MACHINE PERFUSION IN KIDNEY TRANSPLANTATION: CLINICAL APPLICATION & METABOLOMIC ANALYSIS

By ALISON J GUY (MBChB, FRCS)

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Department of Immunity and Infection

University of Birmingham

&

Department of Renal Surgery

University Hospitals Birmingham

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ABSTRACT

Kidney Transplantation is the gold standard treatment for patients with end-stage renal failure. Most kidneys used for transplantation are from deceased donors and ensuring successful outcomes depends on many factors. One of these is organ storage.

Hypothermic Machine Perfusion (HMP) of deceased donor organs has been shown to have several benefits. However, it has not been widely adopted and the underlying mechanism is poorly understood.

The first section of this thesis examines the introduction of HMP into clinical practice. HMP outcomes were similar to those of standard storage techniques but with the additional benefit of increasing safe storage times. This was likely due to inherent benefits of the machine itself, improved recipient preparation and better peri-operative conditions.

The second part of this study analysed HMP perfusate using metabolomics (Nuclear Magnetic Resonance) to identify potential predictors of graft outcome. Differences were identified in the metabolic profiles of perfusate from kidneys with immediate and delayed graft function. These may have a future role in viability assessment. Improved understanding of metabolism during storage may help target optimization strategies for deceased donor organs.

The final part of this study describes the development of a porcine model of transplantation to test future hypotheses.

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ABBREVIATIONS

Acetyl CoA	acetyl coenzyme A
ACR	albumin creatinine ratio
ADP	adenosine diphosphate
AER	albumin excretion rate
AMP	adenosine monophosphate
AST	aspartate transaminase
ATP	adenosine triphosphate
AUROC	area under the curve receiver operating characteristics
BMPS	Belzer machine perfusion solution
BPAR	biopsy proven acute rejection
Ca	calcium
CC	correlation coefficient
CIT	cold ischaemic time
CKD	chronic kidney disease
CMV	cytomegalovirus
D2O	deuterium oxide
DBD	donation after brain death
DCD	donation after cardiac death
DD	deceased donor
DGF	delayed graft function
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

EC	Eurocollin's
ECMO	extracorporeal membrane oxygenation
ECD	extended criteria donor
EM	electron microscopy
ER	endoplasmic reticulum
ESRF	end stage renal failure
FCS	fetal calf serum
fDGF	functional delayed graft function
GBM	glomerular basement membrane
GFR	glomerular filtration rate
GST	glutathione-S-transferase
HAV	hepatitis A virus
HBV	hepatitis B virus
HCV	hepatitis C virus
HD	haemodialysis
H&E	haematoxylin and eosin
HIV	human immunodeficiency virus
HES	hydroxyethyl starch
HMP	hypothermic machine perfusion
HOC	hyperosmolar citrate
HTK	histidine-trytophan-ketoglutarate
IGF	immediate graft function
IGL-1	Insitute Georges Lopez-1
IQ	interquartile

IR	ischaemia reperfusion
K	potassium
KDIGO	kidney disease improving global outcomes
KLF2	krüppel-like factor 2
KPS-1®	kidney perfusion solution-1®
LDH	lactate dehydrogenase
LM	light microscopy
mPT	non selective pore
MS	mass spectroscopy
Na	sodium
NADPH	nicotinamide adenine dinucleotide phosphate
NHS	National Health Service
NHSBT	National Health Service Blood and Transplant
NMR	nuclear magnetic resonance
ORS	organ recovery systems
PAS	Periodic-acid Schiff
PD	peritoneal dialysis
PGE1	prostaglandin-1
PNF	primary non function
PRA	panel reactive antibodies
RNA	ribonucleic acid
ROC	receiver operating characteristics
RPM	rotation per minute
RRT	renal replacement therapy

SCS	static cold storage
TSP	(3-trimethylsilyl)propionic-(2,2,3,3-d4)-acid sodium salt
UHB	University Hospitals Birmingham
UK	United Kingdom
US	United States
UW	University Of Wisconsin
WIT	warm ischaemic time

CHAPTER ONE: INTRODUCTION

1.1 CHRONIC KIDNEY DISEASE (CKD)

1.1.1 CKD - DEFINITIONS & STAGING

Chronic kidney disease (CKD) was defined by the US National Kidney Foundation in 2002 as the presence of kidney damage or decreased kidney function for three months or more, irrespective of the underlying cause. This can manifest with various markers of kidney damage or reduction in the glomerular filtration rate (GFR < 60 mL/min/1.73m^2) (National Kidney Foundation, 2002).

A staging system based on GFR has also been defined:

Table 1.1: Staging System for CKD – National Kidney Foundation

Stage	*Description*	*GFR (mL/min/1.73m^2)*
1	Kidney damage with normal or ↑GFR	≥ 90
2	Kidney damage with mild ↓GFR	60-89
3	Moderate ↓GFR	30-59
4	Severe ↓GFR	15-29
5	Kidney Failure	<15 or dialysis

This staging system was revised in 2012 by the Kidney Disease Improving Global Outcomes (KDIGO) CKD Work Group (KDIGO, 2013).

Table 1.2: Revised Staging System for CKD - KDIGO

GFR category	*GFR (ml/min/1.73m^2)*	*Terms*
G1	≥90	Normal or high GFR
G2	60-89	Mildly decreased GFR
G3a	45-59	Mild to moderately decreased GFR
G3b	30-44	Moderately to severely decreased GFR
G4	15-29	Severely decreased GFR
G5	<15	Kidney failure

In addition to GFR, a grading system for the level of albuminuria was also introduced in recognition of its contribution to the prognosis of patients with CKD.

Table 1.3: Grading System for Albuminuria - KDIGO

Category	*AER (mg/24hours)*	*ACR (mg/mmol)*	*ACR (mg/g)*	*Terms*
A1	<30	<3	<30	Normal/mildly increased
A2	30-300	3-30	30-300	Moderately increased
A3	>300	>30	>300	Severely increased

AER: Albumin Excretion Rate, ACR: Albumin Creatinine Ratio

This KDIGO group also recommended that a cause should be assigned based on the presence or absence of systemic disease and the location within the kidney of observed or presumed pathological-anatomical findings.

Progression through the stages of CKD results in significant complications such as hypertension and cardiovascular disease, anaemia, malnutrition, neuropathy and a poor quality of life. It can ultimately result in Kidney failure, or End-Stage Renal Failure (ESRF). This is defined as a GFR of less than 15 mL/min/1.73m^2, or the need for Renal Replacement Therapy (RRT) (Levey & Coresh, 2012). This is the most serious complication of CKD and can only be treated by dialysis or transplantation.

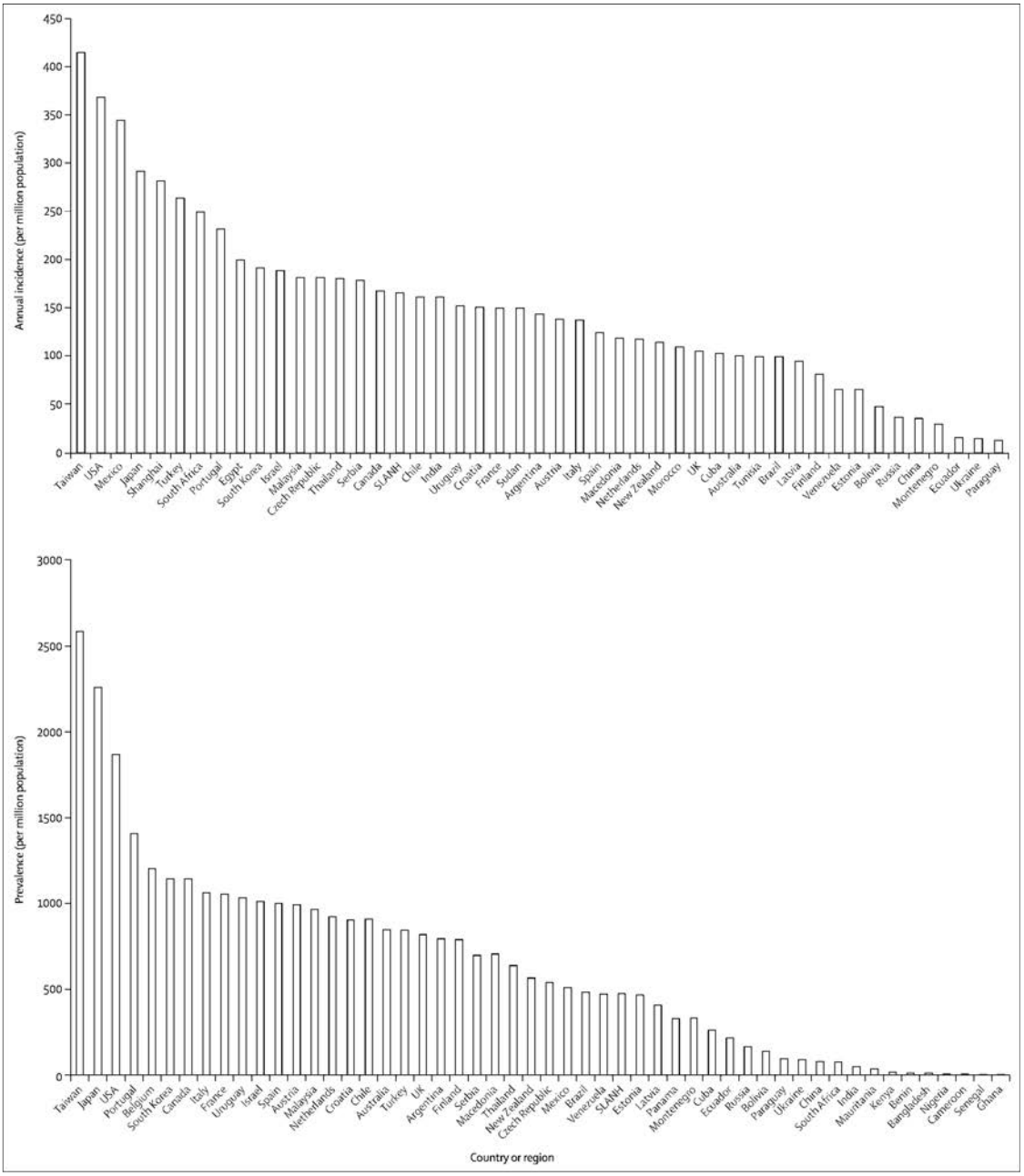
1.1.2 CKD - PREVALENCE, INCIDENCE & ECONOMIC BURDEN

Worldwide

CKD is a worldwide public health problem. A recent estimate suggests that 10% of the world's population is affected by CKD (Eckardt et al., 2013). Prevalence tends to increase with age, exceeding 20% in those over the age of 60 and 35% in those over the age of 70 (Levey & Coresh, 2012).

Prevalence, and incidence, does vary considerably across the world as demonstrated in Figure 1.1 (Jha et al., 2013). These figures can be underestimated in poorer countries where patients are unlikely to be registered on a RRT programme.

Figure 1.1: Annual Incidence (A) & Prevalence Rates (B) of ESRF in Different Countries (Reproduced from Jha et al., 2013)



In countries that can afford to offer RRT, mainly in the form of dialysis, to all patients who have progressed to renal failure, the proportion of health-care expenditure for this group of patients is far out of proportion to its size (Kerr et al., 2012; Vanholder et

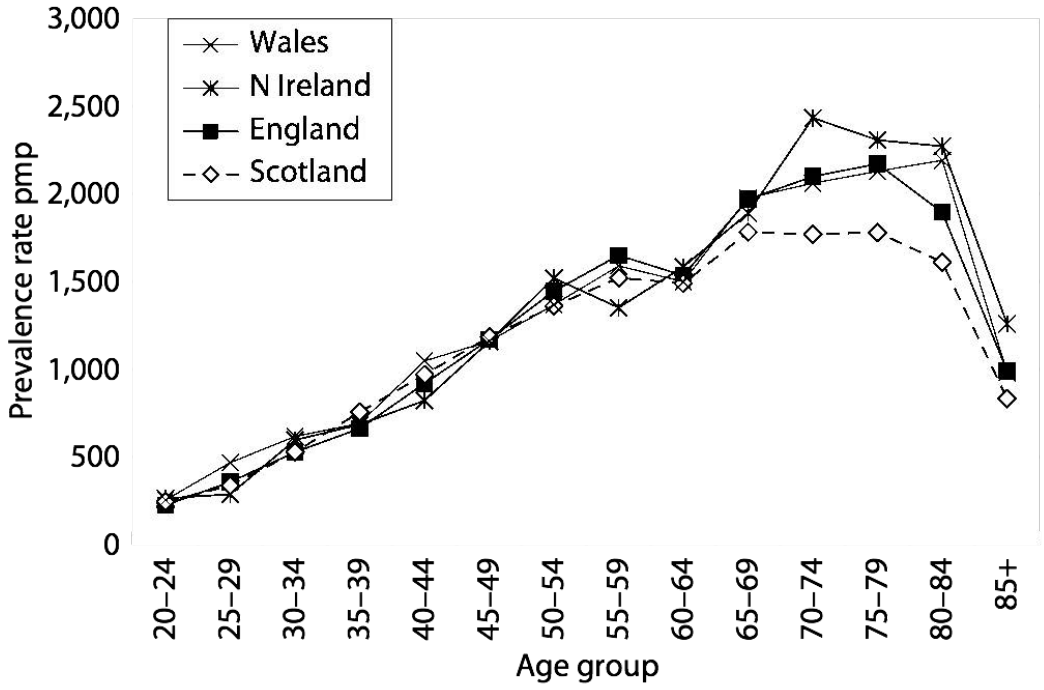
al., 2012). In many countries, economic considerations allow only restricted access, or in some cases no access, to this expensive long term treatment. It is in these poorer parts of the world that the number of patients with CKD is expected to increase most rapidly in the next decade (Couser et al., 2011).

CKD was ranked 18th in the list of causes of total number of global deaths in 2010. This equates to an annual death rate of 16.3 per 100,000 (Lozano et al., 2012). This figure has increased significantly from previous years and is likely to be an underestimate due to the contribution of CKD to death from other causes.

UK

According to the UK renal registry, there were 54,824 adult patients receiving RRT in the UK at the end of 2012, an increase of 3.7% from 2011. This corresponds to a prevalence per million population of 881. The median age of these patients was 58 years, 3 years older than in 2000, and the percentage of RRT patients aged over 70 had increased from 19.2% in 2000 to 24.9% in 2012. Prevalence rates across age groups in the UK are shown in Figure 1.2.

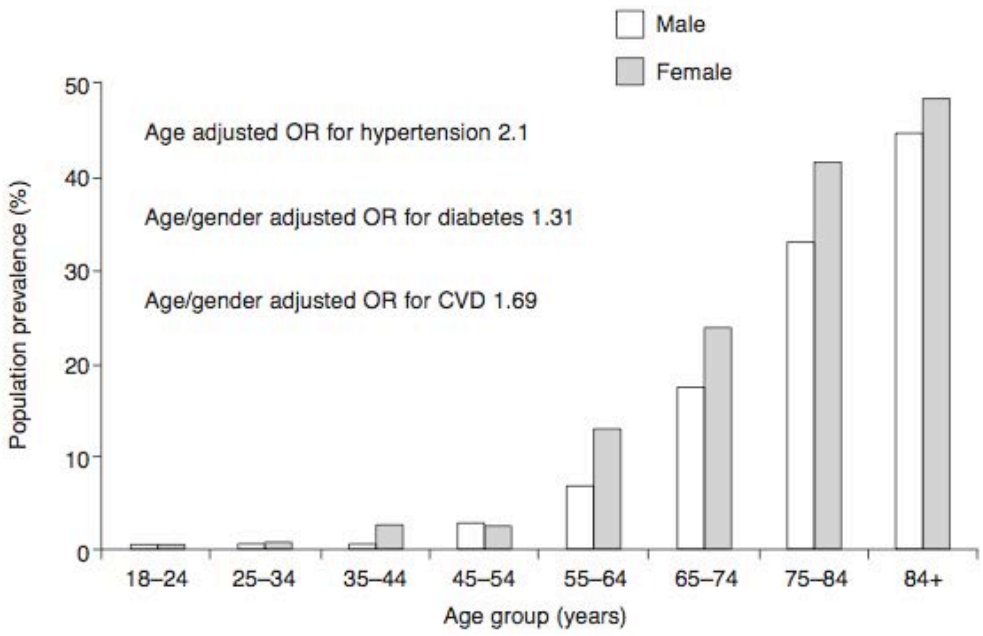
Figure 1.2: Prevalence Rates for RRT per Million Population by Age Group and UK Country on 31/12/2012 (Reproduced from Caskey et al., 2013).



The incidence in the UK has remained relatively stable over the last seven years with 6,891 patients requiring initiation of RRT in 2012 - this equates to an incidence of 108 per million population (Caskey et al., 2013).

The overall number of people with lesser stages of CKD is more difficult to ascertain. A large primary care study suggested that the age standardised prevalence of stage 3-5 CKD was around 8.5% (Stevens et al., 2007). Repeating the trend seen worldwide, the prevalence of CKD rose dramatically with age, Figure 1.3.

Figure 1.3: Prevalence of Stage 3-5 CKD in the UK (Reproduced from Stevens et al., 2007)



Prevalence is likely to increase along with an ageing population and increasing numbers with risk factors such as diabetes (Meguid El Nahas & Bello, 2005).

ESRF has a significant impact on healthcare budgets. More than 2% of the total NHS budget is spent on renal replacement therapy (dialysis and transplantation) for those with established renal failure (Sharif & Baboolal, 2011).

1.1.3 CKD - AETIOLOGY/PATHOLOGY

There are many diseases that can cause kidney damage. Some are renal in origin and others are systemic diseases that affect the kidney. These are summarised in the table below:

Table 1.4: Classification of Causes of CKD (Reproduced from Eckardt et al., 2013)

This classification system is based on the presence or absence of systemic disease and on the location within the kidney of pathological-anatomical findings.

	Examples of primary kidney diseases	*Examples of systemic diseases affecting the kidney*
Glomerular diseases	Diffuse, focal or crescentic proliferative glomerulonephritis; focal and segmental glomerulosclerosis, membranous nephropathy and minimal change disease	Obesity, metabolic syndrome and diabetes, systemic autoimmune diseases, systemic infections, drugs, complement diseases, neoplasia and haemopoietic diseases
Vascular diseases	ANCA-associated renal limited vasculitis, and fibromuscular dysplasia	Hypertension, atherosclerosis, ischaemia, cholesterol emboli, systemic vasculitis, thrombotic microangiopathy, and systemic sclerosis
Tubulointerstitial diseases	Urinary tract infections, stones, and obstruction	Systemic infections, sarcoidosis, drugs, urate, environmental toxins and neoplasia
Cystic and other congenital diseases	Renal dysplasia, medullary cystic disease, and podocytopathies	Autosomal-dominant polycystic kidney disease, Alport syndrome, and Fabry disease

Diabetes and hypertension are the leading causes of CKD in all developed and many developing countries (Jha et al., 2013). In the UK, diabetic renal disease accounts for 26% of cases of renal failure with some variation according to age group, table 1.5.

Table 1.5: Percentage Distribution of Primary Renal Diagnosis by Age, 2012 (Reproduced from Caskey et al., 2013)

	Percentage with Diagnosis		
Diagnosis	*Age < 65*	*Age ≥ 65*	*All Patients*
Diabetes	28.6	22.3	25.6
Glomerulonephritis	17.3	10.4	14.0
Pyelonephritis	6.8	6.4	6.6
Hypertension	6.2	8.8	7.4
Polycystic kidney	10.1	3.1	6.7
Renal vascular	1.7	10.9	6.1
Other	17.4	18.0	17.7
Uncertain aetiology	11.8	20.1	15.9

In developed countries, causes tend to be lifestyle related. In developing or low income countries, infectious diseases continue to be a significant contributor to the development of renal disease along with environmental pollution, pesticides, analgesic abuse, herbal medications and unregulated food additives (Ayodele & Alebiosu, 2010; Jha, 2004).

1.1.4 CKD - DISEASE PROGRESSION

The early stages of kidney disease are often asymptomatic but, if recognised through biochemical testing, may be reversible. Once established, the rate of progression of CKD is variable. Rapidly progressive diseases may lead to kidney failure within months whereas some patients do not progress during many years of follow-up (Levey & Coresh, 2012). Risk factors for disease progression have been identified.

Non- modifiable factors include genetics, race, age, and sex. For example, there is evidence that the rate of progression of CKD is faster among patients who are elderly, male, or African-American (Jungers et al., 1996; Hsu et al., 2003).

Significant modifiable risk factors include hypertension, hyperlipidaemia and cigarette smoking (Jafar et al., 2003; Klahr et al., 1994). There is considerable overlap between factors associated with the progression of CKD and increased cardiovascular risk (KDIGO, 2013). In diabetic nephropathy, poor diabetic control accelerates disease progression (Adler et al., 2003; The Diabetes Control and Complications (DCCT) Research Group, 1995). Obesity has also been linked to progression of CKD caused by glomerulonephritides, focal segmental glomerulosclerosis and IgA nephropathy (Bonnet et al., 2001; de Jong et al., 2002; Kambham et al., 2001; Verani, 1992).

Other suggested progression factors include regular and heavy alcohol consumption (Perneger et al., 1999), recreational opioid use (Perneger et al., 2001) and

analgesics (Klag et al., 1996; Morlans et al., 1990; Perneger et al., 1994). Proteinuria is a well-recognised marker of severity and prediction of progression of CKD, as suggested by the revised guidelines on staging, but controversy exists as to its possible role as a risk factor (Meguid El Nahas & Bello, 2005).

One percent of the CKD population will progress to need RRT. However it remains the most expensive of the chronic diseases and reduces lifespan significantly (KDIGO, 2013).

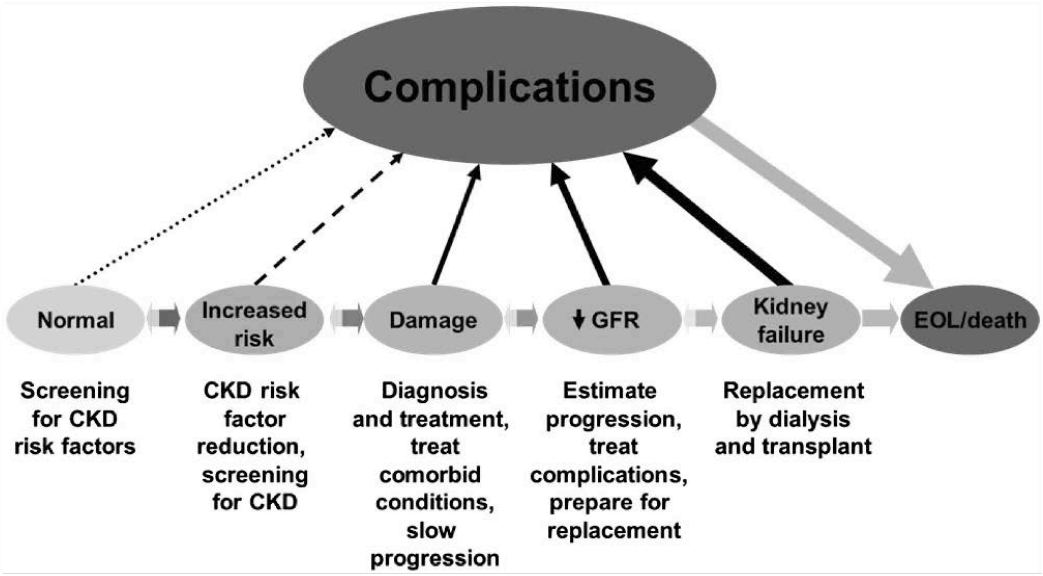
1.1.5 CKD - MANAGEMENT

Treatments for CKD aim to prevent progression, reduce complications of a decreased GFR, reduce the associated risks of cardiovascular disease and improve survival and quality of life (Levey & Coresh, 2012).

Management of all stages of CKD is beyond the scope of this thesis but figure 1.4 represents the different stages of CKD and suggested management strategies at each stage.

Figure 1.4: Conceptual Model of CKD (Reproduced from Levey et al., 2009)

This diagram represents the continuum of development, progression and complications of CKD, along with strategies to improve outcomes. EOL = End of Life.



Once in renal failure, RRT is required to maintain life. This can take the form of dialysis or transplantation. Data from the most recent UK renal registry report (Caskey at al., 2013) indicated that transplantation continued to be the most common treatment modality (50.4%) for renal failure.

1.2 RENAL TRANSPLANTATION (RT)

1.2.1 RT – OVERVIEW / DEFINITIONS

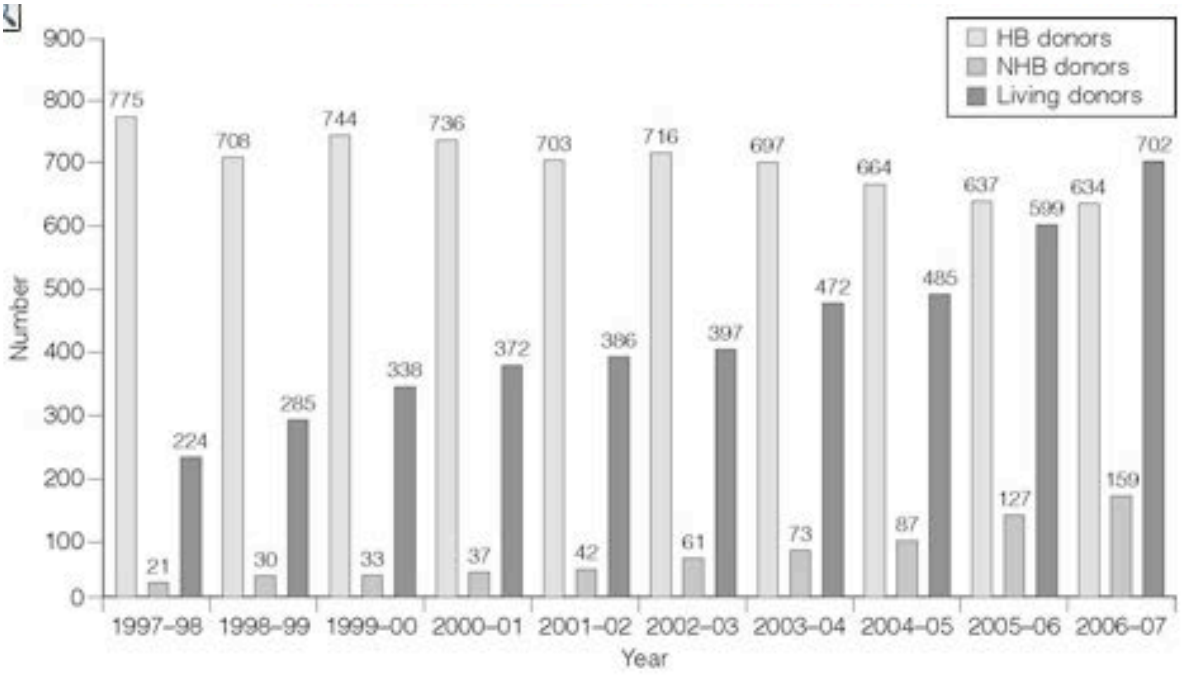
Kidney Transplantation is considered to be the gold standard treatment for patients with ESRF providing both survival benefit and improved quality of life when compared to dialysis (Wolfe et al., 1999). Patient and graft survival rates for transplantation exceed 90% per year (Taylor & Baicu, 2010). Survival on dialysis is poor in comparison with only 35% of patients alive after 5 years (Collins et al., 2008).

There is also an economic advantage. A recent estimate suggested that kidney transplantation provides a cost benefit of £25,800 per year in the second and subsequent years compared to maintaining a patient on dialysis. Over a period of 10 years, a functioning kidney transplant will provide a cost benefit of £241,000 when compared to dialysis over the same period (NHSBT Factsheet 7, www.organdonation.nhs.uk).

Procedures are classified depending on the source of the donor organ. The majority of kidneys used for transplantation are from deceased donors (cadaveric organs) (McAnulty, 2010). However, an increasing number of living-donor transplants are being performed. These can be from genetically related individuals, unrelated individuals or from altruistic donors where donation is non-directed.

Deceased donors (DD) can be further classified into Donation after Brain Death (DBD) donors or Donation after Cardiac Death (DCD) donors. These were previously termed heart-beating or non-heart-beating donors. DBD organs constitute the majority of cadaveric kidneys for transplantation although numbers of DCD organs available are on the increase. The graph below shows the trend for each type of donor procedure between 1997 and 2007 in the UK.

Figure 1.5: Number of Deceased and Living Donors in the UK 1/4/1997-31/3/2007 (Reproduced from Forsythe, 2013)



1.2.2 RT - COLD ISCHAEMIC TIME

Cold Ischaemic Time (CIT) is defined as the time from perfusion of an organ by cold solutions until transplantation into the recipient (Monbaliu et al., 2012).

Initial cold flush of the organ at retrieval aims to clear the vasculature of blood and lower the temperature. Traditionally, cold temperatures are then maintained until the time of transplantation with the aim of slowing down metabolism and preserving kidney function. Cold preservation has been key to the development of clinical transplant programmes; however, there are disadvantages of cooling ischaemic tissue.

CIT, particularly if prolonged, is associated with renal ischaemic injury which impacts negatively on graft function and survival (Watson et al., 2010). It is therefore of key consideration when organising deceased donor transplants.

1.2.3 RT - OUTCOMES

A number of outcomes can be observed following kidney transplantation.

In the early post transplant period, kidneys can display immediate graft function (IGF) or may suffer from delayed graft function (DGF).

DGF is most often defined as the requirement for dialysis within the first post-operative week (Polyak et al., 1999). The criticism of this definition is that it is subjective and over-estimates the true rate of DGF as it includes patients who underwent dialysis following transplantation for any reason. An alternative definition is functional DGF (fDGF) where creatinine fails to decrease by 10% for 3 consecutive days in the first postoperative week. Lack of consistency in the definition of DGF can make it difficult to compare this outcome between studies (Yarlagadda et al., 2008).

DGF is associated with significant morbidity during the peri-transplant period and is an important risk factor for acute rejection (Gjertson, 2002), graft loss (Ojo et al., 1997) and death with a functioning graft (Tapiawala et al., 2010). It also results in prolonged hospital stays with associated increased costs (Yarlagadda et al., 2009).

Primary non-function (PNF) occurs when the transplanted kidney never works sufficiently to allow cessation of dialysis. As mentioned above, graft loss can occur which can result from a variety of causes, such as renal vein thrombosis or recurrent disease, and kidneys can also suffer from rejection.

Survival outcome is often measured as survival with or without a functioning transplant. Long-term function of transplanted kidneys is influenced by donor-dependent factors (such as age, cause of death or mode of death) duration and type of organ preservation and recipient factors (such as co-mobidity, gender, ethnicity) (Polyak et al., 1999).

1.2.4 RT - THE ORGAN SHORTAGE & MARGINAL ORGANS

A shortage of suitable donor organs has always limited clinical transplantation. As solid organ transplantation changed from an experimental procedure with a low chance of success to being the current gold-standard, increasing numbers of patients have required a transplant (Henry & Guarrera, 2012).

For every patient that receives a transplanted organ in the US, there are four more on the waiting list. This pattern is repeated across Europe. In addition, it is said that a patient dies from the lack of a transplant almost every 1½ hours (Lee & Mangino, 2009).

A number of measures have been introduced in order to address the donor shortage. One of these measures is the increased used of organs that may previously have been considered as too high risk for transplantation. These are termed marginal organs. Marginal organs include DCD organs and organs from older donors with co-morbidity (Extended Criteria Donors, ECDs).

All organs for transplantation are exposed to ischaemic injury as a result of interruption to blood supply. DCD organs are exposed to a period of warm ischaemic time (WIT) prior to retrieval. WIT per minute, is much more harmful than the same length of exposure of cold ischaemia - this adversely affects graft outcome (Shiroki et al., 1998). DCD organs essentially experience two insults - the initial warm ischaemia followed by the cold ischaemic storage time. Effects of this combined insult became

more noticeable as increased storage times were required with the growth of clinical transplantation. DGF rates began to approach 60-80% - much higher than with shorter transport times or with DBD organs (Brasile et al., 2001). However, it is of utmost importance to address this mechanism of injury, as DCD organs constitute the deceased donor group with the largest potential for growth (Hayakawa et al., 2006).

ECD kidneys are those from donors aged 60 or over, or those aged between 50-59 with two or more of the following; hypertension, intra-cerebral cause of death or creatinine over 132 μmol (Port et al., 2002).

Both types of marginal organs carry a higher risk of Delayed Graft Function (DGF) and ECD kidney recipients have a 69% higher relative risk for graft failure (Metzger et al., 2003a). However, long-term survival of functioning grafts and survival of recipients are similar in both groups (Gok et al., 2002; Keizer et al., 2005; Nicholson et al., 2000). In addition, patient survival is better in recipients of marginal kidneys than in patients on dialysis, with a gain in life expectancy ranging from 3 to 9 years (Ojo et al., 2001).

To increase the number of patients benefitting from kidney transplantation, use of marginal organs has to be increased and outcomes improved. Some have suggested that the main reason for underutilisation of these organs is because static cold storage (SCS), the main storage method worldwide and in the UK, is inadequate (Lee & Mangino, 2009; McAnulty 2010). This limits the timeframe to organise the

transplant process and seems particularly problematic for DCD organs as SCS becomes ineffective when the warm ischaemic time exceeds 20 minutes (Lee & Mangino, 2009).

1.2.5 RT - ISCHAEMIA-REPERFUSION INJURY

Ischaemia

In order to retrieve organs for transplantation, it is unavoidable that they are subject to a period of ischaemia. Almost immediately after interruption of the blood supply, tissues become completely or partially starved of oxygen and metabolism is very rapidly switched from aerobic to anaerobic (Taylor & Baicu, 2010). Less energy, in the form of adenosine triphosphate (ATP), is produced by this type of metabolism for on-going cellular processes. Metabolic substrates to support anaerobic glycolysis are limited and intermediates from this process, such as lactate and protons, increase cellular osmotic load. ATP and adenosine diphosphate (ADP) are broken down into their constituent parts which can escape from the cell. This means that, even when the blood supply is restored, there is a shortage of substrates to form high energy phosphates (Kosieradzki & Rowiński, 2008). When ischaemia lasts for over 24 hours and energy substrates are lost, ATP can no longer be reformed, leading to lethal cell injury (Sammut et al., 2000).

The depletion of ATP in ischaemic cells has further repercussions. The energy dependent Na+/K+ membrane pump can no longer function effectively to maintain the membrane potential and cell excitability. Sodium can enter the cell followed by water which produces oedema (Ahmad et al., 2004). Oedema is further worsened by the increase in cell osmolarity caused by the intermediates of anaerobic glycolysis and ATP/ADP breakdown, as mentioned previously. Water can then enter by simple diffusion, aquaporin and chloride channels as well as via the glucose transporter (Kosieradzki & Rowiński, 2008).

Oedema results in disruption of all cellular membranes including the membranes of endoplasmic reticulum (ER), Golgi apparatus, mitochondria and cytoskeletal microtubules. This damage is ischaemia time-dependent and results in cell death (Mangino et al., 2008).

Acidosis occurs as an immediate result of anaerobic glycolysis producing lactate. As there is no blood flow to remove the lactate, further energy production is halted, and cell death results (Kosieradzki & Rowiński, 2008). A decreased pH is uniformly observed in ischaemic tissues (Anderson et al., 1999; Soric al., 2007).

Mitochondrial dysfunction is a critical event during ischaemia as it initiates necrosis and apoptosis cascades during reperfusion (Kosieradzki & Rowiński, 2008). Dysfunction of cellular membrane pumps and redistribution from intracellular stores leads to the accumulation of calcium within the cell early in the ischaemic process (Schumacher et al., 1998). Influx of extracellular calcium only occurs during

prolonged ischaemia and reperfusion (Kusuoka et al., 1993). Calcium is retained in the mitochondrial matrix in order to maintain its membrane potential. Mitochondrial calcium overload affects a number of complexes which result in matrix swelling and damage to mitochondrial membranes (Kosieradzki & Rowiński, 2008). It also causes a non-selective pore (mPT), which is impermeable under normal conditions, to open. Prolonged opening of these channels may differentiate reversible from irreversible reperfusion injury (Griffiths & Halestrap, 1995; Halestrap, 2006) and may have a role in attenuating ischaemia-reperfusion (IR) injury.

Free radicals are formed even during global ischaemia if small amounts of oxygen are available. These are believed to be formed by the deamination of adenosine and resultant changes to xanthine dehydrogenase in response to ischaemia (Kosieradzki & Rowiński, 2008). They can also be formed by the respiratory complex (Chen et al., 2007).

Reperfusion

It is considered to be the combination of a long period of cold ischaemia followed by reperfusion that is responsible for tissue injury in this process. Survival or death of the cell depends on the balance between repair and regeneration and harmful processes such as apoptosis, autophagy and necrosis (Kosieradzki & Rowiński, 2008). Apoptosis requires energy and protein synthesis so occurs mainly on reperfusion. The ability of a cell to recover from injury may partly be dependent on

whether calcium concentration can be restored to normal levels (Vanden Hoek et al., 2003).

Rapid release of free radicals on reperfusion is a well-recognised event most probably produced by the respiratory chain. These may be a cause of endothelial injury and also act to worsen cell oedema (Kosieradzki & Rowiński, 2008). Mitochondria are important in the reperfusion process as they have the ability to produce free radicals but also have potent radical-scavenging potential.

Damaged ER fragments increase in reperfused tissues which signal upcoming cellular disintegration (Kirino & Sano, 1984). Lysosomal activity of proteases and cathepsins increase leading to cell death via necrosis or apoptosis. Neutrophils also contribute by causing direct cytotoxicity via the generation of oxygen free radicals and release of cytokines (Kosieradzki & Rowiński, 2008).

Tissues tend to be hyperaemic initially following reperfusion but flow rates soon drop to below normal. This is mostly driven by a decreased metabolic demand and extracellular oedema but neutrophil and platelet accumulation in blood vessels can diminish blood flow (Galiuto & Crea, 2006). The effects of this are not fully understood.

Prevention or suppression of IR injury is important to prevent poor graft function following renal transplant with severe injury being associated with DGF and PNF.

Marginal kidneys are more prone to this type of injury (Dittrich et al., 2004; Dragun et al., 2001; Gok et al., 2004; Harper et al., 2008; Hosgood et al., 2008).

Many components of the transplant procedure can have an effect on injury level and one of those factors is the preservation method.

1.3 ORGAN PRESERVATION

1.3.1 HISTORY OF ORGAN PRESERVATION

The first recorded attempt to perfuse an isolated organ was using the kidney; this was reported as early as 1849 by Loebel (Hoffman, Burger, & Persky, 1965). Also among the cited early experimenters were Le Gallois (Le Gallois, 1812), studying heart perfusion, and Bernard's work on liver perfusion (Bernard, 1855).

In the 1930s, Alexis Carrel collaborated with the aviator Charles Lindbergh to cultivate and perfuse organs with the help of small pumps (Carrel & Lindbergh, 1935). At this time, most work focused on perfusion at normal body temperatures (Fuller & Lee, 2007) with whole blood or cryoprecipitated plasma used as the perfusate (Yuan et al., 2010).

When transplantation became a clinical reality in the 1960s, organ preservation methods were re-evaluated. The potential benefits of hypothermia in clinical practice were beginning to be recognised and renal function had been maintained on a pump oxygenator (Couch, Cassie, & Murray, 1958; Levy, 1959). This provided the basis for the development of hypothermic perfusion.

Further important contributions to the acceptance of hypothermic perfusion techniques were made by the Humphries group (Humphries et al., 1964a, 1964b; Humphries et al., 1968b, 1968c) and Belzer who established the technique as

clinically useful (Belzer, Ashby, & Dunphy, 1967; Belzer et al., 1968; Belzer et al., 1972). Belzer developed one of the first reliable transportable hypothermic perfusion machines (Fuller & Lee, 2007). He also recognised how useful hypothermic machine perfusion (HMP) would be to facilitate an organ matching programme not so limited by transport logistics (Belzer et al., 1972). Major advances in hypothermic perfusion were initially achieved with the kidney; interest in perfusing other organs then followed (Fuller & Lee, 2007).

The majority of kidneys in the US in the 1970s were actually preserved by machine perfusion. However, studies at that time failed to demonstrate superiority of HMP (Clark et al., 1974; Opelz & Terasaki, 1976, 1982; van der Vliet et al., 1983) whilst there were major improvements in immunosuppression which did improve transplant outcomes. This, along with cost implications and ease of use, led to SCS being the most common form of storage in the 1980s (Yuan et al., 2010).

Further developments in preservation solutions improved the storage of organs by SCS. Collins developed a simple, effective SCS solution in 1969 that was modified in 1976 to form the Euro-Collins (EC) solution (Collins, Bravo-Shugarman, & Terasaki, 1969; Yuan et al., 2010). In the 1980s Belzer developed the University of Wisconsin (UW) solution, which although expensive, gradually became the preservation solution of choice for prolonged periods of SCS (Opelz & Döhler, 2007; Ploeg et al., 1988). Bretschneider developed an alternative solution, histidine-tryptophan-ketoglutarate (HTK), which also showed beneficial effects for the preservation of abdominal organs (Groenewoud et al., 1989; Isemer et al., 1988).

During the 1990s, interest in HMP returned due to the increasing success of transplantation and the need to use more DCD and ECD organs. These organs are more prone to cold ischaemic damage and in order to maintain transplant outcomes preservation techniques needed to improve. HMP emerged as a technology with promising results in experimental preservation of abdominal organs and with clinical use in kidney transplantation (Henry & Guarrera, 2012).

1.3.2 PRESERVATION FLUIDS

Preservation solutions are designed to counteract the effects of hypothermia and extend organ preservation time. Most static cold storage fluids are based on three essential constituents: An adequate electrolyte composition, an impermeant to prevent cell swelling and a buffer to prevent acidosis (Fuller & Lee, 2007; McAnulty, 2010).

In terms of HMP, different considerations needed to be addressed. It was recognised early in the development process that balancing the oncotic pressure of the solution with the applied perfusion pressure was vital to prevent tissue oedema (Fuller & Lee, 2007). Use of perfusates without colloid had been sub-optimal when the necessary perfusion pressure has been applied (Humphries et al., 1968a). Diluted blood was used in the initial experiments with HMP but there were problems with increased viscosity and thrombus formation (Humphries et al., 1964a; Humphries et al., 1964b; Humphries et al., 1968c). Similar problems were found with the use of diluted plasma

(Belzer et al., 1968). Methods to fractionate the plasma were developed which helped to address these issues (Belzer et al., 1968; Toledo-Pereyra, Buselmeier, & Najarian, 1975; Toledo-Pereyra et al., 1974). Perfusates based on serum albumin were developed around the same time (Claes et al., 1972; Pegg & Green, 1972).

A completely synthetic perfusate, with hydroxy-ethyl-starch (HES) as the colloid, was developed in the 1980s by Belzer which allowed longer periods of perfusion (McAnulty et al., 1989; Pienaar et al., 1990). Although this was adopted by many North American centres using machine perfusion, the development of UW solution shortly afterwards allowed longer periods of SCS and as such, SCS remained the storage method of choice worldwide (Henry & Guarrera, 2012).

There have been many preservation solutions developed for use in clinical transplantation and many are still in development in the experimental stages. The more common perfusion fluids in clinical use for SCS and HMP are briefly described below:

Collins solution, developed in 1969, is a crystalloid solution containing high concentrations of glucose and the impermeant magnesium sulphate. These compounds acted to decrease cell swelling and allowed longer SCS times. This was modified by the Eurotransplant Organisation in 1976 to *Euro-Collins'* solution. Magnesium sulphate was omitted and the dextrose concentration was increased (Toledo-Pereyra, Palma-Vargas, & Toledo, 2010). Euro-Collins solution has been widely used for SCS with successful clinical results (Squifflet et al., 1981).

Hyperosmolar citrate (HOC) is a basic solution routinely used in the UK. It is less viscous than some other solutions and adequately flushes and cools the kidney (Wilson et al., 2007). It was developed to try to improve upon the Euro-Collins solution. Citrate replaces phosphate and mannitol replaces glucose.

University of Wisconsin (UW) solution was developed by Belzer in the 1980s. It is currently the most widely used preservation solution and is considered to be the gold-standard for SCS (Yuan et al., 2010). It contains metabolically inert substances, lactobionate and raffinose, to maintain the necessary osmotic concentration and HES as a colloid to prevent influx of water into the cell. ATP precursors (adenosine) are present for energy and oxygen free radical scavengers (glutathione and allopurinol) to attenuate IR injury.

SCS with UW provides satisfactory short and long-term outcomes (Opelz & Döhler, 2007). However, it does have high viscosity which can compromise the microcirculation (Tojimbara et al., 1997). It also has a high potassium concentration which can cause vasoconstriction and may contribute to the hyper-aggregation of HES (Morariu et al., 2003; Olschewski et al., 2008). It is also expensive and has fallen out of favour for the flushing of abdominal organs (McAnulty, 2010).

A modified version of UW exists for HMP, *Belzer Machine Perfusion solution* (BMPS), where lactobionate is replaced with gluconate and the potassium concentration is reduced (Bessems et al., 2005; Pienaar et al., 1990). Many of the other HMP fluids have been developed based on this.

Histidine-Tryptophan-Ketoglutarate (HTK) solution was also introduced in the 1980s by Bretschneider, originally for use as a cardioplegic solution (Bretschneider, 1980). Histidine is present as a buffer, tryptophan for membrane stabilisation and ketoglutarate for anaerobic metabolism. HTK has a low viscosity which may allow for improved microcirculation (Maathuis, Leuvenink, & Ploeg, 2007; Mühlbacher, Langer, & Mittermayer, 1999).

Comparative studies between HTK and UW for the SCS of kidneys had failed to consistently show superiority of either solution (Agarwal, Murdock, & Fridell, 2006; de Boer et al., 1999; Lynch et al., 2008; Roels et al., 1998). However, a recent retrospective multi-centre analysis demonstrated an increased risk of graft loss for cadaveric kidneys preserved by HTK compared to those stored in UW solution (Stewart et al., 2009). HTK is less expensive per litre than UW, but increased volumes of HTK are required (Mühlbacher et al., 1999).

Celsior solution was originally developed for heart transplantation (Menasché et al., 1994). It combines the osmotic efficacy of UW (lactobionate, mannitol) and the potent buffering ability of HTK (Yuan et al., 2010). In comparisons with UW, studies have demonstrated similar rates of DGF and graft survival (Marcén et al., 2005; Montalti et al., 2005; Nunes et al., 2007).

Institute-George-Lopez (IGL-1) was developed more recently by a group in France. It contains polyethylene glycol which binds to cell and tissue surfaces, stabilising the underlying surfaces from cell interactions - this may modify donor tissue

immunogenicity and attenuate IR injury (Eugene, 2004). A reduction in DGF has been demonstrated when compared to kidneys stored in UW (Badet et al., 2005) but this was not repeated in a recently published multi-centre study (Codas et al., 2009).

KPS-1® Kidney Perfusion Solution is a solution marketed by Organ Recovery Systems for use with their HMP machine - the LifePort© Kidney Transporter. This is the solution used throughout this study. It has the same composition as the machine solution produced by Belzer. The constituents of KPS-1® are shown below in table 1.6:

Table 1.6: Constituents of KPS-1®

Constituents	*Amount/1000mL*	*Concentration mM*
Calcium chloride (dehydrate)	0.068g	0.5
Sodium hydroxide	0.70g	
HEPES (free acid)	2.38g	10
Potassium phosphate (monobasic)	3.4g	25
Mannitol (USP)	5.4g	30
Glucose, beta D (+)	1.80g	10
Sodium Gluconate	17.45g	80
Magnesium Gluconate (D (-) gluconic acid, hemimagnesium salt)	1.13g	5
Ribose, D (-)	0.75g	5
Hydroxyethyl starch (HES)	50.0g	n/a
Glutathione (reduced form)	0.92g	3
Adenine (free base)	0.68g	5
Sterile water for injection (SWI)	To 1000mL volume	n/a

1.3.3 STATIC COLD STORAGE

Static Cold Storage (SCS) is the most common storage method used for cadaveric kidneys in the UK and worldwide. Following initial cold flush at retrieval, to remove the blood and lower the temperature, kidneys are simply stored in preservation fluid within an ice-box. This has the advantage of being cheap and it requires no special equipment or training.

The standard recommended temperature for cold storage is 4°C. Below these temperatures, freezing can occur which can cause coagulative necrosis upon reperfusion. Temperatures over 4°C in static storage conditions are not sufficient to suppress metabolism. Injury can result from depletion of ATP, accumulation of lactate and mitochondrial dysfunction (Henry & Guarrera, 2012).

SCS has been invaluable in allowing the establishment of sustainable clinical transplant programmes (Calne et al., 1963). However, as the cold ischaemic time (CIT) increases during SCS, so does the risk of suffering from delayed graft function (Koning et al., 1997; Locke et al., 2007; Ojo et al., 1997; Peters et al., 1995; Schold et al., 2005) and subsequent graft failure (Locke et al., 2007; Meier-Kriesche, Schold, & Kaplan, 2004; Opelz & Döhler, 2007). Whilst early experiments with improved preservation solutions in the 1960s allowed SCS time to be extended beyond 24 hours for kidneys (Collins, Bravo-Shugarman & Terasaki, 1969; Collins et al 1969), a safe limit of around 18 hours has been suggested more recently (Opelz & Döhler, 2007). However, in the context of marginal organs, this is probably generous.

1.3.4 HYPOTHERMIC MACHINE PERFUSION

Hypothermic Machine Perfusion (HMP) is a mechanical method of preservation during which cold preservation solution is actively pumped through the vasculature of the kidney during storage time. It is based upon the principle that devices can be designed to facilitate the replacement of blood in the circulation of an ex-vivo organ with specifically designed fluids to maximise the protective effects of hypothermia on the ischaemic tissue (Taylor & Baicu, 2010). Machine perfusion is not a new concept. It was equally in practice in the early days of transplantation but gradually lost favour due to a range of logistical and economic issues (Calne et al., 1963). Several factors have now led to a renewed interest in HMP as an alternative to SCS.

Technology has improved since the early perfusion systems were developed. Belzer developed one of the first reliable, transportable HMP machines (figure 1.6) (Fuller & Lee, 2007) but the equipment was bulky and difficult to operate.

Modern commercially available machines, such as the LifePort© Kidney Transporter 1.0 (Organ Recovery Systems) or the Waters Medical Systems' RM3 Renal Preservation System, are much smaller, easier to transport and, although some training is required, quite easy to use.

Figure 1.6: Transportable Renal HMP Machine Pioneered by Belzer

Professor Belzer with the first 'transportable' HMP machine

Figure 1.7: LifePort© Kidney Transporter 1.0 (Organ Recovery Systems)

Modern HMP machine

There is also increasing evidence of the benefits of HMP. It has been suggested that HMP achieves improved perfusion of the microvasculature along with improved removal of waste products and an increased ability to support oxidative metabolism; this may reduce tissue injury (Fuller & Lee, 2007). Evidence of benefit in clinical application continues to increase (Section 1.3.8) with particular value identified for marginal kidneys (Moers et al., 2009). Some have recommended HMP as the preferred storage for ECD and DCD kidneys (Kosieradzki & Rowiński, 2008).

HMP also provides a unique opportunity for assessment of organ viability prior to transplantation. Accurate evaluation of allograft quality is essential to prevent unnecessary kidney discard, allow maximised donor-recipient matching and to plan appropriate peri-operative care. Viability assessment is even more important in the context of marginal organs where the most common concern is organ quality. The ability to evaluate potentially transplantable organs by machine in-vitro perfusion may significantly increase the number of organs available for use (Taylor & Baicu, 2010). HMP parameters, and/or biomarkers, may be able to indicate the extent of ischaemic injury and may have stronger predictive value for graft function than conventional clinical criteria alone (Polyak et al., 1997; Kosieradzki & Rowiński, 2008).

HMP also permits pharmacological manipulation of the perfusate, for example, with cytoprotective or immune modulating drugs. There is also the possibility of providing metabolic support. One of the most commonly discussed additions is supplementary oxygen. Opinion on this particular addition is divided. Oxygen may have a protective effect of limiting mitochondrial damage and reducing the formation of oxygen free

radicals during reperfusion (Maathuis et al., 2007; Treckmann et al., 2009). Others have reported enhanced free radical production (Fuller, Gower & Green, 1988; Mcanulty & Huang, 1997). There is not a clear consensus on this type of injury.

Some studies have implied that the use of HMP can safely allow longer storage times (Ciancio et al., 2010; Schold et al., 2005). This is of particular interest in this study, as a safe extension of CIT could have significant benefits in providing a safe and successful clinical transplant service.

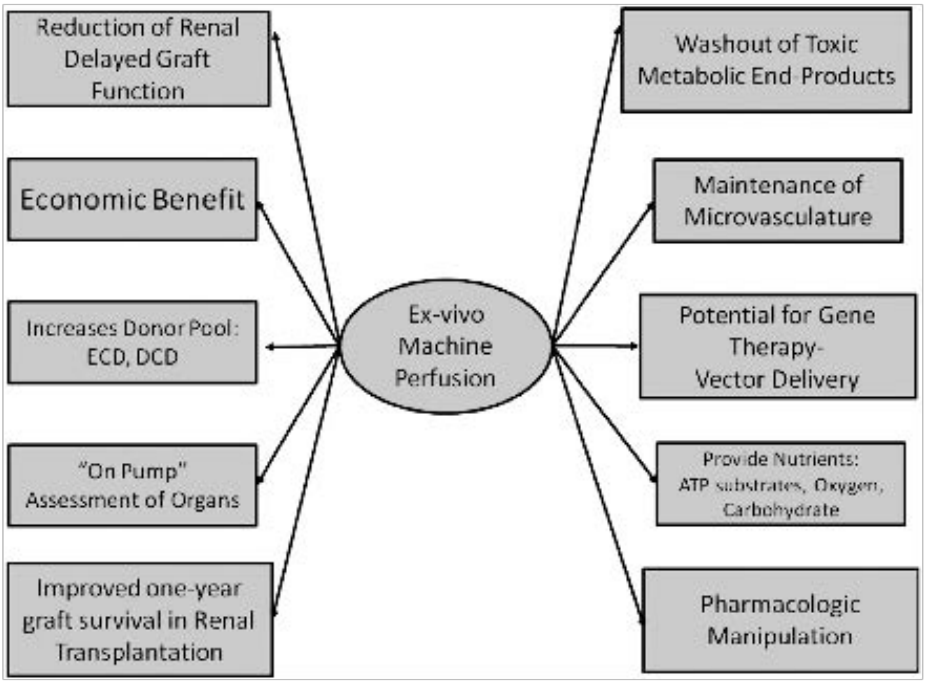
Some training is required to operate the perfusion machines, certainly more than is required to place an organ in SCS. However, the procedure is not difficult. There is also the initial cost associated with purchasing the machine and disposables with some questioning its cost-effectiveness when compared with SCS (Bond et al., 2009). However, there is increasing evidence that HMP lowers DGF rates. Reducing dialysis requirements and hospital stay following the use of HMP could off-set perfusion costs (Wight et al., 2003a; Groen et al., 2012).

There is a suggestion that to gain the most benefit from HMP, organs should be placed on the machine from retrieval (Irish & Katz, 2010). In the UK, retrieval teams do not have this resource available so kidneys are transported to the receiving centre in SCS prior to a decision to use HMP. It could be that the combination of techniques lessens the benefit from HMP (Hosgood & Nicholson, 2011), or differing ratios of HMP to SCS might have different effects.

The diagram below (figure 1.8) summarises the benefits of HMP and the possible underlying mechanisms:

Figure 1.8: Schematic Summary of the Important Benefits of HMP for Clinical Transplantation (Reproduced from Henry & Guarrera, 2012)

Outcome benefits are shown on the left side, mechanistic considerations on the right side



1.3.5 HMP MECHANISMS

Although beneficial effects have been demonstrated using HMP, the mechanisms underlying these effects are poorly understood.

One of the most simplistic contributing factors is likely to be the maintenance of renal vascular bed patency by the regulated flow (Yuan et al., 2010). However, there has been much debate over the years regarding the ideal type of flow provided by HMP and also the best perfusion pressure.

Pumps in HMP machines were developed that produced either continuous or pulsatile flow. Whilst some studies demonstrated comparable results (Kozaki et al., 1995; Kozaki et al., 2000) others have shown improved microcirculation and organ function using pulsatile perfusion for abdominal organs (Dutkowski et al., 1998; Fukae et al., 1996). It was hypothesised that pulsatile flow could overcome the initial opening pressure of the capillary bed without requiring continuous high pressure.

High-pressure flow has been linked to endothelial injury and the development of oedema: this can reduce perfusion (Fuller & Lee, 2007; Gattone et al., 1985). Mechanical injury associated with HMP can be attenuated by using lower perfusion pressures and appropriate solutions (Cerra et al., 1977). The Newcastle group recommend perfusion pressures of less than 60mmHg (Balfoussia et al., 2012) whilst kidneys included in a large European multi-centre randomised control trial were all perfused at 30mmHg (Moers et al., 2009).

Pulsatile flow and HMP has been linked to the increased expression of endothelial protective genes (Boon & Horrevoets, 2009; Yuan et al., 2010). Of particular interest is the gene Kruppel-like factor 2 (KLF2), which may be responsible for the inhibition of pro-inflammatory responses as part of the innate immune system, which would otherwise cause endothelial damage (Tullius & Garcia-Cardena, 2009; Parmar et al., 2006; Sebzda et al., 2008). KLF2 is also linked to the production of endothelial-derived nitric oxide, a potent vasodilator, and the expression of anti-thrombogenic genes (Tullius & Garcia-Cardena, 2009).

HMP is able to provide efficient delivery of nutrients and oxygen to support tissue metabolism and is able to do this over a prolonged period (Maathuis et al., 2007; Yuan et al., 2010). It is also able to facilitate recovery of energy stores to maintain cellular processes (McAnulty, 2010). Interestingly, in porcine liver experiments using nuclear magnetic resonance (NMR) imaging, ATP levels were identifiable in HMP livers but not those stored by SCS (Changani et al., 1997). In addition to supply of nutrients, HMP may also improve the 'washout' removal to toxic metabolites (Yuan et al., 2010).

1.3.6 HMP VIABILITY ASSESSMENT

Accurate assessment of graft viability is essential to prevent unnecessary kidney discard, ensure ideal matching of donor and recipient and to plan post-operative

care. This is becoming increasingly important with the use of marginal organs and their higher risk of poor graft function.

Whilst donor information, such as patient age, co-morbidity or terminal serum creatinine, along with kidney biopsy data can be useful, these methods have limited accuracy for prediction of graft outcomes (Jochmans & Pirenne, 2011; Louvar et al., 2009). Predictive models have been developed using different combinations of these parameters some of which are in clinical use (Anglicheau et al., 2008; Cockfield et al., 2010; Munivenkatappa et al., 2008; Nyberg et al., 2003; Rao et al., 2009; Schold et al., 2005).

Biomarkers measured in urine and perfusate have an advantage over biopsy data of being non-invasive and can be measured more frequently and objectively (Bhangoo et al., 2012). HMP is an ideal method of storage for biomarker assessment providing easy and safe access to perfusate and tissue during storage time.

A recent review highlighted the biomarkers that have been assessed in urine and machine perfusate in regards to graft outcome (Bhangoo et al., 2012). Few studies were recent, and even fewer were deemed to be of good quality. Several perfusate markers were significantly associated with allograft outcomes; Lactate dehydrogenase (LDH), glutathione-S-transferase (GST) and aspartate transaminase (AST) were linked to DGF in the greatest number of studies. Further validation was recommended. Other markers investigated in kidney perfusate included glucose,

lactate, electrolytes, pH, gas pressures, osmolarity, redox active iron, malondialdehyde and total antioxidant status.

Machine perfusion parameters, such as flow rate and vascular resistance, are generally accepted as good indicators of graft quality (Kozaki et al., 2000; Matsuno et al., 2000; Polyak et al., 2000). Kidneys showing the best functional outcome during HMP tend to have reduced intra-renal resistance during the perfusion process, implying that the machine improves renal micro-vascular circulation (Wight et al., 2003a). Several studies, however, have warned of the dangers of using resistance values to determine kidney discard and although it has been demonstrated as an independent predictor of DGF, its predictive value is poor (Jochmans & Pirenne, 2011; Jochmans et al., 2011; Sonnenday et al., 2003; de Vries et al., 2011).

The Newcastle clinical viability protocol devised for HMP kidneys from DCD donors uses a combination of HMP parameters and a perfusate biomarker. It requires a perfusion flow rate of 0.4mL/min/100g/mmHg or more and a perfusate GST concentration of less than 100 IU/L/100g renal mass (Navarro et al., 2008). Other similar protocols also exist (Matsuno et al., 2006).

Despite considerable interest in various indicators and markers of graft quality, few are used routinely in clinical practice, and no single biomarker or parameter has yet proven to be definitive.

1.3.7 HMP PHARMACOLOGICAL MANIPULATION

In addition to viability assessment during HMP time, there is also the opportunity to modify the perfusate with various pharmacological agents.

Several groups have studied the use of prostaglandin-1 (PGE1) added to the perfusate. PGE1 may act to stabilise endothelial cell membranes, inhibit platelet aggregation that can cause microvascular thrombi and attenuate neutrophil sequestration and degranulation (Fantone & Kinnes, 1983; Schlondorff, Yoo, & Alpert, 1978). Other studies have cited its role in ameliorating mitochondrial ischaemic injury and reducing release of calcium ions into the perfusate as its beneficial mechanism (Polyak et al., 2000; Polyak et al., 1999). Its use with HMP has been shown to markedly improve early graft function compared with SCS (Polyak et al., 1999).

Other groups have examined the use of nitric oxide, an important factor in maintaining vascular endothelium. Its addition has been shown to be beneficial in experimental models of kidney transplantation (Erkasap & Ates, 2000).

Machine perfusate has also been modified with nitroglycerin and polyethylene glycolsuperoxide dismutase - these additions to Belzer Machine Perfusion Solution (BMPS) have been shown to improve DGF when compared to the solution alone (Guarrera et al., 2004). Phentolamine mesylate, hydralazine and calcium channel blockers have also been used experimentally (Henry & Guarrera, 2012).

Manipulation of the perfusate during HMP may allow modification of injuries acquired during the retrieval and transport period with the potential to improve graft outcomes.

1.3.8 HMP CLINICAL OUTCOMES

There is an increasing body of evidence to support the benefits of HMP over SCS as the preservation method of choice for cadaveric renal transplantation. However, early evidence was equivocal.

Initial studies comparing HMP to SCS failed to show consistent benefits of either storage method in terms of DGF or graft survival (Barber et al., 1988; Burdick et al., 1997; Clark et al., 1974; Halloran & Aprile, 1987; Light et al., 1995; Sellers et al., 2000). In the studies that showed reduced DGF with HMP, the possibility of reduced costs associated with dialysis requirements and hospital stay was also suggested. However, many of these studies were retrospective, non-randomised and compared a different donor and recipient profile compared to the current transplant population.

Some of the earlier studies compared paired kidneys from the same donor, one stored in SCS and the other by HMP, in an attempt to control for donor factors. One of these studies found a significant decrease in post-transplantation dialysis in kidneys undergoing HMP, but only 29 kidney pairs were analysed (Alijani et al., 1985). Two larger studies of kidney pairs failed to show any difference in dialysis

rates post-transplantation in the different storage groups (Merion et al., 1990; Mozes et al., 1985).

Recent studies have been better powered and more accurately represent the current situation regarding marginal donors, more challenging recipients and advances in transplantation, such as immunosuppression.

In 2003, a meta-analysis and systematic review demonstrated a 20% reduction of DGF with the use of HMP (Wight et al., 2003b). In supporting studies, not only was DGF reduced, but HMP led to an increased utilisation of ECD kidneys (Matsuoka et al., 2006; Schold et al., 2005). It was also noted that by decreasing DGF, overall costs should consequently decrease. This was supported by a review of cost-effectiveness (Wight et al., 2003a).

A recent study lent strong support to the beneficial effects of HMP over SCS. Moers et al (as part of the Eurotransplant trial) conducted the first multi-centre, prospective, randomised clinical trial comparing 336 consecutive deceased donors from which one kidney was stored in SCS and the paired kidney preserved by HMP (Moers et al., 2009). The study demonstrated a reduced DGF rate for HMP kidneys compared to SCS (20.8% v 26.5%) and an improved 1-year graft survival (94% v 90%). Similar findings were reported in smaller studies (Kwiatkowski et al., 2007; Moustafellos et al., 2007; Polyak et al., 2000; Shah et al., 2008a).

The Eurotransplant trial group later reported on subgroup analysis and long-term outcomes. They found a significantly reduced rate of DGF and an improved 1-month graft survival in DCD kidneys stored by HMP rather than SCS (Jochmans et al., 2010). In terms of 3-year graft survival, this was better in HMP kidneys, particularly for those from ECD donors. A number of studies have also demonstrated improved outcomes with HMP for ECD donors (Matsuoka et al., 2006; Stratta et al., 2006; Stratta et al., 2007; Sung et al., 2008) along with lower costs when compared to SCS (Buchanan, et al., 2008). However, whilst 3-year graft survival was better for HMP kidneys from DBD donors, the initial improved DGF rates for DCD kidneys did not translate into an improved long-term graft survival (Moers et al., 2012).

Interestingly, a UK multi centre study conducted at a similar time to the Eurotransplant trial, demonstrated conflicting results for DCD kidneys. Watson et al examined DCD kidneys only and found that HMP had no benefit over SCS in the reduction of DGF (Watson et al., 2010).

This was surprising given the evidence from the Eurotransplant trial and other supporting studies (Moustafellos et al., 2007; Reznik et al., 2008). Differences between the UK and European trial may have arisen for several reasons. The rate of DGF was significantly lower in the SCS arm of the UK trial (56%) than in the SCS arm of the European trial (70%). There was also a variation in the number and geographical spread of centres involved, possible differences in blinding and different reporting of ischaemic times. Importantly, the UK trial placed kidneys on HMP following transport in SCS whereas the European trial kidneys were placed on HMP

at retrieval. It has been suggested that kidneys may benefit from HMP only if used for the entire preservation period (Irish & Katz, 2010).

Goldstein et al presented a retrospective analysis of 1067 deceased donor kidneys at the American Transplant Congress in 2011 (Goldstein et al., 2011). They examined the ratio of SCS to HMP where combined storage was used. They found that DGF directly correlated with increasing SCS time and that detrimental effects of prolonged CIT were ameliorated with increasing the HMP component of CIT. They suggested that employing HMP early after recovery would reduce the negative effects of CIT and increase the utilisation of kidneys with prolonged CIT.

Other studies have suggested the benefits of HMP for longer preservation times. Equivocal or reduced rates of DGF have been demonstrated with HMP where the HMP group has had a longer CIT than the SCS group (Ciancio et al., 2010; Kwiatkowski et al., 2007; Lodhi et al., 2012; Schold et al., 2005). Ciancio also found that HMP pump times of over 24 hours were associated with significantly lower rates of biopsy-proven acute rejection (BPAR) and favourable graft survival outcomes. They suggested that 'in the presence of relatively short SCS times, use of machine perfusion provides a truer, more successful form of hypothermic preservation and superior early outcomes, even with pump times as long as 48 hours'. A recent publication suggests however that although HMP is associated with reduced DGF, it is still important to minimise CIT in deceased donor transplants (Gill et al., 2014).

Overall, the evidence has swung in favour of HMP, rather than SCS, as the preferred storage method for deceased donor kidneys. HMP clearly reduces the risk of DGF in deceased donor kidneys, particularly in the ECD group, and long-term outcomes look promising. Some controversy still remains over the use of HMP in DCD organs, particularly in terms of graft survival. Although HMP equipment and training is associated with an initial cost, evidence clearly suggests reduced overall costs associated with reduced DGF and shorter hospital stay.

Still of interest is the ability of HMP to extend CIT without detriment to graft function. The ideal duration of HMP, and the ratio of it to SCS, is yet to be established, as is the maximum safe HMP time. Safe extension of CIT could have a significant impact on clinical practice.

1.3.9 OTHER PRESERVATION METHODS

Alternatives to SCS and HMP are warm/normothermic perfusion and oxygen persufflation. These are briefly summarised below:

Warm/Normothermic Perfusion

Early experiments in organ perfusion were often conducted at normothermic or warm temperatures. However, its use was limited due to available perfusates and problems with thrombus formation, vascular damage and infection (Fuller & Lee, 2007). The

benefits of cold temperatures were beginning to be discovered, so storage methods focused on hypothermic techniques. With the increased use of marginal organs, which are particularly susceptible to cold ischaemic damage (Saba, Munusamy, & Macmillan-Crow, 2008), there has been a recently renewed interest in perfusion at warmer temperatures.

The main aim of warmer perfusion temperatures is to maintain the kidney in a near normal physiological state providing oxygen and nutrients to support aerobic metabolism. Table 1.7 summarises the proposed advantages and disadvantages of normothermic preservation. In addition to the disadvantages listed in the table, the current techniques of warm/normothermic perfusion demand an additional human resource requirement.

Table 1.7: Proposed Advantages & Disadvantages Of Normothermic Preservation (Reproduced from Hosgood & Nicholson, 2011)

Advantages	*Disadvantages*
Aerobic metabolism	Technical support/equipment
Restoration of function	Logistics of transportation
Avoid/reduce hypothermic injury	Cost
Organ assessment	
Resuscitation	
Regeneration and repair	
Treatment and modification	

Before retrieval, organs are normally perfused in-situ with cold preservation solution. However, some benefit has been shown with the use of extracorporeal membrane oxygenation (ECMO) at normal body or room temperature prior to cold flush. A study using 60 minutes of normothermic recirculation in DCD donors before cold flush showed a reduced rate of DGF and PNF compared to standard cooling (Valero et al., 2000). Similar results have been reproduced (Gravel et al., 2004) and there have been promising results with normothermic recirculation and long-term graft survival (Lee et al., 2005).

It was demonstrated in the 1970s in an animal model that warm flush prior to cold flush limits the vasoconstrictive effect of hypothermia (Das et al., 1979). Flushing rat kidneys with normothermic UW solution was also shown to be superior to cold flush with other solutions (Hughes et al., 1996). More recently, no detrimental effect was found in porcine kidneys flushed with a novel non-phosphate buffered solution called AQIX (AQIX Ltd, London,UK) at 32°C (Kay et al., 2011). This solution has sufficiently maintained viability in porcine kidneys flushed and statically stored for 2 hours at 32 32°C (Kay et al., 2007).

There has been some evidence of the usefulness of combined hypothermic and normothermic techniques. Canine kidneys were preserved for a total of 144 hours by a combination of autologous blood at normal body temperatures and hypothermic perfusion (van der Wijk et al., 1980). In a similar study, perfusion time was extended to 6 days (Rijkmans, Buurman, & Kootstra., 1984). Periods of warmer perfusion seemed to 'resuscitate' the kidneys and this was supported by other experimental

trials on canine kidneys (Maessen et al., 1989a, 1989b). These extended preservation times are not currently required in clinical practice but kidneys with a higher injury burden, such as DCD and ECD kidneys, may benefit from this type of repair.

Further work with a canine autotransplant model demonstrated that a period of ex-vivo normothermic perfusion could allow functional recovery following severe warm and cold ischaemic injury and that prolonged periods of normothermic preservation were more beneficial than hypothermic techniques (Brasile et al., 2005; Brasile et al., 2002). Normothermic perfusion of porcine kidneys demonstrated enhanced renal blood flow during reperfusion and a porcine transplant model was developed to demonstrate that this technique could be safely and feasibly applied to clinical practice (Bagul et al., 2008; Hosgood et al., 2011b).

Since then, promising results have been achieved clinically. The Leicester group reported on the first case of ex-vivo normothermic renal transplant perfusion in man (Hosgood & Nicholson, 2011b). Each kidney was retrieved from an ECD following cardiopulmonary arrest with a significant period of warm ischaemia; one kidney was stored in SCS and the other underwent normothermic perfusion. The kidney preserved by normothermic perfusion exhibited superior early graft function. This was followed up by the first in-man clinical study comparing 18 ECD kidneys that underwent normothermic perfusion prior to transplantation to a control group of SCS kidneys (Nicholson & Hosgood, 2013). A reduced rate of DGF was shown in the normothermic group and the technique was deemed to be feasible and safe.

The main disadvantages of systems that use warm perfusion temperatures are their complexity and the level of supervision required. This makes them less attractive for use in the clinical setting with the current available technology. This is likely to change in the future, with advances in available systems and the need to improve preservation for marginal organs.

Oxygen Persufflation

This technique uses gaseous oxygen that is bubbled through the vasculature of an organ. The gas then escapes through small perforations in the organ's surface (Lee & Mangino, 2009). Oxygen Persufflation (OP) was first tested on canine kidneys and has also undergone pilot clinical studies in kidney and liver preservation (Rolles, Foreman, & Pegg, 1989; Sachweh et al., 1972; Stegemann et al., 2009). Most work using this technique has been performed on animal models of kidney and liver perfusion, where it has shown some benefit in treating DCD organs (Fischer et al., 1978; Lee & Mangino, 2009; Rolles, Foreman, & Pegg, 1984).

1.4 OVERALL AIMS

Aims

The aims of this study were as follows:

1. Assessment of Clinical Application

To examine the effects of the introduction of HMP into existing clinical practice on the renal surgery unit at University Hospitals Birmingham (UHB). Of particular interest was outcome data compared to kidneys stored by SCS and the ability of HMP to safely extend CIT to allow improved management of logistics.

2. Analysis of HMP Perfusate

Could analysis of HMP perfusate predict graft outcome following kidney transplantation? Perfusate samples were collected during HMP of DD kidneys for analysis by Nuclear Magnetic Resonance (NMR) imaging (metabolomics). The aim of which was to interrogate the metabolic profile of kidney perfusate during HMP and identify any differences between the perfusate of DGF and IGF kidneys which might aid in prediction of outcome. If possible, harmful processes in the metabolism of DGF kidneys would be identified.

3. Development & Assessment of a Porcine Model

In order to test hypotheses and modify the metabolism occurring during HMP, an animal model needed to be developed. A porcine model representing DCD conditions was designed and perfusate and histology analysed to ascertain the validity of the model.

CHAPTER TWO: ASSESSMENT OF CLINICAL UTILITY

2.1 BACKGROUND

Cold Ischaemic Time (CIT) is recognised as a major factor influencing early post-transplant graft function (Irish et al., 2003; Koning et al., 1997; Lee et al., 2000; Ojo et al., 1997; Peters et al., 1995). As CIT increases during SCS so does the risk of subsequent DGF and graft failure. It is often this consideration that drives the logistics of clinical transplantation. There are many other factors, however, that demand equal consideration and optimisation if the best outcomes in renal transplantation are to be achieved - particularly in an era when DD renal transplantation is increasingly complex.

As previously discussed, kidneys available for transplantation have become increasingly marginal (Cohen et al., 1997; Cohen et al., 2005). A growing number of ECD kidneys are being utilised and these, along with DCD kidneys, are associated with an increased risk of delayed graft function (DGF) when compared to SCD kidneys (Audard et al., 2008; Metzger et al., 2003b; Ojo et al., 2001; Port et al., 2002).

The recipient population of DD kidneys is also more challenging. Recipients are frequently older with significant comorbidity, often previously considered inappropriate for transplantation (Ojo et al., 2001; Forsythe, 2013}. This is exacerbated by the observation that many patients with less significant co-morbidity now receive early living donor transplants. In consequence, it is now common experience that patients waiting for a DD transplant have no living donor option and

long histories of dialysis with its associated complications. Patients may also deteriorate between listing and admission for transplantation (Danovitch et al., 2002). Optimising these patients during an emergency admission for renal transplantation can be problematic and the need for new investigations or interventions can extend CIT.

DD organ transplantation is frequently undertaken outside of normal working hours in an attempt to minimise CIT. In this time-critical situation, recipient issues may receive less attention than is ideal. Furthermore, in an attempt to minimise CIT, transplants may be performed on emergency operating lists staffed by general teams with limited experience of managing the specific needs of renal failure patients (Seow, Riad, & Dyer, 2006; Shaw et al., 2012). Such compromise carries the inherent risks that care may fall below a level of excellence.

Means by which acceptable extensions in CIT could be achieved without impacting upon early graft function would therefore be of value. Against this background, the role of Hypothermic Machine Perfusion (HMP) has been investigated as a tool by which this might be obtained.

2.1.1 AIMS

The objective for this section of the study was to examine the effects of the introduction of HMP into existing clinical practice on our renal surgery unit. In particular:

Could graft function be maintained or even improved during longer cold ischaemia times using HMP?

Could we simultaneously acquire the benefits of an optimised recipient with improved use of logistics and expertise?

2.1.2 SETTING

This assessment of clinical utility was performed in a large teaching hospital providing a regional transplant service to a mixed demographic population of over 6 million. Approximately 190 kidney transplants are performed annually, 120 of which are DD transplants.

After recovery, DD kidneys arrive at our unit in SCS. Emergency operating theatre space is available on a competitive basis with other specialties and consequently it is not always possible to ensure a short CIT. Furthermore, emergency teams often consist of staff who do not regularly deal with renal failure patients. Incoming

transplant recipients have multifaceted medical problems and may have been managed at peripheral dialysis units. Accordingly, they may need considerable pre-operative re-evaluation and preparation that may include peri-operative dialysis.

As a compromise to 24-hour operating theatre availability, the unit has a dedicated renal transplant theatre team available between 8am and 8pm. Where possible such complex renal cases are directed towards this resource.

2.2 METHODS

2.2.1 PATIENT RECRUITMENT

All DD kidneys accepted for transplantation were included except for paediatric cases, which are performed on a different site, and kidneys transplanted as part of a combined liver or heart transplant procedure, which involve combined care.

2.2.2 KIDNEY PRESERVATION

All DD kidneys arrive on the unit in SCS. In this study, as in normal departmental practice, the decision to transfer to HMP was based on donor - recipient readiness and theatre availability. If the predicted time to theatre was within elective hours (8am to 8pm), allowing transplant to take place in the dedicated renal transplant theatre, the kidney was transplanted from SCS. If the predicted time to theatre was outside of these hours (8pm to 8am) the kidney was transferred to HMP and then transplanted at the earliest opportunity on the next available dedicated transplant list.

Machine Perfusion

All HMP kidneys were preserved using the Lifeport© Kidney Transporter 1.0 from Organ Recovery Systems. Perfusion pressure was set at 30mmHg and not altered

during perfusion time. This setting is recommended by the manufacturer and used in the Eurotransplant trial (Moers et al., 2009).

The machine was prepared for each case by filling the insulated ice container with ice and cold water. The disposable perfusion circuit was then loaded into the machine and fitted into the pump mechanism. The organ tray was filled with 1 litre of cold KPS-1® (kidney perfusion solution). The machine was then set to 'flush' to ensure any air bubbles were removed from the tubing. When complete, the machine was 'primed' ready for use.

Meanwhile, the kidney was prepared as standard for a transplant procedure. This ensures that the vessels are clearly visible. Depending on the arrangement and number of arteries on the donor kidney, the kidney was attached to an appropriate cannula.

The kidney was then transferred into the organ tray and connected to the tubing via the cannula. Perfusion was commenced ensuring there were no leaks present. Sterile techniques were used throughout.

2.2.3 DATA COLLECTION & OUTCOME MEASURES

Clinical data were collected prospectively including donor and recipient demographics, CIT, operative factors and post-operative outcomes. HMP parameters

(flow, resistance, pressure, temperature) were also recorded throughout perfusion time.

The primary outcome measure was DGF (defined as the requirement for dialysis, for any reason, within the 1st post-operative week following kidney transplant).

Secondary outcomes were CIT, timing of surgery, HMP parameters, complications, length of hospital stay and post-operative creatinine measurements.

2.2.4 STATISTICAL ANALYSIS

Data were analysed using GraphPad Prism 6.0c (GraphPad Software, La Jolla California USA) and IBM SPSS 19 (IBM Corp. Armonk, NY). Continuous variables were expressed as arithmetic or geometric means with 95% confidence intervals, or as medians and interquartile ranges, as appropriate. Comparisons of demographics and complication rates between HMP and SCS kidneys were made using Mann-Whitney tests or Fisher's Exact test for continuous and categorical data respectively. The trends over time in resistance and creatinine levels were assessed using Repeated Measures ANOVA models, with the dependent variables being $\log_{10}$-transformed where necessary. Throughout the analysis, $P<0.05$ was considered to be indicative of statistical significance.

2.3 RESULTS

During the observation period (January 2012 - December 2013 inclusive) 196 cadaveric kidneys were accepted to the unit for transplantation.

Kidneys transplanted as part of a combined heart or liver transplant procedure were excluded (n=5). Three kidneys were discarded due to severe atherosclerosis of the renal artery with an adverse donor history, a hypoplastic cystic kidney and inadequate flush at recovery. No kidneys were discarded based on machine parameters. Paediatric cases were also excluded (n=13).

Of the 175 kidneys included in the study, 74 (42%) underwent HMP and 101 (58%) were transplanted from SCS. These two groups are compared throughout.

2.3.1 DONOR & RECIPIENT DEMOGRAPHICS

Donor and recipient demographics for the two storage groups are shown in table 2.1.

Table 2.1: Donor and recipient demographics

Demographic	*HMP (n=74)*	*SCS (n=101)*	*P Value*
Donor			
Male gender	42 (57%)	47 (47%)	0.221
Median age (IQ range)	52 (40-60)	51 (35-61)	0.831
CMV +ve	32 (43%)	49 (49%)	0.541
DCD	9 (12%)	26 (26%)	0.035*
ECD	27 (36%)	35 (35%)	0.873
Marginal	34 (46%)	55 (54%)	0.287
Recipient			
Male gender	47 (64%)	62 (61%)	0.875
Median age (IQ range)	51 (44 - 57)	50 (39-57)	0.423
Ethnicity: British	45 (61%)	48 (48%)	0.041*
Asian	16 (22%)	33 (33%)	
Black	9 (12%)	6 (6%)	
Other	4 (5%)	14 (14%)	
Virology: CMV+	38 (51%)	70 (69%)	0.019*
HAV+	10 (14%)	20 (20%)	0.315
HBV cAb+	6 (8%)	7 (7%)	0.778
HBV sAb+	1 (1%)	0 (0%)	0.423
HCV Ab+	1 (1%)	1 (1%)	1.000
HIV+	1 (1%)	2 (%)	1.000
Dialysis: HD	48 (65%)	68 (67%)	0.967
PD	21 (28%)	27 (27%)	
Pre	5 (7%)	6 (9%)	
Previous transplant ≥1	8 (11%)	8 (8%)	0.599
PRA level >5%	32 (43%)	34 (34%)	0.210
0.0.0 mismatches	11 (15%)	10 (10%)	0.352
Immunosuppression standard	65 (88%)	94 (93%)	0.291

ECD = defined as age >60 or 50-59 with 2 or more of hypertension, intracranial cause of death, creatinine >132, Marginal = DCD or ECD, Standard immunosuppression: Basiliximab, Tacrolimus, Mycophenolate mofetil, Prednisolone. Data reported as: "Median (Quartiles)" or "N (%)", with p-values from Fisher's exact tests or Mann-Whitney tests, as appropriate *Significant at $p < 0.05$

The two groups were generally well matched, although the HMP group had significantly lower rates of DCD kidneys (12% vs. 26%, p=0.035) and less recipients who were cytomegalovirus (CMV) positive (51% vs. 69%, p=0.019). There was also a difference in recipient ethnicity between the two groups (p=0.041), with increased numbers of Asian recipients in the SCS arm.

2.3.2 DELAYED GRAFT FUNCTION (DGF)

DGF was the most frequently occurring graft complication with 67 transplant recipients overall (38%) requiring dialysis within the first week. Twenty HMP kidneys suffered from DGF (27%) compared to 47 (47%) in the SCS group (p=0.012).

For donation after brain death (DBD) kidneys only, 15 of the 65 (23%) kidneys in the HMP group developed DGF compared to 30 of 75 (40%) kidneys in the SCS group (p=0.046).

For DCD kidneys only, 5 of 9 (56%) kidneys in the HMP group developed DGF compared to 17 of 26 (65%) in the SCS group (p = 0.698).

These findings are summarised in the table 2.2:

Table 2.2: Delayed Graft Function by Donor Type and Storage Group

Kidney Group	*DGF in HMP Group (Total 74)*	*DGF in SCS Group (Total 101)*	*P Value*
All	20 (27%)	47 (47%)	0.012*
DBD only	15 (23%)	30 (40%)	0.046*
DCD only	5 (56%)	17 (65%)	0.698

P values: Fischer's Exact 2-tailed test

The median duration of DGF, from operation to the last dialysis session, was similar for both storage groups (p=0.628). For both groups the duration was 5 days with an IQ range of 2.25–6.75 and 2.00–8.50 for HMP and SCS respectively. The median number of dialysis sessions required was identical across the two groups (IQ range 1.00-3.00 and IQ range 1.00–5.00 for HMP and SCS respectively).

2.3.3 COLD ISCHAEMIC TIME AND TIMING OF SURGERY

Overall, the median CIT in the HMP group was 23.85 hours (IQ range 19.30–26.62) which was significantly longer than the median of 13.00 hours (IQ range 11.79–15.36) in the SCS group (p=<0.0001).

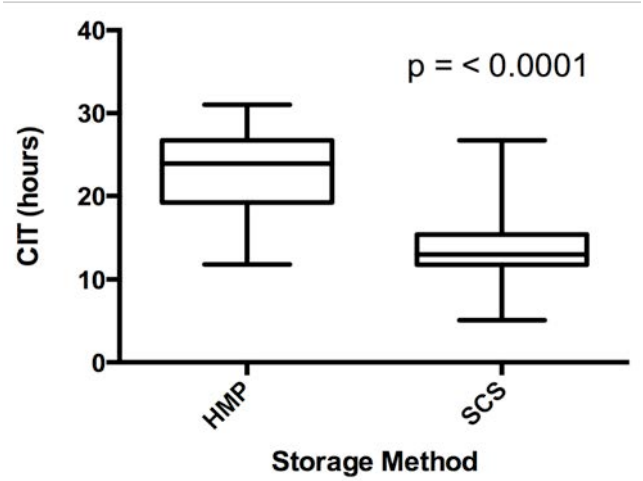
For DBD kidneys only, the median CIT in the HMP group was 24.18 hours (IQ range 20.73-26.93) which was significantly longer than the median of 13.32 hours (IQ range 10.66-16.13) in the SCS group (p=<0.001).

For DCD kidneys only, the median CIT in the HMP group was 16.60 hours (IQ range 13.27-26.53) which was significantly longer than the median of 12.72 hours (IQ range 12.00-14.04) in the SCS group (p=<0.0070).

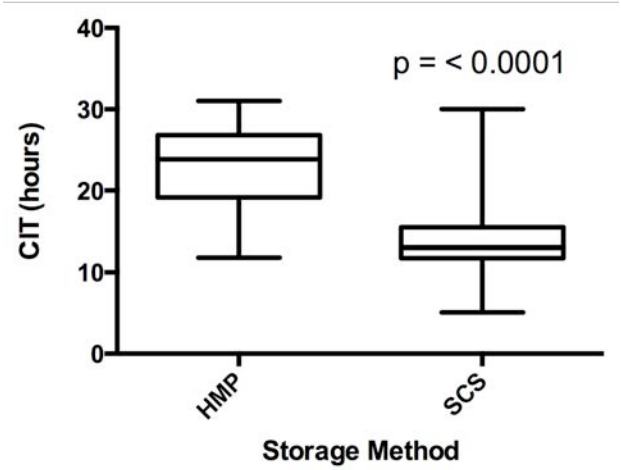
This is represented in Figure 2.1.

Figure 2.1: Cold Ischaemic Time by Donor Type and Storage Group

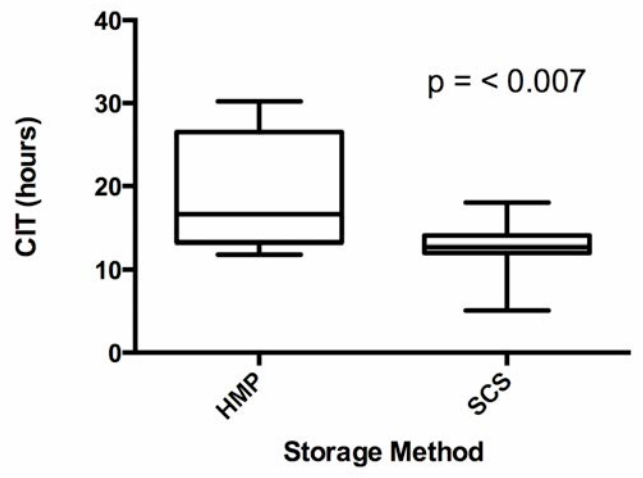
All Kidneys



DBD Kidneys Only



DCD Kidneys Only



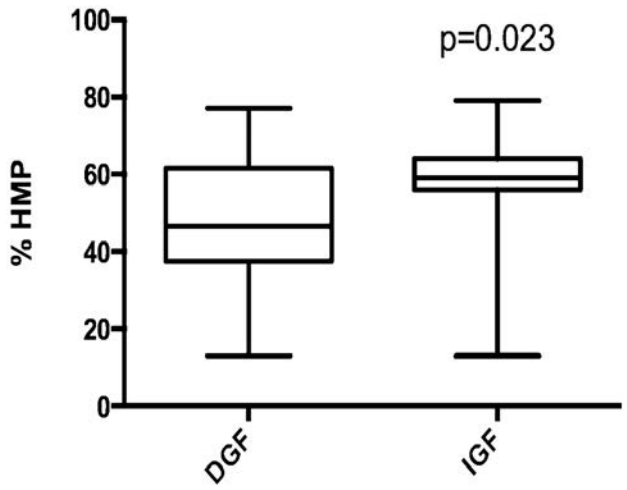
In the HMP group, 58 of 74 kidneys (78%) were transplanted during elective hours (8am to 8pm) which was significantly more than the 58 of 101 kidneys (57%) in the SCS group (p = 0.006).

2.3.4 HMP PARAMETERS

Overall, kidneys spent a median of 58% (IQ range 48–64) of their total CIT undergoing HMP. The median time of HMP during CIT was 14.5 hours (IQ range 9.75–15.75). Perfusion pressure was constant at 30mmHg. Temperature remained at or below 4°C.

HMP kidneys exhibiting IGF had a significantly higher proportion of HMP time within their total CIT with a median of 59% (total range 13-79%) compared to 46.5% (total range 13-77%) for DGF kidneys (figure 2.2).

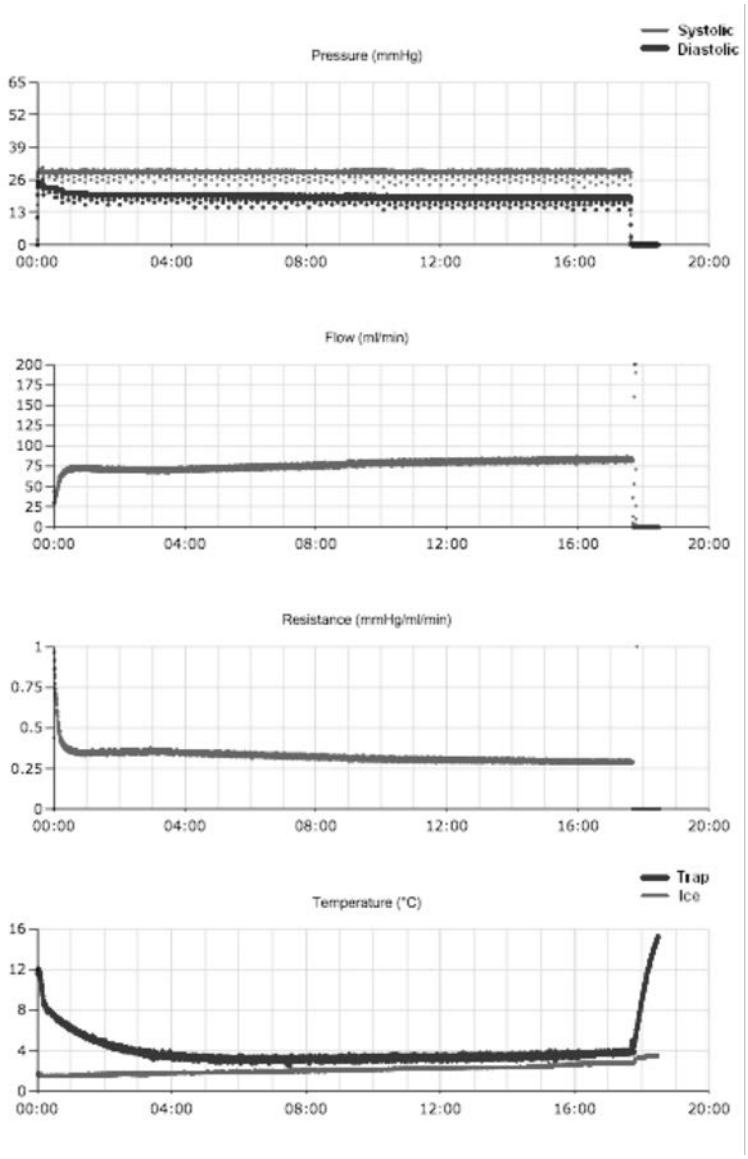
Figure 2.2: Proportion of HMP Time During CIT for Storage Groups



Changes in flow and resistance during machine perfusion mostly occurred within the first 60 minutes. An example of this can be seen in figure 2.3 below.

Figure 2.3: LifePort Kidney Transporter Case Report Showing Machine Parameters

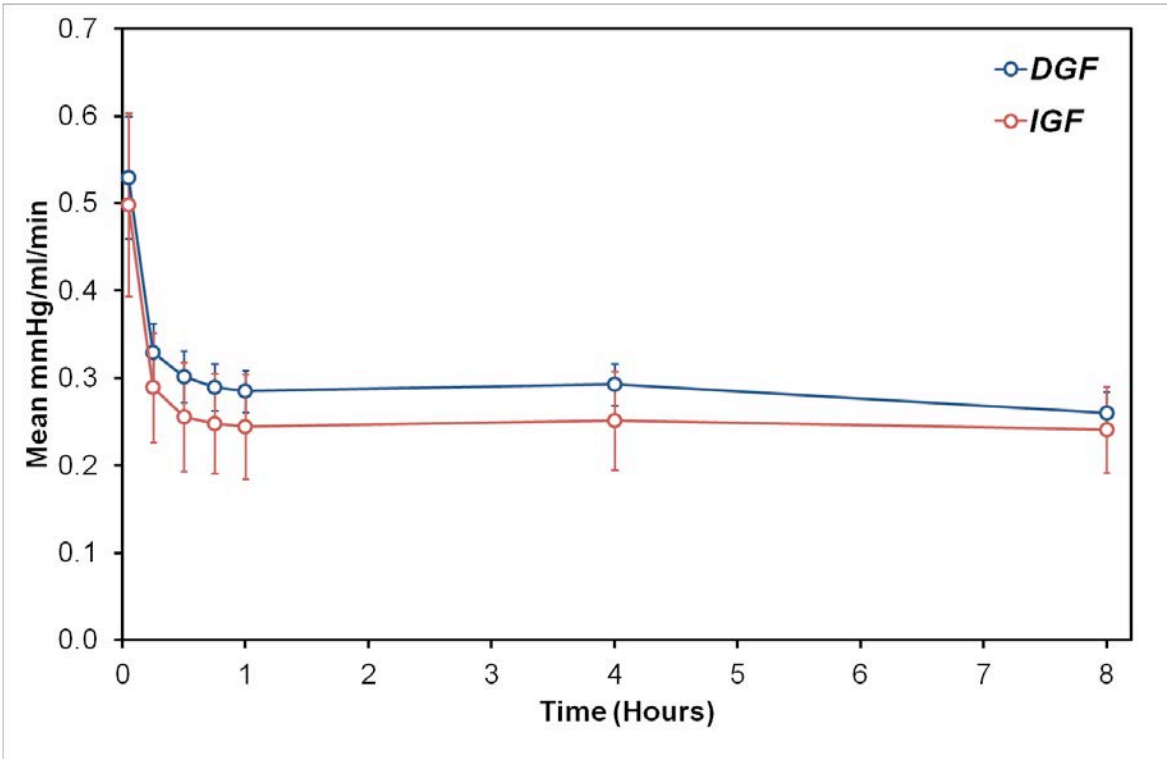
These graphs illustrate the machine parameters that are recorded during perfusion time. These are systolic and diastolic perfusion pressures (mmHg), flow (mL/minute), resistance (mmHg/mL/min) and the temperature within the ice and the 'trap' which contains the kidney (°C). The machine will alarm and halt perfusion if the temperature rises above 7°C. Data can be recorded for a 48-hour period.



Overall, flow increased from a median of 53 mL/min (IQ range 39.5–76.0) to 100 mL/min (IQ range 81.0–128.5). Resistance decreased from a median of 0.48 mmHg/mL/min (IQ range 0.33–0.66) to 0.22 mmHg/mL/min (IQ range 0.18-0.29).

A repeated measures ANOVA model found that, whilst the change in resistance over time was significant (p=<0.001) reflecting the large decline in resistance over the first hour, there was no significant difference in resistance between immediate graft function (IGF) or DGF kidneys (p=0.827). The interaction term in the model was also non-significant (p=0.841) hence there is no evidence that the trend in resistance over time is related to DGF (figure 2.4).

Figure 2.4: Repeated Measures ANOVA Model for Change in Resistance During HMP Time in IGF & DGF Kidneys



2.3.5 COMPLICATIONS & LENGTH OF STAY

There was one recipient death in the HMP group due to a cardiac event. Two of the 175 patients developed Primary Non-Function (PNF), one of which required graft excision. These were both in the SCS group. Ten patients overall suffered graft loss (HMP n=5, SCS n=5): vessel thrombosis (n=7), renal vein tear (n=1), PNF (n=1) and graft failure of unknown cause (n=1).

Table 2.3 provides a summary of other in-patient complications across both patient cohorts.

Table 2.3: In-patient Complications Following DD Renal Transplantation

Complication	*Overall (n=175)*	*HMP (n=74)*	*SCS (n=101)*	*P Value*
Respiratory Infection	12 (7%)	3 (4%)	9 (9%)	0.243
Diarrhoea/Ileus	10 (6%)	4 (5%)	6 (6%)	1.000
*Bleeding**	9 (5%)	5 (7%)	4 (4%)	0.497
NODAT	8 (5%)	1 (1%)	7 (7%)	0.141
Cardiac dysrhythmia	5 (3%)	0 (0%)	5 (5%)	0.074
Wound infection/ Superficial dehiscence	4 (2%)	2 (3%)	2 (2%)	1.000
*Hydronephrosis***	4 (2%)	2 (3%)	2 (2%)	1.000
*Abdominal collection***	3 (2%)	1 (1%)	2 (2%)	1.000
DVT/PE	2 (1%)	2 (3%)	0 (0%)	0.177
Drug Reaction	2 (1%)	1 (1%)	1 (1%)	1.000

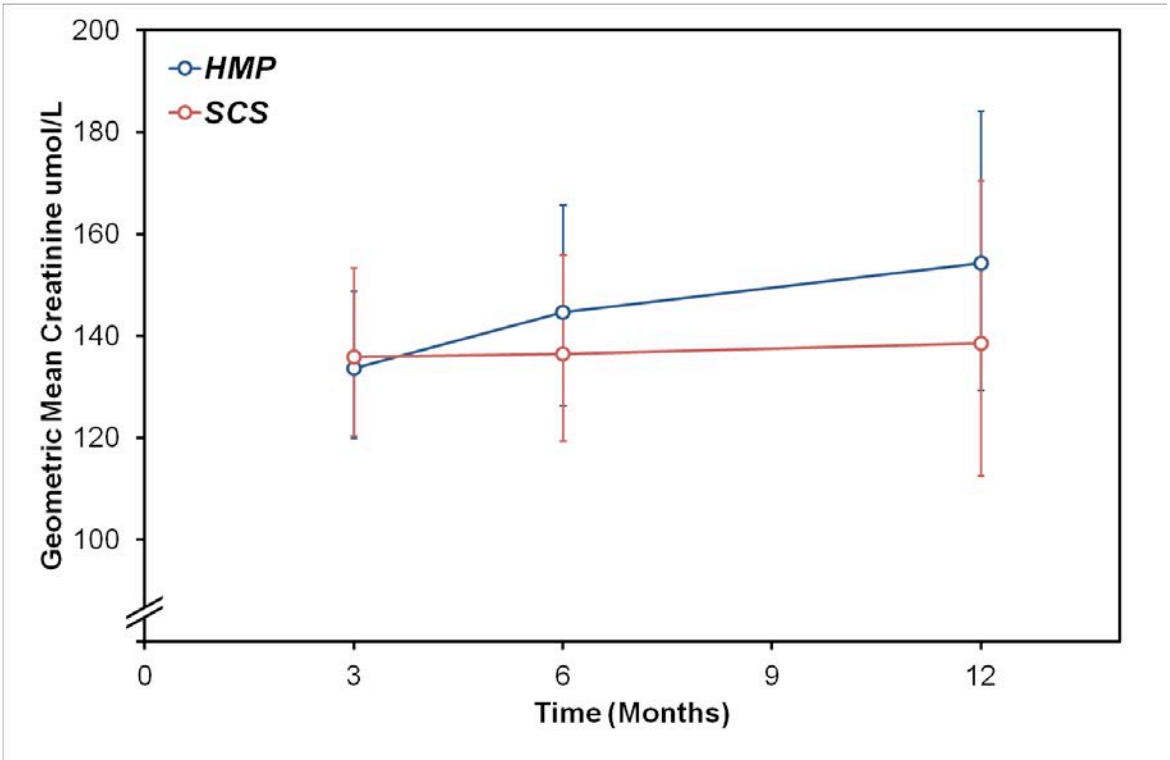
Data reported as: "N (%)", with p-values from Fisher's exact tests, * requiring further surgery or transfusion, ** Requiring intervention

Median length of in-patient stay was 9 days in both groups (IQ range 7-12 and 8-14 in the HMP and SCS groups respectively)(p=0.310).

2.3.6 POST-OPERATIVE CREATININE

Geometric mean creatinine levels for HMP kidneys were 134 μmol/L, 144 μmol/L and 154 μmol/L at 3, 6 and 12 months respectively. This compared to levels of 136 μmol/L, 136 μmol/L and 138 μmol/L in the SCS group (Figure 2.5). A repeated measures ANOVA model found that that these values neither differed between the groups (p=0.717), nor over time (p=0.594), and that this lack of a significant trend over time was common to both groups (interaction p=0.489).

Figure 2.5: Repeated Measures ANOVA Model for Post-operative Creatinine in HMP & SCS Kidneys



2.3.7 HISTOLOGY

Of the 74 HMP kidneys, 17 (23%) underwent a transplant biopsy within the first month of the procedure compared to 26 (26%) from the SCS group. Biopsies are performed on clinical indication rather than being protocol driven. Results are shown in table 2.4.

Table 2.4: Histology of Post-transplant Kidney Biopsies

Biopsy Finding	*HMP (17)*	*SCS (26)*	*P value*
No rejection	12 (70%)	14 (53%)	0.35
Borderline	1 (6%)	2 (8%)	1.00
Rejection	2 (12%)	8 (31%)	0.27
Inadequate sample	2 (12%)	2 (8%)	1.00

Fisher's Exact 2-tailed test

2.4 DISCUSSION

DD renal transplantation is a process with many variables relating to the donor kidney, recipient and the hospital environment. Whilst CIT has traditionally been regarded as the most significant factor influencing post-transplant outcome, the growing complexity of DD transplantation now mandate that other factors should be given equal consideration.

2.4.1 DONOR & RECIPIENT CONSIDERATIONS

Growing transplant waiting lists have driven the increased use of higher risk ECD and DCD organs, despite their greater risk of poor initial graft function when compared to SCD kidneys (Metzger et al., 2003b; Ojo et al., 2001; Port et al., 2002). Similarly, recipients have become more complex with live donor transplantation selecting out less co-morbid patients; those who receive pre-emptive grafts or have shorter periods of time on dialysis. Inevitably, the higher-risk patients remain on the DD waiting list.

Ensuring that complex recipients of DD kidneys are suitable for surgery can be difficult. They often require re-evaluation and additional preparation on admission to ensure safe surgery. The combined responsibility of attending to increasingly intricate kidney and recipient factors may prolong CIT, increasingly into out-of-hours operating time. This compounds an already apparent paradox; living donor recipients, with

lower levels of co-morbidity, are transplanted on elective lists with the highest level of expertise available. More complex DD recipients are invariably managed outside of normal working hours by emergency teams with variable experience of the anaesthetic and operative management of complex renal failure patients. In the UK, NCEPOD (National Confidential Enquiry into Peri-operative Deaths) reports have clearly demonstrated that patients undergoing surgery under such circumstances experience a less favourable outcome (Campling, Devlin, & Hoile, 1997; Cullinane, 2003) .

This being the case, it would appear evident that to achieve the highest standards of care either the level of expertise in 'out-of-hours' operating must be upgraded to equal that provided for our living donor recipients or DD renal transplantation must be an 'in hours' procedure. The former is difficult: providing the highest level of expertise round-the-clock, 365 days of the year poses a problem due to the finite limitations on human and financial resources. Moving renal transplantation into elective hours would reconcile some of the logistic issues but would only be acceptable if CIT could be prolonged without detriment to the kidney.

2.4.2 USE OF HMP & CIT

In this study, a dedicated renal transplant theatre and team was available between 8am to 8pm. This is specific to UHB but could be extrapolated to other situations. Ensuring surgery is performed on this list would prolong CITs. To address this, HMP

was utilised to bridge longer CITs in the anticipation that this would minimize the impact of CIT on graft function.

HMP is an increasingly recognised alternative form of kidney preservation (St Peter, Imber, & Friend, 2002). Improved graft outcomes have been achieved using HMP although the exact mechanism by which this occurs remains unclear. Studies have reported both reduced DGF and improved graft survival using HMP to preserve DD kidneys, as previously discussed (Cannon et al., 2013; Kwiatkowski et al., 2007; Lodhi et al., 2012; Matsuoka et al., 2006; Moers et al., 2009; Schold et al., 2005; Shah et al., 2008b; Stratta et al., 2007; Wight et al., 2003b).

Others studies have implied that HMP may permit longer periods of CIT without increasing DGF rates (Ciancio et al., 2010; Schold et al., 2005). This study strongly supports this concept. Despite the significantly longer CITs in the HMP group, less DGF was experienced than by kidneys stored for shorter periods using SCS. This was true overall and for DBD kidneys. In the DCD kidney group, CIT was also significantly longer for HMP kidneys but the reduction in graft function just failed to reach statistical significance.

HMP kidneys displaying IGF tended to have a higher proportion of HMP time compared to SCS time within the total CIT. This indicates that in cases when a period of SCS is unavoidable, it may be necessary to employ a longer period of HMP. This has been observed in other reports (Goldstein et al., 2011).

The study also demonstrates that longer storage periods can be utilised to resolve assessment and logistical issues, most specifically permitting more transplants to be performed during daylight hours when maximum expertise is available. This may be a more efficient use of human resource and may even help to promote recruitment into transplantation. The reduced number of cardio-respiratory problems in the HMP group may reflect the specialist anaesthetic and recovery care available during elective hours, although these numbers were small. The effects of HMP during organ storage, increased time for preparation and improved peri-operative conditions may all have contributed to the improved outcomes.

2.4.3 RESISTANCE

Kidneys showing the best functional outcome during HMP tend to have reduced intra-renal resistance during the perfusion process, implying that the machine process improves renal microvascular circulation (Wight et al., 2003a). Several studies have warned of the dangers of using resistance values to determine kidney discard and although it has been demonstrated as an independent predictor of DGF, its predictive value is poor (Jochmans & Pirenne, 2011; Jochmans et al., 2011; Sonnenday et al., 2003; de Vries et al., 2011). In this study, there was no significant difference in resistance or change in resistance between the DGF and IGF kidneys and no kidneys were discarded based on these measurements.

2.4.4 POST-OPERATIVE CREATININE

Creatinine levels following transplantation were similar in both groups. Longer-term follow-up of graft function in the two groups is clearly necessary but if 1-year creatinine level is accepted as a surrogate marker of future outcome, then HMP treated kidneys would appear to have similar potential to SCS kidneys despite longer CITs at the time of transplantation.

2.4.5 BIOPSY-PROVEN ACUTE REJECTION RATES

BPAR rates did not vary significantly between the two preservation methods. Similar proportions of HMP and SCS kidneys also underwent a biopsy. This is unexpected as increased rates of DGF in the SCS kidneys might be expected to result in more biopsies in this group. It might be that other reasons were identified as causes of DGF in the SCS group negating the need for histology or that clinical decision-making is inconsistent.

2.4.6 LIMITATIONS

There are several limitations to this study. Allocation of kidneys to storage type was non-randomised therefore subject to selection bias. The purpose of this study, however, was not to prove superiority of HMP over SCS - this has been

demonstrated in larger trials. The aim was to investigate how HMP could be utilised within our existing clinical practice to improve or maintain outcomes by optimising as many peri-operative factors as possible.

A further limitation is the small number of DCD kidneys and their increased representation in the SCS group. This almost certainly demonstrates a bias to transplant DCD kidneys more expeditiously despite some studies suggesting that DCD kidneys may be a group likely to benefit most from a period of HMP (Jochmans et al., 2010; Moers et al., 2009). The increased number of DCD donors in the SCS group could be anticipated to bias the results in favour of HMP, but if DBD kidneys alone are analysed then the beneficial results of HMP on graft function are still significant. For DCD kidneys alone there was no difference between the storage methods in terms of early graft function. Even equivalence with an extended CIT may be of benefit to allow optimisation of other factors.

In the UK, DD kidneys arrive at the transplanting centre from the organ recovery centre in SCS. Consequently, in this study and according to departmental guidelines, the decision to take kidneys from SCS and place them on HMP was made. This decision was based upon the prediction of CIT, which was itself based on a consideration of other logistical factors. It is common experience that these predictions are frequently inaccurate. Equally, logistics rarely change to reduce timescales. In this study, despite unit guidelines, not all HMP kidneys were transplanted during elective hours as planned and a number of SCS kidneys were also transplanted out-of-hours. These guideline breaches were most often due to

predicted theatre times becoming unavailable due to delays with recipient preparation, theatre team unavailability or previously unexpected competing emergency cases. Furthermore, the complex logistics associated with multiple kidney offers occasionally resulted in HMP kidneys originally designated to elective lists being transplanted in emergency theatre time. Such unpredictability is inherent in the workings of a busy hospital, and thus largely unavoidable. As this study demonstrates that HMP protects kidneys against increased DGF with longer CITs, the case could be made that the decision to leave some kidneys in SCS and put others on HMP becomes redundant. If all kidneys had been put on HMP at the time of arrival in the unit they would have been pre-emptively protected against all reasonable eventualities. Since evidence suggests that the effect of HMP on DGF is heightened if perfusion is commenced immediately after recovery, it would seem even more reasonable to commence all deceased donor kidneys on HMP at source rather than at the implanting unit (Moers et al., 2009; Watson et al., 2010). This would maximise the benefit of HMP on DGF and also permit a reasonable prolongation of CIT, allowing for improved assessment of recipients and more flexible operating logistics to ensure the highest level of available expertise.

2.4.7 CONCLUSION

Traditional concerns about CIT have dictated that surgery must be expedited, possibly at the cost of other factors. However, these patients demand careful assessment and sometimes intervention on the day of transplantation. This study

demonstrates that comparable outcomes can be achieved with longer CITs by utilizing HMP storage rather than traditional SCS. This effect is likely to be multi-factorial including the inherent effects of HMP itself, improved recipient preparation and possibly better peri-operative conditions. Additional larger studies of subgroups and long-term outcomes would be beneficial in further defining the utility of HMP.

CHAPTER THREE: ANALYSIS OF HMP PERFUSATE

3.1 BACKGROUND

HMP provides a unique opportunity for assessment of deceased donor kidneys during storage with easy and safe access to perfusate samples, kidney tissue and information regarding flow dynamics. As previously discussed, accurate evaluation of allograft quality is essential to prevent unnecessary kidney discard, allow maximised donor-recipient matching and to plan appropriate peri-operative care. Viability assessment is of increasing importance with the higher utilisation rates of marginal DD kidneys. Despite considerable interest in various indicators and markers of graft quality, few are used routinely in clinical practice, and no single biomarker or parameter has proven to be definitive.

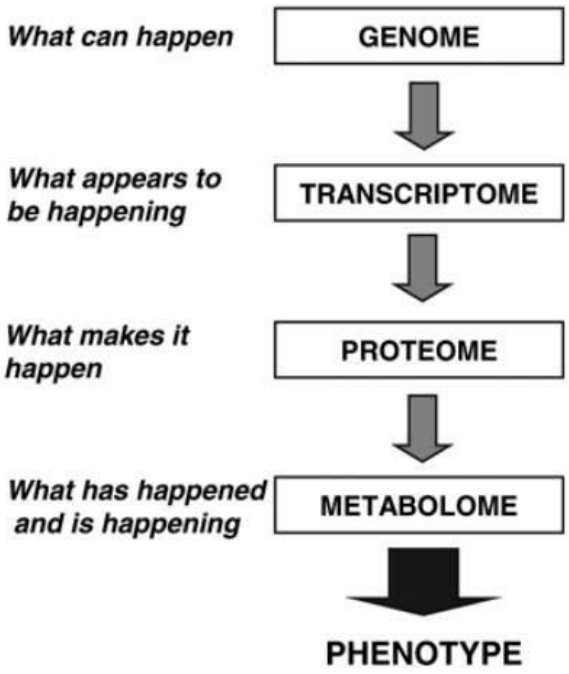
Although the clinical benefits of HMP are well documented, recent advances in normothermic perfusion have led some to question whether HMP will remain relevant. For the foreseeable future, HMP has the advantage of being far less labour intensive, therefore cheaper, and is much easier to operate than the complex arrangement requirement for normothermic perfusion. The exact mechanism by which HMP produces its beneficial effects remains unclear. Whilst initially the mechanism of action was thought to be largely due to maintaining the patency of vascular beds, there is increasing evidence that substantial metabolic activity occurs (Fuller & Lee, 2007). However the metabolic activity in this ex vivo, hypoxic, hypothermic environment is poorly understood.

‘Metabolomics’, or in this case NMR analysis of HMP perfusate, could provide an insight into this activity and identify potential biomarkers for graft quality. With a better understanding of the underlying processes occurring in the HMP kidney, it may be possible to ‘resuscitate’ and modify an injured kidney thereby improving outcomes and maximising the number of good kidneys available for transplantation.

3.1.1 METABOLOMICS

Systems biology, aiming to understand living organisms at the molecular systems level, is still in its early stages of development. However, it is emerging as an area of research that will use metabolomics, along with other 'omics' including genomics, transcriptomics and proteomics, as key investigative processes (Dettmer, Aronov, & Hammock, 2007).

Figure 3.1: The “Omics” Cascade



'Metabolomics' is defined as the systematic analysis of the chemical 'fingerprints' of specific cellular processes. It tends to refer to metabolites in one sample, which might be derived from only one cell type, whereas 'metabonomics' is the global study of systems that regulate metabolism, including variations over time (Hunter, 2009). Often these terms are used interchangeably.

Metabolomics has a number of potential applications including diagnostics, drug treatment and discovery and nutritional research. As a diagnostic tool it is powerful, providing a snapshot of an organism's metabolic state through the systematic analysis of its metabolites (Hunter, 2009). Any deviations from the normal range indicate possible pathological aberrations or changes due to environmental stimuli.

Metabolomics is the endpoint of the "omics cascade" and therefore provides the closest indication of phenotype (Dettmer, Aronov, & Hammock, 2007).

The metabolome represents a vast number of components that belong to a wide variety of compound classes such as amino acids, lipids, organic acids and nucleotides (Dettmer, Aronov, & Hammock, 2007). It is estimated that in humans there are around 2000 major metabolites - these are diverse in their physical and chemical properties and occur in a wide concentration range (Beecher, 2003).

A variety of analytical techniques can be used for metabolomic analysis, most commonly mass spectrometry and/or nuclear magnetic resonance (NMR) spectroscopy. These methods can identify compounds and their quantities in fluids or tissue samples. Given the diversity of metabolic compounds no single-instrument platform can currently analyse all metabolites, however, this is not normally necessary (Dettmer, Aronov, & Hammock, 2007).

Mass spectroscopy (MS) uses electrical fields to separate compounds, whereas NMR relies on magnetic fields. NMR spectroscopy provides the most comprehensive information about a wide range of metabolites and is generally preferred for large-scale applications (Hunter, 2009). NMR spectroscopy can simultaneously detect, identify and quantify hundreds of metabolites, some of which may have prognostic or therapeutic value (Serkova et al., 2005).

3.1.2 METABOLOMIC INVESTIGATIONS

There are two methods to approaching metabolomic analysis: metabolic profiling and metabolic fingerprinting (Dettmer & Hammock, 2004). Both of these can be used to identify new biomarkers.

Metabolic Profiling

Metabolic profiling aims to examine a specific cellular pathway via the involved metabolites or compounds (Dettmer, Aronov, & Hammock, 2007). A specific analyte, for example a biomarker of a particular disease, can also be targeted, identified and quantified (Fiehn, 2002). This technique is mostly hypothesis-driven. The main disadvantage of this approach is that due to its focused methodology, it may exclude, as yet, unrecognised pathways or metabolites (Dettmer, Aronov, & Hammock, 2007).

*Metabolic Fingerprintin*g

This alternative approach aims to identify specific patterns of metabolites, known as ‘fingerprints’, which might change in response to trigger factors. These factors might be, for example, a change in disease stage. This method has been applied to examine a number of substances such as urine, blood products, saliva and cells or tissues. The analysis of substances excreted into or taken up from these fluids can provide important information on cellular processes and the condition of the tissues being examined. The use of metabolic fingerprinting using NMR techniques has

proven to be an extremely powerful tool for screening samples and has been widely used in the field of biochemistry. An example of its use is as a diagnostic test: the metabolic fingerprint of an individual can be compared to one with and without a disease. Alternatively, it could be used to measure the success of a treatment if a fingerprint changes from ‘abnormal’ back to that of a healthy individual (Dettmer, Aronov, & Hammock, 2007).

3.1.3 PRINCIPLES OF NUCLEAR MAGNETIC RESONANCE (NMR)

NMR is an effect whereby nuclei in a magnetic field absorb and re-emit electromagnetic energy.

Each nucleus has one or more protons and neutrons, except for the hydrogen nucleus ^{1}H which only contains a single proton. Protons and neutrons both have an intrinsic property known in quantum mechanics as 'spin'. Inside an oriented magnetic field, the spin of the nuclei can either be parallel or anti-parallel with the external magnetic field. The energy absorbed or emitted by the nuclei is at a specific resonance frequency, distinct to each nucleus.

Molecules respond differently to the resonating magnetic field according to the electron density distribution around the nuclei. Each proton resonates at a slightly different frequency, called the chemical shift. This can be used to generate highly

refined spectral patterns that yield a great deal of data about the metabolites in a sample (Hunter, 2009).

3.1.4 NMR IN DISEASE STUDIES

NMR-based metabolomics is widely used in disease studies (Nicholson, Lindon, & Holmes, 1999). For example, ^{1}H-NMR has been used to distinguish markers in human serum predictive of coronary artery disease and hypertension (Brindle et al., 2002). It has also been used extensively to investigate diabetes both in rat models (Serkova et al., 2005; Zhao et al., 2010) and in human subjects for diagnosis and management of diabetic patients (Griffin & Vidal-Puig, 2008) and for identification of complications such as nephropathy (Mäkinen et al., 2006).

A full review of its experimental and clinical applications is beyond the scope of this study but NMR has the potential to diagnose disease, monitor the success of interventions and provide new biomarkers to assess human health (Dettmer, Aronov, & Hammock, 2007).

3.1.5 NMR IN TRANSPLANTATION

Several studies have demonstrated the use of NMR-based metabolomics within transplantation. Various isolated metabolites in the blood and urine have been found

to be indicators of kidney injury following transplantation. These metabolites are intermediates or end products of cellular processes and, therefore, reflect the global integrated response of an organ or entire biological system to pathophysiologic stimuli (Nicholson et al., 1999).

Hauet et al investigated the use of ^{1}H-NMR on urine to assess the level of renal damage at retrieval in a porcine transplant model (Hauet et al., 2000a). They concluded that "NMR spectroscopy, which is a non-invasive and non-destructive technique, is more efficient in assessing renal damage than conventional histology and biochemical analysis."

NMR based analysis has also been used in rat models to establish markers of Ischaemia-Reperfusion (IR) injury (Serkova et al., 2005) and various NMR-based publications have reported trimethylamine-N-oxide (TMAO) to be a urine marker for nonspecific medullar injury (Foxall et al., 1993; Hauet et al., 2000a; Hauet et al., 2000b; Richer et al., 2000).

HMP perfusate has been examined by NMR techniques in liver transplantation experiments. Liu et al examined the use of ^{1}H-NMR to discriminate warm ischaemic injury of the liver during HMP in a porcine model (Liu et al., 2009). They discovered that AST, alanine and histidine in HMP perfusate discriminated warm ischaemic injury in porcine liver grafts and suggested them as potential biomarkers for viability. They also concluded that ^{1}H-NMR is a valid screening tool to analyse the composition of the perfusate during liver HMP.

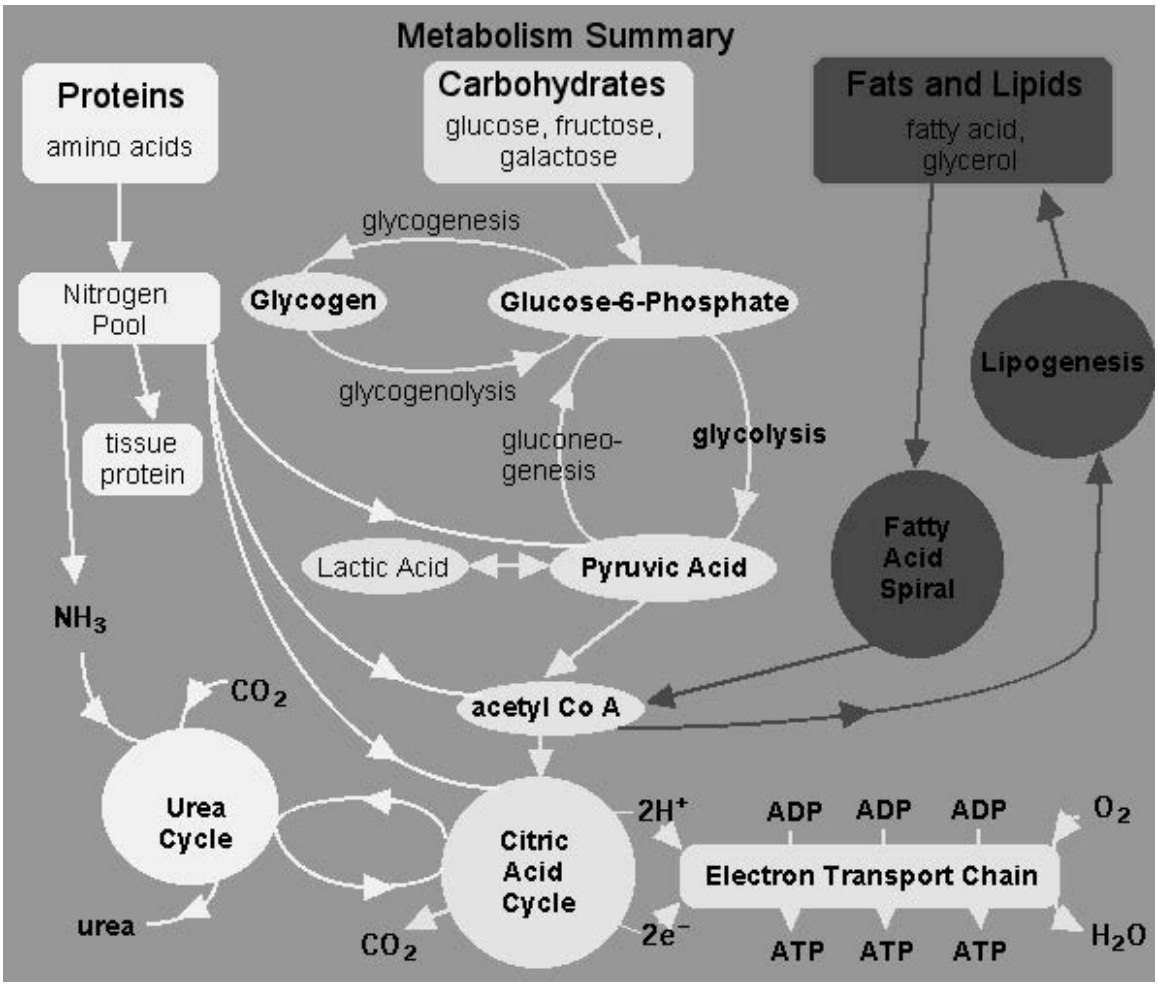
More recently, Bon et al used NMR spectroscopy to examine HMP perfusate in a porcine autotransplant model (Bon et al., 2014). They concluded that multivariate analysis of biomarkers during perfusion using NMR data could be an interesting tool to assess graft quality. They also reported that NMR analysis could be performed in a timeframe that allows clinical utility.

Whilst several publications have demonstrated the feasibility of NMR for evaluating different clinical scenarios in transplantation no previous studies, to our knowledge, have examined the perfusate of human cadaveric kidneys during HMP using NMR.

3.1.6 KEY METABOLIC PATHWAYS

Metabolism encompasses all biological and chemical reactions that can be carried out by cells. It is a balance between anabolism and catabolism controlled by regulatory thermodynamic mechanisms (Ophardt, 2003). Whilst a complete review of cellular metabolism is well beyond the scope of this study, there are a number of important cellular pathways that might be relevant in the cold stored kidney. The most significant pathways in cellular metabolism are outlined below and in figure 3.2.

Figure 3.2: Summary of the Main Metabolic Pathways (Reproduced from Ophardt, 2003)

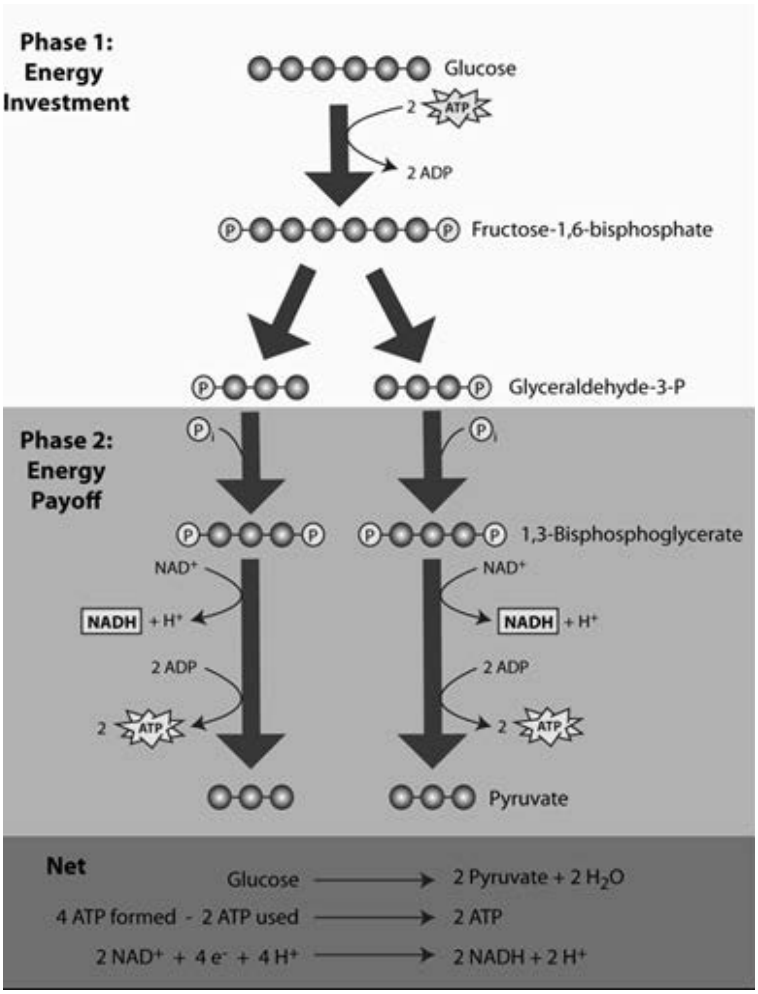


3.1.6.1 GLYCOLYSIS

The most important requirement of all cells in the body is for an immediate source of energy, often in the form of glucose. Glucose is transported into cells as needed where a series of reactions can take place. The three major carbohydrate energy-producing reactions are glycolysis, the citric acid cycle and the electron transport chain (Ophardt, 2003).

The major steps of glycolysis are outlined in figure 3.3. Each glucose molecule is converted into two pyruvic acid molecules. Whilst energy is required to initiate this process a net gain of energy, in the form of ATP, is produced (Ophardt, 2003).

Figure 3.3: Glycolysis (Reproduced from www.moodle2.halesowen.ac.uk)



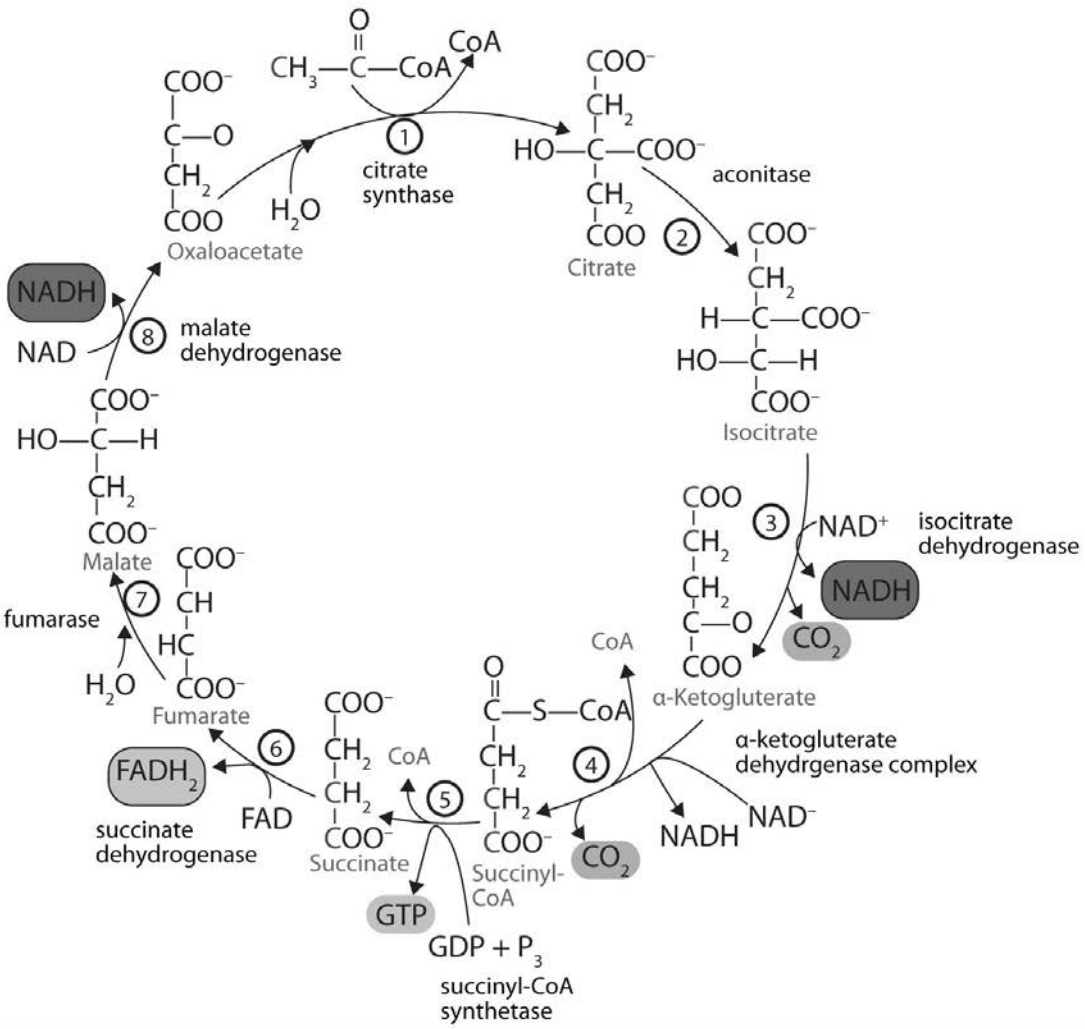
However, glycolysis releases relatively little ATP so reactions continue to convert pyruvic acid to acetyl coenzyme A (acetyl CoA) and then to citric acid in the citric acid cycle.

3.1.6.2 THE CITRIC ACID CYCLE

As mentioned above, the Citric Acid Cycle begins with acetyl CoA. Acetyl CoA, sourced from either glycolysis or the fatty acid cycle, joins with a four-carbon compound to form citric acid. The cycle, along with the electron transport chain, is responsible for the synthesis of most of the body's ATP (Ophardt, 2003).

Acetyl CoA is converted from pyruvic acid prior to entry into the citric acid cycle. If necessary, pyruvic acid can instead be transformed back into glucose or glycogen (gluconeogenesis) or converted by transamination into alanine, an amino acid. If conditions are anaerobic, pyruvic acid is converted into lactc acid. This allows for continued energy production, in the form of ATP, despite the unavailability of oxygen (Ophardt, 2003).

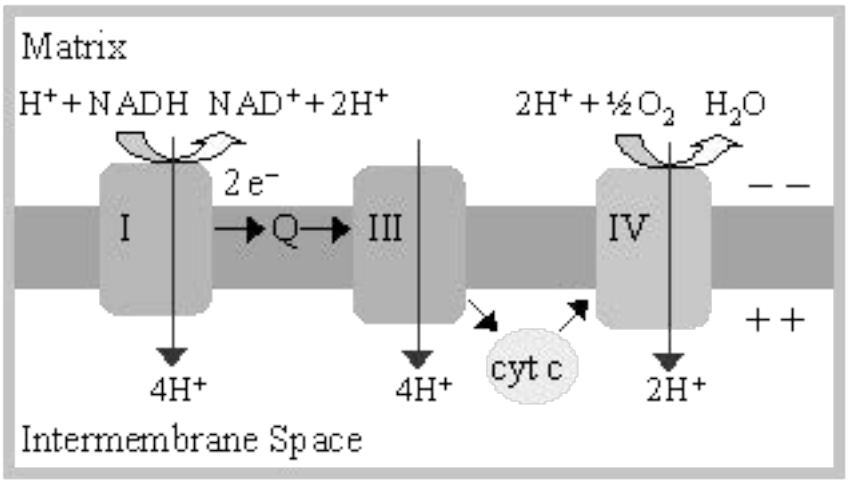
Figure 3.4: The Citric Acid Cycle (Reproduced from Ball, 2011)



3.1.6.3 OXIDATIVE PHOSPHORYLATION

Oxidative phosphorylation uses electrons released by other processes, for example glycolysis and the citric acid cycle, to produce and store energy in the form of ATP (Berg, Tymoczko and Stryer, 2002).

Figure 3.5: Oxidative Phosphorylation (Reproduced from Hinkle, 2005)



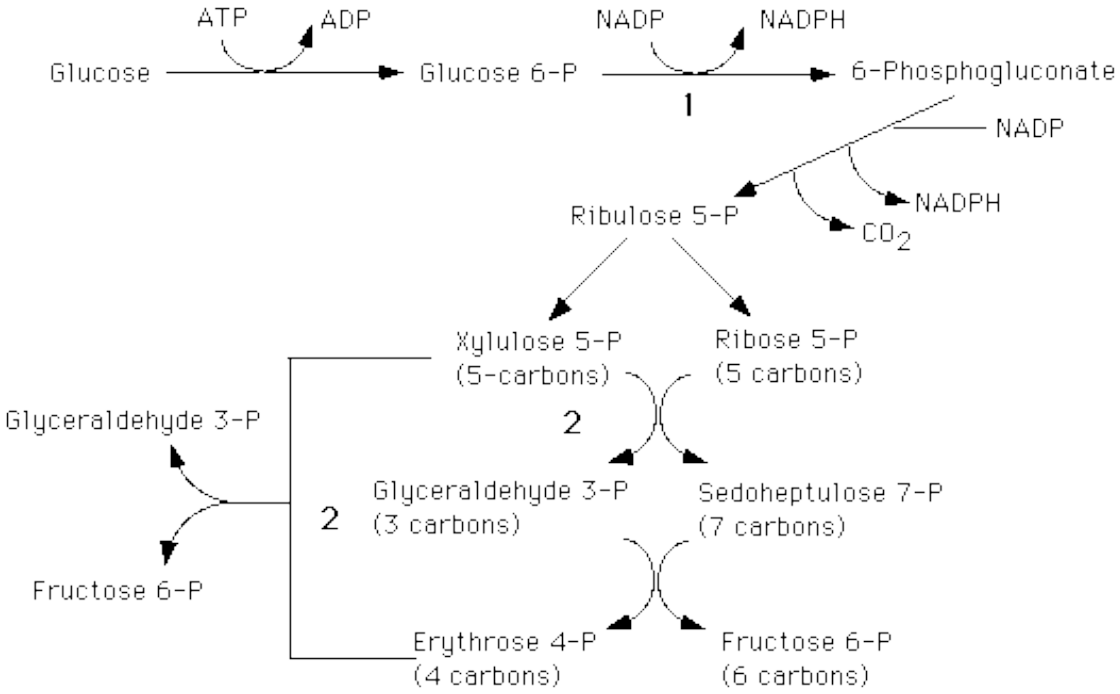
3.1.6.4 THE PENTOSE PHOSPHATE PATHWAY

This pathway is primarily a cytoplasmic anabolic pathway that converts the six carbons of glucose to five carbon (pentose) sugars and reducing equivalents. This process oxidises glucose and its products can be completely oxidised to glucose and water. The pathway has both oxidative and non-oxidative arms.

The oxidative part occurs at the beginning of the pathway, utilising glucose-6-phosphate and producing NADPH. Non-oxidative reactions generate ribose 5-phosphate. The pentose phosphate pathway also converts 5 carbon sugars into 6 and 3 carbon sugars that can then be used for glycolysis (Sheriff, 2004).

Figure 3.6: The Pentose Phosphate Pathway (Reproduced from Sheriff, 2004)

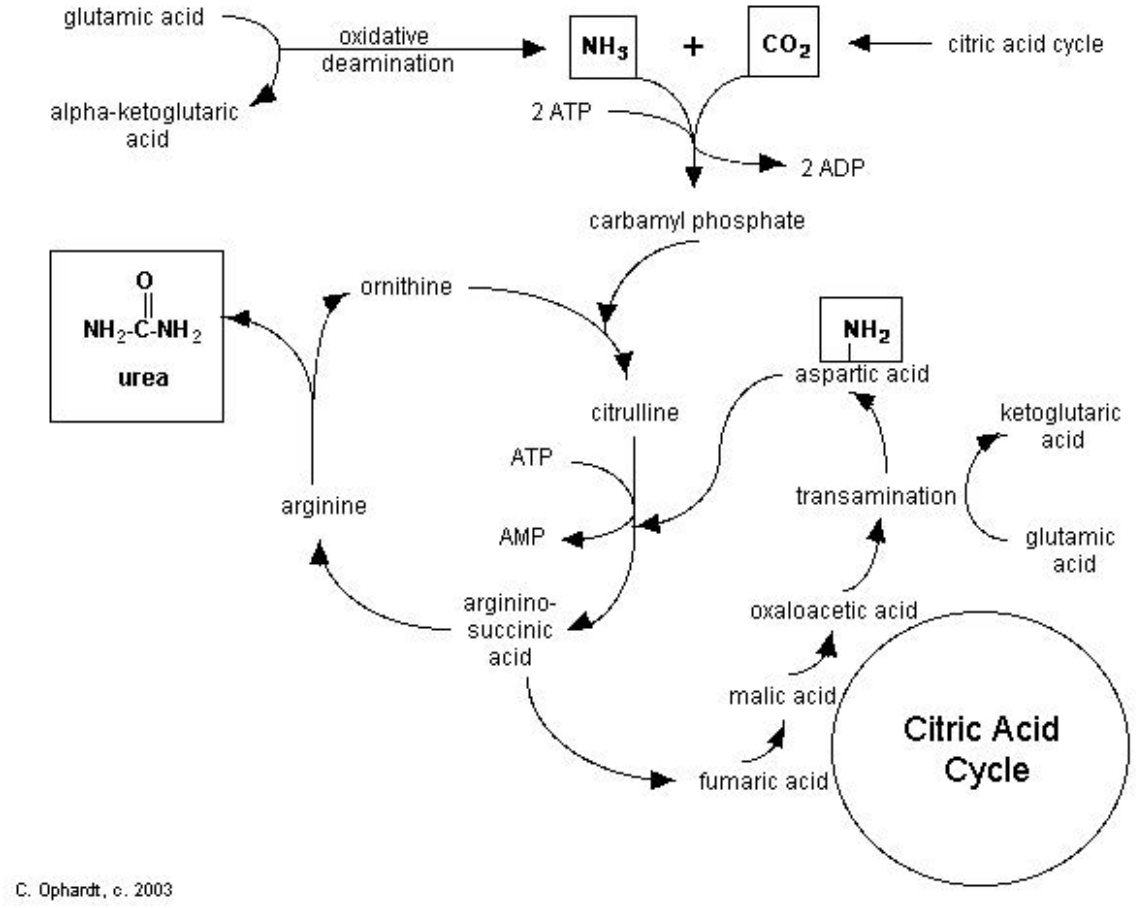
The upper part of the pathway to ribulose-5-P is the oxidative arm. The lower part of the pathway provides a link from ribose-5-P to the glycolytic pathway, gluconeogenesis, and back.



3.1.6.5 THE UREA CYCLE

Urea is the main end product of nitrogen metabolism in humans and mammals. Ammonia, the product of oxidative deamination reactions, is toxic in even small amounts. The urea cycle converts ammonia into urea. This process is shown in figure 3.7.

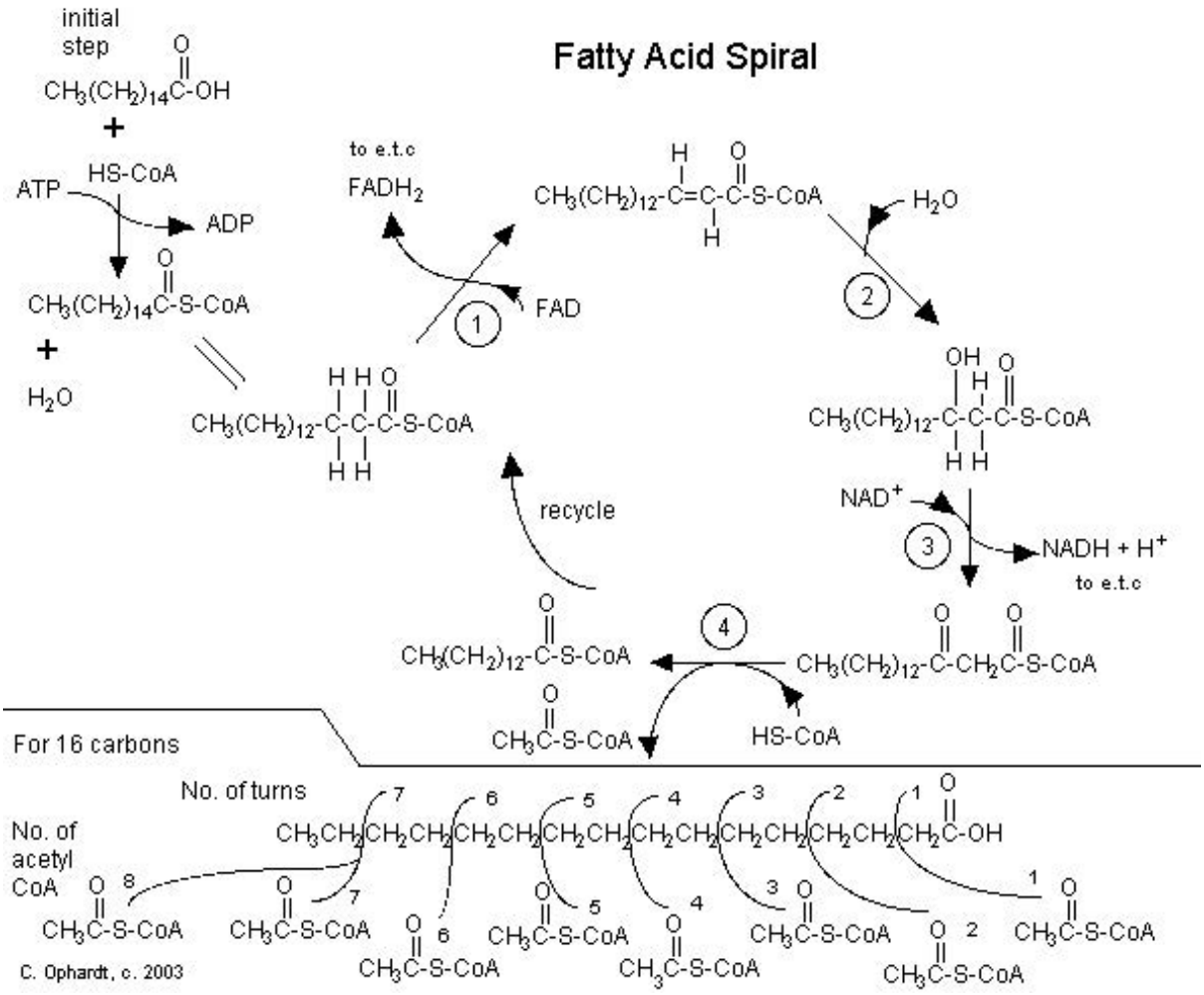
Figure 3.7: The Urea Cycle (Reproduced from Ophardt, 2003)



3.1.6.6 FATTY ACID BETA-OXIDATION

The major reaction to produce energy from lipids is the fatty acid spiral. The beta-oxidation of fatty acids occurs by the removal of two carbons at a time as acetyl CoA in a spiral type reaction. These reactions occur in the mitochondria and thus are closely associated with the electron transport chain to produce energy in the form of ATP. In addition, the acetyl CoA which is produced is fed mostly into the citric acid cycle.

Figure 3.8: Fatty Acid Beta-Oxidation (Reproduced from Ophardt, 2003).



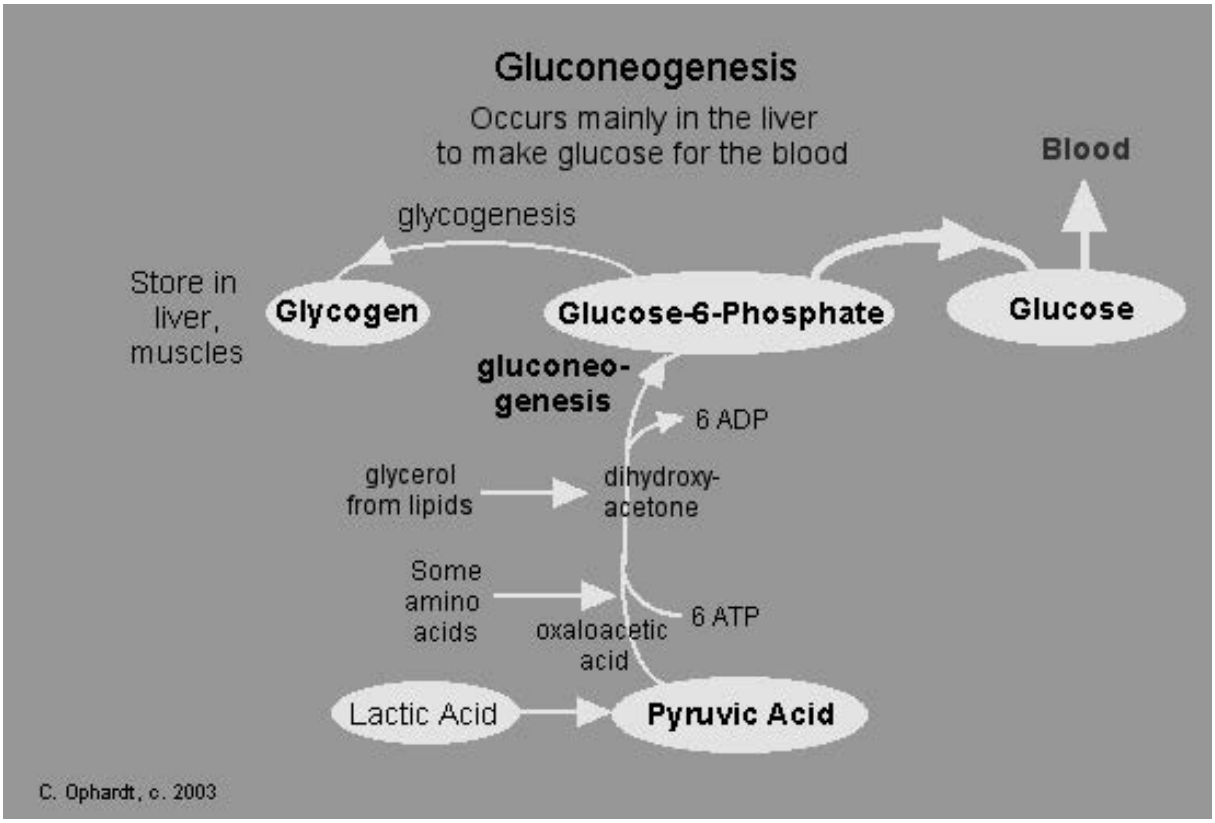
3.1.6.7 GLUCONEOGENESIS

Gluconeogenesis is the mechanism by which glucose is produced from sources other than carbohydrates. There are a variety of molecules from which this process can begin, such as oxaloacetic acid and dihydroxyacetone phosphate, but most often the

pathway starts with pyruvic acid. Other substances that can be converted into glucose are lactic acid, glycerol and some amino acids (Ophardt, 2003).

If the process starts with pyruvic acid, this is first converted into oxaloacetic acid. In the presence of low levels of acetyl CoA and high concentrations of ATP, oxaloacetic acid proceeds along the gluconeogenesis pathway. Alternatively, oxaloacetic acid can enter the citric acid cycle and interact with acetyl CoA. This is summarised in the diagram below.

Figure 3.9: Gluconeogenesis (Reproduced from Ophardt, 2003).



3.2 AIMS

The aim of this part of the study was to use NMR spectroscopy (metabolomics - metabolic fingerprinting) to examine the metabolic profile of the perfusate of human cadaveric kidneys for transplantation and to identify possible discriminators between the profiles of kidneys with delayed and Immediate Graft Function (IGF).

3.3 METHODS

3.3.1 ETHICS & SPONSORSHIP

Ethical approval was obtained from the West of Scotland Research Ethics Service for this study on the 10th of July 2012 (REC reference number: 12/WS/0166).

A research project authentication for this study was obtained from UHB on 25th April 2012 (RRK 4459 Machine Perfusion in Transplantation).

3.3.2 PATIENT RECRUITMENT

Adult cadaveric kidneys accepted for transplantation and undergoing HMP at UHB between July 2012 and August 2013 were included. This was subject to agreement for the use of tissue/fluid for research during the standard donor consent process and recipient agreement for follow-up. Paediatric cases were excluded.

Demographic and clinical data were collected prospectively. Delayed Graft Function (DGF) was defined as the requirement for dialysis within the first post-operative week. IGF kidneys were those not requiring dialysis support post-operatively.

3.3.3 KIDNEY PRESERVATION

Kidneys were cold stored in the period following retrieval and transferred to the LifePort© Kidney Transporter 1.0 (Organ Recovery Systems) at UHB under aseptic conditions. The decision to perfuse kidneys was determined by clinical protocol taking into account donor-recipient issues and theatre availability as per the clinical part of the study. Perfusion pressure was set at 30mmHg and kidneys were perfused with 1L of KPS-1® at 4°C. No additional oxygen was supplied.

3.3.4 SAMPLE COLLECTION

The LifePort© Kidney Transporter has a sampling port as part of the disposable tubing mechanism. 12 mL of perfusate was sampled from the port during perfusion time at the following time intervals:

- 0 minutes, 15 minutes, 30 minutes, 45 minutes, 60 minutes
- 4 hours, 8 hours
- The end of perfusion (a variable time-point)

2 mL of perfusate from each sample was transferred into a cryogenic vial and stored at -20°C until thawed at room temperature, prepared and processed for NMR analysis.

The remaining 10 mL of each sample was transferred into a centrifuge tube and stored in a standard fridge. It was then centrifuged at 4°C for 5 minutes at 1700 RPM. The supernatent was separated and stored at -80°C. The remaining pellet was mixed with 1 mL of freezing media (5mL DMSO/45mL FCS) and also stored at -80°C. These were stored as reserve samples for repeat or further analysis.

3.3.5 SAMPLE PROCESSING

NMR samples were prepared by mixing 150 µL of a 400 mM pH 7.0 phosphate buffer solution containing 2 mM TSP [(3-trimethylsilyl)propionic-(2,2,3,3-d4)-acid sodium salt] with 390 µL of each perfusate sample and 60 µl of deuterium oxide (D_2O) to reach a final phosphate buffer concentration of 100 mM and a final TSP concentration of 500 µM. D_20 provides a field-frequency lock, whereas TSP is used as a chemical shift as well as a concentration reference. After mixing, the 600 µL samples were pipetted into NMR tubes and centrifuged to remove any air bubbles.

3.3.6 SPECTRAL ACQUISITION

^{1}H-NMR spectra were acquired using a Bruker AVII 500 MHz spectrometer equipped with a 5 mm inverse Cryoprobe. The sample temperature was set to 300 K, excitation sculpting was used to suppress the water resonance (Hwang & Shaka, 1995). One-dimensional spectra were acquired using a 6 kHz spectral width, 32768 data points,

4 s relaxation delay and 128 transients. Matching was manual prior to acquisition of first sample and each sample was automatically shimmed (1D-TopShim) to a TSP line width of less than 1 Hz prior to acquisition. Samples with a TSP line width >1 Hz were acquired again after manual shimming where the TSP half height line width was shimmed below 1 Hz. Total experimental time was approximately 15 minutes per sample.

All data sets were processed using the MATLAB based MetaboLab software (Ludwig & Günther, 2011). Data sets were zero filled to 65536 data points. An exponential line broadening of 0.3 Hz was applied before Fourier transformation. The chemical shift axis was calibrated by referencing the TMSP signal to 0 ppm. Spectra were manually phase corrected and baseline correction using a spline before segmental alignment of all resonances using Icoshift (Savorani, Tomasi, & Engelsen, 2010). Spectra were then exported into Bruker format.

3.3.7 SPECTRAL ANALYSIS

Resultant spectra were examined at two time-points for each set of perfusate samples - 45 minutes and 4 hours.

Spectra were examined using Chenomx 7.0 (Professional edition, version 7, Chenomx, Edmonton, AB, Canada). This allows peaks on the spectrum to be identified and quantified. Firstly, the peaks corresponding to compounds known to be

present in KPS-1® were assigned. Other peaks on the spectrum were than assigned where possible. Chemical shift assignments are shown in table 3.1 and figure 3.10. Each signal annotation and quantification was checked manually.

Table 3.1: Chemical Shifts References Used for Metabolite Quantification

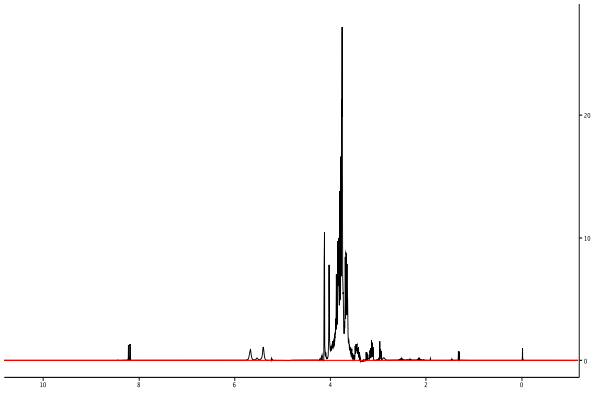
Some metabolites are represented by more than one peak on the NMR spectrum. The concentration of a metabolite is measured by the height of one of these peaks above the baseline. To ensure consistent measurement across samples, the same peak must be used for each sample. The chemical shift references shown here represent the position of the peak on the spectrum that has been used for quantification of each metabolite.

Metabolite	*NMR Chemical Shift Reference (ppm)*	*Metabolite*	*NMR Chemical Shift Reference (ppm)*
3-Hydroxybutyrate	1.204	Hippurate	7.82
3-Methylxanthine	8.02	Hypoxanthine	8.20
Acetate	1.91	Inosine	6.055
Adenine	8.11	Isoleucine	0.997
Alanine	1.46	Isopropranolol	1.162
Citrate	2.67	Lactate	1.40
Ethanol	1.17	Leucine	0.948
Formate	8.44	Malonate	3.11
Fumarate	6.51	Mannitol	3.840
Gluconate	4.12	N-Phenylacetylglycine	7.412
Glucose	3.524	Ribose	2.21
Glutamate	2.341	Tyrosine	6.877
Glutathione	2.97	Uracil	7.52
Glycine	3.54	Valine	1.029

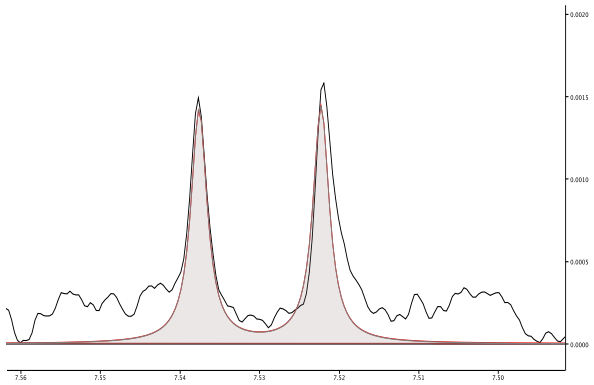
Figure 3.10: Examples of Spectral Assignments

These images show an example spectrum along with magnified areas of the spectrum demonstrating the use of Chenomx software. The black line represents the spectrum produced from sample analysis. The shaded areas are ‘ghost’ images produced by the software that can be matched to spectral patterns to identify peaks.

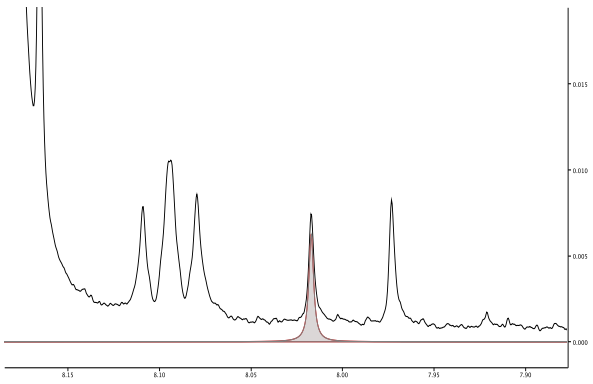
Whole Spectra



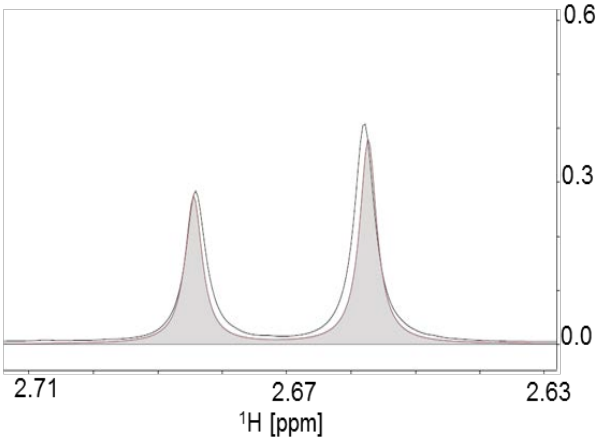
Uracil



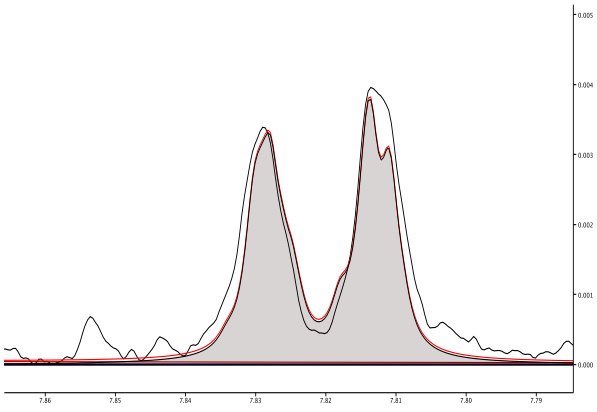
3-Methylxanthine



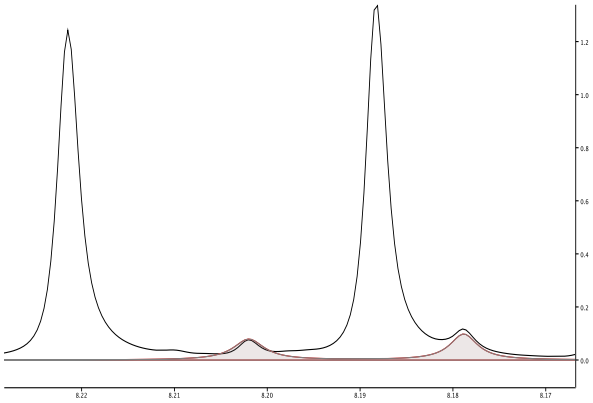
Citrate



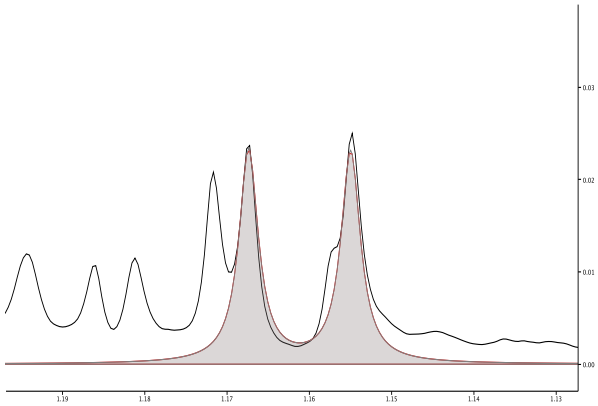
Hippurate



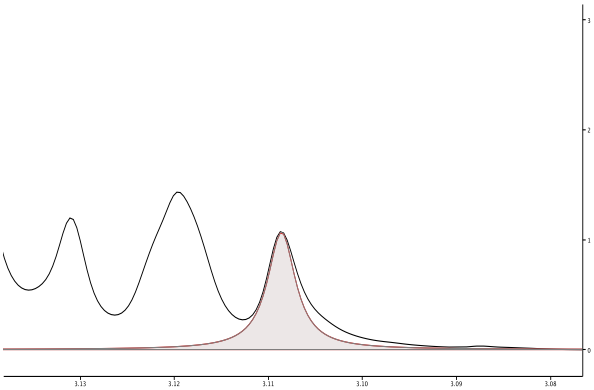
Hypoxanthine



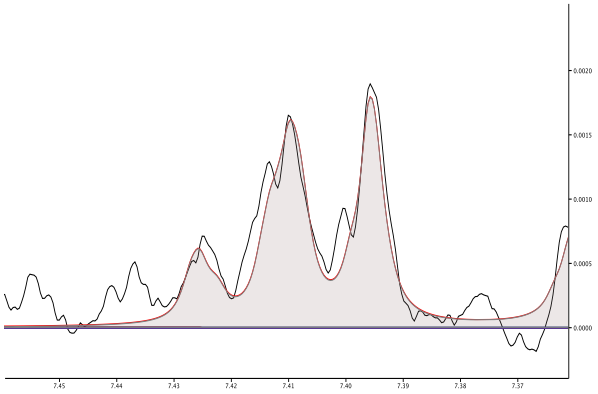
Isopropranolol



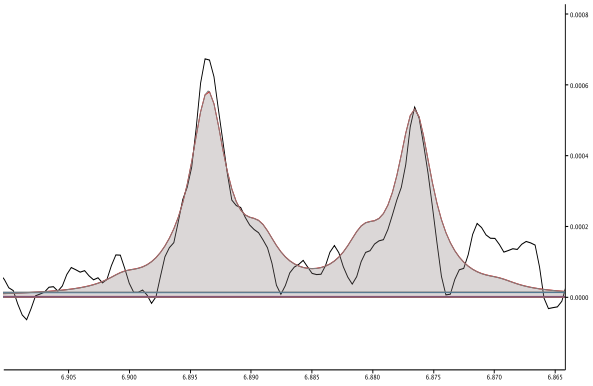
Malonate



N-Phenylglycine



Tyrosine



3.3.8 STATISTICAL ANALYSIS

Data were analysed using GraphPad Prism version 6.0c for Mac (GraphPad Software, La Jolla California USA, www.graphpad.com) and IBM SPSS 19 (IBM Corp. Armonk, NY).

Metabolite averages are stated as median values due to non-parametric data distribution. Change in metabolite concentration over time was analysed by Wilcoxon signed rank test. Metabolite concentrations were compared using Mann Whitney-U test.

The rules for outliers in the boxplots are the ones used by default in SPSS. The outliers represented by circles on the plot are those cases that are greater than 1.5 times the height of the box above or below the box. The stars are classified as extreme outliers, which are greater than three times the height of the box above or below the box. All available data (including the "outliers") were used in the significance tests. The tests used were non-parametric (i.e. based on ranks), and so were insensitive to these outliers.

Comparison between glucose measurements using NMR and glucometer readings were analysed using non-parametric Spearman correlation. $P<0.05$ was considered to be indicative of statistical significance.

3.4 RESULTS

Twenty-nine kidneys were included in the study. Three kidneys were rejected for implantation following HMP. Reasons included severe atherosclerosis of the renal artery with an adverse donor history, a hypoplastic cystic kidney and inadequate initial flush of the kidney at recovery. No kidneys were rejected on HMP parameters. Transplantation proceeded in the remaining 26 kidneys. Donor and recipient characteristics are shown in table 3.2. HMP parameters and storage times are shown in table 3.3. There were no statistically significant differences in characteristics or parameters between the IGF and DGF kidneys.

Table 3.2: Donor and Recipient Characteristics for HMP Kidneys

Characteristic	*Overall (26)*	*IGF (19)*	*DGF (7)*	*P Value*
Donor				
Male gender	16 (62%)	11 (58%)	5 (71%)	0.67
Median age (IQ range)	47 (40-60)	47 (42-59)	51 (31-61)	1.00
CMV +ve	12 (46%)	8 (42%)	4 (57%)	0.67
DBD	23 (88%)	18 (95%)	5 (71%)	0.17
DCD	3 (12%)	1 (5%)	2 (29%)	0.23
SCD	16 (62%)	13 (68%)	3 (18%)	0.37
ECD	10 (38%)	6 (32%)	4 (57%)	0.37
Recipient				
Male gender	18 (69%)	12 (63%)	6 (86%)	0.37
Median age (IQ range)	50 (44-54)	50 (44-55)	50 (44-53)	0.94
Ethnicity: British	18 (69%)	14 (74%)	4 (57%)	0.64
Asian	5 (19%)	4 (21%)	1 (14%)	1.00
Black	3 (12%)	1 (5%)	2 (29%)	0.17
Virology: CMV+	12 (46%)	7 (37%)	5 (71%)	0.19
HAV+	2 (8%)	1 (5%)	1 (14%)	0.47
HBV cAb+	3 (12%)	1 (5%)	2 (29%)	0.17
HBV sAb+	1 (4%)	1 (5%)	0 (0%)	1.00
Dialysis: HD	18 (69%)	12 (63%)	6 (86%)	0.37
PD	6 (23%)	5 (26%)	1 (14%)	1.00
Pre	2 (8%)	2 (11%)	0 (0%)	1.00
Previous transplant ≥1	3 (12%)	3 (16%)	0 (0%)	0.54
PRA level >5%	13 (50%)	9 (47%)	4 (57%)	1.00
0.0.0 mismatches	4 (15%)	4 (21%)	0 (0%)	0.55
Immunosuppression standard	24 (92%)	19 (100%)	5 (71%)	0.06

CMV: cytomegalovirus. HAV: Hepatitis A virus, HBV: hepatitis B virus. cAb: core antibody, sAb: surface antibody. PRA: panel reactive antibodies. Standard immunosuppression: Basiliximab, Tacrolimus, Mycophenolate mofetil, Prednisolone. Data reported as: “Median (Quartiles)” or “N (%)”, with p-values from Fisher’s exact tests or Mann-Whitney tests, as appropriate
*Significant at $p < 0.05$

Table 3.3: HMP Parameters & Storage Times

Characteristic	*Overall (26)*	*IGF (19)*	*DGF (7)*	*P Value*
Storage Times (hours)				
Cold Storage time	7 (6-11)	8 (6-11)	7 (5-10)	0.33
HMP time	15 (7-17)	15 (10-17)	15 (5-22)	1.00
Total CIT	23 (16-27)	24 (17-27)	22 (15-29)	0.92
HMP Parameters				
Initial Flow mL/min	64 (43-88)	67 (49-92)	47 (39-65)	0.20
End Flow mL/min	108 (86-138)	111 (92-135)	95 (74-170)	0.72
Increase in Flow mL/min	45 (26-58)	45 (22-57)	44 (32-69)	0.43
Initial Resistance mmHg/mL/min	0.42 (0.30-0.64)	0.37 (0.28-0.56)	0.58 (0.39-0.66)	0.18
End Resistance mmHg/mL/min	0.21 (0.16-0.27)	0.20 (0.17-0.25)	0.25 (0.15-0.31)	0.52
Decrease in Resistance mmHg/mL/min	0.21 (0.08 -0.34)	0.16 (0.07-0.29)	0.26 (0.21-0.40)	0.19

3.4.1 IDENTIFIED METABOLITES

Twenty-eight metabolites were identified in the perfusate of HMP kidneys at both examined time-points. Some of these were known to be constituents of KPS-1® (table 1.6) and some were 'new' metabolites i.e. not known constituents of the perfusion fluid.

3.4.2 METABOLITES IN KPS-1®

Six of the 28 identified metabolites were recognised constituents of the KPS-1® fluid. These were adenine, gluconate, glucose, glutathione (reduced form), mannitol and ribose. Median concentrations of these metabolites measured by ^{1}H-NMR at each time-point and the p-values for change over time are shown in table 3.4. Notably, there was a significant change in concentration of glucose and glutathione between the two time points.

Table 3.4: Metabolite Concentrations (mM) for Constituents of KPS-1® Identified in Kidney Perfusate

Metabolite	*Median Conc at 45 mins of HMP*	*Interquartile range*	*Median Conc at 4 hours of HMP*	*Interquartile range*	*P-value (change over time)*
Adenine	4.82	4.12-5.51	4.69	3.92-5.57	0.804
Gluconate	53.05	50.69-59.02	56.31	51.08-61.87	0.340
Glucose	9.07	8.32-10.04	9.75	8.35-10.79	0.041*
Glutathione	0.89	0.79-0.94	0.50	0.40-0.60	<0.001*
Mannitol	32.13	31.13-34.68	34.84	29.65-40.50	0.111
Ribose	0.00	0.00-0.00	0.00	0.00-0.00	0.129

Statistically significant at <0.05, Wilcoxon signed rank test

3.4.3 NEW METABOLITES IN PERFUSATE

The remaining 22 identified metabolites are not listed as constituents of KPS-1®. Median concentrations of these metabolites at each time-point and p-values for change over time are shown in Table 3.5. Most of these metabolites changed significantly (highlighted in bold).

Table 3.5: Metabolite Concentrations (mM) Measured in Kidney Perfusate Not Listed as Constituents of KPS-1®

Metabolite	*Median Conc at 45 mins of HMP*	*Interquartile range*	*Median Conc at 4 hours of HMP*	*Interquartile range*	*P-value (change over time)*
3-Hydroxybutyrate	**0.008**	**0.006-0.014**	**0.021**	**0.014-0.030**	**<0.001***
3-Methylxanthine	0.016	0.014-0.018	0.017	0.013-0.019	0.363
Acetate	0.100	0.082-0.113	0.087	0.062-0.102	0.085
Alanine	**0.047**	**0.034-0.065**	**0.121**	**0.077-0.147**	**<0.001***
Citrate	**0.477**	**0.000-0.830**	**0.621**	**0.000-1.299**	**<0.001***
Ethanol	0.017	0.014-0.020	0.015	0.011-0.019	0.282
Formate	**0.051**	**0.037-0.071**	**0.055**	**0.037-0.086**	**0.013***
Fumarate	**0.002**	**0.001-0.003**	**0.003**	**0.001-0.004**	**0.005***
Glutamate	**0.145**	**0.065-0.325**	**0.646**	**0.375-0.807**	**<0.001***
Glycine	**0.469**	**0.391-0.541**	**1.363**	**0.902-1.792**	**<0.001***
Hippurate	0.003	0.000-0.005	0.002	0.000-0.006	0.093
Hypoxanthine	**0.041**	**0.020-0.067**	**0.086**	**0.034-0.157**	**<0.001***
Inosine	**0.006**	**0.002-0.015**	**0.008**	**0.003-0.020**	**0.014***
Isoleucine	**0.003**	**0.002-0.004**	**0.004**	**0.003-0.006**	**<0.001***
Isopropranolol	0.015	0.010-0.021	0.015	0.012-0.021	0.164
Lactate	**0.602**	**0.481-0.694**	**1.158**	**0.932-1.322**	**<0.001***
Leucine	**0.008**	**0.006-0.010**	**0.012**	**0.008-0.016**	**<0.001***
Malonate	2.136	1.050-2.344	1.975	0.000-2.408	0.568
N-Phenylacetylglycine	**0.003**	**0.000-0.006**	**0.004**	**0.000-0.012**	**0.009***
Tyrosine	**0.003**	**0.002-0.003**	**0.004**	**0.003-0.006**	**<0.001***
Uracil	**0.007**	**0.003-0.008**	**0.009**	**0.006-0.013**	**<0.001***
Valine	**0.005**	**0.004-0.006**	**0.003**	**0.006-0.010**	**<0.001***

Statistically significant at <0.05, Wilcoxon signed rank test

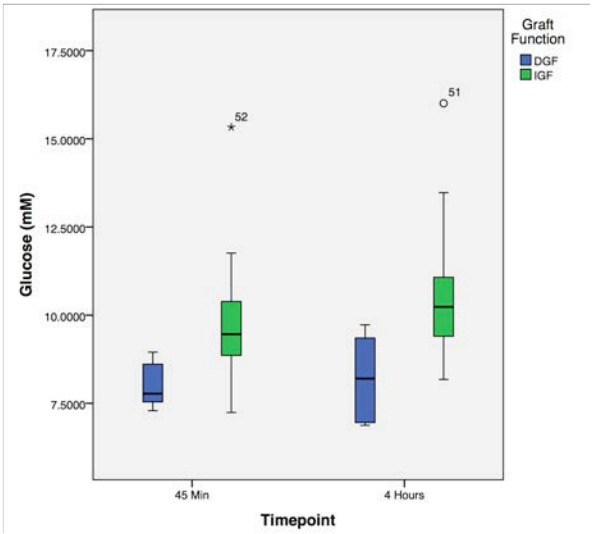
3.4.4 GRAFT FUNCTION & METABOLOMIC PROFILE

Of the 26 transplanted kidneys following HMP, 19 (73%) kidneys displayed IGF post transplant and 7 (27%) suffered from DGF. There were differences between the metabolomic profile of these groups – metabolites that were significantly different at one or both timepoints between the groups were glucose, inosine, leucine and gluconate.

3.4.4.1 GLUCOSE

Glucose concentrations were significantly lower in DGF kidney perfusate compared to the perfusate of kidneys with IGF at both 45 minutes (7.772 v 9.459 mM, p=0.006) and 4 hours (8.202 v 10.235 mM, p=0.003) (figure 3.11).

Figure 3.11: Box and Whisker Plot to Represent Glucose Concentrations in HMP Kidney Perfusate



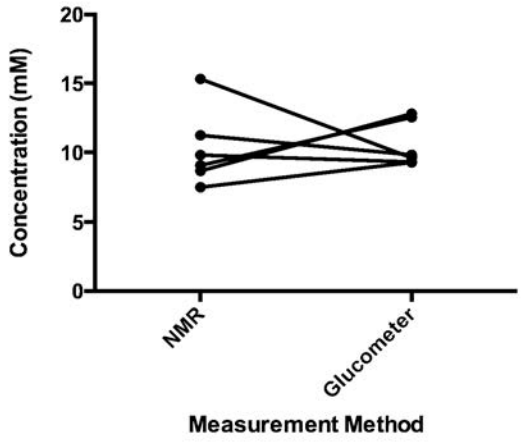
3.4.4.1.1 CORRELATION OF GLUCOSE MEASUREMENTS

Following the finding that glucose levels were significantly different between the perfusate of DGF and IGF kidneys, the reserve samples were thawed and tested with a standard bedside glucometer (Codefree^{TM} blood glucose monitoring system). Seven sets of human perfusate samples (45 minute and 4 hours samples for each) were selected at random and thawed at room temperature. Readings were performed three times on each sample and the mean average calculated. This was then compared with the NMR measured concentrations.

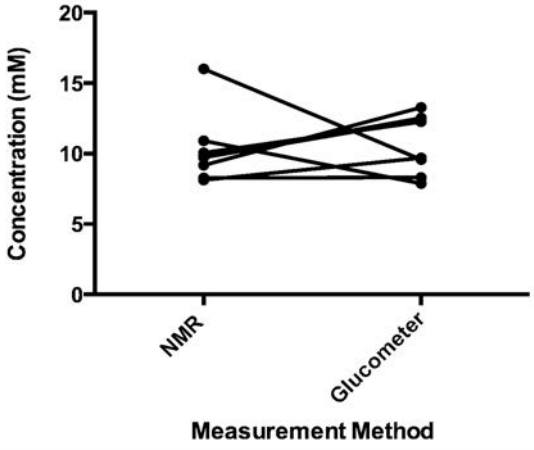
Glucose levels measured using the bedside glucometer and by NMR spectroscopy did not correlate at 45 minutes (correlation coefficient, CC 0.029; p-value = 1.000) or at 4 hours (CC -0.238; p-value = 0.582). This is demonstrated in figure 3.12.

Figure 3.12: Correlation of Glucose Levels Measured by NMR and a Standard Glucometer

At 45 Minutes



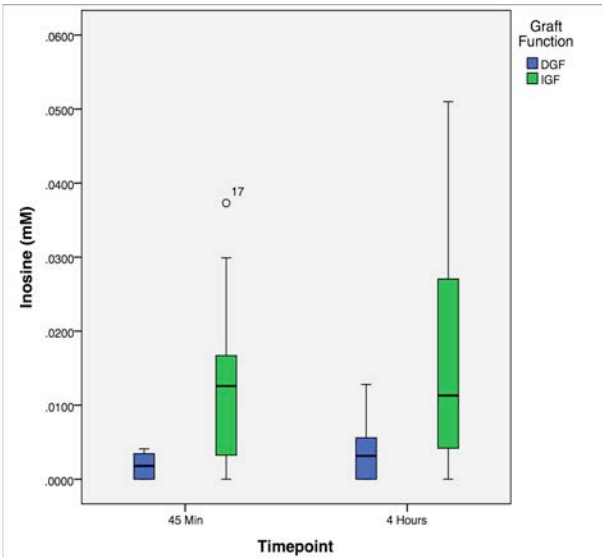
At 4 Hours



3.4.4.2 INOSINE

Concentrations of inosine were significantly lower in DGF than IGF kidney perfusate at 45 mins (0.002 v 0.013 mM, p=0.009) but not at 4 hours (figure 3.13).

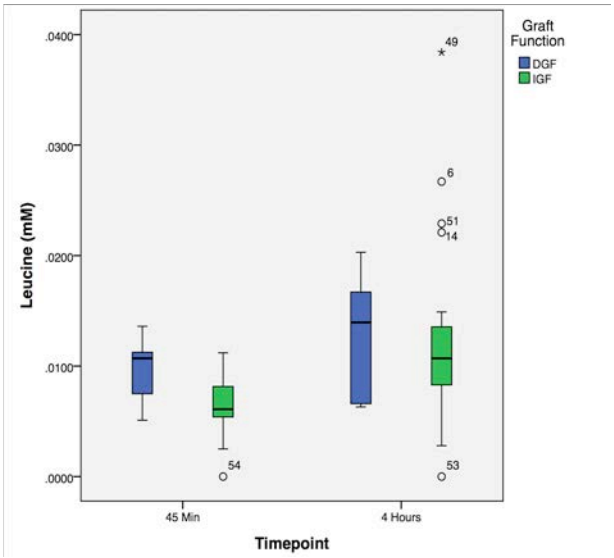
Figure 3.13: Box and Whisker Plot to Represent Inosine Concentrations in HMP Kidney Perfusate



3.4.4.3 LEUCINE

Concentrations of leucine were significantly higher in DGF than IGF kidney perfusate at 45 mins (0.011 v 0.006 mM, p=0.036) but not at 4 hours (figure 3.14).

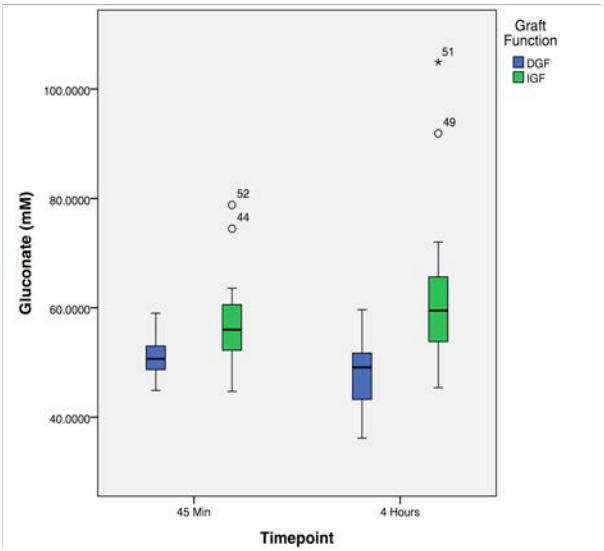
Figure 3.14: Box and Whisker Plot to Represent Leucine Concentrations in HMP Kidney Perfusate



3.4.4.4 GLUCONATE

Gluconate levels were significantly lower in DGF kidney perfusate than in the perfusate of IGF kidneys at 4 hours (49.099 v 59.513 mM p=0.009) but not at 45 minutes (figure 3.15).

Figure 3.15: Box and Whisker Plot to Represent Gluconate Concentrations in HMP Kidney Perfusate

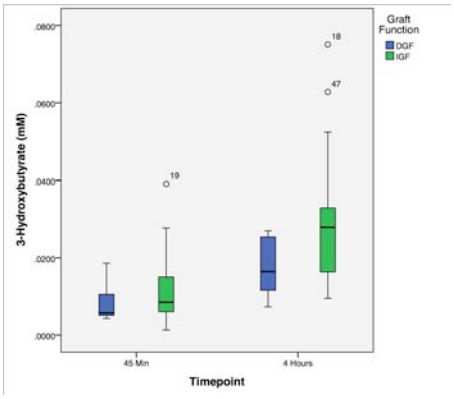


3.4.4.5 OTHER METABOLITES

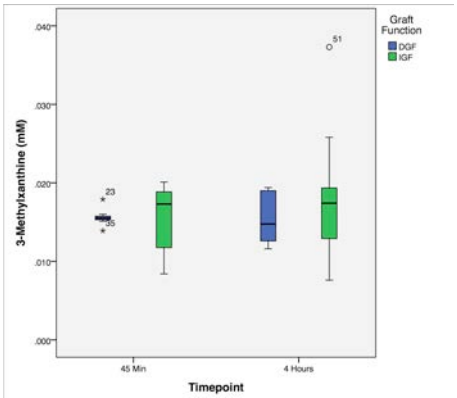
Levels of the other identified metabolites did not differ significantly between the perfusate of IGF and DGF kidneys at the two timepoints measured. Median levels and interquartile ranges are shown in the box and whisker plots in figure 3.16.

Figure 3.16: Box and Whisker Plot to Represent Concentrations of Other Metabolites in HMP Kidney Perfusate

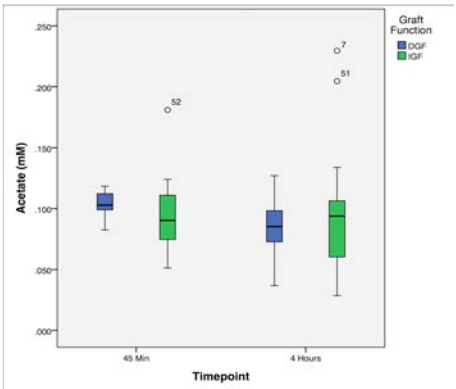
3-Hydroxybutyrate



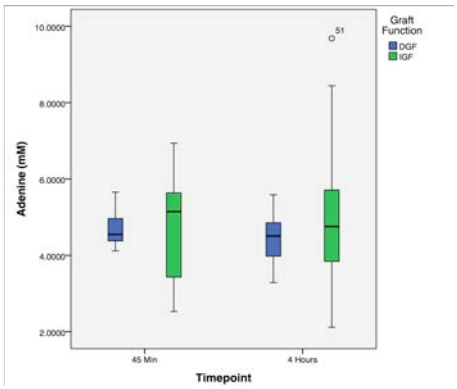
3-Methylxanthine



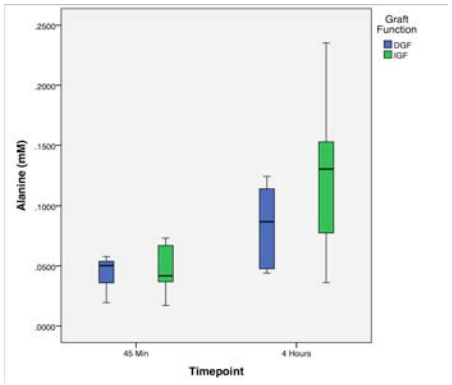
Acetate



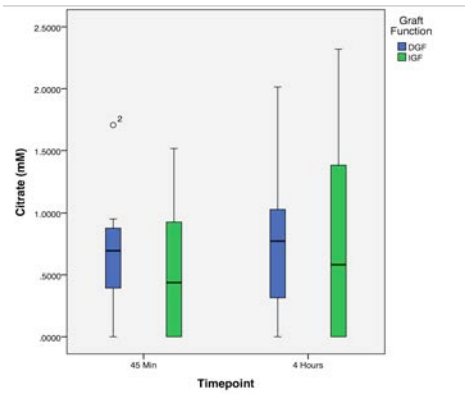
Adenine



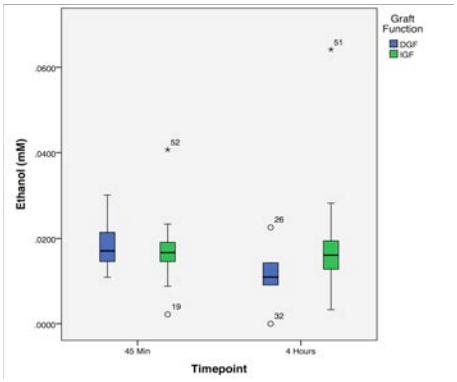
Alanine



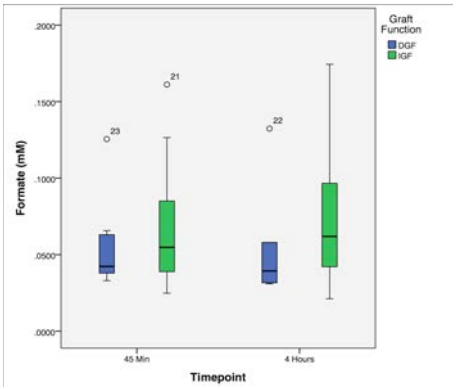
Citrate



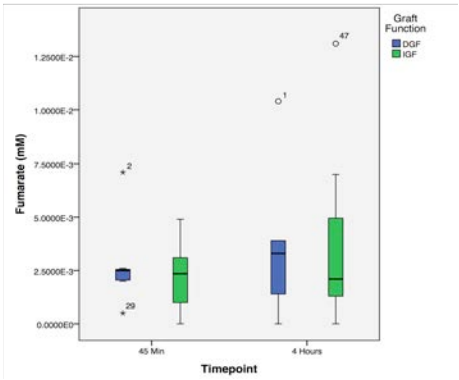
Ethanol



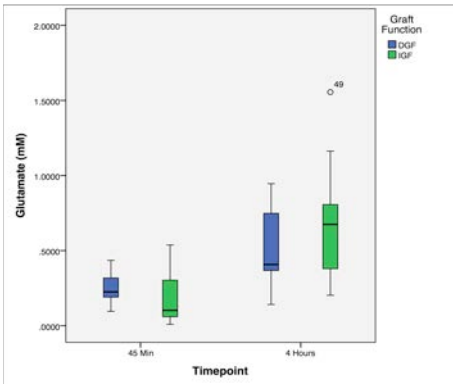
Formate



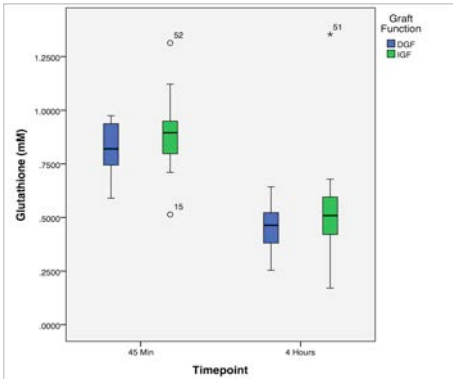
Fumarate



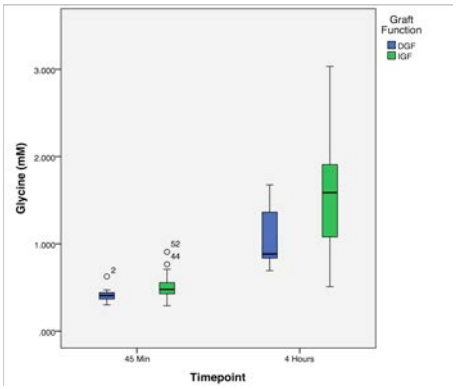
Glutamate



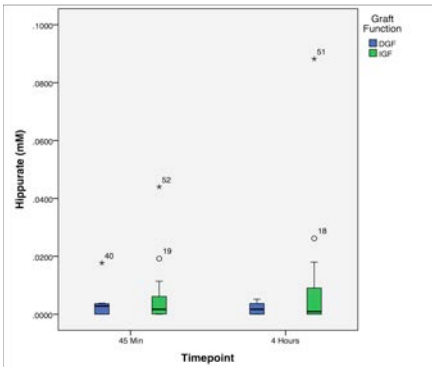
Glutathione



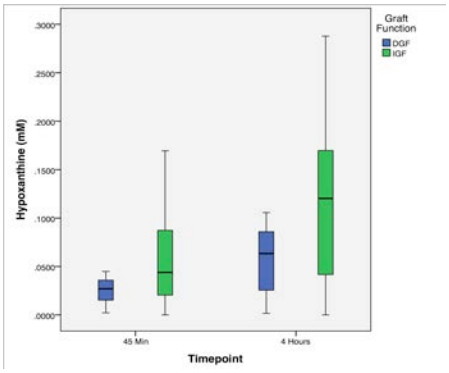
Glycine



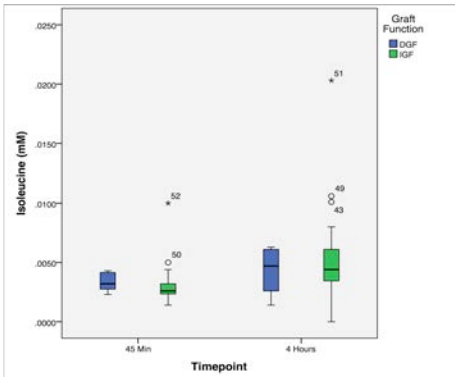
Hippurate



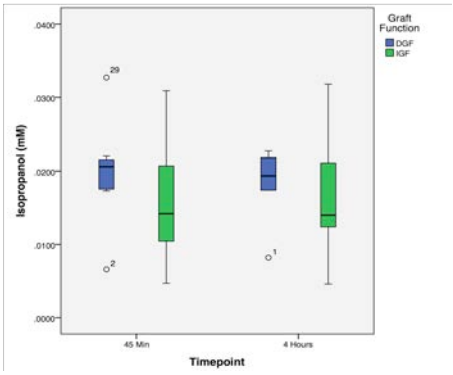
Hypoxanthine



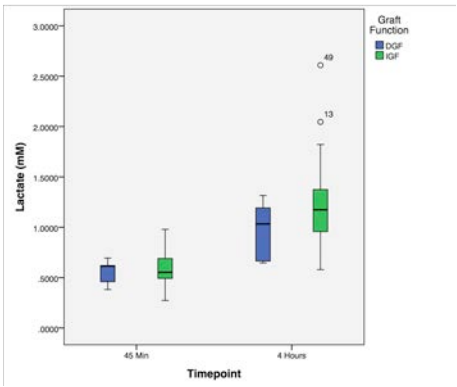
Isoleucine



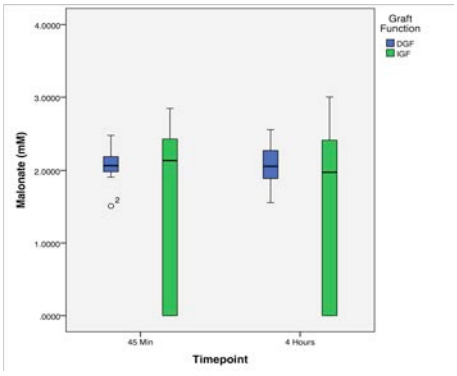
Isopropranolol



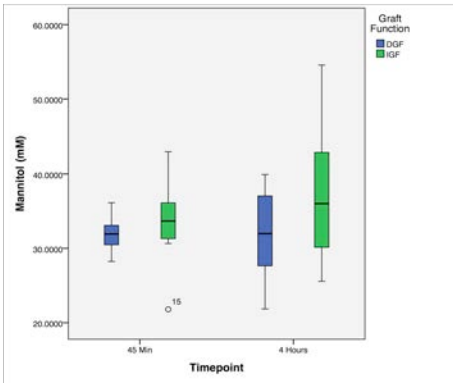
Lactate



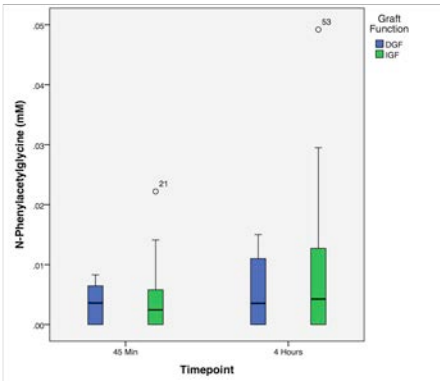
Malonate



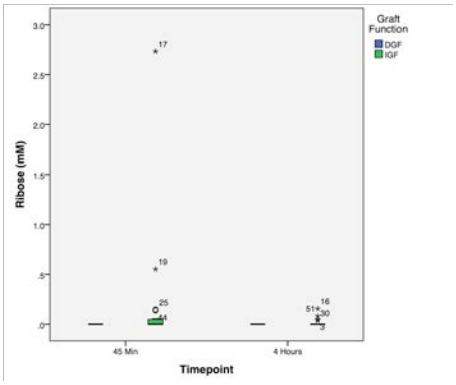
Mannitol



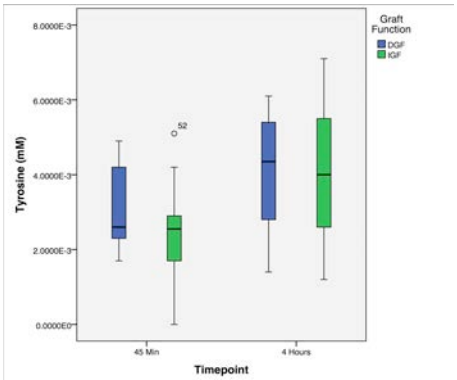
N-Phenylacetylglycine



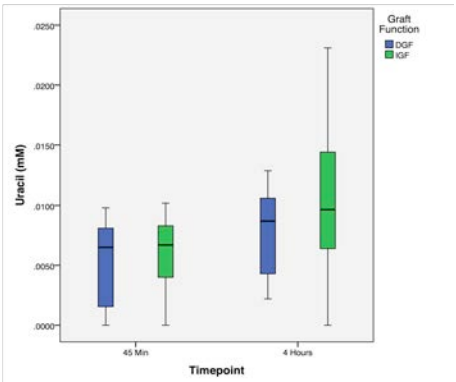
Ribose



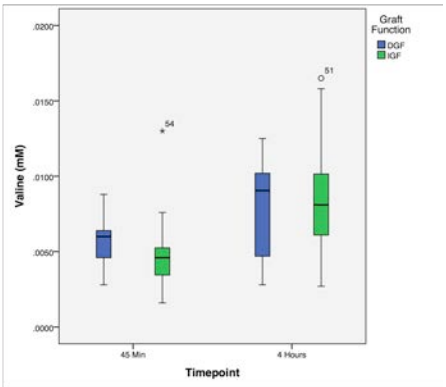
Tyrosine



Uracil



Valine

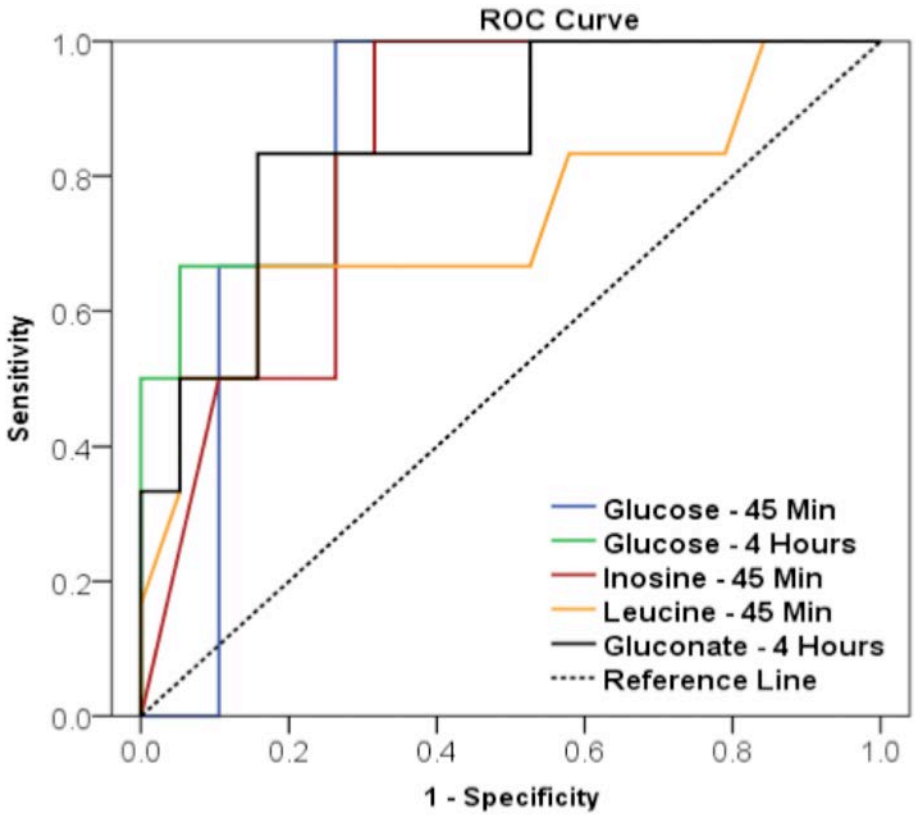


3.4.5 ROCS FOR PREDICTION OF DGF

A Receiver-Operator Characteristic (ROC) curve is a graphical plot of sensitivity versus 1- specificity. The area under the ROC (AUROC) is a combined measure of these indices and is representative of the overall performance of a diagnostic test. The AUROC can be a value between 0 and 1, and the closer the value to 1, the better the overall performance of the test. A value of greater than 0.7 is considered to be 'fair' and over 0.8 considered to be 'good'.

Receiver-operator Characteristic (ROC) curves assessing the predictive accuracy of glucose, inosine, leucine and gluconate for DGF are shown in figure 3.17. Glucose yielded an area under the ROC (AUROC) curve of 0.842 (Standard Error; SE 0.080) at 45 minutes and 0.895 (SE 0.069) at 4 hours. The AUROC for inosine at 45 minutes was 0.833 (SE 0.082) and for leucine at 45 minutes was 0.732 (SE 0.135). Gluconate at 4 hours had an AUROC of 0.851 (SE 0.089). Cut-off values for the ROC curves can be seen in the appendices.

Figure 3.17: Receiver-operator Characteristic (ROC) Curves and Areas Under the ROC Curves (AUROCs) for Significant Metabolites



Area Under the Curve

Test Result Variables	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Glucose 4 hours	0.895	0.069	0.004	0.759	1.000
Gluconate 4 hours	0.851	0.089	0.011	0.676	1.000
Glucose 45 minutes	0.842	0.080	0.013	0.686	0.998
Inosine	0.833	0.082	0.016	0.673	0.993
Leucine	0.732	0.135	0.092	0.468	0.997

[a] Under the non-parametric assumption

[b] Null hypothesis: true area = 0.5

3.5 DISCUSSION

This is the first report to detail the metabolomic profile of perfusate during HMP of human cadaveric kidneys. Changes in the perfusate composition during this time may represent substances being removed by the kidney to supply ongoing cell processes or products of metabolism or degradation being released from the kidney. This experiment demonstrates that after only 45 minutes of machine perfusion, the perfusate is markedly different from the original preservation solution. Furthermore, this study has identified differences in the metabolomic profile of IGF and DGF kidney perfusate that may allow prediction of functional graft outcome following transplantation.

Accurate assessment of graft quality is increasingly important to achieve the highest levels of success in transplantation. Whilst donor information, such as patient age, co-morbidity or terminal serum creatinine, along with kidney biopsy data is important, they still have a limited capacity for accurate prediction of graft outcomes (Jochmans & Pirenne, 2011; Louvar et al., 2009). Machine perfusion parameters, such as resistance, are generally accepted as good indicators of graft quality but several studies have warned of the dangers of using resistance values to determine kidney discard (Jochmans et al., 2011; Sonnenday et al., 2003; de Vries et al., 2011).

Theoretically, biomarkers measured in urine and perfusate have an advantage over biopsy data of being non-invasive and can be measured more frequently and objectively. A recent review highlighted the biomarkers that have been assessed in

regards to graft outcome (Bhangoo et al., 2012). Few studies were recent and even fewer were deemed to be of good quality. Levels of lactate dehydrogenase (LDH), glutathione-S-transferase (GST) and aspartate transaminase (AST) were significantly associated with DGF in the greatest number of studies but further validation was recommended. Other markers investigated in kidney perfusate include glucose, lactate, electrolytes, pH, gas pressures, osmolarity, redox active iron, malondialdehyde and total antioxidant status.

Many potential biomarkers have and are being investigated across a variety of medical fields. These might predict or assess clinical outcomes or help to monitor treatments and responses. Few biomarkers, however, meet the strict criteria that are required for them to accurately predict or act as a substitute for clinical outcomes (Fleming, 2005). Many criteria have been suggested but a valid biomarker can be defined as ‘a biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is an established scientific framework or body of evidence that elucidates the physiological, toxicological, pharmacological, or clinical significance of the test results (www.fda.gov/cder/guidance/6400fnl.pdf).

Validation of a biomarker is the process by which it is assessed and its performance characteristics defined, including the determination of the conditions under which the biomarker will give reproducible and accurate results (Wagner, 2002; Lee et al., 2006). The process can be time-consuming and expensive but is necessary to ensure high-quality and reliable biomarkers. Different criteria can be assessed but

typically include measurement of sensitivity, specificity, bioanalytical assessment, probability of false positives, probability of false negatives and apharmacokinetic/pharmacodynamic model (Lesko & Atkinson, 2001).

NMR-based metabolomics is a novel approach for rapidly identifying the changes in global metabolite profiles of biological samples and is widely used in disease studies (Nicholson et al., 1999). Several studies have demonstrated possibilities for the use of NMR-based metabolomics within transplantation. It has shown promise as a tool to predict long-term graft outcome based on the energy state of the kidney in-vivo and it may be able to assess biomarkers of kidney injury ex-vivo (Hauet et al., 2000c; Richer et al., 2000; Seto et al., 2001). More recently, NMR has been used to examine HMP perfusate in a pre-clinical DCD porcine model (Bon et al., 2014). This study concluded that analysis of biomarkers during HMP using NMR could be an interesting tool to assess graft quality and was compatible with clinical application.

3.5.1 CONSTITUENTS OF KPS-1®

In this study, the main constituents of KPS-1® were identified by ^{1}H-NMR, except for HEPES and HES which the software cannot recognise. Of the identified metabolites, median levels of adenine, ribose, mannitol and gluconate did not change significantly over HMP time for all perfused kidneys.

3.5.1.1 ADENINE & RIBOSE

Adenine is a purine base found in both DNA and RNA. When attached to ribose, it becomes the nucleoside adenosine. Adenosine triphosphate (ATP) is formed when three phosphate groups are added to adenosine. Adenosine triphosphate is used in cellular metabolism as one of the basic methods of transferring chemical energy between chemical reactions (www.HMDB,ca Wishart et al., 2013).

Ribose is a 5-carbon sugar found in all living cells. As mentioned above, it is a constituent part of ATP. When ATP stores are rapidly depleted, ribose can help to restore levels by bypassing the rate-limiting step in the *de-novo* pathway. Ribose can be synthesised by many tissues in the body from other substances, such as glucose (www.HMDB,ca Wishart et al., 2013).

Adenine and ribose were historically added to perfusion fluids, in preference to adenosine, to increase the synthesis of adenine nucleotides (McAnulty, Southard, & Belzer, 1987). Ribose was almost undetectable in the perfusate samples even at 45 minutes so not detecting a change over time is unsurprising. This could be a true reflection of ribose levels or it could due to changes in labelled concentrations during the storage period. Ribose also has a complex pattern on spectral analysis and the peaks overlap with neighbouring spikes making quantification difficult. Adenine concentrations, however, also did not alter over time or between the perfusate of kidneys with DGF or IGF possibly reflecting that this is not an important source of energy in HMP kidneys.

3.5.1.2 GLUCONATE & MANNITOL

Gluconate (like mannitol) is present in preservation solution to provide osmotic stability, so a significant change in concentration would be unexpected. Gluconate levels were, however, significantly lower in the perfusate of DGF kidneys at 4 hours. It is possible that cellular damage in the DGF kidneys allows influx of gluconate into the cell which would not normally occur within healthy tissue.

3.5.1.3 GLUTATHIONE

Glutathione is included in many preservation solutions, including KPS-1®, and acts as a free radical scavenger to attenuate ischaemia-reperfusion injury. It contains the crucial thiol (-SH) group that makes it an effective antioxidant (www.HMDB,ca Wishart et al., 2013).

Concentrations of reduced glutathione decreased significantly over HMP time in both DGF and IGF kidney perfusate. Glutathione in its oxidised form was not detected. The oxidisation of glutathione during HMP time could account for this apparent decrease.

3.5.1.4 GLUCOSE

Glucose is a monosaccharide containing six carbon atoms and an aldehyde group. It is a primary source of energy for living organisms. Glucose can be synthesised in the kidneys from non-carbohydrate intermediates, such as pyruvate and glycerol, by gluconeogenesis (www.HMDB,ca Wishart et al., 2013).

Glucose is included in preservation solutions to prevent energy loss and increase osmotic pressure. It actually increased just significantly over perfusion time when all kidneys were examined. However, glucose levels in the perfusate of DGF kidneys were significantly lower at both time-points than those found in the perfusate of IGF kidneys, with good AUROCs for prediction of DGF. Although the reason for this is not known, it is possible that more glucose is utilized for repair by damaged DGF kidneys or that DGF kidneys are not effectively suppressed by hypothermia and require more glucose for metabolism.

Glucose levels measured by NMR did not correlate with levels measured with the bedside glucometer. This was attempted to assess the possibility of a simple clinical test in comparison to the more complex metabolomics analysis. The glucometer is designed for use with blood so the constituents or viscosity of the perfusion solution may have affected its accuracy. Samples tested had also been stored for varying levels of time at -70ºC. This could have degraded the samples. The accuracy of the glucometer also varies with only 66% of readings being within 10% of a standard measured by a laboratory glucose monitor.

3.5.2 NEW METABOLITES IN PERFUSATE

Of the metabolites discovered that are not part of the preservation solution two of these, leucine and inosine, were significantly different in the perfusate of DGF kidneys compared to IGF kidney perfusate at 45 minutes.

3.5.2.1 LEUCINE

Leucine is one of three branched chain amino acids, an essential amino acid whose carbon structure is marked by a branch point. This type of amino acid is particularly involved with stress, energy and muscle metabolism (www.HMDB,ca Wishart et al., 2013). The role of the kidney in the metabolism of branched chain amino acid remains incompletely understood (van de Poll et al., 2003).

Leucine was detected in significantly higher levels in the perfusate of DGF kidneys at 45 minutes. It is known that larger proteins are released into the perfusate during HMP as a sign of cellular damage (Hoogland et al., 2013). Raised concentrations of amino acids could indicate increased cellular breakdown in the more ischaemically damaged DGF kidneys.

Other amino acids identified in the perfusate included the other two branched chain amino acids valine and isoleucine along with alanine, glycine, glutamate and tyrosine. The concentrations of all of these increased significantly over time in the

perfusate of all kidneys but were not significantly different between the DGF and IGF groups.

3.5.2.2 INOSINE

Inosine is formed from the breakdown of adenosine nucleotides such as adenosine monophosphate, adenosine diphosphate and adenosine triphosphate.

Concentrations of inosine were significantly lower in DGF kidney perfusate at 45 minutes compared to IGF perfusate. The lower levels of inosine detected in the more ischaemically damaged DGF kidneys would suggest that this is not being released as a product of cellular degradation but is a product of cellular metabolism. Although the pathway involved is not clear, it is unlikely that the detected inosine was formed from the adenine in the preservation fluid, as adenine levels did not decrease over time to correspond with this. The AUROCs for leucine and inosine at 45 minutes were good at 0.833 and 0.732.

3.5.2.3 OTHER METABOLITES

Other metabolites of interest identified in HMP perfusate include the ketone body 3-hydroxybutyrate, increasing concentrations of which may indicate ongoing fatty acid

metabolism which is the main source of energy in the renal cortex during hypothermia (Ayala-García et al., 2012).

Levels of lactate increased in the perfusate of HMP over time, as might be expected, due to the production of lactate by glycolysis in anaerobic conditions. Also detected to be increasing over time were citrate and glutamate which are both intermediates of the citric acid cycle.

Glycine concentrations increased over time and, although just failing to reach statistical significance, glycine levels were higher in the perfusate of IGF kidneys compared to that from DGF kidneys. Glycine has been shown to prevent renal tubular cell injury in a rat model by reducing mild IR injury (Yin et al., 2002).

3.5.3 PREDICTION OF DGF

The metabolites identified as significantly different between the HMP perfusate of DGF and IGF kidneys show promise as predictors for DGF. The ROC curve analysis demonstrates AUROCs of between 0.732 and 0.895 which is considered to be ‘fair’ to ‘good’.

3.5.4 LIMITATIONS

To further elucidate the processes occurring during HMP, examination of additional timepoints would be useful. Two early timepoints were chosen for this study to examine the potential for indicators of graft function that would be applicable in a clinically useful timeframe and to ensure that samples at comparable times were available for all kidneys. Furthermore, HMP parameters changed most markedly within the first hour of perfusion which might have been reflected in the metabolic profile of the perfusate. A combined analysis of HMP parameters and metabolomic data might be of interest but has not been performed in this study.

At present, HMP is not used as a standard preservation technique at organ recovery in the UK. In this study, kidneys were placed on HMP at our unit and remained at the centre for transplantation. If HMP were utilised from recovery, sample collection for studies such as this would be more complicated. However, some evidence suggests that using HMP from recovery might be more beneficial than using a combination of SCS and HMP (Moers et al., 2009; Watson et al., 2010).

NMR has revealed many metabolites that may help to elucidate the underlying metabolic processes occurring during HMP. However, not surprisingly, there are limitations. Not every signal produced on the spectra can, as yet, be identified and complex molecules can produce spectral patterns that overlap each other. This can sometimes make identification and quantification difficult. Furthermore, it is unclear how accurately levels of perfusate metabolites reflect intracellular activity. This study

has used the technique to screen perfusate and has identified specific metabolites (glucose, inosine, leucine, gluconate) that might be predictive of graft function. These would require further investigation and validation, as discussed earlier. Studies have now commenced to determine whether routine biochemical assays of these metabolites would be helpful in providing real time data to support clinical practice.

This type of analysis may not yet be transferrable into general clinical practice. A recent study suggested that analysis of perfusate using NMR could be performed in less than 2 hours and within accepted timeframes for clinical application (Bon et al., 2014). This relied on rapid processing of samples, a limited selection of metabolites and access to a local spectrometer. Many centres will not have an on-site NMR facility with 24-hour access and the necessary expertise to process and analyse the samples. This could change in the future but at present, at our facility, would be impractical.

3.5.5 CONCLUSION

In this study, it has been possible to identify differences in the metabolomic profiles of perfusate from kidneys with immediate and delayed graft function. These differing metabolites may prove to have a useful predictive role in viability assessment. With a better understanding of the underlying metabolic processes occurring in damaged kidneys, it may be possible to modify harmful metabolic processes, support cell function and possibly extend storage periods prior to transplantation.

CHAPTER FOUR: DEVELOPMENT & ASSESSMENT OF A PORCINE MODEL

4.1 BACKGROUND

In order to further investigate the mechanisms of HMP and to test future hypothesis, an experimental model needed to be developed. Animal models continue to be used in medical research to characterize human diseases and to design and test therapeutic interventions (Giraud et al., 2011).

Rodent, canine and porcine organs have all been used in such experiments in transplantation. The first renal transplantation was performed by Emerich Ullmann in a dog in 1902 and the first pig kidney transplant was reported in 1965 (Mery et al., 1965). The porcine kidney structure, being multilobular, is more similar to the structure of human kidneys than rodent or dog kidneys, which have a unilobular structure. The similarity in size, physiology, and in organ development and disease progression make the pig an ideal model for human disease research (Bon et al., 2014; Giraud et al., 2011; Sachs 1994).

Porcine organs have been used not only in perfusion experiments on the kidney but also in the investigation of HMP on liver (Monbaliu et al., 2007) and pancreas (Taylor et al., 2008). They have been used extensively to investigate several areas relevant to clinical transplantation: to compare SCS and HMP preservation (Hosgood et al., 2010; La Manna et al., 2009), to investigate normothermic preservation techniques (Hosgood & Nicholson, 2011) and to test novel preservation solutions (Kay et al., 2011). More recently, perfusate from a porcine HMP auto-transplant model has been examined with NMR (Bon et al., 2014).

Under normal physiological conditions, the metabolomic profile of porcine blood, kidney tissue, urine and serum has been shown to be comparable to humans (Merrifield et al., 2011; Nielsen et al., 2014). These experiments, utilising NMR, have demonstrated that metabolites observed in pigs are qualitatively comparable to that of humans. This has not been validated in the ex-vivo hypothermic environment as encountered during HMP.

4.2 AIMS

For this section of the study, a porcine kidney model needed to be developed which was representative of the retrieval and storage process in humans. If this could be achieved, numerous repeated experiments would be possible and modifications could be attempted which would not be appropriate with human kidneys.

Using a paired kidney model, the metabolomic profile of perfusate from porcine kidneys stored by SCS was compared to the perfusate of those stored by HMP. Biopsies for histology were also taken to assess for changes present at the cellular level for each storage method.

Then, the parameters and metabolic profile of porcine kidneys during HMP were compared to that of the previously examined HMP human kidneys in an attempt to assess the validity of the porcine model.

4.3 METHODS

4.3.1 SOURCING OF PORCINE KIDNEYS

Facilities for animal retrieval and transplantation are not available at the University of Birmingham. Therefore, pig kidneys were sourced from a local abattoir. Initial and on-going discussion with the abattoir staff ensured that the retrieval process represented the human retrieval process as closely as possible.

4.3.2 PORCINE KIDNEY RECOVERY

Large white pigs, at 22-26 weeks of age, [redacted] Kidneys were recovered within 20 minutes of in-situ warm ischaemia. A pair of kidneys from each pig was retrieved per experiment.

4.3.3 PORCINE KIDNEY FLUSH

Kidneys were taken to a sterile area for preparation. Both kidneys were flushed simultaneously and as soon as possible following recovery. Each organ was flushed with 1 litre of Soltran® kidney perfusion fluid at 4ºC via a giving set and cannula at a pressure of 150mmHg.

Each kidney was then placed in a bag with 500 ml of KPS-1® and then transferred to an ice-box for transport to the laboratory. Once transported, one kidney was placed in the fridge in an ice-box (SCS) and the other transferred to HMP. The choice of kidney designated to each storage method was performed at random.

4.3.4 HMP TECHNIQUE

The kidney allocated to HMP was transferred to a LifePort© Kidney Transporter 1.0 (Organ Recovery Systems) at the laboratory. This machine was acquired for research use only, but was the same as the machine used clinically. The machine was prepared as stated in the clinical section of the study with the perfusion pressure set at 30mmHg. Kidneys were perfused with a fresh 1L of KPS-1® at 4°C. HMP parameters were monitored throughout perfusion time.

4.3.5 SAMPLE COLLECTION

Samples were collected at the following time-points from the beginning of HMP:

- start of HMP, 15 minutes, 30 minutes, 45 minutes, 60 minutes
- 4 hours, 8 hours, 18 hours, 24 hours, 28 hours

At each time-point, 10 mL of perfusate was collected from the SCS fluid and from the HMP perfusate. For SCS sampling, a 3-way tap was connected to the renal artery and 60 mL of the SCS fluid was flushed through the kidney vasculature prior to sampling the perfusate. This was to try to ensure that the sample was representative of the fluid retained within the kidney.

2 mL of the perfusate was transferred into a cryogenic vial and stored at -20°C until thawed at room temperature, prepared and processed for NMR analysis.

The remaining 8 mL was centrifuged at 4°C for 5 minutes at 1700 RPM. The supernatant was separated and stored at -80°C. The remaining pellet was mixed with 1 mL of freezing media (5ml DMSO/45ml FCS) and also stored at -80°C. These were stored as reserve samples for repeat or further analysis.

In addition to this, trucut biopsy samples were taken with a 16-gauge needle at 0 minutes, 18 hours and 28 hours. These were stored in formalin and processed at the UHB clinical laboratory for examination by light microscopy (LM).

4.3.6 MODIFICATIONS

Following the use of this model with the first five pairs of pig kidneys, a number of modifications were made to the design. Several attempts and modifications were required to improve the protocol:

Attempts were made to increase the sterility of the procedure. Whilst the processing at the abattoir was of the standard required by the British Meat Processors Association, it was not theatre standard. Theatre drapes were used at the abattoir workstation to minimise possible contamination and the pressure bag handles were covered with sterile gloves.

To try to minimise contamination from the kidney itself, kidneys were dipped in iodine and washed with Soltran®. Iodine was chosen as it has an easily identifiable spike on the NMR spectrum so would not cause interference during metabolomic analysis.

The HMP kidney from the second pair of pig kidneys did not perfuse well on the machine. It appeared that a distal division had been perfused following recovery rather than the main artery. This resulted in inadequate clearance of the vasculature in about 40% of the inferior part of the kidney. Following this, kidneys were rejected at the abattoir if they didn't perfuse globally.

Examination of the first few sets of histology samples showed that light microscopy was insufficient to detect any changes between SCS and HMP kidney tissue. Following this fewer samples were taken, at 4 and 28 hours only, but they were stored in 4% formaldehyde/10% normal saline to allow more detailed examination with electron microscopy (EM).

Additional timepoints were added for collection of samples for NMR analysis. Using porcine kidneys allowed increased experimental time as the kidneys were being

discarded following use in the model. Additional samples were collected at 48, 72 and 120 hours when possible.

In the initial protocol, the SCS kidney remained in the same 500mL of preservation fluid from recovery through storage time. The HMP kidney was stored with a fresh 1 litre of fluid when transferred to the machine from storage and transport. This made calculations of concentrations more difficult than necessary and changing the fluid following transport could have altered the detected concentrations. For further experiments, each kidney was placed in 500ml from the same bag of KPS-1® at recovery. When back at the laboratory, a further 1L bag of KPS-1® was divided between the kidney pair for continued storage.

4.3.7 PREPARATION FOR NMR

4.3.7.1 SAMPLE PROCESSING

Sample processing was performed by the same method as described for the human perfusate samples in section 3.3.5.

4.3.7.2 SPECTRAL ACQUISITION

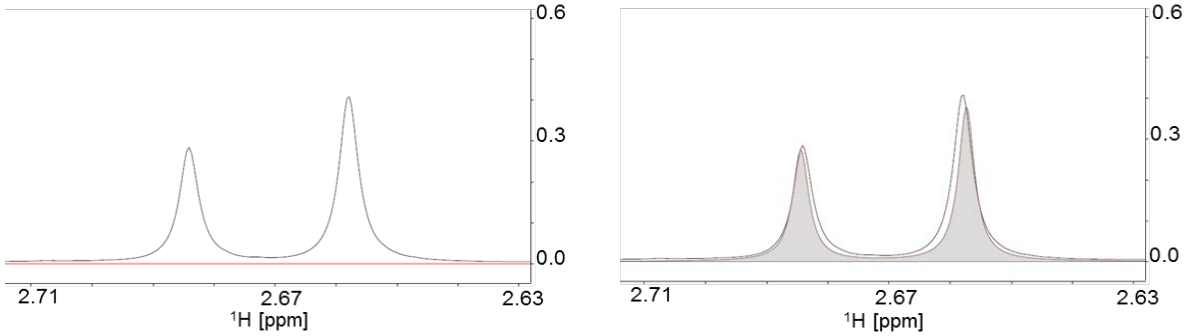
Spectral acquisition was performed by the same method as described for the human perfusate samples in section 3.3.6.

4.3.7.3 SPECTRAL ANALYSIS

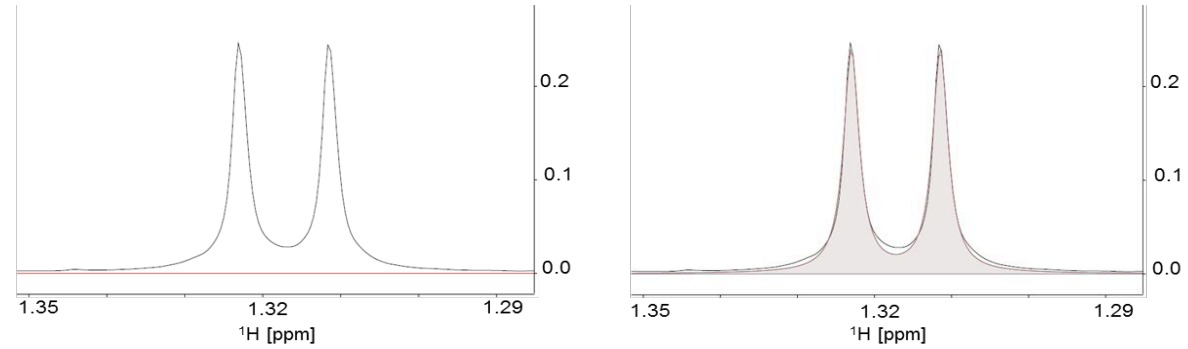
Resultant spectra were examined at three time-points for the first five pairs of porcine perfusate samples - 45 minutes, 4 hours and 28 hours. For the following five pairs, additional timepoints were examined – 48, 72 and 120 hours. Spectra were examined as previously described in section 3.3.8 using Chenomx 7.0 (Professional edition, version 7, Chenomx, Edmonton, AB, Canada). Further examples of spectral assignments are shown in figure 4.1.

Figure 4.1: Examples of Porcine Spectral Assignments

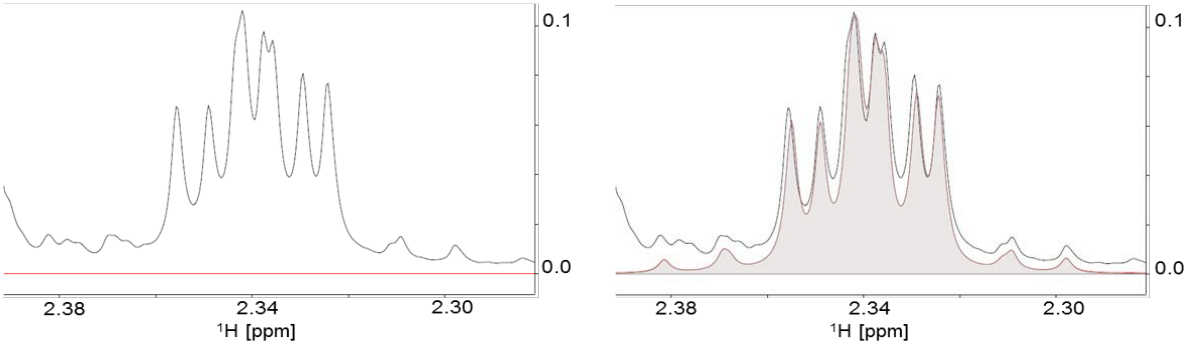
Citrate



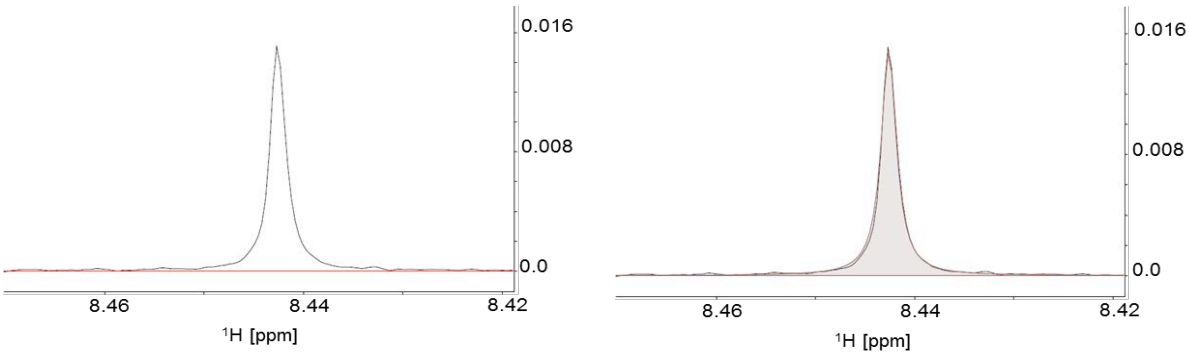
Lactate



Glutamate



Formate



4.3.7.4 STATISTICAL ANALYSIS OF PORCINE DATA

Data were analysed using GraphPad Prism version 6.0c for Mac (GraphPad Software, La Jolla California USA, www.graphpad.com) and IBM SPSS 19 (IBM Corp. Armonk, NY). A p-value <0.05 was deemed to be indicative of statistical significance throughout.

Prior to analysis, the distribution of metabolites was examined. Where non-normality was detected, Log_{10}-transformations were applied. Since there were some zero measurements, a constant was added to each of the measurements before the transformation. This was a multiple of ten one order of magnitude lower than the mean value of the metabolite being considered. For example, if the mean was 1, then 0.1 would be added to all of the values before the Log_{10}-transformation.

Repeated measures ANOVA models were then used to compare metabolite concentrations, between HMP and SCS, and across the three timepoints. In addition to the main effect terms in the models, interaction terms were also included, in order to compare the rate of change over time in the metabolite concentrations between HMP and SCS kidneys. Where the assumption of sphericity was violated i.e. when the variances of the differences between all combinations of related groups were not equal, the Greenhouse-Geisser corrected p-values were used. The Greenhouse-Geisser calculation corrects the degree of freedom of the *F*-distribution for a more accurate significance value.

Data were reported as arithmetic means and 95% confidence intervals for the normally-distributed data. Where Log-transformations were used, the resulting summary statistics were back transformed, and reported as geometric means and 95% confidence intervals.

4.3.8 HISTOLOGICAL PREPARATION FOR LIGHT MICROSCOPY

Samples were prepared for examination with light microscopy using two different stains: haematoxylin and eosin and Periodic acid Schiff. These were prepared using the established, standard techniques.

4.3.9 HISTOLOGICAL PREPARATION FOR ELECTRON MICROSCOPY (EM)

The protocols used to prepare the porcine kidney samples for electron microscopy in the UHB laboratory are described briefly here.

The main ultramicrotome in the unit is the Leica UC7. This is used for cutting semi-thin and ultra-thin sections of resin embedded tissue. Tissue has to be sectioned very thinly in order to obtain optimal resolution of ultrastructural detail. All semi-thin survey sections (0.4-0.7µm thick), for examination at the light microscope (LM) level, are cut using a glass knife. Ultra-thin (70-90nm thick) sections for examination at the

EM level can be cut using glass knives, but experienced ultramicrotomists usually use a diamond knife.

Semi-thin survey sections: toluidine blue staining

Semi-thin resin sections are used for two purposes. Because the EM uses very high magnifications, the area of tissue that can be examined in a reasonable length of time is small. The area available for individual ultra-thin sections is also strictly limited. To reduce as much as possible the sampling problem, several larger pieces of tissue are embedded in resin and semi-thin sections are cut from a number of these and stained with toluidine blue for examination by LM. Areas of particular interest are selected for ultra-thin sectioning based on the clinical diagnosis and any features of interest.

Formvar coated grids

Ultra-thin sections are mounted on slot grids for examination by EM. These are coated with a thin film of Formvar which spans the slot and acts as a support for sections. It is then possible to examine the entire section.

Ultra-thin sections

Unstained ultra-thin sections have no contrast. Uranyl acetate followed by lead citrate (an alkaline solution of lead citrate) is the routine staining sequence used for these sections. The heavy atoms of uranium and lead become attached to subcellular

structures, increasing their electron density and thus enhancing the contrast of the electron image. Uranyl acetate is used dissolved in methanol.

Staining ultra-thin sections with uranyl acetate

1 Clean the grid staining area with alcohol.

2 Put the grid holder in the glass dish and the dish in the square plastic box.

3 Carefully place grids to be stained in the grid holder, usually three grids per block.

4 Check name labels in each Petri dish of grids and make a record of which grid is in which position on form.

5 Make sure the grids do not protrude above the level of the holder.

6 Put the staining dish on the tray.

7 Pour the methanol from the "Used methanol" into the glass dish around the grid holder and cover with the square plastic lid. This provides a methanolic atmosphere and helps reduce the evaporation of methanol from the stain solution which could lead to stain deposit on the sections.

8 Put the bottle of uranyl acetate stain on the tray. Withdraw approximately half a pipette full of stain and transfer quickly to cover the grids; use all the stain in the pipette, do not return excess stain to the bottle. Have the lid off the box for as little time as possible.

9 Wash out the glass pipette with water directly down the plughole. Set timer for 18 - 20 minutes for muscle, nerve and renal tissue.

10 Set timer for 6 minutes for renal tissue reclaimed from wax. Formalin-fixed tissue may require reduced staining times.

11 Check periodically that the stain has not evaporated, top up with fresh uranyl acetate as necessary.

12 Replace uranyl acetate stain in the fridge making sure the lid is screwed on tightly.

13 Put the three glass rinsing vials on the metal tray and fill with AnalaR methanol.

14 Rinse each grid by dipping into the three vials in turn, approx. 30 dips per vial.

15 Blot off excess from between tines with filter paper.

16 Leave grid in fine forceps to dry; place on bench and cover with Petri dish lid.

17 Leave all grids to air dry for at least half an hour with card indicating Uranyl only.

18 Pour methanol from third rinse into the 'used methanol' vial for next time.

19 Filter paper contamination should be negligible, dispose of into clinical waste.

Staining ultra-thin sections with Reynold's lead citrate

1 Clean the demarcated grid staining area of the bench with alcohol.

2 Place the wax sheet over the sodium hydroxide pellets in the Petri dish and put the dish on the tray.

3 Put the bottle of Reynolds lead citrate solution on the tray close to the dish.

4 Withdraw about 1mL of the staining solution with a glass pipette and put out drops on the wax; grids from the same block can share a drop of stain. Do not return excess stain to the bottle.

5 Blot tip of pipette with paper.

6 Place the grids section side down on the drops of stain.

7 Set timer for 18 - 20 minutes for renal tissue. Formalin-fixed material, or samples reclaimed from wax may require reduced staining times.

8 At the end of the staining period, pick up each grid in turn and rinse thoroughly with deionised water, e.g. by dipping into a beaker of fresh water for about 10 seconds, and then placing them under a stream of about 50ml of water from the wash bottle.

9 Draw off excess water from between tines of forceps with filter paper.

10 Leave grid in fine forceps to dry; place on bench and cover with plastic lid. Leave all grids to air dry for at least half an hour.

Tissue fixation

Glutaraldehyde followed by osmium tetroxide as the secondary fixative is the generally recommended fixation sequence for routine electron microscopy.

Glutaraldehyde is a very good fixative for subcellular structures but it has a slow rate of penetration. Formaldehyde is included in the primary fixative as it penetrates more quickly. Tissue is fixed in a mixture of glutaraldehyde and formaldehyde, "PGP", which is 4% paraformaldehyde, 2% glutaraldehyde in 0.1M sodium phosphate buffer,

pH 7.2-7.4. Samples trimmed for EM in the Histopathology lab will go into phosphate buffered glutaraldehyde (2.5%)

The osmium tetroxide helps to stabilise lipid components of the tissue, decreasing their extraction during subsequent processing with organic solvents. Osmium tetroxide also acts as an electron stain because osmium is a heavy atom.

Tissue Processing

For the examination of tissues by EM, it is necessary to cut very thin sections. In order to do this without the specimen being compressed beyond usefulness, pieces of tissue are embedded in resin after the subcellular structure has been preserved by a routine fixation sequence of glutaraldehyde followed by osmium tetroxide. The water in the tissue is replaced by increasing concentrations of alcohol and the alcohol is in turn replaced by propylene oxide. This is miscible with Araldite, a thermosetting resin in which the tissue is blocked out.

4.3.10 COMPARISON OF PORCINE & HUMAN HMP KIDNEYS

In order to enable a valid comparison of HMP parameters and metabolomics, 10 porcine kidneys were compared with 12 standard criteria cadaveric human kidneys, all of which were successfully transplanted with immediate graft function.

4.3.11 STATISTICAL ANALYSIS OF PORCINE & HUMAN KIDNEYS

All analysis was performed using GraphPad Prism 6.0c (GraphPad Software, La Jolla California USA) and IBM SPSS 19 (IBM Corp. Armonk, NY), with $p<0.05$ deemed to be indicative of statistical significance.

Prior to analysis, the distribution of metabolites was examined. Where data was non-parametric, Log_{10}-transformations were applied, after adding 1 to remove zero values. Repeated measures ANOVA models were then used to compare metabolite concentrations, both between pig and human samples, and between 45 minute and 4 hour timepoints. In addition to the main effect terms in the models, interactions were also included, in order to compare the rate of change over time in the metabolite concentrations between pig and human samples.

Data were reported as arithmetic means and 95% confidence intervals for the normally-distributed data. Where Log-transformations were used, the resulting summary statistics were back transformed, and reported as geometric means and 95% confidence intervals.

4.4 RESULTS

Ten pairs of porcine kidneys were examined in total - five pairs using the initial protocol and the following five pairs with the modified protocol. The HMP kidney from pig 2 was excluded from analysis due to inadequate flush at recovery.

4.4.1 ISCHAEMIC TIMES

All porcine kidneys underwent a combination of WIT and CIT as comparable to a model of DCD donation.

Median WIT during recovery was 18 minutes (IQ range 18-18 minutes). Average CIT during transport was 152.5 minutes (IQ range 146.25 - 157.5 minutes).

The first five pairs of pig kidneys then underwent a further 28 hours of HMP or SCS depending on their designated storage method, with the following five pairs undergoing up to 120 hours of their designated storage method.

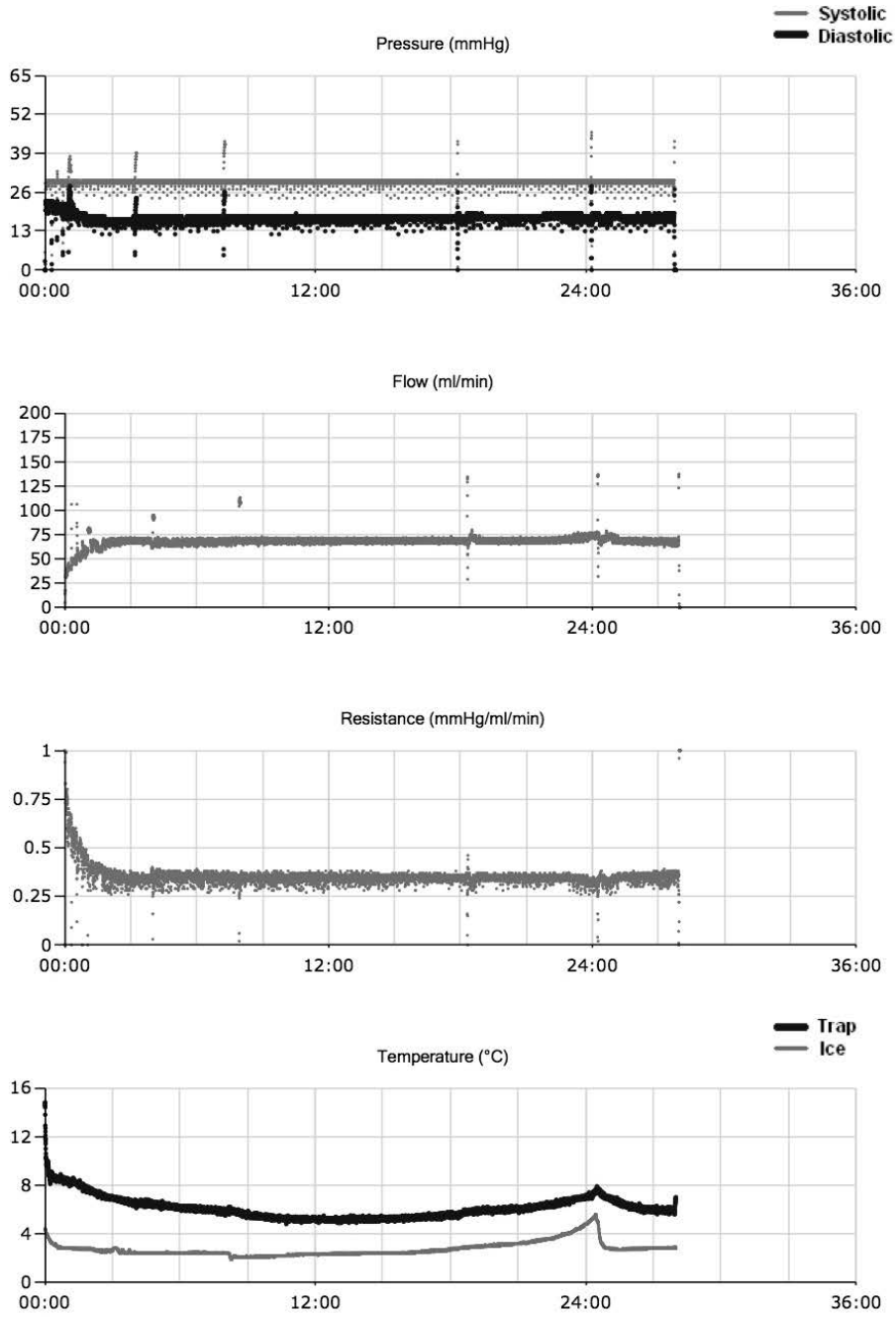
4.4.2 PORCINE HMP PARAMETERS

Median flow rate at the start of perfusion for all porcine kidneys was 34 mL/min (IQ range 29.0-41.0) increasing to a median of 52 mL/min (IQ range 40.0-71.5) at 28

hours of perfusion. Median resistance at the start of perfusion was 0.82 mmHg/ml/min (IQ range 0.67-1.02) decreasing to 0.46 mmHg/mL/min (0.34-0.66) by 28 hours of perfusion.

Figure 4.2 shows an example of a typical case report from a porcine HMP kidney:

Figure 4.2: LifePort Kidney Transporter Case Report Showing Machine Parameters for Porcine HMP Kidneys



4.4.3 PORCINE NMR ANALYSIS

A total of 30 metabolites were identified in the perfusate of porcine kidneys. The following tables and graphs represent the metabolite concentrations and changes over time for the SCS & HMP porcine kidneys. Only metabolites with statistically significant results are shown. The complete table and figures can be seen in the appendices.

Table 4.1: Metabolite Concentrations (mM) Measured in Kidney Perfusate of SCS & HMP Porcine Kidneys (Significant Metabolites Only)

This table shows the arithmetic or geometric mean (with confidence intervals) for metabolites at each timepoint and according to storage method. "Time" is testing whether there is a change in the metabolite levels over time (independent of storage type), "Storage" is comparing the metabolite concentrations for the two different types of kidney storage (independent of timepoint) and "Int." is the interaction term in the model. This tests whether the rate of change over time differs between HMP and SCS.

		Timepoint			p-Values		
	Storage	*45 Minutes*	*4 Hours*	*28 Hours*	*Time*	*Storage*	*Int.*
Ribose[#]	HMP	2.5 (1.9 - 3.0)	2.3 (1.7 - 2.9)	2.4 (2.0 - 2.7)	0.846	0.004*	0.696
	SCS	3.3 (2.8 - 3.8)	3.4 (3.0 - 3.9)	3.4 (3.1 - 3.8)			
Glutathione[#]	HMP	0.96 (0.80 - 1.11)	0.39 (0.24 - 0.54)	0.00 (0.00 - 0.00)	<0.001*	<0.001*	<0.001*
	SCS	1.59 (1.33 - 1.86)	1.64 (1.38 - 1.91)	1.55 (1.31 - 1.79)			
Lactate[#]	HMP	0.58 (0.16 - 0.99)	0.65 (0.47 - 0.83)	1.23 (0.92 - 1.55)	<0.001*	0.126	0.755
	SCS	0.43 (0.18 - 1.04)	0.19 (0.12 - 0.27)	0.83 (0.44 - 1.22)			
Citrate	HMP	2.3 (1.4 - 3.9)	2.6 (1.7 - 4.1)	3.1 (2.2 - 4.4)	<0.001*	0.737	0.236
	SCS	1.6 (0.8 - 3.4)	2.3 (1.1 - 4.7)	3.4 (1.7 - 6.8)			

Alanine	HMP	0.065 (0.049 - 0.085)	0.107 (0.075 - 0.152)	0.201 (0.152 - 0.266)	<0.001*	<0.001*	0.056
	SCS	0.025 (0.015 - 0.041)	0.031 (0.019 - 0.051)	0.103 (0.069 - 0.152)			
Glutamate	HMP	0.38 (0.24 - 0.61)	0.98 (0.74 - 1.30)	2.15 (1.89 - 2.45)	0.001*	<0.001*	0.733
	SCS	0.06 (0.03 - 0.15)	0.11 (0.05 - 0.23)	0.25 (0.11 - 0.58)			
Hypoxanthine	HMP	0.12 (0.07 - 0.18)	0.18 (0.12 - 0.28)	0.26 (0.22 - 0.32)	0.015*	0.003*	0.704
	SCS	0.04 (0.02 - 0.07)	0.04 (0.02 - 0.09)	0.08 (0.03 - 0.20)			
Fumarate	HMP	0.0033 (0.0016 - 0.0068)	0.0053 (0.0030 - 0.0094)	0.0161 (0.0124 - 0.0209)	<0.001*	0.022*	0.362
	SCS	0.0014 (0.0006 - 0.0036)	0.0011 (0.0004 - 0.0031)	0.0064 (0.0039 - 0.0106)			
Leucine	HMP	0.017 (0.012 - 0.023)	0.023 (0.016 - 0.034)	0.035 (0.027 - 0.044)	<0.001*	0.013*	0.247
	SCS	0.004 (0.002 - 0.008)	0.008 (0.004 - 0.017)	0.017 (0.008 - 0.035)			
Valine	HMP	0.016 (0.012 - 0.020)	0.024 (0.016 - 0.036)	0.035 (0.027 - 0.046)	<0.001*	0.008*	0.256
	SCS	0.005 (0.002 - 0.009)	0.008 (0.004 - 0.013)	0.017 (0.008 - 0.037)			
Glycine	HMP	0.58 (0.45 - 0.75)	1.47 (1.12 - 1.93)	2.63 (2.32 - 2.98)	<0.001*	<0.001*	0.504
	SCS	0.10 (0.04 - 0.23)	0.18 (0.13 - 0.24)	0.52 (0.37 - 0.74)			
Pyruvate	HMP	0.006 (0.003 - 0.012)	0.009 (0.005 - 0.019)	0.017 (0.007 - 0.043)	0.022*	0.169	0.414
	SCS	0.005 (0.002 - 0.013)	0.003 (0.001 - 0.006)	0.009 (0.004 - 0.021)			
Hippurate	HMP	0.0002 (0.0001 - 0.0007)	0.0007 (0.0001 - 0.0038)	0.0015 (0.0003 - 0.0067)	0.025*	0.532	0.620
	SCS	0.0002 (0.0001 - 0.0005)	0.0005 (0.0002 - 0.0018)	0.0005 (0.0001 - 0.0021)			
Isoleucine	HMP	0.009 (0.007 - 0.011)	0.014 (0.010 - 0.020)	0.021 (0.016 - 0.027)	0.004*	0.024*	0.348
	SCS	0.005 (0.002 - 0.011)	0.005 (0.003 - 0.009)	0.015 (0.009 - 0.023)			
Tyrosine	HMP	0.009 (0.007 - 0.012)	0.013 (0.009 - 0.020)	0.020 (0.016 - 0.024)	<0.001*	0.012*	0.211
	SCS	0.004 (0.002 - 0.006)	0.005 (0.003 - 0.008)	0.013 (0.007 - 0.024)			
3-Methylxanthine	HMP	0.009 (0.003 - 0.028)	0.008 (0.002 - 0.028)	0.017 (0.016 - 0.018)	0.190	0.007*	0.579
	SCS	0.002 (0.001 - 0.003)	0.002 (0.001 - 0.004)	0.002 (0.001 - 0.005)			

Data reported as geometric means and 95% confidence intervals, unless stated otherwise.

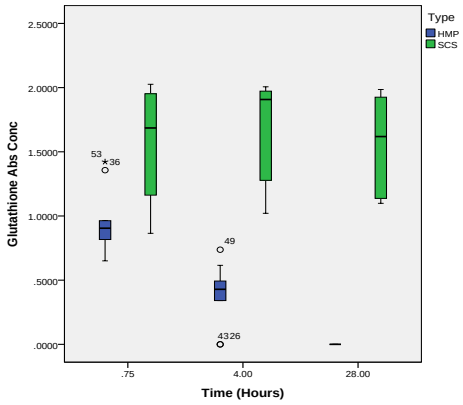
#Arithmetic means and 95% confidence intervals.

p-values from repeated measures ANOVA.

Figure 4.3: Box and Whisker Plot to Represent Concentrations of Metabolites in HMP and SCS Porcine Kidney Perfusate (significant metabolites only)

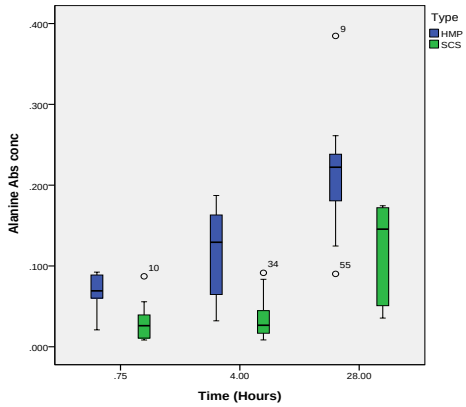
Metabolites with a significant change in concentration over time and a significant difference in concentration and rate of change between storage groups

Glutathione

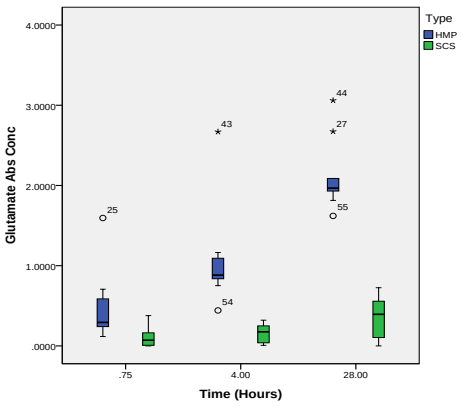


Metabolites with a significant change in concentration over time and a significant difference in concentration between storage groups (no significant difference in the rate of change between storage goups).

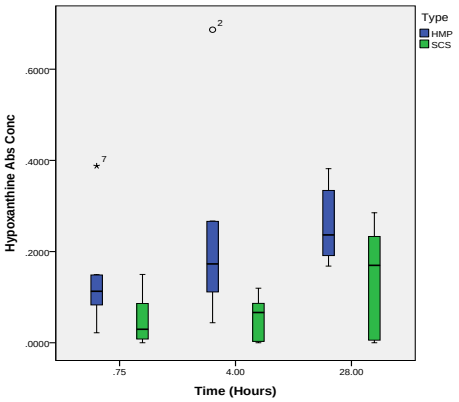
Alanine



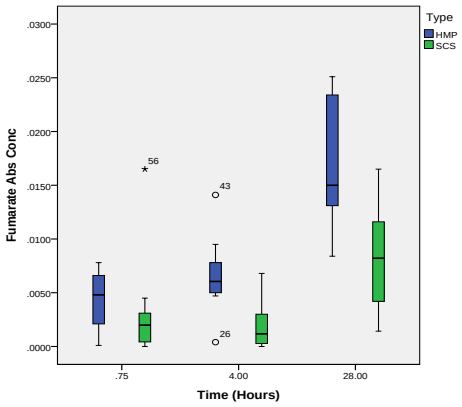
Glutamate



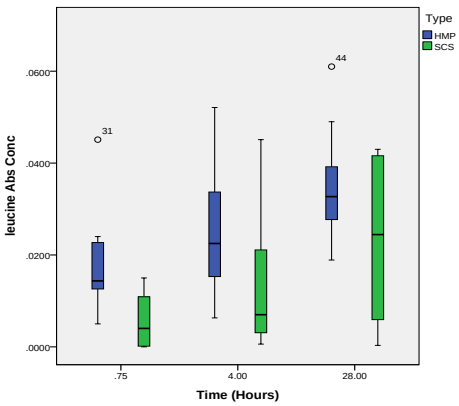
Hypoxanthine



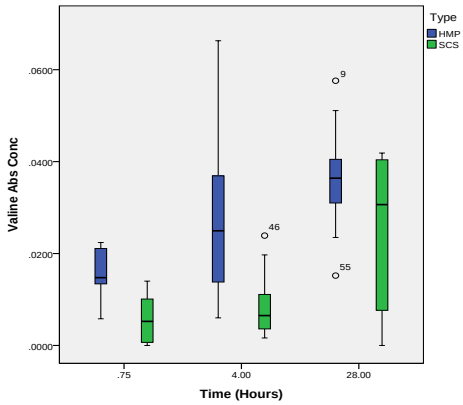
Fumarate



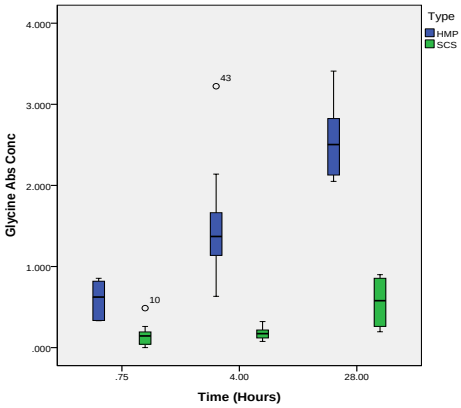
Leucine



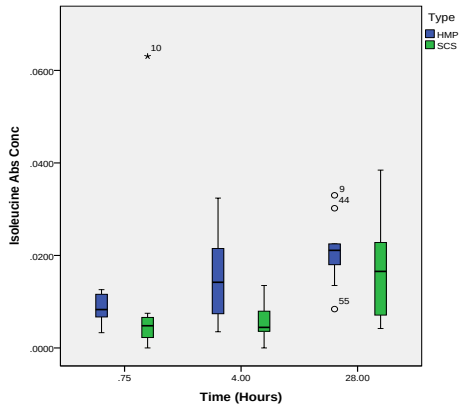
Valine



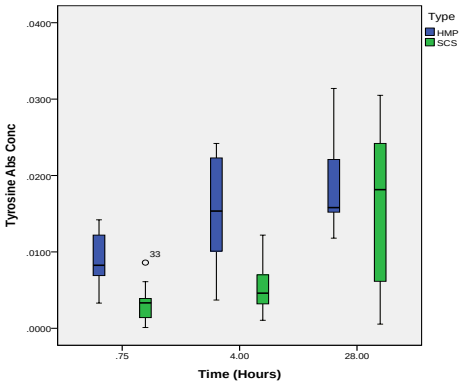
Glycine



Isoleucine

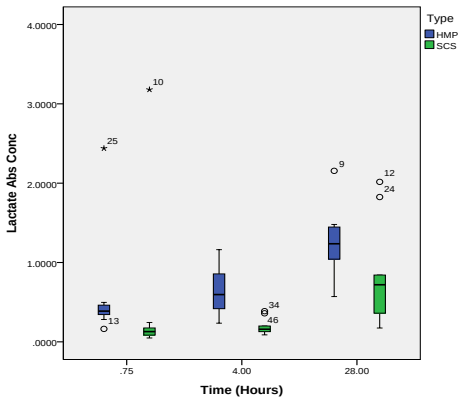


Tyrosine

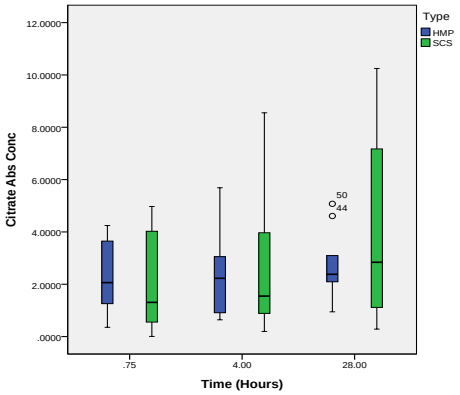


Metabolites with a significant change in concentration over time (no significant difference in concentration or rate of change between storage groups).

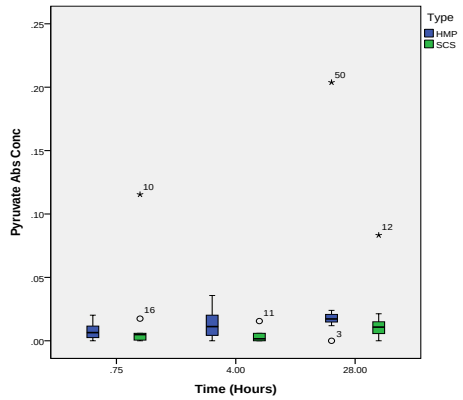
Lactate



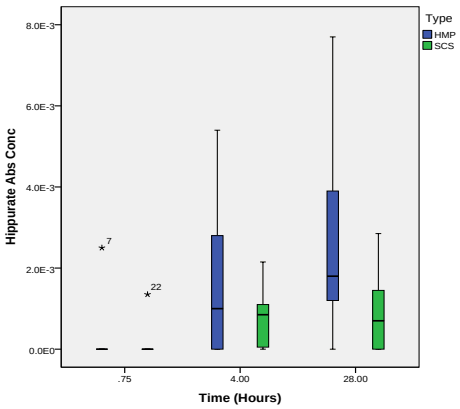
Citrate



Pyruvate

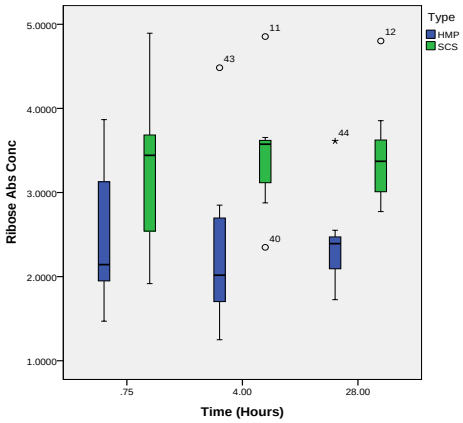


Hippurate

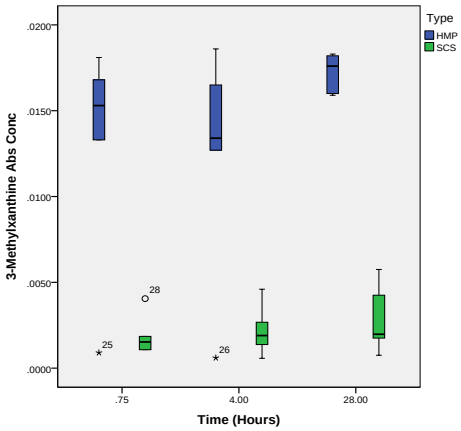


Metabolites with a significant difference in concentration between storage groups (no significant change in concentration over time or in the rate of change between storage groups).

Ribose



3-Methylxanthine



4.4.4 PORCINE HISTOLOGY

4.4.4.1 LIGHT MICROSCOPY

Light microscopy performed on samples from the first five pairs of pig kidneys was of inadequate power to demonstrate any cellular differences between SCS and HMP kidneys.

4.4.4.2 ELECTRON MICROSCOPY

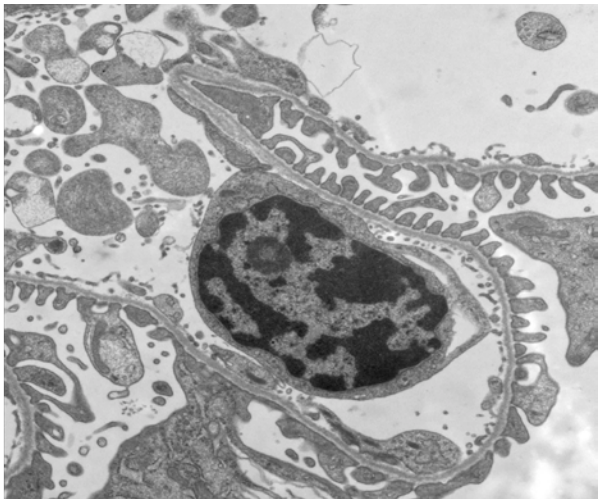
Biopsies were examined by electron microscopy at 4 and 28 hours of perfusion for two pairs of pig kidneys (pig 8 & 10). Elements examined were endothelial cells, epithelial cells, glomerular basement membrane and endothelium, arteriolar smooth muscle, tubules and peri-tubular capillaries.

4.4.4.2.1 ENDOTHELIAL CELLS

There was little difference between endothelial cells in either of the samples from SCS and HMP porcine kidneys. Nuclei were comparable and patterns of chromatin were similar (figure 4.4).

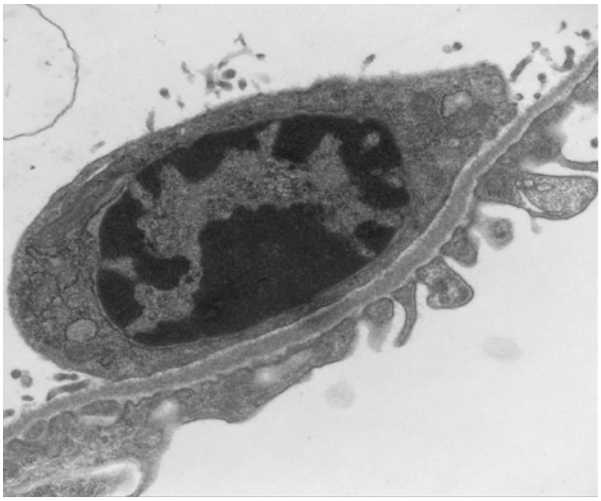
Figure 4.4: Electron Microscopy Images of Endothelial Cells in SCS & HMP Porcine Kidney at 4 & 28 Hours

SCS 4 Hours



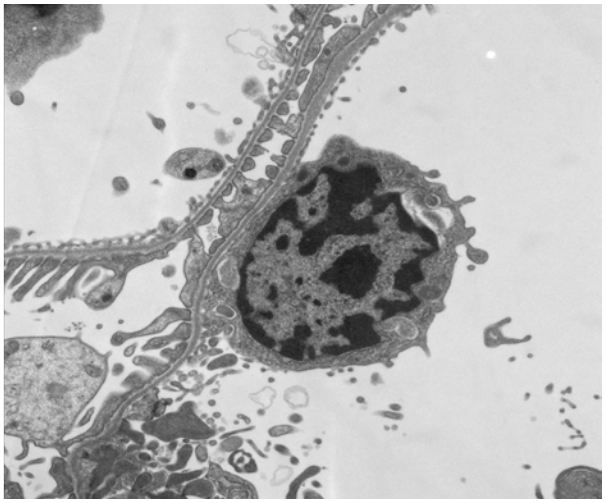
Magnification 9300x

SCS 28 Hours



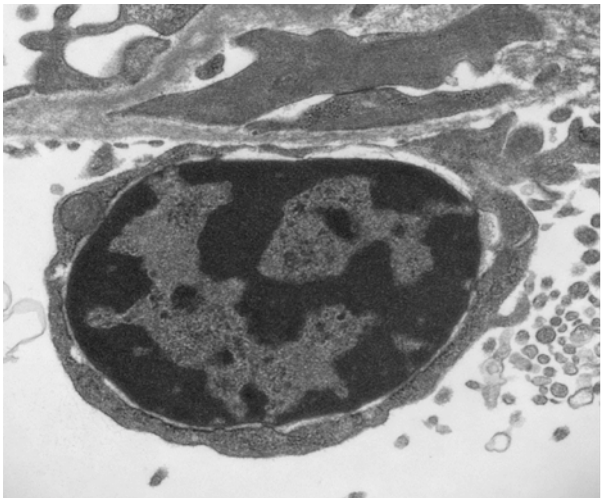
Magnification 23000x

HMP 4 Hours



Magnification 11000x

HMP 28 Hours



Magnification 30000x

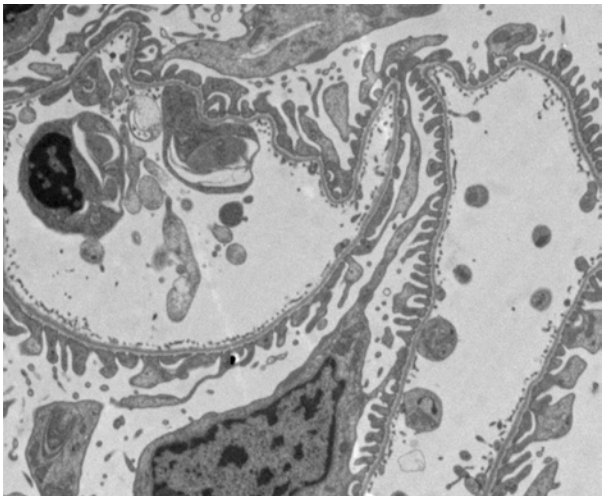
4.4.4.2.2 EPITHELIAL CELLS

In epithelial cells, there was little difference detected between biopsies from SCS and HMP kidneys at the 4 hour time-point with similar nuclei and normal foot processes seen. However, by 28 hours, the SCS cells showed increased evidence of cytoplasmic swelling with pale nuclei. This was evident in both pairs of porcine kidneys (figure 4.5).

Higher magnification pictures show these changes in more detail with the pale swollen cytoplasm evident in the SCS epithelial cells (figure 4.6).

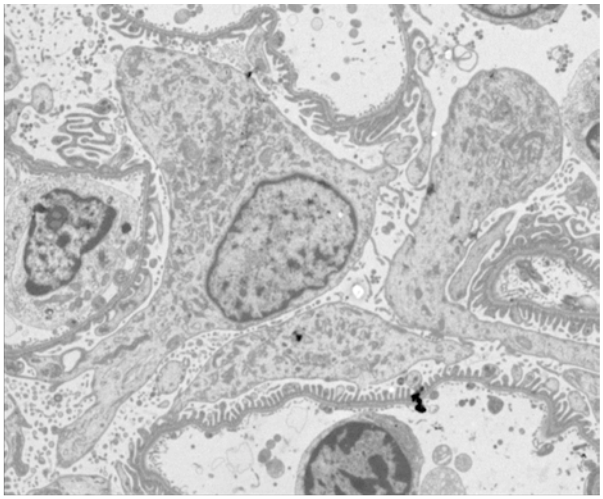
Figure 4.5: Electron Microscopy Images of Epithelial Cells in Porcine SCS & HMP Kidney at 4 & 28 Hours

SCS 4 Hours



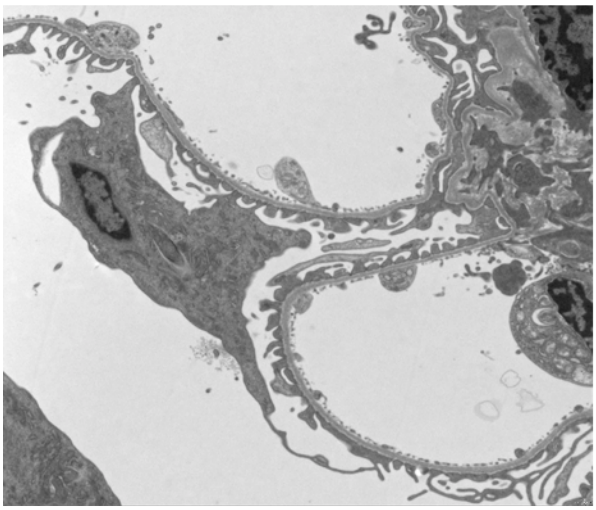
Magnification 1900x

SCS 28 Hours



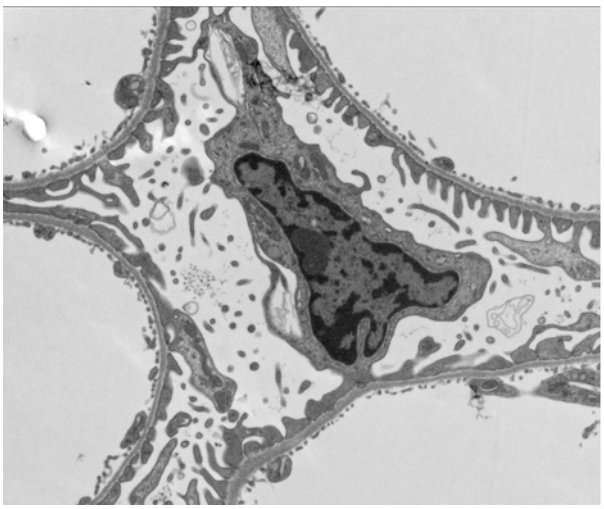
Magnification 1900x

HMP 4 Hours



Magnification 1900x

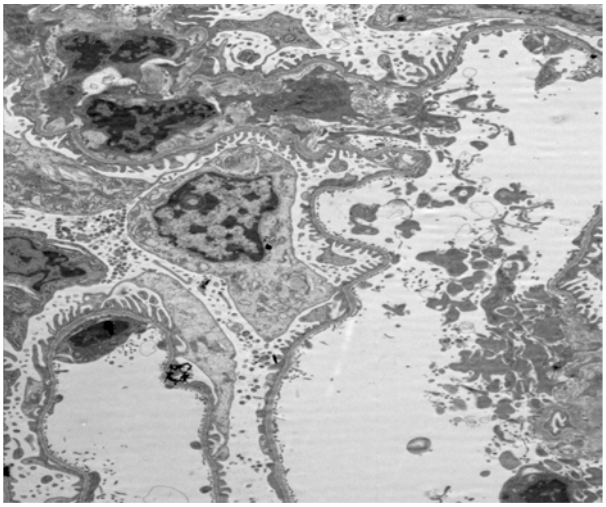
HMP 28 Hours



Magnification 2900x

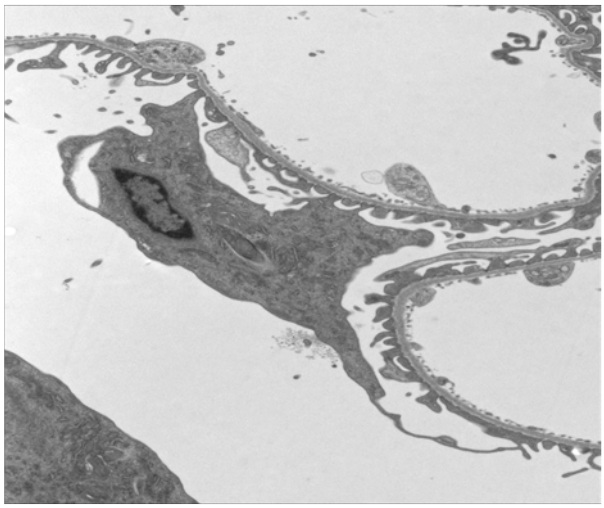
Figure 4.6: Higher Magnification Electron Microscopy Images of Epithelial Cells in SCS Porcine Kidney at 28 Hours & HMP Porcine Kidney at 4 Hours

SCS 28 Hours



Magnification 11000x

HMP 4 Hours



Magnification 9300x

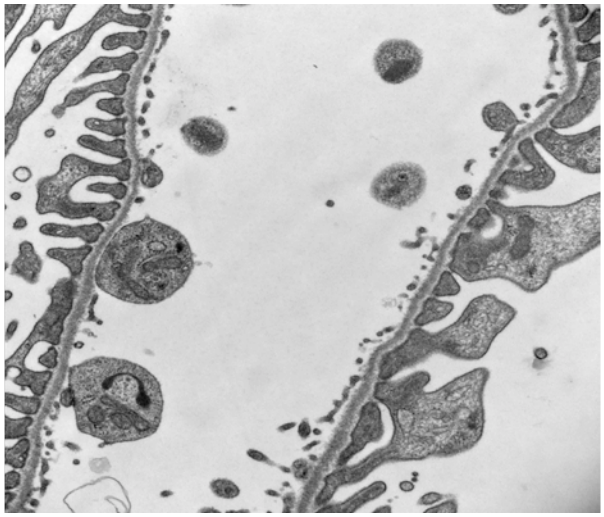
4.4.4.2.3 GLOMERULAR BASEMENT MEMBRANE AND ENDOTHELIUM

There were some differences in the glomerular basement membrane (GBM) of the HMP and SCS kidneys in pig 8 but these were not reproduced in pig 10.

The fenestrae in the GBM of the HMP kidneys in pig 8 looked flatter and closer together compared to the SCS fenestrae which appeared to be more separated (figure 4.7).

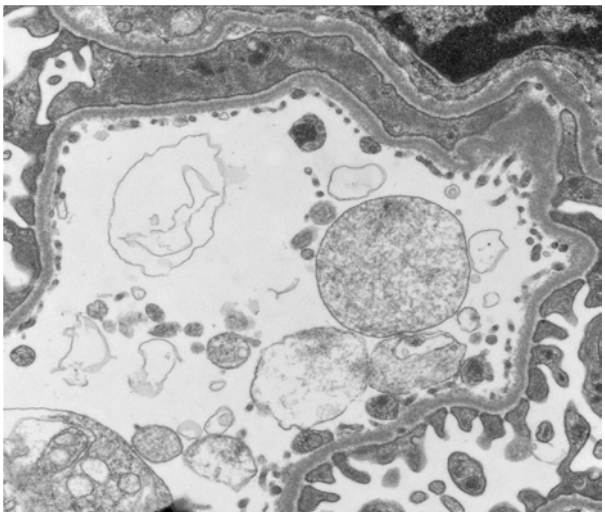
Figure 4.7: Electron Microscopy Images of the Glomerular Basement Membrane in Porcine SCS & HMP Kidney at 4 & 28 Hours (pig 8 only)

SCS 4 Hours



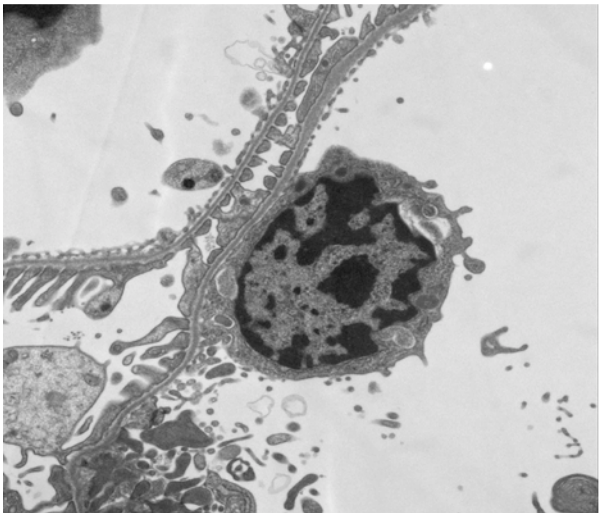
Magnification 18500x

SCS 28 Hours



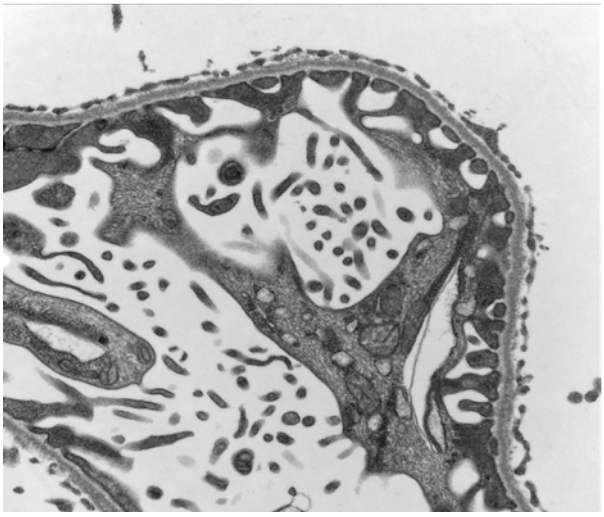
Magnification 18500x

HMP 4 Hours



Magnification 11000x

HMP 28 Hours



Magnification 18500x

4.4.4.2.4 ARTERIOLAR SMOOTH MUSCLE

In the HMP kidneys, there appeared to be damage to the smooth muscle layer in the arterioles. The endothelium was intact at 4 hours but by 28 hours there was increased vacuolation in the smooth muscle layer of the HMP arterioles which appeared to be detached from the endothelium. These changed were present in both HMP porcine kidneys and not in the paired SCS samples (figure 4.8).

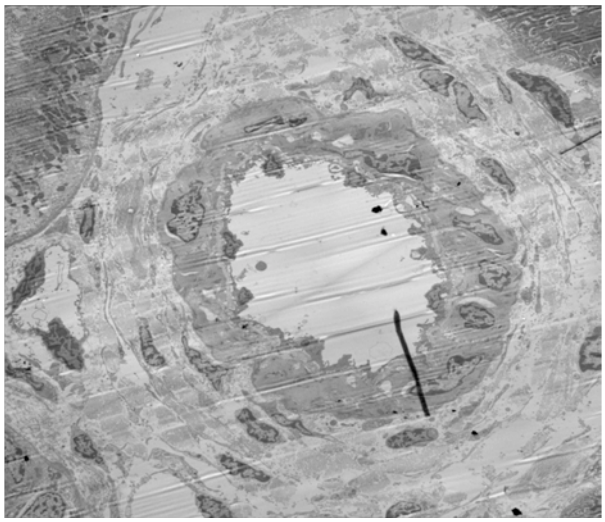
These changes are clearer at higher magnification (figure 4.9).

Figure 4.8: Electron Microscopy Images of Arteriolar Smooth Muscle in Porcine SCS & HMP Kidney at 4 & 28 Hours

SCS 4 Hours

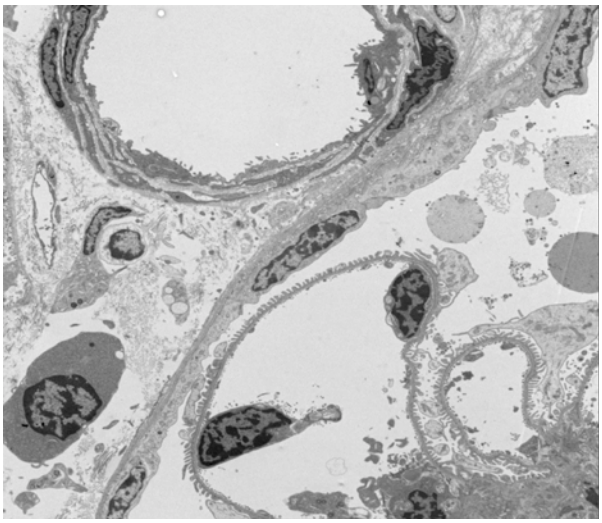
No arterioles detected

SCS 28 Hours



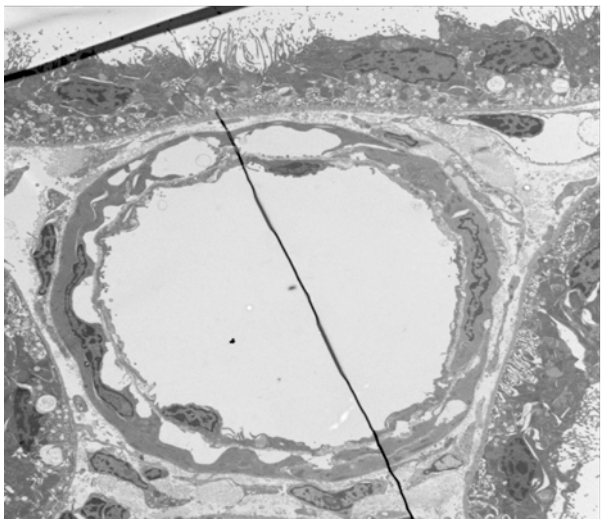
Magnification 1900x

HMP 4 Hours



Magnification 2900x

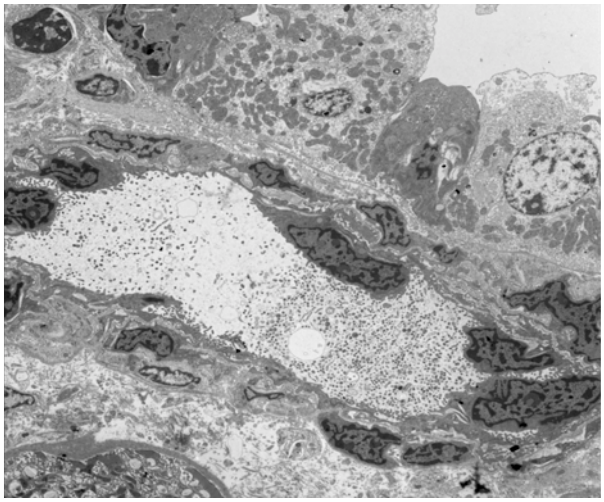
HMP 28 Hours



Magnification 2900x

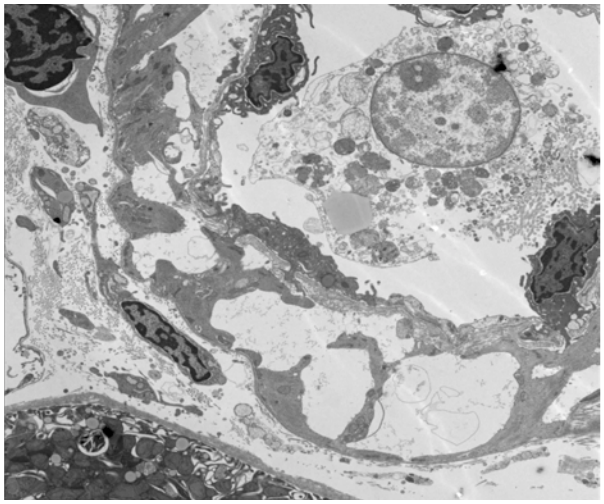
Figure 4.9: Higher Magnification Electron Microscopy Images of Arteriolar Smooth Muscle in SCS & HMP Porcine Kidney at 28 Hours

SCS 28 Hours



Magnification 2900x

HMP 28 Hours



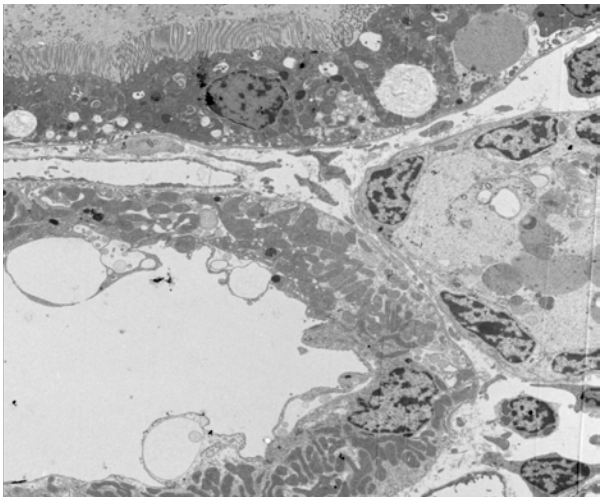
Magnification 4800x

4.4.4.2.5 TUBULES

Tubular changes were variable. In pig 8, there were no identifiable differences. In pig 10, the brush border was lost by 28 hours in the SCS kidney but intact in the HMP kidney (figure 4.10).

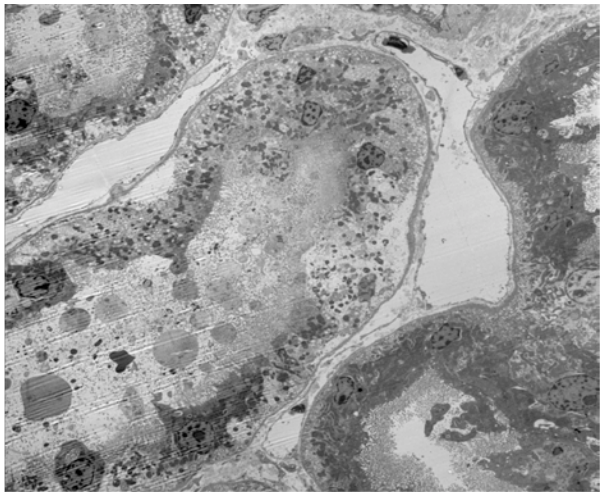
Figure 4.10: Electron Microscopy Images of Tubules in Porcine SCS & HMP Kidney at 4 & 28 Hours (pig 10 only)

SCS 4 Hours



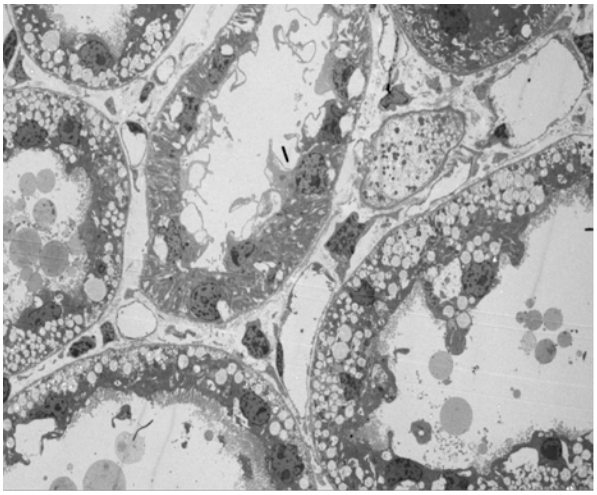
Magnification 2900x

SCS 28 Hours



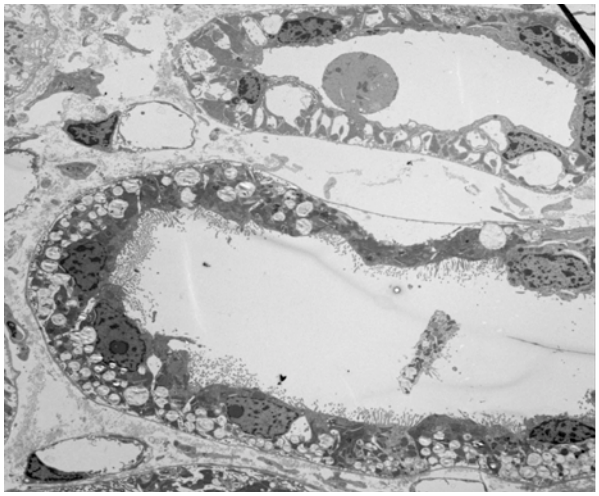
Magnification 1400x

HMP 4 Hours



Magnification 1400x

HMP 28 Hours



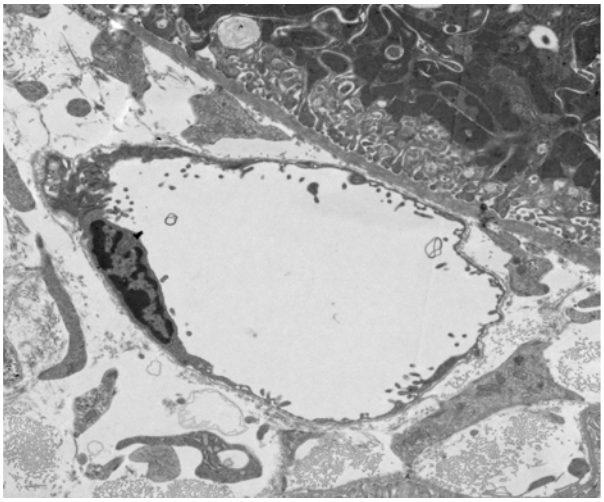
Magnification 1900X

4.4.4.2.6 PERITUBULAR CAPILLARIES

There were no visible difference in the peri-tubular capillaries of HMP and SCS porcine kidney samples (figure 4.11).

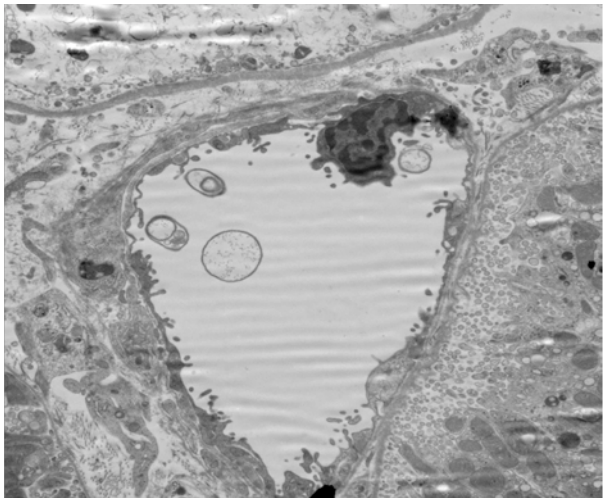
Figure 4.11: Electron Microscopy Images of Peri-Tubular Capillaries in Porcine SCS & HMP Kidney at 4 & 28 Hours

SCS 4 Hours



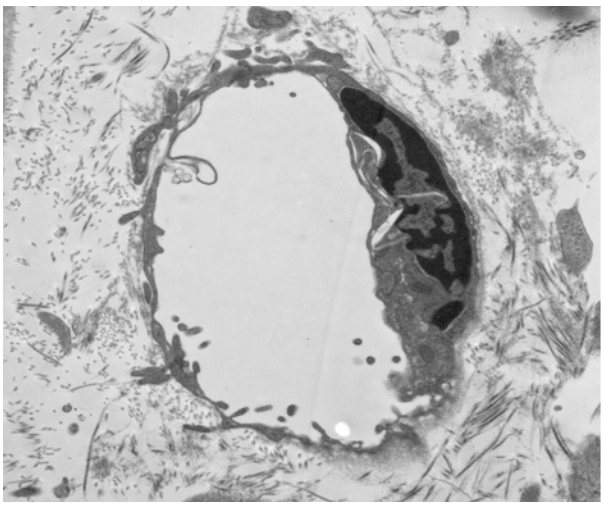
Magnification 6800x

SCS 28 Hours



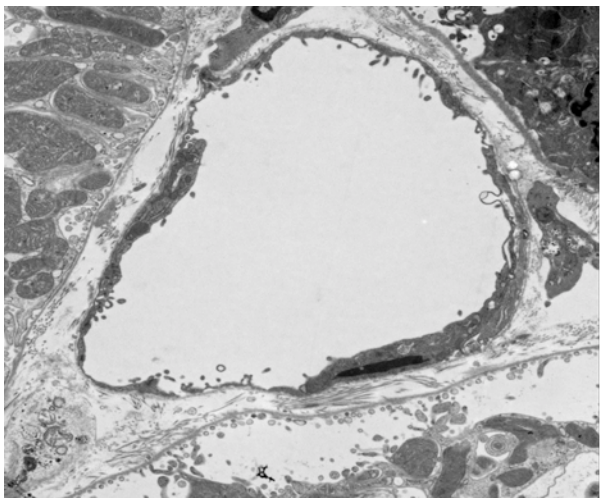
Magnification 6800x

HMP 4 Hours



Magnification 11000x

HMP 28 Hours



Magnification 6800x

4.4.5 COMPARISON OF PORCINE AND HUMAN HMP KIDNEYS

4.4.5.1 ISCHAEMIC TIMES

The median WIT for porcine kidneys during recovery was 18 minutes (IQ range 18-18 minutes). None of the human kidneys used for comparison were subjected to a warm ischaemic insult prior to storage as they were cooled in-situ during recovery (DBD donors).

Average CIT during transport was 152.5 minutes (IQ range 146.25 - 157.5 minutes) for the porcine group compared to 465 minutes (IQ range 360 - 679) for the human group (p=0.0005).

Porcine kidneys underwent a further 28 hours of HMP. HMP period was variable for the human group. Samples were compared at the 45 minutes and 4 hours timepoints.

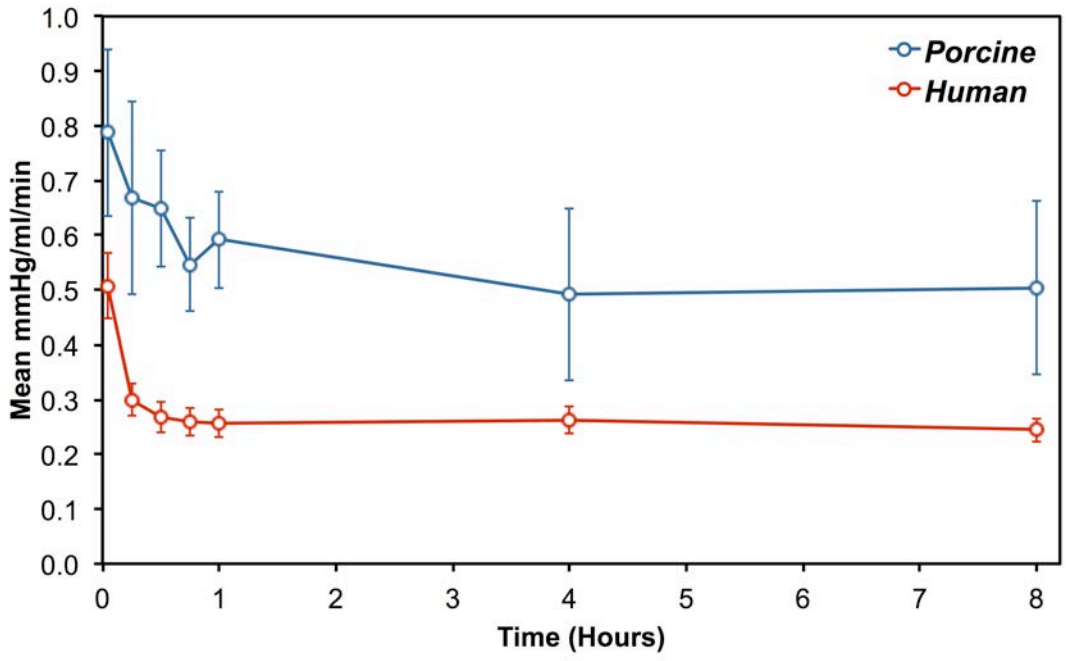
4.4.5.2 HMP PARAMETERS

Median flow rate at the start of perfusion for all porcine kidneys was 34 mL/min (IQ range 29.0-41.0) compared to 67mL/min (IQ range 53.0-89.0) for the human comparator group (p=0.0008).

At the end of perfusion time, median flow rate for the porcine kidneys was 52 mL/min (IQ range 34.0-82.5) compared to 116 mL/min (IQ range 98.0-129.0) in the sample of human kidneys (p=0.0002).

These changes are reflected in the corresponding resistance measurements. The graph below shows the change over time for resistance in the porcine kidneys compared to the human kidneys. A repeated measures ANOVA model (figure 4.12) found that the change in resistance over time was significant in both groups (p=0.000). There was also a significant difference between the resistance of porcine and human kidneys at all time-points (p=0.000). The interaction term in the model was non-significant (p=0.067), hence there is no evidence that the trend in resistance over time is related to whether the kidney is human or porcine.

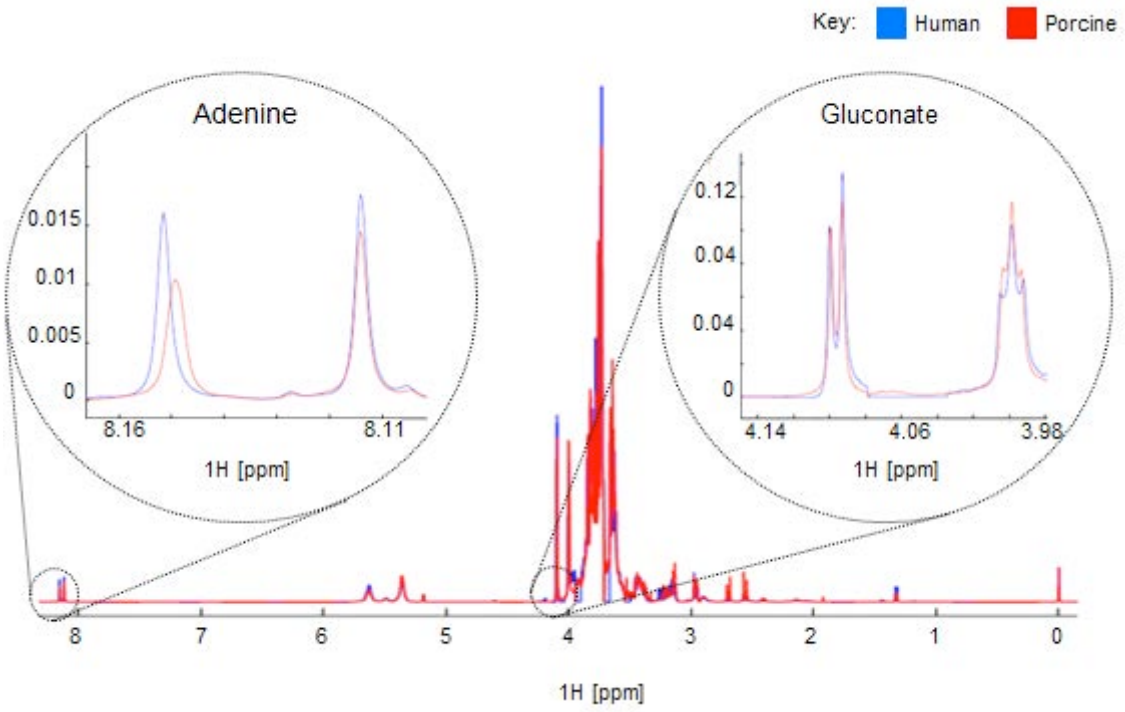
Figure 4.12: Repeated Measures ANOVA Model for Change in Resistance During HMP Time in Human & Porcine Kidneys



4.4.5.3 METABOLOMICS

A spectral overlay analysis was performed for the mean spectra for pig and human samples at 45 minutes and 4 hours. There were similar profiles for both pig and human groups (figure 4.13). There were no metabolites present in significant quantities that were not detected in the other group.

Figure 4.13: NMR Spectral Overlay Plot Demonstrating The Similarity Of HMP Perfused Pig (Red) And HMP Perfused Human (Blue) Kidneys After 4 Hours Of Perfusion



There were a total of 30 metabolites identified in the perfusate of both pig and human kidneys during hypothermic machine perfusion. Of these six (gluconate, mannitol, glucose, adenine, ribose and glutathione) were constituents of the original KPS-1® perfusion fluid. There was consumption of glutathione in both pig and human groups but no other significant interspecies or time effect for the other metabolites present in the preservation fluid.

For the 24 metabolites present de novo, there was an overall change over time for 12 (50%) with production of lactate, glycine, glutamate, hypoxanthine, alanine, 3-hydroxybutryate, inosine, N-phenylacetylglycine, leucine, valine, isoleucine and fumarate.

When concentrations were analysed according to species, there was no difference during HMP between pig and human kidneys for 16 metabolites (53.3%) following a repeated measures analysis.

The rate of change of concentration for 3-Hydroxybutyrate was greater in human kidneys compared to pig kidneys (0.017 to 0.040mM vs 0.012 to 0.013mM) ($p<0.001$) For the other 29 metabolites (96.7%), there was no difference in the rate of change of concentration between pig and human samples (table 4.2). Figure 4.14 demonstrates several of the metabolites, that although detected in different concentrations, the rate of change was the same.

Table 4.2: Concentrations of Metabolites at 45 Minute & 4 Hours Timepoints with Repeated Measures Analysis and Comparison of Concentrations Between Human & Porcine Perfusate

This table shows the arithmetic or geometric mean (with confidence intervals) for metabolites at each timepoint and according to species. "Time" is testing whether there is a change in the metabolite levels over time (independent of species), "Species" is comparing the metabolite concentrations for the two species (independent of timepoint) and "Int." is the interaction term in the model. This tests whether the rate of change over time differs between human and porcine perfusate.

		Timepoint		p-Values		
	Species	*45 Minutes*	*4 Hours*	*Time*	*Species*	*Int.*
Gluconate	Human	92.9 (85.3 - 100.4)	96.6 (84.1 - 109.2)	0.799	0.342	0.616
	Pig	89.6 (78.4 - 100.7)	88.3 (76.4 - 100.3)			
Mannitol	Human	48.8 (45.6 - 52.1)	52.3 (46.0 - 58.5)	0.543	0.368	0.667
	Pig	53.3 (45.8 - 60.7)	53.9 (45.0 - 62.7)			
Glucose	Human	9.8 (9.0 - 10.6)	10.7 (9.6 - 11.7)	0.158	0.088	0.709
	Pig	11.4 (9.3 - 13.5)	12.9 (9.7 - 16.1)			
Adenine	Human	7.0 (5.8 - 8.1)	7.1 (5.7 - 8.5)	0.924	0.681	0.816
	Pig	6.7 (5.7 - 7.7)	6.7 (5.4 - 7.9)			
Ribose	Human	3.0 (2.8 - 3.3)	3.0 (2.4 - 3.6)	0.548	0.147	0.718
	Pig	3.7 (2.9 - 4.5)	3.4 (2.6 - 4.3)			
Glutathione	Human	1.3 (1.2 - 1.4)	0.8 (0.6 - 0.9)	<0.001*	0.731	0.1
	Pig	1.4 (1.2 - 1.7)	0.6 (0.4 - 0.8)			
Malonate	Human	2.36 (2.04 - 2.67)	2.42 (1.90 - 2.95)	0.778	0.855	0.91
	Pig	2.26 (1.26 - 3.26)	2.41 (1.58 - 3.23)			
Citrate[#]	Human	1.0 (0.6 - 1.6)	1.1 (0.5 - 1.8)	0.478	0.005*	0.672
	Pig	2.8 (1.7 - 4.5)	3.1 (1.9 - 4.8)			
Lactate[#]	Human	0.94 (0.80 - 1.09)	1.88 (1.49 - 2.33)	0.002*	0.005*	0.057
	Pig	0.73 (0.37 - 1.17)	0.93 (0.69 - 1.22)			
Glycine[#]	Human	0.58 (0.47 - 0.70)	1.86 (1.40 - 2.41)	<0.001*	0.086	0.683
	Pig	0.87 (0.67 - 1.09)	2.20 (1.65 - 2.85)			
Glutamate[#]	Human	0.26 (0.22 - 0.30)	1.05 (0.80 - 1.34)	<0.001*	0.013*	0.545
	Pig	0.63 (0.34 - 0.99)	1.48 (1.07 - 1.96)			
Hypoxanthine[#]	Human	0.17 (0.12 - 0.22)	0.29 (0.22 - 0.36)	0.005*	0.782	0.888
	Pig	0.19 (0.10 - 0.28)	0.30 (0.16 - 0.45)			

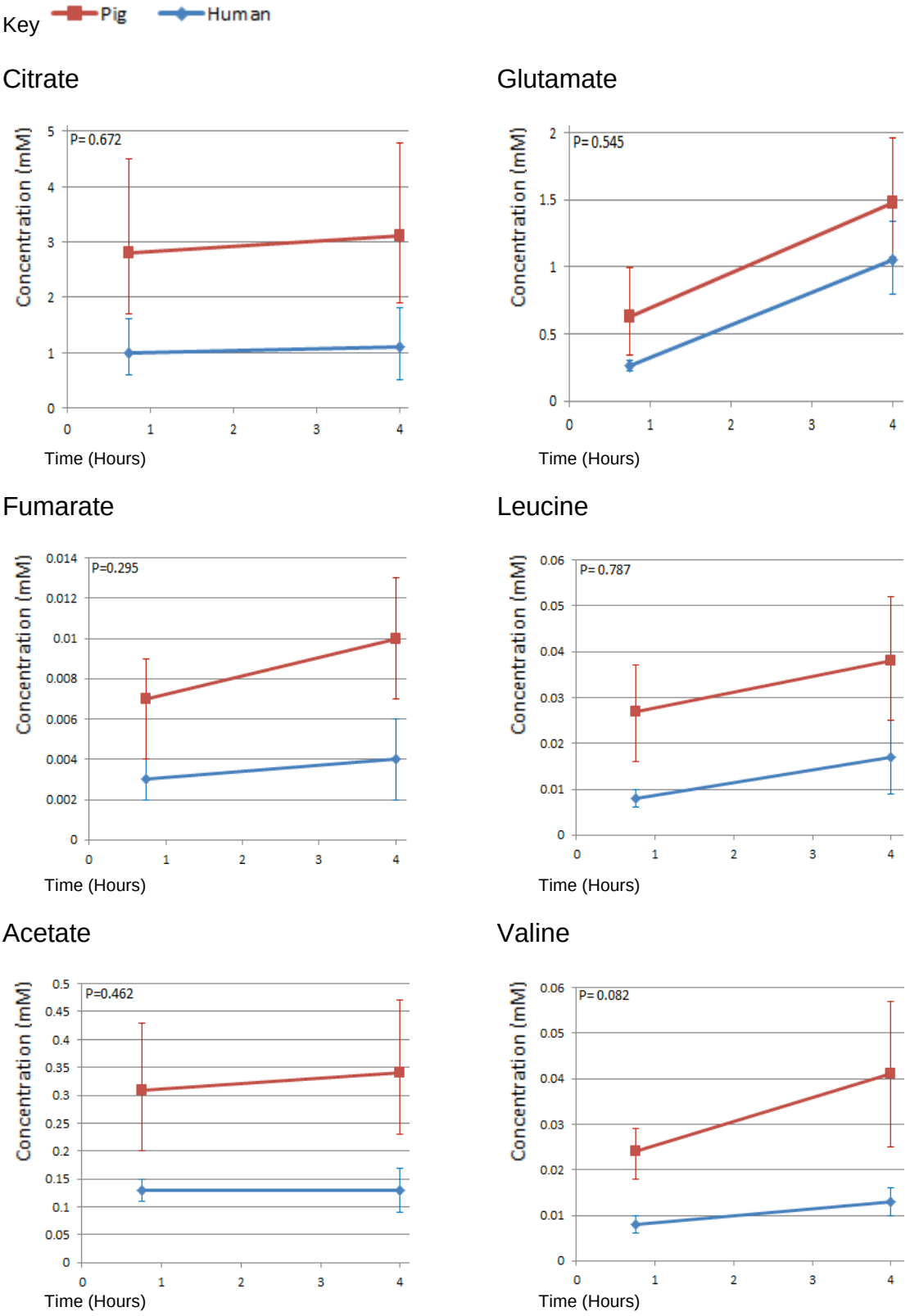
Acetate[#]	Human	0.13 (0.11 - 0.15)	0.13 (0.09 - 0.17)	0.507	<0.001*	0.462
	Pig	0.31 (0.20 - 0.43)	0.34 (0.23 - 0.47)			
Formate[#]	Human	0.10 (0.07 - 0.13)	0.11 (0.08 - 0.15)	0.594	0.306	0.411
	Pig	0.13 (0.09 - 0.18)	0.13 (0.09 - 0.18)			
Alanine[#]	Human	0.08 (0.07 - 0.09)	0.20 (0.16 - 0.24)	<0.001*	0.961	0.133
	Pig	0.10 (0.08 - 0.12)	0.18 (0.13 - 0.23)			
Creatinine[#]	Human	0.031 (0.020 - 0.043)	0.057 (0.049 - 0.065)	0.084	0.031*	0.558
	Pig	0.080 (0.029 - 0.133)	0.133 (0.040 - 0.233)			
Ethanol[#]	Human	0.024 (0.021 - 0.027)	0.036 (0.010 - 0.063)	0.385	0.018*	0.573
	Pig	0.076 (0.040 - 0.114)	0.079 (0.037 - 0.123)			
Isopropanol[#]	Human	0.023 (0.017 - 0.030)	0.025 (0.018 - 0.032)	0.792	0.063	0.167
	Pig	0.017 (0.015 - 0.019)	0.016 (0.014 - 0.018)			
3-Methylxanthine	Human	0.020 (0.016 - 0.025)	0.024 (0.019 - 0.028)	0.361	0.118	0.44
	Pig	0.017 (0.010 - 0.023)	0.017 (0.010 - 0.023)			
3-Hydroxybutyrate	Human	0.017 (0.013 - 0.021)	0.040 (0.031 - 0.048)	<0.001*	<0.001*	<0.001*
	Pig	0.012 (0.006 - 0.017)	0.013 (0.005 - 0.020)			
Inosine[#]	Human	0.017 (0.008 - 0.025)	0.023 (0.011 - 0.035)	0.038*	0.008*	0.241
	Pig	0.003 (0.001 - 0.004)	0.005 (0.002 - 0.007)			
Uracil	Human	0.011 (0.010 - 0.012)	0.018 (0.013 - 0.023)	0.05	0.020*	0.258
	Pig	0.007 (0.005 - 0.008)	0.009 (0.000 - 0.017)			
N-Phenylacetyl glycine[#]	Human	0.011 (0.005 - 0.016)	0.022 (0.010 - 0.034)	0.041*	0.18	0.094
	Pig	0.009 (0.003 - 0.014)	0.010 (0.004 - 0.016)			
Pyruvate[#]	Human	0.011 (0.006 - 0.016)	0.010 (0.005 - 0.015)	0.371	0.112	0.24
	Pig	0.012 (0.005 - 0.019)	0.020 (0.010 - 0.030)			
Leucine[#]	Human	0.008 (0.006 - 0.010)	0.017 (0.009 - 0.025)	0.020*	<0.001*	0.787
	Pig	0.027 (0.016 - 0.037)	0.038 (0.025 - 0.052)			
Valine[#]	Human	0.008 (0.006 - 0.010)	0.013 (0.010 - 0.016)	0.002*	<0.001*	0.082
	Pig	0.024 (0.018 - 0.029)	0.041 (0.025 - 0.057)			
Tyrosine[#]	Human	0.007 (0.001 - 0.012)	0.006 (0.005 - 0.008)	0.132	0.001*	0.083
	Pig	0.014 (0.010 - 0.017)	0.020 (0.014 - 0.026)			
Hippurate[#]	Human	0.005 (0.002 - 0.007)	0.027 (0.000 - 0.067)	0.298	0.198	0.337
	Pig	0.001 (0.000 - 0.002)	0.002 (0.000 - 0.004)			
Isoleucine[#]	Human	0.004 (0.004 - 0.005)	0.008 (0.006 - 0.010)	0.001*	<0.001*	0.074
	Pig	0.014 (0.010 - 0.017)	0.024 (0.016 - 0.032)			
Fumarate[#]	Human	0.003 (0.002 - 0.004)	0.004 (0.002 - 0.006)	0.012*	0.002*	0.295
	Pig	0.007 (0.004 - 0.009)	0.010 (0.007 - 0.013)			

Data reported as "Arithmetic Mean (95% Confidence Interval), unless stated otherwise

#Analyses were log-transformed in the analysis, hence are reported as "Geometric mean (95% CI)

*Significant at p<0.05

Figure 4.14: A Comparison Between Concentrations of Selected Metabolites in Pig & Human Kidney Perfusate at 45 Minutes and 4 Hours of HMP



4.5 DISCUSSION

4.5.1 THE PORCINE MODEL

Development of a porcine model was the first requirement for this part of the study. In designing the model, consideration had to be given to the facilities available.

Ideally, a porcine auto-transplant model would have been developed. This would have allowed a kidney to be removed from a pig, stored as required, and then transplanted back into the same pig with simultaneous nephrectomy of the other kidney. The major advantage with such a model is the ability to measure graft function after storage through biochemistry, urine measurements and/or histology. Reproducible models of this type have been reported (Jochmans et al., 2009). Unfortunately, this type of facility is not available locally and, even if it were, it would most likely have been prohibitively expensive. This was a reasonable first step and corresponds to the clinical situation.

An alternative option was to source pig kidneys from a local abattoir. Although an element of warm ischaemic time is unavoidable in this situation, pairs of pig kidneys could be retrieved and flushed in a relatively short time frame and with little expense. There were some initial issues with trial retrievals where damage to kidneys or vessels occurred. This meant that some kidneys had to be rejected at source but the procedure was improved and refined as more experiments were performed. As there was no option to transplant stored kidneys back into pigs, for the purposes of this

study it was assumed that all kidneys would be of similar quality and likely to display immediate graft function.

A number of modifications were made to the model as the experiment progressed. The level of sterility was improved after concerns were raised regarding possible bacterial contamination of the porcine samples. Amino acids detected by NMR analysis could have been produced by breakdown of bacteria. Efforts were made to improve the retrieval and transport process. However, these amino acids were also detected in the human samples were levels of sterility are very high so these were unlikely to be contaminants.

Use of the preservation fluid for the first five kidney pairs was not ideal. Initially, a 1-litre bag of KPS® was split between the pair of kidneys at retrieval. Then, the HMP kidney was transferred to the machine with a fresh 1-litre bag of fluid. This could have diluted true concentrations and also meant that calculations were more complicated than necessary. This was adjusted for further experiments.

Porcine HMP kidneys were stored on the laboratory research machine by the same method as used for the human kidneys. As the porcine kidneys were stored for longer, there was some difficulty keeping the kidney cool for the extended length of storage time in a warm laboratory. The machine stops if the temperature rises above 7°C. This problem was solved by moving the machine to a cold room. The machine also only records HMP parameters for 48 hours, a problem not arising with human kidneys.

4.5.2 PORCINE ISCHAEMIC TIMES

Warm ischaemic time in the porcine model was a median of 18 minutes followed by a period of SCS or HMP, mostly 48 hours but occasionally up to 120 hours. Using a porcine model allowed storage to be extended beyond what would be appropriate for human kidneys used for transplantation.

This type of model is a reasonable representation of the DCD kidney retrieval & storage process in the UK, albeit with extended storage times for the porcine kidneys. It may not be as applicable to the DBD donation and storage process.

4.5.3 PORCINE HMP PARAMETERS

Flow increased and resistance decreased during porcine kidney HMP time. As previously experienced with the human kidneys, these changes occurred mostly within the first hour.

Machine perfusion pressure was set at 30mmHg. This was selected as it is the recommended perfusion pressure for human kidneys. This is supported by additional work on a porcine auto-transplant model which showed that perfusion at 30mmHg improved kidney viability whereas perfusion at a higher pressure of 60mmHg was detrimental to the kidney (Maathuis et al., 2007).

4.5.4 PORCINE NMR ANALYSIS

A total of 30 metabolites were identified in the perfusate of porcine kidneys. Concentrations were measured at three standard timepoints – 45 minutes, 4 hours and 28 hours during SCS or HMP. Some samples were examined at extended timepoints but the numbers analysed were insufficient for statistical analysis

Glutathione was the only metabolite with statistically significant differences for all the measured parameters – change in overall concentration over time, concentration between storage groups and rate of change between storage groups. Overall, Glutathione levels decreased over experimental time. Concentrations were lower in HMP porcine kidneys compared to SCS porcine kidneys and concentrations decreased over time during HMP but remained fairly constant during SCS.

Glutathione, in its reduced form, is present in the perfusion fluid (3mM) as a free radical scavenger. When exposed to oxygen or light, glutathione is altered to its oxidised form. Levels detected by NMR in this experiment represent reduced glutathione only. Improved oxygenation and increased likelihood of exposure to light in the HMP group, could have resulted in increased oxidisation of reduced glutathione and hence the lower levels detected. It should also be noted that when oxidised glutathione loses its scavenging abilities. It has been suggested that oxidisation can affect glutathione levels in perfusate prior to use and that supplemental glutathione may be necessary (van Breussegem et al., 2011). This

may be of particular importance during HMP given the changes shown in the porcine model.

Several metabolites changed significantly over time and were significantly different between storage groups. These included amino acids (alanine, glutamate, leucine, valine, glycine, isoleucine and tyrosine), hypoxanthine and fumarate.

Concentrations of all of these amino acids increased over experimental time but more so in the HMP group. It is known that larger proteins are released into the perfusate during HMP as a sign of cellular damage (Hoogland et al., 2013) and the release of amino acids could be due to the same process. However, the results from the clinical part of this study (and other published material) suggest that HMP kidneys show improved function post-transplantation compared to SCS kidneys and might therefore have been exposed to less tissue damage. Concentrations of cellular damage markers in the perfusate might therefore be expected to be less in the HMP group. These results could be a true reflection of cellular injury or it could be that the pulsatile flushing of the kidney during HMP leads to a more efficient washout than the ‘one off’ flush prior to sampling in the SCS kidneys.

Hypoxanthine, and inosine, are end-products of ischaemic adenine nucleotide catabolism in isolated renal tissue (Buhl & Buhl, 1979). Hypoxanthine levels increased over experimental time, more so in the HMP group. Again, the increased levels detected in the HMP perfusate could be a true reflection of increased cellular damage or, more likely, could reflect improved washout of breakdown products.

Inosine concentrations, however, were no different between porcine SCS and HMP perfusate.

Fumarate is a metabolite of the citric acid cycle. Exogenous fumurate has been shown to have a role in preserving cell integrity in studies of cardiac IR injury (Laplante et al., 1997). Its role in this case is unclear, but levels in HMP perfusate were higher than in SCS perfusate.

Concentrations of lactate, citrate, pyruvate and hippurate all increased over time in both storage groups at similar rates. Lactate and pyruvate are involved in glycolysis and their accumulation in ischaemic tissue is expected. Citrate is an intermediate of the citric acid cycle and its excretion into the urine in a porcine model has been shown to correlate with favourable graft outcomes post transplant (Hauet et al., 2000b). Levels in the perfusate were similar for both storage methods in this porcine experiment but transplantation was not performed. In the human kidneys, pre-transplant perfusate levels of citrate did not differ between IGF and DGF kidneys. It might be that citrate has a role in reperfusion injury rather than ischaemic injury alone. Hippurate is an acyl glycine and a normal constituent of urine.

Levels of ribose and 3-methylxanthine were different between the storage groups although the rate of change was similar. Ribose levels were lower in the HMP perfusate. If ribose is being used as an energy source then this could reflect increased metabolic activity in the HMP stored kidneys but, as with the human HMP kidneys, adenine levels did not decrease to correspond with this mechanism. 3-

Methylxanthine levels were higher in the perfusate of HMP porcine kidneys – this again probably represents improved wash out of waste products.

Overall concentrations, rate of change and difference between storage groups was not found to be significant for the other measured metabolites.

4.5.5 PORCINE HISTOLOGY

Use of the porcine model allowed multiple biopsies to be performed. It became clear following examination of the first few samples that light microscopy lacked the power to define any differences between the SCS and HMP kidneys. Similar limitations of LM have been reported in other studies examining porcine kidney histology (Gallinat et al., 2012). Electron Microscopy, however, did suggest some interesting differences between kidneys stored by the different storage methods.

There were no differences found between SCS and HMP kidneys when endothelial cells and peri-tubular capillaries were examined. There was evidence of increased histological injury in the SCS kidneys when epithelial cells, GBM and tubules were examined.

The epithelial cells in the 28 hour samples, showed increased cytoplasmic swelling and paler nuclei in the SCS group compared to the HMP group. Cellular swelling is one of the consequences of depletion of energy sources in anaerobic conditions

(Hosgood et al., 2010). HMP may act to better preserve energy levels by improved delivery of nutrients to the tissue and therefore reduce cellular swelling compared to SCS.

There appeared to be some damage to the GBM of the SCS kidneys but not the HMP kidneys. This was only present in one of the pig pairs but similar changes have been shown in other studies (Hosgood et al., 2010).

Changes to the tubules were variable but there was some brush border loss in one of the SCS kidneys by 28 hours that was not evident in the HMP paired kidney. This has been demonstrated in other models of porcine preservation (Hosgood et al., 2010).

In terms of injury related to HMP, there was evidence of damage to the arteriolar smooth muscle layer with associated vacuolation in the HMP kidneys. This change was distinctive in both kidneys pairs. It is possible that this is related to the perfusion pressure during HMP. This has not previously been reported.

4.5.6 COMPARISON OF PORCINE & HUMAN HMP KIDNEYS

In an attempt to make a valid comparison, porcine kidneys were compared with SCD human kidneys that displayed IGF. Two assumptions were made in comparing these groups. Firstly, that porcine kidneys were equivalent to SCD human kidneys. This

seems reasonable as all kidneys were from young pigs and were disease free. Secondly, that the porcine kidneys would have had IGF. Unfortunately, the model cannot verify this as there is no method to measure graft outcome post-storage.

The porcine model described in this thesis, more accurately represents DCD retrieval in humans. The numbers of DCD human kidneys in this study were too small to use as a comparator group, so DBD human kidneys were used in the analysis. The main difference between the HMP human and porcine kidneys, in terms of pre-preservation factors, was therefore the WIT.

The median WIT for porcine kidneys during recovery was 18 minutes whereas none of the comparator human group experienced a warm ischaemic insult. Average CIT during transport and prior to storage was also much shorter for the porcine group (152.5 minutes) compared to the human group (465 minutes).

The pattern of flow and resistance change whilst on HMP was the same for human and porcine kidneys – it was only the absolute measurements that were different at each timepoint. Human kidneys displayed higher flow and lower resistance readings at all timepoints compared to the porcine group. It is unknown whether this would have been reflected in outcome. It may simply be a product of the size of the vessels in the different species. Increasing perfusion pressure may have resulted in increased flow and lower resistance within the porcine kidneys. However, evidence suggests that this may be detrimental to the kidney (Maathuis et al., 2007).

Although there were some differences between the species, the metabolomic profile of human and porcine kidney perfusate was comparable. The majority of metabolites were present in similar concentrations in both species (53.3%).

For the metabolites present in different concentrations for the two species, all metabolites other than 3-hydroxybutryate had comparable rates of change in concentration for pig and human samples. This would imply that the active metabolic pathways or processes of cellular damage during HMP in both human and porcine kidneys are broadly similar. The kidney cannot synthesise the ketone body 3-hydroxybutyrate to any significant extent but can consume it as an energy source and this process is likely to be more pronounced in hypothermic conditions. Interspecies differences of levels of the degradatory enzyme 3-hydroxybutyrate dehydrogenase with in the renal cortex and plasma levels of 3-hydroxybutyrate have been reported and may account for this finding (Koundakjian & Snoswell, 1970).

Differences in metabolite concentrations between the two species could well be due to the variation in retrieval conditions mentioned previously. Porcine kidneys were subjected to WIT during retrieval whereas human kidneys were not in this experiment. In the clinical situation, WIT would occur at the time of transplantation. The CIT during transport for the pig kidneys was shorter than the human group. In addition, human kidneys were subjected to the global metabolic changes associated with brain death prior to retrieval including thyroid, catecholamine and glycaemic effects (Gramm et al., 1992; Powner et al., 1990; Smith, 2004; Wood et al., 2004). Such difference in retrieval conditions is likely to account for many of the interspecies

differences found such as the trend towards higher levels of lactate in the human kidneys.

4.5.7 LIMITATIONS

There are several limitations to this part of the study, some of which have already been alluded to.

The main issue is the lack of kidney outcome for the porcine model i.e. no auto-transplant model. The assumption is therefore made that the porcine kidneys would all display IGF so be comparable to the human IGF kidneys. This is likely to be true but there is no proof of this.

Histology findings are interesting and potentially represent some insight into the mechanism of action of HMP. Again, sample numbers were small due to limited resources and logistical issues but further study of these findings might be very useful.

A short WIT was unavoidable for the porcine kidneys. A better human comparator might therefore have been human DCD kidneys but numbers of these were too small to be statistically useful.

Perfusate analysis determines the concentration of metabolites in the extracellular environment of the kidney. Whilst the intracellular activity of many metabolites can be inferred from this, metabolomic analysis of kidney tissue would provide a more detailed account of the intracellular activity. It would also address the issue of whether increased levels of metabolites in HMP compared to SCS kidneys are 'true' or result from the improved flushing mechanism.

In terms of the statistical analysis of the porcine metabolites, there is a high likelihood of both false positive and false negative errors due to the number of comparisons and small sample size. Also, where human and porcine metabolites are compared, small numbers may mean that it lacks the power to detect small interspecies differences. There are likely to be several false positive results at the p=0.05 significance level.

4.5.8 CONCLUSIONS

This study demonstrates that the behaviour of human and porcine kidneys during HMP is similar. Machine parameters show comparable trends and NMR based metabolomics profiles are also similar. This implies that the same underlying metabolic pathways and cellular processes are occurring during preservation in the two species. This appears to validate the porcine HMP model as a valid comparator for human studies and would suggest that a defined optimal metabolic support protocol for HMP in a pig model would be translatable into clinical practice

CHAPTER 5: CONCLUDING REMARKS

Improving transplant opportunities for kidney failure patients and optimising care for renal transplant recipients has the potential to have a huge impact on a significant proportion of the population. This is demonstrated in a better survival and quality of life for patients receiving a functioning kidney transplant. Improvements in all aspects of transplantation can also have a major impact on healthcare budgets.

Organ storage is only one of many steps in the complex process of clinical transplantation. Donor and recipient demographics, organ viability, procurement methods and warm/cold ischaemic times are only a few of the other factors that impact on transplant outcomes. However, the method of storage used during CIT and its application can have a significant effect on outcome. Hypothermic Machine Perfusion is an alternative form of organ storage with many potential benefits. This thesis aimed to investigate its role and underlying mechanism.

The introduction of HMP into clinical practice was examined in the first part of this study. This was initiated by developing and instigating an organ storage protocol based on donor-recipient readiness and theatre availability. The aim of this was to use HMP as a tool to extend CIT and allow surgery to take place under optimum conditions without detriment to graft function. As already discussed, a number of preceding trials had demonstrated improved graft function with HMP. Others had suggested that HMP might allow longer, safe storage times. Our department recognised the potential benefits of this time extension for improving service provision and patient safety.

Managing the process of kidney transplantation from the acceptance of a deceased donor organ through to operation and discharge from hospital is a detailed process. Many difficulties recognised at UHB will be relevant to other transplant units. Renal failure patients are a high risk group with significant co-morbidity which increases their peri-operative risk compared to that of the general population. In addition UHB, as with other regional transplant units, has a large catchment area with patients being managed by different healthcare providers. Often patients require dialysis or further optimization which will delay surgery. Many reports, such as the CEPOD documents, outline the risks of operating out-of-hours. Attempting to perform as many transplants as possible during normal working hours when maximal expertise is available, therefore, seems logical.

The introduction of HMP at UHB proved to have several benefits. These resulted from the principal finding that HMP allowed extended periods of CIT whilst maintaining post transplant graft function. This provided more time to optimise peri-operative conditions and operate during normal working hours with a specialist team.

Despite the protocol, a number of transplants were still performed out of hours. This highlighted the difficulty in predicting time to theatre when so many factors have to be addressed and ‘emergency’ theatre time is shared between multiple specialties. As an observation, there also appeared to be some concern in extending CIT for the small number of DCD kidneys examined. Traditionally, much importance had been placed on minimising CIT for these organ types. However, marginal organs, such as DCD kidneys, might be the type to benefit the most from the use of HMP. Further

dissemination of the evidence might be useful to reassure medical staff that HMP is a safe option for DCD kidneys.

Along with further investigation of kidney subgroups, long-term outcomes will be essential to prove the benefits of HMP. These were not available in the required timeframe but would be a useful addition to the data.

Consistent HMP outcomes might be achieved, both locally and UK-wide, by adopting HMP at recovery. It is not currently used routinely and it's introduction would require re-organisation at a national level and evidence of financial benefit.

Cost-effectiveness was discussed but not investigated as part of this thesis. This is key to introducing any new technology, especially on a national scale. Previous health economic reviews have suggested that there is an overall cost benefit to HMP. Despite the initial cost outlay, return is gained by lower rates of DGF and reduced hospital stays.

There has also been increasing interest in normothermic perfusion which has gained favour in some specialists units. Currently, the required techniques are much more complex and labour intensive and would be difficult to institute on a wider scale. This may change in the future as technology improves.

The second part of the study involved analysing the perfusate of HMP kidneys using NMR. The aim of this was to identify differences between IGF and DGF kidney

perfusate and find potential biomarkers relating to kidney injury. Accurate assessment of graft quality is essential to achieve good outcomes and maximise the use of valuable donor organs. Current methods have limited capacity to predict graft function and no single biomarkers has proven to be reliable.

The metabolomic profile of HMP perfusate from human cadaveric kidneys has not previously been examined. In this study, the metabolomic profile of the perfusate changed markedly within only 45 minutes with significant differences identified between IGF and DGF kidneys.

Several metabolites showed promise as predictors of DGF – glucose, inosine, leucine and gluconate – with good values on ROC curves. This was a small unique study and these require further examination and validation.

NMR appears to be a useful tool to identify potential markers or biological pathways of interest. From my experience, time required for processing and analysis prevents NMR from being clinically useful at present. Other groups have suggested that it is possible to utilise it in a viable timeframe for transplantation. It might be that NMR can identify markers that can then be measured with more simple bedside tests. It may also be possible to combine these markers with machine parameters to create a prediction index. Similar prediction methods do already exist and some are in clinical use but a single simple marker would be an improvement.

If further metabolomics examination can identify harmful pathways within stored kidneys it might then be possible to modify these processes. This may allow salvage of damaged kidneys which might otherwise have suffered from dysfunction or been discarded. Further information might be gained by examining kidney tissue using NMR. This may help to determine which processes are actively occurring within the kidneys and which substances are being excreted only. It is also possible to ‘tag’ substances, such as carbon, which are involved in several potential pathways which could also help to elucidate metabolic activity in the stored kidney.

The final part of the study aimed to establish a porcine model to test future hypothesis and allow modifications to stored kidneys. A porcine model of HMP was established which proved to be an acceptable comparator for human studies. This will allow further investigation to continue.

Different perfusion patterns and pressures could be tested along with varying perfusates at different temperatures. Initial examination of histological samples also produced some interesting findings which could be further examined. The ability to transplant the stored organ back into a pig to test for graft outcomes would be most useful.

Organ perfusion is still in its infancy. It seems likely that with further refinement of the methodology longer CIT can be achieved. This might allow for manipulation of immunogenicity and modulation of repair and regeneration within the stored kidney. Organ discard would be reduced allowing more life-changing transplants to take

place. In the future, safe extended storage times might allow for further reaching, even worldwide, patterns of organ exchange.

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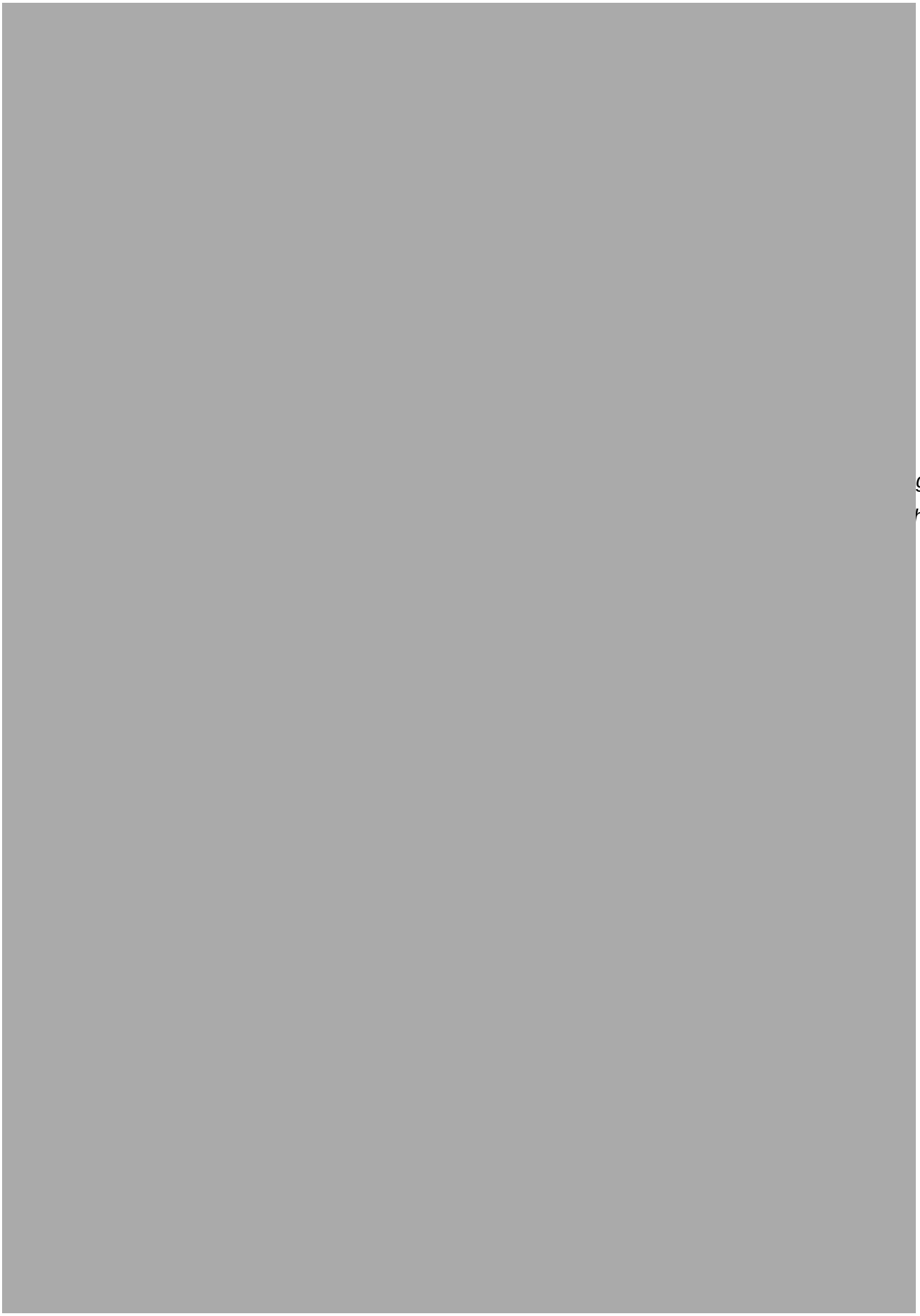
CHAPTER 7: APPENDICES

7.1 RESEARCH PROTOCOL



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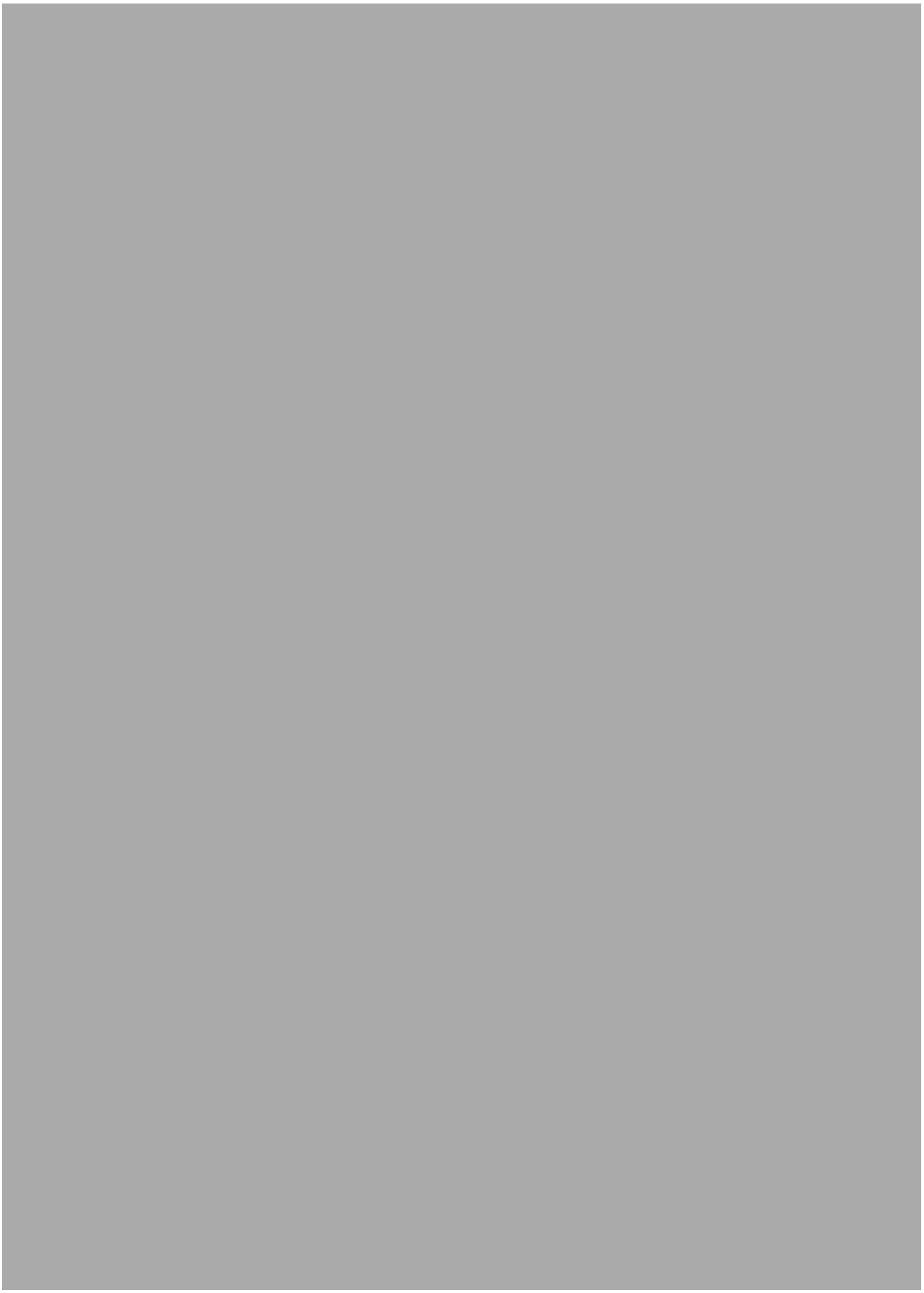

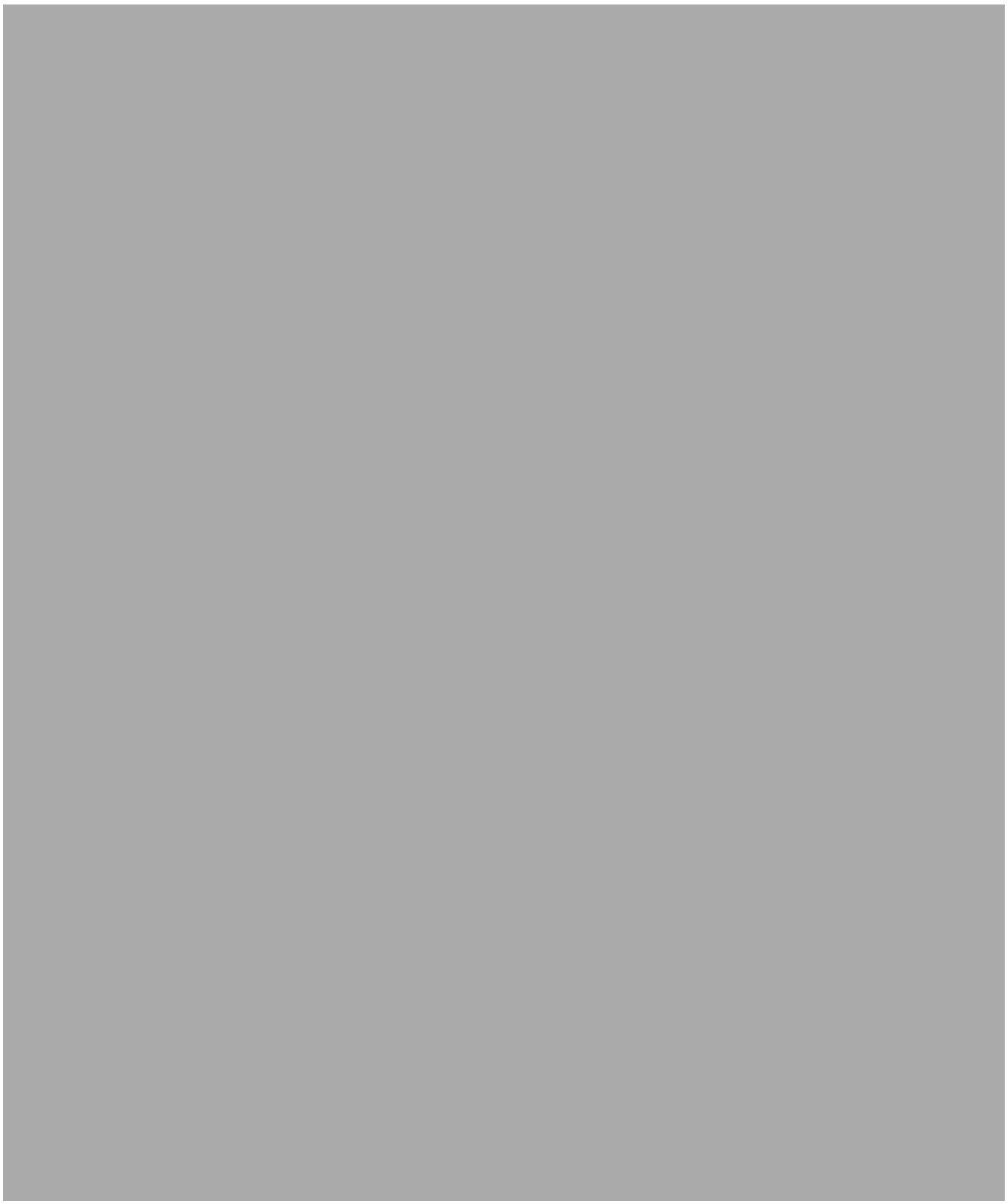

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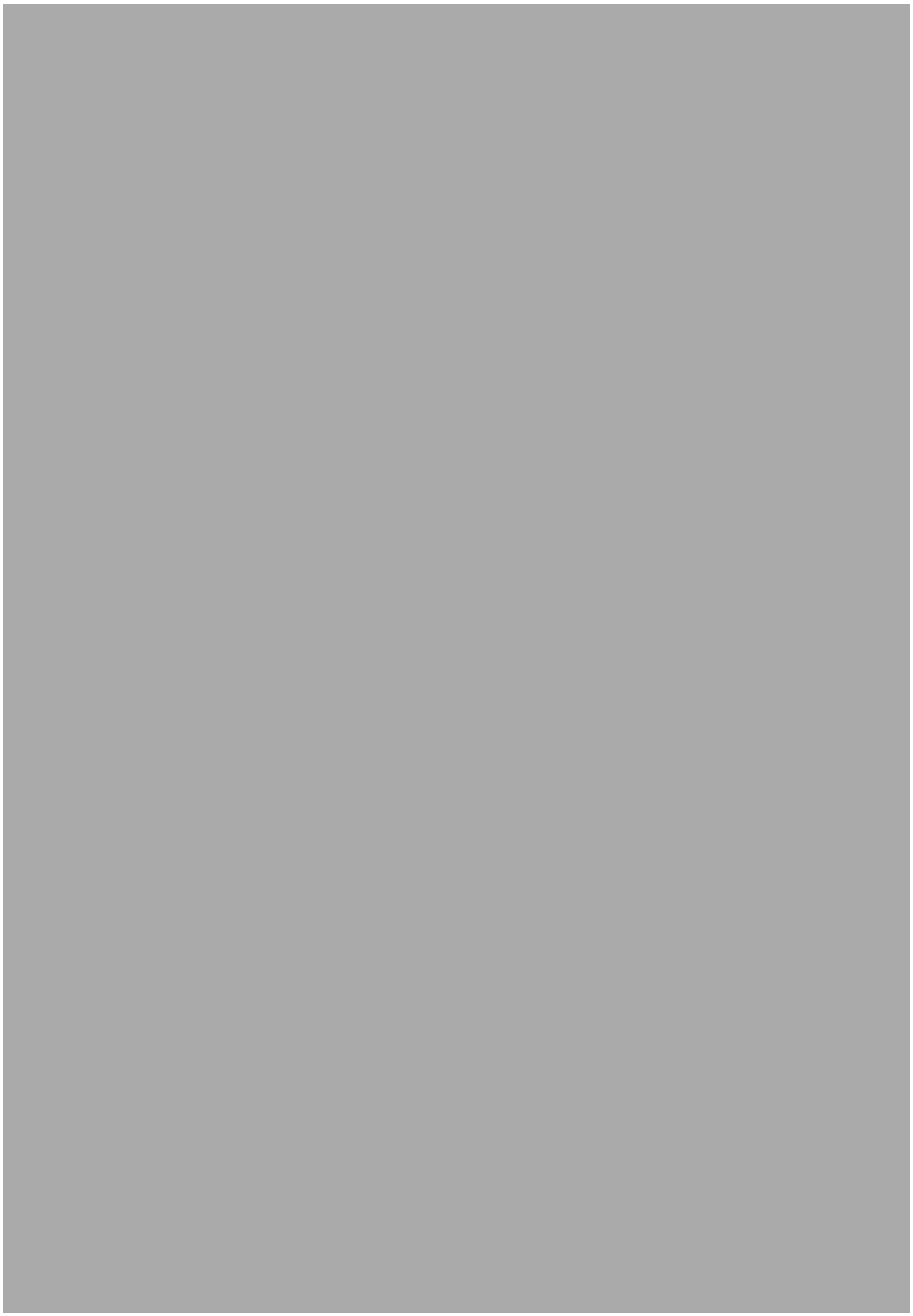
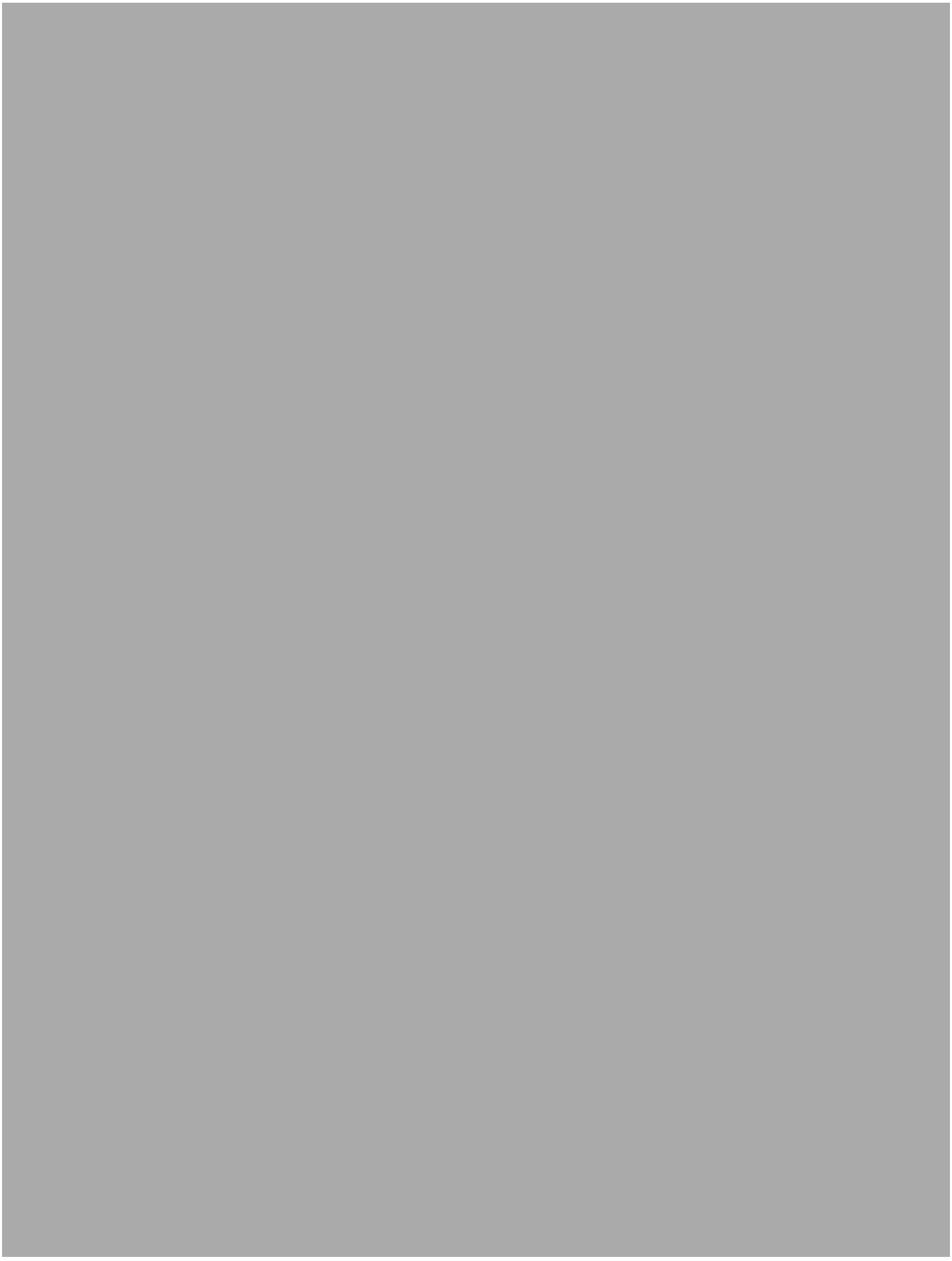
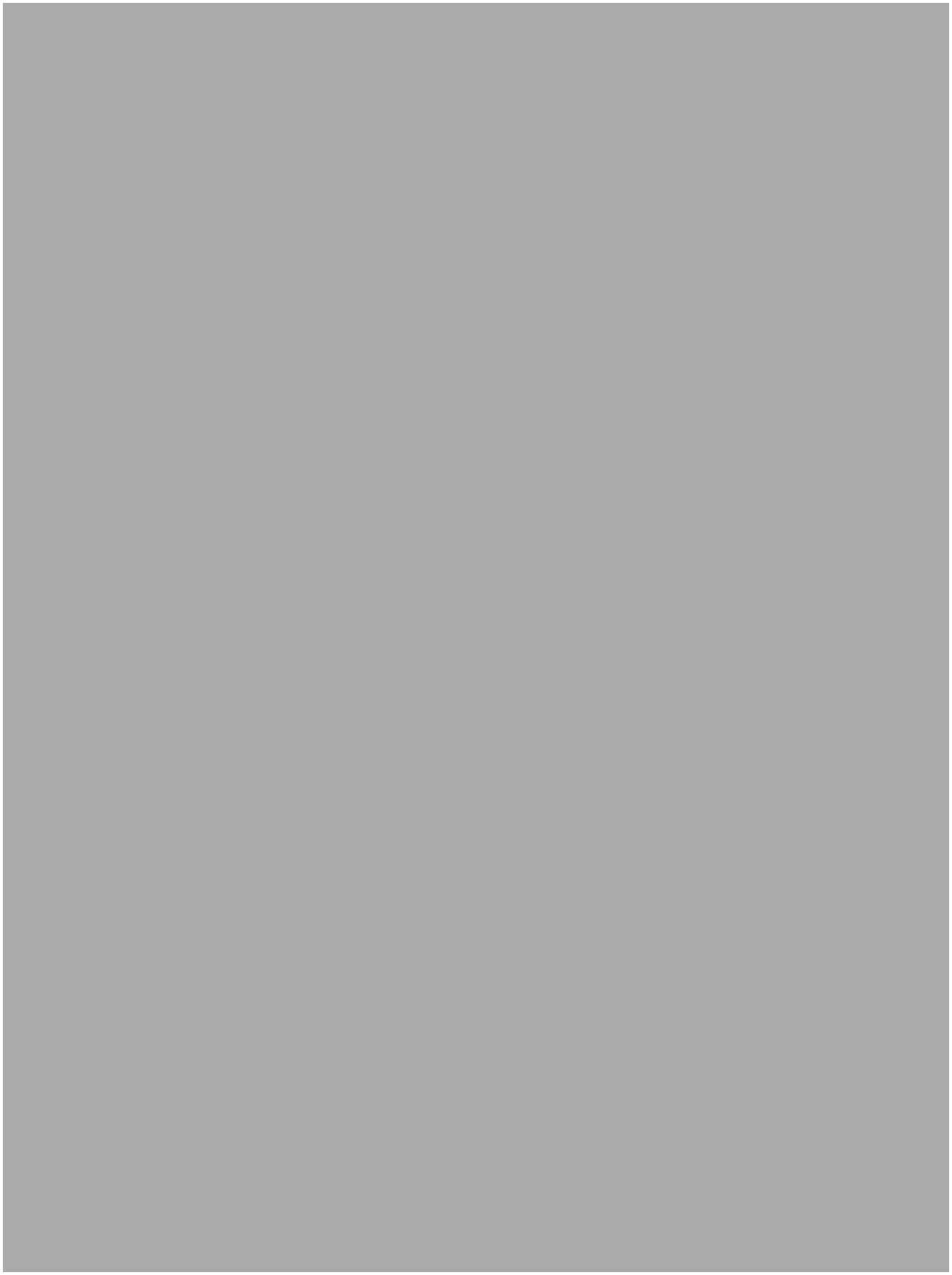
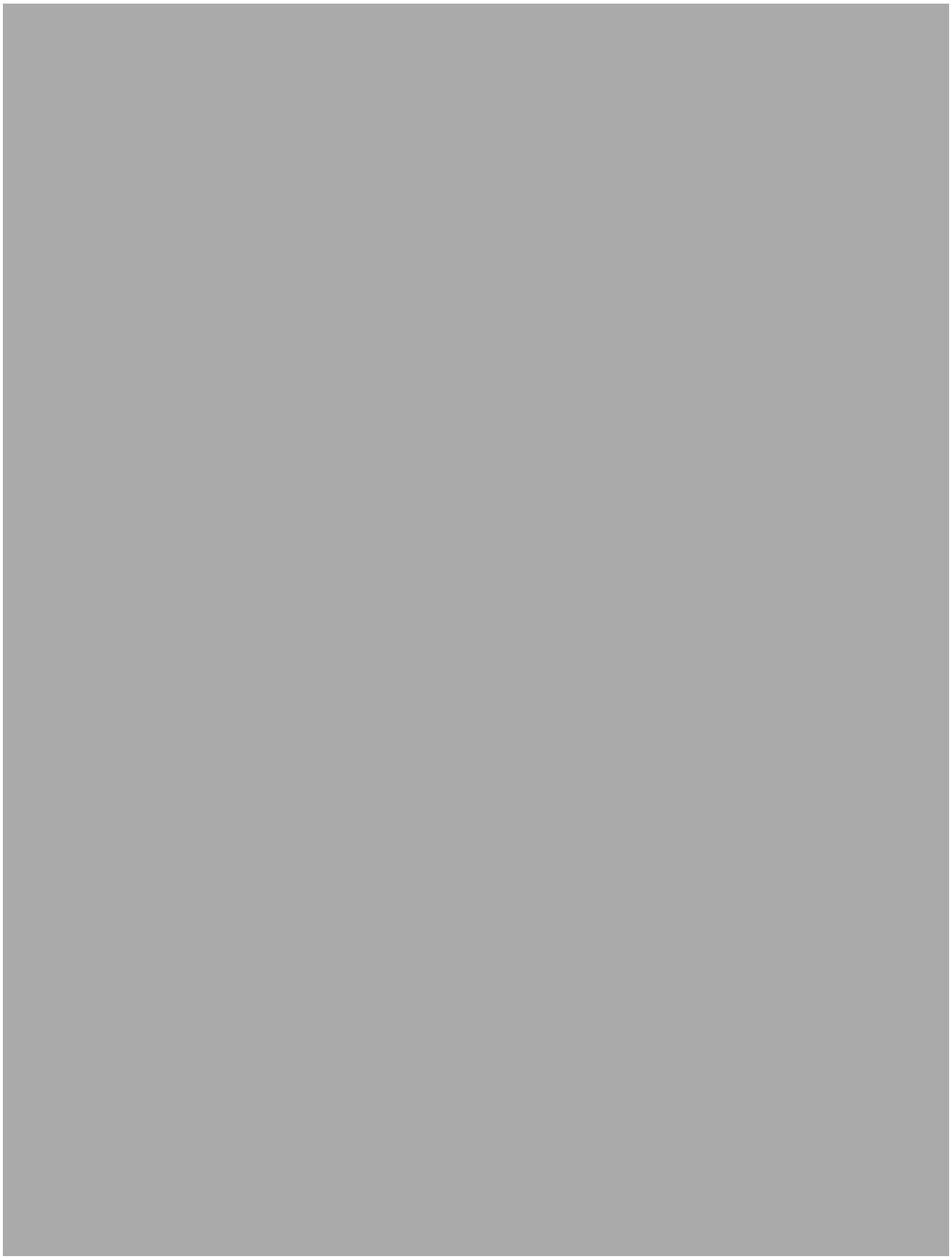
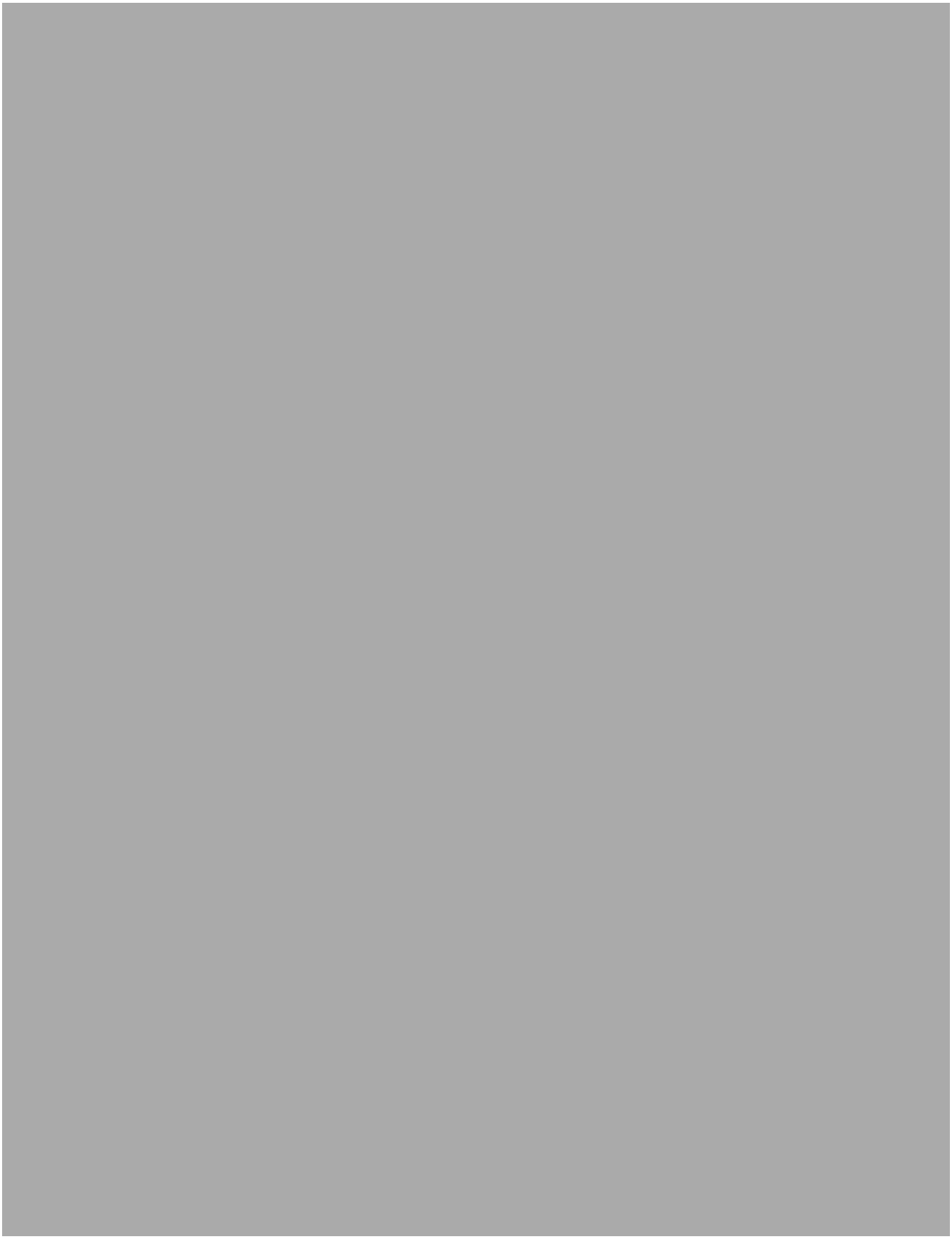
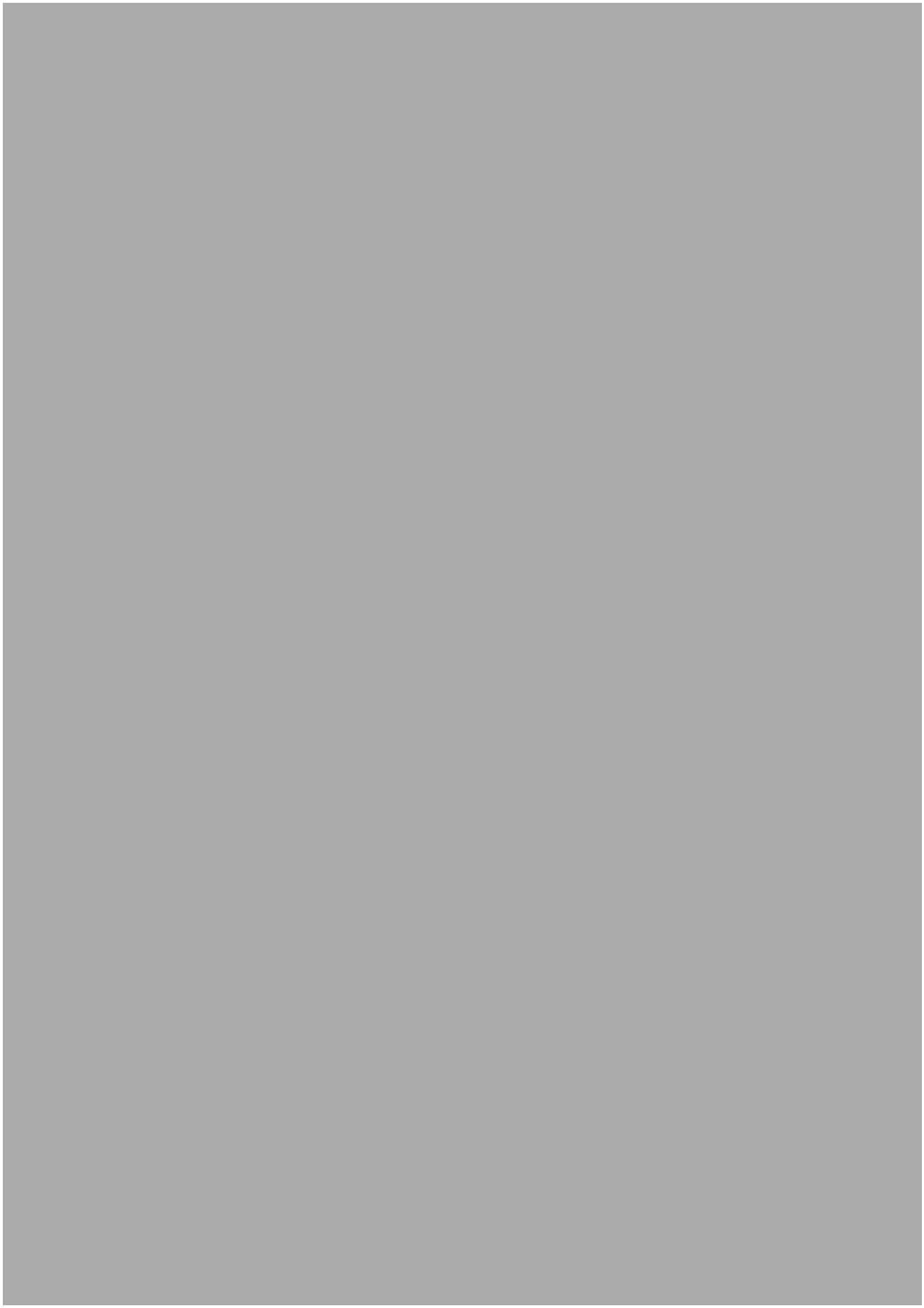


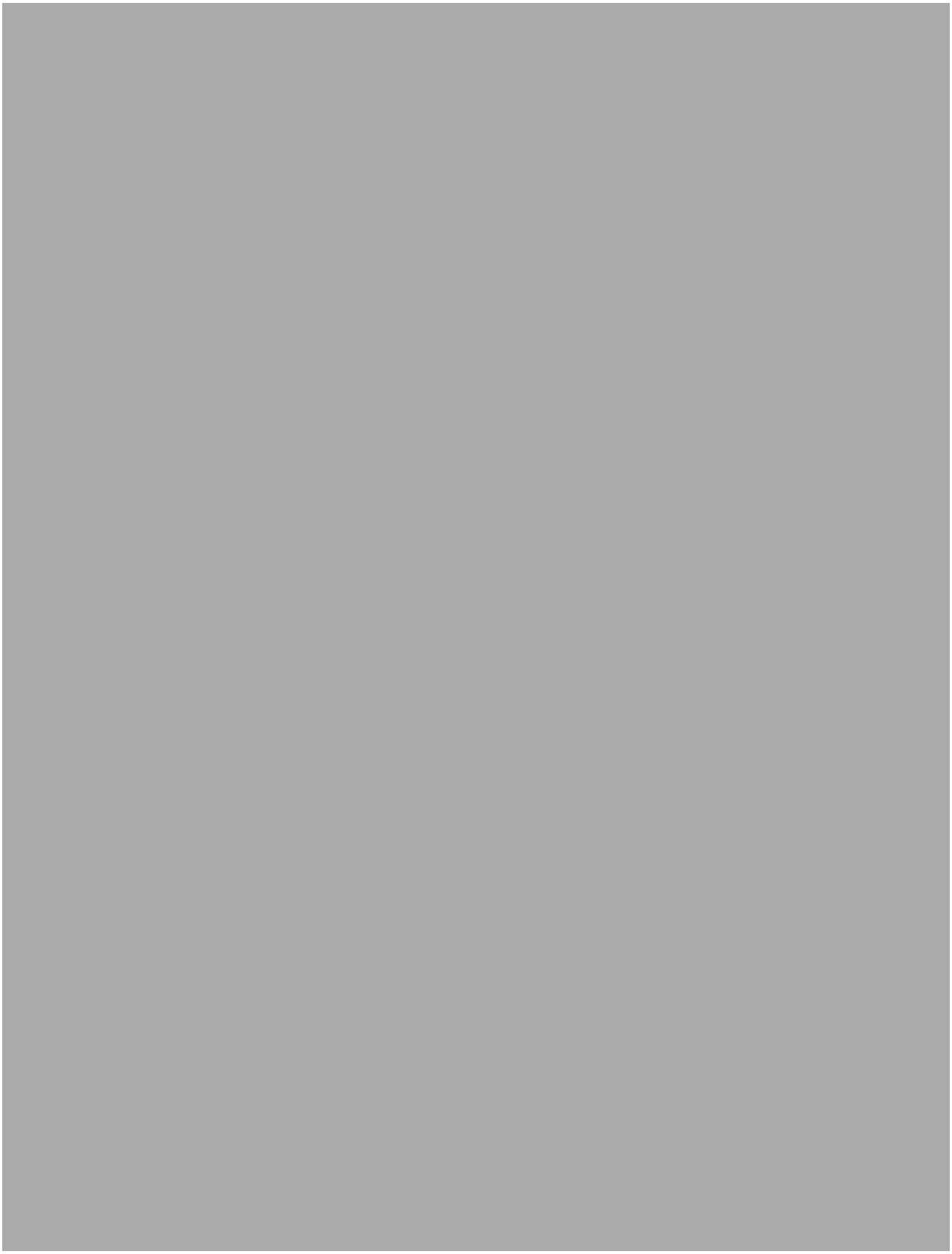


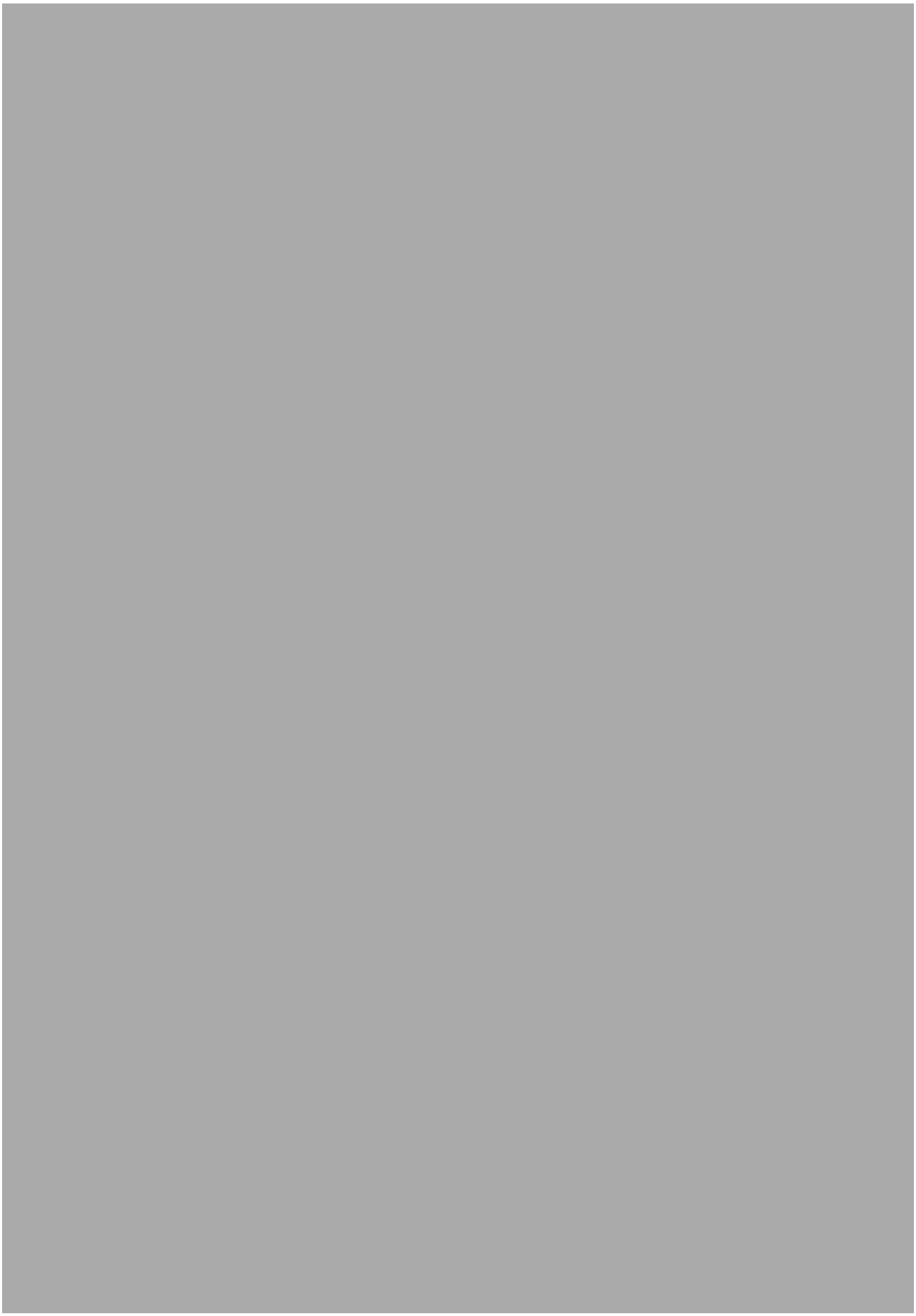


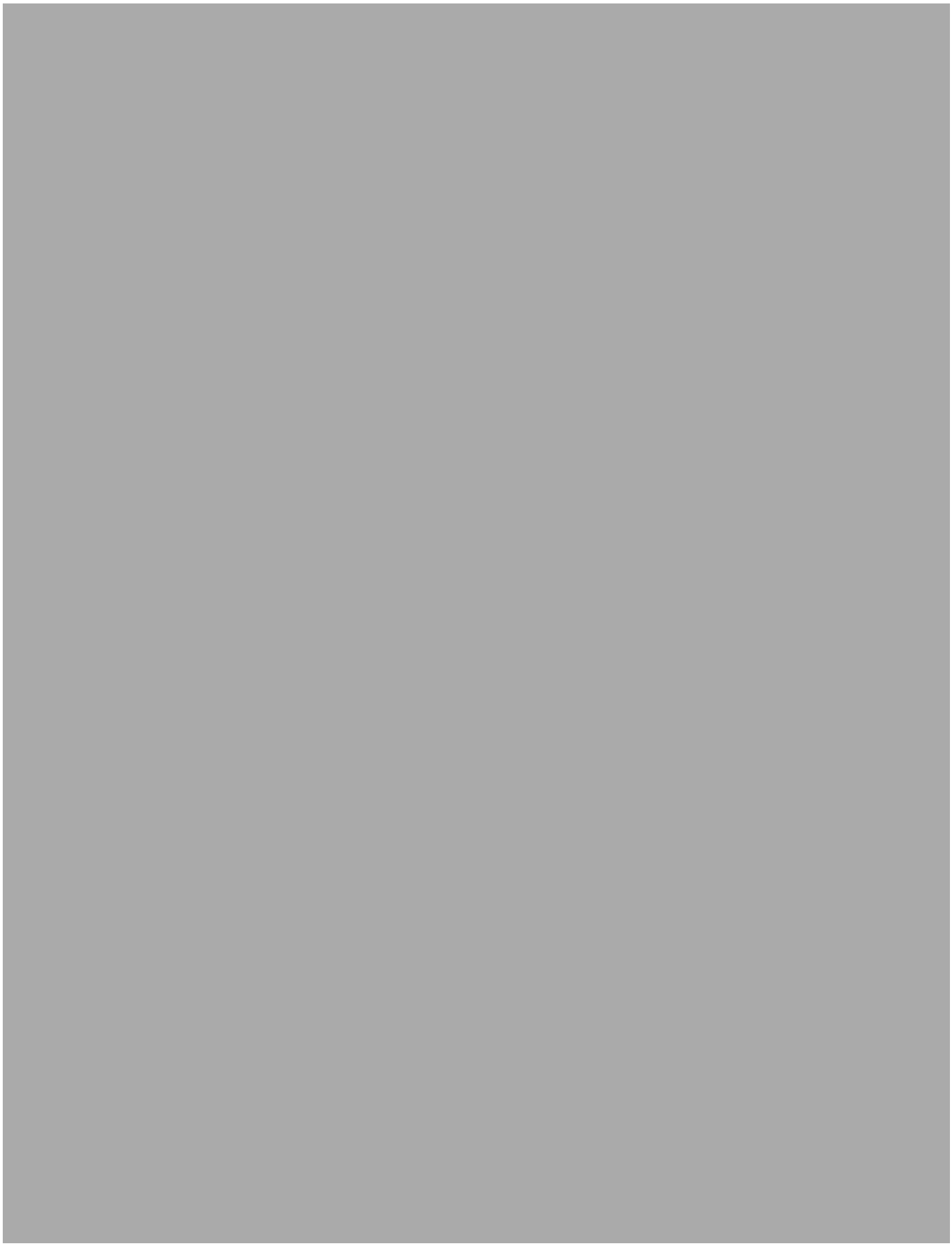


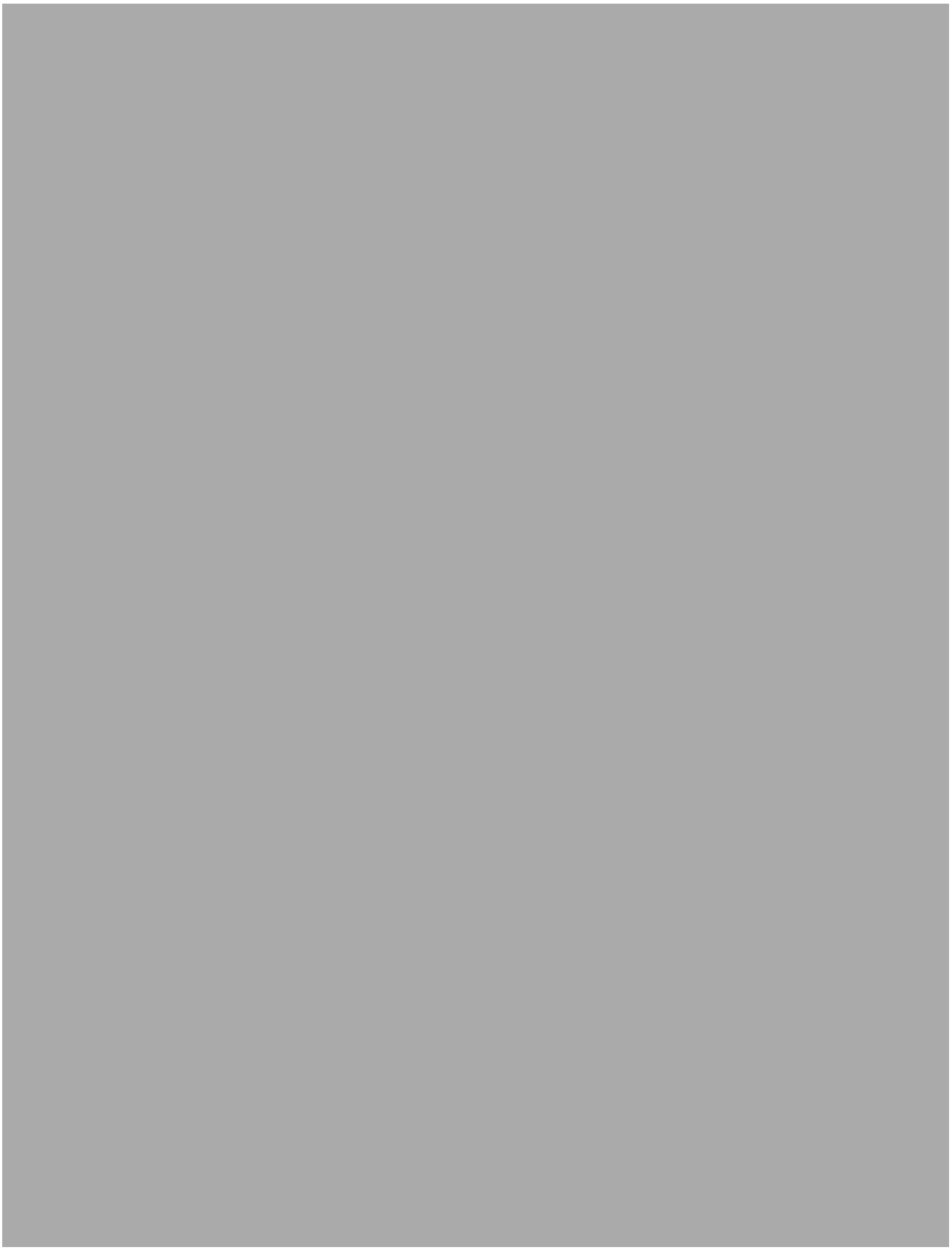


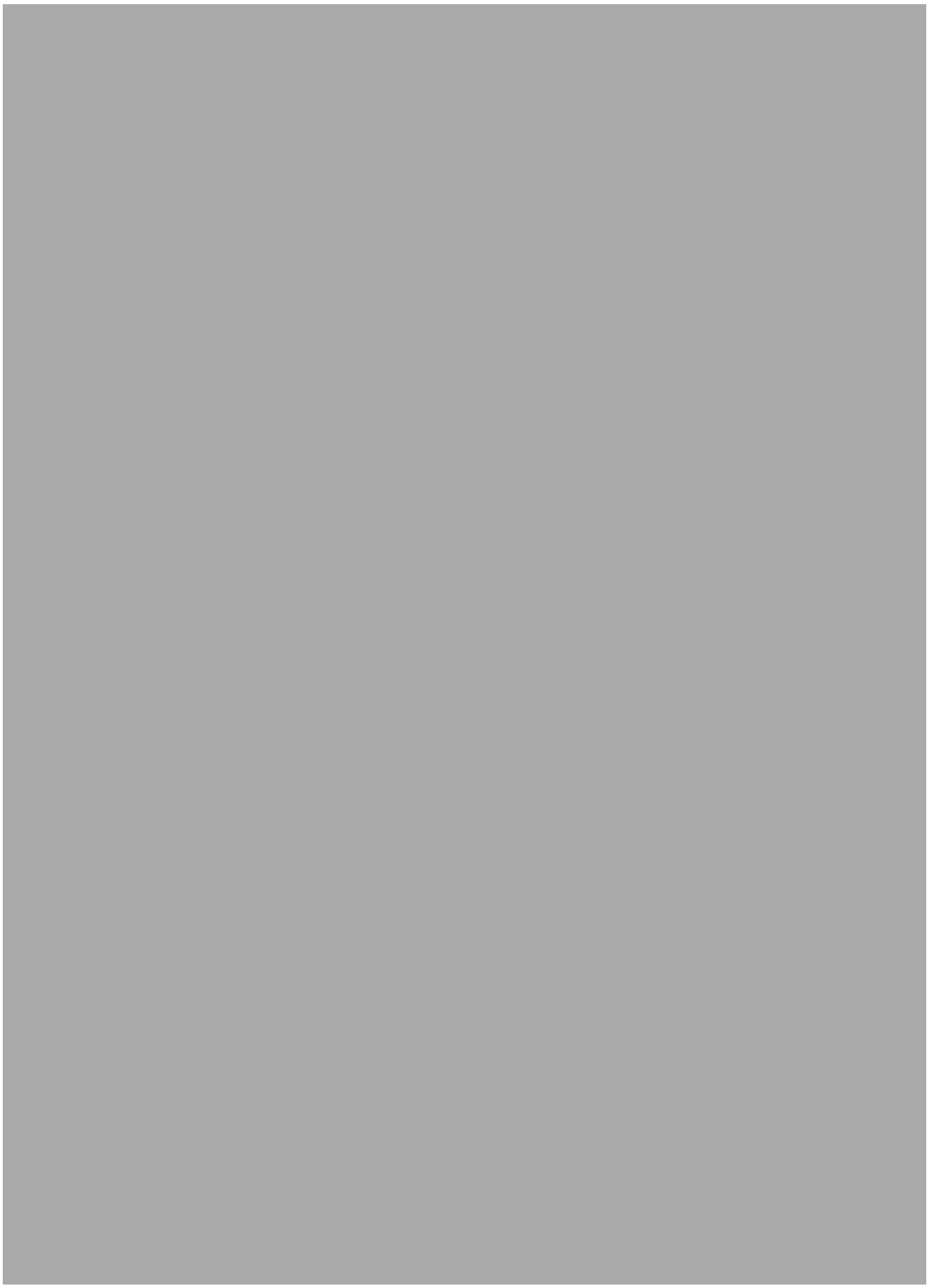






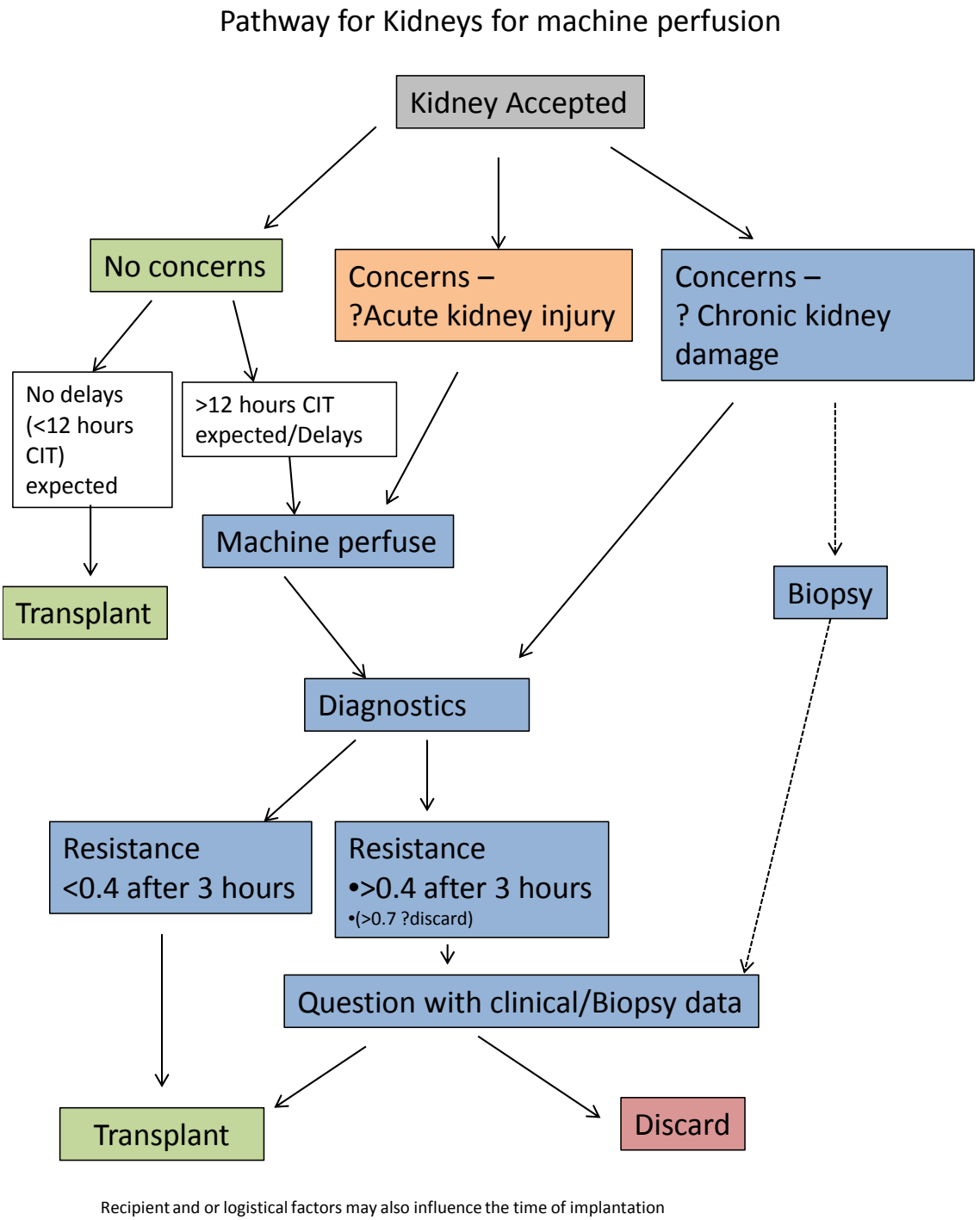






Appendix 1

Algorithm for Machine Perfusion of Kidneys at QEH



Appendix 2 Recipient Consent Form

University Hospitals Birmingham NHS
NHS Foundation Trust

CONSENT FORM FOR RESEARCH STUDY

Title of Project: Machine Perfusion in Kidney Transplantation

Name of Researchers:

Please initial to confirm

- I confirm that I have read and understand the information sheet for **Machine Perfusion in Kidney Transplantation** ☐
- I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐☐
- I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐☐
- I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by researchers, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐☐
- I agree to take part in the above research study. ☐☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

Consent to Research Participation form V1
06/06/2012

Appendix 3

Recipient Information Sheet

Machine Perfusion in Kidney Transplantation

Thank you for reading this leaflet which explains the study we are running and that hopefully you will take a part in.

What is the study?

This study is looking at how well kidneys function following transplantation depending on how they are stored and transported prior to the operation.

There are two methods of storing and transporting kidneys prior to transplantation. One is in an ice box (static cold storage) and the alternative is in a perfusion machine. In addition to providing a cold environment, the perfusion machine continuously circulates fluid through the kidney. Both methods are acceptable and safe.

Several large international studies have suggested that machine perfusion is beneficial and improves outcomes for patients receiving these kidneys. The machine may also improve kidneys which might otherwise have been rejected for transplantation allowing more kidney transplants to take place. Kidneys can also be stored for longer on the machine which gives us more time to prepare recipients for their surgery. Because of these results, we are trying to machine perfuse most kidneys that are transplanted at the Queen Elizabeth Hospital. We are hoping to better understand how the perfusion machine works and improve outcomes for our kidney transplant patients.

The results of the study will also be used towards an educational qualification.

Why are you doing it?

You will be receiving a kidney which has been stored and transported by the perfusion machine. In order for us to ensure we are achieving the best outcomes possible for our patients, we would like to collect some data to record how well your transplant kidney works. This will include results of your blood tests for kidney function and any problems you may encounter following your transplant. We hope this will help us to improve outcomes for kidney transplant patients and increase the number of kidney transplants that we can do.

What would I have to do?

We need your permission to record the results of your blood tests after your operation and record information on how well your kidney is working and any problems you might have.

There are no extra tests, needles or extra appointments. The results we normally use to take care of you following your transplant will be recorded separately and securely. These will be used to help us determine how we get the best results for our patients.

Will being in the study change my treatment at all?

Whether you take part in the study or not will not influence the treatment you receive in any way. You are also free to stop being in the study at any point. If you choose to stop participating in the study your treatment will remain exactly the same.

Can I get the results of the study?

If you are interested in the results of the study, a summary can be sent to you once all the analysis is completed.

What do I need to do if I do want to be in the study?

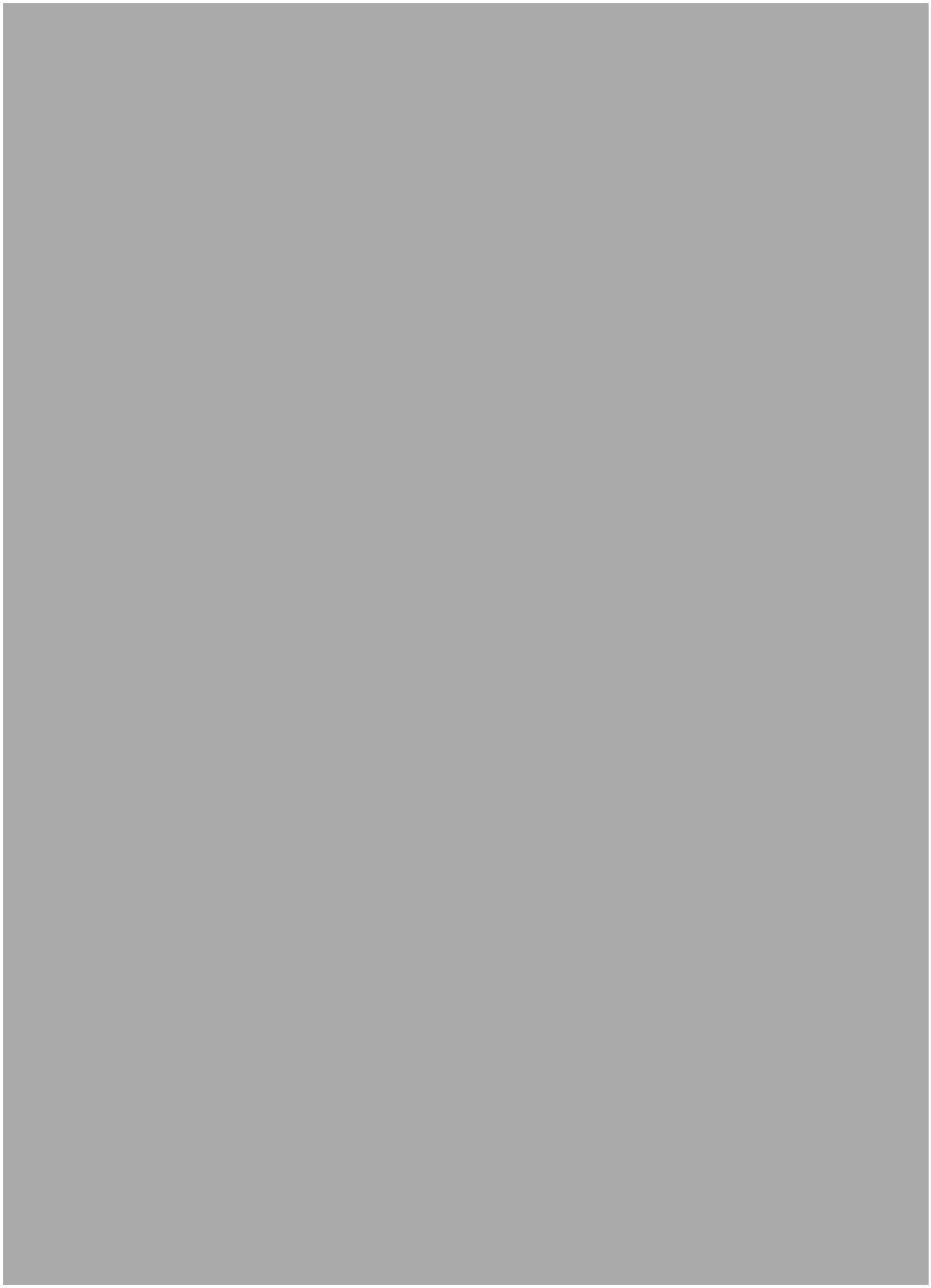
All you need to do to take part is to agree and sign the consent form. It is important to take the time to read this information and ask any questions you have. Once you have agreed to be in the study the information will be collected. Once the study is finished all data will be destroyed.

Thank you very much for reading this information and we hope you will be part of the study. Feel free to ask any questions.

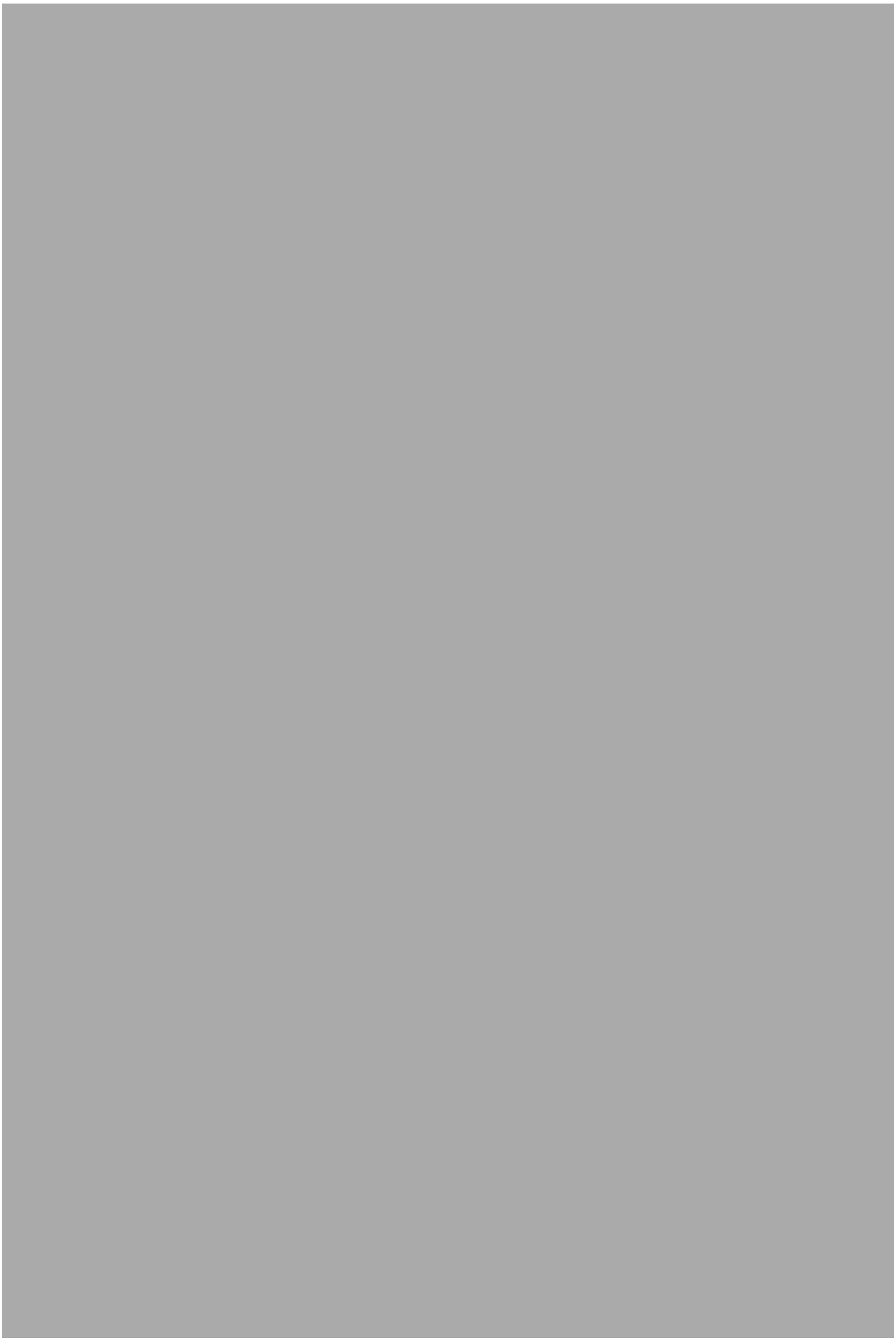
If you would like general, impartial advice on taking part in research studies we would suggest contacting the PALS office on

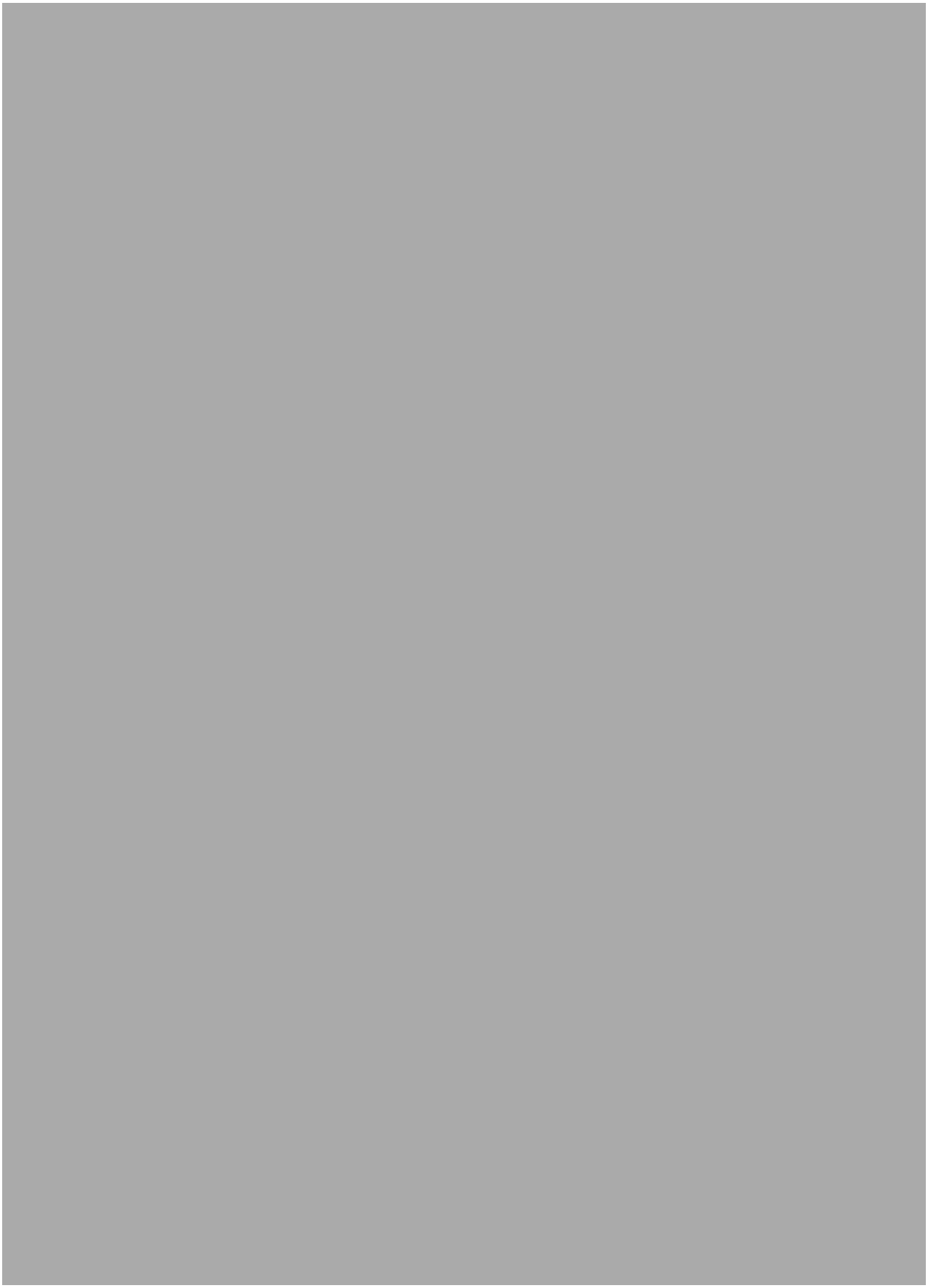
Appendix 4

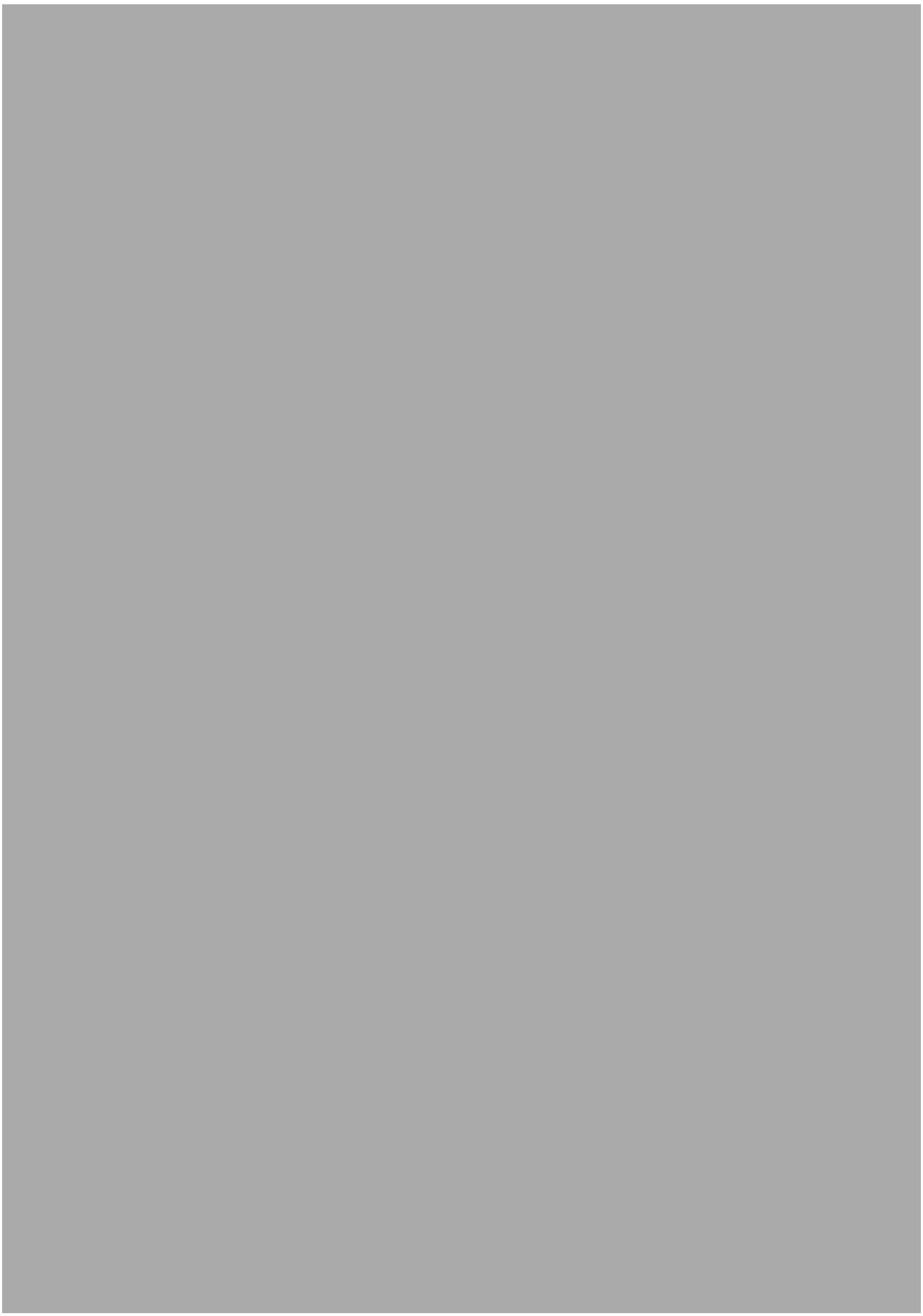
Ethics Advice



7.2 ETHICS FAVOURABLE OPINION LETTER







7.3 CUT-OFF VALUES FOR ROC CURVES

Predictor	**Cutoff**	**Sensitivity**	**Specificity**	**Youden's J**
Glucose (45 Mins)	9.0	100%	74%	0.737
Glucose (4 Hours)	9.7	100%	68%	0.684
Inosine (45 Mins)	0.0056	100%	68%	0.684
Leucine (45 Mins)	0.0087	67%	84%	0.509
Gluconate (4 Hours)	52.0	83%	84%	0.675

Based on Youden's J statistic

Due to small sample size, cutoffs are likely to have a wide degree of error

7.4 METABOLITE CONCENTRATIONS (MM) MEASURED IN KIDNEY PERFUSATE OF SCS & HMP PORCINE KIDNEYS (ALL METABOLITES)

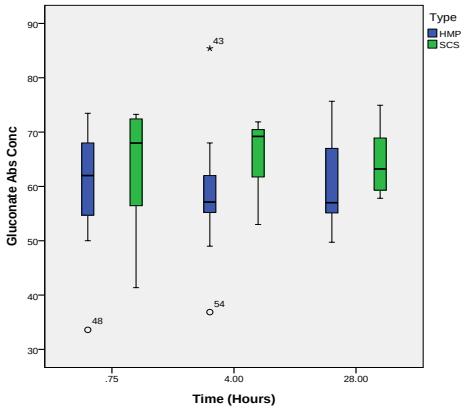
		Timepoint			p-Values		
	Storage	*45 Minutes*	*4 Hours*	*28 Hours*	*Time*	*Storage*	*Int.*
Gluconate#	HMP	59.7 (52.3 - 67.0)	58.8 (50.9 - 66.8)	61.1 (55.3 - 66.8)	0.954	0.143	0.659
	SCS	64.3 (57.7 - 70.8)	65.8 (61.3 - 70.3)	64.7 (60.9 - 68.5)			
Mannitol#	HMP	35.7 (30.7 - 40.7)	35.9 (30.0 - 41.7)	38.7 (34.3 - 43.0)	0.106	0.628	0.481
	SCS	31.5 (27.9 - 35.0)	35.0 (30.0 - 40.1)	40.5 (34.1 - 46.8)			
Glucose#	HMP	7.6 (6.2 - 9.0)	8.6 (6.4 - 10.7)	9.8 (7.9 - 11.6)	0.084	0.947	0.432
	SCS	8.2 (7.1 - 9.3)	8.8 (7.8 - 9.9)	9.1 (8.5 - 9.7)			
Adenine#	HMP	4.5 (3.8 - 5.2)	4.5 (3.6 - 5.3)	4.7 (4.3 - 5.1)	0.955	0.407	0.500
	SCS	4.8 (4.3 - 5.4)	4.9 (4.6 - 5.2)	4.9 (4.3 - 5.5)			
Ribose#	HMP	2.5 (1.9 - 3.0)	2.3 (1.7 - 2.9)	2.4 (2.0 - 2.7)	0.846	0.004*	0.696
	SCS	3.3 (2.8 - 3.8)	3.4 (3.0 - 3.9)	3.4 (3.1 - 3.8)			
Glutathione#	HMP	0.96 (0.80 - 1.11)	0.39 (0.24 - 0.54)	0.00 (0.00 - 0.00)	<0.001*	<0.001*	<0.001*
	SCS	1.59 (1.33 - 1.86)	1.64 (1.38 - 1.91)	1.55 (1.31 - 1.79)			
Lactate#	HMP	0.58 (0.16 - 0.99)	0.65 (0.47 - 0.83)	1.23 (0.92 - 1.55)	<0.001*	0.126	0.755
	SCS	0.43 (0.18 - 1.04)	0.19 (0.12 - 0.27)	0.83 (0.44 - 1.22)			
Citrate	HMP	2.3 (1.4 - 3.9)	2.6 (1.7 - 4.1)	3.1 (2.2 - 4.4)	<0.001*	0.737	0.236
	SCS	1.6 (0.8 - 3.4)	2.3 (1.1 - 4.7)	3.4 (1.7 - 6.8)			
Alanine	HMP	0.065 (0.049 - 0.085)	0.107 (0.075 - 0.152)	0.201 (0.152 - 0.266)	<0.001*	<0.001*	0.056
	SCS	0.025 (0.015 - 0.041)	0.031 (0.019 - 0.051)	0.103 (0.069 - 0.152)			
Glutamate	HMP	0.38 (0.24 - 0.61)	0.98 (0.74 - 1.30)	2.15 (1.89 - 2.45)	0.001*	<0.001*	0.733
	SCS	0.06 (0.03 - 0.15)	0.11 (0.05 - 0.23)	0.25 (0.11 - 0.58)			
Acetate	HMP	0.21 (0.15 - 0.28)	0.23 (0.17 - 0.30)	0.25 (0.21 - 0.29)	0.141	0.437	0.266
	SCS	0.17 (0.07 - 0.43)	0.12 (0.07 - 0.21)	0.24 (0.11 - 0.51)			
Hypoxanthine	HMP	0.12 (0.07 - 0.18)	0.18 (0.12 - 0.28)	0.26 (0.22 - 0.32)	0.015*	0.003*	0.704
	SCS	0.04 (0.02 - 0.07)	0.04 (0.02 - 0.09)	0.08 (0.03 - 0.20)			
Formate	HMP	0.09 (0.07 - 0.13)	0.09 (0.06 - 0.13)	0.10 (0.08 - 0.13)	0.124	0.798	0.428
	SCS	0.09 (0.03 - 0.27)	0.06 (0.03 - 0.12)	0.11 (0.04 - 0.29)			
Fumarate	HMP	0.0033 (0.0016 - 0.0068)	0.0053 (0.0030 - 0.0094)	0.0161 (0.0124 - 0.0209)	<0.001*	0.022*	0.362
	SCS	0.0014 (0.0006 - 0.0036)	0.0011 (0.0004 - 0.0031)	0.0064 (0.0039 - 0.0106)			
Leucine	HMP	0.017 (0.012 - 0.023)	0.023 (0.016 - 0.034)	0.035 (0.027 - 0.044)	<0.001*	0.013*	0.247
	SCS	0.004 (0.002 - 0.008)	0.008 (0.004 - 0.017)	0.017 (0.008 - 0.035)			

Valine	HMP	0.016 (0.012 - 0.020)	0.024 (0.016 - 0.036)	0.035 (0.027 - 0.046)	<0.001*	0.008*	0.256
	SCS	0.005 (0.002 - 0.009)	0.008 (0.004 - 0.013)	0.017 (0.008 - 0.037)			
Glycine	HMP	0.58 (0.45 - 0.75)	1.47 (1.12 - 1.93)	2.63 (2.32 - 2.98)	<0.001*	<0.001*	0.504
	SCS	0.10 (0.04 - 0.23)	0.18 (0.13 - 0.24)	0.52 (0.37 - 0.74)			
Pyruvate	HMP	0.006 (0.003 - 0.012)	0.009 (0.005 - 0.019)	0.017 (0.007 - 0.043)	0.022*	0.169	0.414
	SCS	0.005 (0.002 - 0.013)	0.003 (0.001 - 0.006)	0.009 (0.004 - 0.021)			
3-Hydroxybutyrate	HMP	0.02 (0.01 - 0.02)	0.02 (0.01 - 0.02)	0.02 (0.01 - 0.02)	0.390	0.515	0.397
	SCS	0.04 (0.00 - 0.33)	0.01 (0.01 - 0.01)	0.03 (0.01 - 0.05)			
Ethanol	HMP	0.04 (0.02 - 0.07)	0.04 (0.02 - 0.07)	0.03 (0.01 - 0.07)	0.178	0.984	0.163
	SCS	0.02 (0.01 - 0.06)	0.03 (0.01 - 0.08)	0.06 (0.02 - 0.19)			
Hippurate	HMP	0.0002 (0.0001 - 0.0007)	0.0007 (0.0001 - 0.0038)	0.0015 (0.0003 - 0.0067)	0.025*	0.532	0.620
	SCS	0.0002 (0.0001 - 0.0005)	0.0005 (0.0002 - 0.0018)	0.0005 (0.0001 - 0.0021)			
Inosine	HMP	0.0003 (0.0001 - 0.0014)	0.0008 (0.0001 - 0.0041)	0.0016 (0.0004 - 0.0069)	0.181	0.693	0.588
	SCS	0.0003 (0.0001 - 0.0008)	0.0013 (0.0010 - 0.0018)	0.0006 (0.0001 - 0.0040)			
Isoleucine	HMP	0.009 (0.007 - 0.011)	0.014 (0.010 - 0.020)	0.021 (0.016 - 0.027)	0.004*	0.024*	0.348
	SCS	0.005 (0.002 - 0.011)	0.005 (0.003 - 0.009)	0.015 (0.009 - 0.023)			
Isopropranolol	HMP	0.014 (0.013 - 0.015)	0.013 (0.012 - 0.014)	0.013 (0.012 - 0.015)	0.274	0.087	0.314
	SCS	0.021 (0.002 - 0.189)	0.007 (0.006 - 0.007)	0.004 (0.002 - 0.008)			
N-Phenylacetylglycine	HMP	0.0002 (0.0000 - 0.0014)	0.0010 (0.0001 - 0.0067)	0.0007 (0.0001 - 0.0031)	0.648	0.287	0.651
	SCS	0.0003 (0.0001 - 0.0011)	0.0003 (0.0001 - 0.0010)	0.0003 (0.0001 - 0.0013)			
Malonate	HMP	1.4 (0.6 - 3.3)	1.3 (0.6 - 2.8)	1.7 (0.9 - 3.2)	0.885	0.188	0.231
	SCS	0.7 (0.3 - 1.8)	1.3 (0.7 - 2.5)	1.0 (0.5 - 2.4)			
Tyrosine	HMP	0.009 (0.007 - 0.012)	0.013 (0.009 - 0.020)	0.020 (0.016 - 0.024)	<0.001*	0.012*	0.211
	SCS	0.004 (0.002 - 0.006)	0.005 (0.003 - 0.008)	0.013 (0.007 - 0.024)			
Uracil	HMP	0.002 (0.000 - 0.010)	0.003 (0.000 - 0.019)	0.002 (0.000 - 0.009)	0.845	0.450	0.743
	SCS	0.003 (0.002 - 0.006)	0.004 (0.002 - 0.007)	0.005 (0.002 - 0.009)			
3-Methylxanthine	HMP	0.009 (0.003 - 0.028)	0.008 (0.002 - 0.028)	0.017 (0.016 - 0.018)	0.190	0.007*	0.579
	SCS	0.002 (0.001 - 0.003)	0.002 (0.001 - 0.004)	0.002 (0.001 - 0.005)			

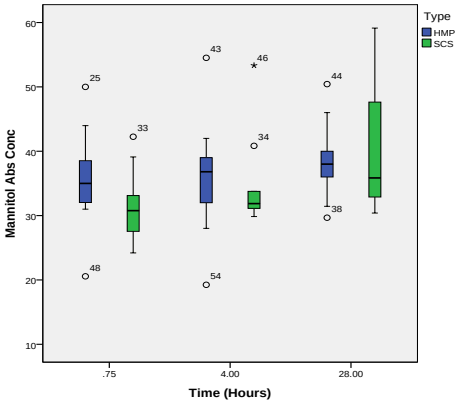
7.5 BOX AND WHISKER PLOT TO REPRESENT CONCENTRATIONS OF METABOLITES IN HMP AND SCS PORCINE KIDNEY PERFUSATE (NON-SIGNIFICANT METABOLITES)

Metabolites with no significant change in concentration over time and no significant difference in concentrations between concentration or rate of change in concentration between storage groups

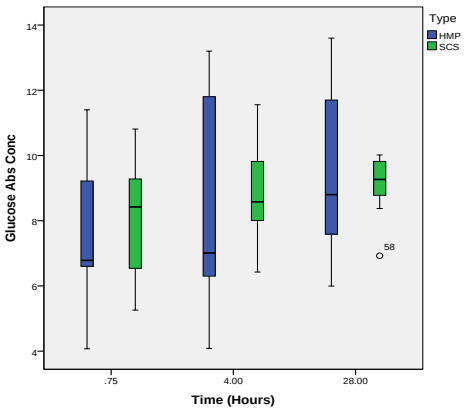
Gluconate



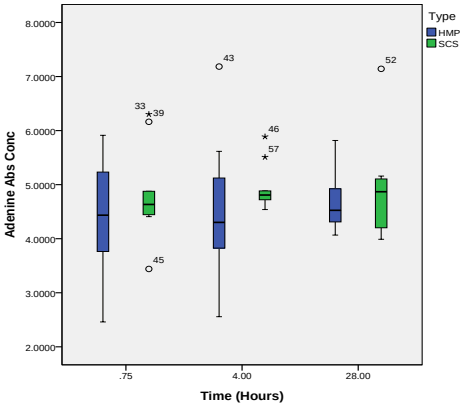
Mannitol



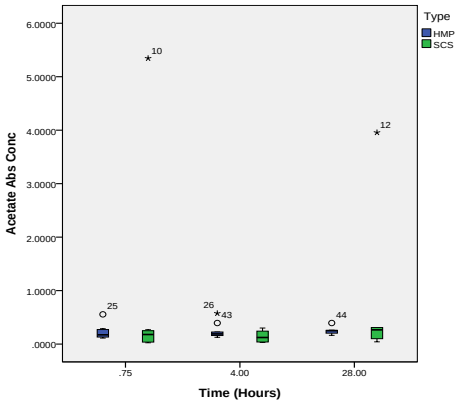
Glucose



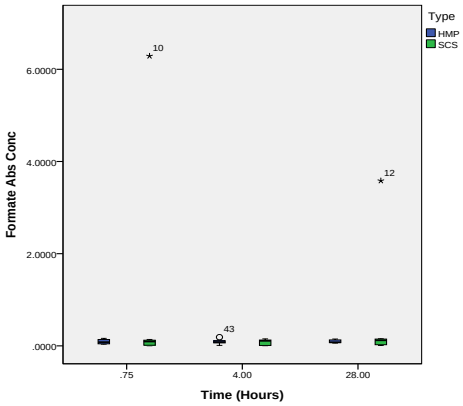
Adenine



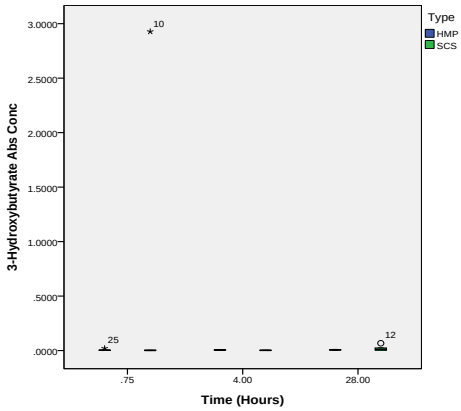
Acetate



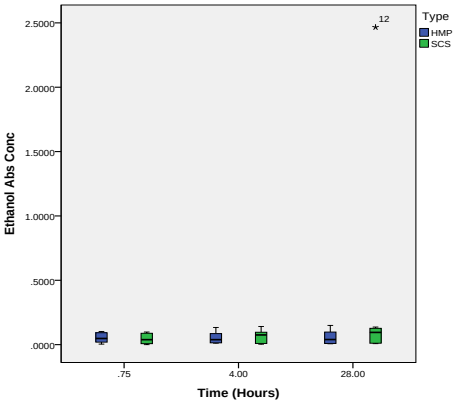
Formate



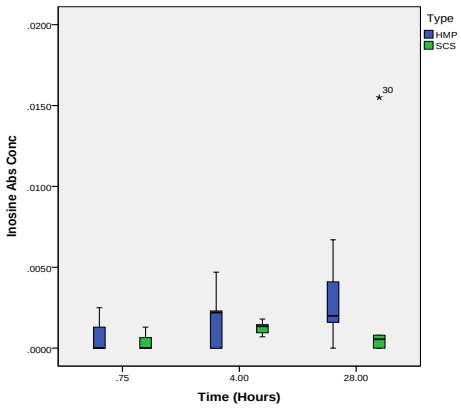
3-Hydroxybutyrate



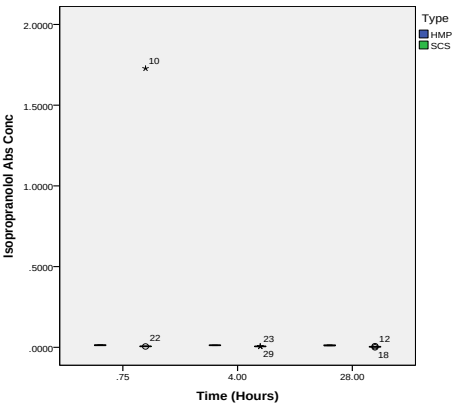
Ethanol



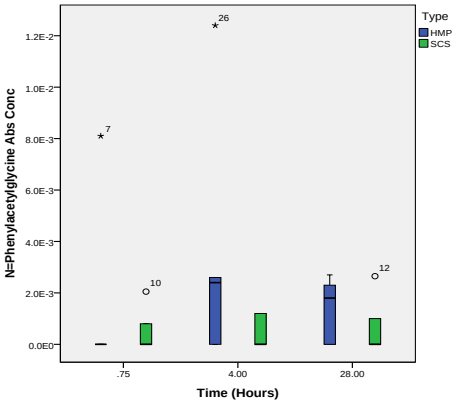
Inosine



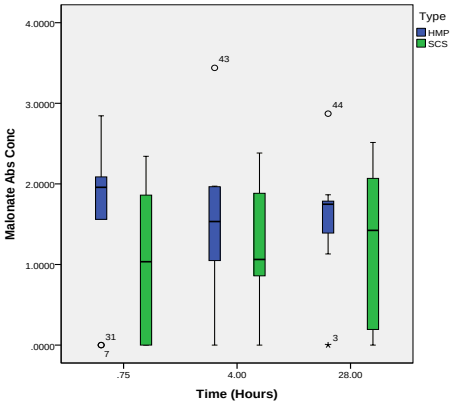
Isopropranolol



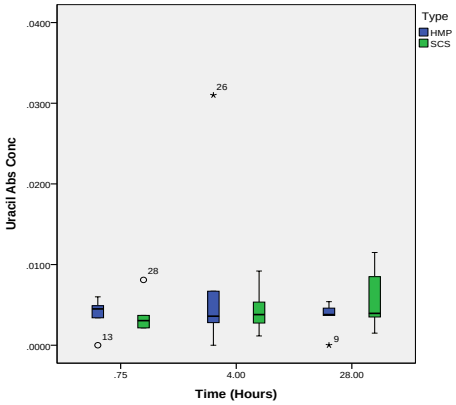
N-Phenylacetylglycine



Malonate



Uracil



CHAPTER 8: PUBLICATIONS & PRESENTATIONS TO DATE

Publications

Hypothermic Machine Perfusion Permits Extended Cold Ischaemic Times With Improved Early Graft Function
A Guy, D McGrogan, N Inston, A Ready
Experimental & Clinical Transplantation: Accepted 2014 December.

Metabolomic Perfusate Analysis During Kidney Machine Perfusion: the Pig Model Provides an Appropriate Model for Human Studies
Jay Nath, Alison Guy, Thomas B Smith, Mark Cobbold, Nicholas G Inston, James Hodson, Daniel A Tennant, Christian Ludwig, Andrew R Ready.
PLOS ONE: Accepted 2014 October.

Metabolomic Analysis of Perfusate During Hypothermic Machine Perfusion of Human Cadaveric Kidneys
A Guy, J Nath, M Cobbold, C Ludwig, D Tennant, NG Inston, AR Ready
Transplantation – Published ahead of print. 2014 September.

Abstracts

Metabolomic Analysis of Perfusate from Cadaveric Kidneys Stored by Hypothermic Machine Perfusion
A Guy, Nath J, C Ludwig, D Tennant, N Inston, M Cobbold, A Ready
Transplant International. 2014 May;27(S1):p12

Hypothermic Machine Perfusion During Extended Cold Ischaemic Times – An Opportunity to Improve Pathways of Care in Cadaveric Renal Transplantation
A Guy, D McGrogan, J Nath, C Pattenden, A Hamsho, S Mellor, N Inston, A Ready
American Journal of Transplantation. 2014 June;14(S3):p269

Hypothermic Machine Perfusion to Optimise the Timing of Renal Transplantation
A Guy, M Field, H Krishnan, N Inston, A Ready
American Journal of Transplantation. 2013 May;13(S5):207- 554

International Presentations

Hypothermic Machine Perfusion During Extended Cold Ischaemic Times – An Opportunity to Improve Pathways in Cadaveric Renal Transplantation (Poster)
A Guy, D McGrogan, J Nath, C Pattenden, A Hamsho, S Mellor, N Inston, A Ready
World Transplant Congress, San Francisco, July 2014
***Poster of Distinction**

Metabolomic Analysis of Perfusate from Cadaveric Kidneys Stored by Hypothermic Machine Perfusion (Oral)
A Guy, Nath J, C Ludwig, D Tennant, N Inston, M Cobbold, A Ready
2nd International IMIRT Congress, Poitiers, April 2014

Structured Use of Hypothermic Machine Perfusion (HMP) in Cadaveric Kidney Transplantation – An Algorithmic Approach *(Oral)*
A Guy, Mellor SJ, Hamsho AH, NG Inston, AR Ready
South African Transplantation Congress, Durban, July 2013

Hypothermic Machine Perfusion to Optimise the Timing of Renal Transplantation (Poster)
A Guy, M Field, H Krishnan, N Inston, A Ready
American Transplant Congress, Seattle, USA, May 2013

National Presentations

Metabolomic Analysis of Cadaveric Kidneys Stored by Hypothermic Machine Perfusion (Oral)
A Guy, J Nath, C Ludwig, D Tennant, N Inston, M Cobbold, A Ready
British Transplant Society Annual Conference, Glasgow, Feb 2014

Hypothermic Machine Perfusion During Extended Cold Ischemic Times – An Opportunity to Improve Pathways of Care in Cadaveric Renal Transplantation (Poster)
A Guy, D McGrogan, J Nath, C Pattenden, A Hamsho, S Mellor, N Inston, A Ready
British Transplant Society Annual Conference, Glasgow, Feb 2014

Hypothermic Machine Perfusion to Optimise the Timing of Renal Transplantation (Poster)
A Guy, M Field, H Krishnan, N Inston, A Ready
British Transplantation Society Annual Conference, Bournemouth, March 2013

Regional Presentations

Hypothermic Machine Perfusion Reduces Delayed Graft Function in Kidney Transplantation (Oral)
A Guy, Y Marie, H Krishnan, A Hamsho, S Mellor, N Inston, A Ready
West Midlands Surgical Society Meeting, Shrewsbury, November 2012

Machine Perfusion in Kidney Transplantation (Poster Presentation)
A Guy, P Mistry, M Field, M Cobbold, N Inston, A Ready
Research Showcase Event, University of Birmingham, Sept 2012

DECLARATION

This thesis includes text similar to that contained within the following publication:

Metabolomic Perfusate Analysis During Kidney Machine Perfusion: the Pig Model Provides an Appropriate Model for Human Studies

Jay Nath, Alison Guy, Thomas B Smith, Mark Cobbold, Nicholas G Inston, James Hodson, Daniel A Tennant, Christian Ludwig, Andrew R Ready.

PLOS ONE: Accepted 2014 October.

This paper was co-authored with a significant contribution by myself to all aspects of producing the paper including experiment design, performing the experiments, analysing the data, providing reagents, materials, tools, and writing the paper. Much of the content was present in this thesis prior to paper submission.

Metabolomic Analysis of Perfusate During Hypothermic Machine Perfusion of Human Cadaveric Kidneys

Alison J. Guy,[1,5] Jay Nath,[1] Mark Cobbold,[2] Christian Ludwig,[3] Daniel A. Tennant,[4] Nicholas G. Inston,[1] and Andrew R. Ready[1]

Background. The metabolic processes occurring within the preserved kidney during hypothermic machine perfusion (HMP) are not well characterized. The aim of this study was to use nuclear magnetic resonance (NMR) spectroscopy to examine the metabolomic profile of HMP perfusate from human cadaveric kidneys awaiting transplantation and to identify possible discriminators between the profiles of kidneys with delayed graft function (DGF) and immediate graft function (IGF).
Methods. Perfusates from HMP kidneys were sampled at 45 min and 4 hr of preservation with the LifePort Kidney Transporter 1.0 (Organ Recovery Systems, Chicago, IL) using KPS-1. Prepared samples underwent 1-D Proton-NMR spectroscopy, and resultant spectra were analyzed. Clinical parameters were collected prospectively.
Results. Perfusate of 26 transplanted cadaveric kidneys was analyzed; 19(73%) with IGF and 7(27%) with DGF. Glucose concentrations were significantly lower in DGF kidneys compared to those with IGF at both 45 min (7.772 vs. 9.459 mM, $P = 0.006$) and 4 hr (8.202 vs. 10.235 mM, $P = 0.003$). Concentrations of inosine and leucine were significantly different between DGF and IGF kidneys at 45 min (0.002 vs. 0.013 mM, $P = 0.009$ and 0.011 vs. 0.006 mM, $P = 0.036$), and gluconate levels were also significantly different between DGF and IGF kidneys at 4 hr (49.099 vs. 59.513 mM, $P = 0.009$).
Conclusion. Significant metabolic activity may be occurring in kidneys during HMP. The NMR spectroscopy of the perfusate can identify differences in the metabolomic profiles of DGF and IGF kidneys that might have a predictive role in viability assessment. Modification of harmful metabolic processes may improve outcomes for HMP kidneys.

Keywords: Cadaveric kidney, Hypothermic machine perfusion, Metabolomics, NMR, Transplantation.

(*Transplantation* 2014;00: 00–00)