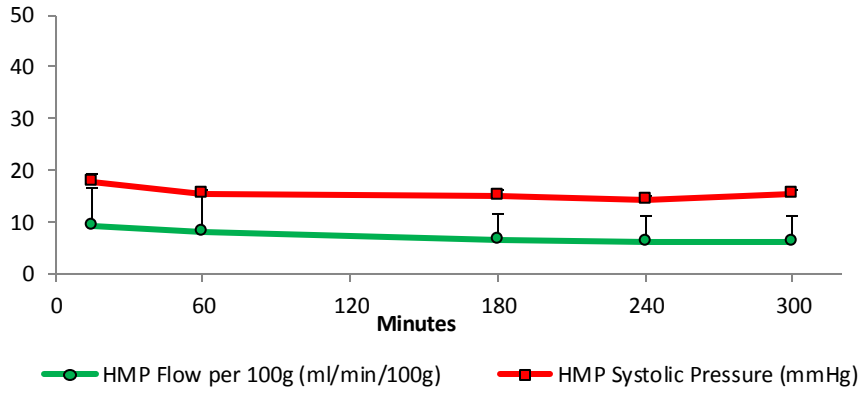
Of three human grafts used in the development of the pre-clinical pancreatic perfusion model, one was successfully perfused using the experimental HMP protocol, but during failed to perfuse during reperfusion with KHB, developing massive oedema with limited venous outflow. This pancreas was excluded from all analysis.

WITs were the similar for all human grafts, and based on normal clinical retrieval times in the UK. In the remaining two grafts (HP1, HP2) CIT was 26.8 and 56 hours respectively (Average: 28.7 ± 30.5 hours).

7.3.3.1 Hypothermic Machine Perfusion

Pancreases were perfused at low pressures of 15.8 ± 0.8 mmHg with a flow rate of 6.8 ± 5.8 ml/min/100g, resistance of 0.4 ± 0.2 mmHg/ml/min and PFI of 0.44 ± 0.34 ml/min/100g/mmHg (see Figure 97).

Comparing the two pancreases directly perfusion was superior in pancreas HP1 vs. HP2 (HP1= 10.9 ± 1.9 ml/min/100g, HP2= 2.6 ± 0.4 ml/min/100g; measured resistance: HP1= 0.24 ± 0.04 mmHg/ml/min, HP2= 0.56 ± 0.06 mmHg/ml/min; and calculated PFIs: HP1= 0.69 ± 0.1 ml/min/100g/mmHg, HP2= 0.18 ± 0.03 ml/min/100g/mmHg).



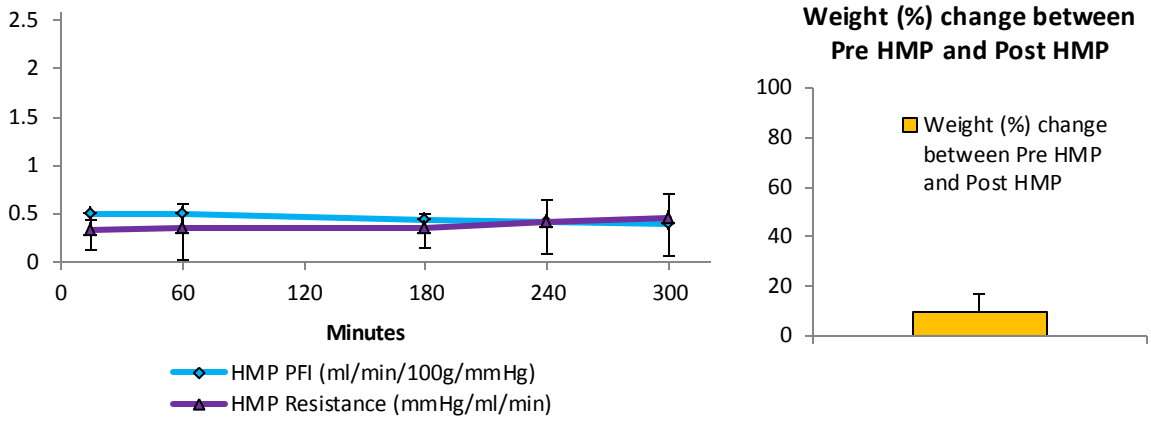


Figure 97 Perfusion dynamics and weight change in human grafts during 5 hours of low pressure HMP.

7.3.3.2 Normothermic Machine Perfusion

During NMR pancreases were perfused at a systolic pressure of 37.3 ± 5.7 mmHg, with a flow rate flow rate of 42.7 ± 11 ml/min/100g, resistance of 0.17 ± 0.15 mmHg/ml/min and PFI of 1.18 ± 0.47 ml/min/100g/mmHg (see Figure 98).

Comparing NMR perfusion dynamics of the two pancreases directly it was evident that, in contrast to HMP dynamics, perfusion was better in HP2 despite a lower perfusion pressure (HP1: 40 ± 2.8 mmHg vs. HP2: 32 mmHg; flow rate: HP1= 32.2 ± 3.5 ml/min/100g, HP2= 49.8 ± 3.1 ml/min/100g; measured resistance: HP1= 0.29 ± 0.07 mmHg/ml/min, HP2= 0.07 ± 0.03 mmHg/ml/min; and calculated PFIs: HP1= 0.81 ± 0.14 ml/min/100g/mmHg, HP2= 1.56 ± 0.1 ml/min/100g/mmHg). The average perfusate amylase measured during NMR was 3475 ± 1068 U/L. HP1 NMR amylase was 4229.9 U/L, while HP2 amylase was lower at 2719.2 U/L.

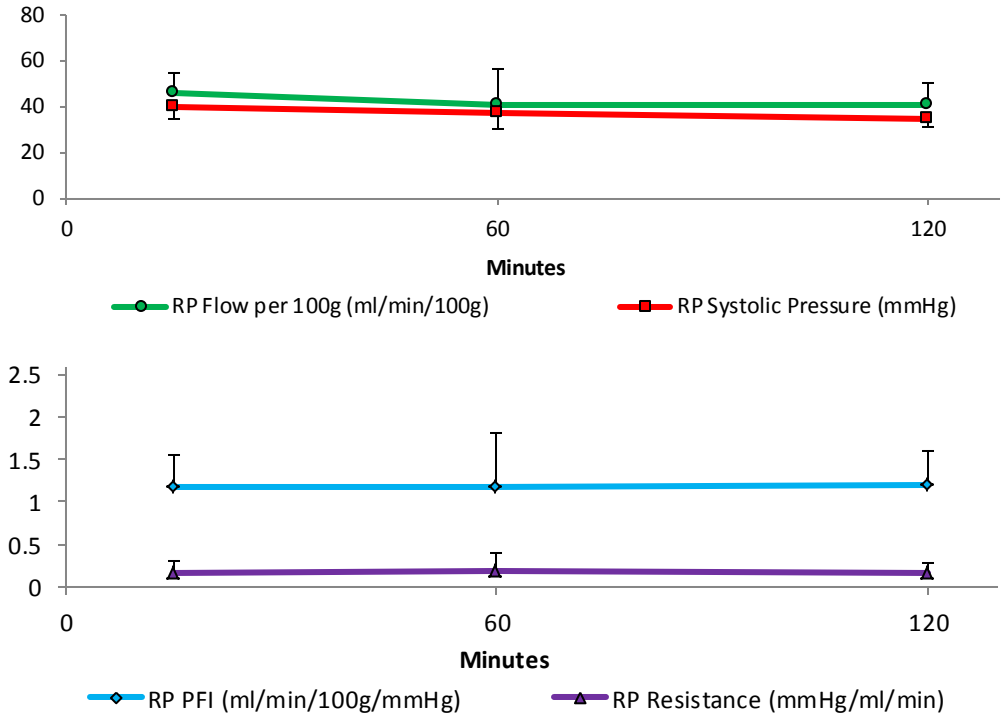


Figure 98 Reperfusion dynamics in human grafts during 2 hours of NMR, demonstrating stable reperfusion parameters.

7.3.3.3 Functional Assessment

After 30 minutes of NMR glucose was added to the NMR circuit to stimulate insulin secretion. In HP2 insulin was detected at 15 minutes (16.8 uU/ml) but none during the remainder of NMR. In contrast in HP1 insulin was detected at 15 minutes (70.4 uM/ml), peaked at 75 minutes (199.2 uM/ml) before decreasing to the end of perfusion (120mins: 73 uM/ml). Exocrine and duodenal secretions were produced by both pancreases (1 hour: 176 ± 106 ml/hr; 2 hour: 113 ± 19 ml/hr), see Figure 99. Secretions were analysed for amylase (HP1: 1 hour=2116 U/L/g, 2 hour=3327 U/L/g; HP2: 1hour=1497 U/L/g, 2 hour=1410 U/L/g) and insulin (HP1: 1 hour=1.61 uM/ml/g, 2 hour=0.89 uM/ml/g; HP2: 1hour=4.2 uM/ml/g, 2 hour=3.5 uM/ml/) demonstrating both endocrine viability and confirming exocrine functionality.



Figure 99 Volume of exocrine secretions during NMR.

7.3.3.4 Histology

Core biopsies taken after SCS and before HMP demonstrated normal pancreatic structures and cells with intact nudei, with similar observations after HMP. After reperfusion there were focal scattered areas of central necrosis (10% of tissue) and universally at the periphery of the specimens, however there was overall good preservation of the cellular architecture (see Figure 100).

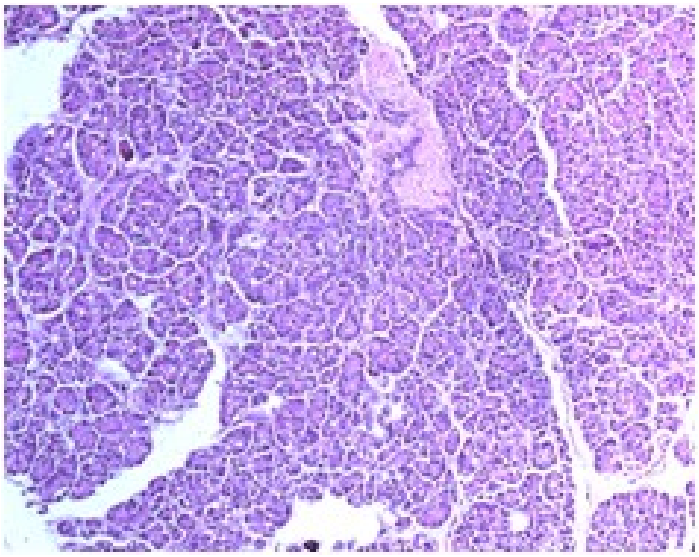


Figure 100 Photomicrographs of light microscopy of an human pancreas. After 5 hours of HMP and then 2 hours of NMR. H&E staining, x10. Demonstrating focal areas of central necrosis but also areas of intact cellular architecture.

7.4.1 Comparison of Renal and Pancreatic HMP Perfusion

Pancreatic graft preservation by machine organ perfusion has been attempted experimentally by a number of groups over the past four decades. In canine models HMP was shown to be potentially feasible, but complex and with no benefit on graft preservation and function after experimental allotransplantation. However the increasing use of ECD and DCD donor organs to expand donor pools in recent years has renewed interest in HMP as potential preservation modality for these organs as HMP in renal setting has shown to provide a functional and survival advantage to allografts compared to simple SCS.

The majority of research in machine organ perfusion has remained focussed on renal perfusion. In developing a model of pancreatic perfusion it is necessary to delineate the important differences and characteristics of the pancreas compared to the kidney.

Physiologically the kidney is a high flow organ [896], while the pancreas is not[1136]. Tersigni et al. observed that graft oedema is detrimental for the pancreas, and despite lowering perfusion pressures (ranging from 5 to 20 mmHg) to reduce barotrauma was unable to ameliorate oedema during HMP[1028]. An antomical cause for this occurance is potentially the lack of a organ capsule unlike the kidney, presence of which could limit fluid accumulation. Modification of their cryoprecipitated plasma perfusate with human albumin however allowed perfusion at a higher 25mmHg pressure with minimal oedema. The canine pancreas however has a distinct anatomy compared to the porcine pancreas, which is relatively more complicated[1029,1043]. In the UK it is logistically preferable to use porcine models for experimental organ preservation.

This project demonstrated that using Belzer UW supplemented with mannitol it is feasible to hypothermically perfuse at a low pressures (<20mmHg) a porcine pancreas for 5 hours. Using this protocol produced significantly lower flow rates but similar PFI profiles as use of higher pressures (30mmHg), but without detrimental oedema. Furthermore such PFI profiles and low oedema were comparable to a high flow, high pressure model of renal perfusion.

The flow rates observed in this model were lower than those seen in historical attempts at pancreatic HMP. The University of Wisconsin group perfused kidneys at a series of pressures ranging from 5 to 25 mmHg, which produced flow rates of 10-40 mls/min[1028]. While Brynger et al.'s perfusion of pancreases at 50mmHg produced high flow rates of 90ml/min[1030]. The differences

may be explained by several reasons: 1) use of perfusates of differing viscosity will generate different flow rates through an graft, 2) in this project flow rate was calculated per 100g of pancreatic tissue, while in the described reports only observed flow was reported.

It has been suggested that both perfusion pressure and the nature of the perfusate are key to achieving successful organ perfusion[1137], as these results have demonstrated. Belzer UW has a composition similar to Belzer MPS the only commercially HMP preservation solution[878]. It was initially designed as both a SCS and a HMP solution by Belzer and Southard at the University of Wisconsin, however with the specific development of Belzer MPS for HMP, Belzer UW remained only in use for SCS organ preservation[722]. Belzer UW is the preservation solution of choice for pancreatic SCS [682,768], but is theoretically suitable for pancreatic HMP. Belzer UW was compared to Belzer MPS for HMP in canine kidneys with Belzer UW conferring a survival and functional advantage after auto-transplantation in compromised allografts[1138]. Belzer UW may be preferable to Belzer MPS for pancreatic HMP as the pancreas is more susceptible to ischaemic stress than the kidney. Furthermore the omission of glucose from the perfusate may further limit unnecessary stimulation of insulin secreting islet cells during preservation. The addition of mannitol (a polysaccharide and potent impermeant) to the perfusate in this project intended to further limit cellular oedema in the pancreatic graft.

Mannitol is used in numerous organ preservation solutions, including HTK and UW solution, and has also has been used in clinical practice as osmotic diuretic. Its use however in has been associated with osmotic nephrosis and vacuolization in proximal tubules of kidneys with the development of acute kidney injury [1139]. Vacuolization in proximal tubular cells as a histologic entity has been documented with other compounds, including those used in preservation solutions (e.g. HES and sucrose) [1140]. No evidence of vacuolization was found during microscopy of pancreases undergoing HMP.

7.4.2 Short Post-ischaemic Pancreatic HMP vs. Pancreatic SCS

A short period of post-ischaemic HMP has been shown to confer a functional benefit in experimental porcine renal transplant models[1141]. Experimental studies of long duration (≥24 hours) of HMP in pancreatic allografts has not demonstrated a benefit compared to SCS preservation [1137]. However the feasibility and benefits of a short period of post-ischaemic HMP in the pancreas after 24 hours of SCS has not been investigated.

The viability of pancreases preserved via 24 hours of SCS was compared to pancreases that underwent 24 hours of SCS and then a short 5 hour period of HMP. Using the low pressure protocol with modified Belzer UW 5 hours of HMP in porcine pancreases demonstrated stable perfusion characteristics, and limited weight change of approximately 20%. Islet and tissue oedema has been reported in experimental perfusion of porcine pancreases and is an expected occurance in grafts post HMP [1032]. Despite the oedema encountered during HMP however there was no evidence of tissue or islet cell oedema seen at microscopy.

The NMR circuit then allowed a short duration of ex-vivo viability assessment using perfusion metrics, insulin release, and histology[1033].

During NMR pancreatic blood flow rates were overall higher in SCS only, compared to SCS-HMP pancreases. However blood flow decreased during perfusion in the SCS only preserved grafts, while it remained stable in SCS-HMP pancreases. Suggesting a stability in endothelial resistance and integrity in SCS-HMP pancreases but potentially progressive microvascular disruption in the SCS pancreases and severe dysfunction.

Administration of glucose to stimulate insulin secretion is the most sensitive test for determining functional viability of the preserved pancreatic grafts [1028].

The NMR circuit employed was a closed loop circuit, as such the blood perfusate would act as a sink for insulin secreted by the graft. Insulin levels in SCS pancreases peaked at 15 minutes, prior to administration of glucose, and despite a higher presence of glucose insulin levels decreased for the remainder of NMR. It is likely that this decrease reflects both a decline in insulin secretion by islet beta cells and also degradation of circulating insulin by insulin degrading enzymes (IDE) known to be present within blood plasma of the perfusate [1142,1143]. In the SCS-HMP pancreases however insulin levels peaked by 15 minutes and remained relatively stable for the duration of NMR, and that despite the likely presence of IDE in the blood perfusate plasma insulin levels remained stable due to a persistent and stable secretion by islet cells.

Florak et al used a canine allotransplant model when they compared SCS to HMP preservation of pancreatic allografts, thus assessing graft viability by determining the endocrine capacity of the pancreas by the ability of the pancreaticoduodenal segments to maintain a previously pancreatectomized dog[1137].

However experimental normothermic models of isolated pancreatic perfusion have used insulin levels to gauge organ viability. Bartel et al. demonstrated that longer cold ischaemic injury sustained

by the porcine pancreas the greater the deterioration in normothermic perfusion dynamics, as well as the decline in insulin release in response to a glucose load during viability assessment on the isolated haemoperfusion circuit [1035]. Interestingly 3-6 hours of CIT did not affect normothermic reperfusion parameters, nor endocrine viability, however SCS CIT of 24 hours or greater produced severe pancreatic dysfunction. In our model pancreases with 24 hours of SCS only had deteriorating perfusion during NMR, and poor insulin secretion. In contrast pancreases that had also been subjected to 5 hours of HMP had lower, but stable perfusion characteristics during NMR and sustained insulin release, suggesting better viability.

In the porcine pancreases there was no evidence of exocrine or duodenal segment secretions during NMR. The porcine pancreas is a very fragile organ compared to the native human pancreas, in both cases (SCS and SCS-HMP) 24 hours of CIT may have been extreme and the damage sustained unrecoverable. Barthel et al. document that less CIT damage is required for exocrine and duodenal segment functional failure than with the endocrine failure [1035].

rsMD for lactate during HMP of pancreases demonstrated stable levels throughout perfusion. While tissue lactate was higher during NMR of SCS only pancreases compared to those having been subjected to HMP after cold storage. The pancreas is not an endogenous source of physiological lactate like the liver or kidney[1051]. Thus lactate will solely originate from glycolytic metabolism during ischaemic stress, which is the most likely source of the low levels of lactate detected during HMP. During NMR higher levels of lactate were detected in SCS only vs. SCS-HMP preserved pancreases suggesting cells of SCS only pancreases were unable to effectively restart aerobic metabolism, relying on glycolytic energy production to a greater degree than the SCS-HMP grafts.

Histological assessment of the pancreatic grafts in this project after NMR showed a similar degree of severe pancreatic necrosis in both SCS and SCS-HMP grafts. As suggested from the model by Barthel et al. 24 hours of SCS may induce irreversible damage in pancreatic grafts upon reperfusion[1035]. Interestingly this damage was evident in all pancreases despite SCS-HMP pancreases demonstrating better functional viability.

7.4.3 Application of experimental pancreatic HMP protocols to human grafts

Use of porcine models are a crucial step in the translational application of experimental protocols into the clinical arena[1144]. Porcine organs in particular are of a similar anatomical and physiological construct to those in the human[1136]. Low pressure perfusion protocols with modified Belzer UW solution have been demonstrated to successfully perfuse porcine pancreases

with minimal weight gain, however the feasibility of HMP in human pancreases needs to be demonstrated. In the development of novel organ perfusion and preservation technologies the gap between porcine work and clinical application is routinely bridged by experimentation using human grafts that are retrieved but not used for clinical transplantation and instead donated for research[1145].

Recent developments in the UK national system of organ retrieval for transplantation has led to the formation of the National Organ Retrieval Service under the aegis of NHS Blood and Transplant (the national overseeing body), with regional centralisation of this service. Furthermore responsibility of consent for deceased donor donation has been placed with the donor coordinators. Despite efforts to place all organs donated some retrieved grafts are unable to be used for conventional clinical therapies due to physiological status of the organs which places them outside of the suitable criteria for transplantation. Relatives to the donor of such organs however often feel that donation for research can maximise the benefit from the sad events that led to the stage requiring such decisions to be made[1146]. In the UK a national process and framework under NHS Blood and Transplant is in place for organs that are retrieved but subsequently declined for clinical transplantation to be used for ethically and nationally approved research projects.

Pancreatic grafts not used for clinical transplantation, obtained through NHS Blood and Transplant were used in this project to determine the feasibility of applying perfusion protocols developed the translational porcine model to human pancreases. Recently early attempts to apply normothermic perfusion preservation to human pancreases was described with limited success[1147]. There are no reports in the literature of applying HMP to human pancreases, with the aim of preservation, nor then using isolated whole organ haemoreperfusion to assess graft viability [1148]. This project has successfully shown for the first time that using human pancreas HMP is feasible at low pressures for 5 hours producing stable perfusion dynamics with minimal oedema.

Functional assessment during NMR indicated that similar to results seen in the porcine model human pancreases produce insulin in response to a glucose load - despite extreme cold ischaemia prior to HMP and subsequent NMR assessment.

Furthermore unlike the porcine model, human pancreases produced exocrine and duodenal secretions during NMR, despite longer CITs than in the porcine grafts. Analysis of the exocrine secretions revealed secretion of both amylase, and insulin[1149] which has been shown to indicate graft exocrine and endocrine functionality respectively in experimental settings [1028,1035].

Histologically despite longer SCS CIT prior to HMP and NMR in the human models than in the porcine pancreases there was qualitatively less focal necrosis and better preservation of the tissue. This further demonstrates that despite the anatomical and physiological similarities between human and porcine pancreases[1043], they are more susceptible to ischaemic injury[1150], and even in light of promising initial data in the porcine model, application of these HMP techniques to unused human grafts is necessary.

7.4.4 The Next Step for Pancreatic MP

There has been a renewed interest in applying the success of HMP enjoyed in renal preservation to the pancreas. Historically experimental attempts at pancreatic HMP in animal models have been complex with limited scope and merit compared to simpler SCS methods[1028,1137]. The predominate problem of the pancreas is that it is physiologically a 'low flow' organ, with a delicate vasculature that is easily damaged with deleterious oedema a result[1150]. We have shown that using current commercially available technology a low pressure perfusion protocol using modified Belzer UW solution is capable of perfusing a porcine pancreas for 5 hours with stable perfusion dynamics and minimal oedematous weight change. Comparing viability of porcine pancreases that underwent SCS only or SCS and HMP demonstrated potentially better endocrine viability and functionality in the HMP subjected grafts.

Further more for the first time a HMP protocol has successfully been applied to human pancreatic grafts, with isolated ex-vivo viability assessment demonstrating both endocrine and exocrine graft functionality despite prior exposure to an extreme period of cold ischaemia.

The development of these perfusion models and demonstration of their feasible applicability in human grafts is key to exploring further strategies to allow 1) better preservation of pancreases, 2) assessment of graft viability, and 3) use of HMP as method of organ pre-conditioning and delivery of pharmacological agents into grafts to optimize viability. Such strategies will be useful in the context of using ECD and DCD donor pancreases to further expand the donor pool by allowing selection and usage of suitably viable grafts, and minimising unnecessary discard.

Chapter 8:

Conclusions

This thesis focussed on the development of number of pre-clinical translational ex-vivo strategies in assessing and optimising organ viability during dynamic HMP and normothermic reperfusion of kidney and pancreas grafts.

In recent years efforts to expand donor pools has necessitated use of ECD and DCD kidneys and pancreases. Use of such organs is set to grow to address the growing demand by patients on transplant lists. The inherent nature of the donors of these organs renders these grafts at higher risk of post-transplant complications particularly of thrombosis, delayed-function, non-function, subsequent rejection and longer-term graft-failure, with the mechanism of damage through IR injury. IR is characterised by endothelial injury, and the development of pro-inflammatory and pro-coagulant milieu within the transplanted organ leading to micro-vascular dysfunction, reduced tissue perfusion and organ failure. To enable effective use of these organs in clinical transplantation advances in organ preservation need to focus on techniques to both assess and optimise organ viability prior to transplantation.

Five project themes were prevalent in this research: 1) use of real-time rsMD for tissue viability assessment during SCS and HMP preservation; 2) pre-conditioning organs by targeting novel localising anti-coagulants to the endothelium of kidneys in an addressive strategy to prevent microvascular dysfunction; 3) developing a new HMP preservation solution from a novel adenosine/lidocaine based cardioplegia; 4) exploring the potential for a period of post-ischaemic HMP to recover porcine kidneys after extreme length of SCS; and 5) developing an experimental model of HMP for pancreases.

The majority of work was conducted using large animal porcine kidney and pancreas grafts. However permission and ethical approval was obtained for use of nationally retrieved but subsequently unused human kidney and pancreatic grafts for applying and testing several of these strategies in a pre-clinical human model.

8.1

Application of Rapid Sampling Microdialysis in Organ Viability Assessment During Preservation: A Comparison of SCS vs. HMP

This study characterises for the first time in real time metabolic differences between SCS and HMP preserved allograft which can help elucidate why HMP is clinically superior to SCS allowing development of interventions to augment these benefits. HMP enables ongoing anaerobic metabolism, but may also facilitate a resumption of normal kidney physiological processes by recycling substrates between cortico-medullary beds and energy production. Furthermore this data provides the baseline ischaemic profile for porcine kidneys whilst demonstrating the technique of rsMD as a tool for organ viability assessment during preservation, enabling further use of this technology in experimental ex-vivo strategies to optimise organ viability.

The recent developments in normothermic machine perfusion organ preservation is an area where real-time assessment of tissue biochemistry will be most useful. Furthermore downscaling of the size of component in the rsMD system to be more portable and with probes that allow a larger region of tissue to be sampled will enable this technology to be more applicable to the clinical arena. Newer technologies for organ preservation must be matched by progress in organ viability assessment and that of the tissue itself and rsMD is one such novel technique that could provide this.

8.2

Addressive Targeting of Anti-thrombin Agents to Graft Vasculature during HMP: Preconditioning Organs to Ameliorate Post Transplant Micro-vascular Thrombosis

The interventional potential of using HMP for pre-conditioning the endothelium of organs during preservation with localising anticoagulants has been successfully demonstrated for the first time. Such pre-conditioning ameliorates the deterioration in macro- and micro-vascular perfusion dynamics seen in an *ex-vivo* haemo-reperfusion model, with less clotting activity and metabolic evidence of superior tissue perfusion. There is high potential for further development of this translational strategy to deliver targeted and locally-active anti-coagulants directly within allografts

where they are needed, decrease the development of microvascular thrombophilia and perfusion deficits, while avoiding systemic anticoagulation and its associated risks.

Such a therapy will be of most interest in the pancreas which is an organ very prone to thrombosis. As newer organ preservation techniques emerge, in particular machine perfusion for organs normally preserved predominantly by SCS, such as the pancreas the potential to pharmacologically manipulate the graft to optimise viability and prevent potential complications related to reperfusion injury will more fully be recognised. Targeting key processes only now appreciated to be involved in reperfusion such as the serine proteases will be feasible and beneficial.

8.3

Development of Adenocaine as a Novel Hypothermic Machine Perfusion Preservation Solution

UW solution is the gold standard HMP solution in use, however its composition, developed 40 years ago in an era where the majority of allografts were from SCD and DBD donors may not be the optimal HMP perfusate for ECD and DCD kidneys. AL is a novel cardioplegia and cardiac preservation solution that has recently demonstrated superiority in cardiac surgery models compared to conventional solutions. This solution may offer benefits in renal preservation, in particular HMP. Using a modified version of AL solution renal HMP is feasible and produces a more rapid stabilisation of perfusion dynamics to viable levels, with higher flow rates and PFI compared to the gold standard UW, potentially allowing earlier viability assessment and categorisation. NMR viability assessment demonstrated similar renal function and viability profiles between AL and UW perfused kidneys. Development of AL as an alternative HMP solution to UW is a step towards maximising the benefits of HMP for marginal grafts. By improving both organ viability and its assessment there is potential for cost-savings in relation to current techniques, while potentially expanding donor pools. Further refinement and modfication of ALs components will aim to achieve the success seen in cardiac preservation with both the kidney, and other abdominal organs that are also very susceptible to ischaemic injury and the subsequent damage during reperfusion - in particular the pancreas.

8.4

Can Post Ischaemic Reconditioning by HMP Recover Kidneys from Extreme Cold Ischaemia?

Post-ischaemic HMP reconditioning has the potential for benefit in the use of ECD and DCD organs for transplantation. Kidneys with exposure to an extremely prolonged period of SCS for 52 hours demonstrated similar perfusion and viability dynamics during post-ischaemic HMP as less injured kidneys with only 4 hours of SCS. This observation is attributed to detected graft weight loss after prolonged SCS, which may facilitate better HMP perfusion dynamics and thus incorrectly suggesting suitable viability. Such a phenomenon deserves recognition by clinicians utilising HMP to reduce the risk of inadvertent use of unsuitable grafts for transplantation. Despite clinical success with conventional HMP, in its present form however utilising passive oxygenation HMP may not be able to recover grafts exposed to extreme SCS, suggesting that HMP interventions utilising active oxygenation of the perfusate may be better to provide benefit in such organs. The ability to salvage grafts exposed to prolonged ischaemia, by application of a period of 'revitalization' via oxygenated HMP has the potential to improve logistical access and organizational constraints in the use of marginal and DCD donor kidneys, and allow a much needed and welcomed expansion of the donor pool to meet the increasing demands for grafts on national waiting lists throughout the world.

8.5

Development of Pancreatic Machine Perfusion Models

HMP is in routine clinical use for kidney preservation but historical technical difficulties have limited the expansion of HMP into pancreatic preservation. With the increasing use of ECD donor allografts HMP may be of benefit in pancreatic preservation allowing opportunities to improve and assess viability. This project has successfully established a pre-clinical model of pancreatic HMP using porcine grafts. HMP at low pressures is feasible in the pancreas, with stable perfusion dynamics and minimal oedema. Importantly these protocols were applied to a series of human pancreatic grafts demonstrating the key translational potential of this perfusion strategy. These *ex-vivo* perfusion models will allow further development of strategies to improve graft preservation by 1) optimising

the HMP perfusate, 2) determining the appropriate viability assessment criteria, and 3) allowing for and testing various pharmacological interventions, for example use of TLN in prevention of pancreatic graft thrombosis, the Achilles heel of pancreas transplantation. The era of machine organ preservation started approximately 100 years ago with Lindberg and Carrel, and now a century on the interest in the novel and potentially revolutionary possibility of ex-vivo preservation and assessment of organ function prior to transplantation is becoming a reality. Despite this challenges await in the development and application of these techniques to the clinical arena, never the less the potential benefits envisioned out weigh the current obstacles and dynamic machine perfusion of the pancreas is likely only a few years away.

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