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THE CLINICAL BENEFITS AND METABOLIC MECHANISMS OF *EX VIVO* MACHINE PERFUSION OF KIDNEYS PRIOR TO TRANSPLANTATION.

By

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A thesis submitted to

The School of Medicine and Dentistry

The University of Birmingham

For the degree

DOCTOR OF PHILOSOPHY

Institute of Metabolism and Systems Research

The University of Birmingham

March 2017

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Abstract

Hypothermic machine perfusion (HMP) is well established in the practice of renal transplantation and is associated with improved short and long-term outcomes for selected organs. Despite the advantages of this therapy during the period prior to transplantation, the mechanisms by which these benefits occur are not entirely clear and are likely to include factors additional to improved flow dynamics.

In the first part of this thesis, using both a local and national transplant dataset, I aim to establish the benefits (if any) and rationale for HMP relevant to current UK practice for both deceased and live donor kidneys.

In the second part of this thesis the mechanisms by which HMP exert benefit are further interrogated, with a particular focus on metabolism. Nuclear magnetic resonance (NMR) spectroscopy is the principal modality through which this is investigated and in addition to established 1D ^{1}H NMR protocols, glucose tracer studies using 2D ^{13}C NMR are described. Whole organ *ex vivo* perfusion is studied in this work using human kidneys (both transplanted and non-transplanted) and porcine organs, with the porcine model validated for metabolic studies.

In the final section, methods to modify metabolism during HMP are attempted, with the effects of supplemental oxygenation described.

Acknowledgements

There are a multitude of people without whose help this work would not have been possible. I am sincerely grateful to all of them. My apologies if I have forgotten to mention anybody that has contributed.

First and foremost, I would like to thank my clinical and academic mentor, Andrew Ready, whose vision and determination it was to develop the academic group to complement the successful clinical renal transplant unit in Birmingham. Your guidance has been invaluable, and I hope that your approach to science with equal measures of humour and scepticism rubs off on to me.

Secondly, my lead academic supervisor, Christian Ludwig. Without your expertise in NMR spectroscopy, this thesis would not have been possible. The hours that you have invested in this project has gone above and beyond.

To the other members of the renal research team, including my co-supervisors Dan Tennant and Nick Inston and my predecessor Alison Guy, who laid the foundations for this project, I am most grateful. Much of the painstaking, and often antisocial, hard work contained within this thesis has been aided by my colleagues in the research group. This includes the undergraduate students, Alex Hollis, Sefa Canbilen and Samuel Ebbs as well as my successor Kamlesh Patel. However, as the longest serving member of the group, a special thank you is due to Thomas Smith who, more than anybody, has kept the project progressing over the past three years.

Collectively we are grateful to all of those associated with the University Hospitals Birmingham Charities who had the vision to invest in this research. In addition, our industry collaborators and friends at Organ Recovery Systems, particularly Peter

DeMuylder and Gunther Vanwezer as well as the Royal College of Surgeons Edinburgh.

Assistance with statistical analysis was provided by the UHB statistics department, and in particular James Hodson, to whom I am very grateful.

In addition, thanks to the staff at the abattoir FA Gill Ltd and in particular Darren who has helped us develop a useful animal perfusion model.

Finally, I would like to thank my wife Carley who has not only tolerated, but has encouraged and supported me over the past three years.

Relevant publications

Published academic journal papers

Nath J, Field M, Ebbs SR, Smith T, McGrogan D, Al-Shakarchi J, Hodson J, Mellor S, Ready A. Evolution of Renal Transplant Practice Over the Past Decade: A U.K. Center Experience. *Transplantation Proceedings* 2015; **47**(6): 1700-4 (1).

Nath J, Hodson J, Canbilen SW, Al Shakarchi J, Inston NG, Sharif A, Ready AR. Effect of cold ischaemia time on outcome after living donor renal transplantation. *The British Journal of Surgery* 2016; **103**(9): 1230-6 (2).

Guy AJ, **Nath J**, Cobbold M, Ludwig C, Tennant DA, Inston NG, Ready AR. Metabolomic analysis of perfusate during hypothermic machine perfusion of human cadaveric kidneys. *Transplantation* 2015; **99**(4): 754-9 (3).

Nath J, Guy A, Smith TB, Cobbold M, Inston NG, Hodson J, Tennant DA, Ludwig C, Ready AR. Metabolomic perfusate analysis during kidney machine perfusion: the pig provides an appropriate model for human studies. *PloS One* 2014; **9**(12). (4)

Nath J, Smith T, Hollis A, Ebbs S, Canbilen SW, Tennant DA, Ready AR, Ludwig C. (13)C glucose labelling studies using 2D NMR are a useful tool for determining *ex vivo* whole organ metabolism during hypothermic machine perfusion of kidneys. *Transplant Res* 2016; **5**: 7. (5)

Nath J, Smith TB, Patel K, Ebbs S, Hollis A, Tennant DA, Ludwig C, Ready AR. Metabolic differences between cold stored and machine perfused porcine

kidneys: A ^{1}H-NMR based study. *Cryobiology*. 201;74:115-20. (6)

Published book chapter

Heude C*, **Nath J***, Carrigan JB, Ludwig C. Nuclear Magnetic Resonance Strategies for Metabolic Analysis. Chapter 3. *Advances in Experimental Medicine and Biology.* Springer Publishing 2017;965:45-76. (7)

*joint first author

This chapter is included in the appendix p1-32.

Abstracts/Presentations

Copies of the following abstracts are available appendix p33-37.

Nath J, Smith T, Tennant D, Ludwig C, Ready AR. Deciphering de novo metabolism of kidneys during hypothermic machine perfusion using isotopic NMR tracer studies. *Transplant International*. 2015 **28**(S4):p178

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

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

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

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

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

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

Canbilen S, **Nath J**, Hodson J, Inston NG, Sharif A, Ready AR. Effect of cold ischaemia time on outcome after living donor renal transplantation. Oral presentation at West Midlands Surgical Society Meeting May 2016.

Project funding

Grant awards

This project has been supported by the generous grants listed below. A copy of the award letters are available Appendix p129-135.

£60,000 from University Hospital Charities Birmingham (2012)

LifePort© Kidney Transporter 1.0 perfusion machine (circa £25k) donated by Organ Recovery Systems (2012)

£4,786 from University of Birmingham small research grant award (2014)

£20,00 from University Hospital Charities Birmingham (2015)

£60,000 from University Hospital Charities Birmingham (2015)

£9,564 from Royal College of Surgeons Edinburgh surgical research grant award (2014)

£3,980 from University of Birmingham research development fund (2016)

£15,000 Project support grant Organ Recovery Systems (2016)

£65,000 (circa.) Supply of second LifePort© Kidney Transporter 1.0 machine and ^{13}C labelled fluid Organ Recovery Systems (2016).

Grant applications

Wellcome - local training fellowship application Nov 2013

MRC - clinical research training fellowship application Jan 2014

Wellcome - research training fellowship Jan 2013

University of Birmingham - surgical research grant Mar 2014

Royal College of Surgeons Edinburgh - surgical research grant Oct 2014

University Hospitals Birmingham Charities - project-specific grant Sep 2015

Kidney Research UK - student bursary Sep 2015

Royal College of Surgeons England - small research grant Nov 2015

University of Birmingham - research development grant Mar 2016

Institute of Translational Medicine – starter fellowship grant Mar 2016

University of Birmingham - student summership Apr 2016

Kidney Research UK - student bursary Sep 2016

Student supervision

During my PhD, I have co-supervised six students that have completed BMedSci degrees at the University of Birmingham with a further four students due to complete their course in 2017 (listed below). As a reflection of this, I was nominated for and won first prize for the best postgraduate teacher within the school of medical and dental sciences at the University of Birmingham (appendix p136).

Sefa Canbilen BMedSci (2:1) awarded July 2016

Samuel Ebbs BMedSci (1st) awarded July 2016

Alex Hollis BMedSci (2:1) awarded July 2016

Sanna Tahir BMedSci (1st) awarded July 2016

Francesca Jackson Spence BMedSci (1st) awarded July 2016

Holly Gillot BMedSci (1st) awarded July 2016

Charlotte Turnbull BMedSci (1st) awarded July 2017

William Ries BMedSci (1st) awarded July 2017

Adam Arshad BMedSci (1st) awarded July 2017

Imogen Chappelow BMedSci (1st) awarded July 2017

This work has not been submitted, considered or accepted for any degree other than doctorate of philosophy at the University of Birmingham.

List of abbreviations

ATP adenosine triphosphate

ADP adenosine diphosphate

AMP adenosine monophosphate

Ca^{2+} calcium

CIT cold ischaemia time

D_2O deuterium oxide

DMSO dimethyl sulfoxide

DBD donation after brain death

DCD donation after cardiac death

Da Dalton

DGF delayed graft function

EPO erythropoietin

EC Eurocollins

ECD extended criteria donor

EM electron microscopy

ESRF end stage renal failure

GCMS gas chromatography mass spectrometry

GBM glomerular basement membrane

GFR glomerular filtration rate

GST glutathione-s-transferase

H&E haematoxylin and eosin

HES hydroxyethyl starch

HIF hypoxia inducible factor

HTK histidine-tryptophan-ketoglutarate

HPLC high performance liquid chromatography

HMP hypothermic machine perfusion

IV intravenous

IGL-1 Istitute-George-Lopez-1

IGF immediate graft function

IQ interquartile

K potassium

KPS-1® kidney perfusion solution-1

LDH lactate dehydrogenase

LM light microscopy

MS mass spectrometry

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

PCA principal component analysis

PHD prolyl hydroxylase enzymes

PNF primary non-function

PEG polyethylene glycol

ROC receiver operating characteristic

ROS reactive oxygen species

SOD superoxide dismutase

SMR standard metabolic rate

SCS static cold storage

TCA tricarboxylic acid

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

VEGF vascular endothelial growth factor

WIT warm ischaemia time

List of tables

Table of figures

Table of contents

Chapter 5

Chapter 6

Chapter 7

Chapter 8

Chapter 9

Chapter 1

General introduction

Contents of this chapter have been published in the following textbook (7).

Heude C, **Nath J***, Carrigan JB, Ludwig C. Nuclear Magnetic Resonance Strategies for Metabolic Analysis. Chapter 3. *Advances in experimental medicine and biology.* Springer Publishing 2017;965:45-76.

*joint first author

As joint first author, any section that is identical to the published version has been written by myself. The entire text of this chapter is included in the appendix p1-32.

Overview

Benefits of transplantation

The evolution of renal transplantation over the past sixty years is a triumph of modern medicine, with one-year graft survival rates in the contemporary era of over 90%(8). Transplantation has become irrefutably the treatment of choice for patients with end stage renal failure (ESRF), improving both quality and quantity of life (9). Such are the benefits of transplantation that even in high-risk patients with complex medical problems there is a distinct survival advantage compared with the alternative of dialysis (10, 11).

In addition, from a health economics perspective, kidney transplantation is advantageous with a cost saving of £250,000 per kidney compared with haemodialysis (based upon a median graft survival of ten years) (12).

Types of donor organs for transplantation

Donor organs can be broadly classified by donor type, either deceased or living donor. Whilst the numbers of living donor kidneys transplanted annually are increasing, and such organs have the best outcomes, deceased donor organs still account for the majority of organs transplanted in the UK (8). Deceased donor organs can be further categorised according to the physiology of the donor; donation after brain death (DBD) and donation after cardiac death (DCD). By definition, donors with brain stem death have an irreversible neurological injury that, amongst other things, annihilates the respiratory centres and neurological impetus to breathe. However, such patients do have a cardiac output, provided that they are

sufficiently oxygenated via a ventilator. DBD donors are considered both medically and legally dead in the UK and resultantly the organ donation procedure for these patients can occur in a controlled fashion whilst the donor organs are being perfused. In contrast DCD donors also have a grave neurological injury but do not fulfil brain stem death criteria. As such patients are neither medically nor legally dead, organ retrieval can not commence until the cessation of cardiorespiratory output has occurred (plus five minutes in the UK). This cardiorespiratory arrest is typically precipitated by the cessation of all supportive therapy (e.g. inotropes, ventilation). Needless to say, for DCD donors, there is a period between the cardiac arrest and cold perfusion of organs whereby the organs are both warm and ischaemic, termed warm ischaemia time (WIT). This warm ischaemia is known to be more detrimental than equivalent periods of cold ischaemia. Historically DBD and DCD donors were called heart beating and non heart beating donors, but such terms were deemed over-simplistic.

Increased usage of extended criteria kidneys

Despite the ever-growing demand, transplantation is limited by the paucity of available organs and considerable efforts have been made to expand the donor pool, reflected by increased transplant numbers both in the UK and worldwide (8). Patients not fortunate enough to have a suitable live donor must rely on a deceased donor organ. Such are the benefits of transplantation and the discrepancy between supply and demand that organs previously deemed as unsuitable for transplantation are now being utilised, including extended criteria donor (ECD) organs. A definition

of an extended criteria donor is given in Table 1.

- Donors aged ≥ 60 years
- Donors aged 50-59 plus at least two of the following
  - Hypertension
  - Intra-cerebral cause of death
  - Pre-terminal creatinine > 132 μmol/L

Table 1 Definition of extended criteria donor (ECD)

Techniques for organ preservation

Organs have been traditionally cold stored in the interval between organ retrieval and implantation. This period of cold ischaemia or cold ischaemia time (CIT) is known to cause significant detriment to graft survival if excessive and is therefore kept to a minimum (13-15). Whilst there is some evidence that a controlled period of normothermic or sub-normothermic perfusion may have a beneficial effect in selected kidneys prior to transplantation (16-18), this has not been validated in high volume studies and is not used outside of specialist academic centres. Thus cold storage to minimise any metabolic function and rapid implantation to minimise CIT continue to drive the practice of kidney transplantation to date. The two methods for cold preservation of kidneys, static cold storage (SCS) and hypothermic machine perfusion (HMP), are discussed below.

Static cold storage (SCS)

Static cold storage (SCS) is the traditional modality of organ preservation and

remains the most commonly used method worldwide (19). The governing principles are simple and have undergone little change since the inception of renal transplantation some sixty years ago, namely the deceleration of metabolism that occurs as the temperature of the kidney is reduced. At storage temperatures below 4°C, the metabolic function within the kidney is reportedly only 5-8%, compared with *en vivo* conditions (20). During periods of SCS, the kidney is essentially stored in hypertonic solution surrounded by ice within a polystyrene storage container.

History of static cold storage

Whilst Belzer and colleagues developed the machinery and perfusates for mechanical perfusion in the 1960's, Dr. Collins and his team were championing a much simpler kidney storage technique; namely SCS. Following the initial cold flush to remove any remaining blood, organs were preserved in the eponymous Collins solution in which the high concentration of potassium and low concentration of sodium resembled the intracellular environment. A high concentration (140 mM) of glucose also ensured that the solution was slightly hypertonic (350 mOsm/L). Despite the lack of impermients in Collins solution, such as lactobionate, hydroxyethyl starch (HES), or mannitol, the solution proved effective at storing canine kidneys for up to 30 hours (21). Although unacceptable by today's standards, human trials later proved cold storage with Collins solution to be superior to the Belzer perfusion technique for kidneys with prolonged preservation times, with one year graft survival of 53% vs. 40% ($P<0.001$) (22). This results were equivalent for machine perfused kidneys in other studies from same era (23, 24). Given the lack of clinical benefit for HMP, combined with the easily reproducible, transportable, low-cost advantages of SCS, this quickly gained favour as the organ preservation modality of choice. Indeed only

2% of all kidneys transplanted between 2000 and 2007 were preserved using machine perfusion (25).

Although the fluid used for SCS preservation of kidneys changed over the subsequent decades, from Collins solution to EuroCollins to University of Wisconsin (UW) solution, the principle did not. Clinical outcomes for renal transplantation improved dramatically over the subsequent decades, owing largely to improved immunological therapy including the widespread usage of calcineurin inhibitors as part of the immunosuppressant regimen. The relative benefit, if any, of machine perfusion seemed to be small compared to the huge improvements achieved through improved perioperative medical care.

Clinically used preservation fluids for SCS

It was quickly established that fluids commonly used for intravenous replacement (e.g. 0.9% NaCl, Ringers lactate, etc.) were unsuitable for organ preservation and resultantly, a multitude of fluids were designed for this purpose. Clinically utilised preservation fluids with their constituent ingredients are listed in Table 2**Error! Reference source not found.**. The University of Wisconsin solution (UW) was first designed over thirty years ago but is still considered the gold standard in most of the world for the initial cold flushing of organs during procurement as well as for static organ preservation. UW contains lactobionate and hydroxyethyl starch (HES) as impermeants. Similar to Collins solution, the Na^+/K^+ ratio of UW resembles intracellular concentrations to minimise the ATP requirement necessary in maintaining transcellular concentration gradients via Na^+/K^+ ATPase pumps. The amount of HES present in UW is 50 g/L making a 5% solution (molecular weight range 100-1000 kDa, mean 250 kDa) (26). Other constituents include raffinose,

glutathione to limit the harmful effects of reactive oxygen species, and adenosine to enhance ATP synthesis. A systematic review and meta-analysis of preservation solutions used for SCS of kidneys concluded that there were higher rates of delayed transplant function for EC (Eurocollins) preserved kidneys than those stored in either UW or histidine-tryptophan-ketoglutarate solution (HTK). There was no difference found between UW, Celsior and HTK preserved kidneys (27).

Components (mmol/L)	IC	Euro-Collins	UW	EC	Celsior	SCOT	IGL-1
KCl	118	15	–	5	15	5	–
KOH	–	–	100	–	–	–	–
NaCl	5	–	–	118	–	118	120
$NaHCO_3$	25	10	–	25	–	25	–
NaOH	–	–	27	–	100	–	–
KH_2PO_4	–	15	25	–	–	–	25
K_2HPO_4	–	32.2	–	–	–	–	–
$CaCl_2$	1.75	–	–	1.75	0.25	1.2	–
$MgCl_2$	1.2	–	–	1.2	13	2.2	–
$MgSO_4$	–	–	5	–	–	–	5
Glucose	11	194	–	11	–	11	–
Mannitol	–	–	–	–	60	–	–
Raffinose	–	–	30	–	–	–	30
Lactobionic acid	–	–	105	–	80	–	100
Glutathione	–	–	3	–	3	–	3
Adenosine	–	–	5	–	–	–	5
Allopurinol	–	–	1	–	–	–	1
Histidine	–	–	–	–	30	–	–
Glutamate	–	–	–	–	20	–	–
HES (g/L)	–	–	50	–	–	–	–
PEG20 (g/L)	–	–	–	–	–	30	–
PEG35 (g/L)	–	–	–	–	–	–	1
Osmolality (mOsm/kg)	280	355	320	280	320	320	290
pH1	7.4	7.0	7.4	7.4	7.3	7.5	7.4

Table 2 Composition of different preservation solutions

Hypothermic machine perfusion (HMP)

Hypothermic machine perfusion (HMP) involves the recirculation of chilled preservation fluid through the renal vasculature in the period prior to transplantation. The device used in clinical practice in the UK (LifePort© kidney transporter) delivers pulsatile flow through the renal artery of specialized perfusion fluid (KPS-1®) at a fixed maximal pressure (commonly 30 mmHg).

History of machine perfusion

Ex vivo organ perfusion is not a new concept but has recently been propelled back into the domain of clinical interest for two reasons. Firstly there is a recognition that the quality of deceased donor organs is inferior to previous generations, and therefore the optimisation of organs during the preservation window is paramount for ensuring the best outcome for these marginal kidneys. Secondly, the clinical benefits of HMP have been validated by a high quality multinational randomised controlled trial (28, 29).

The basic technique that facilitated renal transplantation was the vascular anastomosis as described by the French surgeon Alexis Carrell at the beginning of the 20th Century, for which he was later awarded a Nobel prize. Carrell himself, along with the aviator Charles Lindbergh performed multiple large animal transplant experiments, which were largely doomed due to immunological barriers such as HLA incompatibility that would only be described decades later. However, Carrell and Lindbergh were advocates of machine perfusion and felt it was the most physiological way to preserve organs *ex vivo*. The organ preservation system that they described consisted of an organ chamber and mechanical pump that remains

the basis of machine perfusion devices to this day (30, 31).

Further animal studies demonstrated the feasibility of hypothermic machine perfusion of kidneys, with refinements of both machinery and greater understanding of preservation fluids. Groups including the one lead by Arthur Humphries were able to achieve good functional outcomes in a canine model following prolonged perfusion times (32-34). An early machine device is shown in Figure 1.

Much credit for the translation of machine perfusion from the laboratory to clinical practice is owed to teams lead by Folkert Belzer in the 1960's. Belzer found that he was able to achieve excellent graft and animal survival in a canine autotransplant model following 72 hours of machine perfusion (35). This was performed at 6-8°C using an oxygenated autologous plasma based perfusate solution. The problem of hypothermic aggregation was circumvented by ultrafiltration to remove lipoproteins and similar molecules (36). As the perfusion fluids became more sophisticated, plasma was exchanged in favour of albumin as a colloid. Much of this work was performed by Dr Claes in Gothenburg, whose albumin-based solution was far easier to prepare than the plasma ultrafiltrate. Further modifications to their Gambro perfusion machine included surface oxygenation in favour of membrane oxygenation (37). This perfusion technique was utilised with moderate success in the early 1970's (38). Despite recognising the advantages of a synthetic perfusion fluid, including a reduction in disease transmission, early perfusates largely contained human products. Indeed, it was not until the early 1980's that a fully synthetic perfusate solution was developed by Belzer containing hydroxyethyl starch (HES) as the colloid component in place of albumin (39). This was successful in preserving canine kidneys for up to seven days (40, 41).

However, as mentioned earlier, despite some encouraging results, SCS continued to be the organ preservation modality of choice as dramatic improvements in immunosuppressive therapy lead to significant improvements in outcome. Any gains, if any, from the cumbersome, labour intensive, expensive process of machine perfusion were deemed to be minimal by comparison.

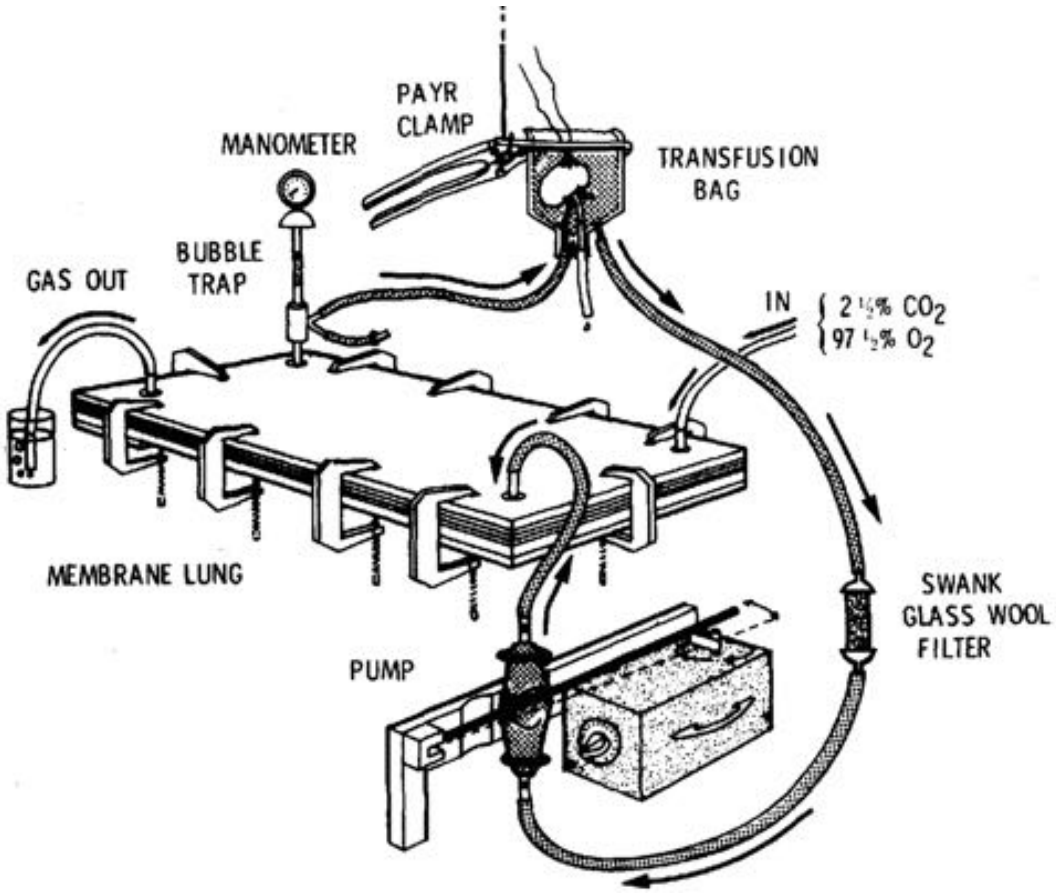

Figure 1 Early example of an oxygenated HMP device

Reproduced from Humphries et al. (32) with permission from Wiley & Sons Inc.

Clinical evidence to support HMP

Although the benefits of HMP are now widely accepted, for several decades there was insufficient evidence to support its use. Whilst there were some reports to the contrary, multiple studies reported no significant difference in the rates of delayed graft function (DGF) between HMP and SCS kidneys (42-45). Similarly, no advantage for one-year graft survival was demonstrated (44, 46) or reduction in primary graft non-function (43, 44, 47).

To provide robust scientific evidence as to the benefits, if any, of HMP in the modern

era of transplantation, a randomised controlled trial (the machine preservation trial) was commissioned and in 2009 the results from this well-designed prospective paired analysis randomised control trial were published. The machine preservation trial group reported reduced rates of DGF (20.8% vs. 26.5%, p=0.05) and improved 1-year graft survival (94% vs. 90% p=0.04) for consecutive kidneys preserved with HMP compared to the contralateral organ preserved with SCS (28). In a thorough meta-analysis published in 2012, which included the machine preservation trial results, the use of HMP was supported for reducing the incidence of DGF post transplantation (48) but was unable to identify superior graft survival from the studies analysed. However, follow on findings reported after three years found that overall, 3-year graft survival was better for machine-perfused kidneys (91% vs. 87%; adjusted hazard ratio for graft failure, 0.60; P=0.04) (29). This study provided the most robust evidence to date to support the usage of HMP in kidney transplantation. However, whilst clearly demonstrating that HMP can be utilized in kidney transplantation with beneficial effect, there are several important points regarding the study that warrant further elaboration. Firstly, the kidneys in this study were randomized to either the SCS or HMP group, and if allocated to the HMP group, this was commenced at the retrieval centre itself. This differs drastically to transplant practice in the UK whereby all kidneys are placed in SCS conditions at the retrieval centre and then transferred to the recipient centre. Thus HMP occurs after a period of SCS organ preservation, which can be protracted depending on the distance travelled. Therefore the superiority of HMP following SCS over an equivalent period of SCS was not determined in the machine perfusion study and this warrants further investigation (see discussion and future work Chapter 10). Secondly, the SCS

and HMP kidney groups in the machine perfusion study had equivalent durations of CIT prior to transplantation (median 15.0 hours). Therefore, the study does not help the clinician faced with the decision of whether to commence HMP when a kidney arrives at the recipient centre. Whether it is better to implant the kidney as soon as it arrives and therefore keep CIT to a minimum or whether it is advantageous to commence HMP at this point and effectively prolong the CIT is unanswered by the machine perfusion study. Furthermore, if a period of HMP is advantageous, the optimal time for which organs should be perfused is also not established. This poses an interesting question from a mechanistic perspective regarding HMP, namely whether HMP results in actual improvement in organ function in the pre-transplant period or whether it merely decelerates the destructive processes that occur once the organ has been removed from the donor.

Interestingly, the results for DCD kidneys undergoing HMP are less clear than for DBD organs. Indeed subgroup analysis based on the machine preservation trial failed to demonstrate a graft survival advantage for DCD kidneys undergoing HMP (29). This finding was further verified by a UK-based randomised multicentre paired trial which found no advantage for HMP in DCD kidneys compared with cold storage (49). The reasons for this are unclear and warrant further investigation.

Mechanisms of HMP

Whilst the clinical benefits of HMP are now supported by high quality evidence, the mechanism behind these benefits are less clear and is likely to be multifactorial. In a review article by Taylor in 2010, he listed three probable major physiological benefits of HMP over static cold storage as well as a further seven potential, financial or

logistical benefits (50) (Table 3).

"*Maintains the patency of the vascular bed*

Provides nutrients, and low demand O_2 to support reduced energy demands

Removes metabolic by-products and toxins

Provides access for administration of cytoprotective agents and/or immunomodulatory drugs

Increases available assays for organ viability checks

Can facilitate change from emergency to elective surgery with reduced costs and improved outcomes

Improves outcomes as demonstrated by reduced Primary Non-Function and Delayed Graft Function

Permits use of expanded criteria kidneys, or organs from non-heart-beating donors to increase donor pool

Economic benefit for transplant centers & reduces health care costs

Provides technology for ex vivo use of non-transplanted organs for pharmaceutical research."

Table 3 Beneficial effects of HMP (Taylor 2010)

Commercially available HMP machines

There are several commercially available HMP machines; the LifePort© Kidney Transporter (Organ Recovery Systems) (Figure 2), RM3 (Waters Medical Inc.) (Figure 3), and the Organ Assist devices. The LifePort© is the device utilised in clinical practice in the UK and is approved by the UK body the National Institute for Health and Care Excellence (NICE) (25). The key mechanical difference between the LifePort© (and Organ Assist) and the RM3 is that the latter is a flow-driven device (i.e. flow rates can be set), in comparison to a pressure-driven device. Flow through the kidney is directly proportional to the driving pressure and inversely proportional to the resistance (flow = pressure / resistance). Therefore when perfusing a high resistance kidney, the flow-driven RM3 device would deliver fluid at a high pressure

in order to meet the flow demands. Although perfusion pressures during HMP are largely subphysiological, there is some evidence that high pressure can have deleterious consequences. Maathuis et al. performed a porcine autotransplant experiment comparing the effect of two different pulsatile perfusion pressures (60/40 mmHg and 30/20 mmHg) versus cold storage in a hypothermic, oxygen supplemented model (51). Levels of von Willebrand factor (vWF) and monocyte chemotactic peptide-1 were higher in the 60/40 mmHg perfused kidneys compared to 30/20 mmHg group. The authors postulated that increased shear stress at these pressures induces endothelial injury release of vWF and could lead to graft thrombosis (51).

Conversely, LifePort© delivers perfusion at a constant pressure (adjustable between 30-60 mmHg). Whilst this would avoid potential endothelial injury from a high perfusion pressure within a high resistance organ, the flow would be limited, and thus the beneficial effects of perfusion would likely be diminished.

In a small prospective randomised trial comparing the RM3 flow-driven device and LifePort© pressure-driven device the authors concluded that the pressure-driven system gave superior results. However, there was no significant difference in rate of DGF or renal function between the two preservation methods (52).

Figure 2 LifePort© Kidney Transporter 1.0 (Organ Recovery Systems)

Figure 3 Walters RM3 Renal Preservation System

Clinically used fluids for HMP

The fluid licensed for use with the LifePort© hypothermic machine perfusion device is Kidney Perfusion Solution-1 (KPS-1®). In many ways, this resembles UW solution, and is also known as UW machine perfusion solution (UW-MPS). However, there are several key differences between conventional UW and the solution used for HMP. Firstly, KPS-1® has an extracellular-like Na^+/K^+ balance in contrast to the intracellular concentrations in UW and Eurocollins. Secondly, raffinose and lactobionate which are present in UW as impermeants have been substituted with gluconate and mannitol. Glucose is also included in the KPS-1® solution at a concentration of 10 mM (Table 4). The role of impermeants in preservation fluids is discussed further below.

Constituents	**Amount/1000 mL**	**Concentration, mM**
Calcium chloride (dehydrate)	0.068 g	0.5
Sodium hydroxide	0.70 g	
HEPES (free acid)	2.38 g	10
Potassium phosphate (monobasic)	3.4 g	25
Mannitol (USP)	5.4 g	30

Glucose, beta D (+)	1.80 g	10
Sodium gluconate	17.45 g	80
Magnesium gluconate D (–) gluconic acid, hemimagnesium salt	1.13 g	5
Ribose, D (–)	0.75 g	5
Hydroxyethyl starch (HES)	50.0 g	n/a
Glutathione (reduced)	0.92 g	3
Adenine (free base)	0.68 g	5
Sterile water for injection	To 1000 mL volume	n/a

Table 4 Concentrations and constituents of KPS-1® perfusion fluid.

Source: http://www.organ-recovery.com/kps-1

Histological changes following HMP

Maathuis et al. were unable to determine any histological differences between machine-perfused and cold stored porcine kidneys using light microscopy (20x magnification) following haematoxylin and eosin and periodic acid-Schiff staining (51). Renal cortical biopsies were compared with control pigs using an ischaemia reperfusion injury scale (IRI) (53). Markers of injury include mesangial matrix expansion, brush border loss in proximal tubule, oedema, tubular dilatation, tubular cell vacuolisation, necrosis, endothelial cell oedema and interstitial fibrosis. Although the time points used for analysis are not well defined in the manuscript, the authors report that the IRI scores were low across both study groups and that there were no significant differences between the HMP and cold stored kidneys (51). Similarly, there was no difference seen on light microscopy of porcine kidneys after either 4 or 28 hours in a paired SCS HMP models as reported by my predecessor Ms Guy (54).

Metabolic support during organ preservation

The concept that organ preservation is harmful is ingrained into the practice of organ transplantation. Damage limitation of these destructive processes is achieved through hypothermia (thus restricting any cellular metabolism) and minimising the duration of preservation (i.e. the cold ischaemia time). Even with the advent of HMP, cold ischaemia times are still kept to a minimum though the optimal duration of machine perfusion has not been established.

The metabolism within the kidney is thought to be halved for every 10°C decrease in temperature with only 5-8% of normal metabolic function occurring at 4°C (20), with a similar decrease in oxygen requirement (36). This deceleration of cellular metabolism during hypothermia is though to preserve organ ATP reserves and limit the accumulation of harmful by-products such as lactic acid.

However, hypothermia does not cause a uniform deceleration of all metabolic pathways (50) and furthermore, the effect on cellular metabolism of hypothermia within different organs is not uniform (55). If indeed certain enzymes are particularly thermo-sensitive, then manipulation of such pathways may offer a mechanism for protective metabolic support.

There are surprisingly few recent reports detailing renal metabolism during either SCS or HMP conditions and, as discussed, is likely to reflect the belief that such metabolic activity during organ preservation is deleterious. Indeed the beneficial effects of HMP were largely thought to be mechanical, with the potential benefits of metabolic support during HMP only reported relatively recently (50, 56).

The concept that preservation is an intrinsically harmful process is in contrast to the scientific approach to other biological systems whereby cells can be supported in a

hypoxic media environment for extensive time periods. The metabolic simplicity of currently used perfusion fluids, which have been designed principally to prevent cellular lysis (through osmotic and pH stability) and maintain transmembrane electrolyte balance, is in stark contrast to cell culture fluids which are designed with the intention of supporting all of the metabolic needs of the cell in question. A typical composition of cell culture fluid is listed in Table 5. In addition to the salt solution and buffers present in perfusion fluids, cell culture media typically consist of essential and non-essential amino acids, growth factors and hormones (e.g. insulin). These media can be serum-free or serum-based, and there are advantages of each (57).

Several groups have aimed to preserve kidneys with perfusion fluid more akin to culture media in order to provide necessary nutrients and facilitate metabolism. In a canine autotransplant model, Brasile et al. used ischaemically damaged kidneys and either re-implanted them immediately or subjected them to sub-normothermic (32°C) oxygenated machine perfusion with their culture media-like fluid before autotransplantation. The dogs in the perfusion group all survived in comparison to the non-perfused group in whom a rapid postoperative rise in creatinine was observed (58). This study demonstrated the principle that, provided adequate nutritional support is present, organ preservation can improve the function of damaged organs and is not merely a damage limitation exercise. Subsequent studies have suggested that prolonged preservation times (up to 48 hrs) can be achieved and that growth factors such as fibroblast growth factor (FGF-1 and FGF-2) also improved the outcomes in this canine model (58, 59).

In a non-oxygenated porcine HMP model, Baicu et al. measured concentrations of

isolated metabolites in the perfusion fluid over 72 hours (60). Although the perfusate used was replaced every 24 hours, they demonstrated an increase in perfusate glutamate concentrations during perfusion with both UW-MPS (KPS-1®) and their own solution (Unisol-UHK). A similar rise in the perfusate ammonium ($NH4^+$) levels was noted although less pronounced in the UW-MPS (KPS-1®) group. The suggested mechanism for the increase in glutamate was the reduction (and deamination) of glutamine to glutamate by glutaminase (releasing ammonium as a by-product). The source of glutamine in both fluids was thought to be glutathione (reduced) which had been largely oxidised in the UW-MPS group by the time of fluid usage and resulted in lower levels of glutamate in this group (60-62). The authors also conclude that the deamination of glutamate occurs (releasing ammonium) to form alpha-ketoglutarate, an important TCA cycle intermediate (60).

In contrast to the renal setting, there have been extensive attempts to describe hepatic metabolism during machine perfusion (63). Whilst such studies are useful, caution should be exercised when comparing these two systems (liver and kidney) during isolated *ex vivo* single organ perfusion, not least as both have independent metabolic and excretory properties. Further comparisons are weakened as liver perfusion systems are largely oxygen enriched, which is not the case with HMP used in clinical practice (64-67). However, in both human and animal models, hypothermic liver perfusion has been shown to increase ATP levels (64-68) and aid glutathione recovery (65, 67).

Considerations for HMP metabolic studies

Evaluation of kidney metabolism during preservation using perfusate appears attractive. The HMP device widely used in clinical practice (LifePort© Kidney

Transporter 1.0 (Organ Recovery Systems, Chicago, IL)) has a designated sampling port to remove samples of perfusion fluid or perfusate in an easy and sterile fashion. Furthermore, the volume of fluid required for most metabolic analysis is small (several ml) in comparison to the volume of circulating fluid (1 L). However, a major limitation of using perfusate as a tool for organ metabolism assessment is that it determines the concentration of metabolites in perfusion fluids circulating through the intravascular environment of the kidney. Metabolic conditions within the interstitial extracellular fluid and more importantly the intracellular compartment may be quite different. The inference of the intracellular environment from perfusate concentrations is therefore limited, and would depend on the cellular permeability for that specific metabolite amongst other factors. For organs preserved via a SCS method, analysis of the fluid bathing the kidney is even less useful as it is very unlikely to represent the intrarenal environment. Alternatives to perfusate have been suggested including analysing the metabolites in fluid obtained via a microdialysis catheter inserted into the extracellular interstitial space (69) (Figure 4). However, there are also limitations of this approach as the metabolic microenvironment surrounding the catheter is altered as a consequence of the dialysis process itself.

Determination of intracellular metabolism would seem to be imperative in understanding the complex process occurring during organ preservation. Cell extraction of tissue samples is the best described way of doing this and various cell extraction techniques have been proposed for this purpose. Largely these involve the cessation of metabolism via rapid freezing of tissue (-80°C) and mechanical homogenisation in the presence of methanol. However thorough metabolic analysis often requires a reasonable mass of tissue (e.g. 1 g) in order to perform the multiple

analyses to establish metabolite type, concentration, and flux. Whilst 'metabolomic' modalities such as nuclear magnetic resonance (NMR) spectroscopy analysis have enhanced this process, some metabolites are technically difficult to distinguish on conventional ^{1}H-NMR (e.g. pyruvate). In addition, tissue biopsy during HMP is problematic as removal of tissue from this pressurised system results in perfusate leak through the biopsy site and decrease in the intrarenal resistance. In effect, only one large volume tissue sample is achievable during HMP without jeopardising the metabolic integrity of the system. This is one of the limitations of whole organ metabolic HMP studies and is one of the reasons that we propose a HMP cell line model to complement experimental work (Chapter 10). The problems of fluid leakage are not encountered in the clinical setting if tissue biopsy occurs at the end of HMP, as once the kidney is reperfused with blood following transplantation, activation of the clotting cascade rapidly 'plugs' any tissue discontinuity. Repeated biopsy on the same kidney during SCS would appear less problematic, although following biopsy and breach of the capsule, an inflow and efflux of metabolites into the bathing fluid would occur, which could confound subsequent samples.

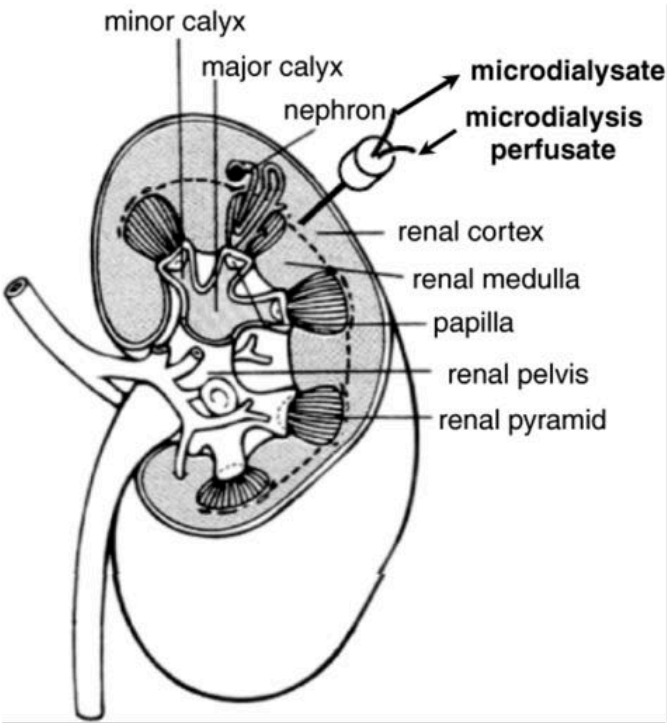

Figure 4 Placement of microdialysis catheter during HMP.

Reproduced from Baicu et al. (69) with permission from Wiley & Sons Inc.

Inorganic Salts	**Amino acids**
Calcium Chloride	L-arginine•HCl
Ferric nitrate	L-cystine•2HCl
Magnesium sulphate (anhydrous)	Glycine
Potassium Chloride	L-histidine•HCl•H_2O
Sodium bicarbonate	L-isoleucine
Sodium chloride	L-leucine
Sodium phosphate monobasic (anhydrous)	L-lysine•HCl
Vitamins	L-methionine
Choline chloride	L-phenylalanine
Folic acid	L-serine
Niacinaminde	L-tryptophan
D-pantothenic acid (hemicalcium)	L-Tyrosine•2Na•2H_2O
Pyridoxal•HCl	L-valine
Pyridoxine•HCl	**Other**
Riboflavin	D-glucose
Thiamine•HCl	Phenol red•Na

Table 5 Example of culture fluid composition

Perfusate biomarkers

Although great advances have been made in the field of transplantation, to date, there remains no single reliable pre-transplant predictor of post-transplant graft function. This is of increasing importance in the current era whereby marginal donor kidneys are being increasingly utilised in an attempt to address the problems of organ shortage (70-73). As such, there is a clinical need for a reliable test to determine the 'usability' of such marginal organs. Although scoring systems have been proposed for this purpose, often using histological parameters from a kidney biopsy (74), they are not used widely. In practice, donor information such as patient age, comorbidity and pre-mortal creatinine is used to judge the 'transplantability' of a kidney, often in conjunction with any histological information. However even this combined approach has a limited capacity for accurate prediction of graft outcomes (75, 76). Furthermore, clinically used measurements for the monitoring of post-transplant graft function also have limitations as rejection can occur in the presence of normal creatinine values and structural changes on renal biopsy only present after significant injury has occurred (77).

The identification of a biomarker during the pre-transplant period would be attractive and useful but to date has proved elusive. HMP seems well suited for biomarker analysis as perfusate samples are readily available, non-invasive and should offer insight into the cellular components within the organ. Furthermore, machine perfusion also provides the opportunity for repeated measurements, which may predict graft function with greater accuracy. Low intrarenal resistance during perfusion has been shown to correlate with improved post transplant function, but several studies have demonstrated that organs should not be rejected solely on flow

dynamic information, which strengthens the need for a perfusate biomarker (78-81). In a systematic review by Bhangoo et al. (82), there was found to be a paucity of high quality HMP biomarker studies. Elevated levels of glutathione-S-transferase (GST), lactate dehydrogenase (LDH) and aspartate transaminase (AST) were significantly associated with delayed graft function in the majority of studies. AST is expressed from injured renal parenchymal cells. GST is a marker of renal tubular injury. LDH is a non-specific marker of cellular injury (82). However, there was insufficient evidence to recommend any single parameter as a sensitive pre-transplant biomarker. Given the complex nature of whole organ metabolism during *ex vivo* perfusion a combination of biomarkers, or biomarker panel may be more sensitive than any metabolite in isolation and this concept is further explored in Chapter 5.

Oxygenation

The purpose of *ex vivo* oxygenation during organ perfusion is to deliver an adequate amount of oxygen at a cellular level to support aerobic metabolism and prevent ischaemic injury. Oxygenation of organs during the preservation period would seemingly provide a more physiological storage environment. However, the benefit, if any, of oxygenation during HMP is not clear with evidence to date largely inferred from small scale animal experiments (83).

Aside from the technical challenge of oxygen delivery, there are concerns that unregulated oxygenation may pose risks to the perfused kidney (56). High levels of oxygenation have been shown to result in the formation of free radicals leading to tissue injury (84). Furthermore, specific protective mechanisms such as the hypoxia inducible factor (HIF) pathway are active in ischaemic conditions with concerns that supplemental oxygenation may ameliorate such beneficial processes.

The role of oxygenation during HMP for transplant kidneys is further explored in Chapter 9.

Oxygenators

The technology involved in *ex vivo* oxygenation has been largely driven by the development of cardiothoracic surgery and the need for cardiopulmonary bypass. The ambition to replicate the physiological process by which carbon dioxide is removed from blood and oxygen is delivered through diffusion at the alveolar membrane led to the evolution of modern day oxygenators as it was quickly established that direct oxygenation of blood *ex vivo* resulted in blood trauma and the potential for gas emboli (85). The early bypass circuits used bubble oxygenators, but these have been superseded by modern membrane oxygenators through which

gaseous diffusion occurs across membrane micropores (85). Indeed modern day perfusion experiments largely use membrane oxygenators to achieve the required oxygen tensions within the perfusate. The oxygenator incorporated into the OrganAssist device as well as the oxygenators used in this thesis are membrane oxygenators.

Oxygen carriers

Whilst red blood cells containing haemoglobin are the natural, and probably most efficacious oxygen carrier, there are a variety of interesting synthetic oxygen carriers available, which could potentially be useful during organ perfusion. Perfluorocarbons (PFCs) are stable synthetic compounds containing fluorine. They have a high affinity for gases such as oxygen, but themselves are insoluble in water and need to be emulsified in a surfactant (86). PFCs have been used in animal machine perfusion experiments as an oxygen substitute (87). Pyridoxylated haemoglobin polyoxyethylene (PHP) is a form of haemoglobin that has been pyridoxylated, stabilising it for use in the absence of red cells and has also been used in perfusion models (59, 88). Several other oxygen carrier molecules have been used for perfusion studies including Hemarina-M101, which is an extracellular form of haemoglobin derived from a marine invertebrate. This product has proven to be efficacious at oxygen delivery over a range of temperatures and has been used in a pig autotransplant model during HMP with positive results (89). The advantages, if any, of oxygen carriers over active oxygenation of perfusion fluid without such carriers has not been established.

Free radical formation

Free radicals are atoms or groups of atoms with unpaired electrons. These can have

a positive, neutral or negative charge. When such free radicals are derived from oxygen, they are termed reactive oxygen species (ROS) and includes the superoxide ($O_2^{\bullet -}$), peroxide ($O_2^{\bullet 2-}$), and hydroxyl radical ($^{\bullet}HO$).

The formation of ROS is a normal physiological phenomenon and occurs as a consequence of the electron transport chain within cell mitochondria. A common property of free radicals is that they are highly reactive. Whilst some of these reactions can be protective, there are also many deleterious effects of unregulated ROS. The toxic effects of ROS are well described in damaging the phospholipid layer of the cell membrane, a process known as lipid peroxidation (90). To counter the harmful effects of ROS, a parallel system of antioxidants has evolved to protect cells from oxidative stress including enzymatic and non-enzymatic processes.

Enzymatic antioxidants include superoxide dismutases (SOD), which catalyse the conversion of superoxides into the more stable compounds of hydrogen peroxide and oxygen. This reaction is depicted in Figure 5. Upregulation of SOD occurs in the presence of high oxygen concentrations, and its potential beneficial antioxidant effects have been exploited in human studies. In a prospective randomised kidney transplant trial, 200 mg of SOD given intraoperatively was found to significantly reduce the incidence of postoperative acute rejection (91). However, these beneficial effects were not observed when a similar study using SOD was performed on a different cohort (92).

Another important antioxidant is the tripeptide glutathione, composed of glutamic acid, glycine, and cysteine (Figure 6). This contains an exposed sulphydryl group that is highly reactive and therefore a target for free radical interaction, resulting in oxidation (Figure 7). Reduced glutathione is a central component of the

preservation solution UW-MPS/KPS-1®, and studies have demonstrated inferior graft function following preservation of kidneys in the absence of glutathione (93).

Activation of antigen presenting cells via a ROS mediated process during kidney ischaemia and reperfusion is thought to be central to the development of acute rejection (94, 95). The accumulation of ROS precursors occurs as a result of ischaemia. Based on a tourniquet limb model of ischaemia reperfusion, Freidl et al. postulated that the mechanism for this is largely through the utilisation of intracellular ATP, resulting in the increase in ADP levels which are then catabolised into hypoxanthine (and inosine and adenosine) (96). Levels of xanthine oxidase were found to increase through conversion from xanthine dehydrogenase via calcium-dependent proteases that are activated in the hypercalcaemic environment resultant from ischaemia (96). The oxidation of hypoxanthine and xanthine during reperfusion is thought to be the principal pathway during this ischaemic process (90).

Figure 5 Enzymatic effect of superoxide dismutase on the superoxide radicals

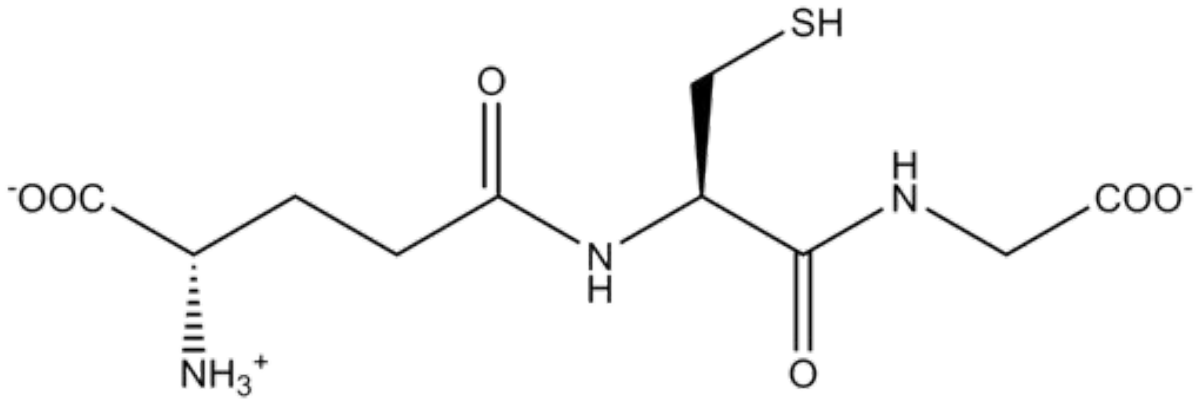

Figure 6 Chemical composition of reduced glutathione molecule

By Jjexpat - Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=49205211

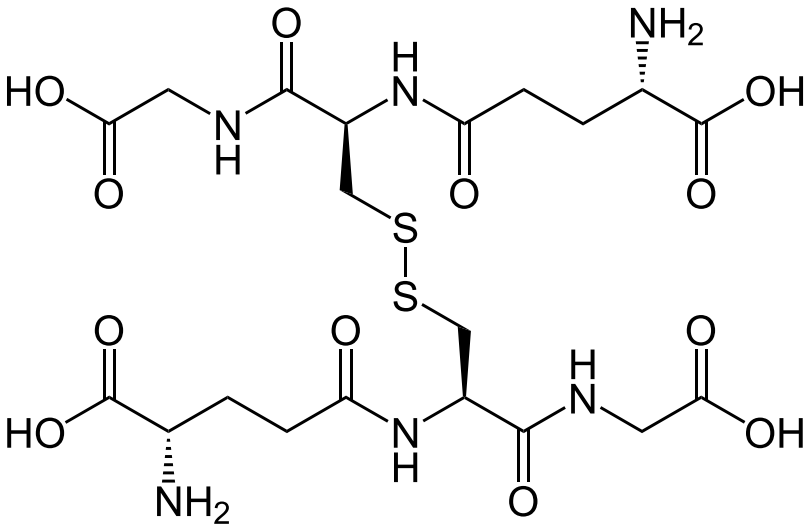

Figure 7 Chemical composition of oxidised glutathione

Hypoxia inducible factor (HIF)

Hypoxia inducible factors (HIF) are a family of oxygen sensitive heterodimeric transcription factors that play a major role in the cellular response to periods of hypoxia (97). Given the significant periods of both hypoxia and ischaemia during the organ donation and preservation period as well as the subsequent ischaemia-reperfusion injury, the HIF pathway may provide a target for organ optimisation.

The hypoxia inducible factor (HIF) pathway is activated as part of the cellular

response to hypoxia and exerts its beneficial effects via multiple mechanisms. Upregulation of the HIF pathway has been shown in an animal model to reduce ischaemia reperfusion injury (IRI) (98-100) which is known to be deleterious during renal transplantation and is associated with an increased risk of delayed graft function (DGF) and reduced graft survival (101). Furthermore, in hypoxic conditions, activation of the HIF pathway is associated with upregulation of mechanisms that facilitate glycolysis such as facultative glucose transporter-1, aldolase A, phosphoglycerate kinase 1 and pyruvate kinase M (102).

The increased delivery of glucose to the intracellular environment and subsequent glycolysis to produce ATP (103, 104) would be seemingly beneficial during HMP given the glucose rich environment of clinically used perfusion fluids (KPS-1® /UW-MPS) in the absence of oxygen carriers or exogenous oxygen.

Findings from animal studies indicate that HIF pathway activation is not exhausted even after total mechanical ischaemia (such as during DCD retrieval conditions) (98). Given that there are potential benefits to further HIF stimulation following organ retrieval and HMP can deliver pharmaceutical agents to the cells within an organ, it is perhaps surprising that there are no previous reports detailing the role of pharmacological HIF pathway stimulators during HMP.

HMP provides a novel opportunity to pre-condition organs prior to transplantation. Given these two potentially protective mechanisms of HIF activation during organ preservation, namely amelioration of the IRI phenomenon and promotion of glycolytic activity, the HIF pathway appears to be a target for metabolic optimization during the organ preservation period.

Metabolomics and NMR spectroscopy

Metabolomics is the identification and quantification of the complete set of metabolites, or metabolome, within a biological system (105). The complete number of human metabolites present (or human metabolome) is not known, but there have been over 40,000 identified to date (106). Metabolites are small molecules (<1500 Daltons) which participate in general metabolic reactions that are required for the maintenance, growth and function of a cell (107). The metabolites within a system reflect intrinsic biochemical activity, which is known to be highly sensitive to environmental factors. Metabolomics, like other 'omics' studies such as genomics and proteomics, have been the subject of great scientific interest in recent years. NMR spectroscopy is a valid method for identification of constituent metabolites within a biological sample, with proponents highlighting the highly sensitive, reproducible nature of this technique (7). Metabolic pathways are reflective of the genome and proteome with up to 10,000-fold increase in metabolite concentration resulting from a single amino acid change in a protein or base change in a gene (108). Furthermore, metabolic changes are apparent within minutes of a biological event and therefore provides an almost 'real-time' feedback (105). Whilst NMR metabolomics experiments have been described since the early 1980s (109), the development of high throughput NMR spectrometers with the necessary software for analysis has been relatively recent and has greatly improved its scientific utility (105). Indeed, the development of modern NMR spectrometers combined with the set-up of standardized sample preparation and acquisition protocols (110) have enabled the analysis of thousands of molecules with high sensitivity and reproducibility within a few minutes (111).

Whilst conventional metabolomic analysis provides an excellent snapshot of the biochemical milieu at a given time point, interpretation of pathway activity is difficult, given that most metabolites can be the product of multiple precursors. Metabolic flux analyses have been used successfully to determine pathway activity within isolated cell lines. However, the applicability to complex whole organ systems with structural and biochemical heterogeneity is limited and introduces large errors into the metabolic modelling software programmes. Isotopic tracer studies have been developed in order to gain mechanistic information of biological progression within a system, which is much more applicable to whole-organ analysis. Such isotopic metabolomic studies have been undertaken in the human clinical setting and are discussed in further detail below.

There are two commonly employed techniques for performing metabolomic analysis, namely nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). A comprehensive review of the physico-chemical principles of each modality and their relative merits is outside the scope of this thesis, but a summary of the modality (NMR) used throughout this thesis is given below. Needless to say, there are limits to both techniques but when used in conjunction they can provide a reliable, highly sensitive picture of the type, concentration and biochemical structure of a given compound.

NMR spectroscopy is a technique dependent on the magnetic properties of specific atomic nuclei. The nuclei that are most commonly exploited for NMR analysis (e.g. ^{1}H, ^{13}C, ^{15}N, ^{31}P) have an imbalance of protons and neutrons and therefore impose a small magnetic field from their spinning nuclei. NMR metabolomic techniques have been used to gain clinical insight into a multitude of pathological conditions, ranging

from cancer (112) to neurodegeneration (113) and a full range of the clinical applications of NMR is outside the remit of this chapter.

Mechanical and quantum principles of NMR spectroscopy

In the absence of an external magnetic field, the nuclei of 'NMR detectable' atoms are not aligned in any preferential direction and therefore have no net 'lie' or magnetisation vector (Figure 8). However, when in the presence of an orientated homogeneous external magnetic field, the nuclei align themselves either parallel or antiparallel to the direction of the field lines, with a slight preference for nuclei aligned in the lower energy parallel direction (Figure 9).

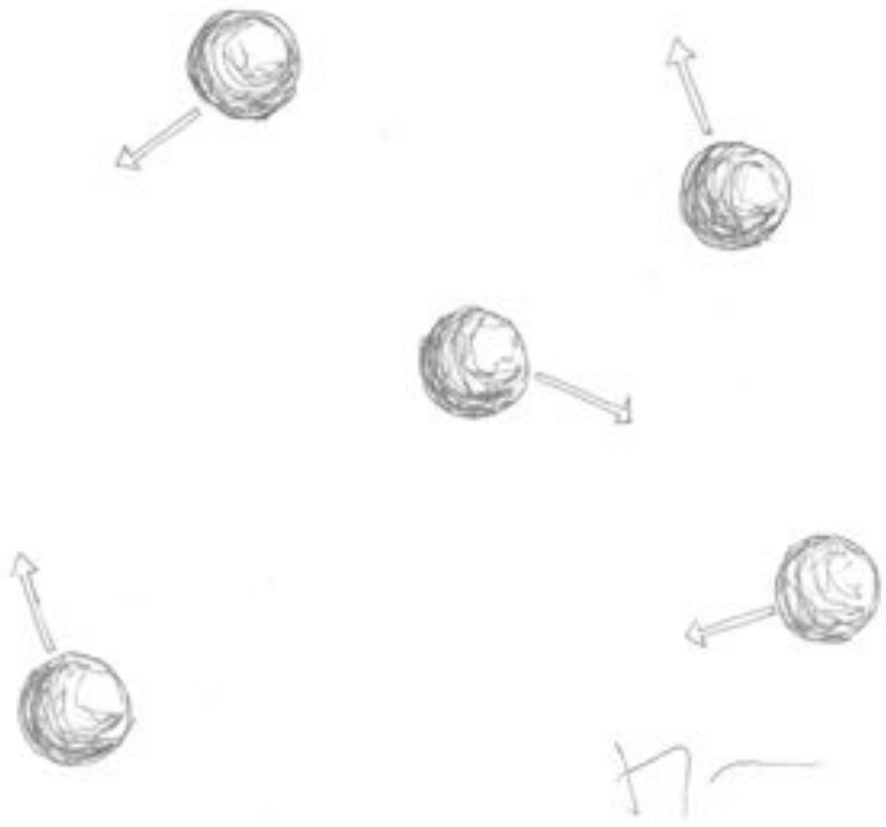

Figure 8 Alignment of ^{1}H nuclei in the absence of external magnetic field

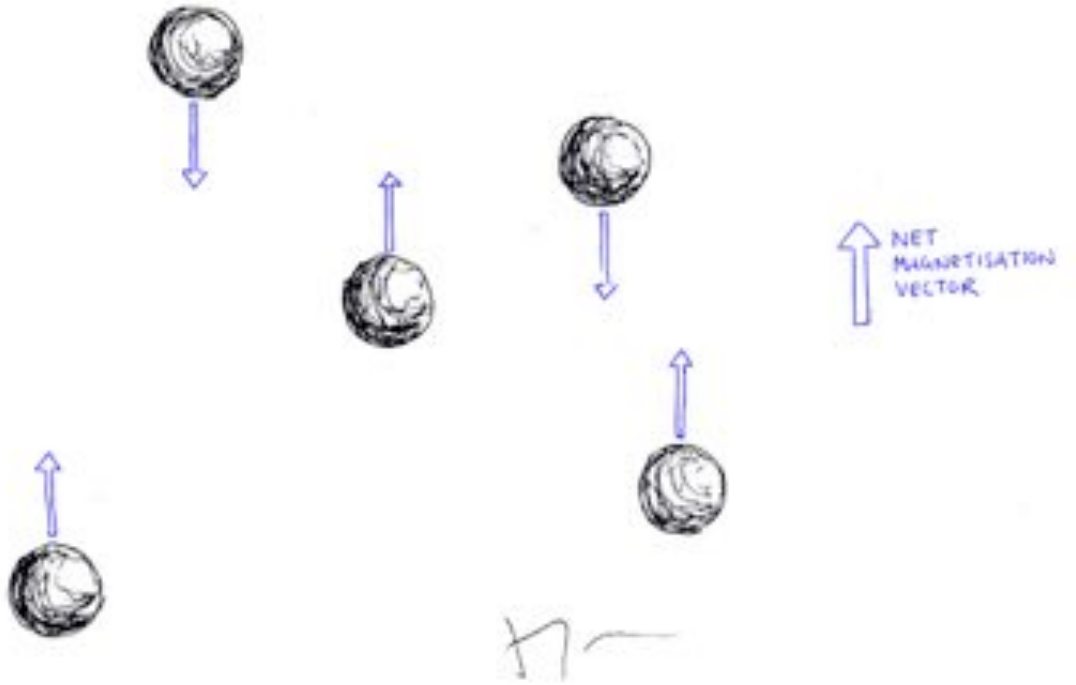

Figure 9 Magnetisation vectors of ^{1}H nuclei in an external magnetic field

As nuclei carry charge, a spinning nucleus possesses a magnetic moment ($\boldsymbol{\mu}$) which is a product of the magnetogyric ratio (γ) and the angular momentum (P) such that:

$\boldsymbol{\mu} = \gamma P$

Although the net magnetisation of any single nucleus over time is parallel or antiparallel to the field lines, the individual nuclei actually precess around this magnetic axis, much like a spinning top in the earth's gravitational field. This circular motion or precession rate is dependent on the magnetogyric ratio (γ) which is constant for any given nucleus and is a measure of how 'strongly magnetic' it is (114). Precession rate is also proportional to the external magnetic field strength (B_0) such that precession rate (v_0)

$v_0 = \gamma B_0 / 2\pi$

In quantum mechanical terms, all nuclei possess a spin quantum number I, which may be zero (e.g. ^{12}C), half (e.g. ^{1}H) or one (e.g. ^{2}H) or more. Where I=0, the nucleus has no net spin and is therefore not detectable by NMR spectroscopy. For protons, the most widely exploited nucleus in NMR spectroscopy, the spin quantum number I = 1/2 which means that any nucleus can exist in the +1/2 (up or α state) or -1/2 (down or β-state). As discussed, when an external magnetic field is applied the spins align in the direction of the magnetic field (either parallel α or antiparallel β), with slightly more nuclei in the α state compared to the β state. The difference in energy between the α and β states is proportional to the strength of the external magnetic field applied (B_0). Nuclei in the lower energy α state can absorb a discrete photon of energy and transform into the higher energy β state. As the energy required is discrete, this corresponds to a specific frequency of electromagnetic radiation. This energy of the photon (ΔE) is a product of the precession frequency (v_0) and Planck's Constant (h) such that:

$\Delta E = hv_0 = h\gamma B_0/2\pi$

Indeed, the early NMR spectrometers relied on a radio-frequency electromagnetic radiation that was gradually changed in the vicinity of the specimen. When the frequency of radiation correlated to the resonance frequency of a nucleus in the specimen, energy was absorbed (ΔE) and this was recorded as a peak.

Chemical shift

The resonance frequency of a particular nucleus within a sample depends largely on the composition of the nucleus itself. However, due to the surrounding electron bonds, this frequency is also influenced by the chemical environment around it. The

alteration in resonance frequency from the fundamental frequency of the nucleus due to the surrounding electron environment is in the order of parts per million (ppm), and this concept of chemical shift is essential to the identification of compounds in NMR spectroscopy. Thus in ^{1}H NMR spectroscopy, a proton within a methyl group attached to a methylene group has a much smaller chemical shift than a proton within a methyl group attached to an electronegative oxygen containing group.

An NMR spectrum is a graphical demonstration of the shift in resonance frequencies from the fundamental resonance frequency for the nucleus of interest. Each peak within the spectrum is resultant from a proton with resonance frequency different to the fundamental proton resonance frequency and therefore represents a unique chemical environment. Each molecule, therefore, may have multiple distinct peaks in the spectrum; a simplified spectrum for methanol is demonstrated in Figure 11.

Splitting

Close examination of NMR spectra will reveal that many 'peaks' are actually two or more peaks closely spaced. This phenomenon of splitting can be explained by considering two protons (proton 1 and 2) attached to adjacent carbon atoms within an organic compound. The protons attached to the adjacent carbons are close enough to exert a magnetic influence on each other. As all nuclei within a substance exist in either the up (α) or down (β) state, then the magnetic field strength experienced by proton 1 will be either slightly more (external field + α) or slightly less (external field + β) than the external field alone, depending on the magnetic orientation of proton 2, with almost half of the molecules of the substance in each

state. As the resonance frequency (v_0) of a nucleus is proportional to the magnetic field strength experienced by that nucleus, there will be two distinct resonance frequencies for the protons in this example resulting in spectral splitting. In an identical fashion, proton 1 will split the peak of proton two into two distinct peaks. The small frequency difference between the doublet of peaks in this example is known as the coupling constant (J), which is measured in Hz.

For a methyl group (CH_3) the three protons can all be in either the up (α) or down (β) state, resulting in four different perturbations of magnetic influence (all α, all β, two α one β, two β one α). The scenario of two α or two β is the most likely by a factor of three. Therefore a proton on an adjacent carbon is split by the methyl group into a quartet in the ratio of 1:3:3:1 (Figure 12).

Pulsed NMR

During modern NMR analysis, a strong pulsed radio frequency is applied to the sample, perpendicular to the external magnetic field. This is exerted at a single frequency (approximately the midpoint of the required spectrum). The strength of the pulse is such that it exerts a bandwidth phenomenon affecting a range of nuclei. The effect of the strong pulsed wave is twofold. Firstly the nuclei are able to move freely between the α (low energy) and β (high energy) state as the specific energy required (photon) corresponds to the frequency of radiation. Secondly, the precession of the nuclei synchronise such that the net effect of all nuclei is a rotating magnetic dipole in the *xy* plane (Figure 13). NMR spectrometers contain a coil surrounding the sample probe, and much like a dynamo on a bicycle, the rotating magnetic dipole of the 'pulsed' nuclei induces a small electric current in the

surrounding coil. The frequency of the induced current (Hz) is dependent on the rate of precession of the nuclei. As the rate of precession for a nucleus is dependent on the chemical environment surrounding it, each group of protons within a sample with a unique surrounding chemical environment will induce an electric current with a slightly different frequency. The overall signal induced will be a summation of all of the individual signals.

If the RF irradiation was continuous, a continuous alternating current would be induced with a sinusoidal waveform. However, the RF pulse applied to the sample is typically only 10 microseconds for a standard ^{1}H spectrum. Upon cessation of the RF pulse, the precession of nuclei once again become desynchronised with restoration of the α / β discrepancy - a phenomenon known as R1 longitudinal nuclear relaxation. The net magnetisation vector of the nuclei realigns to the external magnetic field with 'petering out' of the induced current, a phenomenon known as the free induction decay (FID).

Fourier transformation

For a given sample, the waveform of the induced current is a summation of all the electrical current produced from all the nuclei with differing resonance frequencies within the sample (Figure 14). To convert this information into something interpretable, Fourier transformation (FT) is applied. This converts the summated waveforms with time and amplitude on the *x* and *y* axis to a waveform with frequency and amplitude on the *x* and *y* axis (Figure 15). The frequency is denoted in parts per million (ppm) and represents the chemical shift from the absolute resonance frequency of a standard reference compound. Each nucleus with a

different magnetic resonance is represented by a peak at a different frequency (or ppm). The number of peaks for a particular compound depends on the number of nuclei within that compound surrounded by a unique chemical environment. The height of each peak, or more precisely the area underneath it, is a function of the concentration of nuclei with that specific resonance and permits accurate quantification of compounds.

The deconstruction of summated rapidly decaying waveforms into individual peaks within the 1D spectrum requires significant computation and can lead to artefact errors from random 'noise'. To minimise this, and improve the signal to noise ratio, this sequence of pulse excitation and FID recording is repeated multiple times for each sample and significantly enhances the sensitivity of this technique.

Of the NMR 'visible' nuclei, ^{1}H the considerably the most sensitive and resultantly accounts for the majority of NMR-based metabolomic studies. ^{13}C studies, by comparison, are 5700 times less sensitive than ^{1}H NMR due to the low natural abundance of ^{13}C (1.1%) and a much lower resonance frequency. The sensitivity is dependent on the natural abundance levels, the magnetogyric ratio of the nucleus in question at start of the pulse sequence times and the magnetogyric ratio of the nucleus acquired factored by effects of probe geometry (e.g. inner versus outer coil).

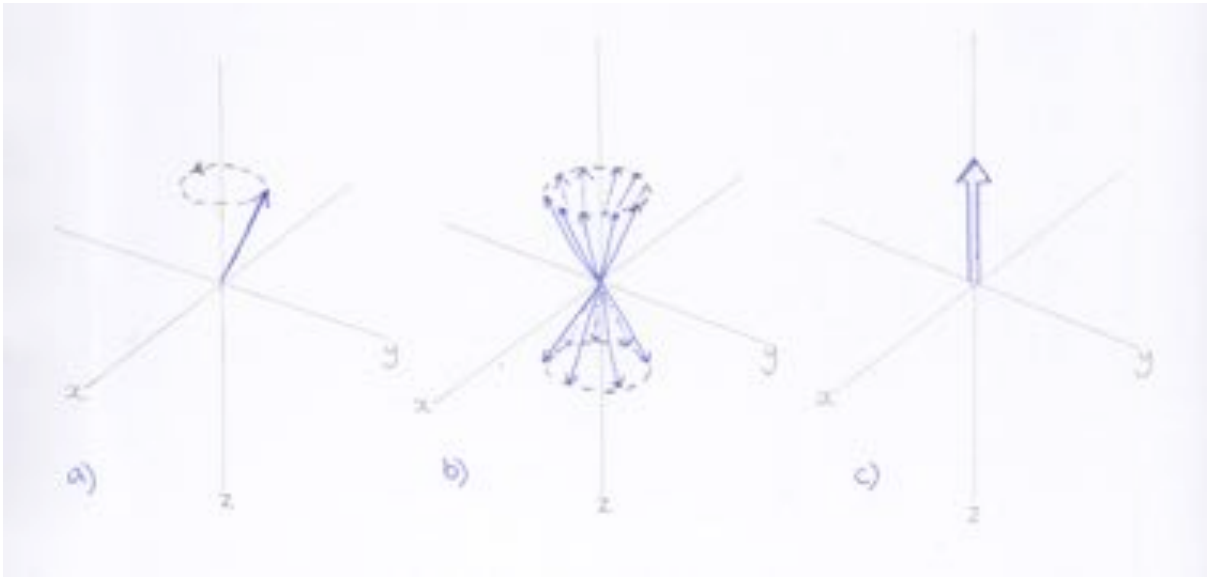

Figure 10 Mechanical vector model demonstrating the magnetisation vector of a single nucleus, multiple nuclei and the bulk magnetisation

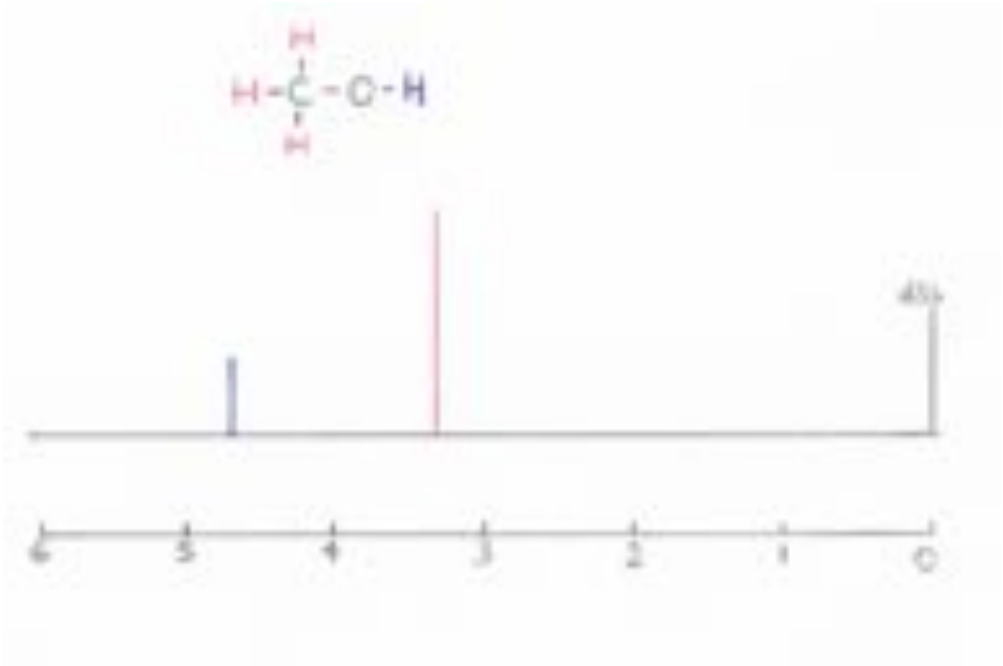

Figure 11 1D ^{1}H NMR spectrum of methanol (schematic)

Sample at 30°C with chemical shift demonstrated (x-axis) The two unique environments of the ^{1}H nucleus produce two peaks on the spectra, with a greater peak at shift location of 3.35 due to the greater number of protons (x3).

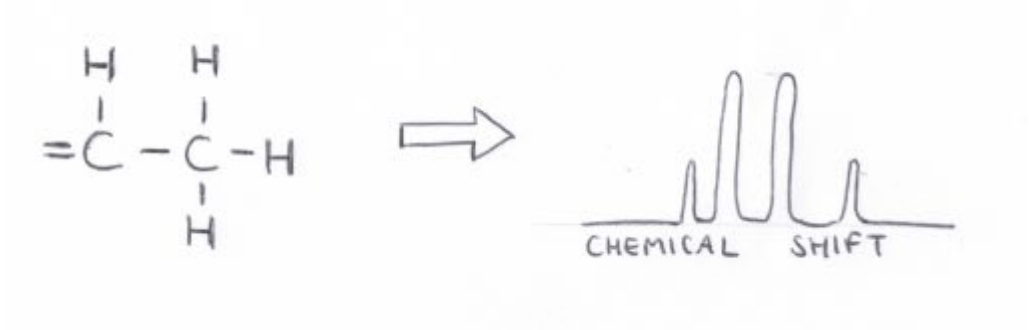

Figure 12 Chemical splitting effect of a methyl group

Chemical splitting effect of the three protons of a methyl group on a proton attached to an adjacent carbon. A quartet pattern is observed with intensity ratio 1:3:3:1

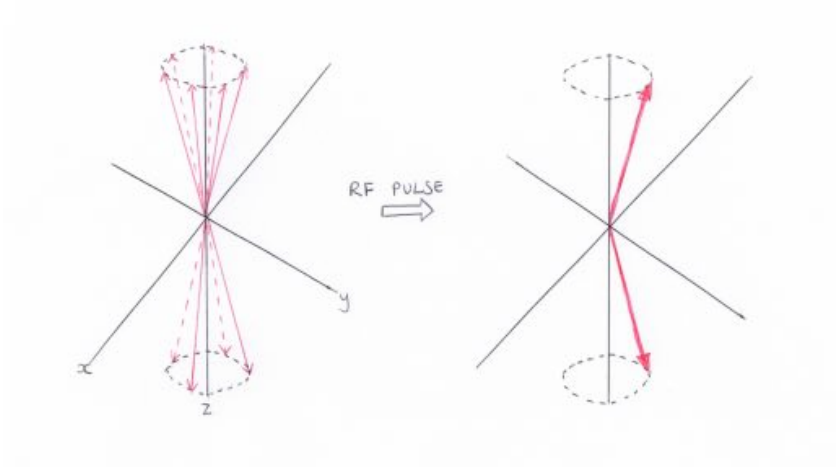

Figure 13 Schematic effects of RF pulse applied to a sample

Equal numbers of nuclei exist in the alpha and beta state following irradiation with synchronisation of precession such that the net magnetic field follows a circular path in the xy plane.

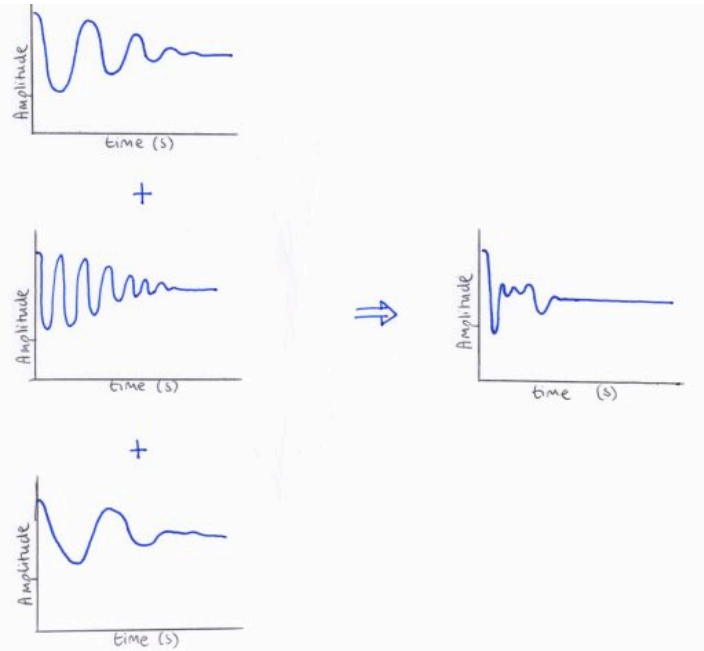

Figure 14 FID waveform

A schematic example of FID waveform (right) as a summation of multiple different ones (three in this example).

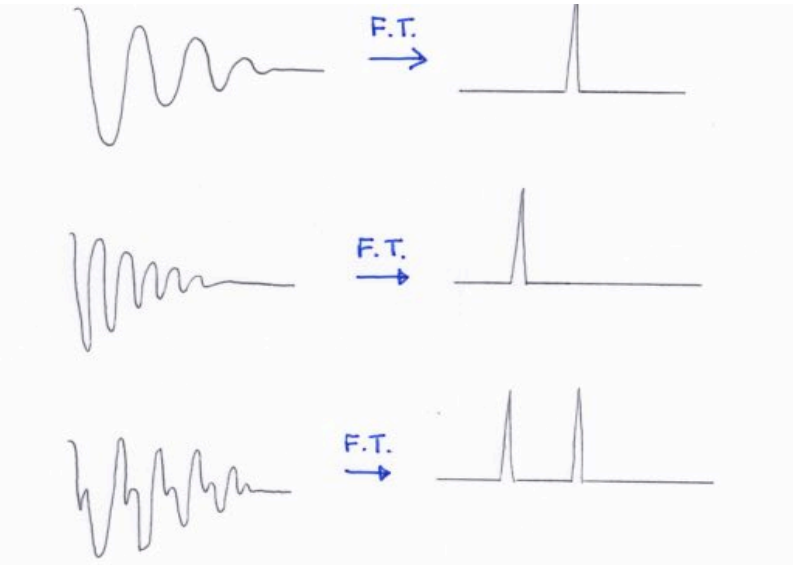

Figure 15 Schematic representation of a Fourier transformation (FT)

A waveform with amplitude (*y*) and time (*x*) is converted to amplitude (*y*) and frequency (*x*) in cycles/sec or Hz. In this example, a summation of two induced currents undergoes FT to produce two individual peaks on the spectrum.

The rationale for 2D NMR spectroscopy

In one dimensional (1D) NMR spectroscopy there is only one time dimension utilised, corresponding to the FID, which undergoes Fourier transformation to produce a typical NMR spectrum.

One of the great limitations of 1D NMR spectroscopy is that it can be difficult to distinguish between compounds if the nuclei within them have similar chemical shifts. This is particularly problematic for smaller compounds whereby there are few peaks on the spectrum to utilise and particularly when present in low concentrations and near to other compounds with a resonant frequency at high concentrations.

Two dimensional (2D) NMR spectra can be extremely helpful in determining the structure of compounds which have spectra that overlap. In 2D NMR spectroscopy, two distinct time dimensions are involved. Essentially there is a variable wait time between initial RF pulse and a subsequent one. This wait time effects the FID signal generated and hence, variation of the wait time produces a range of FID signals.

There are two different classes of 2D NMR spectroscopy experiments: correlation experiments and Overhauser effect experiments. Correlation experiments provide information regarding the connections or coupling within a molecule. Overhauser effect experiments provide information on spatial proximity within a molecule.

Experiments contained within this thesis utilise one type of correlation spectroscopy: heteronuclear single quantum coherence (HSQC). However, needless to say, multiple types of 2D spectroscopy can be performed in the analysis of any sample and can give complementary information to determine the quantity and structure of a compound.

2D HSQC experiments (Figure 16) are used in this thesis to determine mechanistic metabolic information regarding metabolism during HMP. [U-^{13}C] glucose (i.e. ^{13}C nuclei in all six carbon positions of the glucose molecule) is used as a metabolic tracer.

In 2D ^{1}H,^{13}C-HSQC NMR, the ^{1}H chemical shift is presented on the *x*-axis and is correlated with the ^{13}C chemical shift on the *y*-axis. Where there is a proton attached to a ^{13}C carbon nucleus, a signal appears on the spectrum at the intersection of the two chemical shifts. Because both ^{1}H and ^{13}C chemical shifts are used, in comparison to 1D NMR, the likelihood of signal overlap is significantly reduced in HSQC spectra. This type of 2D NMR experiment exploits the spin properties of both ^{1}H and ^{13}C nuclei. The pulse sequence used involves heteronuclear (^{1}H-^{13}C) decoupling procedures, but ^{13}C-^{13}C couplings remain active. As a result, the peak on the spectrum resulting from a ^{13}C nucleus is altered if an adjacent ^{13}C nucleus is present such that the signal is split into two. Similarly, if there is a ^{13}C nucleus positioned on each side of the ^{13}C nucleus of interest, the signal is split into four components. The natural abundance level of ^{13}C is well known and consistent (1.1%) and the probability of two neighboring carbon nuclei being ^{13}C is very low ($1.1\%^2$ = 0.0121%).

Using quantum mechanical simulations, the amount of ^{13}C-labelled substrates can be calculated relative to the amount of molecules in which a single nucleus will exist as the ^{13}C isotope due to natural abundance levels (1.1%). Absolute concentrations of a given metabolite are easily determined using conventional 1D ^{1}H NMR and therefore using a combination of 2D ^{1}H,^{13}C-HSQC and 1D ^{1}H NMR, the absolute concentration of isotopic ^{13}C-labelled substrates can be determined. The 2D HSQC

theory and protocols are discussed in further detail in Chapter 8 (p142).

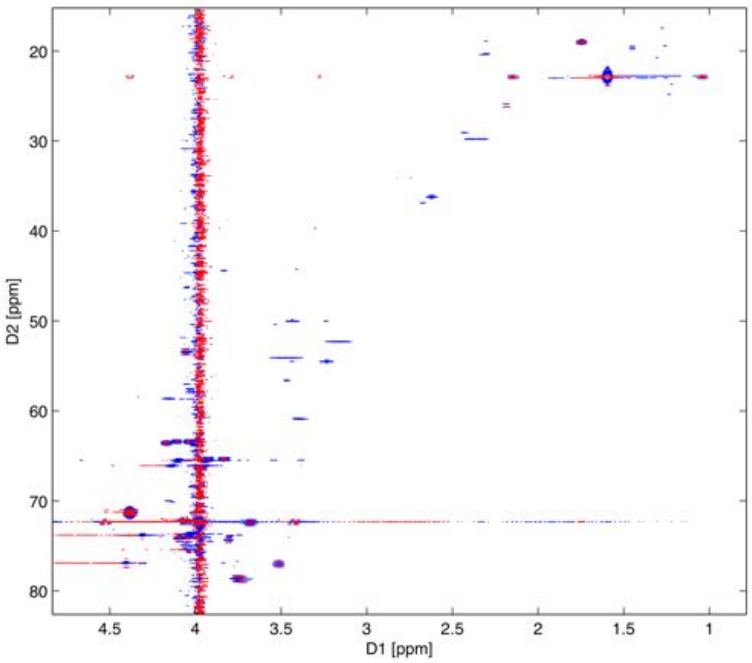

Figure 16 Example 2D $^{1}H,^{13}C$-HSQC

Region depicted from a heteronuclear single quantum coherence NMR spectrum (2D-HSQC)

NMR in transplantation studies

There has been great interest in the application of metabolomics to renal transplantation as a tool for identification of biomarkers applicable to transplantation, largely driven by the need for a clinically useful biomarker to predict post-transplant graft function.

Proteomic studies have been performed extensively, and a multitude of potential biomarkers have been identified, many through non-targeted proteomic studies (115, 116). The most promising of these are neutrophil gelatinase-associated lipocalin (NGAL) (117, 118), kidney injury molecule-1 (KIM-1) (119-121), interleukin-

18 (118), α- and π-glutathione S-transferase (GST) (122), liver fatty-acid-binding protein kidney (L-FABP) (123), and N-acetyl-β-d-glucosaminidase (NAG) (117, 124). However, whilst such studies are encouraging, it must be remembered that these biomarkers are yet to transcend into clinical practice.

Hypothermic machine perfusion appears to be particularly well suited to metabolomic analysis as the circulating perfusate biofluid is plentiful, easily accessible, contains a range of metabolites and would be suited to repeat analysis. Indeed Bon and colleagues have suggested that 1D ^{1}H NMR spectroscopy of perfusate could be used to provide real-time analysis to make organ viability decisions (125). However, despite the obvious attractions of metabolomic analysis of perfusate samples, there are no reports detailing the metabolomic profile of human perfused kidneys using ^{1}H-NMR.

Repeated studies have demonstrated a significant increase in the concentration of trimethylamine-N-oxide (TMAO) in urine and serum of dysfunctional grafts in both animal and human transplant models (126-129). TMAO is produced by the renal medulla with raised levels thought to be the consequence of ischaemic injury to medullary cells (105). However, the concentration of TMAO may also be related to transplant immunosuppression medication with higher blood levels (compared to controls) demonstrated in a rats treated with combination immunosuppression (130).

Rush et al. demonstrated a correlation between the metabolomic profile of urine post-transplantation and histological rejection (131). They suggested that urinary metabolomics could be used a non-invasive way of determining major post-transplant morbidity such as rejection. This concept has been echoed by other

similar studies (132), and characteristic urinary metabolic profiles have been shown to correlate with other histological abnormalities such as acute tubular necrosis (ATN) (133).

Hene et al. (134) performed ^{31}P NMR analysis on 42 human kidneys preserved using cold storage in HTK solution. Organs were subsequently transplanted and whole-organ analysis performed. The group found there were differences in the phosphomonoesterase / inorganic phosphate ratios (PME / Pi) between kidneys, with the highest ratios observed in live donor kidneys and the lowest from donation after cardiac death (DCD) donors. They concluded that the PME/Pi ratio was a marker of the high energy phosphate status of the kidney. Interestingly the group did not compare ATP levels between the storage groups using NMR and reported that ATP was hard to detect using their NMR protocol (134).

Despite the attraction of using a metabolomics approach for analysing various transplant models, some degree of caution must be adopted. The metabolomic spectra obtained from a sample of urine or plasma can contain hundreds of different data points with large numbers of discrete metabolites present. Metabolomic transplant studies are often small with patient numbers normally between ten and one hundred and often statistically underpowered to identify pertinent metabolic differences (77). Further methodological problems arise, particularly for non-targeted metabolomic studies. Given the potential for large numbers of metabolites to be identified within a sample, there is a risk of detecting false positive significance, particularly at a significance level of 0.05 as is the case with most studies. Metabolomic studies only provide a metabolic 'snapshot' of the type and concentration of metabolites present at a particular time point. Conventional 1D ^{1}H-

NMR and MS merely identifies metabolites but does not provide mechanistic information regarding metabolic pathway activity. To determine pathway activity within entire transplant organs, isotopic tracer studies have been proposed and are further discussed below (p45, p51).

Patients undergoing renal transplantation are an extremely heterogeneous group often with significant comorbidities (e.g. diabetes, hypertension). Furthermore, deceased donors are also a heterogeneous group with markedly different pre-mortal conditions (length of time in intensive care, nutritional status, cause of death, etc.), all of which invariably affect metabolism and therefore the exquisitely sensitive metabolome. In addition, from the moment of transplantation patients are commenced on a multitude of immunosuppression medications. Some of these drugs (such as the calcineurin inhibitor tacrolimus) need dose titration, and some (such as the steroid prednisolone) are fixed dose irrespective of body habitus. Immunosuppressive drugs have been shown to affect the metabolomic profile of urine (135-138) and blood (130), with detectable changes present after a single dose (137).

Metabolomic analysis of urine poses further problems as often patients undergoing transplant do have some residual renal function and therefore native urine output. The volume of urine produced by the transplanted kidney is also variable both chronologically and between patients. The concentrating effect of the kidney is not uniform for all metabolites and for many this parameter is not known. Although many studies have tried to circumvent this issue by normalising the data to urinary creatinine levels, this metod is also likely to be inaccurate (139). Therefore drawing meaningful conclusions from the urinary metabolites identified in the mixture of

native and transplant urine can be difficult.

In summary, metabolomics studies in renal transplantation have identified potential biomarkers in both the plasma and urine that correlate with both post-transplant graft function and histological injury. However, none of these have been validated in a large patient series, or transcended into clinical practice. Interestingly, there are relatively few publications whereby a metabolomics-based approach has been used to try and determine the type and degree of metabolism during hypothermic machine perfusion. The most significant was a recent report by Bon et al. (125), whereby the perfusate of pig kidney was analysed using 1D ^{1}H NMR. They found that the perfusate metabolites corresponded to post-transplant graft function in their auto-transplant pig model. The metabolomics-based approach to organ preservation is something that is explored in detail in this thesis in Chapters 5, 6 and 7 (p105, p116, p132). We hope that through a detailed understanding of metabolism during machine preservation the possibility of metabolic manipulation in order to improve post-transplant graft function could be explored. Furthermore, metabolic studies could reveal a clinically useful biomarker to denote organ viability. However, given the resources already invested to date, the hunt for an elusive biomarker of post-transplant function may well be futile, and a metabolic profile rather than a single marker will prove to be the most robust indicator of organ viability.

Isotopic NMR tracer studies

Identification and quantification of metabolites provide an understanding as to the properties of a biological system. This may be sufficient to identify disease states and identify certain active metabolic pathways. However, many metabolites can be

an intermediate in multiple different pathways with extensive interactions present. For example, the amino acid alanine is commonly produced from pyruvate through the enzyme alanine aminotransferase, which is known to be present in renal tubules (140, 141). However, alternative metabolic pathways can also lead to the accumulation of alanine, such as the catabolism of larger amino acids such as tryptophan (142). The metabolic 'snapshot' provided by conventional 1D ^{1}H NMR / MS has limitations for the understanding of cellular metabolism in isolated *ex vivo* organ perfusion models. Identification and quantification of a metabolite is insufficient to determine mechanistic information. For a given metabolite in the perfusion fluid, metabolism of pre-existing intracellular substrate stores, metabolism of substrates derived from the perfusion fluid, and release of metabolites secondary to cellular lysis are all credible explanations for the substrate precursor. Even with serial metabolite levels, such relationships are unclear. For this purpose, isotopic studies provide valuable additional information.

Incorporation of stable isotopes such as ^{13}C or ^{15}N into common compounds enables metabolic tracer studies within a biological system. This is not a new concept with initial studies reported over 40 years ago (143). Glucose in which the carbons 1 and 2 are in the ^{13}C isotopic form ([1,2-^{13}C] glucose) and in all six carbon positions ([U-^{13}C] glucose) are examples of compounds used for NMR tracer studies and are readily available and relatively inexpensive. The principle of tracer studies is simple, irrespective of the modality used for detection. For example, if [1,2-^{13}C] glucose is added to the perfusion fluid of a kidney during HMP and [2,3-^{13}C] lactate is detected then there is strong evidence of *de novo* glycolysis (Figure 17). Although theoretically possible, the relative population of naturally occurring [2,3-^{13}C] lactate

is less than 1/8,000 (the natural abundance of ^{13}C is 1.1%).

In a study of the metabolism of human lung cancer tissue, patients underwent intravenous infusion of fluid containing universally labelled [U-^{13}C] glucose before lung cancer resection (144). The metabolomic profiles of resected cancerous tissue was then compared to the surrounding non-involved lung tissue using both NMR and gas chromatography-mass spectrometry (GCMS). Increased levels of ^{13}C-labelled lactate, alanine, succinate, glutamate, aspartate and citrate were noted in the tumour tissue. The authors concluded that this was representative of greater glycolytic and TCA cycle activity in the tumour tissue and further postulated that TCA intermediates were required for the synthesis of proteins and other substrates for the growing tumour (144).

In a rat lung transplant model, rat lungs were flushed and then preserved with [U-^{13}C] glucose and/or [3-^{13}C] pyruvate following inflation with oxygen (145). After 6 hours of preservation, tissue extracts were analysed using 1D ^{13}C NMR spectroscopy. There was evidence of acetyl-CoA in both groups, highlighting TCA cycle activity. However, the proportion of labelled acetyl-CoA was significantly higher in the pyruvate labelled group with evidence of gluconeogenesis and glycogen synthesis in the ^{13}C pyruvate-only group and the authors conclude that the addition of pyruvate in lung preservation fluid would be more useful than glucose as both an anabolic and catabolic substrate (145). There are multiple reports using ^{13}C and other NMR detectable isotope tracer studies on cell lines including those specific to the kidney. However, to our knowledge, there are no published reports of *ex vivo* kidney perfusion whereby isotopic ^{13}C substrates are introduced into the perfusion fluids and tracer studies performed using NMR techniques which is described in Chapter 8

(p142).

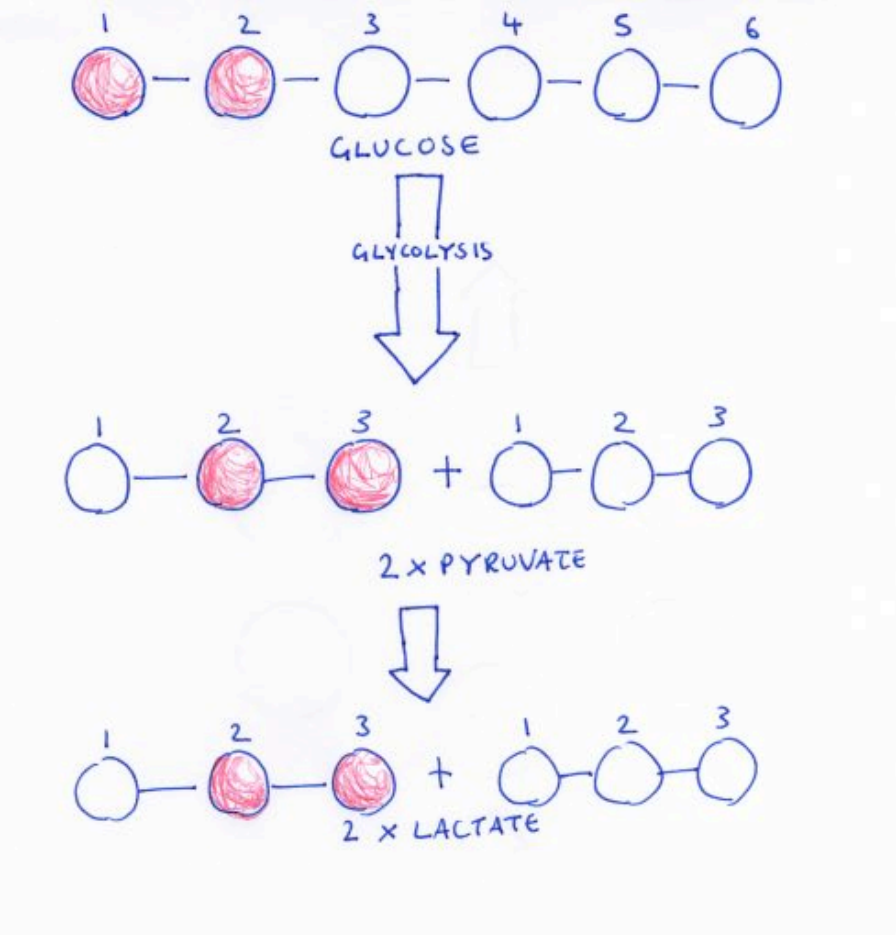

Figure 17 Conversion of ^{13}C labelled glucose into lactate

In this example, glucose labelled with the isotope ^{13}C in the 1 and 2 carbon nucleus positions ([1,2-^{13}C] glucose) are converted into two pyruvate and two lactate molecules with isotopic labelling in the 2 and 3 carbon positions [2,3-^{13}C] pyruvate and [2,3-^{13}C] lactate in one half of the molecules.

Hypothermia and hypoxia in biology overview

Unlike certain mammals, humans have few natural mechanisms to cope with either severe hypoxia or hypothermia. Prolonged exposure to such conditions culminates in cell death and principally occurs when the cellular ATP reserves are insufficient to sustain the ATP-dependent processes necessary to maintain transmembrane ionic gradients (55). This can result in membrane depolarisation with a catastrophic influx of Ca^{2+}, leading to membrane rupture and cellular necrosis (146). The brain and heart appear particularly sensitive to such extremities as evidenced by either brain dysfunction or cardiac arrhythmia as the predominant cause of death in such conditions. In contrast, human skeletal muscle is amongst the most refractory tissues to hypoxic conditions (55).

Under normal physiological conditions, the kidneys are metabolically highly active. The renal blood flow per gram of tissue is amongst the highest in the body and the kidneys receive 20-30% of the cardiac output and consume 10% of the bodies oxygen. The functions of the kidney are multiple and many, such as the filtration and selective reabsorption of electrolytes, are highly ATP-dependent. As a result, the kidney has evolved to supply the necessary energy substrates through intrinsic synthetic activity. As the static energy reserves within the kidney, such as glycogen, are relatively low, the kidney is reliant on the metabolism of circulating substrates. These include glucose, lactate, glutamine, free fatty acids, ketone bodies, citrate and glycerol (147).

After filtration of circulating blood across the glomerular membrane in the cortex, most of the water and electrolytes are reabsorbed in the proximal convoluted tubule (also in the cortex). The cortex, therefore, is the region with the highest energy

requirement and resultantly is the site of highest mitochondrial oxidative phosphorylation, with only a small proportion occurring in the outer medulla. The cells of the proximal convoluted tubules are also unique within the kidney as they contain all the enzymes needed for gluconeogenesis (147).

Cellular response to hypoxia

Adenosine triphosphate (ATP) is the principal energy source within the cell. Protein synthesis and maintenance of the Na^+/K^+ APT-ase pump are the two principal consumers of ATP in mammalian systems during standard metabolic rate (SMR) (148). It is thought that 90% of the oxygen consumption of mammalian cells in the at SMR is utilised by mitochondria, of which 80% is coupled to ATP synthesis, with 20% subject to proton leak (148).

In stress conditions such as hypoxia, human cells are unable to downregulate the amount of ATP necessary to maintain cellular functions and therefore an ATP deficit is quickly established which is only partially and temporarily abridged by anaerobic ATP production such as glycolysis. In contrast, animals such as the common frog (*Rana temporaria*) can downregulate their energy turnover in hypoxic conditions and thus conserve vital sources of ATP. Indeed there is surprisingly little variation in the intracellular ATP concentration in certain species despite significant shifts in oxygen tension (149). This effective ATP equilibrium in such species is sustained by a selective metabolic depression via the down-regulation of energy consuming processes and up-regulation of the efficiency of energy-dependent processes (148), (150). Protein synthesis, in particular, has been shown to be drastically reduced in certain species in hypoxic conditions with prioritisation of more essential energy-

dependent functions such as membrane ionic stability via the Na^+/K^+ ATP-ase (151) (152). Although ATP-dependent Na^+/K^+ pumps remain the dominant site of ATP consumption, even in species refractory to hypoxia, this is minimised through a phenomenon known as 'channel arrest' (146). Suppression of ion channel densities effectively results in a reduction in cell membrane permeability with maintenance of transmembrane ion gradients and electrochemical potentials (153-155). The concept of anoxic ion channel arrest is well established, but the precise mechanism by which this occurs is not known. There is some evidence that adenosine may play a role, which in itself is an end product of ATP breakdown.

In contrast, in oxygen-sensitive species such as humans, ion channels often become more porous in hypoxic conditions, even further increasing the ATP necessary to fuel the ATP-ase dependent ion channels.

Oxidative phosphorylation occurring in the mitochondria of cells in oxygen-rich conditions produces the vast majority of cellular ATP. Of the potential 38 molecules of ATP produced from a single glucose molecule, 34 of these are derived through mitochondrial oxidative phosphorylation (142). The final step of ATP production within the inner mitochondrial matrix relies on the conversion of ADP to ATP via the enzyme ATP synthase, in conjunction with the influx of a proton via the F_1F_0 ATPase channel (55). However during periods of anoxia, the reversible enzyme, ATP synthase, that normally results in ATP production, works in the contralateral direction to pump protons from the mitochondrial matrix and maintain membrane stability. Thus during periods of anoxia, the mitochondria become an ATP consuming organelle, further exacerbating the hypoxic strain within the cell (156). However, only a small concentration of oxygen is necessary to ameliorate this backwards flow

of ATP synthase, with some evidence that mitochondrial efficiency is actually improved in hypoxic conditions due to the reduction of protein leak and uncoupled respiration (157).

Provided there is sufficient oxygen to diffuse into the mitochondria and facilitate oxidative phosphorylation, certain tissues exposed to chronic hypoxia in mammalian systems display adaptive properties, with both liver and heart cells reducing their metabolic rate by up to 50% (158, 159). Some cells, such as cardiac myocytes display a reduced functional activity in hypoxic conditions (160). Other adaptive mechanisms are less clearly defined but include changes in neurotransmitter release, ion channel activation and gene expression (161-163). Examples of hypoxia-induced genetic induction include the hormone erythropoietin (EPO), which stimulates red blood cell production, and vascular endothelial growth factor (VEGF). Furthermore, there is up-regulation of the enzymes involved in anaerobic glycolytic pathways and heat-stress proteins which are protective against intracellular stress (55). The mechanisms of gene up-regulation are not fully clear but it may be that the hypoxia sensor is a haem protein that initiates a cascade that culminates in reactive oxygen species (ROS) (55).

Cellular response to hypothermia

There is also a profound effect of uncontrolled hypothermia at a cellular level in humans and other cold-sensitive mammals. Many transcellular ion-regulatory mechanisms are thermosensitive with some evidence that cell membrane permeability is also increased at low temperatures and that membrane stabilisers such as polyethylene glycol (PEG) or butylated hydroxytoluene may be protective

(164, 165). The term 'cold swelling' has been coined to describe the harmful effect of hypothermia on neural tissue and is largely a consequence of the increase in intracellular Na^+ concentration. Reduction in Na^+/K^+ ATP-ase activity occurs although it is not clear whether this is purely due to a supply / demand discrepancy of ATP in hypothermia or a thermodynamic effect of decreased ATP production (146, 166). Intracellular hypernatraemia is further exacerbated by activation of the Na^+/H^+ exchanger and by a relatively greater passive influx of Na^+ compared to efflux of K^+ (167).

Although the hypothermic effect of the intracellular accumulation of Na^+ is perhaps best understood, many other active and passive ion transport mechanisms are also thermosensitive. Elevated cytosolic concentrations of Ca^{2+} have been demonstrated in animal models during hypothermia (168, 169). Disruption of the cell membrane Na^+/Ca^{2+} exchange process and reduced uptake (ATP dependent) and decreased efflux of Ca^{2+} from the sarcoplasmic reticulum are the likely causative factors (146) (170). Further massive calcium influx results if the rise in cytosolic Na^+ occurs to such an extent so as to trigger membrane depolarisation and the opening of voltage-dependent Ca^{2+} channels, which often results in irreversible cellular injury (146, 166, 170).

Chapter 2

Materials and methods

Abattoir kidney studies

Abattoir pig kidneys were used for all animal experiments in this thesis. Organs were obtained from a local abattoir. The rationale for using these organs is that they are cheap, easily obtainable and do not involve animal slaughter purely for research purposes. Porcine kidneys are a widely used large animal model in transplantation studies owing to their similar physiological and anatomical properties to human organs (171, 172). The use of abattoir kidneys has certain advantages and disadvantages compared to the other commonly used porcine model; namely the autotransplant model.

Whilst the use of abattoir kidney makes thorough interrogation of functional outcome difficult (in contrast to autotransplant studies), for many studies, the purpose was to interrogate the metabolic output. Furthermore, multiple studies have reported that when young and otherwise healthy porcine kidneys (e.g. abattoir kidneys) are transplanted following periods of up to 24 hours of machine perfusion they have good functional outcome (173-177). One of the great advantages of using abattoir kidneys is that it permits a paired experiment model (i.e. the two kidneys from the same animal can be separately subjected to the control and experimental conditions). This is in contrast to the autotransplant setup whereby only one kidney from each animal is used for analysis. Abattoir kidneys in these experiments were obtained from FA Gill's abattoir in Wolverhampton and 22–26 week old 'bacon weight' pigs, weighing 80–85 kg were used.

Workstation setup

Prior to receiving organs, a workstation was created in the abattoir to receive and

rapidly flush the organs in order to cool them and remove any remaining blood. All equipment used for this purpose was sterile, and the equipment setup is demonstrated in Figure 18 and 19. The environment was not truely sterile in the same vain as the operating theatre, not least due to the lack of laminar air flow. The basic equipment needed included bowls, surgical tools, 14G cannulae with an attached giving set and 4°C fluid for flush (either UW or Soltran® in these studies). For some studies, a pressure bag was utilised to obtain constant flush pressure and in others a drip-stand was utilised for this purpose. Much of the equipment utilised was disposable (e.g. cannulae, giving sets, perfusion fluids, etc.). Non-disposable items (e.g. drapes, metal bowl sets, etc.) were sterilised using autoclave before usage.

Animal slaughter

Pigs were killed by electrical stunning, following which they were hung by their hind legs and exsanguinated via transection of their jugular veins. Any blood required was collected at this stage in a heparinised vessel. The deceased animal then passed through several processing stages of hair removal before laparotomy (sternopubic) and removal of abdominal viscera. Kidneys were retrieved by abattoir staff with maximal lengths of renal vein, artery, and ureter attached. Male pigs were used in preference owing to their larger size and therefore vessel calibre. Organ retrieval was performed by the abattoir staff with pairs of kidneys used.

Treatment of kidneys

Following removal of a pair of kidneys and associated anatomy by the abattoir staff, organs were quickly transported to the designated workstation within the abattoir.

Organs were rapidly inspected to ensure that no gross abnormality/damages/lacerations were present. They were then weighed in order to determine the pre-perfusion weight. Kidneys were then placed into a bowl of ice and the renal artery cannulated. Perfusion of the organ with prechilled (4°C) preservation solution was rapidly commenced through a cannula sited in the renal artery (14 G). 5,000 units of heparin were introduced into each bag of perfusion fluid prior to receiving the kidneys in order to minimise intra-organ thrombosis. Based on the fairly fixed time periods each stage of the abattoir process takes, we estimate that the cold flush of pig kidneys was commenced 14 minutes after the animal's death. The perfusion pressure was fixed using an inflatable 'IV pressure bag' or dripstand with slight variation in pressure depending on flush fluid and experiment. The fluid utilised in these studies was either Soltran® or UW, which are both used in clinical practice during the organ retrieval process. Each kidney was perfused with 1 L of fluid and the effluent fluid free from blood before perfusion ceased.

Initial period of static cold storage

Following the rapid cooling and flush-out of any remaining blood from the kidneys they were then re-weighed and the excess tissue around each organ resected. Kidneys were then placed in a fresh litre of fluid (either UW or Soltran®), placed in a sterile bag with air expelled and transported in a cooler box surrounded by ice back to the laboratory. 10 ml of Penstrep antibiotics was added to the cold stored kidneys to minimise bacterial contamination. The average period that organs were stored in these SCS conditions before starting HMP was 2 hours and reflects the period of SCS

that human organs undergo between retrieval centre and transplant centre.

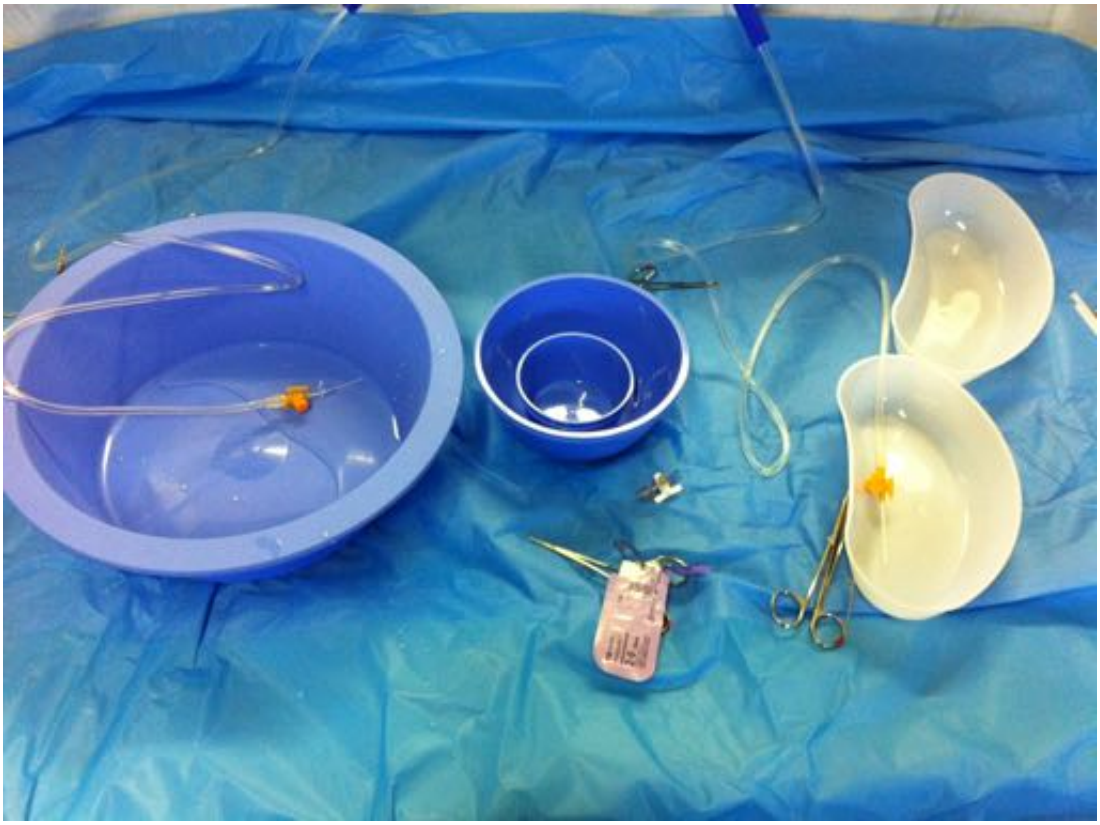

Figure 18 Abattoir workstation setup (1)

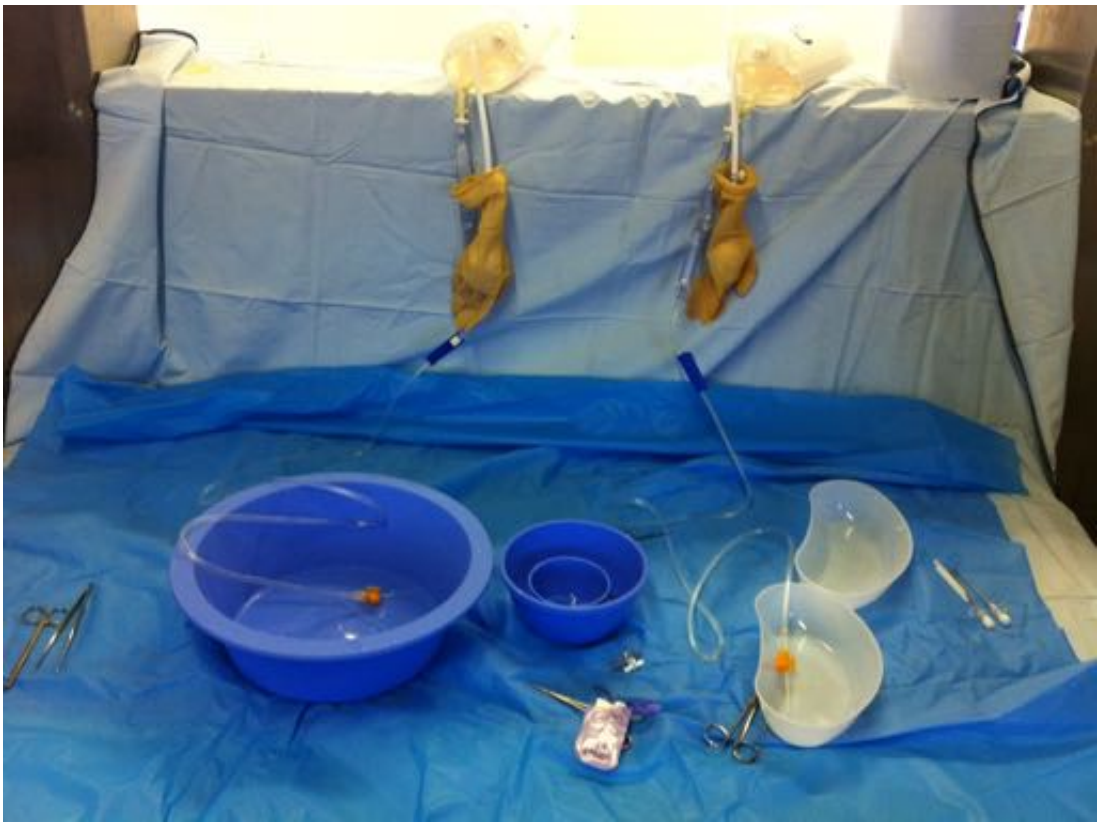

Figure 19 Abattoir workstation setup (2)

Hypothermic machine perfusion

Kidneys were reweighed prior to any perfusion experiments. The LifePort© Kidney Transporter 1.0 (Organ Recovery Systems) was utilised for all organ perfusion experiments (Figure 2) with two laboratory machines facilitating paired perfusion experiments. Where the kidney had an associated aortic patch (normally the left), a SealRing® patch connector (Organ Recovery Systems, Chicago, IL) was used to connect the kidney to the LifePort© machine (Figure 20). For the contralateral kidney, a T-connector was utilised for this purpose with a 5 mm T connector being the appropriate size for the majority of experiments (Figure 21). The accompanying LifePort© cassettes were used for all studies. Ice was inserted into the ice reservoir and the perfusion circuits primed prior to perfusion. The LifePort© ceases to perfuse at or above 8°C, and the temperature of most perfusion experiments was

consistently below 4°C. The default perfusion pressure of 30 mmHg was utilised for experiments in this thesis (Figure 22). For some experiments (e.g. the oxygenation experiments), additional equipment was required in the perfusion circuit such as an oxygenator (Figure 23 and 24). For this, additional tubing (1/4 inch internal diameter) was connected to the existing perfusion circuit and is further discussed in Chapter 9.

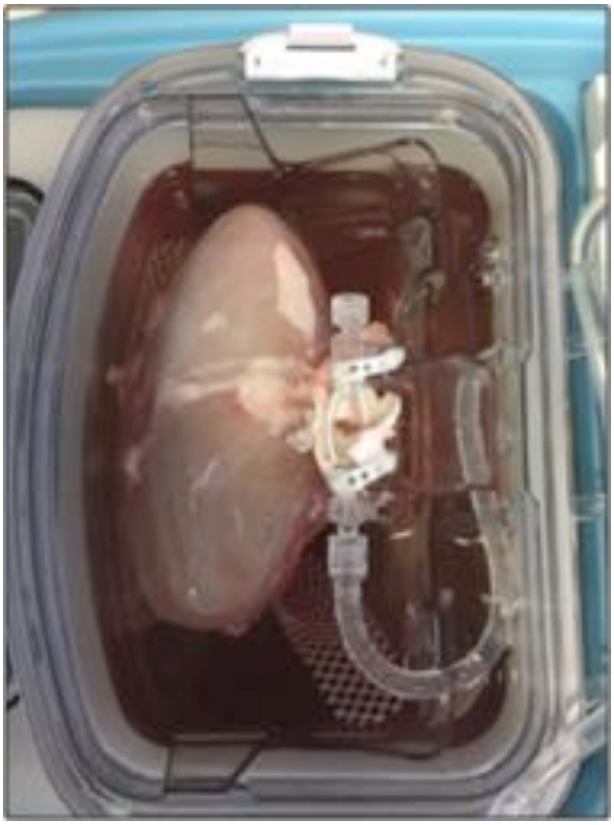

Figure 20 Kidney connected via a SealRing® device

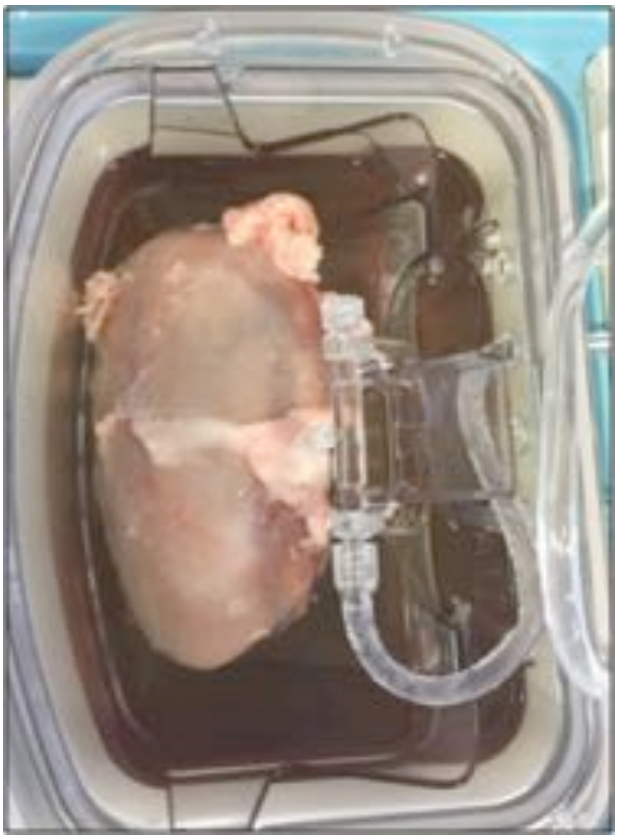

Figure 21 Kidney connected using a 5 mm T connector

Figure 22 Display settings LifePort© device

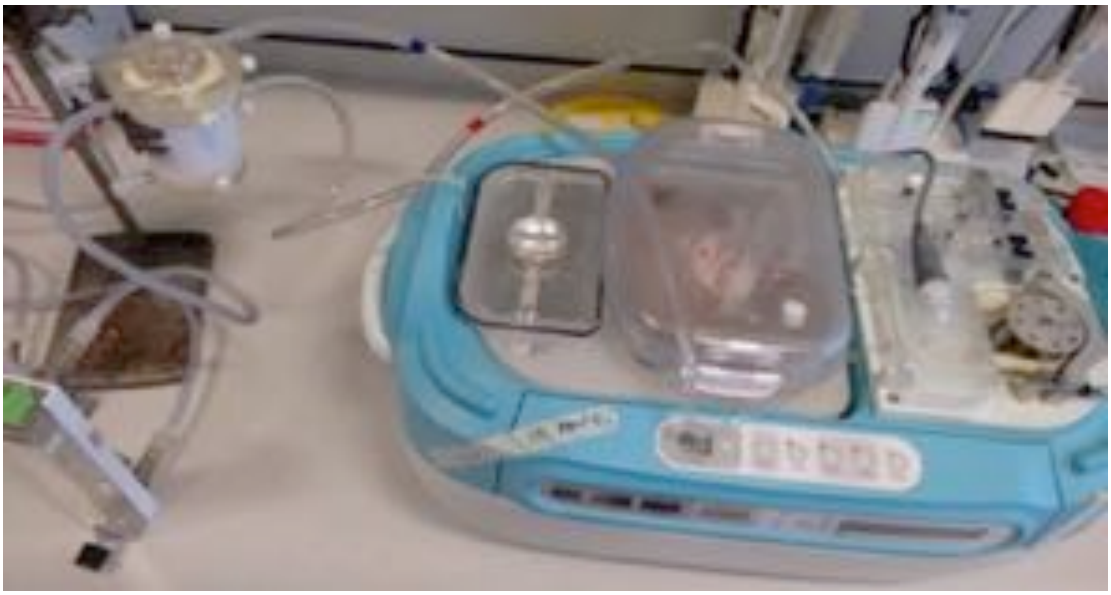

Figure 23 Modified LifePort© device with Hilite LT 800 paediatric oxygenator

Figure 24 Hilite 2800 paediatric oxygenator

Perfusion fluid

The perfusion fluid utilised in this thesis depended on the experiment being performed. For some measurements, industry standard KPS-1® fluid was used (e.g. for comparing SCS and HMP conditions). In other experiments, particularly where 2D NMR studies were used, a home-made variant of KPS-1® was formulated, and the

exact composition of this is discussed in the relevant chapter. For all experiments, 1L of fluid was used for perfusion. For most porcine perfusion experiments, the flow rate after the first hour was approximately 50 ml/minute resulting in the entire litre of fluid circulating through the kidney every twenty minutes.

Perfusate sampling

Perfusion fluid sampling (perfusate) was performed through the designated sampling port on the LifePort© device (Figure 25). Only small volume samples (circa 2 ml) were necessary for analytical purposes at each time point which was insignificant compared to the total volume of circulating fluid (circa 1 L)

Figure 25 Sampling port for LifePort© device (arrowed)

Tissue sampling

Immediately after the designated experiment end time, kidneys were removed from the LifePort© and were laterally bisected (Figure 26) to enable the clear identification and tissue sampling of the kidney cortex and medulla (Figure 27).

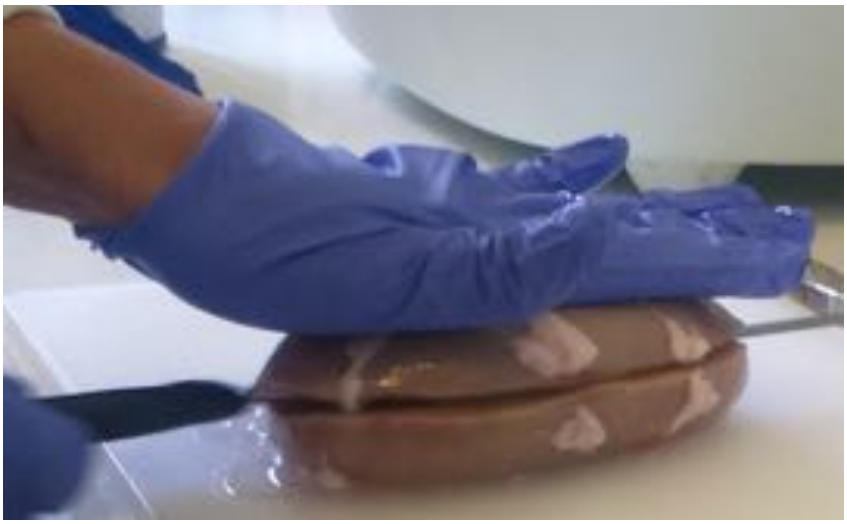

Figure 26 Lateral bisection of kidney

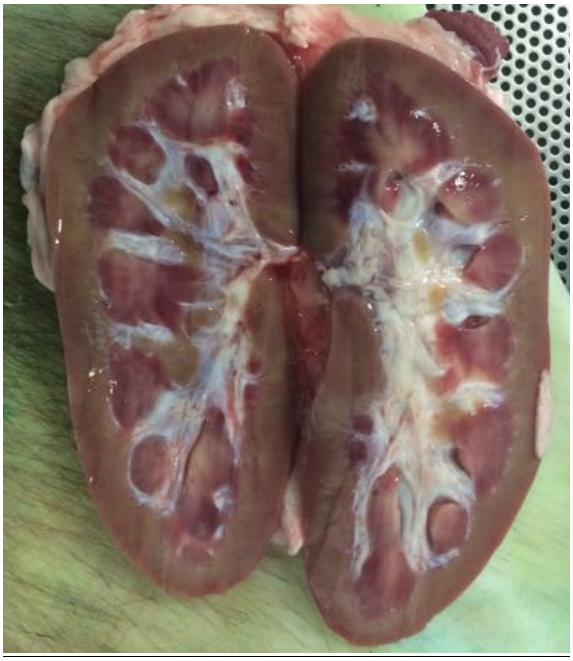

Figure 27 Demonstration of cortex (outer) and medulla of bisected kidney

This kidney was a control kidney and was not perfused.

Metabolic studies

From the laterally bisected kidney, chunks (circa 5 g) of renal cortex were dissected and snap frozen in liquid nitrogen in order to immediately cease metabolism. From the bisected kidney, areas of renal medulla were clearly identifiable, and these were

also dissected and snap frozen. All tissue samples were stored in -80°C conditions prior to metabolic analysis. Protocols for metabolic studies are discussed below.

Histological studies

From the bisected kidney, a wedge shape area of kidney encompassing all regions of the renal cortex and medulla was dissected (Figure 28) and stored in formalin. Sections of renal artery were similarly preserved. Samples were prepared for examination by light microscopy using two common stains: haematoxylin and eosin and periodic acid shiff's. These were prepared using published standard techniques.

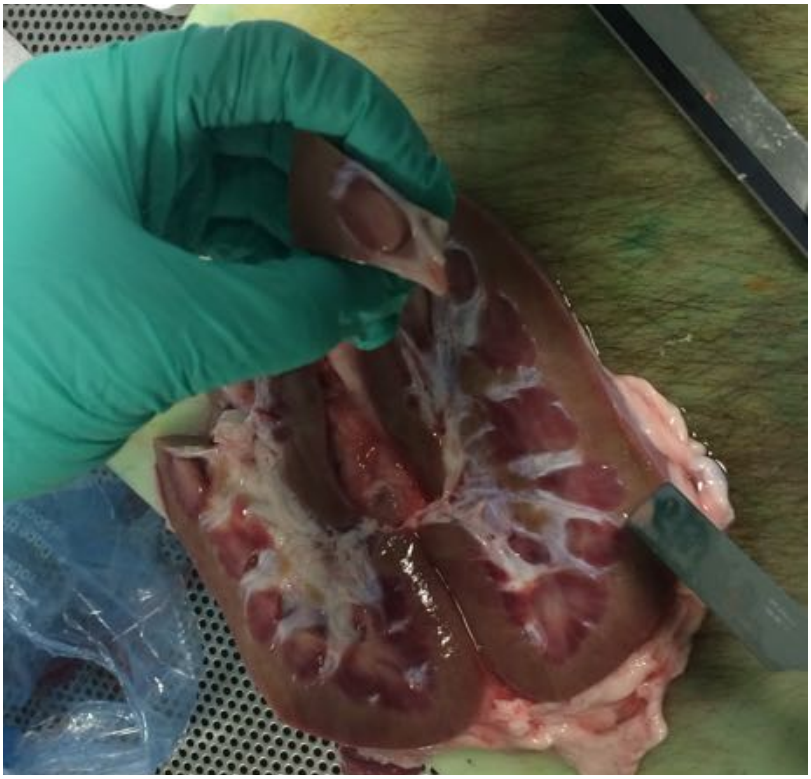

Figure 28 Specimen of cortex and medulla for histological analysis

NMR sample preparation - perfusion fluid

Non-extracted samples

Each perfusate sample was stored in a 2 ml cryovial and frozen (-20°C) prior to sample preparation. When required this was then thawed at room temperature for preparation. A stock NMR buffer solution was prepared for each study.

The stock solution composed of the following

60 ml of 0.4 M NaH_2PO_4 (+2 mM DSS + 8 mM imidazole)

90 ml of 0.4 M Na_2HPO_4 (+2 mM DSS + 8 mM imidazole)

60 ml of Deuterated water (D_2O)

(DSS = 4,4-dimethyl-4-silapentane-1-sulfonic acid)

To do this, 150 ml of ultrapure water was prepared to which the two phosphate buffer reagents were added (2.88 g of NaH_2PO_4 and 5.11 g of Na_2HPO_4) along with 0.067 g of DSS and 0.082 g imidazole. Following addition of D_2O, the solution was mixed thoroughly before use.

In a microcentrifuge tube, 231 µL of stock solution was added to 429 µL of perfusate to make a final volume of 660 µL with a final phosphate buffer concentration of 100 mM. The lid was closed on the microcentrifuge tube prior to vortex. 5 mm NMR tubes were used for perfusion fluid analysis. The lid cap was removed from the NMR sample tube, and 600 µL of sample (stock + perfusate mixture) manually pipetted into this. Samples were re-capped and centrifuged using a hand centrifuge to remove any bubbles. Following this, samples were sonicated for 10 minutes to ensure even sample distribution within the NMR tube. The external surface of the NMR tube was then dried and re-centrifuged. A lint cloth was used to wipe the NMR

sample tubes prior to placing into an NMR sample rack and sealing the tube orifice with a polyoxometalate (POM) ball to prevent evaporation. NMR samples were stored at 4°C prior to processing or if delays of more than 24 hours anticipated they were stored at -80°C.

Extracted samples

In some studies, extraction of perfusion fluid was performed. Whilst this did introduce an additional step to sample preparation, it enabled the removal of large protein molecules from the sample, as well as concentrating the samples. Extraction was performed using a chloroform/methanol extraction method.

Either 1.7 mm or 5 mm NMR tubes were used for analysis of extracted perfusion fluid depending on the study in question. An example protocol for preparation of 1.7 mm NMR sample tube is given below. Any variation of this protocol is discussed further in the relevant chapter.

Frozen perfusate samples were thawed and 1.5 ml was mixed with 1.5 ml methanol (-80°C) and 1.5 ml of chloroform (-20°C). The resulting 1:1:1 ratio solution was vigorously mixed for 10 minutes and then centrifuged at 1300 g at 4°C for 15 minutes. This resulted in the biphasic separation of the solution, with the top layer containing the water, methanol, and polar metabolite fraction of the perfusate sample. From the upper fraction (polar), 2 ml was aspirated and dispensed into two microcentrifuge tubes (i.e. 1 ml in each). All polar fractions were dried overnight in a vacuum dryer set to 30°C. Following this, extracted samples were frozen until needed. To prepare samples for analysis, the dried perfusate extracts were retrieved from the freezer and resuspended in 480 µL of 0.1 M phosphate buffer (pH 7.0),

containing 0.5 mM DSS, 2 mM imidazole and 10% D_2O. The dried pellet was vortexed until completely dissolved in a microcentrifuge tube and then sonicated to dissolve any remaining microparticles. Following this, the perfusate extracts were added to champagne vials and 35 µl (<10% of sample volume) of this was transferred to 1.7 mm NMR tubes using a Gilson robot. The NMR tubes were centrifuged, wiped with methanol and a lint free cloth with inserted POM ball and stored at 4 °C.

NMR sample preparation - tissue

To prepare kidney tissue (cortex or medulla) for NMR analysis, chloroform/methanol extraction was also performed. Tissue samples were removed from the -80°C freezer and then submerged in liquid nitrogen and pulverised to a fine powder using a manual cryogrinder. 0.5 g of the resulting powder was then added to a 7 ml Precellys homogenisation tube containing 5.1 ml of methanol at -80°C.

The prepared 7 ml Precellys tubes were all processed on the Precellys 24 Dual homogenisor, and run time was standardised to 8 runs at the lowest RPM/time (i.e. 5000 RPM for 1 x 10 s) as we have observed this is the minimum required to homogenise renal tissue completely. Sample tubes were cooled on dry ice between runs to prevent sample overheating.

The homogenised tissue in 5.1 ml methanol (-80°C) was mixed with 4.65 ml deionised water (rather than 5.1 ml, to account for the volume of water already present in the tissue, and 5.1 ml chloroform).

From the upper fraction (polar), 4.5 ml was aspirated and polar fractions were dried overnight in a vacuum dryer set to 30°C. Following the drying procedure, samples

were prepared analysis as for the extracted perfusate samples above.

NMR data acquisition

NMR data acquisition was performed at the Henry Wellcome Building NMR facility, University of Birmingham. The two most common NMR spectroscopic methods used in this thesis were one-dimensional proton NMR spectroscopy (1D-^{1}H NMR) and two-dimensional proton-carbon heteronuclear single quantum coherence spectroscopy (2D ^{1}H,^{13}C-HSQC). The spectrometers used for acquisition were the Bruker AVII 500 MHz and 600MHz spectrometers, with either a 5 mm or 1.7 mm inverse Cryoprobe.

1D ^{1}H NMR acquisition protocols

The sample temperature was set to 300 K. Excitation sculpting was used to suppress the water resonance (178). Spectra were acquired with a 6 kHz spectral width 32768 data points, a 4 s relaxation delay and 128 transients. Manual probe tuning and matching was performed before the first sample acquisition. Each sample was automatically shimmed (1D-TopShim) to a reference compound (TMSP or DSS) line width of less than 1 Hz before acquisition. Samples with a line width of more than 1 Hz were re-acquired again after manual shimming where the TSP half height line width was shimmed below 1 Hz. The experimental time to acquire 1D spectra was approximately 15 minutes per sample. Technical replicates for most experiments were performed. All data sets were processed using the MATLAB-based MetaboLab software (179). Data sets were zero-filled to 131072 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/DSS signal to 0 ppm. Spectra were

manually phase corrected and baseline corrected using a spline before segmental alignment of all resonances using Icoshift (180). Spectra were then exported into Bruker format.

Samples for NMR analysis were prepared by myself, T.S. or A.G. The data acquisition was performed by my supervisor C.L. Phase correction was conducted by myself, T.S., or A.G. under the supervision of C.L.

2D ^{1}H,^{13}C-HSQC acquisition protocols

Spectral widths were set to either 7,812.5 Hz (^{1}H) or 24,155 Hz (^{13}C). 512 complex data points were acquired for the ^{1}H dimension of 2D ^{1}H,^{13}C-HSQC NMR spectra. 30% out of 8192 complex data points (2458) were sampled for the ^{13}C dimension using an exponentially weighted non-uniform sampling scheme. 128 transients were recorded for the 1D-NMR spectra using a 4 s interscan relaxation delay. 2 transients per increment were recorded for the 2D ^{1}H,^{13}C-HSQC NMR spectra. The interscan relaxation delay was set to 1.5 s. Each sample was automatically tuned, matched and then shimmed (TopShim) to a DSS line width of < 1 Hz prior to acquisition of the first spectrum. The chemical shift was calibrated by referencing the DSS signal to 0 ppm. 1D-spectra were manually phase corrected before correcting the spectral baseline using a spline function (179). 2D ^{1}H,^{13}C-HSQC NMR spectra were reconstructed with compressed sensing using the MDDNMR and NMRpipe software (181-183). The spectra were zero-filled to 1024 (^{1}H) times 16384 (^{13}C) real data points prior to multiplet simulation analysis.

Samples for NMR analysis were prepared by myself or T.S. The data acquisition was performed by my supervisor C.L. Phase correction was performed under the supervision of C.L.

1D ^{1}H NMR metabolite identification

For 1D ^{1}H NMR analysis, metabolite identification and concentration was determined using Chenomx 7.0 or 8.1 (Chenomx INC., Edmonton, AB, Canada). Chenomx software quantifies metabolites based upon the area under the peak for known spectral peaks for each particular metabolite. The software has a function for automated quantification. The quantification of all metabolites in this thesis was checked and corrected manually although automated quantification was often used as an initial fit process. Where metabolites have multiple peaks within a spectrum, there is inevitably some discrepancy in the reported concentration depending on which peak from the spectrum is used for this purpose. Therefore, to maintain consistency, for a given metabolite the same peaks were used for quantification throughout all experiments. The spectral peaks that I have utilised in this thesis for quantification purposes are depicted in Appendix p139-145 with examples shown below (Figure 29). To further minimize bias during this manual quantification process, samples were given unique identification numbers that did not reveal the experimental condition, effectively blinding the researcher from the study group. Metabolite quantifications were corrected to allow for sample dilution with sample buffer.

All metabolite quantification for 1D ^{1}H spectra was performed or supervised by myself.

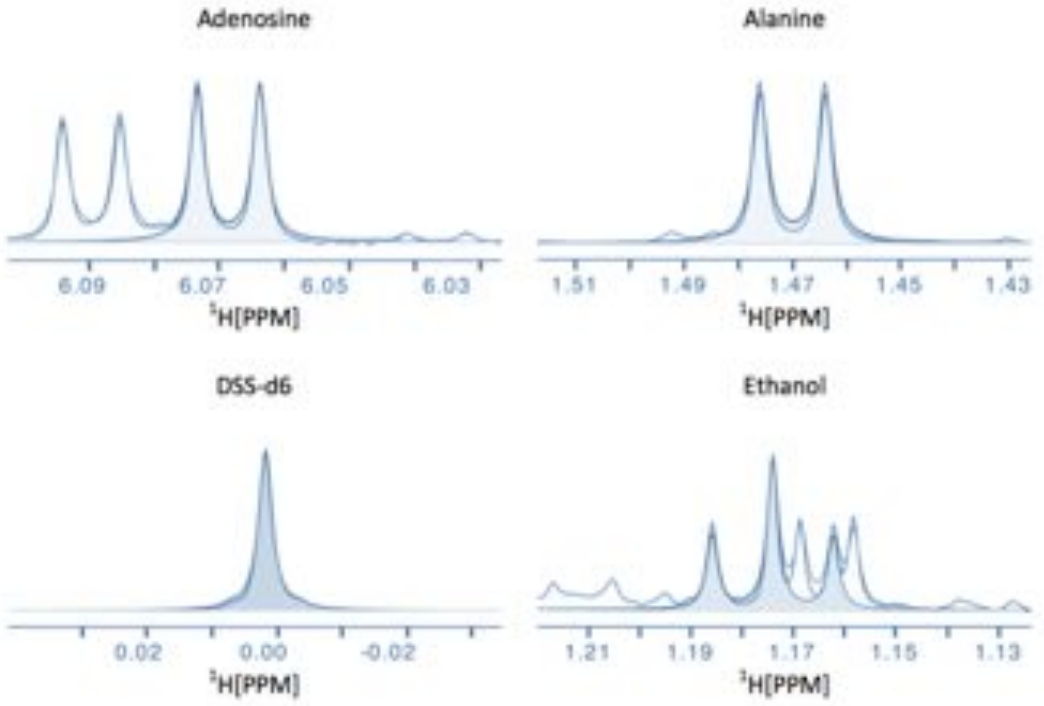

Figure 29 Chemical shift used for quantification of example metabolites

2D $^{1}H,^{13}C$-NMR metabolite analysis

HSQC spectra were analysed using MetaboLab software, which has been designed and developed for this purpose by my supervisor C.L. The MetaboLab programme uses pyGamma software for multiplet simulations (184). The methyl group of L-lactate is used to calibrate chemical shift based on the assignment in the human metabolome database (185).

Following chemical shift calibration, potential metabolites are selected from the reference library, and the chemical shifts for each constituent ^{13}C nuclei (with attached ^{1}H) displayed (Figure 30). Subsequent to manually attributing peaks in the spectrum to particular metabolites, a fitting process occurs whereby a best-fit model is applied to the observed region based upon known and theoretical ^{13}C-^{13}C spin-spin J-coupled splitting algorithms (Figure 31).

Based upon the splitting patterns from adjacent ^{13}C nuclei, the distribution of constituent isotopomers can be calculated in MetaboLab, correcting for the natural ^{13}C abundance quantity. Thus the relative proportion of a labelled versus unlabelled metabolite can be obtained, but not the absolute concentrations using this software alone. For example, from a particular sample using MetaboLab software one can deduce that of the lactate present, 5% is universally labelled [U-^{13}C] lactate (i.e. ^{13}C in all 3 carbon positions).

Whilst this NMR approach gives unparalleled detail regarding the structural isotopologues within a sample, unlike mass spectrometry techniques, the determination of this structural composition becomes challenging when there are extensive regions of ^{12}C nuclei within molecules. Thus using this HSQC approach, it would not be possible to differentiate the presence of molecules of a six

hydrocarbon chain with ^{13}C in positions 1, 2, 5 and 6 from equal proportions of 1, 2 - only and 5, 6 - only labelled molecules. Such challenges are easily resolved with MS and serve to highlight the potential benefits of a combined NMR and MS approach to isotopologue analysis.

All fitting and isotopomer analysis using MetaboLab was performed by or supervised by myself or my supervisor C.L.

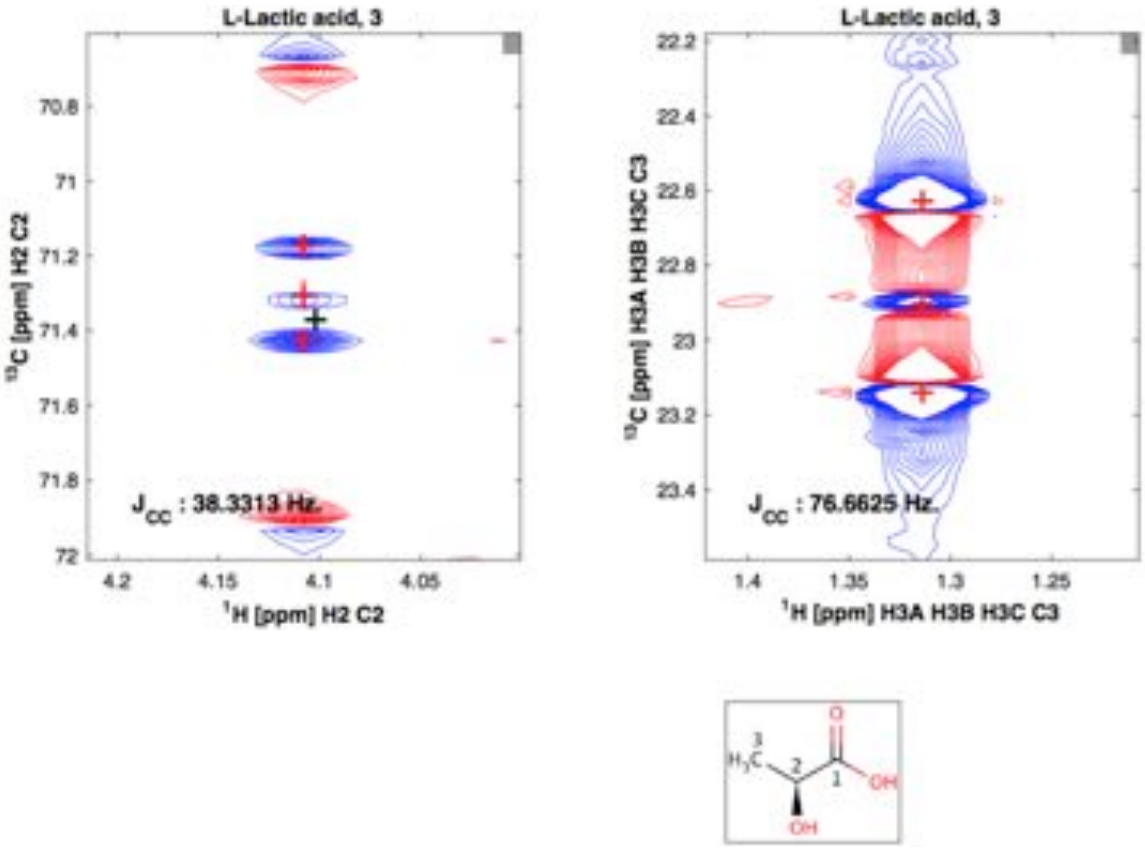

Figure 30 MetaboLab software demonstrating chemical shifts for L-Lactic acid

Only the chemical shift regions correlating to carbon 2 and 3 nuclei are seen as carbon 1 has no attached proton and therefore does not produce a signal. In this example, clear signal splitting is seen, indicating the presence of adjacent ^{13}C nuclei.

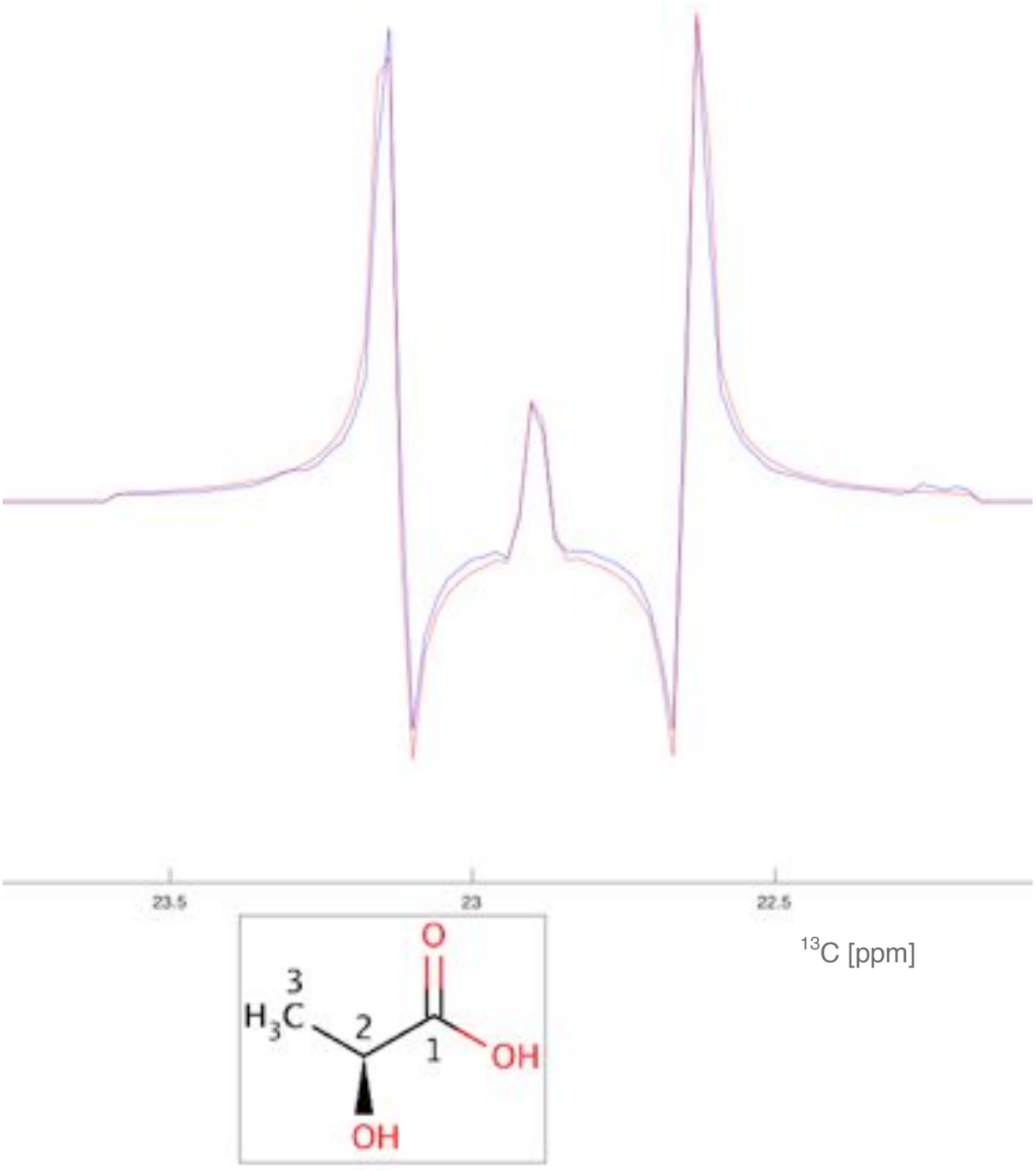

Figure 31 MetaboLab fitting process

The splitting pattern for carbon 3 of lactic acid is demonstrated (blue line). A quantum mechanical simulation is applied and the best fit demonstrated (red line).

Chapter 3

HMP for deceased donor kidneys

Paper published (1)

Nath J, Field M, Ebbs SR, Smith T, McGrogan D, Al-Shakarchi J, Hodson J, Mellor S, Ready A. Evolution of Renal Transplant Practice Over the Past Decade: A U.K. Center Experience. *Transplantation Proceedings* 2015; **47**(6): 1700-4.

Permission to incorporate this manuscript into the thesis has been kindly granted by Elsevier (Appendix p57)

Introduction

Kidney transplantation has been an unprecedented success story of modern medicine with well reported patient benefits of survival advantage and improved quality of life compared with the alternative of dialysis (9, 186, 187). In addition, from a health economics perspective, kidney transplantation is advantageous with a cost saving of £250,000 per kidney compared with haemodialysis (assuming median graft survival 10 years) (12).

In the initial five decades since the inception of kidney transplantation, there has been a dramatic progressive improvement in outcomes and is well demonstrated in Figure 32. The reasons for these improvements are multifactorial, but advances in immunosuppression therapy (e.g. introduction of azathioprine and later tacrolimus), and improved perioperative management are undoubted contributory factors. However whilst kidney transplant recipients in the contemporaneous era enjoy excellent outcomes, with 1-year graft survival (death-censored) rates of 94% for deceased donor organs (8) these have not altered over the past decade (188) and is perhaps surprising.

Given that the prognosis for transplant recipients, whilst excellent, appears to be static, it would be reasonable to question whether therapies such as hypothermic machine perfusion (HMP) can offer any clinical benefit, especially given the financial and resource costs of this intervention.

However, such is the success of transplantation that there is a significant disparity between the number of organs available and the demand for such organs. Given the need for further organs (discussed in Chapter 1) efforts have focused on increasing the number of organs available. Living donor kidney transplantation has partially

bridged this gap, with expansion in numbers in the UK over the past decade (Figure 33). However demand for organs still outstrips supply, and as such, a greater reliance on organs from expanded criteria donors, or marginal donors are being used. Whilst once organs such as those from deceased after cardiac death (DCD) organs were infrequently transplanted, these are now commonplace, even from more elderly donors. Indeed whilst the stereotypical donor from several decades ago was a previously fit young male involved in a road traffic accident, the equivalent donor in the contemporaneous era is several decades older with multiple medical comorbidities.

Not only is there a perception that kidneys utilised for transplant in the modern age are from higher risk donors, but that also the recipients of such organs are also generally more high risk. Indeed as kidney transplantation has become more widespread and recipients of transplants living longer than ever before, it is not uncommon for patients to have several previous transplants over their lifetime. Thus recipients of kidney transplants performed in this era are often highly sensitised (have high levels of preformed circulating antibodies), are often more elderly, with resulting cardiovascular comorbidity and many have no easy surgical location in which to site the kidney.

If indeed the organ quality utilised for transplantation has worsened with the development of transplantation and that these organs are indeed transplanted into more medically complex recipients then even stasis in outcome measures reflects significant improvements in clinical practice. Thus the presumption that hypothermic machine perfusion has resulted in little improvement in overall transplant outcome is oversimplistic as the incorporation of this technology into clinical practice has

occurred in an era when both donor and recipients of kidney transplants are more complex.

In this chapter, I aim to determine whether there has been a global worsening of deceased donor organ quality for kidney transplants occurring at the Queen Elizabeth Hospital, Birmingham over the past decade and whether these organs are transplanted into patients with greater medical risks. If indeed this is the case, as suspected, then it might provide a good rationale for hypothermic machine perfusion and for future technologies in order to optimise the function of these damaged grafts.

Methods

In this study, all adult patients receiving a kidney transplant from a deceased donor at the Queen Elizabeth Hospital, Birmingham between January 2004 and January 2014 were included. Data was obtained from an in-house database and the NHS Blood and Transplant Registry (a national registry to whom transplant centres are obligated to submit transplant information to).

To analyse whether donor and recipient characteristics have altered over the past decade, the patients (n=754) were divided into three groups (early, middle and late) depending on when the transplant was performed. Donor, recipient, and transplant specific factors were compared across the three groups and patient survival, graft survival and creatinine values at one year were used as outcome measures.

Findings

We found that there was indeed a global worsening of donor organ quality over the

three eras. One measure of this was the increase in the kidney donor risk index (KDRI), a predictive scoring system used to determine the projected median survival of a kidney transplant based on donor, recipient, and transplant specific factors.

However, despite the increasing complexity of transplantation in the modern era, reassuringly there was no corresponding detriment in transplant outcomes with no difference in patient or graft survival demonstrated at one year. Indeed graft function actually improved between the first and last era, with a decrease in the median creatinine values between the early and later era (median: 161 mmol/L vs. 132 mmol/L, $p < .001$)

Conclusion

In conclusion, in this large single centre analysis, we found outcomes for kidney transplants over the past decade have stayed static or for some indices actually improved. This has occurred in the context of both increasing complexity of kidney donors and recipients and reflects considerable improvements in the perioperative treatment. One of the major advances to occur during this study period was the advent of hypothermic machine perfusion, and as it was our practice to perfuse the majority of marginal kidneys used (roughly one third), this is likely to be a major contributor to this improvement in outcome.

We feel that this study provides circumstantial evidence to support the usage of hypothermic machine perfusion in the deceased donor setting. Furthermore, given the ever increasing demand for kidney transplants, the donor pool is likely to be expanded further in the future. Therapies such as

HMP are likely to become ever more important in the quest to optimise the function of such marginal kidneys.

The following manuscript was published in the journal Transplantation Proceedings in July 2015. I am the first author of this manuscript and was responsible at every stage of its production. I have written the manuscript included.

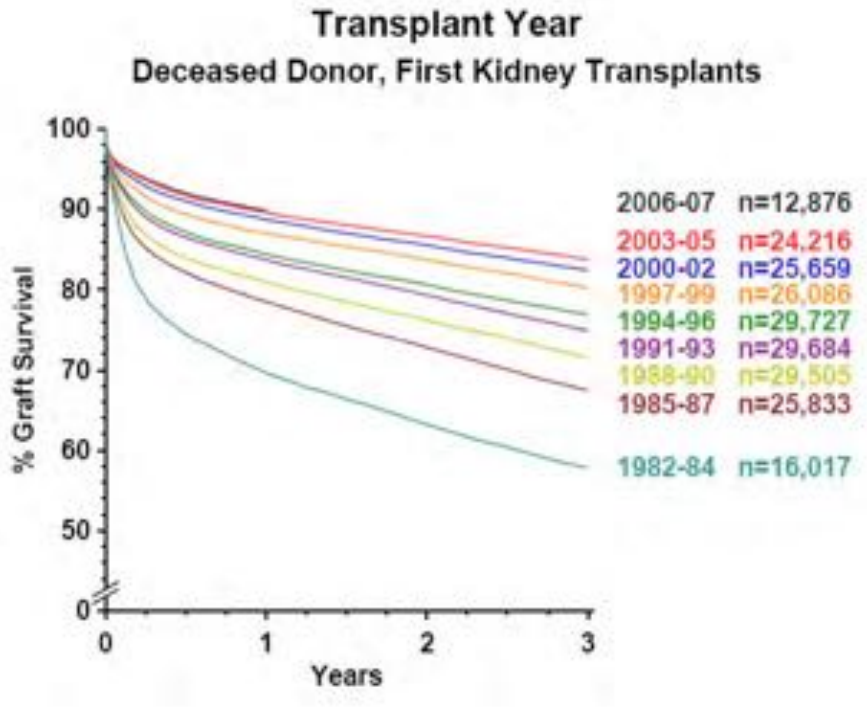

Figure 32 Graft survival by transplantation era.

Clear demonstration of the progressive improvement in outcomes with successive transplant eras (Data from the Collaborative Transplant Study, available at http://ctstransplant.org) (189)

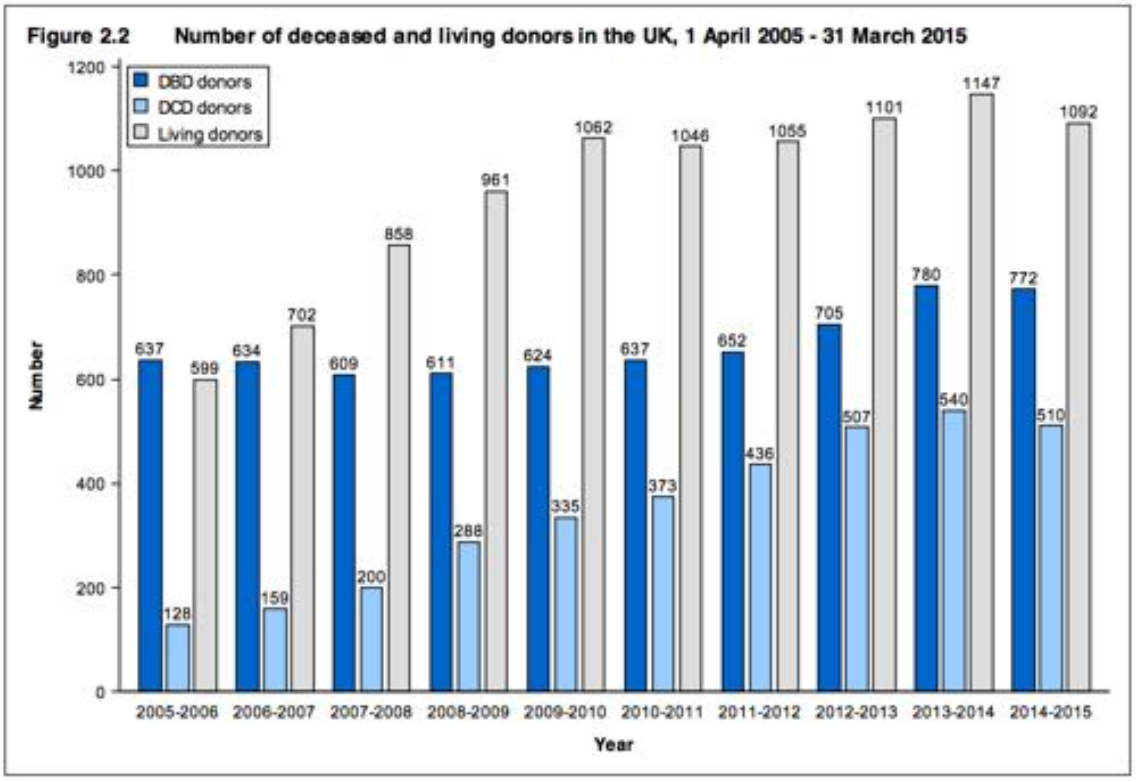

Figure 33 Increase in living donor transplantation

Demonstrating the increase in living donors in the UK over the past decade (grey bars). There has also been an increase in the number of deceased cardiac death (DCD) donors over this period (from NHS Blood and Transplant Organ Donation and Transplantation Activity Report 2014/15(8).

Evolution of Renal Transplant Practice Over the Past Decade: A U.K. Center Experience

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ABSTRACT

Objective. As renal transplantation continues to evolve, there appears to be a change in both donor and recipient populations. Traditional markers of high-risk donor (e.g. donation after cardiac death [DCD]/expanded criteria donor [ECD]) and recipient (e.g. obese, highly sensitized) operations appear to be more common without any noticeable worsening of patient outcome. The present study aimed to compare outcome and define the change in donor and recipient populations for cadaveric transplants over a 10-year period at a large U.K. center.

Methods. Single-center analysis of all adult patients undergoing cadaveric renal transplantation between January 2004 and January 2014 (n = 754). Transplants were divided into 3 groups (early, middle, and late) depending on the era, with donor, recipient and outcomes compared.

Results. There were considerable changes in both donor and recipient factors between the 3 eras, with a greater proportion of high-risk operations performed, as reflected by significant increases in Donor Risk Index (median: 1.11–1.16, $P = .022$), and the proportions of ECD (22.2%–33.9%, $P = .003$) and DCD kidneys (10.8%–19.4% $P = .011$). However, 1-year graft survival was comparable between the eras, with a decrease in the average 1-year serum creatinine between the early and late cohort (median: 161 μmol/L vs 132 μmol/L, $P < .001$). There was no significant increase in body mass index (BMI) in either the donor or recipient population across the eras.

Conclusion. Improvement in transplant outcome continues despite a greater proportion of transplants previously considered as high risk being performed. This is likely to reflect a considerable improvement in pre- and postoperative management. BMI remains a major continuing block to transplantation.

THE EVOLUTION of renal transplantation over the past 60 years is a triumph of modern medicine, with patients undergoing organ transplant in the modern era expecting a 90% 1-year graft survival [1]. The decade-upon-decade improvement in outcome is the consequence of many factors, including improved immunosuppressive, surgical, and perioperative standards.

Such are the benefits of renal transplantation that greater numbers of patients are now offered this treatment, including those with complex medical problems, as there is a distinct survival advantage compared to dialysis even in high-risk patients [2–4].

Despite the ever-growing demand, transplantation is limited by the paucity of available organs, and great efforts have been made to expand the donor pool, reflected by increased transplant numbers both in the U.K. and worldwide [3,5]. Expansion of the living kidney program and widespread acceptance of both Extended Criteria Donor (ECD) and Donation after Circulatory Death (DCD) organs as suitable grafts has been central to this.

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0041-1345/15
http://dx.doi.org/10.1016/j.transproceed.2015.06.001

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360 Park Avenue South, New York, NY 10010-1710

Various scoring systems have been derived to predict the likelihood and duration of post-transplant function. These have been based on multivariable analysis of donor and recipient factors on large datasets and have been prompted by the need for optimal organ allocation. The Kidney Donor Risk Index (KDRI) and the transplant calculator are the 2 most used in clinical practice, although the latter is only validated as a predictor of DGF [6,7]. An alternative to the KDRI was proposed based on a U.K. dataset and was based solely on donor characteristics [8].

Despite the encouraging outcomes for modern-day kidney transplants, there is a perception that both the donors and recipients of cadaveric kidneys have become more high risk. Some of the contributing factors (e.g. age, body mass index [BMI]) may simply be reflective of trends in the general population [9]. However, immunological complexity such as highly sensitized patients, often from multiple previous failed grafts, are transplant specific [10].

There is also an opinion that the expansion of the living donation kidney program has altered the overall group demographics of cadaveric recipients. As a group, recipients of living donor kidneys are younger, often with less comorbidity and often preemptive. Therefore, increased live donor numbers may have removed a cohort of straightforward transplants from the list, thus concentrating the more complex cases in the cadaveric pool.

The aim of this study is to compare chronological cohorts of patients undergoing cadaveric renal transplant over the past decade at a single U.K. transplant center to determine whether perceived changed in donor and recipient risk are reflected in clinical practice and transplant outcome.

METHODS

Consecutive adult patients who underwent single-organ renal transplantation at Queen Elizabeth Hospital, Birmingham, between January 2004 and January 2014 were included. Donor and recipient details including age, gender, ethnicity, BMI, donor status, comorbidity, serum creatinine levels, HLA mismatch, peak PRA value, and dialysis status were obtained through the National Health Service Blood and Transplant data registry and a prospectively maintained in-house database.

Renal transplantation was performed via a retroperitoneal approach as described elsewhere, with the right iliac fossa used preferentially [11]. Patients underwent standard transplantation workup, including chest radiograph, electrocardiogram, echocardiogram, myocardial perfusion imaging, coronary angiography, and pulmonary function tests, where appropriate.

Immunosuppression Regime

All patients received 0.5 g methylprednisolone and 20 mg basiliximab (Simulect) at induction of anesthesia. Basiliximab was again given on day 4 postoperatively. Patients were routinely treated with a standardized triple immunosuppressive maintenance regime: mycophenolate mofetil, tacrolimus (Prograf) and prednisolone. However, for some patients in the early cohort, azathioprine, cyclosporine and mycophenolate mofetil were used.

Statistical Methods

The data were initially split into 3 "eras," based on tertiles, to give approximately equal numbers of transplants in each group. A range of factors were then compared across these groups. Jonckheere-Terpstra tests were used for continuous factors, and Kendall's τ for dichotomous ones, in order to consider the chronological ordering of the periods being compared. Categorical variables with more than 2 levels were compared using Fisher's exact test.

Continuous data were assessed for normality prior to analysis, and all were found to follow skewed distributions. Hence, the continuous variables are reported as medians and ranges throughout. All analyses were performed using IBM SPSS Statistics 22 (IBM Corp., Armonk, N.Y., United States), with $P < .05$ deemed to be indicative of statistical significance.

RESULTS

During the 10-year study period, 1276 adult kidney transplants were performed at our center, of which 754 were cadaveric and met the inclusion criteria. These were divided into three chronological eras (n = 251 in the first 2 eras, and n = 252 in the final era) with the comparisons between the 3 eras for donor and recipient factors reported in Tables 1 and 2.

Both donor and recipient age were found to have increased significantly over the period, from medians of 47 to 51 ($P = .035$) and 46 to 49 years ($P = .018$), respectively. There was no significant increase in either donor ($P = .154$) or recipient ($P = .964$) BMI during the study period. For cadaveric donors, the rates of hypertension increased from 16.8% to 25.5% ($P = .017$), but there was no significant increase in the rate of diabetes ($P = .098$). The distribution of causes of death also changed ($P < .001$), with deaths from anoxia increasing (4.8%–15.5%), whereas CVA deaths remained consistent (66.5%, 65.3%, and 67.1% in the 3 eras).

There was a significant increase in the KDRI over the time period, which increased from a median of 1.11 in the first era to 1.16 in the last ($P = .022$). Although modest in absolute terms, this has prognostic implications, with expected median graft survival falling from 10.8 to 9.2 years [6]. Furthermore, the increase in donor KDRI was tampered by a significant decrease in the median cold ischemia time (CIT) between groups from 17.5 to 15.8 hours ($P < .001$). Indeed the KDRI would have risen further if such hastening of CIT had not occurred.

Other markers of high-risk grafts also increased during the study period. The proportion of ECD kidneys increased from 22.2% to 33.9% ($P = .003$) with a similar pattern for DCD kidneys (10.8%–19.4%, $P = .011$). The proportion of highly sensitized patients (PRA >80%) increased from 8.4% to 13.5% ($P < .001$), whereas the proportion of patients with more than 2 HLA mismatches rose from 43% to 60% ($P = .032$).

Of the outcomes considered (Table 3), both patient ($P = .157$) and graft (death censored) ($P = .330$) survival were similar across the 3 periods, changing from 96.4% to 98.4% and 91.3% to 88.7%, respectively. However, renal function

Table 1. Comparison of Donor Factors Across the Three Periods

	Jan 1, 2004 to Jun 10, 2007	Jun 11, 2007 to Dec 25, 2010	Dec 26, 2010 to Jan 1, 2014	*P*-Value
Age (y)	47 (7–74)	48 (12–72)	51 (13–76)	.035*
Sex (male)	142 (56.6%)	126 (50.2%)	131 (52.0%)	.303
Ethnicity				.004*†
Asian	11 (4.4%)	0 (0.0%)	5 (2.0%)	
Black	1 (0.4%)	5 (2.0%)	3 (1.2%)	
Other	2 (0.8%)	4 (1.6%)	6 (2.4%)	
White	237 (94.4%)	242 (96.4%)	233 (94.3%)	
BMI (kg/m^2)	24.9 (12.6–47.3)	25.7 (15.6–50.0)	25.5 (16.0–52.1)	.154
Obese (BMI >30 kg/m^2)	33 (13.1%)	45 (17.9%)	45 (17.9%)	.163
Diabetes	6 (2.4%)	11 (4.5%)	13 (5.2%)	.098
Hypertension	41 (16.8%)	47 (19.3%)	63 (25.5%)	.017*
DCD kidney	27 (10.8%)	52 (20.7%)	49 (19.4%)	.011*
Cause of death†				<.001*†
CVA	167 (66.5%)	164 (65.3%)	169 (67.1%)	
Anoxia	12 (4.8%)	28 (11.2%)	39 (15.5%)	
Other	72 (28.7%)	59 (23.5%)	44 (17.5%)	
Terminal creatinine (μmol/L)	76 (41–408)	74 (18–198)	73 (25–226)	.014*
Total HLA mismatches				.032*
0	36 (15.4%)	37 (14.7%)	33 (13.1%)	
1	14 (6.0%)	14 (5.6%)	9 (3.6%)	
2	83 (35.5%)	63 (25.1%)	58 (23.0%)	
3	49 (20.9%)	92 (36.7%)	97 (38.5%)	
4	39 (16.7%)	45 (17.9%)	54 (21.4%)	
5	7 (3.0%)	0 (0.0%)	1 (0.4%)	
6	6 (2.6%)	0 (0.0%)	0 (0.0%)	
CIT (h)	17.5 (8.0–38.3)	17.0 (7.5–39.0)	15.8 (5.5–30.0)	<.001*
KDRI	1.11 (0.61–2.51)	1.07 (0.51–2.62)	1.16 (0.58–2.19)	.022*
Extended criteria donor	55 (22.2%)	53 (21.9%)	82 (33.9%)	.003*

Data reported as: "Median (Range)", with *P* values from Jonckheere–Terpstra test, or "N (%)", with *P* values from Kendall's τ, as applicable, unless stated otherwise.
*Significant at $P < .05$.
†*P* value from Fisher's exact test.

improved across the study period, with a significant decrease in 1-year creatinine levels between the early and later era noted (median: 161 μmol/L vs. 132 μmol/L, $P < .001$).

Table 2. Comparison of Recipient Factors Across the 3 Periods

	Jan 1, 2004 to Jun 10, 2007	Jun 11, 2007 to Dec 25, 2010	Dec 26, 2010 to Jan 1, 2014	*P* Value
Age (y)†	46 (16–72)	47 (16–77)	49 (17–75)	.018*
Sex (male)	148 (59.0%)	148 (59.0%)	153 (60.7%)	.699
Ethnicity				.457†
Asian	60 (23.9%)	56 (22.3%)	76 (30.2%)	
Black	18 (7.2%)	20 (8.0%)	20 (7.9%)	
Other	2 (0.8%)	4 (1.6%)	2 (0.8%)	
White	171 (68.1%)	171 (68.1%)	154 (61.1%)	
BMI (kg/m^2)	26.1 (15.2–40.6)	26.1 (16.9–38.8)	26.3 (17.1–37.9)	.964
BMI >30 (kg/m^2)	49 (24.0%)	54 (26.7%)	62 (24.8%)	.902
Graft number 2+	28 (11.2%)	33 (13.1%)	28 (11.1%)	.961
PRA				<.001*
0	193 (77.2%)	177 (70.5%)	133 (52.8%)	
1–80	36 (14.4%)	48 (19.1%)	85 (33.7%)	
>80	21 (8.4%)	26 (10.4%)	34 (13.5%)	

Data reported as: "Median (Range)", with *P* values from Jonckheere-Terpstra test, or "N (%)", with *P* values from Kendall's τ, as applicable, unless stated otherwise.
*Significant at $P < .05$.
†*P* value from Fisher's exact test.

DISCUSSION

Our data support the long-held assertion that recipients and donors are becoming increasingly complex. We have demonstrated significant increases in age (in both donor and recipient), donor rates of hypertension, donor deaths to anoxia, increase in KDRI, increase in the number of ECD and DCD kidneys transplanted, increases in the sensitisation level of patients, and increases in the number of HLA mismatches. Despite such increases in marginality of both donors and recipients, outcomes have not only been maintained but have shown improvement, with superior graft function noted in the later cohort. This is reassuring against

Table 3. Transplant Outcome Measures Across the 3 Periods

	Jan 1, 2004 to Jun 10, 2007	Jun 11, 2007 to Dec 25, 2010	Dec 26, 2010 to Jan 1, 2014	*P* Value
1-year patient survival	242 (96.4%)	242 (96.4%)	247 (98.4%)	.157
1-year graft survival†	221 (91.3%)	221 (91.3%)	216 (88.7%)	.330
1-year creatinine	161	127	132	< .001*

Data reported as: "Median" with *P* values from Jonckheere–Terpstra test, or "N (%)", with *P* values from Kendall's Tau, as applicable.
*Significant at $P < .05$.
†Censored at death.

the background of increased complexity and is likely to reflect improvements in perioperative management.

There have been several changes in practice that have occurred during the study period that counter the changes in recipient and donor characteristics. Although no new major class of drug entered into routine use during the study period, the evolution of immunosuppression has continued and may be responsible for some of the improvement in outcome [12].

Over the study period, the proportion of donor deaths attributed to anoxia rose significantly from 4.8% to 15.5%. It is hard to determine exactly the reasons for this. It may be this rise represents an increase in the number of DCD donors in the latter era.

Kidneys in the latter era were less well matched than in the earlier era. It may be this reflects the changes to the allocation system, with greater priority being weighted towards other factors other than the HLA mismatch. It may be this represents an inherent mismatch in our donor population (predominantly Caucasian) and our recipient population (with a high proportion of non-Caucasians).

During the study period, hypothermic machine perfusion was adopted into routine clinical practice. Results from the machine perfusion trial have demonstrated that this can improve 1- and 3-year graft survival for cadaveric kidneys overall [13,14]. The benefit of machine perfusion appears most pronounced for expanded criteria kidneys and may be partly responsible for limiting the effects of these marginal organs within our population [15].

Not all donor/recipient factors worsened over the study period, with an improvement in the donor terminal creatinine and CIT noted. The decrease in terminal creatinine is perhaps surprising, particularly in light of the increasing age of donors and by association the likelihood of reduced glomerular filtration rate. A possible explanation for this finding could be due to improved donor management in the intensive care setting, with goal-directed therapy often begun by specialist teams within the U.K. prior to the organ retrieval process [16].

The reduction in CIT at our center, the only modifiable transplant risk factor, has been an active development and the reasons for this observation are likely to be multifactorial. However, routine implementation of the "virtual crossmatch" for patients with undetectable panel reactive antibodies has negated the need for a lengthy crossmatch period in this cohort of patients and has been a major contributing factor.

Live donor transplants have been excluded from this analysis to avoid nonmeaningful comparisons between inherently different groups, especially given the expansion of the live donor program during the study period. Interestingly, live donors and recipients were found to be younger than for cadaveric kidneys (median: 46 years and 44 years) with comparable BMIs. However, waiting list times for live donor recipients are much lower (384 days vs 1344 days) in our population. The notion that the live donor population has filtered some of the "easier" predialysis patients from the transplant list therefore appears valid, perhaps increasing the complexity in the remaining cadaveric pool. However, to balance this it must also be considered that the live donor pool also absorbs some of the most complex transplants (e.g. ABO-I, HLA-I) and some potential recipients may have technical factors rendering them only suitable for a planned live donor implant.

This study is limited by only looking at relatively short-term outcomes from a single, albeit large, center. However, despite these limitations it does serve to illustrate the point that the characteristics of donor and recipient groups have changed and transplants that were once considered high risk are now performed routinely.

Although overall, donors and recipients have undoubtedly become more complex, there has been no change in the proportion of obese patients transplanted. Reported outcomes for obese patients receiving kidney transplants are inconclusive [17–20], with no clear upper guidelines as to the upper limit of acceptable BMI for transplantation. In our population, outcomes for this group were worse, with risks exaggerated in kidneys with multiple arteries [21]. The static BMI of recipients in this study does not reflect the increase in BMI seen in the general population or the number of obese patients assessed for transplantation at our center and, as such, obesity appears to remain a significant barrier to transplantation.

In the future, both the donor and recipient pool are likely to show further evolution and present further challenges for transplantation. Indeed it is estimated that almost a third of patients commencing dialysis are over 70 [22].

If the changes in the last decade can be maintained, provision for this evolution should be adequate. BMI remains a major continuing block to transplantation and careful consideration will be needed in order to best serve these patients.

CONCLUSION

Although limited to a single center, our results show that both donors and recipients of cadaveric kidneys are increasingly more complex. Despite increased risk, outcomes have improved over the study period, suggesting a significant improvement in transplant care. It is likely that both donors and recipients will become increasingly more complex and that the challenge will be how to improve or maintain renal transplant outcomes in this era of evolution.

ACKNOWLEDGEMENTS

The authors are grateful to NHSBT for supplying some of the data presented. This work was supported through a grant from University Hospital Birmingham Charities.

REFERENCES

[1] NHSBT. Organ Donation and Transplantation: Acitvity Report 2013-2014. 2014.

[2] Wolfe R, Ashby V, Milford E, Ojo A, Ettenger R, Agodoa L, et al. Comparison of mortality in all patients on dialysis, patients on

dialysis awaiting transplantation, and recipients of a first cadaveric transplant. N Engl J Med 1999;341:1725–30.

[3] Gilg J, Rao A, Fogarty D. UK renal replacement therapy Incidence in 2012: national and centre-specific analyses. Nephron Clin Pract 2013;125:1–27.

[4] Oniscu G, Brown H, Forsythe J. How great is the survival advantage of transplantation over dialysis in elderly patients? Nephrol Dial Transplant 2004;19:945–51.

[5] Orandi B, Garonzik-Wang J, Massie A, Zachary A, Montgomery RA, Van Arendonk K, et al. Quantifying the risk of incompatible kidney transplantation: a multicenter study. Am J Transplant 2014;14:1573–80.

[6] Rao P, Schaubel D, Guidinger M, Andreoni K, Wolfe R, Merion R, et al. A comprehensive risk quantification score for deceased donor kidneys: the kidney donor risk index. Transplantation 2009;88:231–6.

[7] Irish W, Ilsley J, Schnitzler M, Feng S, Brennan D. A risk prediction model for delayed graft function in the current era of deceased donor renal transplantation. Am J Transplant 2010;10:2279–86.

[8] Watson C, Johnson R, Birch R, Collett D, Bradley J. A simplified donor risk index for predictin outcome after deceased donor kidney transplantation. Transplantation 2012;93:314–8.

[9] Health &Social Care Information Centre: Statistics on Obesity, Physical Activity and Diet - England, 2013 [updated Feb 20, 2013]. Available at: http://www.hscic.gov.uk/catalogue/PUB10364. Accessed April 2015.

[10] Sharif A, Alachkar N, Kraus E. Incompatible kidney transplantation: a brief overview of the past, present and future. QJM 2012;105:1141–50.

[11] Allen RDM. Vascular complications after kidney transplantation. In: Morris P, Knechtle SJ, editors. Kidney Transplantation: Principles and Practice. 6th ed. UK: Saunders; 2008. p. 439–61.

[12] Ekberg H, Tedesco-Silva H, Demirbas A, Vitko S, Nashan B, Gurkan A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. N Engl J Med 2007;357:2562–75.

[13] Moers C, Smits J, Maathuis M, Treckmann J, van Gelder F, Napieraslski B, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med 2009;360:7–19.

[14] Moers C, Pirenne J, Paul A, Ploeg R, Group MPTS. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med 2012;366:770–1.

[15] O'Callaghan J, Morgan R, Knight S, Morris P. Systematic review and meta-analysis of hypothermic machine perfusion versus static cold storage of kidney allografts on transplant outcome. BJS 2012;94(suppl):277.

[16] Kustogiannis D, Pagliarello G, Doig C, Ross H, Shemie S. Medical management to optimize donor organ potential : a review of the literature. Can J Anaesth 2006;53:820–30.

[17] Chang S, Coates P, Mcdonald S. Effects of body mass index at transplant on outcomes of kidney transplatation. Transplantation 2007;84:981–7.

[18] Bennett W, McEvoy K, Henell K, Valete J, Douzdjian V. Morbid obesity does not preculde successful renal transplantation. Clin Transplant 2004;8:89–93.

[19] Grosso G, Corona D, Mistretta A, Zerbo D, Sinagra N, Giaquinta A, et al. The role of obesity in kidney transplantation outcome. Transplant Proc 2012;44:1864–8.

[20] Meier-Kriesche H, Arndorfer J, Kaplan B. The impact of body mass index on renal transplant outcomes: a signficiant independent risk factor for graft failure and patient death. Transplantation 2002;73:70–4.

[21] Nath J, Mastoridis S, van Dellen D, Guy A, McGrogan D, Krishnan H, et al. Complex kidneys for complex patients: the risk associated with transplantation of kineys with multiple arteries into obese patients. Transplant Proc 2015;47:373–8.

[22] Knoll G. Kidney transplantation in the older adult. Am J Kidney Dis 2013;61(5):790–7.

Chapter 4

HMP in living donor kidneys

Paper published (2).

Nath J, Hodson J, Canbilen SW, Al Shakarchi J, Inston NG, Sharif A, Ready AR.

Effect of cold ischaemia time on outcome after living donor renal transplantation.

The British Journal of Surgery 2016; **103**(9): 1230-6.

Permission to incorporate this manuscript into the thesis has been kindly granted by John Wiley & Sons (Appendix p58)

Introduction

The benefits of HMP in the deceased donor kidney setting includes improved early and late graft function and is discussed in the introduction (p11). Furthermore, given the increasing complexity of kidney transplantation and increasing reliance on high-risk organs, therapies that improve functional outcomes such as HMP are likely to be of increasing clinical importance.

The outcomes for kidney transplants from living donors are superior to those from deceased donors (8). The reasons for this are multifactorial, but a major contributor is that these organs are from healthy donors and therefore the organs are not subjected to the physiological extremis of organs from deceased donors. In addition, the cold ischaemia time for living donor organs has traditionally been very short as the transplant operations have either occurred immediately after or even simultaneous to the organ retrieval (nephrectomy) operation.

One of the major benefits of HMP for deceased donor kidneys is that it reduces the likelihood of delayed graft function (DGF) postoperatively (defined as the need for dialysis in the first week after transplant). Given that the rate of DGF in living donor kidneys has traditionally been very low (<5%), the benefit of the labour intensive and costly intervention of HMP was thought to be limited. Indeed, HMP was not utilised on any living donor kidney transplants performed in the UK between 2001 and 2014 (data obtained following personal communication with NHSBT).

However, practices within living donor transplantation have evolved over the past few years. One of the major developments is that increasing numbers of split site living donor transplants are performed, whereby the kidney donation operation and the transplant operation occur at two different hospitals. Needless to say, the

kidneys utilised in such split-site operations have much longer cold ischaemia time (CIT). The reasons for the increase in split-site operations are partly due to the increased number of altruistic or non-directed donors whereby members of the public donate a kidney to unknown recipients to whom they have no emotional links. The increase in numbers of altruistic kidneys has been dramatic over recent years and is demonstrated in Figure 34.

Given that there is an increasing subset of live donor kidneys which have a significantly prolonged CIT and that prolonged CIT is associated with inferior outcomes for deceased donor kidneys, the following study was performed to determine whether living donor kidneys with prolonged CIT have inferior outcomes. If this were found to be the case, the beneficial role of HMP for living donor kidneys would need to be established, given its benefit in the deceased donor setting.

Methods

In this retrospective study of over 9000 transplant recipients using a national dataset, outcomes for living donor kidneys in the UK were analysed using multivariable modelling based on the duration of CIT (0-2 hours, 2-4 hours and 4-8 hours).

Results

Live donor transplants with modestly prolonged CIT (4-8 hours) were found to have excellent outcomes. Although the risk of DGF on multivariable analysis was greater in the live donor group with prolonged CIT ($p<0.001$), the adjusted rates of DGF were low in the shortest and longest CIT group 4.3% vs. 8.6%. Whilst the impact of

DGF in live donor kidneys is not known, it is unlikely to mirror the prolonged deleterious sequelae sometimes seen in deceased donor transplantation. Even assuming DGF for live donor kidneys is equivalent to deceased donor organs and that HMP would have equal therapeutic benefit, the number of perfused live donor kidneys needed to prevent one occurrence of DGF would be 54 and is therefore probably not justified. (This is based on the reported reduction of DGF in the machine preservation trial for deceased donor kidneys (reduction from 26.5% to 20.8%)) (28).

Conclusion

In conclusion, based on analysis of a large national dataset, there appears to be little evidence to support the routine usage of HMP for live donor kidneys, even for split-site operations with modestly prolonged CIT (4-8 hours). In the albeit rare occurrence where there is an unforeseen delay in transplant operation (e.g. recipient has an anaphylactic reaction prior to anaesthesia) and the projected CIT is likely to be significantly more prolonged than this (e.g. 20 hours), then a decision to use HMP for such organs would seem reasonable although is not validated by this study.

The following manuscript was published in the British Journal of Surgery in June 2016. I am the first author of this manuscript. Statistical analysis was performed by James Hodson (biostatistician) but I was responsible for every other stage of its production and wrote the enclosed manuscript.

The supporting information for this manuscript is in appendix p38-47.

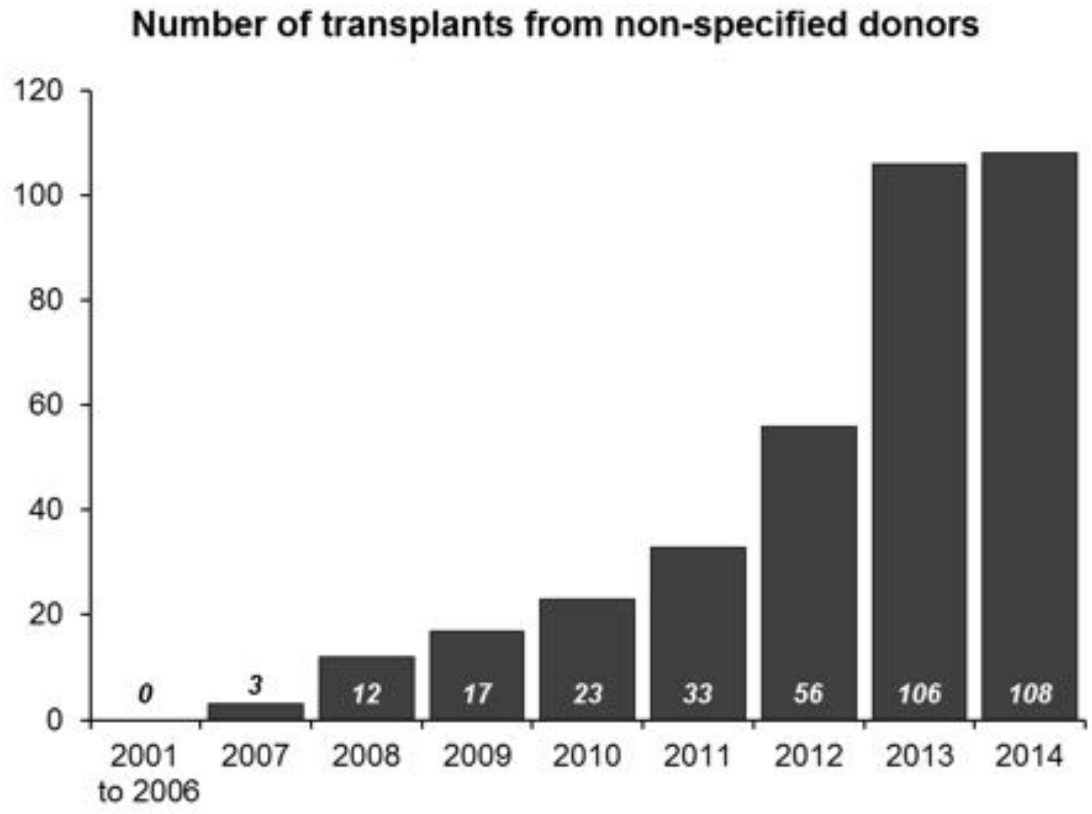

Figure 34 Increase in number of unspecified (altruistic) kidney donors in the UK

Data obtained through NHSBT data request procedures.

Original article

Effect of cold ischaemia time on outcome after living donor renal transplantation

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Background: **The aim of the present study was to determine the effects of cold ischaemia time (CIT) on living donor kidney transplant recipients in a large national data set.**

Methods: **Data from the National Health Service Blood and Transplant and UK Renal Registry databases for all patients receiving a living donor kidney transplant in the UK between January 2001 and December 2014 were analysed. Patients were divided into three groups depending on CIT (less than 2 h, 2–4 h, 4–8 h). Risk-adjusted outcomes were assessed by multivariable analysis adjusting for discordance in both donor and recipient characteristics.**

Results: **Outcomes of 9156 transplants were analysed (CIT less than 2 h in 2662, 2–4 h in 4652, and 4–8 h in 1842). After adjusting for confounders, there was no significant difference in patient survival between CIT groups. Recipients of kidneys with a CIT of 4–8 h had excellent graft outcomes, although these were slightly inferior to outcomes in those with a CIT of less than 2 h, with risk-adjusted rates of delayed graft function of 8·6 *versus* 4·3 per cent, and 1-year graft survival rates of 96·2 *versus* 97·1 per cent, respectively.**

Conclusion: **The detrimental effect of prolonging CIT for up to 8 h in living donation kidney transplantation is marginal.**

Paper accepted 17 February 2016

Published online in Wiley Online Library (www.bjs.co.uk). **DOI:** 10.1002/bjs.10165

Introduction

Living donor (LD) kidney transplantation is associated with excellent clinical outcomes, and has helped to address the disparity between supply and demand of available organs for transplantation. LD transplants now account for one-third of all kidneys transplanted in the UK[1].

Prolonged cold ischaemia time (CIT), the duration for which the organ is cold stored before implantation, has been demonstrated repeatedly to correlate with inferior graft outcome for cadaveric kidney transplants, with higher rates of delayed graft function (DGF), acute rejection and inferior long-term outcome[2–6].

CIT in the LD setting is usually shorter than for cadaveric kidney operations, and CIT times of less than 1 h are achievable when simultaneous donor and recipient operations are performed[7]. However, the effect of a longer interval of cold ischaemia in the LD setting is less clear, and if associated with inferior outcomes would have important consequences.

LD transplantation has evolved significantly over the past decade, with greater numbers of split-site (two hospitals) donor and recipient operations. This is largely a consequence of the increase in paired exchange programmes and non-directed or altruistic donors. Split-site procedures are performed for logistical (to minimize the burden of travel) and ethical (to avoid emotional coercion) reasons. If longer CIT resulting from split-site operations were associated with inferior outcomes, such decisions would have to be rationalized against potential harms. Furthermore, the potential role of hypothermic machine perfusion for LD kidneys would need to be established. This would seem attractive, given that it is associated with reduced rates of DGF[8–10] and improved graft survival for cadaveric organs[9,10].

The aim of this study was to determine whether modest prolongation of CIT (up to 8 h) has any effect on outcome for LD kidney transplants. Excellent outcomes were reported for kidneys with prolonged CIT in a previous study[11] based on US registry data between 1990

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Table 1 Association between cold ischaemia time and patient demographics

	Valid no.	Cold ischaemia time (h)			*P*‡
		<2	2–4	4–8	
Recipient					
Age (years)*	9156	43 (32–53)	44 (33–54)	46 (36–56)	<0·001§
Sex (men)	9149	1630 (61·3)	2829 (60·6)	1049 (56·9)	0·008¶
Ethnicity	9068				0·009
White		2326 (87·7)	3949 (86·0)	1548 (85·0)	
Asian		188 (7·1)	376 (8·2)	174 (9·5)	
Black		86 (3·2)	189 (4·1)	78 (4·3)	
Other		52 (2·0)	80 (1·7)	22 (1·2)	
Diabetes	9156	175 (6·6)	235 (5·1)	124 (6·7)	0·792¶
Dialysis at transplant	8619				<0·001
Haemodialysis		1290 (50·5)	2048 (46·9)	978 (57·4)	
Peritoneal dialysis		589 (23·1)	1015 (23·3)	312 (18·3)	
Not on dialysis		674 (26·4)	1300 (29·8)	413 (24·3)	
Graft no.	9156				0·001¶
1		2322 (87·2)	4107 (88·3)	1528 (83·0)	
2		292 (11·0)	478 (10·3)	257 (14·0)	
≥3		48 (1·8)	67 (1·4)	57 (3·1)	
CMV-positive	8570	1114 (44·5)	1889 (43·8)	865 (49·4)	0·007¶
Blood group	9150				0·822
A		1090 (41·0)	1904 (40·9)	758 (41·2)	
AB		87 (3·3)	162 (3·5)	70 (3·8)	
B		300 (11·3)	558 (12·0)	228 (12·4)	
O		1181 (44·4)	2026 (43·6)	786 (42·7)	
Donor					
Age (years)*	9151	48 (39–56)	47 (38–56)	49 (40–57)	0·055§
Sex (men)	9150	1218 (45·8)	2115 (45·5)	847 (46·0)	0·949¶
Body mass index (kg/m^2)†	8673	26·4(4·0)	26·7(4·0)	26·4(3·9)	0·280§
Ethnicity	9083				0·432
White		2299 (87·8)	4004 (86·5)	1618 (88·1)	
Asian		184 (7·0)	347 (7·5)	128 (7·0)	
Black		83 (3·2)	185 (4·0)	59 (3·2)	
Other		51 (1·9)	93 (2·0)	32 (1·7)	
Blood group	9146				0·018
A		848 (31·9)	1486 (32·0)	644 (35·0)	
B		215 (8·1)	403 (8·7)	178 (9·7)	
O		1595 (60·0)	2758 (59·4)	1019 (55·4)	

Values in parentheses are percentages unless indicated otherwise; values are *median (i.q.r.) and †mean(s.d.). CMV, cytomegalovirus. ‡χ^2 test, except §Jonckheere–Terpstra test and ¶Kendall's τ for binary/ordinal variables to account for the ordering of cold ischaemia time groups.

and 2005. Validation through a second large data set is justified, given the potential implications for clinical practice.

Methods

All adult patients receiving a LD kidney transplant between January 2001 and December 2014 in the UK with CIT between 0 and 8 h were included. Kidney transplant centres within the UK are obliged to submit demographic and clinical data to both NHS Blood and Transplant (NHSBT) and Renal Registry professional bodies for each transplant performed; the data set can be assumed to be complete. The two bodies collect different patient-specific and outcome variables, and therefore the two data sets were amalgamated to try to include all possible variables.

Outcome measures

Primary outcome measures were 1-, 3- and 5-year graft survival (death censored). Secondary outcome measures were rates of DGF, serum creatinine values at 1, 3 and 5 years, patient survival, and rates of acute rejection within 3 months.

Statistical analysis

Initially, univariable analyses were performed to compare factors across the CIT groups, using a combination of

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Table 2 Association between cold ischaemia time and matching/transplant factors

	Valid no.	Cold ischaemia time (h)			*P*†
		< 2	2–4	4–8	
Wait time (days)*	6605	287 (122–600)	285 (114–579)	358 (142–925)	< 0·001‡
Sensitization (PRA)	4701				< 0·001‡
0		601 (59·4)	1565 (63·7)	573 (46·5)	
1–20		78 (7·7)	144 (5·9)	58 (4·7)	
21–80		177 (17·5)	495 (20·2)	336 (27·3)	
81–100		156 (15·4)	252 (10·3)	266 (21·6)	
Year of transplant	9156				< 0·001
2001–2005		879 (46·2)	852 (44·7)	173 (9·1)	
2006–2008		672 (32·6)	1149 (55·8)	238 (11·6)	
2009–2011		649 (26·1)	1297 (52·3)	536 (21·6)	
2012–2014		462 (17·0)	1354 (49·9)	895 (33·0)	
HLA mismatch group	9142				0·001
1		283 (10·7)	540 (11·6)	241 (13·1)	
2		357 (13·5)	619 (13·3)	324 (17·6)	
3		1241 (46·8)	2182 (46·9)	763 (41·5)	
4		773 (29·1)	1308 (28·1)	511 (27·8)	
Related donor	9150	1746 (65·7)	2989 (64·3)	926 (50·3)	< 0·001
Antibody incompatibility	9156				< 0·001§
Not present		2339 (87·9)	4178 (89·8)	1679 (91·2)	
HLAi		175 (6·6)	178 (3·8)	72 (3·9)	
ABOi		126 (4·7)	270 (5·8)	79 (4·3)	
Both HLAi and ABOi		22 (0·8)	26 (0·6)	12 (0·7)	

Values in parentheses are percentages unless indicated otherwise; values are *median (i.q.r.). PRA, panel reactive antibody; HLAi, HLA-incompatible; ABOi, ABO-incompatible. †Kendall's τ, except ‡Jonckheere–Terpstra test and §χ^2 test (factor not ordinal).

Jonckheere–Terpstra, Kendall's τ and χ^2 tests, as appropriate. Multivariable analyses were then done, to account for differences in demographics across the CIT groups. These were based on binary logistic regression, Cox regression and general linear models, depending on the outcome being considered, and used a stepwise approach to select variables for inclusion. Full details of the statistical methodology can be found in *Appendix S1* (supporting information).

Categorical variables are reported as numbers and rates throughout. Continuous variables are presented as geometric means with 95 per cent confidence intervals (c.i.) or median (i.q.r.), as applicable. For multivariable analysis, odds ratios or hazard ratios are reported, as applicable, along with 95 per cent c.i.

All analyses were performed using SPSS® version 22 (IBM, Armonk, New York, USA), with $P < 0{\cdot}050$ deemed indicative of statistical significance.

Results

Data were available for a total of 10 043 LD transplants between 2001 and 2014. Of these, 809 had no data recorded for CIT and 78 had CIT longer than 8 h, so were excluded. This left a total of 9156 transplants, with a CIT of less than 2 h in 2662 (29·1 per cent), 2–4 h in 4652 (50·8 per cent) and 4–8 h in 1842 (20·1 per cent).

The data set was generally well populated, with only two co-variables (sensitization and pretransplant duration on waiting list) being unavailable for more than 10 per cent of transplants. Complete patient demographics and data availability are reported in *Tables S1–S3* (supporting information).

The three CIT groups were markedly different on univariable analysis (*Tables 1* and *2*). The prolonged CIT group was associated with many markers of transplant complexity, and recipients were generally older ($P < 0{\cdot}001$), more likely to be non-white ($P = 0{\cdot}009$), more sensitized ($P < 0{\cdot}001$), with more previous transplants ($P = 0{\cdot}001$), higher rates of haemodialysis ($P < 0{\cdot}001$) and cytomegalovirus (CMV) positivity ($P = 0{\cdot}007$), longer wait times ($P < 0{\cdot}001$) and were less likely to receive organs from related donors ($P < 0{\cdot}001$). However, several factors thought to convey favourable outcome were more prevalent in the longer CIT group, such as female sex ($P = 0{\cdot}008$), fewer HLA mismatches ($P = 0{\cdot}001$) and lower rates of antibody incompatibility ($P < 0{\cdot}001$). More recent transplants were also found to be associated with longer CIT ($P < 0{\cdot}001$).

Owing to these differences in patient demographics, multivariable analyses were used to account for potentially confounding factors. *Table 3* summarizes the results

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Table 3 Adjusted associations between cold ischaemia times and outcomes detemined by multivariable analyses

			Cold ischaemia time			
			2–4 h		4–8 h	
	Valid no.	Overall *P*	Statistic*	*P*	Statistic*	*P*
Patient survival†	8019	0·440	1·08 (0·88, 1·33)	0·468	1·20 (0·91, 1·58)	0·205
Graft survival†	8056	0·049	1·06 (0·91, 1·24)	0·471	1·31 (1·05, 1·62)	0·016
Rejection within 3 months‡	8334	0·004	0·81 (0·71, 0·94)	0·004	1·00 (0·84, 1·20)	0·970
Delayed graft function‡	7168	< 0·001	1·14 (0·88, 1·49)	0·314	2·16 (1·60, 2·92)	< 0·001
Creatinine level at 12 months§	5191	0·025	1·5 (−0·2, 3·2)	0·094	3·1 (0·8, 5·5)	0·007

Values in parentheses are 95 per cent confidence intervals. *Relative to the group with a cold ischaemia time of less than 2 h. †Hazard ratios from multivariable Cox regression model; ‡odds ratios from multivariable binary logistic regression model; §percentage differences from multivariable general linear model. The full multivariable models are reproduced in *Tables S4–S8* (supporting information).

Table 4 Adjusted outcomes from multivariable analyses

	Cold ischaemia time (h)		
	< 2	2–4	4–8
Patient survival			
1 year	99·1	99·1	99·0
3 years	98·0	97·9	97·6
5 years	96·5	96·2	95·8
Graft survival			
1 year	97·1	96·9	96·2
3 years	94·8	94·5	93·3
5 years	92·4	92·0	90·3
Rejection within 3 months	17·2	14·6	17·2
Delayed graft function	4·3	4·9	8·6
Mean creatinine at 12 months (μmol/l)	122	124	126

Adjusted rates generated from the multivariable models in *Table 3*.

of these analyses, indicating the adjusted relationships between CIT and the outcomes considered. The models used to generate these adjusted statistics are reproduced in full in *Tables S4–S8* (supporting information).

Graft survival

After adjusting for confounding factors, there was no significant difference in graft survival between the shorter CIT groups (less than 2 *versus* 2–4 h), with a hazard ratio of 1·06 (95 per cent c.i. 0·91 to 1·24; $P = 0·471$). However, the risk of graft failure was significantly higher in the 4–8-h group relative to the group with a CIT of less than 2 h (hazard ratio 1·31, 1·05 to 1·62; $P = 0·016$) (*Table 3*). Adjusted rates of death-censored 1-year graft survival from this model were 97·1, 96·9 and 96·2 per cent for the groups with a CIT of less than 2, 2–4 and 4–8 h respectively, with similar discrepancy at 3 and 5 years (*Table 4*).

Delayed graft function

DGF was defined as the need for dialysis in the first postoperative week; the overall rate was 5·1 per cent. The association between CIT and DGF was significant on multivariable analysis ($P < 0·001$). Although there was no difference between the groups with a CIT of less than 2 h *versus* 2–4 h ($P = 0·314$), patients with a CIT of 4–8 h were significantly more likely to develop DGF, with an odds ratio of 2·16 (95 per cent c.i. 1·60 to 2·92; $P < 0·001$) relative to the shortest CIT group (*Table 3*). The adjusted rates of DGF were 4·3, 4·9 and 8·6 per cent for the groups with a CIT of less than 2, 2–4 and 4–8 h respectively (*Table 4*).

Patient survival

CIT was not associated with survival on multivariable analysis ($P = 0·440$). The adjusted 1-year patient survival rates were 99·1, 99·1 and 99·0 per cent for the groups with a CIT of less than 2, 2–4 and 4–8 h respectively.

Creatinine

After adjustment for confounding factors, a significant relationship was detected between CIT and creatinine level at 12 months ($P = 0·025$). There was no significant difference in 12-month creatinine values for the two shorter CIT groups ($P = 0·094$), but patients in the 4–8-h group had significantly higher values than those with a CIT of less than 2 h, with a mean difference of 3·1 (95 per cent c.i. 0·8 to 5·5) per cent ($P = 0·007$). The adjusted average creatinine levels at 12 months were 122, 124 and 126 μmol/l in the groups with a CIT of less than 2, 2–4 and 4–8 h respectively.

Acute rejection

The rates of acute rejection (within 3 months) differed significantly by CIT group in multivariable analysis ($P = 0·004$). Interestingly, this difference was not between the shortest (less than 2 h) and longest (4–8 h) CIT groups (odds ratio 1·00, 95 per cent c.i. 0·84 to 1·20; $P = 0·970$).

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Instead, rejection was significantly less common in the middle CIT group (2–4 h), with an odds ratio of 0·81 (0·71 to 0·94; $P = 0·004$) relative to the shortest CIT. The adjusted rates of acute rejection were 17·2, 14·6 and 17·2 per cent for the groups with a CIT of less than 2, 2–4 and 4–8 h respectively.

Discussion

Based on a large UK national data set with good completeness, this study has demonstrated that LD kidneys with moderate prolongation of CIT (4–8 h) have excellent outcomes, although these are marginally inferior to those with shorter CIT.

This report of outcomes for moderately prolonged CIT in UK patients corroborates the findings from the earlier US study[11], which is reassuring given the variable transplant practice and patient outcomes between North American and European centres. The American study reported adjusted odds ratios for DGF of 1·84 and 2·05 in the longest CIT groups (4–6 and 6–8 h), compared with 2·16 in the 4–8-h group in the present study. The US study also observed a similar pattern of rejection, with odds ratios of 0·72, 0·65 and 1·07 for the 2–4-, 4–6- and 6–8-h CIT groups, compared with 0·81 and 1·00 for the 2–4- and 4–8-h groups in the present study. The reproducible finding, that several hours of CIT appears to minimize rates of post-transplant rejection, is interesting and warrants further investigation, as the biological mechanism for this is unclear.

In contrast to the US study, there was a small but significant difference in graft survival between CIT groups, which was worst in the 4–8-h CIT group (hazard ratio 1·31). Omission of era of transplant as a variable, and the lower statistical power in the US study, are likely to be the major reasons for this difference. The identification of CIT as an independent risk factor for graft failure is an interesting finding. However, adjusted graft survival rates of 96·2 per cent at 1 year and 90·3 per cent at 5 years in the 4–8-h CIT group justify planning LD transplants with anticipated CITs of 4–8 h. Similarly, although statistically significant, the minimal rise in serum creatinine concentrations in the prolonged CIT group is unlikely to be of clinical relevance.

The classification of CIT into three groups in this study (less than 2, 2–4 and 4–8 h) was deliberate, to reflect typical CITs in different operative conditions. Times under 2 h are achievable practically only when donor and recipient operations occur simultaneously. Between 2 and 4 h reflects consecutive donor/recipient operations, and 4–8 h is a realistic target for most split-site procedures[7,12]. The outcomes of this study reflect previous reports and suggest that, with regards to CIT reduction, simultaneous procedures offer no clinical benefit compared with sequential operations[7], and that split-site operations confer satisfactory outcomes[12,13]. It must be noted, however, that the 4–8-h CIT group in the present study included a number of sequential same-site procedures.

The donor and recipient differences between the three CIT groups are surprising, with greater proportions of highly sensitized, older, haemodialysed, CMV-positive patients with multiple previous transplants, but with lower levels of mismatch, in the 4–8-h CIT group. This is likely to reflect the greater proportion of altruistic and paired exchange kidneys in this group. Altruistic kidneys are offered to the best match patient from the transplant waiting list, and such patients are inherently more complex than those receiving traditional LD transplants[14]. Similarly, highly sensitized patients are more likely to find a compatible donor via the paired exchange programme, as this effectively increases the size of the donor pool for this hard-to-match group[15,16]. Given the inherent differences in donor and recipient characteristics between CIT groups, univariable (unadjusted) outcome measures are of limited value in this type of study; this highlights the strength of population-based analysis to identify subtle differences in outcome.

Although this data set included a multitude of donor, recipient and transplant factors, the list was not exhaustive. Even though there is no reason to suspect an unequal distribution across CIT groups, initial warm ischaemia time, anastomosis time, vessel anatomy and recipient body mass index have been shown to affect outcome in cadaveric kidney transplants[17–20] and would have been interesting to include.

As anticipated, this study has demonstrated significantly higher rates of DGF in LD kidneys with a CIT of 4–8 h compared with less than 2 h and 2–4 h (adjusted rates 8·6, 4·3 and 4·9 per cent respectively). Although these DGF rates are low compared with those for cadaveric kidneys, the almost twofold increase resulting from modest CIT prolongation is surprising. DGF is defined as the need for dialysis in the first week post-transplant and it is not clear whether this was largely a single postoperative session or more prolonged treatment. As such, the associated patient and graft morbidity of DGF after cadaveric transplants may not be applicable to LD kidneys.

Acknowledgements

The authors are grateful to NHSBT and the Renal Registry for supplying the data. The authors also thank all the UK

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renal units for providing data to the UK Renal Registry and NHSBT, and the Scottish Renal Registry for sharing the data collected and validated in Scotland. Any views or opinions expressed are solely those of the authors and do not necessarily represent those of the UK Renal Registry or NHSBT.
Disclosure: The authors declare no conflict of interest.

References

1 NHS Blood and Transplant. *Organ Donation and Transplantation Activity Report 2013/2014*. https://nhsbtmediaservices.blob.core.windows.net/organ-donation-assets/pdfs/activity_report_2013_14.pdf [accessed 1 October 2015].
2 Ojo AO, Wolfe RA, Held PJ, Port FK, Schmouder RL. Delayed graft function: risk factors and implications for renal allograft survival. *Transplantation* 1997; **63**: 968–974.
3 Debout A, Foucher Y, Trébern-Launay K, Legendre C, Kreis H, Mourad G *et al*. Each additional hour of cold ischemia time significantly increases the risk of graft failure and mortality following renal transplantation. *Kidney Int* 2015; **87**: 343–349.
4 Morris PJ, Johnson RJ, Fuggle SV, Belger MA, Briggs JD. Analysis of factors that affect outcome of primary cadaveric renal transplantation in the UK. HLA Task Force of the Kidney Advisory Group of the United Kingdom Transplant Support Service Authority (UKTSSA). *Lancet* 1999; **354**: 114701152.
5 Quiroga I, McShane P, Koo DD, Gray D, Friend PJ, Fuggle S *et al*. Major effects of delayed graft function and cold ischaemia time on renal allograft survival. *Nephrol Dial Transplant* 2006; **21**: 1689–1696.
6 Salahudeen AK, Haider N, May W. Cold ischemia and the reduced long-term survival of cadaveric renal allografts. *Kidney Int* 2004; **65**: 713–718.
7 Baverstock RJ, Manson AD, Liu L, Gourlay WA. A prospective comparison of simultaneous and sequential live-donor renal transplantation. *Transplantation* 2002; **74**: 1194–1197.
8 O'Callaghan JM, Knight SR, Morgan RD, Morris PJ. Preservation solutions for static cold storage of kidney allografts: a systematic review and meta-analysis. *Am J Transplant* 2012; **12**: 896–906.
9 Moers C, Smits JM, Maathuis MH, Treckmann J, van Gelder F, Napieralski BP *et al*. Machine perfusion or cold storage in deceased-donor kidney transplantation. *N Engl J Med* 2009; **360**: 7–19.
10 Moers C, Pirenne J, Paul A, Ploeg RJ; Machine Preservation Trial Study Group. Machine perfusion or cold storage in deceased-donor kidney transplantation. *N Engl J Med* 2012; **366**: 770–771.
11 Simpkins CE, Montgomery RA, Hawxby AM, Locke JE, Gentry SE, Warren DS *et al*. Cold ischemia time and allograft outcomes in live donor renal transplantation: is live donor organ transport feasible? *Am J Transpl* 2007; **7**: 99–107.
12 Segev DL, Veale JL, Berger JC, Hiller JM, Hanto RL, Leeser DB *et al*. Transporting live donor kidneys for kidney paired donation: initial national results. *Am J Transpl* 2011; **11**: 356–360.
13 Treat EG, Miller ET, Kwan L, Connor SE, Maliski SL, Hicks EM *et al*. Outcomes of shipped live donor kidney transplants compared with traditional living donor kidney transplants. *Transpl Int* 2014; **27**: 1175–1182.
14 Nath J, Field M, Ebbs SR, Smith T, McGrogan D, Al-Shakarchi J *et al*. Evolution of renal transplant practice over the past decade: a U.K. Center Experience. *Transplant Proc* 2015; **47**: 1700–1704.
15 Maggiore U, Oberbauer R, Pascual J, Viklicky O, Dudley C, Budde K *et al*. Strategies to increase the donor pool and access to kidney transplantation: an international perspective. *Nephrol Dial Transpl* 2015; **30**: 217–222.
16 Segev DL, Gentry SE, Warren DS, Reeb B, Montgomery RA. Kidney paired donation and optimizing the use of live donor organs. *JAMA* 2005; **293**: 1883–1890.
17 Osband AJ, Zaki RF. Extraction time of kidneys during organ procurement impacts function. *Clin Transpl* 2011; **25**: 235–238.
18 Hatsuse K, Kinukawa T, Hattori R, Fujita T, Ono Y, Ohshima S. Cadaveric renal transplantations with prolonged warm ischemic times greater than 30 minutes. *Transplant Proc* 1998; **30**: 3787–3789.
19 Heylen L, Naesens M, Jochmans I, Monbaliu D, Lerut E, Claes K *et al*. The effect of anastomosis time on outcome in recipients of kidneys donated after brain death: a cohort study. *Am J Transpl* 2015; **15**: 2900–2907.
20 Nath J, Mastoridis S, van Dellen D, Guy AJ, McGrogan DG, Krishnan H *et al*. Complex kidneys for complex patients: the risk associated with transplantation of kidneys with multiple arteries into obese patients. *Transplant Proc* 2015; **47**: 373–378.

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Chapter 5

Metabolism in human kidneys during HMP

Paper published (3)

Guy AJ, **Nath J**, Cobbold M, Ludwig C, Tennant DA, Inston NG, Ready AR. Metabolomic analysis of perfusate during hypothermic machine perfusion of human cadaveric kidneys. *Transplantation* 2015; **99**(4): 754-9.

Permission to incorporate this manuscript into the thesis has been kindly granted by Walters Kluwer Health Lippincott Williams & Wilkins© (Appendix p59)

Introduction

Initial work, performed largely by my predecessor, Ms Alison Guy focused on metabolomic analyses of human perfusate samples during hypothermic machine perfusion. Ms Guy was awarded an MD at the University of Birmingham (2015), and my role in this research was largely data analyses, interpretation and manuscript preparation.

The rationale for this human perfusate analysis was twofold. Firstly a baseline understanding of the degree and type of metabolite production occurring in a machine perfused kidney would potentially offer a target for metabolic manipulation, with the potential to improve the function of ischaemically damaged kidneys.

Secondly, we were hoping that analyses of perfusate metabolites would identify a metabolite, or more likely a panel of metabolites, that could act as a useful biomarker that could predict graft function. Such a biomarker would prove extremely useful in the clinical setting as greater numbers of high-risk kidneys are being considered for transplantation owing to the profound shortage of organs. Whilst some clinicians use perfusion parameters during HMP to aid decision making as to whether such kidneys are 'transplantable' this is not supported by robust evidence and there remains no sensitive, reproducible test to determine functional outcome in the pre-transplant period (78-81). A simple biomarker or panel of biomarkers detected during the perfusion process which accurately predicted post-transplant function would be invaluable in the context of increasing usage of these marginal organs.

Ethical approval

Ethical approval for this study was obtained from the West of Scotland Research Ethics Service (12/WS/0166) in July 2012. The decision to subject a kidney to HMP was based upon clinical parameters and kidneys were perfused using the LifePort© 1 kidney transporter and 1L of KPS-1® fluid at 4°C. Informed consent was taken for each potential recipient of machine perfused kidneys with a patient information sheet provided. Further hospital Research and Development approval for this project was also attained.

Project funding

This project was funded through a University Hospitals Birmingham charities grant (£60,000) awarded in 2012.

Study overview

During the study period, a total of 29 patients were recruited. 3 kidneys were not perfused for anatomical reasons resulting in machine perfusion of 26 kidneys that were subsequently transplanted. Perfusate samples were taken at 45 minutes and 4 hours with metabolite identification and quantification performed using 1D ^{1}H NMR spectroscopy and Chenomx 7.0 software (Chenomx Inc., Edmonton, AB, Canada). A total of 28 metabolites were identified in the perfusate. Not only were the concentrations of these demonstrated to vary significantly over time but four of the

compounds were highly predictive of post-transplant graft function.

There appears to be a strong correlation between the metabolic phenotype of transplant kidneys during the period prior to transplantation and the functional outcome for that organ. The implications of this are twofold; firstly that a metabolic 'panel' could be used as a useful biomarker to determine which organs are non-viable and should not be transplanted. Secondly, the metabolism could potentially be optimized during perfusion to improve the function of highly damaged organs. NMR spectroscopy is a valid technique for metabolic characterization of organs during perfusion and is likely to be used increasingly for this purpose.

The work was summarised and published in the journal Transplantation in 2015, and the full paper is included in this thesis for review. As the second author, my contribution to this project was largely in data analysis, interpretation, and scientific writing. However, the bulk of the work was performed by Ms. Guy

Supplementary data for this manuscript is also available in the appendix p48-54.

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)

Funding for this research was provided by Organ Recovery Systems and University Hospitals Birmingham Charity.
The authors declare no conflicts of interest.
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A.G. participated in research design, data collection, analysis and interpretation, and writing of the article. J.N. participated in analysis and interpretation and writing of the article. M.C. participated in research design and article review. C.L. participated in research design, analysis and interpretation, and article review. D.A.T. participated in research design, analysis and interpretation, and article review. N.G.I. participated in research design and article review. A.R.R. participated in research design and article review.
Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's website (www.transplantjournal.com).
Received 13 June 2014. Revision requested 8 July 2014.
Accepted 8 July 2014.
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ISSN: 0041-1337/14/0000-00
DOI: 10.1097/TP.0000000000000398

Hypothermic machine perfusion (HMP) is increasingly used to preserve cadaveric kidneys in the period before transplantation (*1*). Studies have demonstrated reduced rates of delayed graft function (DGF) and improved graft survival in machine-perfused kidneys compared to those preserved in traditional static cold storage (SCS) (*2–5*). Hypothermic machine perfusion also provides a unique opportunity for assessment of cadaveric kidneys during storage with easy and safe access to perfusate samples, kidney tissue and information regarding flow dynamics.

Accurate evaluation of allograft quality is essential to prevent unnecessary kidney discard, allow maximized donor-recipient matching and to plan appropriate perioperative care. Viability assessment is of increasing importance in an era where marginal kidneys, with a greater risk of poor graft function, are increasingly being used in an attempt to address the organ shortage (*6–9*). 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 now well documented, the exact processes by which this occurs remain unclear. Although initially the mechanism of action was thought to be the maintenance of vascular bed patency, there is increasing evidence that substantial metabolic activity persists,

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and this may also have a role (*10*). However, the metabolic activity in the ex vivo, hypoxic, hypothermic environment provided by HMP is poorly understood.

Proton-nuclear magnetic resonance (^{1}H-NMR) spectroscopy, or metabolomics, can be used to analyze fluids or tissues and can simultaneously detect, identify, and quantify hundreds of metabolites—some of which may have prognostic or therapeutic value (*11*). Although 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 (*11–14*).

The aim of this study was to use NMR spectroscopy 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).

RESULTS

Twenty-nine kidneys were included in the study. Three kidneys were rejected for implantation after 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, along with HMP parameters, are shown in **SDC1**, http://links.lww.com/TP/B51. There were no statistically significant differences in characteristics or parameters between the IGF and DGF kidneys.

Twenty-eight metabolites were detected in the perfusate of these kidneys at both 45 min and 4 hr of HMP. Of these metabolites, six were recognized constituents of the KPS-1 fluid (**SDC2**, http://links.lww.com/TP/B51): adenine, gluconate, glucose, glutathione (reduced form), mannitol, and ribose. Median concentrations of these metabolites measured by ^{1}H-NMR at each timepoint, and the *P* values for change over time are shown in Table 1. There was a significant change in concentration of glucose and glutathione between the two timepoints.

The remaining 22 metabolites are not listed as constituents of KPS-1. Median concentrations of these metabolites at each timepoint, and *P* values for change over time are shown in Table 2. The majority of these metabolites changed significantly.

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

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, $P = 0.003$) (Fig. 1A). Receiver operating characteristic (ROC) curves assessing the predictive accuracy of glucose for DGF yielded an area under the ROC (AUROC) curve of 0.842 (standard error [SE], 0.080) at 45 min and 0.895 (SE, 0.069) at 4 hr (Fig. 2).

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, $P = 0.036$) (Fig. 1B and C) but not at 4 hr. The AUROC for inosine at 45 min was 0.833 (SE, 0.082) and for leucine at 45 min was 0.732 (SE, 0.135) (Fig. 2).

Gluconate levels were also significantly different between DGF and IGF kidneys at 4 hr (49.099 vs. 59.513 mM, $P = 0.009$) (Fig. 1D) but not at 45 min. The AUROC for gluconate at 4 hr was 0.851 (SE, 0.089) (Fig. 2). Cutoff points for ROC curves are shown in **SDC3** (http://links.lww.com/TP/B51).

DISCUSSION

To our knowledge, 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 study demonstrates that after only 45 min 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 after transplantation.

Accurate assessment of graft quality is increasingly important to achieve the highest levels of success in transplantation. Although donor information, such as patient age, comorbidity, or terminal serum creatinine, along with kidney biopsy data are important, they still have a limited capacity for accurate prediction of graft outcomes (*15,16*). Machine perfusion parameters, such as resistance, are also

TABLE 1. Metabolite concentrations (mM) for constituents of KPS-1 identified in kidney perfusate

Metabolite	Median Conc at 45 min of HMP	Interquartile range	Median Conc at 4 hr of HMP	Interquartile range	*P* (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[a]
Glutathione	0.89	0.79–0.94	0.50	0.40–0.60	<0.001[a]
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

[a] Statistically significant at less than 0.05, Wilcoxon signed rank test.

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TABLE 2. Metabolite concentrations (mM) measured in kidney perfusate not listed as constituents of KPS-1

Metabolite	Median Conc at 45 min of HMP	Interquartile range	Median Conc at 4 hr of HMP	Interquartile range	*P* (change over time)
3-Hydroxybutyrate	0.008	0.006–0.014	0.021	0.014–0.030	<0.001[a]
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[a]
Citrate	0.477	0.000–0.830	0.621	0.000–1.299	<0.001[a]
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[a]
Fumarate	0.002	0.001–0.003	0.003	0.001–0.004	0.005[a]
Glutamate	0.145	0.065–0.325	0.646	0.375–0.807	<0.001[a]
Glycine	0.469	0.391–0.541	1.363	0.902–1.792	<0.001[a]
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[a]
Inosine	0.006	0.002–0.015	0.008	0.003–0.020	0.014[a]
Isoleucine	0.003	0.002–0.004	0.004	0.003–0.006	<0.001[a]
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[a]
Leucine	0.008	0.006–0.010	0.012	0.008–0.016	<0.001[a]
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[a]
Tyrosine	0.003	0.002–0.003	0.004	0.003–0.006	<0.001[a]
Uracil	0.007	0.003–0.008	0.009	0.006–0.013	<0.001[a]
Valine	0.005	0.004–0.006	0.003	0.006-0.010	<0.001[a]

[a] Statistically significant at less than 0.05, Wilcoxon signed rank test.

accepted as good indicators of graft quality, but several studies have warned of the dangers of using resistance values to determine kidney discard (*17–19*).

Theoretically, biomarkers measured in urine and perfusate have an advantage over biopsy data of being noninvasive and can be measured frequently and objectively. A recent review highlighted the biomarkers that have been assessed in regards to graft outcome (*20*). Few studies were recent, and even fewer deemed to be of good quality. Levels of lactate dehydrogenase, glutathione-S-transferase and aspartate transaminase were significantly associated with DGF in the greatest number of studies, but further validation was recommended.

The NMR-based metabolomics is a novel approach for rapidly identifying the changes in global metabolite profiles of biologic samples and is widely used in other disease entities (*21*). 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 markers of kidney injury ex vivo (*22–24*). More recently, NMR has been used to examine HMP perfusate in a preclinical donation after cardiac death porcine model (*14*). 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.

In this study, the main constituents of KPS-1 were identified by ^{1}H-NMR, except for HEPES and hydroxyethyl starch which the software cannot recognize. Of the identified metabolites, median levels of gluconate, mannitol, adenine, and ribose did not change significantly over HMP time for all perfused kidneys. However, gluconate levels were significantly lower in the perfusate of DGF kidneys at 4 hr with a good AUROC for prediction of DGF. Gluconate (like mannitol) is present to provide osmotic stability so a significant change in concentration is unexpected. It is possible that cellular damage in the DGF kidneys allows influx of gluconate into the cell that would not normally occur within healthy tissue.

Glutathione is included in many preservation solutions, including KPS-1, and acts as a free radical scavenger to attenuate ischemia-reperfusion injury. Concentrations of reduced glutathione decreased over time during HMP in both DGF and IGF kidney perfusate. Glutathione in its oxidized form was not detected.

Glucose levels in the perfusate of DGF kidneys were significantly lower at both timepoints 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 used for repair by damaged DGF kidneys or that DGF kidneys are not effectively suppressed by hypothermia and require more glucose for metabolism.

Of the metabolites discovered that are not part of the preservation solution two of these, leucine and inosine, had significantly different concentrations in the perfusate of DGF kidneys compared to IGF kidney perfusate at 45 min. Leucine, a branched chain essential amino acid, was detected in significantly higher levels in the perfusate of DGF kidneys at 45 min. It is known that larger proteins are released into the perfusate during HMP as a sign of cellular damage (*25*). Raised concentrations of amino acids could indicate increased

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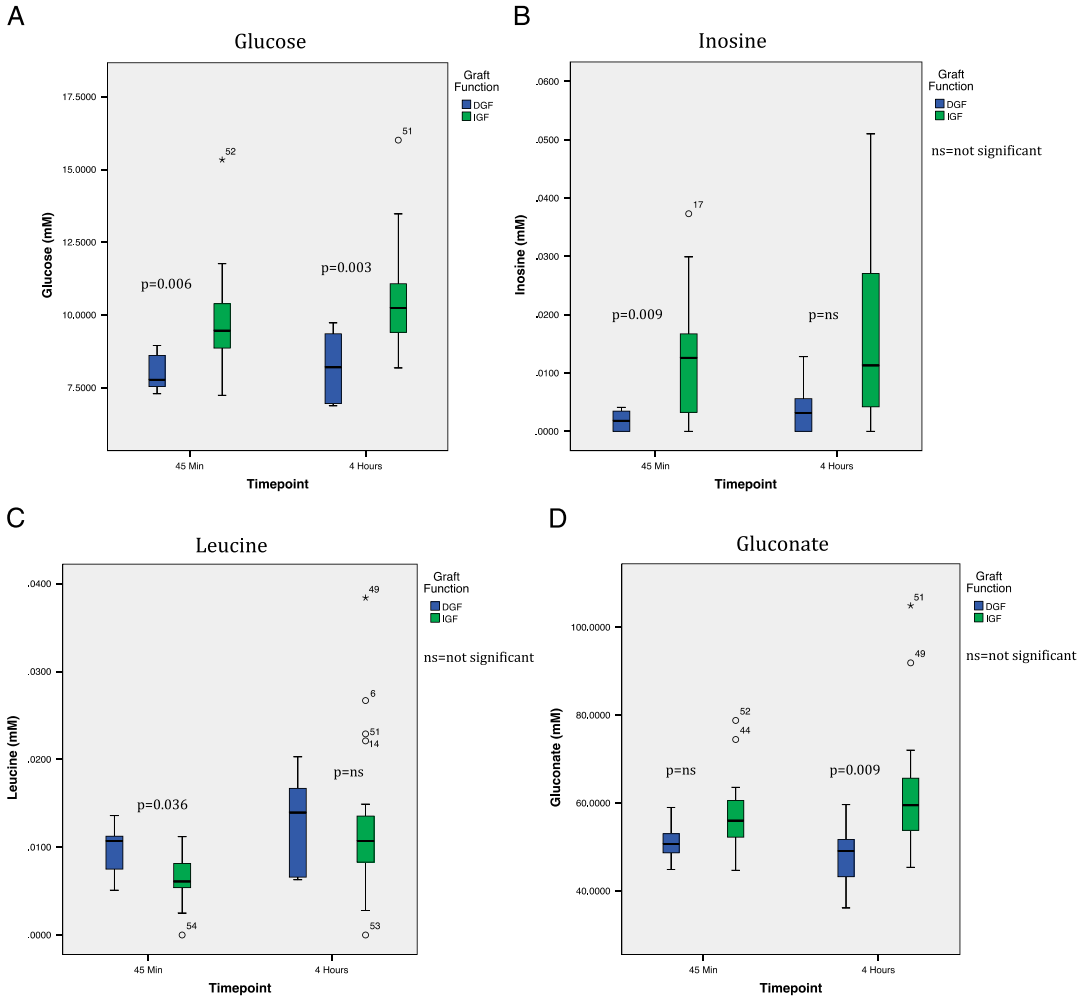

FIGURE 1. Box and Whisker plots representing the significantly different metabolites between the kidney perfusate of DGF and IGF kidneys. DGF, delayed graft function; IGF, immediate graft function.

cellular breakdown in the more ischemically damaged DGF kidneys. Other amino acids identified in the perfusate were alanine, glycine, glutamate, isoleucine, tyrosine, and valine. 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.

In contrast, concentrations of inosine were significantly lower in DGF kidney perfusate at 45 min compared to IGF perfusate. Inosine is formed from the breakdown of adenosine nucleotides, such as adenosine monophosphate, adenosine diphosphate, and adenosine triphosphate. The lower levels of inosine detected in the more ischemically 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 because adenine levels did not decrease over time to correspond with this. The AUROCs for leucine and inosine at 45 min were good at 0.833 and 0.732.

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 (*26*). Levels of lactate increased in the perfusate of HMP over time, as might be expected, because of 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 tricarboxylic acid cycle.

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.

All cadaveric kidneys arrive at our 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 (8:00 A.M. to 8:00 P.M.), the kidney was transplanted from

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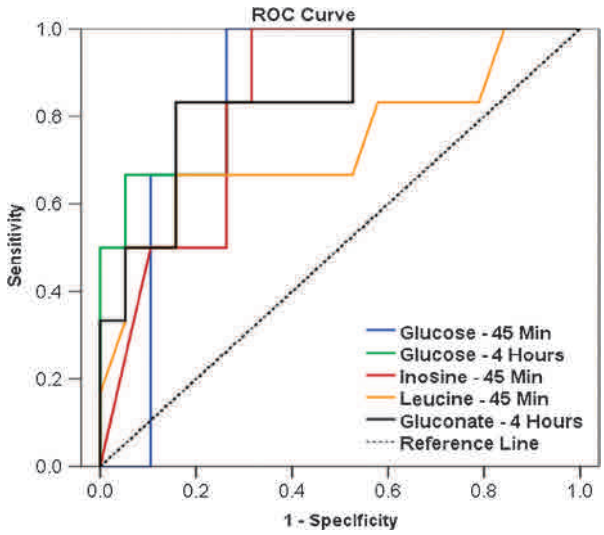

Area Under the Curve

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

FIGURE 2. Receiver-operator Characteristic (ROC) Curves and Areas Under the ROC Curves (AUROCs) for Significant Metabolites.

SCS. If the predicted time to theatre was outside of these hours (8:00 P.M. to 8:00 A.M.), the kidney was transferred to HMP and then transplanted at the earliest opportunity during elective hours. This is to ensure that as many renal transplants as possible are performed in optimal conditions in the dedicated renal transplant theatre.

At present, HMP is not used as a standard preservation technique at organ recovery in the United Kingdom. In this study, kidneys were placed on HMP at the accepting unit and remained at the center for transplantation. If HMP were used 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 (*2,27*).

The 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 predicative of graft function. 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.

In this study, it has been possible to identify differences in the metabolomic profiles of perfusate from kidneys with IGF and DGF. 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 before transplantation.

MATERIALS AND METHODS

Adult cadaveric kidneys accepted for transplantation and undergoing HMP at the Queen Elizabeth Hospital, Birmingham between July 2012 and August 2013 were included, subject to consent and resource availability. Ethical approval was obtained (REC reference number: 12/WS/0166). All kidneys arrived at the unit in SCS. The decision to transfer a kidney to HMP was made according to departmental guidelines. Demographic and clinical data were collected prospectively. Delayed graft function was defined as the requirement for dialysis within the first postoperative week. Immediate graft function kidneys were those not requiring dialysis support postoperatively.

Kidneys were cold stored in the period after retrieval and transferred to the LifePort Kidney Transporter 1.0 (Organ Recovery Systems, Chicago, IL) at the host center under aseptic conditions. The decision to perfuse kidneys was

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determined by clinical protocol taking into account donor-recipient issues and theatre availability. Perfusion pressure was set at 30 mm Hg and not altered during perfusion time. All kidneys were perfused with 1 L of KPS-1 at 4 °C. No additional oxygen was supplied.

Two milliliters of perfusate was sampled at 45 min and 4 hr for each HMP kidney. Perfusate was transferred to a cryogenic vial and stored at −20°C until thawed at room temperature, prepared, and processed.

The NMR samples were prepared by mixing 150 μL of a 400 mM pH 7.0 phosphate buffer solution containing 2 mM (3-trimethylsilyl)propionic-(2,2,3,3-d4)-acid sodium salt (TSP) with 390 μL of each perfusate sample and 60 μL of deuterium oxide to reach a final phosphate buffer concentration of 100 mM and a final TSP concentration of 500 μM. Deuterium oxide 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.

The ^{1}H-NMR spectra were acquired using a Bruker AVII 500 MHz spectrometer equipped with a 5 mm inverse Cryoprobe (Bruker Corporation, Billerica, MA). The sample temperature was set to 300 K, excitation sculpting was used to suppress the water resonance (*28*). One-dimensional spectra were acquired using a 6-kHz spectral width, 32,768 data points, 4 s relaxation delay and 128 transients. Matching was manual before acquisition of first sample, and each sample was automatically shimmed (1D-TopShim) to a TSP line width of less than 1 Hz before acquisition. Samples with a TSP line width greater than 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 min per sample.

All data sets were processed using the MATLAB based MetaboLab software (*29*). Data sets were zero filled to 65,536 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 (*30*). Spectra were then exported into Bruker format.

Resultant spectra were examined using Chenomx 7.0 (Chenomx Inc., Edmonton, AB, Canada) profiling to identify metabolites and their concentrations. Chemical shift assignments are shown in **SDC4**, (http://links.lww.com/TP/B51). Example spectra are shown in **SDC5**, http://links.lww.com/TP/B51. Each signal annotation and quantification was checked manually.

Statistics

Data were analyzed using GraphPad Prism version 6.0c for Mac (GraphPad Software, La Jolla, CA, www.graphpad.com) and IBM SPSS 19 (IBM Corp. Armonk, NY). Metabolite averages stated as median values because of nonparametric data distribution. Change in metabolite concentration over time was analyzed by Wilcoxon signed rank test. Metabolite concentrations were compared using Mann-Whitney *U* test. *P* less than 0.05 was considered to be indicative of statistical significance.

ACKNOWLEDGMENTS

The authors thank James Hodson (University Hospitals Birmingham) for his assistance with the statistical analysis and to Peter DeMuylder & Gunther Vanwezer at Organ Recovery Systems for their help and support.

REFERENCES

1. Henry SD, Guarrera JV. Protective effects of hypothermic ex vivo perfusion on ischemia/reperfusion injury and transplant outcomes. *Transplant Rev (Orlando)* 2012; 26: 163.
2. Moers C, Smits JM, Maathuis M-HJ, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. *N Engl J Med* 2009; 360: 7.
3. Machine Preservation Trial Group. Machine Perfusion or cold storage in deceased-donor transplantation. *N Engl J Med* 2012; 366: 770.
4. Lodhi SA, Lamb KE, Uddin I, et al. Pulsatile pump decreases risk of delayed graft function in kidneys donated after cardiac death. *Am J Transplant* 2012; 12: 2774.
5. Cannon RM, Brock GN, Garrison RN, et al. To pump or not to pump: a comparison of machine perfusion vs cold storage for deceased donor kidney transplantation. *J Am Coll Surg* 2013; 216: 625; discussion 633–4.
6. Audard V, Matignon M, Dahan K, et al. Renal transplantation from extended criteria cadaveric donors: problems and perspectives overview. *Transpl Int* 2008; 21: 11.
7. Metzger RA, Delmonico FL, Feng S, et al. Expanded criteria donors for kidney transplantation. *Am J Transplant* 2003; 3(Suppl 4): 114.
8. Ojo AO, Hanson JA, Meier-Kriesche H, et al. Survival in recipients of marginal cadaveric donor kidneys compared with other recipients and wait-listed transplant candidates. *J Am Soc Nephrol* 2001; 12: 589.
9. Port FK, Bragg-Gresham JL, Metzger RA, et al. Donor characteristics associated with reduced graft survival: an approach to expanding the pool of kidney donors. *Transplantation* 2002; 74: 1281.
10. Fuller BJ, Lee CY. Hypothermic perfusion preservation: the future of organ preservation revisited? *Cryobiology* 2007; 54: 129.
11. Serkova N, Fuller TF, Klawitter J, et al. H-NMR-based metabolic signatures of mild and severe ischemia/reperfusion injury in rat kidney transplants. *Kidney Int* 2005; 67: 1142.
12. Hauet T, Gibelin H, Godart C, et al. Kidney retrieval conditions influence damage to renal medulla: evaluation by proton nuclear magnetic resonance (NMR) pectroscopy. *Clin Chem Lab Med* 2000; 38: 1085.
13. Gibelin H, Eugene M, Hebrard W, et al. Comparison of Euro-Collins and University of Wisconsin solutions in the isolated perfused rat liver model: evaluation by proton nuclear magnetic resonance spectroscopy.*Transplant Proc* 2000; 32: 2792.
14. Bon D, Claire B, Thuillier R, et al. Analysis of perfusates during hypothermic machine perfusion by NMR spectroscopy: a potential tool for predicting kidney graft outcome. *Transplantation* 2014; 97: 810.
15. Jochmans I, Pirenne J. Graft quality assessment in kidney transplantation: not an exact science yet!. *Curr Opin Organ Transplant* 2011; 16: 174.
16. Louvar DW, Li N, Snyder J, et al. "Nature versus nurture" study of deceased-donor pairs in kidney transplantation. *J Am Soc Nephrol* 2009; 20: 1351.
17. Jochmans I, Moers C, Smits JM, et al. The prognostic value of renal resistance during hypothermic machine perfusion of deceased donor kidneys. *Am J Transplant* 2011; 11: 2214.
18. de Vries EE, Hoogland ER, Winkens B, et al. Renovascular resistance of machine-perfused DCD kidneys is associated with primary nonfunction. *Am J Transplant* 2011; 11: 2685.
19. Sonnenday CJ, Cooper M, Kraus E, et al. The hazards of basing acceptance of cadaveric renal allografts on pulsatile perfusion parameters alone. *Transplantation* 2003; 75: 2029.
20. Bhangoo RS, Hall IE, Reese PP, et al. Deceased-donor kidney perfusate and urine biomarkers for kidney allograft outcomes: a systematic review. *Nephrol Dial Transplant* 2012; 27: 3305.
21. Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 1999; 29: 1181.
22. Seto K, Ikehira H, Obata T, et al. Long-term assessment of posttransplant renal prognosis with 31 P magnetic resonance spectroscopy. *Transplantation* 2001; 72: 627.
23. Hauet T, Gibelin H, Richer JP, et al. Influence of retrieval conditions on renal medulla injury: evaluation by proton NMR spectroscopy in an isolated perfused pig kidney model. *J Surg Res* 2000; 93: 1.
24. Richer JP, Baumer TH, Gibelin H, et al. Evaluation of renal medulla injury after cold preservation and transplantation: noninvasive determination of medullar damage by proton nuclear magnetic resonance spectroscopy of urine and plasma. *Transplant Proc* 2000; 32: 47.

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25. Hoogland ERP, de Vries EE, Christiaans MH, et al. The value of machine perfusion biomarker concentration in DCD kidney transplantations. *Transplantation* 2013; 95: 603.
26. Ayala-García M, Hernández M, Ramírez-Barba É, et al. Preservation of Renal Allografts for Transplantation. In: Long L, ed. Renal Transplantation—Updates and Advances. Rijeka, Croatia: Intech Europe; 2012. ISBN: 978-953-51-0173-4. Available from: http://www.intechopen.com/books/renal-transplantation-updates-and-advances/preservation-of-renal-allograft-for-transplantation.
27. Watson CJ, Wells AC, Roberts RJ, et al. Cold machine perfusion versus static cold storage of kidneys donated after cardiac death: a UK multicenter randomized controlled trial. *Am J Transplant* 2010; 10: 1991.
28. Hwang T-L, Shaka AJ. Water suppression that works. Excitation sculpting using arbitrary waveforms and pulsed field gradients. *J Magn Reson* 1995; 112: 275.
29. Ludwig C, Günther UL. MetaboLab—advanced NMR data processing and analysis for metabolomics. *BMC Bioinformatics* 2011; 12: 366.
30. Savorani F, Tomasi G, Engelsen SB. Icoshift: a versatile tool for the rapid alignment of 1D NMR spectra. *J Magn Reson* 2010; 202: 190.

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Chapter 6

The porcine HMP metabolic model

Paper published (4)

Nath J, Guy A, Smith TB, Cobbold M, Inston NG, Hodson J, Tennant DA, Ludwig C, Ready AR. Metabolomic perfusate analysis during kidney machine perfusion: the pig provides an appropriate model for human studies. *PloS One* 2014; **9**(12): e114818.

This is an open access manuscript.

Introduction

The finding that the metabolic profiles within the perfusion fluid of human kidneys predicted outcome was interesting and supported the theory that pre-transplant metabolism correlates with post-transplant graft function. Furthermore, the ability to optimise the metabolic function during the pre-transplant organ preservation period through manipulation of the parameters of perfusion would be a potential target to improve the function of transplanted kidneys.

In order to determine how different factors influence metabolism during the preservation period, an experimental model was needed. Porcine kidneys are now the most widely used large animal model for transplantation studies, although both canine and primate models are also used in preference in some centres (190). Pig kidneys are reported to have similar physiological and anatomical properties to human kidneys (171, 191-193) and the metabolism of such organs at least *in vivo* is thought to be comparable (194, 195). However, the porcine model has not been validated as a metabolic model for human studies during the highly non-physiological conditions of hypothermic machine perfusion.

Aims

In this chapter of this thesis, I aim to determine through comparison of metabolites within the perfusion fluid of perfused pig and human kidneys using ^{1}H NMR spectroscopy, whether the pig is a valid model for such transplantation studies. If indeed the pig is a valid model, then it will justify the use for future experimental porcine studies to try and optimise the metabolism in the pre-transplant period.

Ethical approval

The ethical approval necessary for the analysis of human perfusate samples during the machine perfusion process was valid from the previous human study (12/WS/0166). No specific ethical approval was necessary for experiments on porcine abattoir kidneys.

Methods

The perfusate from 12 standard criteria human kidney during perfusion was compared to 10 porcine kidneys. All kidneys were perfused with 1L of KPS-1® solution as per clinical practice. Perfusate samples were taken at both 45 minutes and 4-hour time points. Metabolite identification and quantification was performed using 1D ^{1}H NMR spectroscopy and Chenomx 7.0 software (Chenomx Inc., Edmonton, AB, Canada).

Results

30 metabolites were identified in both pig and human perfusate. There was no difference in concentration levels identified for the majority of these between pig and human experiments. For 29 of the 30 metabolites, there was no difference in the rate of change with time of the metabolite.

Conclusions

Based on this porcine abattoir DCD kidney model, we concluded that from a metabolomic perspective the pig was a valid model for human studies and provided the platform for further work in this thesis.

The following manuscript was published in the journal PLOSOne in December 2014. I am the first author of this manuscript and was responsible at every stage of its production. I wrote the enclosed manuscript.

PLOS ONE

RESEARCH ARTICLE

Metabolomic Perfusate Analysis during Kidney Machine Perfusion: The Pig Provides an Appropriate Model for Human Studies

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Citation: Nath J, Guy A, Smith TB, Cobbold M, Inston NG, et al. (2014) Metabolomic Perfusate Analysis during Kidney Machine Perfusion: The Pig Provides an Appropriate Model for Human Studies. PLoS ONE 9(12): e114818. doi:10.1371/journal.pone.0114818

Editor: Helge Bruns, University Hospital Oldenburg, Germany

Received: June 30, 2014

Accepted: October 21, 2014

Published: December 12, 2014

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Data Availability: The authors confirm that, for approved reasons, some access restrictions apply to the data underlying the findings. Data are available at Dryad: doi:10.5061/dryad.9jc16.

Funding: University Hospital Birmingham Charities £60,000 Grant number Dkaarcde17496. Organ Recovery Systems, Donation of Lifeport machine (value circa £25,000).

Competing Interests: Mark Cobbold is a PLOS ONE Editorial board member. This does not alter the authors' adherence to PLOS ONE Editorial policies and criteria.

Abstract

Introduction: Hypothermic machine perfusion offers great promise in kidney transplantation and experimental studies are needed to establish the optimal conditions for this to occur. Pig kidneys are considered to be a good model for this purpose and share many properties with human organs. However it is not established whether the metabolism of pig kidneys in such hypothermic hypoxic conditions is comparable to human organs.

Methods: Standard criteria human (n=12) and porcine (n=10) kidneys underwent HMP using the LifePort Kidney Transporter 1.0 (Organ Recovery Systems) using KPS-1 solution. Perfusate was sampled at 45 minutes and 4 hours of perfusion and metabolomic analysis performed using 1-D ^{1}H-NMR spectroscopy.

Results: There was no inter-species difference in the number of metabolites identified. Of the 30 metabolites analysed, 16 (53.3%) were present in comparable concentrations in the pig and human kidney perfusates. The rate of change of concentration for 3-Hydroxybutyrate was greater for human kidneys ($p<0.001$). For the other 29 metabolites (96.7%), there was no difference in the rate of change of concentration between pig and human samples.

Conclusions: Whilst there are some differences between pig and human kidneys during HMP they appear to be metabolically similar and the pig seems to be a valid model for human studies.

Introduction

The use of Hypothermic Machine Perfusion (HMP) in the period between kidney retrieval and implantation is supported by robust clinical evidence with improved early graft outcome [1–9]. Flow dynamics during perfusion is likely to account for some of the benefits of HMP, with reduced intra-renal resistance (and therefore increased flow) a marker of good graft function [10–12]. However, the exact mechanism by which HMP improves outcome remains unclear and there is likely to be a metabolic component underlying these beneficial effects [13, 14]. Accordingly, improved metabolic support during perfusion becomes a target for graft optimisation. Experimental studies are needed to clarify these mechanisms and optimisation of preservation may lead to improved transplant outcomes, especially in marginal kidneys. However the metabolic activity in this ex vivo, hypoxic, hypothermic environment is poorly understood.

Metabolomic analysis using ^{1}H NMR spectroscopy permits identification and quantification of a large number of metabolites within a biological sample and is the subject of great interest. Easy access to perfusate during HMP and ability to perform serial measurements render this an attractive technique with which to establish a reliable biomarker and may even provide the option to improve the metabolic function of organs with obvious potential benefits. We have shown that perfusate analysis of human cadaveric kidneys is feasible and can be used to reliably predict post transplant graft function [15]

Porcine kidneys are a convenient and accessible animal model for experimental studies. They are readily available and have comparable size and physiological properties to human organs [16–19]. Within transplantation, porcine models have been studied extensively and normothermic perfusion is a good example of how this has translated into clinical practice [20]. On a functional level, analysis using ^{1}H-NMR spectroscopy of perfusate from autotransplanted pig kidneys has demonstrated that metabolite concentrations do correspond to graft outcome indicating that there is a strong correlation between pre-transplant metabolism and graft function [21].

Under normal physiological conditions, the metabolic profiles of porcine blood, kidney tissue, urine and serum have been shown to be comparable to humans [22, 23]. As of yet, this has not been validated in the *ex vivo* hypothermic environment as encountered during HMP. The aim of this study is to compare the metabolic profile of human and porcine kidneys using ^{1}H-NMR spectroscopy of HMP-derived perfusate to determine whether the porcine model is a valid surrogate for human studies.

Methods

Human subject research

Ethical approval was obtained from the West of Scotland Research Ethics Service for collection of human perfusate samples and subsequent NMR analysis (REC reference number: 12/WS/0166). Written consent for research purposes was granted from donor next of kin and from prospective kidney recipients to allow for collection and publication of postoperative transplant function information.

Animal Research

Abattoir/slaughterhouse pig kidneys were used in this study, acquired through F.A. Gill, Wolverhampton. No animals were sacrificed solely for the purposes of this study and therefore no ethical board approval was necessary.

Human studies

Standard criteria (e.g. donor age <60) adult cadaveric kidneys ($n=12$) accepted for transplantation and undergoing HMP at the Queen Elizabeth Hospital, Birmingham between July 2012 and August 2013 were included, subject to consent and resource availability. Kidneys from DCD (Donation after Cardiac Death) donors and those fulfilling standard criteria definitions but with predicted delayed graft function (e.g. Cold ischaemia time >16 hours) were excluded to enable valid comparison with the pig group.

Organs were cold stored at 4℃ in the period following retrieval according to local retrieval team protocols and transferred to the perfusion machine at the host centre. Decision to preserve organs with HMP was determined by centre policy.

Pig studies

Experiments were performed on 22–26 week old 'bacon weight' pigs, weighing 80–85 kg ($n=10$). All experiments were performed following the principles of laboratory animal care according to NIH standards. Animal were sacrificed by electrical stunning and exsanguination. Cold perfusion was performed ex-vivo following laparotomy and retrieval and occurred within 14 minutes of death. Kidneys were initially cold flushed (4℃) with 1L Soltran solution under aseptic conditions at pressure of 150 mmHg. Organs were then cold stored in KPS-1 solution for two hours prior to machine perfusion to replicate human organ cold storage conditions.

Hypothermic Machine Perfusion

Perfusion pressure for both animal and human organs was set at 30 mmHg and kidneys were perfused with 1 L of KPS-1 at 4℃ using LifePort Kidney Transporter 1.0 (Organ Recovery Systems). Separate devices were used for human and animal studies. 2 mL of perfusate was sampled at 45 minutes and 4 hours for each

kidney. Perfusate was transferred to a cryogenic vial and stored at −20℃ until thawed at room temperature, prepared and processed.

Sample preparation

NMR samples were prepared by mixing 150 μL of 400 mM (pH 7.0) phosphate buffer containing 2 mM TMSP [(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 TMSP concentration of 500 μM. After mixing, the 600 μL samples were pipetted into NMR tubes and centrifuged to remove any air bubbles.

^{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 [24]. 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 TMSP line width of less than 1 Hz prior to acquisition. Samples with a TMSP line width >1 Hz were acquired again after manual shimming where the TMSP 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 [25]. 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 [26]. Spectra were then exported into Bruker format.

Resultant spectra were examined using Chenomx 7.0 (ChenomxInc) profiling to identify metabolites and their concentrations, as illustrated in Fig. 1. Concentrations were corrected to compensate for the dilutional effect of the buffer.

Statistical Methodology

Prior to analysis, the distribution of metabolites was examined. Where non-normality was detected, 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

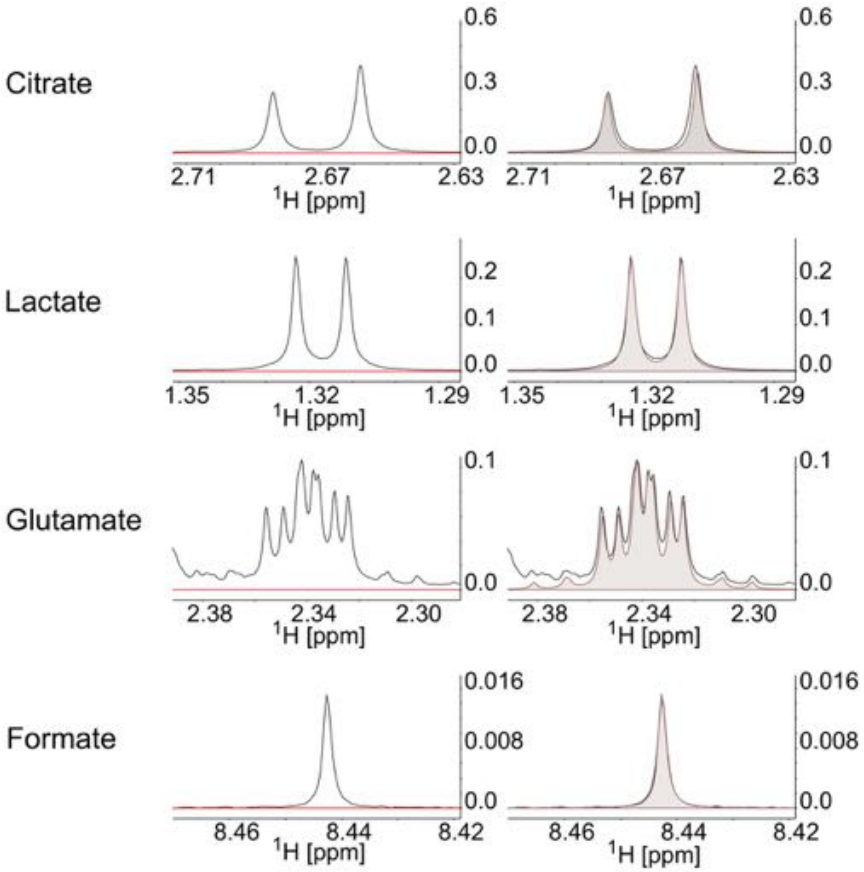

Fig. 1. Example metabolic quantification using Chenomx database. Localised spectral plots for metabolites of interest with shaded figures illustrating metabolite quantification via best fit analysis using Chenomx metabolite database.

doi:10.1371/journal.pone.0114818.g001

summary statistics were back transformed, and reported as geometric means and 95% confidence intervals.

All analysis was performed using IBM SPSS 19 (IBM Corp. Armonk, NY), with $p<0.05$ deemed to be indicative of statistical significance.

Results

Metabolic support may be an important factor in the observed benefit of HMP compared with cold storage for preserving kidneys prior to transplantation. Experimental studies are needed to detail the metabolic activity of kidneys under various storage conditions, but the usage of healthy human kidneys for such research purposes is not justified.

This study seeks to ascertain whether the abundant and accessible porcine kidney can provide a reliable metabolic model for the human kidney during HMP.

Metabolite concentrations as determined from ^{1}H-NMR spectra from 10 porcine kidneys were compared with 12 standard criteria cadaveric human kidneys, all of which were successfully transplanted with immediate graft function.

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 (Fig. 2). There were no metabolites consistently present in significant quantities that were not detected in the other group.

In total, 30 metabolites were identified in the perfusate of both pig and human kidneys during hypothermic machine perfusion. Of these, 6 (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 differences for the other five metabolites present.

For the 24 metabolites present *de novo* (therefore likely produced by the kidney), there was an overall change over time for 12, 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 as assessed using 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 0.040 mM vs 0.012 to 0.013 mM) ($p<0.001$). The vast majority of metabolites detected (29/30) demonstrated no difference in the rate of change between pig and human samples (Table 1, Fig. 3).

Discussion

The aim of this study was to determine whether or not the porcine kidney provides a reliable metabolic model for the human kidney, as assessed by ^{1}H-NMR.

Whilst it is recognized that ^{1}H-NMR can be used to detect metabolic changes present within the perfusates during HMP, this paper demonstrates that in both human and pig studies a significant amount of metabolic activity occurs. Some interspecies metabolite differences are evident but nevertheless, the similarity between the two groups is striking. This appears to validate porcine HMP as a valid metabolic model 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.

The majority of metabolites were present in similar concentrations in both species. For metabolites where concentration differences were observed, all but 3-hydroxybutryate had comparable rates of change in concentration for pig and human samples. This would imply that the active metabolic pathways during HMP in both human and pig kidneys are broadly comparable. The kidney cannot synthesise the ketone body 3-hydroxybutyrate to any significant extent but can

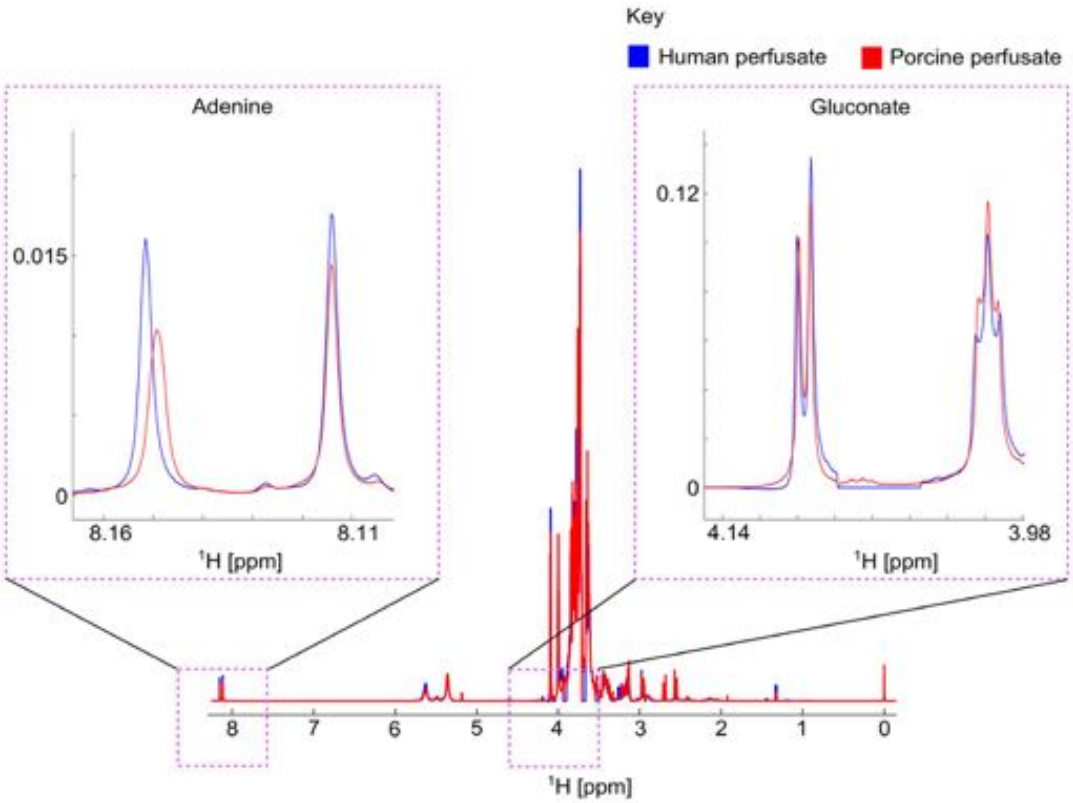

Fig. 2. ^{1}H-NMR spectral overlay plot demonstrating the similarity of HMP perfused pig (red) and human (blue) kidneys after 4 hours of perfusion.

doi:10.1371/journal.pone.0114818.g002

consume it as an energy source and is likely to be more pronounced in stressful conditions such as hypothermia [27, 28]. Interspecies differences in the levels of the enzyme responsible for metabolising 3-hydroxybutyrate, 3-hydroxybutyrate dehydrogenase within the renal cortex and in plasma levels of 3-hydroxybutyrate have been reported and may account for this finding [29].

The pig kidneys were subjected to a warm ischaemia time of 14 minutes which is more prolonged than many Donation after Brain Death (DBD) human kidneys. However the cold ischaemic time for the pig organs (2 hours) was shorter than the human group in this study (mean 8 hr 40 min). There was also a significant age discrepancy between the pig and human organs, with the older human kidneys also likely subject to the global metabolic changes associated with brain death prior to retrieval including thyroid, catecholamine and glycaemic effects [30–35]. 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.

The authors acknowledge that the good quality standard criteria human kidneys in this study are not representative of many of the organs used in clinical practice. Indeed extended criteria organs may have most to gain from metabolic optimistion during machine perfusion and further studies would be of value.

Table 1. Concentration of metabolites present after 45 minutes and 4 hours of perfusion in pig and human kidneys.

		Time point		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

Table 1. Cont.

		Time point		p-Values		
	Species	*45 Minutes*	*4 Hours*	*Time*	*Species*	*Int.*
	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).
p-values: Time – Main effect of measurement time; Species – Main effect of species; Int. – Interaction between measurement time and species.
*Significant at $p<0.05$.

doi:10.1371/journal.pone.0114818.t001

Whilst the number and concentration change of the metabolites in pig and human perfusates are comparable in this study, it lacks the power to detect small interspecies differences. Furthermore in 1-D spectra, metabolites with small single peaks can be obscured by more dominant signals from other metabolites with similar chemical shifts.

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 milieu and is a limitation of this study.

This study demonstrates that ^{1}H-NMR spectroscopy profiles of perfusate samples for porcine and human kidneys during HMP are similar and implies that similar metabolic processes occur during preservation in the two species. This further validates the pig as a model for human transplantation and for HMP in particular.

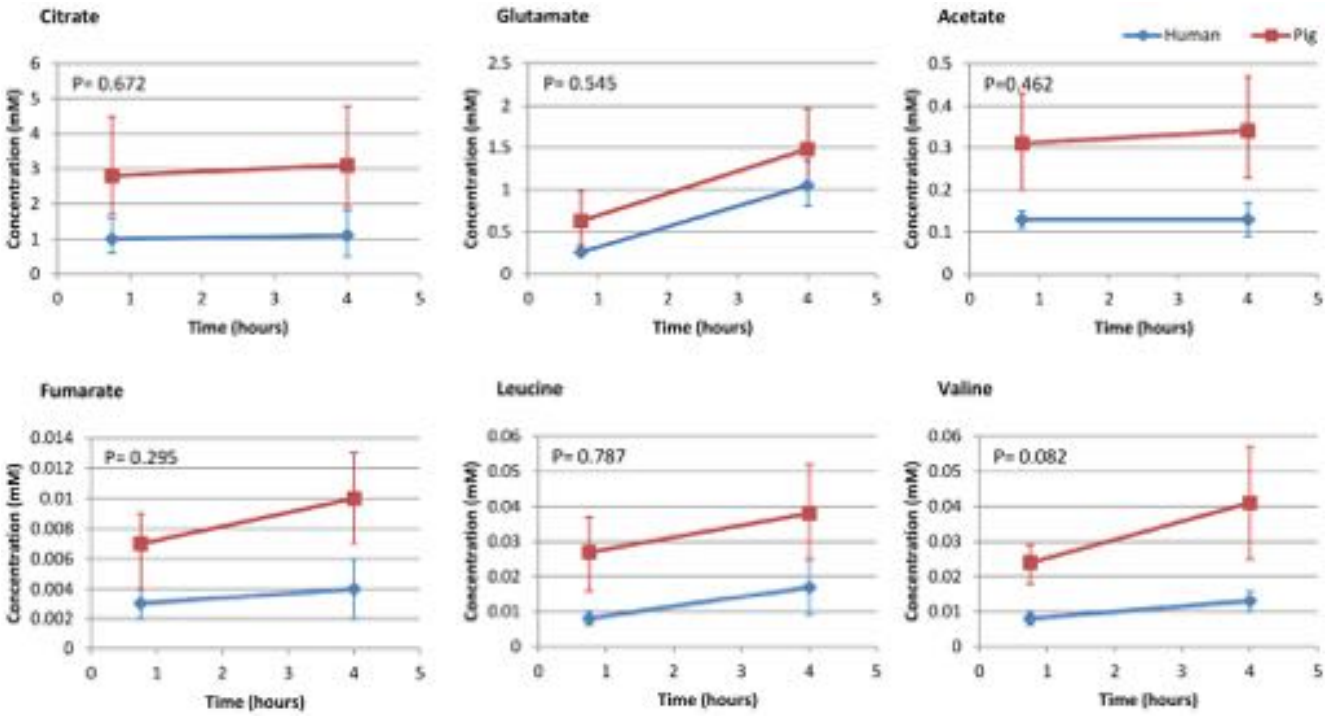

Fig. 3. Comparable rate of change of concentration demonstrated for depicted metabolites during 4 hours of HMP despite absolute interspecies concentration differences.

doi:10.1371/journal.pone.0114818.g003

Acknowledgments

This work was supported by a grant from University Hospital Birmingham charities trust. The authors would like to thank Peter Demuylder and Gunther Vanwezer at Organ Recovery systems for their help and support.

Author Contributions

Conceived and designed the experiments: JN AG MC NGI CL DAT ARR. Performed the experiments: JN AG TBS. Analyzed the data: JN AG TBS JH CL DAT. Contributed reagents/materials/analysis tools: JN AG CL JH. Wrote the paper: JN AG TBS. Paper Review: MC NGI JH CL DAT ARR.

References

1. **Moers C, Smits JM, Maathuis MH, Treckmann J, van Gelder F, et al.** (2009) Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med 360: 7–19.
2. **Cannon RM, Brock GN, Garrison RN, Smith JW, Marvin MR, et al.** (2013) To pump or not to pump: a comparison of machine perfusion vs cold storage for deceased donor kidney transplantation. J Am Coll Surg 216: 625–633; discussion 633–624.
3. **Lodhi SA, Lamb KE, Uddin I, Meier-Kriesche HU** (2012) Pulsatile pump decreases risk of delayed graft function in kidneys donated after cardiac death. Am J Transplant 12: 2774–2780.

4. **Kwiatkowski A, Wszola M, Kosieradzki M, Danielewicz R, Ostrowski K, et al.** (2007) Machine perfusion preservation improves renal allograft survival. Am J Transplant 7: 1942–1947.
5. **Wight JP, Chilcott JB, Holmes MW, Brewer N** (2003) Pulsatile machine perfusion vs. cold storage of kidneys for transplantation: a rapid and systematic review. Clin Transplant 17: 293–307.
6. **Shah AP, Milgrom DP, Mangus RS, Powelson JA, Goggins WC, et al.** (2008) Comparison of pulsatile perfusion and cold storage for paired kidney allografts. Transplantation 86: 1006–1009.
7. **Matsuoka L, Shah T, Aswad S, Bunnapradist S, Cho Y, et al.** (2006) Pulsatile perfusion reduces the incidence of delayed graft function in expanded criteria donor kidney transplantation. Am J Transplant 6: 1473–1478.
8. **Suarez JF, Riera L, Franco E, Ruiz R, Roig M, et al.** (1999) Preservation of kidneys from marginal donors with pulsatile perfusion machine. Transplant Proc 31: 2292–2293.
9. **Sellers MT, Gallichio MH, Hudson SL, Young CJ, Bynon JS, et al.** (2000) Improved outcomes in cadaveric renal allografts with pulsatile preservation. Clin Transplant 14: 543–549.
10. **Polyak MM, Arrington BO, Stubenbord WT, Boykin J, Brown T, et al.** (2000) The influence of pulsatile preservation on renal transplantation in the 1990s. Transplantation 69: 249–258.
11. **Matsuno N, Kozaki K, Degawa H, Narumi Y, Suzuki N, et al.** (2000) A useful predictor in machine perfusion preservation for kidney transplantation from non-heart-beating donors. Transplant Proc 32: 173–174.
12. **Kozaki K, Sakurai E, Uchiyama M, Matsuno N, Kozaki M, et al.** (2000) Usefulness of high-risk renal graft conditioning: functional improvement of high-risk grafts by addition of reagents to continuous hypothermic perfusion preservation solution. Transplant Proc 32: 164–166.
13. **Fuller BJ, Lee CY** (2007) Hypothermic perfusion preservation: the future of organ preservation revisited? Cryobiology 54: 129–145.
14. **Henry SD, Nachber E, Tulipan J, Stone J, Bae C, et al.** (2012) Hypothermic machine preservation reduces molecular markers of ischemia/reperfusion injury in human liver transplantation. Am J Transplant 12: 2477–2486.
15. **Guy AJ, Nath J, Cobbold M, Ludwig C, Tennant DA, et al.** (2014) Metabolomic Analysis of Perfusate During Hypothermic Machine Perfusion of Human Cadaveric Kidneys. Transplantation.
16. **Giraud S, Favreau F, Chatauret N, Thuillier R, Maiga S, et al.** (2011) Contribution of large pig for renal ischemia-reperfusion and transplantation studies: the preclinical model. J Biomed Biotechnol 2011: 532127.
17. **Sachs DH** (1994) The pig as a potential xenograft donor. Vet Immunol Immunopathol 43: 185–191.
18. **Hannon JP, Bossone CA, Wade CE** (1990) Normal physiological values for conscious pigs used in biomedical research. Lab Anim Sci 40: 293–298.
19. **Swindle MM** (2012) The development of swine models in drug discovery and development. Future Med Chem 4: 1771–1772.
20. **Bagul A, Hosgood SA, Kaushik M, Kay MD, Waller HL, et al.** (2008) Experimental renal preservation by normothermic resuscitation perfusion with autologous blood. British Journal of Surgery 95: 111–118.
21. **Bon D, Claire B, Thuillier R, Hebrard W, Boildieu N, et al.** (2014) Analysis of Perfusates During Hypothermic Machine Perfusion by NMR Spectroscopy: A Potential Tool for Predicting Kidney Graft Outcome. Transplantation.
22. **Merrifield CA, Lewis M, Claus SP, Beckonert OP, Dumas ME, et al.** (2011) A metabolic system-wide characterisation of the pig: a model for human physiology. Mol Biosyst 7: 2577–2588.
23. **Nielsen KL, Hartvigsen ML, Hedemann MS, Laerke HN, Hermansen K, et al.** (2014) Similar metabolic responses in pigs and humans to breads with different contents and compositions of dietary fibers: a metabolomics study. Am J Clin Nutr 99: 941–949.
24. **Hwang TL, Shaka AJ** (1995) Water Suppression That Works - Excitation Sculpting Using Arbitrary Wave-Forms and Pulsed-Field Gradients. Journal of Magnetic Resonance Series A 112: 275–279.
25. **Ludwig C, Gunther UL** (2011) MetaboLab - advanced NMR data processing and analysis for metabolomics. Bmc Bioinformatics 12.

26. **Savorani F, Tomasi G, Engelsen SB** (2010) icoshift: A versatile tool for the rapid alignment of 1D NMR spectra. Journal of Magnetic Resonance 202: 190–202.

27. **Laffel L** (1999) Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. Diabetes Metab Res Rev 15: 412–426.

28. **Garber AJ, Menzel PH, Boden G, Owen OE** (1974) Hepatic ketogenesis and gluconeogenesis in humans. J Clin Invest 54: 981–989.

29. **Koundakjian PP, Snoswell AM** (1970) Ketone body and fatty acid metabolism in sheep tissues. 3-Hydroxybutyrate dehydrogenase, a cytoplasmic enzyme in sheep liver and kidney. Biochem J 119: 49–57.

30. **Smith M** (2004) Physiologic changes during brain stem death–lessons for management of the organ donor. J Heart Lung Transplant 23: S217–222.

31. **Barklin A, Larsson A, Vestergaard C, Kjaergaard A, Wogensen L, et al.** (2008) Insulin alters cytokine content in two pivotal organs after brain death: a porcine model. Acta Anaesthesiol Scand 52: 628–634.

32. **Wood KE, Becker BN, McCartney JG, D′Alessandro AM, Coursin DB** (2004) Care of the potential organ donor. N Engl J Med 351: 2730–2739.

33. **Bugge JF** (2009) Brain death and its implications for management of the potential organ donor. Acta Anaesthesiol Scand 53: 1239–1250.

34. **Gramm HJ, Meinhold H, Bickel U, Zimmermann J, von Hammerstein B, et al.** (1992) Acute endocrine failure after brain death? Transplantation 54: 851–857.

35. **Powner DJ, Hendrich A, Lagler RG, Ng RH, Madden RL** (1990) Hormonal changes in brain dead patients. Crit Care Med 18: 702–708.

Chapter 7

Comparison of SCS and HMP metabolism

Paper published (6)

Nath J, Smith TB, Patel K, Ebbs S, Hollis A, Tennant DA, Ludwig C, Ready AR.

Metabolic differences between cold stored and machine perfused porcine

kidneys: A ^{1}H-NMR based study. *Cryobiology*. 2017;**74**:115-20.

Permission to incorporate this manuscript into the thesis has been kindly granted by Elsevier (Appendix p60)

Introduction

Although the superior outcomes resulting from hypothermic machine perfusion are well accepted (28, 29, 48), the precise mechanism by which these benefits are derived is not entirely clear. There is increasing evidence that the promotion of metabolism may be a key component of this and our earlier study elegantly demonstrated that that metabolite concentrations within the perfusate correlate with post-transplant graft function (3). We felt that if we could demonstrate metabolic differences between the two main storage conditions (HMP and SCS), this would provide complementary evidence that the metabolism of the kidney during these perfusion conditions is altered. Surprisingly there are no studies comparing the metabolome of kidneys during these two common kidney storage conditions.

Aims

The aim of this study was to determine whether the type and time-dependent relationship of metabolites in the perfusion fluid of porcine kidneys was similar for HMP and paired SCS kidneys, using ^{1}H 1D NMR spectroscopy.

Ethical approval

No specific ethical approval was necessary for this study using porcine abattoir kidneys.

Project funding

This project was funded through a UHB Charities grant (£60,000) awarded in 2012 and a grant from the Royal College of Surgeons of Edinburgh £9,500 awarded in

2014.

Methods

Twenty kidneys were cold flushed and stored for two hours following retrieval. Paired kidneys then underwent 24 hours of HMP or SCS or served as time zero controls. Metabolite quantification in both storage fluid and kidney tissue was performed using one dimensional ^{1}H-NMR spectroscopy. For each metabolite, the net gain for each storage modality was determined by comparing the total amount in each closed system (i.e. total amount in storage fluid and kidney combined) compared with controls. 26 metabolites were included for analysis

Results

The total system metabolite quantities following HMP or SCS were greater for 14 metabolites compared with controls (all $p<0.05$). In addition to metabolic differences with control kidneys, the net gain in metabolite quantity during HMP was greater than SCS for 8 metabolites (all $p<0.05$). These included metabolites related to central metabolism (lactate, glutamate, aspartate, fumarate and acetate).

Conclusions

The metabolic environments of both perfusion fluid and the kidney tissue are strikingly different between SCS and HMP systems in this animal model. The total amount of central metabolites such as lactate and glutamate observed in the HMP kidney system suggests a greater degree of *de novo* metabolic activity than in the SCS system. Maintenance of

central metabolic pathways may contribute to the clinical benefits of HMP.

The following manuscript has been published in the journal Cryobiology in 2017. I am the first author of this manuscript and was responsible at every stage of its production. I wrote the enclosed manuscript.

Supplementary data for this manuscript is also available in the appendix p55-56.

Cryobiology xxx (2016) 1–6

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Metabolic differences between cold stored and machine perfused porcine kidneys: A ^{1}H NMR based study

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ARTICLE INFO

Article history:
Received 11 July 2016
Received in revised form
18 October 2016
Accepted 21 November 2016
Available online xxx

Keywords:
Hypothermic machine perfusion
Kidney
Transplantation
Metabolism
NMR
Organ preservation

ABSTRACT

Hypothermic machine perfusion (HMP) and static cold storage (SCS) are the two methods used to preserve deceased donor kidneys prior to transplant. This study seeks to characterise the metabolic profile of HMP and SCS porcine kidneys in a cardiac death donor model.

Twenty kidneys were cold flushed and stored for two hours following retrieval. Paired kidneys then underwent 24 h of HMP or SCS or served as time zero controls. Metabolite quantification in both storage fluid and kidney tissue was performed using one dimensional ^{1}H NMR spectroscopy. For each metabolite, the net gain for each storage modality was determined by comparing the total amount in each closed system (i.e. total amount in storage fluid and kidney combined) compared with controls.

26 metabolites were included for analysis. Total system metabolite quantities following HMP or SCS were greater for 14 compared with controls (all $p < 0.05$). In addition to metabolic differences with control kidneys, the net metabolic gain during HMP was greater than SCS for 8 metabolites (all $p < 0.05$). These included metabolites related to central metabolism (lactate, glutamate, aspartate, fumarate and acetate).

The metabolic environments of both perfusion fluid and the kidney tissue are strikingly different between SCS and HMP systems in this animal model. The total amount of central metabolites such as lactate and glutamate observed in the HMP kidney system suggests a greater degree of *de novo* metabolic activity than in the SCS system. Maintenance of central metabolic pathways may contribute to the clinical benefits of HMP.

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1. Introduction

Hypothermic Machine Perfusion (HMP) and Static Cold Storage (SCS) are the two methods of kidney preservation that are used widely in clinical practice during the time period between organ retrieval and implantation [16]. A key concept for both preservation modalities is that cellular metabolism, and therefore cellular metabolic requirements, are minimised in these hypothermic conditions and the rate of metabolism reported to be about 5–8% at temperatures below 4 °C [29] with a similar decrease in oxygen requirement [1].

The superiority of HMP over SCS is well documented [4,17,22,23,27] but the mechanisms by which this occurs are not clear. Improvement in flow dynamics, with fall in the intra-renal resistance is likely to be one factor but the additional metabolic support derived from the circulation of nutrient-containing perfusion fluid may also help preserve organ function and have a beneficial effect [7,30].

Metabolomic analyses of preservation fluid during HMP using 1D-^{1}H NMR (One-dimensional proton nuclear magnetic resonance) spectroscopy, by groups including our own, have demonstrated this to be reproducible and highly specific for metabolite identification and quantification [2,10,24]. However, surprisingly, to our knowledge there are no studies that have sought to compare the metabolomic profiles, or metabolome, of HMP and SCS kidneys.

Porcine kidneys are widely used in transplantation studies owing to their similar physiological and anatomical properties to human organs [9,11]. In addition, the metabolic profiles during

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http://dx.doi.org/10.1016/j.cryobiol.2016.11.006
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Please cite this article in press as: J. Nath, et al., Metabolic differences between cold stored and machine perfused porcine kidneys: A ^{1}H NMR based study, Cryobiology (2016), http://dx.doi.org/10.1016/j.cryobiol.2016.11.006

periods of HMP for porcine and human kidneys are comparable [24], with a correlation between metabolite profiles during storage and post transplant outcome [2]. For HMP preserved human kidneys, the metabolic profile from perfusates of immediate graft function kidneys differs from those with delayed function [10] and reinforces the concept that significant metabolism occurs during HMP and that metabolism reflects functional outcome.

The aims of this study were twofold. Firstly, to determine the distribution of metabolites between the two different compartments (fluid and tissue) during the organ preservation period. Secondly, to determine the total amount of each metabolite within HMP and SCS kidneys systems after 24 h of organ storage and through comparison with control kidneys, the metabolic changes that occur.

2. Methods

2.1. Animal research

Abattoir/slaughterhouse pig kidneys (F.A. Gill, Wolverhampton, UK) were used and no animals were sacrificed solely for the purposes of this study, negating any need for ethical board approval. Experiments were performed on 22–26 week old male 'bacon weight' pigs, weighing 80–85 kg. All experiments were performed following the principles of laboratory animal care according to NIH standards. Animals were sacrificed by electrical stunning and exsanguination. Initial organ preservation was performed following organ retrieval and occurred within 14 min of death, replicating deceased cardiac death (DCD) donor conditions. Kidneys were cold flushed (4 °C) with 1 L SPS-1 (UW) solution at a pressure of 100 mm Hg. Organs were then stored at 4 °C in SPS-1 for 2 h to replicate the clinical period of organ transportation.

2.2. Experimental groups

Paired kidneys were randomly allocated to receive either HMP or SCS for 24 h. HMP kidneys were perfused with 1 L of KPS-1 using the LifePort Kidney Transporter 1.0 (Organ Recovery Systems, Chicago, IL). (Perfusion pressure 30 mm Hg). SCS Kidneys were submerged in 1 L of fresh chilled SPS-1 solution with a surrounding ice bath. Preservation fluid was sampled for each kidney at baseline and 2, 4, 8, 12, 18, and 24 h. After 24 h, organs were rapidly dissected and tissue samples (1 cm^3 sections) flash frozen and stored (−80 °C). All experiments were performed in a cold room (4 °C) to ensure consistency.

2.3. Control kidneys

To ascertain metabolism during SCS or HMP storage conditions, baseline values prior to storage conditions were needed (time 0). Large volume tissue sampling precludes effective organ perfusion and therefore 'Control kidneys' were used to establish baseline metabolite levels. These were (n = 6) flushed and cold transported in identical fashion to experimental kidneys and tissue samples obtained as described above (i.e. not subjected to 24 hr of SCS or HMP).

2.4. Sample processing and metabolite quantification

NMR samples were prepared from storage fluid by mixing 150 μL of 400 mM (pH 7.0) phosphate buffer containing 2 mM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) and 8 mM imidazole with 390 μL of each fluid sample and 60 μL of deuterium oxide (D_2O) to reach a final phosphate buffer concentration of 100 mM and a final DSS concentration of 500 μM. After mixing, the 600 μL samples were pipetted into 5 mm NMR tubes, sonicated and centrifuged. Technical replicates of samples (×3) were prepared for each timepoint.

For cell extract studies, 500 mg of renal cortex was manually cryo-homogenised in liquid nitrogen. 5.1 ml of both methanol (−80 °C) and chloroform was added to the powdered tissue and samples diluted with 4.65 ml of dH_20 at 4 °C. Samples were centrifuged to separate into polar and non-polar layers and 1.5 ml of the upper polar layer was dispensed into a cryovial and dried. Three technical replicates were performed for each tissue sample. Dried polar residue was then dissolved in 390 μL of dH_20 and 210 μL of buffer solution as described above.

The protocol used for ^{1}H NMR analysis has been described previously [10,24]. Briefly, this entailed processing on a Bruker AVII 500 MHz spectrometer, acquisition of one dimensional spectra and then metabolite identification and quantification using Matlab based 'Metabolab' software [18] and Chenomx 8.1 (ChenomxInc) software respectively. Metabolites were deemed to be present if they exhibited non-ambiguous spectral patterns or their presence deemed biologically plausible and confirmed on ultra performance liquid chromatography mass spectrometry. Any metabolites that were present in different concentrations in the SCS and HMP fluid (e.g. glucose, gluconate, mannitol, adenine, adenosine etc.) were excluded from comparative analysis. Metabolite quantifications were corrected to allow for sample dilution with sample buffer. When determining concentrations of metabolites using Chenomx, the researchers were blind to the storage group. Quantification of the total amount of metabolite in the storage fluid, tissue and total system was calculated based upon the weight of the kidney at time of sample acquisition and final volume of storage fluid.

2.5. Statistical analysis

For each timepoint, three results were obtained (technical replicates) and the median value used. For comparison of SCS and HMP conditions, analysis was performed using Wilcoxon paired signed rank test (two tailed) as one kidney from each pair was subjected to each condition and normality was not consistent on prior analysis. When comparing SCS or HMP with control kidneys, Mann-Whitney u test (two tailed) was used, as these were non-paired samples. Data were reported as median concentrations and interquartile (IQ) range. All analysis was performed using GraphPad Prism version 6.00 for Mac OS X, GraphPad Software, La Jolla California USA, with $p < 0.05$ deemed to be indicative of statistical significance.

3. Results

Metabolic optimisation of cadaveric kidneys is a potential target to improve the function of kidneys for transplantation. This study seeks to establish the degree of metabolism, if any, that occurs in the two widely used methods of kidney organ storage prior to transplantation (HMP and SCS).

The total quantity of each metabolite after 24 h of either HMP or SCS was calculated using ^{1}H NMR methods and compared with control organs to determine the net metabolic change during each storage method.

We found evidence of metabolite production for both storage modalities with 14 metabolites present in significantly greater quantities in the HMP or SCS system compared with controls (all $p < 0.05$) (Table 1) (Fig. 1, Fig 1(Suppl)). There were significantly more metabolites with a net increase in the HMP system (13/14) compared with the SCS system (7/14) ($p = 0.033$).

Eight of the metabolites were significantly elevated in the HMP system compared with both the control and SCS systems (all $p < 0.05$), indicating a greater degree of metabolite production.

Please cite this article in press as: J. Nath, et al., Metabolic differences between cold stored and machine perfused porcine kidneys: A ^{1}H NMR based study, Cryobiology (2016), http://dx.doi.org/10.1016/j.cryobiol.2016.11.006

Table 1
Total amount of metabolite present in each of the storage modalities at time zero (controls) or after 24 h of preservation (SCS or HMP). Data reported as Median (Interquartile Range), unless stated otherwise. Statistical test: $^{\Psi}$ Mann-Whitney u test (two tailed) $^{\#}$Wilcoxon paired signed rank test (two tailed). *Significant at $p < 0.05$.

	Storage Modality			p-Values		
	Control System (mmol)	SCS System (mmol)	HMP System (mmol)	Control vs SCS $^{\Psi}$	Control vs HMP $^{\Psi}$	SCS vs HMP $^{\#}$
Glutamate	1.54 (1.12–1.84)	1.38 (1.11–1.66)	3.97 (3.69–4.71)	0.731	0.002*	0.031*
Myoinositol	1.18 (1.16–1.19)	1.29 (1.01–1.52)	2.16 (1.85–2.41)	0.731	0.002*	0.031*
Lactate	1.11 (0.976–1.23)	1.38 (1–1.75)	2.13 (1.67–2.71)	0.138	0.002*	0.031*
Hypoxanthine	0.454 (0.356–0.515)	0.710 (0.641–0.762)	1.05 (0.909–1.17)	0.001*	0.002*	0.156
Formate	0.442 (0.274–0.638)	0.643 (0.589–0.779)	0.842 (0.750–0.943)	0.101	0.004*	0.031*
Acetate	0.210 (0.206–0.212)	0.296 (0.253–0.301)	0.552 (0.494–0.654)	0.234	0.041*	0.031*
Alanine	0.302 (0.243–0.360)	0.486 (0.339–0.499)	0.501 (0.368–0.558)	0.035*	0.015*	0.313
Succinate	0.283 (0.267–0.297)	0.462 (0.312–0.52)	0.434 (0.307–0.541)	0.001*	0.015*	0.844
Inosine	0.588 (0.561–0.628)	1.08 (0.885–1.12)	0.185 (0.146–0.233)	0.001*	0.002*	0.031*
Aspartate	0.114 (0.104–0.118)	0.107 (0.0879–0.11)	0.165 (0.140–0.215)	0.234	0.041*	0.031*
Leucine	0.0476 (0.0441–0.0517)	0.0667 (0.0513–0.0820)	0.0693 (0.0495–0.0773)	0.014*	0.026*	0.688
Niacinamide	0.0196 (0.0181–0.0207)	0.0289 (0.0243–0.0319)	0.0490 (0.0420–0.0557)	0.001*	0.002*	0.031*
Tyrosine	0.0262 (0.0217–0.0302)	0.0434 (0.0339–0.0462)	0.0387 (0.0332–0.0431)	0.001*	0.013*	0.438
Fumarate	0.00412 (0.00339–0.00418)	0.00308 (0.00145–0.00348)	0.0133 (0.0077–0.0212)	0.064	0.002*	0.031*

These included lactate, glutamate, aspartate, fumarate, acetate, myo-inisitol, niacinamide and formate (Fig. 1).

Despite the additional 24 h of organ preservation, albeit in static conditions, the amount of lactate in the SCS system was comparable to controls (1.37 vs 1.11 mmol $p = 0.138$). However the amount in the HMP system (2.13 mmol) was almost twice the amount of either controls or SCS systems ($p = 0.002$ and $p = 0.031$). However, despite greater amounts overall, the amount present in the HMP tissue (0.76 mmol) was actually lower than SCS tissue (1.14 mmol) or control tissue (1.11 mmol) ($p = 0.031$ and $p = 0.002$ respectively), reflective of lower intracellular concentrations for HMP kidneys.

The distribution of metabolites between the extracellular storage fluid and tissue samples for both storage conditions are detailed in Table 2. As expected, there were greater quantities of metabolites in the circulating HMP fluid compared with the static conditions of SCS at most time-points. After 24 h, the total amount of metabolite in the perfusate for the HMP kidneys was significantly greater than the SCS group for (21/26 = 80.8%) of metabolites. Whilst concentrations rose most rapidly in the first 2 h of perfusion and therefore may be in part due a metabolite washout phenomenon, there was an increase in most metabolites over sequential timepoints as would be expected with on-going production (Fig. 2a–c).

Reduced glutathione is a constituent of both KPS-1 (used in HMP) and SPS-1 (used in SCS) fluids at equal concentrations. Whilst this remained at stable in the SCS environment, the glutathione was clearly consumed by the HMP group and after 8 h concentrations were 17.6 fold higher in the SCS fluid (1.60 mM vs. 0.091 mM, $p = 0.001$) (Fig. 2d). Despite apparent organ uptake of reduced glutathione, there was no evidence of this in the tissue samples from either group.

4. Discussion

The aim of this study was to determine any metabolic differences between the two clinically used methods of organ storage in this animal model.

Whilst the calculation of the total amount of metabolite within the system does rely on several assumptions (complete metabolite extraction from tissue and metabolite homogeneity within tissue), we felt this was imperative to draw meaningful comparison between groups and enables the calculation of net metabolite production/consumption in these two closed systems (HMP and SCS).

Although the storage fluid used in each experimental group differs (most notably absence of glucose in the SCS fluid) and therefore caution should be exercised in attributing any differences merely to the parameters of storage (i.e. HMP or SCS), this study was designed to assess metabolism during the two clinically used organ preservation techniques, not merely the storage modality in isolation.

This study clearly demonstrates the presence of major central metabolites such as lactate, glutamate, fumarate, aspartate and acetate at greater levels in the HMP system compared with both controls and SCS (Fig. 1). This strongly suggests that these metabolites are being produced during HMP. Furthermore, the accumulation of these metabolites into the circulating perfusion fluid demonstrates effective homeostatic mechanisms are active to prevent over accumulation within the local cellular environment.

The list of metabolites reported in this study is not exhaustive and is a limitation of this study. Some interesting substrates (eg glucose) were excluded as this is only present in one of the storage fluids (KPS-1). For others the 1D ^{1}H NMR spectral pattern is either ambiguous or can be hidden under more domineering peaks from other compounds.

The increased total lactate in the HMP system is likely to reflect increased glycolysis in the HMP model. Although new glycolytic activity of the glucose within the HMP fluid is one likely contributor, this is unlikely to the singular cause. This is supported by evidence that the HMP fluid glucose concentrations did not decrease during the study period and replicates findings from previous human studies [10]. However conversion of a proportion of perfusion fluid glucose into lactate through glycolytic pathways has been corroborated by work demonstrating activity of these pathways using ^{13}C labelled glucose tracers [25].

The net gain of glutamate, fumarate, aspartate and acetate during HMP is also intriguing. Whilst identification of responsible metabolic pathways is difficult to ascribe solely with ^{1}H NMR studies, one explanation could be increased oxygen dependent tricarboxylic acid (TCA) cycle activity. Although uniform upregulation of TCA intermediates would support this theory, as discussed, many are not easily identifiable using ^{1}H NMR methods [6] and are rarely found in equipoise even *in vivo* [14]. Several (^{13}C) NMR studies have reported glutamate as a valid marker of TCA activity [3,5,20].

For some metabolites, the total system amounts for HMP and SCS kidneys were comparable to the controls, suggesting that either *de novo* production does not occur during the 24 h preservation or that consumption mirrors production (Table 1 *supplementary*). However, for metabolites with similar total amounts, the compartment in which they were located varied per metabolite. Some metabolites were entirely contained within the HMP kidney

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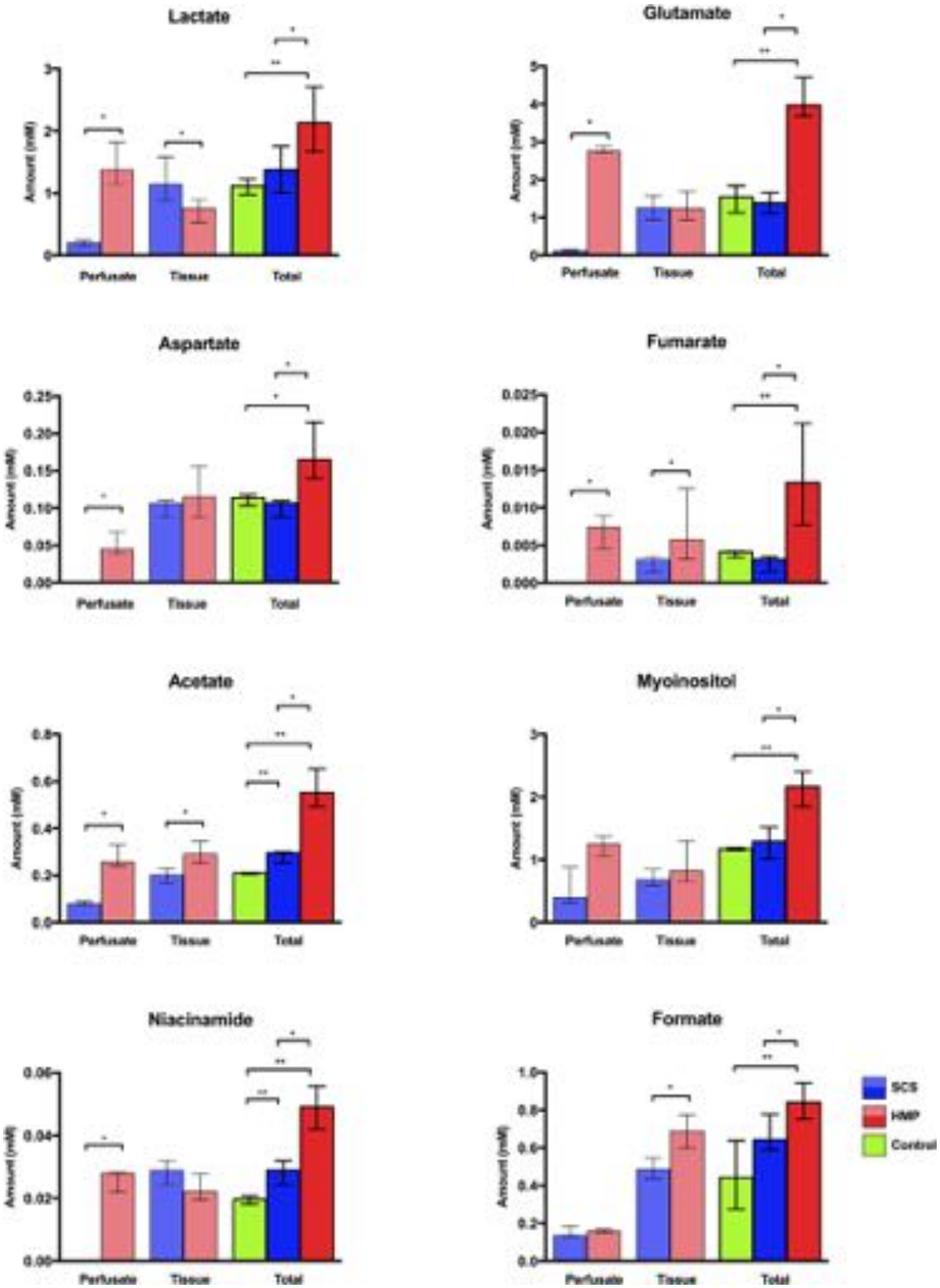

Fig. 1. Metabolites significantly elevated in the HMP system compared with both SCS and control kidneys. Metabolite levels represent total amounts (mmol) in the storage fluid, kidney tissue and entire system for porcine kidneys after 24 hr of HMP or SCS or time zero controls. Highly significant (**$p < 0.01$) and significant (*$p < 0.05$) differences between HMP system versus both controls and SCS kidneys.

tissue (e.g. ADP, AMP, NAD+) and presumably in the intracellular compartment. Other metabolites were evident in both the tissue and storage fluid but at higher concentrations in the HMP fluid. These discrepancies in metabolite location further highlight that cellular transport processes are active in this environment but that movement of metabolites into the extracellular fluid is not

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Table 2
Metabolites present in tissue and storage fluid in HMP or SCS kidney systems at 24 h. Data reported as Median (Interquartile Range), unless stated otherwise. Statistical test: #Wilcoxon paired signed rank test (two tailed). *Significant at $p < 0.05$.

	Storage	Total storage fluid amount (mmol)	p-value#	Total kidney tissue amount (mmol)	p-Value#
Glutamate	SCS	0.0812 (0.125–0.155)	0.0312*	0.952 (1.26–1.58)	0.6875
	HMP	2.72 (2.75–2.89)		0.94 (1.24–1.68)	
Myoinositol	SCS	0.316 (0.399–0.879)	0.0625	0.596 (0.676–0.853)	0.5625
	HMP	1.05 (1.25–1.38)		0.653 (0.816–1.3)	
Lactate	SCS	0.153 (0.205–0.245)	0.0312*	0.89 (1.14–1.59)	0.0312*
	HMP	1.15 (1.38–1.82)		0.521 (0.755–0.895)	
Hypoxanthine	SCS	0.294 (0.328–0.404)	0.0312*	0.289 (0.407–0.424)	0.0625
	HMP	0.705 (0.781–0.867)		0.189 (0.258–0.31)	
Formate	SCS	0.132 (0.136–0.186)	0.4375	0.434 (0.486–0.545)	0.0312*
	HMP	0.151 (0.16–0.169)		0.688 (0.599–0.774)	
Acetate	SCS	0.073 (0.0808–0.0912)	0.0312*	0.167 (0.201–0.229)	0.0312*
	HMP	0.239 (0.257–0.331)		0.252 (0.289–0.344)	
Alanine	SCS	0.0465 (0.0643–0.0815)	0.0312*	0.303 (0.415–0.435)	0.0312*
	HMP	0.253 (0.306–0.358)		0.116 (0.187–0.207)	
Succinate	SCS	0.0104 (0.0155–0.0184)	0.0312*	0.298 (0.446–0.498)	0.0312*
	HMP	0.104 (0.131–0.208)		0.203 (0.294–0.347)	
Inosine	SCS	0.703 (0.852–0.961)	0.0312*	0.145 (0.182–0.201)	0.0312*
	HMP	0.0877 (0.108–0.128)		0.058 (0.0723–0.109)	
Aspartate	SCS	–	0.0312*	0.0879 (0.107–0.11)	0.3125
	HMP	0.039 (0.0452–0.0682)		0.0874 (0.115–0.155)	
Leucine	SCS	0.00442 (0.00506–0.00761)	0.0312*	0.0486 (0.0591–0.0775)	0.0312*
	HMP	0.0285 (0.038–0.0468)		0.0222 (0.0304–0.0318)	
Niacinamide	SCS	–	0.0312*	0.0243 (0.0289–0.0319)	0.0938
	HMP	0.0221 (0.028–0.0282)		0.0194 (0.0221–0.0278)	
Tyrosine	SCS	0.00336 (0.0071–0.00843)	0.0312*	0.0306 (0.0371–0.0391)	0.0312*
	HMP	0.0197 (0.0228–0.0276)		0.0112 (0.0143–0.0171)	
Fumarate	SCS	–	0.0312*	0.00145 (0.00308–0.00348)	0.0312*
	HMP	0.00456 (0.00737–0.00895)		0.00314 (0.00574–0.0126)	

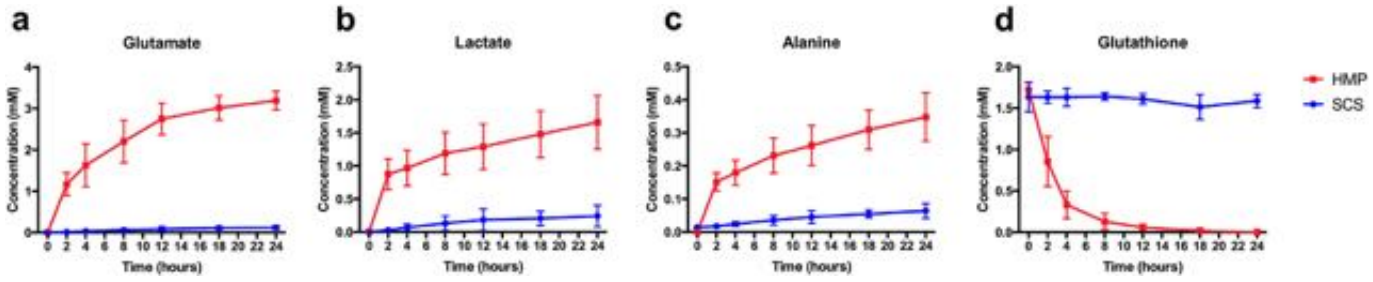

Fig. 2. Concentration of metabolites in the storage fluid of SCS and HMP kidneys over 24 h time period for four example metabolites. Values plotted as median (interquartile range).

indiscriminate.

Reduced glutathione is a constituent of the preservation fluid KPS-1 and is thought to play a role in the removal of Reactive Oxygen Species (ROS) generated during metabolism [19] In contrast to the SCS kidney, there is a rapid decrease in the concentration of glutathione in the preservation fluid of HMP stored kidneys and is about 5% of the SCS values after 8 h (Fig. 2d.). The rate of glutathione depletion observed in this study is similar to a previously reported animal model [28] and is likely to reflect cellular uptake of this protective antioxidant. Interestingly, glutathione concentration remained relatively constant in the SCS kidney group. This further reinforces the concept that HMP exerts its beneficial effects, at least in part, by providing access to the cellular components of the kidney during perfusion. Absence of reduced glutathione in tissue demonstrates that not only is this protective antioxidant readily absorbed by the kidney during perfusion but that even after a few hours it is not longer available in the reduced state.

Although the number of organs in each experimental group is small ($n = 7$), it is comparable to other porcine kidney transplant reports [8,12,15,21,26,30]. To improve validity, samples were processed in triplicate at each timepoint and over 250 NMR spectra were analysed. One strength of this study is that the kidneys stored by HMP or SCS were paired, i.e. from the same pig, thus minimising any metabolic differences arising from polymorphism in cellular mediators of porcine metabolism. Although this approach does not provide functional outcome information for the preserved organ, previous studies have demonstrated good function for otherwise healthy porcine organs stored by either SCS or HMP for similar time periods [2,8,13,15,21,26].

This study demonstrates that in a porcine model, the distribution and amounts of metabolites vary significantly with the storage method (HMP or SCS). The net gain of many central metabolites during HMP conditions further supports the notion that significant metabolism occurs during HMP and this may contribute to the beneficial role of machine perfusion.

Funding

This work was funded through grants from University Hospitals Birmingham Charities and Organ Recovery Systems.

Conflict of interest

As detailed in the funding section, the research was in-part

funded by a grant from Organ Recovery Systems, manufacturers of the LifePort Kidney Transporter used to create conditions of HMP in the laboratory. We do not have any further conflicts of interest to declare.

Acknowledgements

This work was funded through grants from University Hospitals Birmingham Charities (16-5-751) and Organ Recovery Systems.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cryobiol.2016.11.006.

References

[1] F.O. Belzer, J.H. Southard, Organ preservation and transplantation, Prog. Clin. Biol. Res. 224 (1986) 291–303.
[2] D. Bon, C. Billault, R. Thuillier, W. Hebrard, N. Boildieu, O. Celhay, J. Irani, F. Seguin, T. Hauet, Analysis of perfusates during hypothermic machine perfusion by NMR spectroscopy: a potential tool for predicting kidney graft outcome, Transplantation 97 (2014) 810–816.
[3] S.C. Burgess, E.E. Babcock, F.M. Jeffrey, A.D. Sherry, C.R. Malloy, NMR indirect detection of glutamate to measure citric acid cycle flux in the isolated perfused mouse heart, FEBS Lett. 505 (2001) 163–167.
[4] R.M. Cannon, G.N. Brock, R.N. Garrison, J.W. Smith, M.R. Marvin, G.A. Franklin, To pump or not to pump: a comparison of machine perfusion vs cold storage for deceased donor kidney transplantation, J. Am. Coll. Surg. 216 (2013) 625–633 discussion 633–4.
[5] E.M. Chance, S.H. Seeholzer, K. Kobayashi, J.R. Williamson, Mathematical analysis of isotope labeling in the citric acid cycle with applications to 13C NMR studies in perfused rat hearts, J. Biol. Chem. 258 (1983) 13785–13794.
[6] J.C. Chatham, S.J. Blackband, Nuclear magnetic resonance spectroscopy and imaging in animal research, ILAR J. 42 (2001) 189–208.
[7] B.J. Fuller, C.Y. Lee, Hypothermic perfusion preservation: the future of organ preservation revisited? Cryobiology 54 (2007) 129–145.
[8] A. Gallinat, A. Paul, P. Efferz, B. Luer, G. Kaiser, J. Wohlschlaeger, J. Treckmann, T. Minor, Hypothermic reconditioning of porcine kidney grafts by short-term preimplantation machine perfusion, Transplantation 93 (2012) 787–793.
[9] S. Giraud, F. Favreau, N. Chatauret, R. Thuillier, S. Maiga, T. Hauet, Contribution of large pig for renal ischemia-reperfusion and transplantation studies: the preclinical model, J. Biomed. Biotechnol. 2011 (2011) 532127.
[10] A.J. Guy, J. Nath, M. Cobbold, C. Ludwig, D.A. Tennant, N.G. Inston, A.R. Ready, Metabolomic analysis of perfusate during hypothermic machine perfusion of human cadaveric kidneys, Transplantation 99 (2015) 754–759.
[11] J.P. Hannon, C.A. Bossone, C.E. Wade, Normal physiological values for conscious pigs used in biomedical-research, Laboratory Animal Sci. 40 (1990) 293–298.
[12] S.A. Hosgood, M. Patel, M.L. Nicholson, The conditioning effect of ex vivo normothermic perfusion in an experimental kidney model, J. Surg. Res. 182 (2013) 153–160.
[13] I. Jochmans, E. Lerut, V. Heedfeld, T. Wylin, J. Pirenne, D. Monbaliu, Reproducible model for kidney autotransplantation in pigs, Transpl. Proc. 41 (2009) 3417–3421.
[14] H.A. Krebs, Rate control of the tricarboxylic acid cycle, Adv. Enzyme Regul. 8 (1970) 335–353.
[15] G. La Manna, D. Conte, M.L. Cappuccilli, B. Nardo, F. D'Addio, L. Puviani, G. Comai, F. Bianchi, R. Bertelli, N. Lanci, G. Donati, M.P. Scolari, A. Faenza, S. Stefoni, An in vivo autotransplant model of renal preservation: cold storage versus machine perfusion in the prevention of ischemia/reperfusion injury, Artif. Organs 33 (2009) 565–570.
[16] C.Y. Lee, M.J. Mangino, Preservation methods for kidney and liver, Organogenesis 5 (2009) 105–112.
[17] S.A. Lodhi, K.E. Lamb, I. Uddin, H.U. Meier-Kriesche, Pulsatile pump decreases risk of delayed graft function in kidneys donated after cardiac death, Am. J. Transpl. 12 (2012) 2774–2780.
[18] C. Ludwig, U.L. Gunther, MetaboLab–advanced NMR data processing and analysis for metabolomics, BMC Bioinforma. 12 (2011) 366.
[19] R.J. Mailloux, S.L. McBride, M.E. Harper, Unearthing the secrets of mitochondrial ROS and glutathione in bioenergetics, Trends Biochem. Sci. 38 (2013) 592–602.
[20] C.R. Malloy, A.D. Sherry, F.M. Jeffrey, Analysis of tricarboxylic acid cycle of the heart using 13C isotope isomers, Am. J. Physiol. 259 (1990) H987–H995.
[21] T. Minor, A. Paul, P. Efferz, J. Wohlschlaeger, U. Rauen, A. Gallinat, Kidney transplantation after oxygenated machine perfusion preservation with Custodiol-N solution, Transpl. Int. 28 (2015) 1102–1108.
[22] C. Moers, J.M. Smits, M.H. Maathuis, J. Treckmann, F. van Gelder, B.P. Napieralski, M. van Kasterop-Kutz, J.J. van der Heide, J.P. Squifflet, E. van Heurn, G.R. Kirste, A. Rahmel, H.G. Leuvenink, A. Paul, J. Pirenne, R.J. Ploeg, Machine perfusion or cold storage in deceased-donor kidney transplantation, N. Engl. J. Med. 360 (2009) 7–19.
[23] C. Moers, J. Pirenne, A. Paul, R.J. Ploeg, G. Machine Preservation, Trial Study, Machine perfusion or cold storage in deceased-donor kidney transplantation, N. Engl. J. Med. 366 (2012) 770–771.
[24] J. Nath, A. Guy, T.B. Smith, M. Cobbold, N.G. Inston, J. Hodson, D.A. Tennant, C. Ludwig, A.R. Ready, Metabolomic perfusate analysis during kidney machine perfusion: the pig provides an appropriate model for human studies, PLoS One 9 (2014) e114818.
[25] J. Nath, T. Smith, A. Hollis, S. Ebbs, S.W. Canbilen, D.A. Tennant, A.R. Ready, C. Ludwig, (13)C glucose labelling studies using 2D NMR are a useful tool for determining ex vivo whole organ metabolism during hypothermic machine perfusion of kidneys, Transpl. Res. 5 (2016) 7.
[26] M.L. Nicholson, S.A. Hosgood, M.S. Metcalfe, J.R. Waller, N.R. Brook, A comparison of renal preservation by cold storage and machine perfusion using a porcine autotransplant model, Transplantation 78 (2004) 333–337.
[27] J.M. O'Callaghan, R.D. Morgan, S.R. Knight, P.J. Morris, Systematic review and meta-analysis of hypothermic machine perfusion versus static cold storage of kidney allografts on transplant outcomes, Br. J. Surg. 100 (2013) 991–1001.
[28] K. Ormstad, T. Lastbom, S. Orrenius, Evidence for different localization of glutathione oxidase and gamma-glutamyltransferase activities during extracellular glutathione metabolism in isolated perfused rat kidney, Biochim. Biophys. Acta 700 (1982) 148–153.
[29] J.H. Southard, F.O. Belzer, Organ preservation, Annu. Rev. Med. 46 (1995) 235–247.
[30] M.J. Taylor, S.C. Baicu, Current state of hypothermic machine perfusion preservation of organs: the clinical perspective, Cryobiology 60 (2010) S20–S35.

Chapter 8

2D NMR tracer studies during HMP

Published paper (5).

Nath J, Smith T, Hollis A, Ebbs S, Canbilen SW, Tennant DA, Ready AR, Ludwig C. ^{13}C glucose labelling studies using 2D NMR are a useful tool for determining ex vivo whole organ metabolism during hypothermic machine perfusion of kidneys. *Transplant Res* 2016; **5**: 7.

This is an open access manuscript.

Introduction

1D NMR methods (including ^{1}H, ^{13}C, and ^{31}P) have highlighted an array of metabolites that can be used both to understand metabolic processes within the organ and potentially serve as biomarkers of post-transplant function. Whilst metabolic pathway software is available to try and highlight possible pathways involves, such tools have inherent limitations, which are probably exaggerated in the highly non-physiological conditions of *ex vivo* organ preservation.

Although multiple time point analysis can provide further evidence to demonstrate active pathways, ^{1}H 1D NMR essentially details a metabolic snapshot at the time of sampling. It is often difficult to draw meaningful mechanistic metabolic information from this.

Metabolic tracer analysis is an alternative way to highlight active metabolic pathways and can elegantly and unequivocally demonstrate the presence of *de novo* metabolism within these complex systems. NMR spectroscopy is a powerful tool to analyse complex ^{13}C isotopomer/isotopologue distributions in metabolites derived from labelled tracer molecules. There are various spectroscopic methods available of which the simplest is 1D-^{13}C approach. This is not a new concept with initial studies using ^{13}C in NMR tracer experiments reported over 40 years ago (143) with subsequent validation of this model in *ex vivo* organ perfusion models (e.g. heart and lung) (145, 196-198).

Whilst the data requirement (and therefore acquisition time) is much greater for 2 dimensional (2D) ^{1}H,^{13}C NMR tracer studies, they do have significant advantageous compared to 1D ^{13}C NMR experiments (discussed in Chapter 1). In addition to the greater sensitivity, there is increased spectral dispersion using 2D NMR and the

utility of this has been demonstrated in human studies (144).

Methods

In an effort to determine metabolism in porcine kidneys during HMP, we incorporated [U-^{13}C] glucose into the cooled recirculating perfusion fluid. Analysis of perfusion fluid and kidney tissue extracts was performed using both 1D ^{1}H and 2D ^{1}H, ^{13}C heteronuclear single quantum coherence NMR (2D ^{1}H,^{13}C-HSQC). This approach enables both metabolite quantification and proportionate distribution of ^{13}C isotopomers to be calculated.

Results

In this small study, we found that there was significant enrichment of ^{13}C in central metabolites such as [U-^{13}C] lactate during hypothermic machine perfusion. This provides unequivocal evidence of *de novo* glycolytic pathway activity occurring during *ex vivo* organ perfusion.

Conclusion

Both 1D-^{1}H NMR and 2D studies offer metabolic insights into this complex system with the high throughput, reproducible quantification of metabolites determined by 1D-^{1}H analysis complemented by mechanistic information from 2D ^{1}H, ^{13}C studies. This study demonstrates that the 2D NMR techniques described can be successfully applied to the study of metabolism during *ex vivo* whole organ perfusion. Indeed, the 2D NMR

approach described provide the platform for further work described in this thesis.

The following manuscript was published in the journal Transplantation Research in November 2016 I am the first author of this manuscript and was responsible at every stage of its production. I wrote the enclosed manuscript.

Nath et al. Transplantation Research (2016) 5:7
DOI 10.1186/s13737-016-0037-0

Transplantation Research

RESEARCH Open Access

^{13}C glucose labelling studies using 2D NMR are a useful tool for determining ex vivo whole organ metabolism during hypothermic machine perfusion of kidneys

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Abstract

Background: The aim of this study is to determine the feasibility of using nuclear magnetic resonance (NMR) tracer studies (^{13}C-enriched glucose) to detect ex vivo de novo metabolism in the perfusion fluid and cortical tissue of porcine kidneys during hypothermic machine perfusion (HMP).

Methods: Porcine kidneys ($n = 6$) were subjected to 24 h of HMP using the Organ Recovery Systems LifePort Kidney perfusion device. Glucose, uniformly enriched with the stable isotope ^{13}C ([U-^{13}C] glucose), was incorporated into KPS-1-like perfusion fluid at a concentration of 10 mM. Analysis of perfusate was performed using both 1D ^{1}H and 2D ^{1}H,^{13}C heteronuclear single quantum coherence (HSQC) NMR spectroscopy. The metabolic activity was then studied by quantifying the proportion of key metabolites containing ^{13}C in both perfusate and tissue samples.

Results: There was significant enrichment of ^{13}C in a number of central metabolites present in both the perfusate and tissue extracts and was most pronounced for lactate and alanine. The total amount of enriched lactate (per sample) in perfusion fluid increased during HMP (31.1 ± 12.2 nmol at 6 h vs 93.4 ± 25.6 nmol at 24 h $p < 0.01$). The total amount of enriched alanine increased in a similar fashion (1.73 ± 0.89 nmol at 6 h vs 6.80 ± 2.56 nmol at 24 h $p < 0.05$). In addition, small amounts of enriched acetate and glutamic acid were evident in some samples.

Conclusions: This study conclusively demonstrates that de novo metabolism occurs during HMP and highlights active metabolic pathways in this hypothermic, hypoxic environment. Whilst the majority of the ^{13}C-enriched glucose is metabolised into glycolytic endpoint metabolites such as lactate, the presence of non-glycolytic pathway derivatives suggests that metabolism during HMP is more complex than previously thought. Isotopic labelled ex vivo organ perfusion studies using 2D NMR are feasible and informative.

Keywords: Hypothermic machine perfusion, Kidney, Transplantation, Metabolism, NMR, Isotopic tracer study, ^{13}C

Background

Although there is good evidence to support the use of hypothermic machine perfusion (HMP) in clinical practice [1–3], there are surprisingly few reports detailing renal metabolism during this process. This is likely to reflect the widely held belief that metabolism is deleterious during organ preservation and should be therefore minimised by both hypothermia and keeping cold ischaemia times (CIT) as short as possible. The concept that metabolic support may have beneficial effects during HMP in addition to the mechanical effects is relatively recent [4, 5].

Using a one-dimensional (1D) ^{1}H nuclear magnetic resonance (NMR) metabolomic approach, we have previously identified a panel of metabolites within the perfusion fluid during HMP that are predictive of post-transplant graft function [6]. We have also demonstrated that porcine and

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human metabolic profiles are similar, validating the pig as a valid metabolic model for kidney transplant studies [7].

However, the metabolic 'snapshot' provided by this conventional NMR metabolomic approach has limitations for the understanding of cellular metabolism in complex models such as ex vivo organ perfusion. Merely identifying and quantifying a metabolite is often insufficient in the determination of any reliable mechanistic information: even with serial measurements, such relationships are often unclear. For example, the detection of lactate within the perfusate of a machine-perfused kidney could be secondary to the metabolism or release of pre-existing intracellular substrate stores as well as de novo metabolism of substrates derived from the perfusion fluid. Thus, the appearance of a particular metabolite within the perfusion fluid does not confirm de novo metabolism. For this reason, we propose performing isotopic ^{13}C-labelled glucose studies.

Incorporation of stable isotopes such as ^{13}C into common compounds enables metabolic tracer studies within a biological system. This is not a new concept, with initial studies reported over 40 years ago [8]. Introduction of labelled metabolic precursors can highlight specific pathways within the metabolic network. For example, after addition of [U-^{13}C] glucose to the fluid of a kidney during HMP, detection of uniformly labelled ^{13}C lactate provides unequivocal evidence of glycolytic pathway activity.

NMR spectroscopy is a powerful tool to analyse complex ^{13}C isotopomer/isotopologue distributions in metabolites derived from labelled tracer molecules. There are various spectroscopic methods available, of which the simplest is a 1D ^{13}C approach. 1D NMR organ perfusion studies have been reported in ex vivo heart and lung animal models using perfusate labelled with ^{13}C glucose and pyruvate [9–12]. This demonstrates the feasibility of using ^{13}C-labelled metabolic precursors to determine de novo metabolism in a whole organ perfusion model. 2D ^{1}H,^{13}C HSQC NMR is an alternative to 1D ^{13}C NMR, benefitting from higher sensitivity and increased spectral dispersion. Whilst previous authors have demonstrated the utility of a human 2D NMR approach using ^{13}C-labelled glucose [13], to our knowledge there are no reports of this technique in the field of transplantation.

The aim of this study is to assess whether metabolic pathway activity within a machine-perfused porcine kidney can be determined using ^{13}C-labelled glucose tracer experiments and 2D NMR.

Methods

Pig studies

Experiments were performed on 22–26-week-old 'bacon weight' pigs, weighing 80–85 kg ($n = 6$) as described elsewhere [7]. All experiments were performed following the principles of laboratory animal care according to NIH standards. To replicate human conditions, kidneys were initially cold flushed (4 °C) with 1 l Soltran solution under aseptic conditions at a pressure of 150 mmHg, followed by 2 h of cold storage. Organs were then perfused with 1 l of a modified version of KPS-1 solution containing 10 mM of [U-^{13}C]-labelled glucose and 30 g of PEG 35 kDa as an impermeant in place of hydroxyethyl starch. The LifePort Kidney Transporter 1.0 (Organ Recovery Systems) was used for perfusion at a pressure of 30 mmHg (Fig. 1).

Kidneys were perfused for 24 h with perfusate sampled at times 6, 12, 18 and 24 h from each kidney via the designated LifePort sampling port and stored at −20 °C. The samples were thawed at room temperature, prepared and processed as described below. Kidney cortex tissue samples were also taken at the study endpoint and flash frozen prior to homogenization.

2D NMR spectroscopy

2D HSQC NMR experiments were performed in this study. This type of 2D NMR exploits the spin properties of both protons (^{1}H) and isotopic carbon (^{13}C) such that only ^{13}C nuclei with an attached ^{1}H produce a signal on the resulting spectrum (Fig. 2). Whilst the pulse sequence used here actively decouples ^{1}H and ^{13}C, inter-proton and inter-carbon couplings are still active. However, the only coupling resolved in the resulting NMR spectra is the ^{13}C–^{13}C coupling. Thus the spectral peak of a ^{13}C nuclei is altered if there is an adjacent ^{13}C nuclei, in that the signal is split and two peaks exist, splitting into four if flanked by a ^{13}C either side (Fig. 3). In these experiments, [U-^{13}C] glucose was used as a tracer (i.e. ^{13}C nuclei in all six carbon positions of the glucose molecule) therefore forming lactate

Fig. 1 LifePort Kidney Transporter 1.0 (Organ Recovery Systems) containing a porcine kidney. **a** Bubble chamber. **b** Reservoir for ice. **c** Aortic patch connector for perfusion. **d** Peristaltic pump. **e** Controls (pressure, prime, wash, infuse). **f** Sampling port

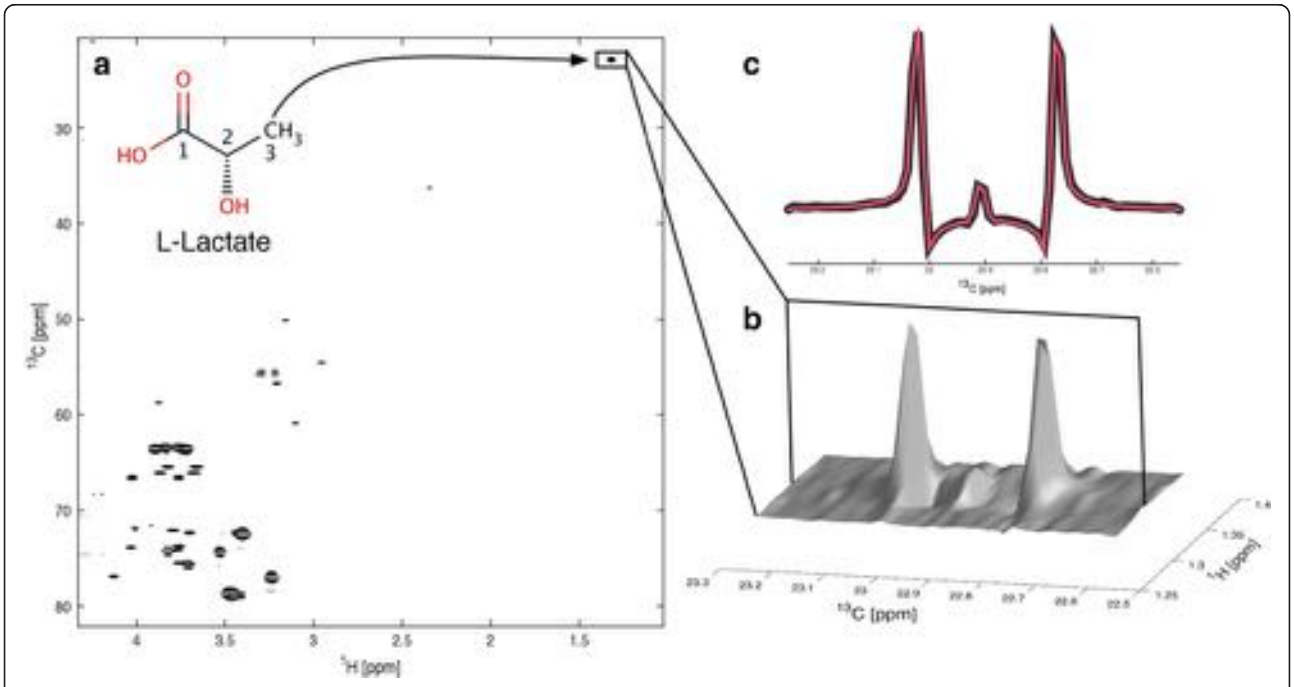

Fig. 2 A 2D HSQC spectrum of extracted pig kidney cortex is shown in **a**. A 3D plot of the methyl group (carbon 3) signal of lactate is displayed in **b. c** A cross section through the middle of the methyl group signal. The recorded spectrum is plotted in *black*, whereas a quantum-mechanical simulation of the same multiplet is shown in *red*. The plot demonstrates that the simulation allows for a fully quantitative analysis of the NMR multiplets, which forms the base for a subsequent isotopomer/isotopologue analysis

with all three carbons in the isotopic ^{13}C version produced through glycolysis. As the natural abundance of ^{13}C is known (1.1 %) and the likelihood of adjacent ^{13}C nuclei occurring naturally very low (1.1 $\%^2$ = 0.0121 %), it is possible to determine the relative concentration of isotopic carbon molecules compared to the natural abundance levels by performing quantum-mechanical simulations of the different components of an experimental HSQC multiplet (Fig. 3). Absolute concentrations of metabolites can be determined using 1D ^{1}H NMR, and therefore, absolute concentrations of labelled metabolites can be calculated using a combination of these two NMR approaches.

Sample preparation

Perfusates

Perfusate samples were extracted using a chloroform/methanol extraction. Briefly, 1.5 ml of perfusate was combined with 1.5 ml of chilled methanol (at −80 °C) and 1.5 ml chloroform (i.e. a 1:1:1 ratio). This was mixed vigorously for 10 min and then cooled on wet ice for 10 min. The polar and non-polar phases were separated by centrifugation (1300*g* for 15 min at 4 °C) as described elsewhere [14, 15] and 2 ml of the polar fraction aspirated and dried at 30 °C overnight. The dried samples were resuspended in 480 μl NMR phosphate buffer containing 0.1 M phosphate buffer (pH 7.0), 0.5 μM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid), 2 mM imidazole, and 48 μl D_20 (deuterium oxide), and vortexed until the dried pellet had completely dissolved. Following this, 35 μl of the sample was added to a 1.7-mm NMR tube, sonicated for 10 min to dissolve any remaining micro particles and then centrifuged to remove air bubbles. The sample was stored at 4 ° C until processed.

Tissues

Immediately after storage, the kidneys were laterally bisected and sections of cortex were excised and snap frozen in liquid nitrogen. Sections of cortex were then pulverised to a fine powder using a manual cryogrinder. After this, 0.5 g of the powdered sample was added to a 7-ml Precellys homogenisation tube containing 5.1 ml of chilled methanol (−80 °C) to quench ongoing metabolism. The sample was homogenised using the Precellys 24 Dual homogeniser set at the lowest mode (5000 rpm for 15 s) until fully homogenised (this was standardised to eight courses of the lowest setting with cooling in dry ice between runs to prevent overheating). Once homogenised, the samples were mixed with 4.65 ml ultrapure water accounting for the estimated 79 % water (around 395 μl) present in the tissue already [16] and 5.1 ml chloroform (i.e. a 1:1:1 ratio as with the perfusate samples).

Metabolite extraction and resuspension in NMR buffer was performed as in the perfusate sample preparation, with the exception that 4.5 ml of the top polar layer was

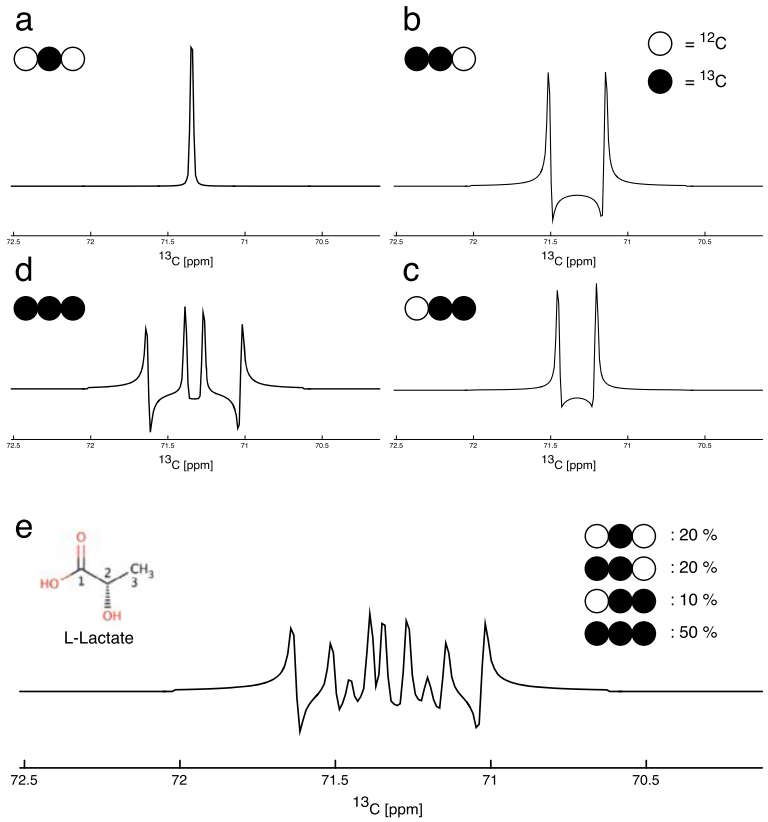

Fig. 3 Possible multiplet components for the central carbon (carbon 2) of lactate. Clockwise (**a–d**), the four possible multiplet components are shown. The spectrum shown in **a** contains ^{13}C in position 2 only, whereas positions 1 and 3 carry a ^{12}C nucleus. In both **b** and **c**, one of the adjacent carbon nuclei carries an additional ^{13}C nucleus in the same molecule. Therefore, the NMR signal is split into two resonance lines (doublet). Because of the different chemistry of carbons 1 and 3, the amount of splitting depends on which neighbour carries the ^{13}C nucleus. This is why splittings can be used to uniquely assign which neighbouring carbon is labelled even though it may be impossible to directly observe that carbon, such as carbon 1, in a HSQC spectrum. The multiplet for a fully labelled lactate molecule is plotted in **d**. Because both neighbouring carbons are ^{13}C, the signal is now split into four peaks (doublet of doublets). Under normal circumstances, multiplets will contain a mixture of those patterns. Such a mixture is shown in **e**. Despite the rather complex appearance of this signal, it can be fully quantified by a quantum-mechanical signal deconvolution

aspirated and dried before resuspension in 60 μl of NMR phosphate buffer and 35μl of this was added to a 1.7mm NMR tube as before.

NMR spectroscopy

Both 1D ^{1}H NMR and 2D $^{1}H,^{13}C$ HSQC NMR spectra were acquired on a 600-MHz Bruker Avance III NMR spectrometer equipped with a TCI 1.7-mm z-PFG cryogenic probe at 300 K. NMR acquisition time was circa 5 h per sample (Fig. 4). The spectral widths for all spectra were set to either 7812.5 Hz (^{1}H) or 24,155 Hz (^{13}C). For the 1D ^{1}H NMR spectra, 16,384 complex data points were acquired. For the ^{1}H dimension of 2D $^{1}H,^{13}C$ HSQC NMR spectra, 512 complex data points were acquired. Out of 8192 complex data points (2458), 30 % were sampled for the ^{13}C dimension using an exponentially weighted non-uniform sampling scheme. Using a 4-s interscan relaxation delay, 128 transients were recorded for the 1D NMR spectra. Two transients per increment were recorded for the 2D $^{1}H,^{13}C$ HSQC NMR spectra. The interscan relaxation delay was set to 1.5 s. Each sample was automatically tuned, matched and then shimmed (1D TopShim) to a DSS line width of <1 Hz prior to acquisition of the first spectrum. 1D ^{1}H NMR spectra were processed using the MATLAB-based MetaboLab software [17]. All 1D data sets were zero-filled to 131,072 data points prior to Fourier transformation. The chemical shift was calibrated by referencing the DSS signal to 0 ppm. 1D spectra were manually phase corrected before correcting the spectral baseline using a spline function [17]. 1D ^{1}H NMR spectra were then exported into Bruker format for metabolite identification and concentration determination using Chenomx 8.1 (ChenomxINC). 2D $^{1}H,^{13}C$ HSQC NMR spectra were reconstructed with compressed sensing using the MDDNMR and NMRpipe software [18–20]. The spectra

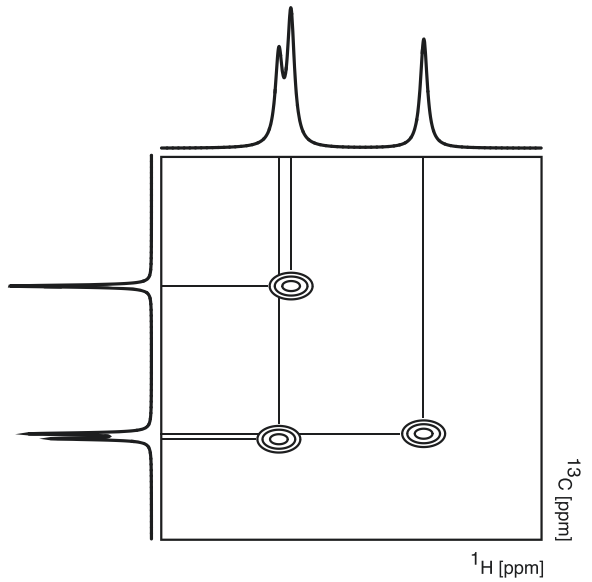

Fig. 4 Schematic representation of 2D HSQC NMR spectrum. Chemical shift for ^{1}H depicted on the *x*-axis and ^{13}C on the *y*-axis. Whilst in both the projected 1D ^{1}H and the 1D ^{13}C spectrum display signal overlap, all signals are well resolved in the 2D spectrum

were zero-filled to 1024 (^{1}H) times 16,384 (^{13}C) real data points. HSQC spectra were then analysed using MetaboLab, which uses the pyGamma software for multiplet simulations [21]. The methyl group of lactate was used to calibrate the chemical shift based on its assignment in the human metabolome database [22].

Statistical analysis

Data were analysed using GraphPad Prism v6 (GraphPad Software, San Diego, USA). Data were reported as mean ± SD, unless stated otherwise. Statistical significance was tested using two-tailed Student's *t* test. A *p* value of <0.05 was considered significant.

Results

An understanding of the active metabolic pathways during HMP could potentially facilitate the development of a more nutritionally supportive perfusion fluid enabling the metabolic optimisation of these valuable organs prior to transplantation.

In this donation after cardiac death (DCD) large animal model of kidney transplant preservation, enrichment of ^{13}C in a number of metabolites was observed in both perfusate solution and kidney cortex tissue. This confirms that de novo metabolism occurs during HMP and that the proposed NMR methodology is useful to demonstrate this. Even after 6 h of perfusion, glycolytic activity was evident with enriched [U-^{13}C] lactate, enriched [U-^{13}C] alanine and enriched [U-^{13}C] acetate detected in the perfusion fluid.

The proportion of [U-^{13}C] enriched lactate (as a fraction of the total lactate present) increased over the study period (3.25 % ± 1.02 after 6 h and 7.74 % ± 1.57 after 24 h $p < 0.001$). In addition, the total concentration of lactate present (enriched and non-enriched) increased (1.51 ± 0.20 mM at 6 h and 2.04 ± 0.57 mM at 24 h $p < 0.05$). Given that the total amount of enriched lactate present at a given time point is the product of the relative proportion (e.g. 3.25 %) and total concentration (e.g. 1.51 mM), it is self-evident that as both of these are increasing; the amount of enriched lactate being produced is also increasing (total amount of labelled [U-^{13}C] lactate (per sample) 31.1 ± 12.2 nmol at 6 h vs 93.4 ± 25.6 nmol at 24 h $p < 0.01$).

There is a similar pattern observed with alanine with the enriched proportion increasing (1.25 % ± 0.44 at 6 h and 2.83 % ± 1.19 at 24 h $p < 0.05$) and the total concentration increasing by a similar proportion (0.24 mM ± 0.018 at 6 h and 0.41 mM ± 0.13 at 24 h $p < 0.01$) (total [U-^{13}C] alanine per sample (1.73 ± 0.89 nmol at 6 h vs 6.80 ± 2.56 nmol at 24 h $p < 0.05$)) (Fig. 5).

Whilst enriched acetate was present at all time points in the perfusion fluid (circa 1.25 %), there was no change in concentration over time. Similarly, the total concentration of acetate did not change over time, suggesting that either the production of acetate was limited or being converted to other enriched substrates.

The same enriched metabolites (lactate, alanine and acetate) were also present in the extracted kidney cortex samples at all time points. In addition, glutamate with ^{13}C enrichment in the 4 and 5 carbon position was present in several samples in small amounts (<0.5 % of total present). The presence of this labelling pattern in glutamate indicates mitochondrial tricarboxylic acid (TCA) cycle activity (via pyruvate dehydrogenase and complex 1).

Discussion

This paper provides further evidence that a significant amount of metabolism occurs during HMP and that NMR methods are useful for identifying these [6, 7, 23]. However, unlike ^{1}H NMR reports, this paper provides unequivocal evidence that de novo metabolism occurs in these organs during HMP with the glucose present in the perfusate used as a substrate. To our knowledge this is the first report to demonstrate such de novo metabolism in a whole organ ex vivo kidney model using NMR tracer methods.

The labelled tracer study techniques described herein highlight active metabolic pathways during the machine perfusion period. This could be particularly useful as clinicians seek to alter perfusion characteristics in order to optimise kidney graft function prior to transplant.

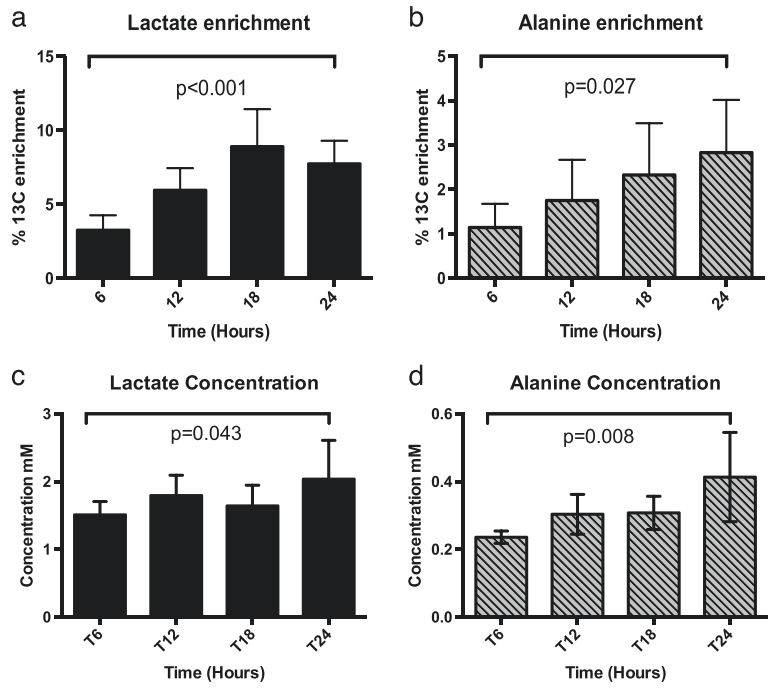

Fig. 5 Changes over time for lactate and alanine in the perfusion fluid of HMP kidneys. **a**, **b** The % enrichment present and **c**, **d** the total concentration of these metabolites. In all cases, there was a significant increase both in ^{13}C enrichment and in concentrations between the 6- and 24-h samples

The metabolic effects of such perfusion modification (e.g. temperature, oxygenation, oxygen carriers) can be elegantly demonstrated using ^{13}C NMR, with analysis of perfusion fluid permitting non-invasive metabolic monitoring.

The appearance of uniformly labelled lactate and alanine in the perfusate and tissue samples demonstrate that de novo glycolysis is occurring in this non-physiological environment and that alternative metabolic pathways such as the pentose phosphate pathway do not appear to be active. Although cellular metabolism is reported to only be about 5–8 % at temperatures below 4 °C [24] with similar oxygen requirements [25], hypothermia does not cause uniform deceleration of all metabolic pathways [4] and the metabolic effects of hypothermia within different organs is not uniform [26]. There is some evidence that the energy substrate utilised to support metabolism in a hypothermic canine ex vivo model is predominantly glucose entering glycolysis [27], highlighting the importance of this pathway during machine preservation of organs. Interestingly, other studies demonstrate an increase in perfusate metabolites such as glutamate during perfusion. The suggested mechanism was the reduction (and deamination) of glutamine to glutamate by glutaminase. The authors concluded that the deamination of glutamate occurs to form alpha-ketoglutarate, an important TCA cycle intermediate [28].

The presence of labelled acetate within the perfusate is interesting. Whilst acetate can be formed from multiple pathways, the early appearance of the labelled substrate in these experiments may indicate acetyl-coA hydrolase activity [29].

The labelled metabolites in this study were detectable in the extracellular perfusion fluid within 6 h and demonstrates that cellular uptake of glucose, glycolysis and transport/efflux of resulting metabolites occurs within this time period. Although the proportion of de novo metabolites is modest, it does occur independently of any glucose uptake stimulators such as insulin.

There has been great interest in the role of oxygenation and oxygen carriers during kidney preservation, with proponents suggesting that oxygenation replenishes cellular ATP levels [30] and detractors highlighting the perils of uncontrolled oxygenation exacerbating the reactive oxygen species (ROS) generation [31] and the ischaemia reperfusion phenomenon [32]. This study demonstrates that even in the absence of supplemental oxygen, small amounts of glucose-derived glutamic acid were observed in several tissue samples indicative of aerobic TCA cycle activity. Although this is surprising, it must be remembered that HMP is not an anoxic environment and oxygen solubility increases at lower temperatures and increased salinity and the preservation solution is further saturated by the physical fluid agitation that occurs during perfusate cycling. Whilst other

metabolites indicative of TCA activity were commonly identified using NMR such as citrate, succinate and fumarate (by 2D and/or 1D), they were not labelled to indicate de novo formation.

One of the benefits of the 2D NMR methodology used in this paper is that the sensitivity (per unit time) is increased compared with 1D ^{13}C NMR. However, ^{13}C NMR is inherently less sensitive than ^{1}H NMR, and therefore, it is possible that labelled metabolites present in very small quantities would not be identified using this method. Furthermore, our 2D database of splitting patterns and chemical shift parameters for different metabolites is evolving and other labelled metabolites may be identified once these have been further clarified. Whilst we feel the 2D NMR methods in this paper relay unparalleled amounts of metabolic information, they are both time consuming and resource intensive and would not be suitable for real-time analysis to inform clinical decisions, as proposed for 1D ^{1}H NMR studies [23].

The experimental conditions in these experiments were standardised as much as possible to reduce inter-experimental variability and designed to replicate clinical conditions. Whilst previous studies have repeatedly demonstrated the viability of porcine kidneys after similar periods of HMP [23, 33], this study does not seek to corroborate this and as such we are unable to correlate de novo metabolism with functional outcome. Given the findings from earlier studies [6], we hypothesise that glycolytic activity during perfusion may correlate with post-transplant function and propose a human observational study to clarify this, which is possible due to the non-radioactive nature of the ^{13}C isotope used in this study.

Conclusions

We conclude that the described ^{13}C labelling study convincingly demonstrates de novo metabolism during kidney storage by HMP, and this can be used to highlight active metabolic pathways in this hypothermic, hypoxic environment.

We postulate that although most of the supplied ^{13}C-enriched glucose is converted to endpoint metabolites associated with glycolytic metabolism (i.e. lactate), the presence of labelled non-glycolytic pathway derivatives suggests that kidney metabolism during HMP storage is more complex than previously thought.

Isotopic labelled ex vivo organ perfusion studies using 2D NMR are therefore informative and feasible. Metabolic manipulation is a potential target for therapeutic intervention during the preservation period, and accurate understanding of active metabolic pathways could potentially facilitate studies to optimise this, potentially improving the function of the kidneys post-transplant.

Abbreviations

CIT, cold ischaemia time; HMP, hypothermic machine perfusion; HSQC, heteronuclear single quantum coherence; NIH, National Institute of Health; PEG, polyethylene glycol

Acknowledgements

Not applicable.

Availability of data and materials

Raw data was uploaded on to the MetaboLights repository at http://www.ebi.ac.uk/metabolights: accession nos. are MTBLS367. Available at http://www.ebi.ac.uk/metabolights/MTBLS367.

Funding

This work was funded through grants from University Hospitals Birmingham Charities, Organ Recovery Systems and the Royal College of Surgeons Edinburgh.

Authors' contributions

The experiments were designed by JN, DT, CL and AR. These experiments were performed by JN, TS, AH, SE, SC and CL. The paper itself was written by JN, TS and CL. Finally, the paper was reviewed by all authors JN, TS, AH, SE, SC, DT, AR and CL. All authors read and approved the final manuscript.

Competing interests

This work was part funded through a grant from Organ Recovery Systems Inc.

Consent for publication

Not applicable.

Ethics approval and consent to participate

As animals were slain for meat purposes at F.A. Gills (ltd) abattoir, ethical approval was not required for animal experiments. All experiments were performed following the principles of laboratory animal care according to NIH standards.

Received: 31 March 2016 Accepted: 27 July 2016
Published online: 05 August 2016

References

1. Moers C, Smits JM, Maathuis MH, Treckmann J, van Gelder F, Napieralski BP, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 2009;360(1):7–19.
2. Moers C, Pirenne J, Paul A, Ploeg RJ, Machine Preservation Trial Study G. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 2012;366(8):770–1.
3. O'Callaghan JM, Morgan RD, Knight SR, Morris PJ. Systematic review and meta-analysis of hypothermic machine perfusion versus static cold storage of kidney allografts on transplant outcomes. Br J Surg. 2013;100(8):991–1001.
4. Taylor MJ, Baicu SC. Current state of hypothermic machine perfusion preservation of organs: the clinical perspective. Cryobiology. 2010;60(3 Suppl):S20–35.
5. Fuller BJ, Lee CY. Hypothermic perfusion preservation: the future of organ preservation revisited? Cryobiology. 2007;54(2):129–45.
6. Guy AJ, Nath J, Cobbold M, Ludwig C, Tennant DA, Inston NG, et al. Metabolomic analysis of perfusate during hypothermic machine perfusion of human cadaveric kidneys. Transplantation. 2015;99(4):754–9.
7. Nath J, Guy A, Smith TB, Cobbold M, Inston NG, Hodson J, et al. Metabolomic perfusate analysis during kidney machine perfusion: the pig provides an appropriate model for human studies. PLoS One. 2014;9(12):e114818.
8. Dorman DE, Roberts JD. Nuclear magnetic resonance spectroscopy: 13C spectra of some common nucleotides. Proc Natl Acad Sci U S A. 1970;65(1):19–26.
9. Peltz M, He TT, Adams GA, Chao RY, Meyer DM, Jessen ME. Characterizing lung metabolism with carbon-13 magnetic resonance spectroscopy in a small-animal model: evidence of gluconeogenesis during hypothermic storage. Transplantation. 2005;80(3):417–20.
10. Gilbert NF, Meyer PE, Tauriainen MP, Chao RY, Patel JB, Malloy CR, et al. Effects of hypothermia on myocardial substrate selection. Ann Thorac Surg. 2002;74(4):1208–12.

11. Peltz M, He TT, Adams GA, Koshy S, Burgess SC, Chao RY, et al. Perfusion preservation maintains myocardial ATP levels and reduces apoptosis in an ex vivo rat heart transplantation model. Surgery. 2005;138(4):795–805.
12. Rosenbaum DH, Peltz M, DiMaio JM, Meyer DM, Wait MA, Merritt ME, et al. Perfusion preservation versus static preservation for cardiac transplantation: effects on myocardial function and metabolism. J Heart Lung Transplant. 2008;27(1):93–9.
13. Fan TW, Lane AN, Higashi RM, Farag MA, Gao H, Bousamra M, et al. Altered regulation of metabolic pathways in human lung cancer discerned by (13)C stable isotope-resolved metabolomics (SIRM). Mol Cancer. 2009;8:41.
14. Serkova N, Fuller TF, Klawitter J, Freise CE, Niemann CU. H-NMR-based metabolic signatures of mild and severe ischemia/reperfusion injury in rat kidney transplants. Kidney Int. 2005;67(3):1142–51.
15. Young JF, Larsen LB, Malmendal A, Nielsen NC, Straadt IK, Oksbjerg N, et al. Creatine-induced activation of antioxidative defence in myotube cultures revealed by explorative NMR-based metabonomics and proteomics. J Int Soc Sport Nutr. 2010;7(1):9.
16. Mitchell HH, Hamilton TS, Steggerda FR, Bean HW. The chemical composition of the adult human body and its bearing on the biochemistry of growth. J Biol Chem. 1945;158(3):625–37.
17. Ludwig C, Gunther UL. MetaboLab—advanced NMR data processing and analysis for metabolomics. BMC Bioinf. 2011;12:366.
18. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. Nmrpipe—a multidimensional spectral processing system based on Unix pipes. J Biomol NMR. 1995;6(3):277–93.
19. Kazimierczuk K, Orekhov VY. Accelerated NMR spectroscopy by using compressed sensing. Angew Chem Int Edit. 2011;50(24):5556–9.
20. Orekhov VY, Jaravine VA. Analysis of non-uniformly sampled spectra with multi-dimensional decomposition. Prog Nucl Mag Res Sp. 2011;59(3):271–92.
21. Smith SA, Levante TO, Meier BH, Ernst RR. Computer-simulations in magnetic-resonance—an object-oriented programming approach. J Magn Reson Ser A. 1994;106(1):75–105.
22. Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, et al. HMDB: the human metabolome database. Nucleic Acids Res. 2007;35:D521–6.
23. Bon D, Billault C, Thuillier R, Hebrard W, Boildieu N, Celhay O, et al. Analysis of perfusates during hypothermic machine perfusion by NMR spectroscopy: a potential tool for predicting kidney graft outcome. Transplantation. 2014;97(8):810–6.
24. Southard JH, Belzer FO. Organ preservation. Annu Rev Med. 1995;46:235–47.
25. Belzer FO, Southard JH. Organ preservation and transplantation. Prog Clin Biol Res. 1986;224:291–303.
26. Boutilier RG. Mechanisms of cell survival in hypoxia and hypothermia. J Exp Biol. 2001;204(Pt 18):3171–81.
27. Huang JS, Downes GL, Childress GL, Felts JM, Belzer FO. Oxidation of 14C-labeled substrates by dog kidney cortex at 10 and 38 degrees C. Cryobiology. 1974;11(5):387–94.
28. Baicu SC, Taylor MJ, Brockbank KG. Modulating biochemical perturbations during 72-hour machine perfusion of kidneys: role of preservation solution. Cryobiology. 2007;54(1):114–20.
29. Rae C, Fekete AD, Kashem MA, Nasrallah FA, Broer S. Metabolism, compartmentation, transport and production of acetate in the cortical brain tissue slice. Neurochem Res. 2012;37(11):2541–53.
30. Buchs JB, Lazeyras F, Ruttimann R, Nastasi A, Morel P. Oxygenated hypothermic pulsatile perfusion versus cold static storage for kidneys from non heart-beating donors tested by in-line ATP resynthesis to establish a strategy of preservation. Perfusion. 2011;26(2):159–65.
31. Llarrull MS, Pizarro MD, Scandizzi AL, Bottai H, Guibert EE, Rodriguez JV. Cold preservation of isolated hepatocytes in UW solution: experimental studies on the respiratory activity at 0 degrees C. Cryo Letters. 2007;28(5):313–28.
32. Rodriguez F, Bonacasa B, Fenoy FJ, Salom MG. Reactive oxygen and nitrogen species in the renal ischemia/reperfusion injury. Curr Pharm Des. 2013;19(15):2776–94.
33. Hosgood SA, Yang B, Bagul A, Mohamed IH, Nicholson ML. A comparison of hypothermic machine perfusion versus static cold storage in an experimental model of renal ischemia reperfusion injury. Transplantation. 2010;89(7):830–7.

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Chapter 9

Effects of oxygenation during HMP

Introduction

The benefit, if any, of oxygenation during hypothermic kidney preservation is yet to be established. There are no high quality studies comparing the effect of oxygenation versus non-oxygenation of perfusate during HMP in human kidneys, and evidence is largely derived from small scale animal experiments (83, 199-201).

Hypothermia results in a deceleration of metabolism, and as we have shown in a previous chapter (Chapter 7), the metabolism during standard HMP (non-oxygenated) differs considerably from SCS conditions, with the promotion of multiple central metabolic pathways (6). Thus the oxygen requirement, or at least the therapeutic value of oxygenation, is likely to differ between kidneys stored in HMP and SCS conditions. Furthermore, the promotion of metabolic pathways during HMP could potentially be harmful in the absence of oxygen, or could limit the duration for which it is effective. Indeed, greater levels of lipid peroxidation, a marker of oxidative stress have been demonstrated in HMP kidneys despite improved function, which may reflect these hypoxic conditions (202, 203).

Seemingly, oxygenation would appear to provide a more physiological storage environment for the preserved kidney with the promotion of aerobic metabolism, improving cellular ATP stores and facilitating the ATP-dependent Na^+/K^+ pump (56, 199, 204, 205). The beneficial effects of oxygenation during machine perfusion on ATP levels have been demonstrated in multiple animal organ models including liver (murine) (205), heart (porcine) (206, 207) and kidney (199). There is also some evidence that ATP generation is dependent on 'dosage' of oxygen. In a highly oxygenated (100 kPa) porcine kidney perfusion model, greater ATP levels were demonstrated compared to lower partial pressures (50 kPa) (199, 208).

The principal HMP device used in clinical transplantation in the UK (LifePort© Kidney Transporter, Organ Recovery Systems, US) provides non-oxygenated pulsatile perfusion. However, the KidneyAssist Transport device (Organ Assist, NL) does have an integrated membrane oxygenator enabling hypothermic (4°C) oxygenated perfusion of kidneys.

The benefits of oxygenation during HMP in the human setting are currently being assessed by two European trials using the KidneyAssist device. The COPE POMP trial is comparing the outcomes of extended criteria kidneys undergoing oxygenated HMP (2 hours) after a period of static cold storage to investigate the role, if any, of oxygenated preconditioning (209). The COPE COMPARE trial is comparing the outcomes of oxygenated vs. non-oxygenated HMP in donation after cardiac death kidneys (209).

Aims

The aim of this study was to determine the effects of oxygenation during the HMP process by comparing the metabolic profiles of paired porcine kidneys perfused with oxygenated and aerated perfusion fluid. A combination of analytical technologies were used including using both ^{1}H and ^{13}C NMR, gas chromatography mass spectrometry (GCMS), and high performance liquid chromatography (HPLC).

Participants

I was responsible for the conceptualisation of this project and was involved in all of the perfusion experiments. However a number of other persons were involved in this project without whom it would not have been possible. These include Thomas Smith,

Kamlesh Patel, Alex Hollis, Samuel Ebbs, Sefa Canbilen (Perfusion experiments, sample preparation, NMR analysis), Yugo Tsuchiya (HPLC analysis), Alpesh Thakker (GCMS analysis), and Christian Ludwig (NMR analysis).

Methods

Eight paired abattoir porcine kidneys were retrieved and cold flushed as per the protocol described in Chapter 2. At the laboratory these kidneys were connected to the LifePort© perfusion machine as previously described. The perfusion experimental setup performed differed from that described in Chapter 6 in two distinct ways. Firstly, instead of perfusing the kidneys with 1 L of chilled (4°C) homemade KPS-1®-like perfusion fluid containing 10 mM of [U-^{13}C] glucose, factory KPS-1® fluid was used with 5 mM of [U-^{13}C] glucose added to it. As factory KPS-1® contains glucose at a concentration of 10 mM, the final concentration of the glucose in the fluid was therefore 15 mM, of which only a third was the [U-^{13}C] isomer. The second modification was that the fluid was either oxygenated or aerated.

Oxygenation/aeration setup

A paediatric membrane oxygenator was used for this purpose and incorporated into the LifePort© circuit as depicted below (Figure 35). The oxygenator used was the Hilite® 800 LT series hollow fibre oxygenator (Medos, Medizintechnik AG). This was selected as the permissible flow rate for both perfusion fluid and oxygen through the device was appropriate for HMP. In these experiments we wanted to compare the effects of oxygenation versus aeration. For the oxygen group, Carbogen (BOC Medical) was used which is composed of 95% oxygen and 5% carbon dioxide. For the aerated group, atmospheric air was used and pumped through a filter into the

oxygenator using a peristaltic pump. The flow of air or oxygen into the Hilite® 800 LT hollow fibre oxygenator was maintained at 0.1 L/min in order to reduce the formation of gas microemboli in the perfusion fluid. Flow meters (Platon™) were used to regulate the flow of oxygen / air.

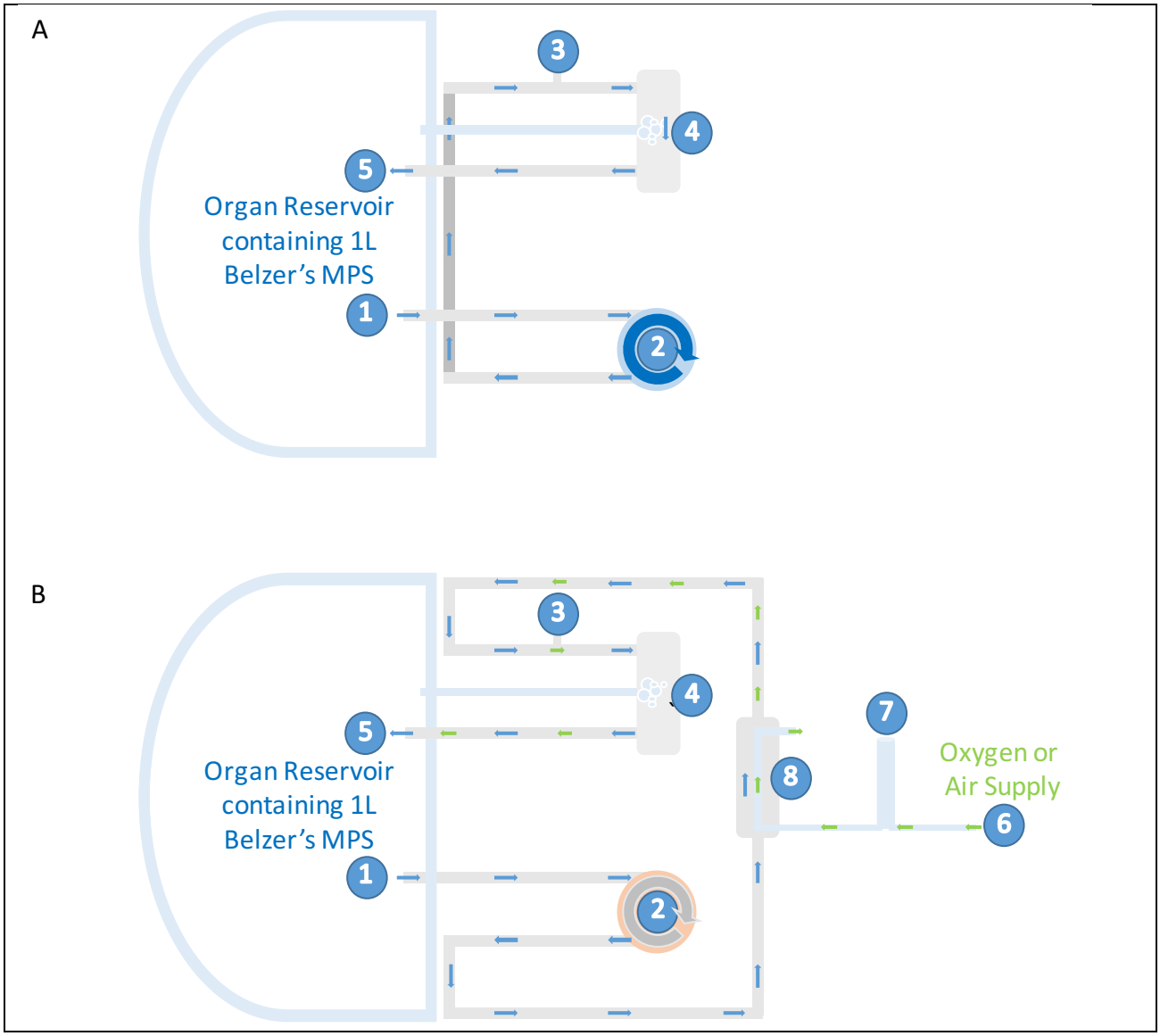

Figure 35 Standard LifePort© device(A) and modified device with oxygenator(B)

(1) Fluid aspirated from reservoir, (2) peristaltic pump, (3) sampling port, (4) bubble chamber, (5) fluid entering renal artery. Additional equipment used in the modified circuit include (6) gas inlet (oxygen or air), (7) flow meter, (9) oxygenator.

Oxygen measurements

Oxygen levels were measured relative to a control (4°C deionised water) using the Microx TX3 oxygen sensor (PreSens- Precision Sensing GmbH, Regensburg, Germany). To calibrate, deionised water at 4°C left open to the air overnight was used as the calibration positive control (i.e. this was the 100% O_2 level). For the 0% calibration control, a 1% solution of sodium sulphite vigorously agitated was used, as per manufacturer's instructions. The negative control was validated against deionized water at room temperature, deoxygenated by bubbling nitrogen gas through it for 1 hour, which returned a value of around 0% relative to the 100% and 0% oxygen calibrators. The phase values for each calibrator was phase:59.37 at 21.5°C for the 0% oxygen control and phase: 32.87 at 4°C for the 100% control.

NMR

Both 1D ^{1}H and 2D $^{1}H,^{13}C$-HSQC NMR spectra were acquired for this study as described in Chapter 2 and Chapters 5-8.

GCMS

GCMS analysis was carried out by a collaborator (Alpesh Thakker) according to a protocol used within his group and is briefly described below. Tissue was extracted as per the protocol described in Chapter 2 (chloroform/methanol). 90% of the extracted sample was used for NMR analysis with the remainder used for GCMS to ensure homogeneity between GCMS and NMR. 40 µl of 2 % methoxyamine HCl in pyridine (Sigma-Aldrich, Dorset, UK) was added and incubated at 60°C. 60 µl of N-tertbutyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) with 1% (w/v)

tertbutyldimethyl-chlorosilane (TBDMSCI) (Sigma-Aldrich, Dorset, UK) derivatization reagent was then added and further incubated for 60 mins prior to centrifugation (13000 rpm for 5 mins). The supernatant was then transferred to a chromatography vial prior to GC-MS analysis.

For analysis of the derivatized samples an Agilent 7890B Series GC/MSD gas chromatograph with a polydimethylsiloxane GC column coupled with a mass spectrometer (GC-MS) (Agilent Technologies UK Limited, Stockport, UK) was used.

Prior to sample analysis the GC-MS was tuned to a full width at half maximum (FWHM) peak width of 0.60 atomic mass units (amu) in the mass range of 50 to 650 mass to charge ratio (m/z) using PFTBA tuning solution. 1 µl of sample was injected into the GC-MS in split mode 1:10 with helium carrier gas at a rate of 1.0 ml/min. The inlet liner containing glass wool was set to a temperature of 270°C. Compound detection was carried out in full scan mode in the mass range 50 to 650 m/z, with 2-4 scans/sec, a source temperature of 250°C, transfer line temperature 280°C and a solvent delay time 6.5 minutes.

Raw GC-MS data was converted to common data format (CDF) using acquisition software (Masshunter, Agilent) and further processing of the isotope data including isotope correction and mass isotopomer analysis was performed on Metabolitedetector software (210).

Combined NMR/GCMS methodology

As described previously in this thesis (Chapter 8), the MetaboLab software package was used to determine the proportion of differing ^{13}C isotopologues based upon the 2D $^{1}H,^{13}C$-HSQC spectra acquired. However, in this chapter, for metabolites readily

detectable by both 2D NMR and GCMS (e.g. lactate, alanine and glutamate) we have used a combined approach that incorporates data from both of these techniques, which is also part of the MetaboLab program. This combination of MS/NMR enhances the utility compared to either technique in isolation. Analysis of the same sample using two different techniques serves as an internal validation for the experiment. In addition to the highly detailed information regarding the local environment surrounding ^{13}C nuclei within a molecule as determined using NMR, the combination of MS removes structural ambiguity where one ^{13}C nuclei is outside the range of magnetic influence of the next ^{13}C nuclei. This combined GCMS/2D NMR approach using MetaboLab software has been recently published by supervisors (C.L. & D.T.)(211).

HPLC

AMP/ADP/ATP analysis was performed by a collaborator (Yugo Tsuchiya) at University College London. Adenine nucleotides were extracted from frozen powdered tissue with ice-cold 0.35 M perchloric acid and analysed by ion-pair reverse phase HPLC as described by Sellevold et al (212).

Results

Oxygenation in standard HMP

Prior to commencing experiments looking at the oxygen concentrations in aerated or oxygen enriched conditions, we initially wished to established the amount of oxygen present during HMP under standard (non-oxygenated) conditions. Therefore, we

perfused several porcine abattoir kidneys with KPS-1® solution as described in multiple chapters of this thesis and measured the oxygen levels relative to chilled deionised water. The oxygen levels were measured at two sites: in the arterial limb (i.e. in the perfusion fluid prior to entering the renal artery and perfusing the kidney) and in the venous effluent of fluid exiting the kidney.

Having performed this simple experiment during 10 hours of HMP we made several important observations that are clearly demonstrated in the figure below (Figure 36). Firstly that at the start of perfusion (time zero) the oxygen levels in the KPS-1® are the same as that of the control (deionised water) and hence KPS-1® and water appear to have the same amount of dissolved oxygen at 4°C. Secondly, the amount of oxygen in the venous effluent is substantially lower (circa 10%) than the amount of oxygen in the arterial fluid. This clearly demonstrates that oxygen is consumed by the kidney during HMP under standard conditions (213) and would seemingly corroborate findings in previous chapters that oxygen-dependent metabolism does occur during HMP. Thirdly the amount of oxygen in the arterial limb perfusion fluid falls rapidly during HMP and after only two hours is less than 20% of that at the start. Given that HMP in clinical practice is commonly employed for much greater durations than two hours, the effects of supplemental oxygen after this time point are interesting to ascertain and provide further justification for this current study.

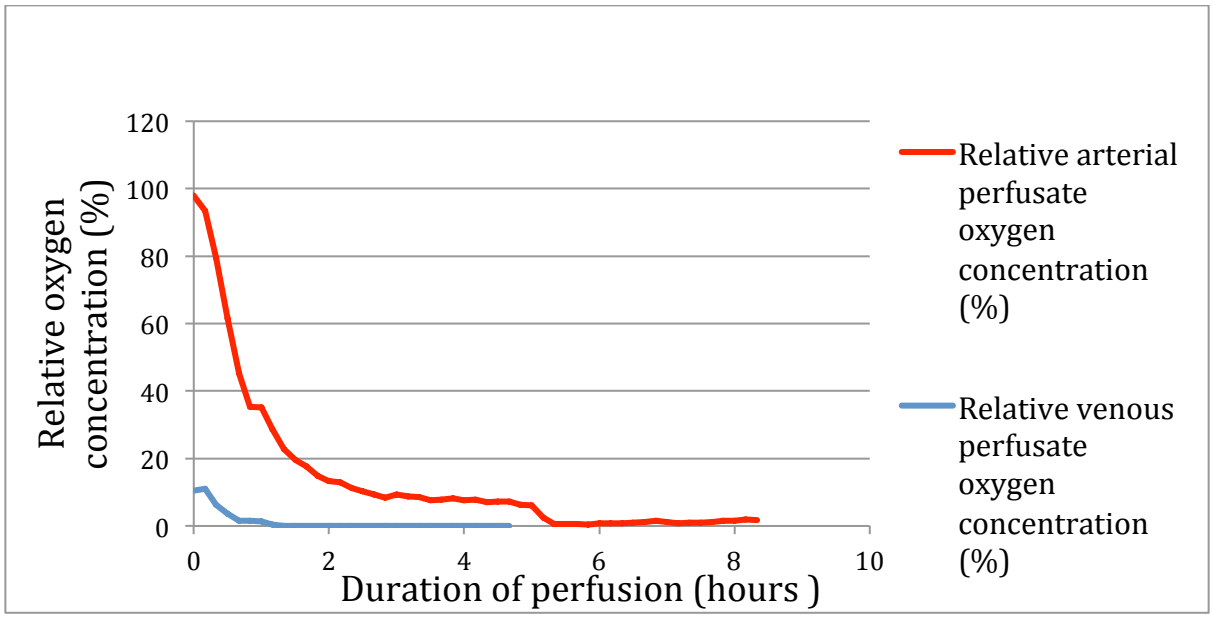

Figure 36 Diminishing oxygen levels under standard HMP conditions

Oxygen levels in experimental groups

Using the same oxygen measurement system, we sought to determine the oxygen levels in the perfusion fluid of our two experimental groups (oxygenated and aerated) during HMP. Paired porcine kidneys (n=8) underwent HMP with either supplemental oxygen or air provided via a membrane oxygenator. Oxygen levels in the perfusion fluid were measured in both the arterial (fluid entering the kidney) and venous limbs (fluid exiting the kidney). Chilled deionised water was again used as the 100% standard. The results are shown in Figure 37 below. As expected, the oxygen levels were highest in the fluid supplemented with oxygen with readings 4 fold higher than the aerated group. In both groups, the oxygen present in the venous fluid is lower than the arterial. This demonstrates that even after prolonged periods of HMP (18 hours), oxygen consumption still occurs within the kidney. Indeed oxygen levels in both groups remained static throughout the perfusion

process. Consumption appeared to be slightly greater in the group where supplemental oxygen was provided (410% to 305% vs. 102% to 31%). The fluid oxygen level in the aerated group after 18 hours mirrored the readings found under standard HMP non-oxygenated conditions at the start of perfusion i.e. 100% in arterial limb and 31% in venous (see Figure 36). Thus, it would appear that aerating perfusion fluid replenishes the oxygen consumption occurring in the kidney even for prolonged periods of HMP.

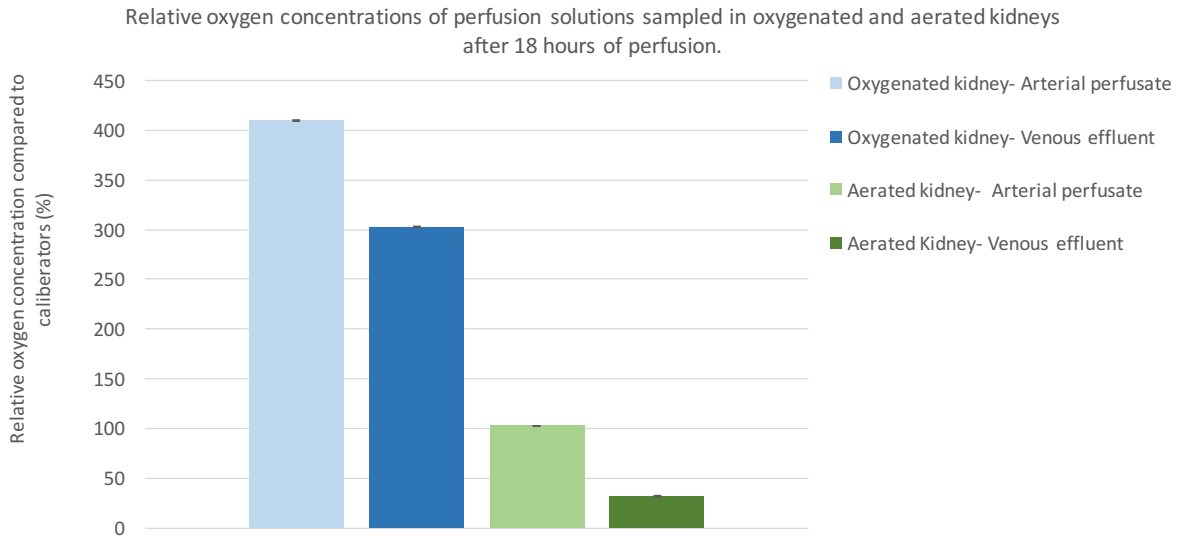

Figure 37 Relative oxygen concentration after 18 hours of HMP

Metabolic profiles using 1D ^{1}H NMR

Metabolite quantification was performed on neat perfusion fluid and extracted kidney cortex tissue as described in Chapter 2 and Chapters 5-7 using Chenomx software 8.1 (Chenomx Inc., Edmonton, AB, Canada). The metabolite concentrations between the two groups (aerated and oxygenated) were compared using a Wilcoxon matched pairs signed rank test. All analysis was performed using GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, La Jolla California USA), with $p < 0.05$ deemed to be indicative of statistical significance.

In total, 46 metabolites were reliably detected in the perfusion fluid or tissue using ^{1}H NMR. Of these, there were five metabolites (formate, glutamate, hypoxanthine, lactate, succinate) in the perfusion fluid at different concentrations between the oxygenated and aerated groups (Figure 38) and three metabolites in the kidney cortex samples present in different concentrations (choline, glutamate, succinate).

For all the metabolites that differed in concentrations between the two perfusion conditions (oxygenated or aerated), the concentrations were greater in the aerated perfusion group, with no metabolite present in greater concentration in the oxygenated group (Figure 38).

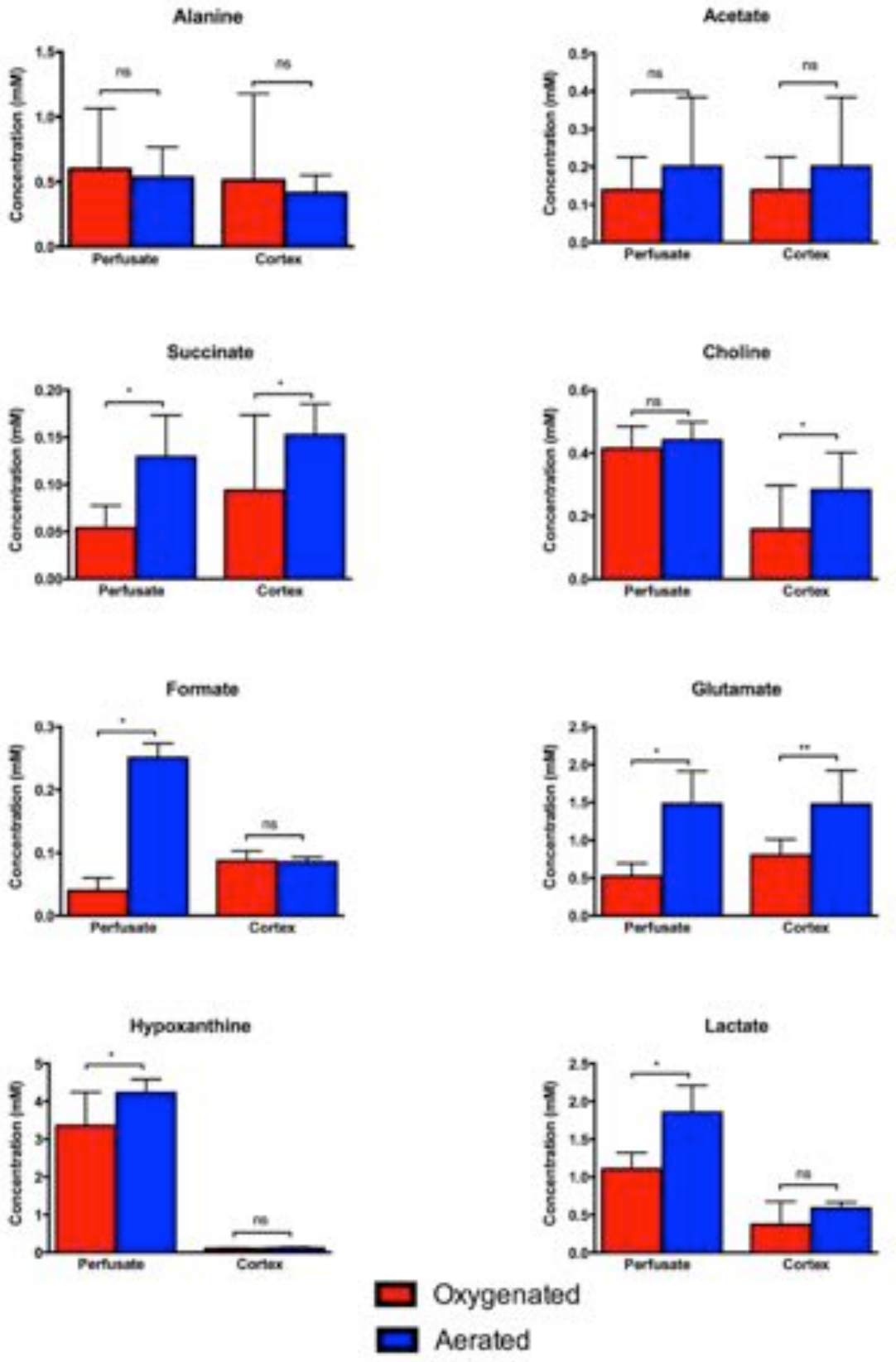

Figure 38 Concentrations of major metaboilites perfusate and cortex T=18 hrs

Data presented as mean + standard deviation and comparison using Wilcoxon matched pairs signed rank test. *p≤0.05, **p≤0.01.

AMP/ADP/ATP analysis

Adenine nucleotide (AMP, ADP and ATP) concentrations were determined using HLPC analysis of extracted kidney cortex and medulla samples from the oxygenated and aerated experiments. The two groups (aerated and oxygenated) were compared using a Mann-Witney test (unpaired, non parametric). All analysis was performed using GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, La Jolla California USA), with $p < 0.05$ deemed to be indicative of statistical significance.

This analysis demonstrated strikingly different concentrations of adenine nucleotides present. Although the amount of AMP was not significantly different between the oxygenated and aerated kidneys, the amount of ADP (both cortex and medulla) and ATP (cortex) was significantly higher in the kidneys perfused with oxygenated solution. Indeed the ATP present after 18 hours of perfusion in the cortex samples of oxygenated kidneys was over seven-fold greater than for aerated (mean 20.0 vs. 2.8 mmol/mg protein, $p<0.0001$).

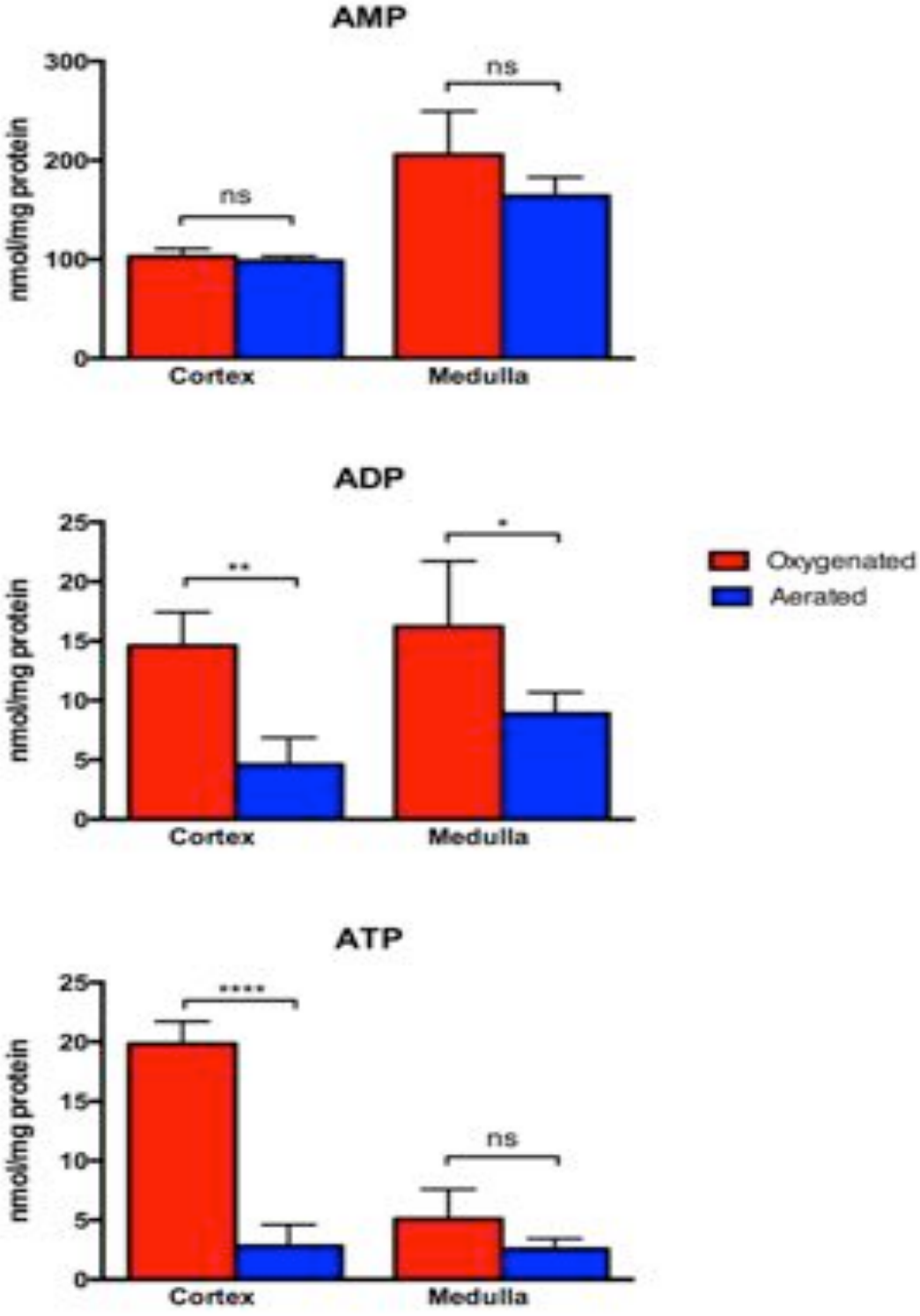

Figure 39 Adenine nucleotide levels for oxygenated and aerated tissue samples.

HPLC analysis of adenine nucleotides in oxygenated and aerated porcine kidneys. Data presented as mean + standard deviation and comparison using Mann-Witney U test.

Combined 2D $^{1}H,^{13}C$-HSQC NMR and GCMS analysis

Using both 2D [^{1}H, ^{13}C]-NMR and GCMS, splitting patterns for the metabolites alanine, glutamate and lactate were consistently seen in the perfusion fluid, extracted kidney cortex and medulla samples for kidneys undergoing both aerated and oxygenated perfusion. As mentioned previously the samples were analysed by both analytical techniques and a combined presentation of the results for these metabolites is demonstrated in the figure below (Figure 40-Figure 42). The two groups (aerated and oxygenated) were compared using a Mann-Witney test (unpaired, non parametric). All analysis was performed using GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, La Jolla California USA), with $p < 0.05$ deemed to be indicative of statistical significance.

Data are presented as mean (standard deviation) for the percentage ^{13}C enrichment for the isotopomers of each metabolite. As only a third (5 mM/L) of the glucose in the perfusion fluid was [U-^{13}C] glucose, the proportion of labelled metabolites demonstrated underestimates the absolute amount of *de novo* metabolism from perfusate glucose (by two-thirds).

Figure 40 displays the different (^{13}C) isotopomers for alanine. This clearly demonstrates (as expected) that the most abundant was [U-^{13}C] alanine although small amounts of [2,3-^{13}C], [2-^{13}C] and [3-^{13}C] alanine were also present. The predominant [U-^{13}C] alanine is derived through glycolysis of [U-^{13}C] glucose in the perfusion fluid and conversion from pyruvate into alanine via the enzyme alanine transaminase. Interestingly, the proportion of [U-^{13}C] alanine was greater in aerated kidneys (perfusate, cortex and medulla) and this was significant for both perfusate

and cortex ($p<0.001$). The biochemical mechanisms responsible for the production of the additional ^{13}C isotopomers (e.g. [2,3-^{13}C] alanine) is not clear but indicate activity of alternate glucose-consuming pathways (e.g. the pentose phosphate pathway).

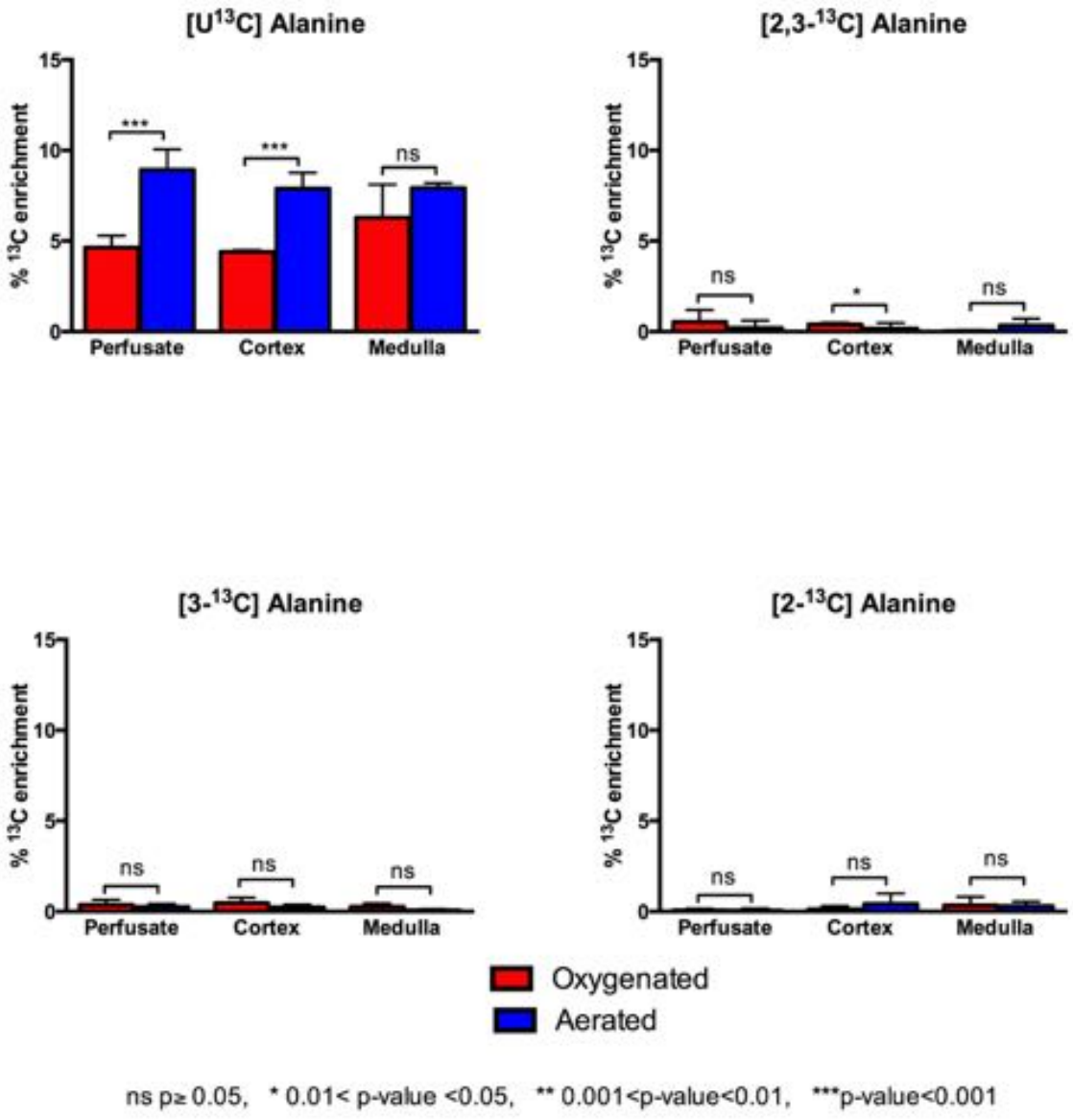

Figure 40 ^{13}C enrichment for different alanine isotopomers

Similar to alanine, a range of (^{13}C) isotopomers was also detected for lactate (Figure 41), with [U-^{13}C] lactate predominating as, similar to [U-^{13}C] alanine, this is

produced from [U-^{13}C] pyruvate following glycolysis of the perfusion fluid [U-^{13}C] glucose. The conversion of pyruvate to lactate via lactate dehydrogenase, occurs in more anaerobic conditions and therefore, as expected, the proportion of [U-^{13}C] lactate was significantly greater in the perfusion fluid, kidney cortex and kidney medulla for aerated samples compared the oxygenated ones (all $p<0.01$) (Figure 41).

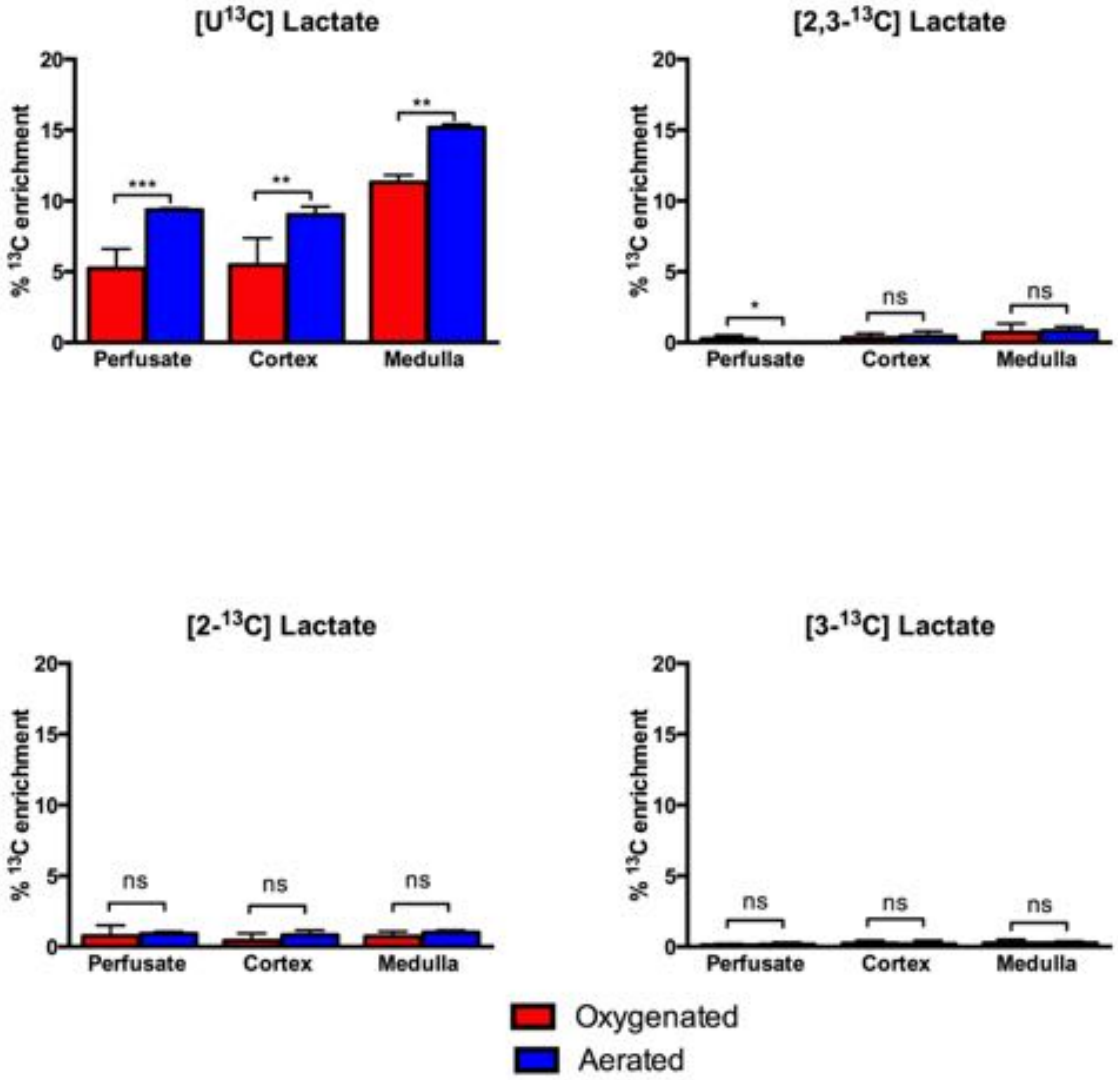

Figure 41 ^{13}C enrichment for different lactate isotopomers

There were also striking differences in the proportions of labelled glutamate between

the oxygenated and aerated kidneys on combined MS/NMR analysis (Figure 42). The ^{13}C tracer studies clearly demonstrate a significantly greater proportion of labelled glutamate (both [4,5-^{13}C] and [4-^{13}C] glutamate) in the oxygenated experiments compared with aerated. The proposed aetiology of the [4,5-^{13}C] glutamate in this study is via glycolysis of the [U-^{13}C] glucose in the perfusion fluid and subsequent formation of 2 x [U-^{13}C] pyruvate molecules which is then converted to acetyl-CoA within the cell mitochondria via pyruvate dehydrogenase. Propagation of the TCA cycle results in the formation of α-ketoglutarate and glutamate formed by the reductive amination of α-ketoglutarate by glutamate dehydrogenase. Indeed the metabolite [4,5-^{13}C] glutamate is seen due to early cycles of TCA activity with more complex labelling patterns formed with subsequent revolutions. The greater proportion of labelled glutamate observed in the oxygenated experiments is biologically anticipated given the requirement of oxygen to potentiate the TCA cycle. However, clearly the amount of oxygen present in the aerated perfusion fluid is sufficient do support some (albeit lesser) oxygen-dependent TCA activity which was greater than that seen when kidneys were perfused without air or oxygen (Chapter 8). The absence of [2,3-^{13}C] and [1,2,3-^{13}C] glutamate is also interesting and suggests that there is no significant conversion of pyruvate into TCA intermediates via pyruvate carboxylase activity. The discrepancy between the proportion of [4,5-^{13}C] glutamate present in the aerated and oxygenated samples was greatest in the medulla samples compared with cortex (mean 3.7% vs 0.45% and 1.5% vs 0.56%, respectively). Indeed the difference in proportion of [4,5-^{13}C] glutamate between oxygenated cortex and medulla samples is highly significant ($p<0.001$) and may reflect the predilection for TCA metabolism within the mitochondria of the porcine

medulla compared with the cortex (214). Interestingly, overall the total (^{13}C labelled and unlabelled) amount of glutamate was significantly lower for oxygenated kidneys, both in the perfusion fluid and tissue extracts on 1D ^{1}H analysis mentioned above (Figure 38). Thus, whilst the highly ATP efficient processes of glutamate production via TCA activity is more active in the oxygenated pig kidney, alternative pathways of glutamate production are greater in an non-oxygenated environment.

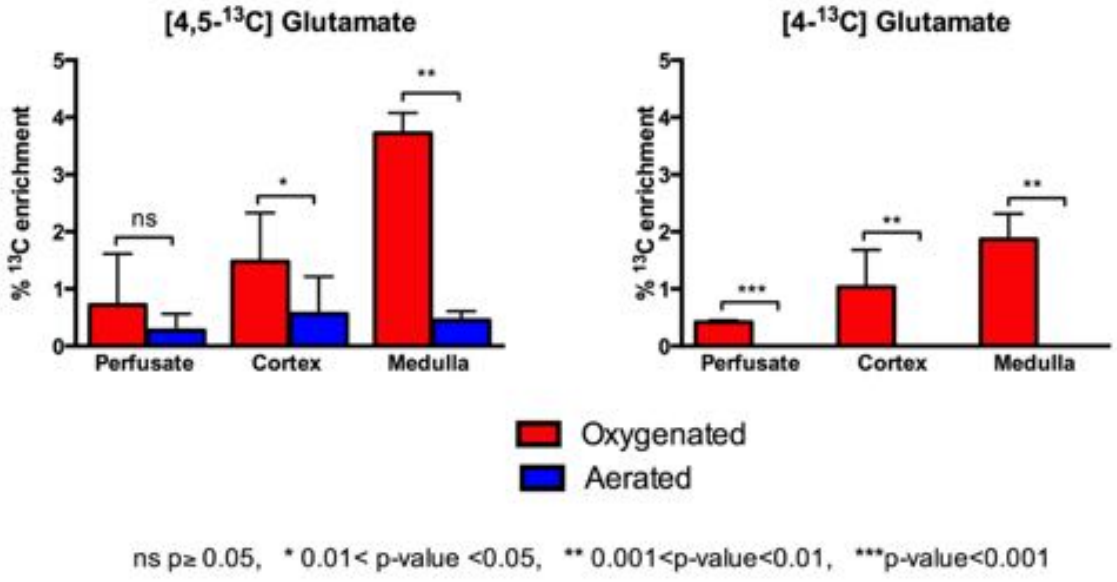

Figure 42 ^{13}C enrichment for different glutamate isotopomers

Discussion

The therapeutic value of active oxygenation during hypothermic machine perfusion of kidneys has yet to be established in the human setting. In an earlier chapter of this thesis (Chapter 7), we demonstrated that the kidney is metabolically active during HMP, and even during the presumably hypoxic conditions of HMP there is some evidence of aerobic metabolic activity (Chapter 8). The aim of this study was to determine whether aeration of perfusion fluid would be sufficient to propagate

maximal aerobic metabolic processes within the HMP environment or whether oxygenation was needed for this purpose. Given that evidence from other disease settings as to the harmful effects of high dose oxygen, it would seem prudent that if oxygen provision is beneficial during HMP, it should be provided at the minimum level necessary in order to achieve the desired results.

A modified perfusion device (LifePort© Kidney Transporter 1.0 Organ Recovery Systems) was used for these experiments, with a paediatric membrane oxygenator incorporated into the circuit. Using a simple oxygen probe to measure oxygen levels compared to a control, we demonstrated through comparison of the oxygen levels in the 'arterial' and 'venous' limb of the LifePort© circuit that the kidney is an oxygen consuming organ during HMP conditions. Furthermore, the amount of oxygen present in the perfusion fluid under standard HMP conditions falls rapidly within the first two hours and that the organ is rendered severely hypoxic after this time period. However, the amount of oxygen present in the fluid can be maintained at constant levels, comparable to those at the start of perfusion through aeration of the perfusion fluid using a paediatric membrane oxygenator. Indeed if low flow oxygen (0.1 L/min) is used instead of air, the oxygen levels in the fluid increase roughly four-fold. Thus this chilled preservation fluid can be rendered hyperoxic by the provision of supplemental oxygen. Whether this active oxygenation is sufficient to derive maximal benefit for the perfused kidney in comparison to oxygen carriers is not explored in this study but would be interesting to determine.

This study demonstrates that the metabolic profiles for aerated and oxygenated kidneys are different. This has been shown using multiple different laboratory techniques including 1D ^{1}H NMR, 2D ^{1}H-^{13}C NMR, GCMS and most strikingly HPLC

analysis of adenine nucleotides.

Glutamate is a useful molecule for determining TCA cycle activity using 2D NMR methods. Indeed when [U-^{13}C] glucose is incorporated into the perfusion fluid and [4,5-^{13}C] labelled glutamate is demonstrated, this indicates initial TCA activity. However with further revolutions of the TCA cycle, alternative labelling patterns occur. In this study, we report a significantly increased proportion of [4,5-^{13}C] labelled glutamate present in the kidney cortex and medulla in the oxygenated samples (compared with aerated), which clearly demonstrates a greater degree of TCA activity. Conversely, the proportion of [U-^{13}C] lactate and alanine was greater for the aerated kidneys. The promotion of aerobic TCA metabolism within the mitochondria of oxygenated kidneys at the expense of lactate forming anaerobic pathways would seem the likely explanation for this.

Interestingly, upon 1D ^{1}H NMR analysis, the metabolic profiles of oxygenated and aerated kidneys appeared very similar. However for a number of metabolites (including glutamate and succinate) the total concentrations (i.e. ^{13}C labelled and non labelled) present in both perfusion fluid and tissue were actually lower for the oxygenated group. The reasons for this are not fully clear but it may be that in the absence of additional oxygen, only minimal TCA cycle and electron transport chain activity occurs with a reliance on glycolytic pathways to generate the necessary ATP required for cell survival. It may be, that in oxygen abundant states, the pathways central to cellular maintenance or ATP generation predominate and that whilst some pathways are upregulated, there is a global metabolic quiescence under such oxygen enriched conditions.

Regardless whether individual metabolic pathways are upregulated or not, this study

does clearly demonstrate that, using the experimental setup we describe, oxygenation of perfusate ultimately results in a significantly greater levels of cellular ATP (over seven-fold) following prolonged periods of perfusion. This corroborates findings from other *ex vivo* animal perfusion experiments (199, 205-208) and supports the hypothesis that a greater degree of aerobic metabolism occurs under these conditions.

Conclusions

The kidney consumes oxygen during hypothermic machine perfusion. Membrane oxygenators can be incorporated into the LifePort© circuit to provide additional oxygen and that the metabolic profiles of oxygenated kidneys differs from those that are aerated with a greater amount of ATP generated.

Chapter 10

Thesis Conclusion and Future work

Future work – experimental

Human cell line model

The experimental animal work within this thesis has used a whole organ (abattoir pig) kidney as the model for transplantation. This model represents a DCD kidney with an associated WIT of around 14 minutes. We have demonstrated that from a metabolic standpoint, the porcine kidney is a valid model for human studies (4), in addition to being of comparable size with similar physiology. Whilst the porcine abattoir model is a paired system (i.e. one kidney from each animal can be perfused under control and experimental conditions), which is an inherent strength, it does have some limitations. The most notable limitation is that logistical factors (i.e. number of LifePort© machines) dictate that only one pair of kidneys can undergo perfusion experiments at any time. Whilst the effects of major differences in perfusion conditions (e.g. oxygen vs. no oxygen) can be determined using this model through repetition of experiments, more subtle differences are not possible to establish (e.g. optimal concentration of glucose in the perfusion fluid). For this type of study, we propose that a cell line would be a more appropriate investigative modality. We are therefore in the process of establishing a cell line model of HMP. We have cultured a human proximal tubular cell line and are attempting to replicate the conditions of HMP through a custom-built motile perfusion device. Furthermore, the feasibility of creating a 3D cell line structure to enhance the similarly with the whole organ model is under investigation. This work will contribute to the Ph.D. thesis of Thomas Smith. The high degree of homogeneity of cell lines should enable reproducible experimental conditions. Clearly, any promising avenues of HMP

optimization will be verified on a whole organ model.

Ex-vivo normothermic perfusion model

During my Ph.D. tenure, we developed a primitive *ex vivo* normothermic autologous blood reperfusion device, based largely on the setup reported by Nicholson et al (18, 215, 216). We aim to develop this in future laboratory experiments in order to provide functional outcome results for future experimental work.

Further labelling studies

In this thesis, we successfully describe how information regarding active central metabolic pathways can be determined using [U-^{13}C] glucose in the perfusion fluid. [U-^{13}C] glucose was the logical initial tracer to introduce into the HMP system and is present in high concentrations in the HMP fluid (10 mM in KPS-1®). Furthermore, as was confirmed by these studies (5), metabolism of glucose through glycolytic pathways was biologically probable. Having confirmed that the kidney is metabolically active despite the *ex vivo* hypothermic conditions, the downstream metabolism of alternative substrates would be fascinating. Additional labelling studies could include ^{15}N-glutamine.

^{13}C human perfusion study

Work contained within this thesis has successfully demonstrated several important concepts regarding ^{13}C studies. Firstly that when [U-^{13}C] glucose is introduced into the perfusion fluid of HMP preserved kidneys that *de novo* metabolic activity occurs (5). Secondly, that NMR studies (principally 2D ^{1}H,^{13}C-HSQC and 1D ^{1}H spectra) can be used to reliably identify these metabolites and calculate both relative proportions

of isotopologues and infer absolute concentrations (5). Finally that when the parameters of perfusion are changed (supplemental oxygen in this case), so does the type and isotopologue distribution of labelled products indicating differences in metabolic activity. Kidneys studied in this thesis are largely porcine abattoir kidneys (DCD model) with smaller numbers of discarded (non-transplantable) human kidneys. We have also demonstrated in the human setting, using ^{1}H NMR of perfusion fluid, that the metabolomic profile of kidneys with immediate graft function differs from those with delayed graft function highlighting that pre-transplant metabolism dictates post-transplant function (3). However, as mechanistic information regarding metabolism is difficult to deduce from such 1D studies reliably, the next logical step for this project would be to perform ^{13}C labelling studies on human kidneys for transplantation. The degree of *de novo* metabolic activity could then be correlated with the functional outcome of that kidney.

Ethical approval

In September 2015, ethical approved was granted to perform a single centre study at the Queen Elizabeth Hospital Birmingham using KPS-1® perfusion fluid, whereby the fluid glucose has been substituted with a [U-^{13}C] glucose version (Appendix p93-97). Ethical approval has been granted to analyse perfusion fluid, urine, and transplant kidney biopsy tissue. The study has been called the ^{13}CHAMPION study (^{13}**C H**uman **A**llograft **M**achine **P**erfus**ION** study). The study motif is depicted below.

Study overview

We aim to perfuse 30 kidneys with the [U-^{13}C] KPS-1® fluid over an 18 month period. The 2D labelling studies will be correlated with the kidney outcome (DGF, GFR, etc.). The study commenced in October 2016 and is due to complete in April 2018. Sample collection and data analysis is being performed largely by my successor, Mr. Kamlesh Patel. This study should provide clarification of active metabolic pathways for transplanted kidneys and is a world first study of its type. A copy of the study protocol, consent form and patient information sheet is in Appendix p98-128

Industry collaboration

This study is reliant on a collaboration between ourselves and an industry collaborator (namely Organ Recovery Systems). As the producers of KPS-1® perfusion fluid, following discussions with ORS, they have kindly agreed to produce (and pay for) 30 litres of clinical grade [U-^{13}C] KPS-1®. This fluid was manufactured in August 2016. A Material Transfer Agreement for this study was signed by representatives from Organ Recovery Systems, myself and Mr. Andrew Ready.

Conclusions

During this three year Ph.D. degree, I have attempted to further understand the mechanisms and role of hypothermic machine perfusion for kidney preservation.

Through a retrospective large (albeit single centre) clinical dataset analysis, I have demonstrated that transplantation from deceased donors is increasingly more complex with an increase in a multitude of high-risk markers. Although this study does not directly demonstrate that HMP is beneficial in this population cohort, evidence that HMP improves the short and longer term function of deceased donor kidneys is well established. As such, the demonstration of increasing deceased donor kidney complexity would suggest that therapies that maximise the potential function of such complex organs such as HMP will be of increasing importance.

The potential role of HMP in living donor kidney transplantation was also explored through a retrospective analysis of a national dataset. We found that no live donor kidney in the UK had undergone HMP. Furthermore, we found that even in kidneys that had a comparatively prolonged cold ischaemia time (4-8 hours), excellent outcomes were observed (without HMP). As such, under normal clinical conditions, we concluded that there is little evidence to support the use of HMP in the live donor setting.

Having explored the potential role of HMP in the deceased and live donor setting, experimental studies were performed to detail the metabolism during HMP further. NMR spectroscopy was largely used for this purpose. When the perfusion fluid of human kidneys was analysed using 1D ^{1}H NMR, we found that the metabolome (or metabolic profile) of kidneys that proceeded to have immediate graft function differed from those that had delayed function. This highlights a fundamental

principle, namely that the metabolic phenotype during HMP denotes the functional outcome for that organ. Whilst this study was limited by a relatively small sample size (n = 26) and therefore robust analyses of kidney subgroups (e.g. DCD vs. DBD) difficult, it does suggests that a metabolic profile (or biomarker panel) could be clinically useful to predict the function of kidneys pre-transplantation.

Given the need for experimental studies to complement the human observational studies, a porcine abattoir kidney model was used extensively throughout this thesis. Although porcine kidneys are known to resemble human organs both anatomically and physiologically, it was important to ensure the validity of this model from a metabolic standpoint during the highly non-physiological conditions of HMP. The metabolic profile of human and porcine kidneys was therefore compared at equivalent time points during HMP using 1D ^{1}H NMR. We demonstrated that the metabolome of human and porcine organs are strikingly similar and even where absolute concentrations of metabolites differed, the rate of change during perfusion was equivalent, suggesting that similar metabolic pathways are active.

Having demonstrated that the metabolome during HMP reflects functional outcome and that porcine organs are a valid model of human metabolism, further studies were performed to gain further insight into HMP metabolism. The metabolic profile of porcine organs stored using either HMP or static cold storage conditions were compared. A paired model was utilised for this purpose and based on the weight of organ, tissue and perfusate concentrations, the net metabolic gain for each metabolite of interest was calculated. We found evidence of significantly greater metabolic activity in the HMP kidney compared with SCS counterparts. These included metabolites related to central metabolism: lactate, glutamate, aspartate,

fumarate and acetate. This study highlights significant metabolic differences between HMP and SCS preserved kidneys. Although this does not provide direct evidence that the benefits of HMP over SCS are solely attributable to metabolic improvements, these results do support the hypothesis that HMP improves the metabolism of preserved kidneys.

Whilst 1D ^{1}H NMR analysis enables the quantification of a vast number of metabolites, it does not provide definitive evidence of active metabolic pathways within a system. Even with repeated measures over successive time points, pathway activity is inferred but not proven. In order to provide unequivocal evidence of *de novo* metabolic activity during HMP, metabolic tracer studies were undertaken. Porcine kidneys were perfused under clinical conditions with a perfusate containing isotopic glucose [U-^{13}C] glucose. Metabolites were analysed using a combined 2D $^{1}H,^{13}C$-HSQC and 1D ^{1}H NMR approach. In this unpaired proof of concept study, we demonstrated that a significant amount of *de novo* metabolic activity occurs during HMP, with a progressive increase in the amount of labelled lactate and alanine present at sequential time points.

Having demonstrated that HMP induces metabolism and that ^{13}C studies can be used to demonstrate *de novo* activity, we sought to determine the metabolic effects of active oxygenation during HMP. The LifePort© perfusion circuit was altered to incorporate a paediatric membrane oxygenator, with an aerated group serving as the control. [U^{13}C] glucose was added to KPS-1® fluid for isotopic labelling. We found several findings from this study involving both porcine and human organs (deemed unfit for transplantation): firstly that, the amount of oxygen present in the perfusate of oxygenated kidneys was several-fold higher than in the aerated group,

highlighting the increase in oxygen carriage of KPS-1® following active oxygenation, even in the absence of any oxygen carrier; secondly that the metabolism of oxygenated kidneys differed from the paired aerated counterparts; and thirdly that oxygenation significantly improves the amount of ATP in kidney tissue (both cortex and medulla). Whilst this study does not seek to determine the clinical effects of oxygenation during HMP, it does serve to highlight mechanistic differences between the two groups.

Figure 43 First litre of ^{13}C KPS-1® perfusion fluid

References

1. Nath J, Field M, Ebbs SR, Smith T, McGrogan D, Al-Shakarchi J, et al. Evolution of Renal Transplant Practice Over the Past Decade: A U.K. Center Experience. Transplant Proc. 2015;47(6):1700-4.
2. Nath J, Hodson J, Canbilen SW, Al Shakarchi J, Inston NG, Sharif A, et al. Effect of cold ischaemia time on outcome after living donor renal transplantation. Br J Surg. 2016;103(9):1230-6.
3. Guy AJ, Nath J, Cobbold M, Ludwig C, Tennant DA, Inston NG, et al. Metabolomic analysis of perfusate during hypothermic machine perfusion of human cadaveric kidneys. Transplantation. 2015;99(4):754-9.
4. Nath J, Guy A, Smith TB, Cobbold M, Inston NG, Hodson J, et al. Metabolomic perfusate analysis during kidney machine perfusion: the pig provides an appropriate model for human studies. PLoS One. 2014;9(12):e114818.
5. Nath J, Smith T, Hollis A, Ebbs S, Canbilen SW, Tennant DA, et al. (13)C glucose labelling studies using 2D NMR are a useful tool for determining ex vivo whole organ metabolism during hypothermic machine perfusion of kidneys. Transplant Res. 2016;5:7.
6. Nath J, Smith TB, Patel K, Ebbs SR, Hollis A, Tennant DA, et al. Metabolic differences between cold stored and machine perfused porcine kidneys: A 1H NMR based study. Cryobiology. 2017;74:115-20.
7. Heude C, Nath J, Carrigan JB, Ludwig C. Nuclear Magnetic Resonance Strategies for Metabolic Analysis. Adv Exp Med Biol. 2017;965:45-76.
8. NHS Blood and Transplant. Organ Donation and Transplantation Activity Report 2014/15 2015 [cited 2015 Nov 15th]. Available from: http://nhsbtmediaservices.blob.core.windows.net/organ-donation-assets/pdfs/activity_report_2014_15.pdf.
9. Wolfe RA, Ashby VB, Milford EL, Ojo AO, Ettenger RE, Agodoa LY, et al. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. N Engl J Med. 1999;341(23):1725-30.
10. Gilg J, Rao A, Fogarty D. UK Renal Registry 16th annual report: chapter 1 UK renal replacement therapy incidence in 2012: national and centre-specific analyses. Nephron Clin Pract. 2013;125(1-4):1-27.
11. Oniscu GC, Brown H, Forsythe JL. How great is the survival advantage of transplantation over dialysis in elderly patients? Nephrol Dial Transplant. 2004;19(4):945-51.
12. Transplantation Cost Effectiveness 2013 [1st June 2016]. Available from: http://www.kidney.org.uk/archives/news-archive-2/campaigns-transplantation-trans-cost-effect/.
13. Kyllonen LE, Salmela KT, Eklund BH, Halme LE, Hockerstedt KA, Isoniemi HM, et al. Long-term results of 1047 cadaveric kidney transplantations with special emphasis on initial graft function and rejection. Transpl Int. 2000;13(2):122-8.
14. Ojo AO, Wolfe RA, Held PJ, Port FK, Schmouder RL. Delayed graft function: risk factors and implications for renal allograft survival. Transplantation. 1997;63(7):968-74.

15. Quiroga I, McShane P, Koo DD, Gray D, Friend PJ, Fuggle S, et al. Major effects of delayed graft function and cold ischaemia time on renal allograft survival. Nephrol Dial Transplant. 2006;21(6):1689-96.
16. Hosgood SA, Nicholson ML. First in man renal transplantation after ex vivo normothermic perfusion. Transplantation. 2011;92(7):735-8.
17. Hosgood SA, van Heurn E, Nicholson ML. Normothermic machine perfusion of the kidney: better conditioning and repair? Transpl Int. 2014.
18. Nicholson ML, Hosgood SA. Renal transplantation after ex vivo normothermic perfusion: the first clinical study. Am J Transplant. 2013;13(5):1246-52.
19. Opelz G, Dohler B. Multicenter analysis of kidney preservation. Transplantation. 2007;83(3):247-53.
20. Southard JH, Belzer FO. Organ preservation. Annu Rev Med. 1995;46:235-47.
21. Collins GM, Bravo-Shugarman M, Terasaki PI. Kidney preservation for transportation. Initial perfusion and 30 hours' ice storage. Lancet. 1969;2(7632):1219-22.
22. Opelz G, Terasaki PI. Advantage of cold storage over machine perfusion for preservation of cadaver kidneys. Transplantation. 1982;33(1):64-8.
23. van der Vliet JA, Vroemen JP, Cohen B, Lansbergen Q, Kootstra G. Preservation of cadaveric kidneys. Cold storage or machine perfusion? Arch Surg. 1983;118(10):1166-8.
24. Clark EA, Terasaki PI, Opelz G, Mickey MR. Cadaver-kidney transplant failures at one month. N Engl J Med. 1974;291(21):1099-102.
25. NICE. Machine perfusion systems and cold static storage of kidneys from deceased donors (ta165). In: Excellence NIfHaC, editor. London2009.
26. Morariu AM, Vd Plaats A, W VO, NA TH, Leuvenink HG, Graaff R, et al. Hyperaggregating effect of hydroxyethyl starch components and University of Wisconsin solution on human red blood cells: a risk of impaired graft perfusion in organ procurement? Transplantation. 2003;76(1):37-43.
27. O'Callaghan JM, Knight SR, Morgan RD, Morris PJ. Preservation solutions for static cold storage of kidney allografts: a systematic review and meta-analysis. Am J Transplant. 2012;12(4):896-906.
28. Moers C, Smits JM, Maathuis MH, Treckmann J, van Gelder F, Napieralski BP, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 2009;360(1):7-19.
29. Moers C, Pirenne J, Paul A, Ploeg RJ, Machine Preservation Trial Study G. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 2012;366(8):770-1.
30. Carrel A, Lindbergh CA. The Culture of Whole Organs. Science. 1935;81(2112):621-3.
31. Lindbergh CA. An Apparatus for the Culture of Whole Organs. J Exp Med. 1935;62(3):409-31.
32. Humphries AL, Jr., Russell R, Christopher PE, Goodrich SM, Stoddard LD, Moretz WH. Successful Reimplantation of Twenty-Four-Hour Stored Kidney to Nephrectomized Dog. Ann N Y Acad Sci. 1964;120:496-505.
33. Humphries AL, Jr., Russell R, Gregory J, Carter RH, Moretz WH. Hypothermic Perfusion of the Canine Kidney for 48 Hours Followed by Reimplantation. Am Surg. 1964;30:748-52.

34. Humphries AL, Jr., Russell R, Stoddard LD, Moretz WH. Successful five-day kidney preservation. Perfusion with hypothermic, diluted plasma. Invest Urol. 1968;5(6):609-18.
35. Belzer FO, Ashby BS, Dunphy JE. 24-hour and 72-hour preservation of canine kidneys. Lancet. 1967;2(7515):536-8.
36. Belzer FO, Southard JH. Organ preservation and transplantation. Prog Clin Biol Res. 1986;224:291-303.
37. Claes G, Blohme I, Engeset J. Simplifications in the use of continuous perfusion for renal preservation. Transplant Proc. 1974;6(3):261-5.
38. Stephenson TP, O'Donoghue EP, Hendry WF, Wickham JE. Preservation of human kidneys for transplantation: preliminary results with a Gambro perfusion machine. Br Med J. 1973;1(5850):379-81.
39. Hoffmann RM, Southard JH, Lutz M, Mackety A, Belzer FO. Synthetic perfusate for kidney preservation. Its use in 72-hour preservation of dog kidneys. Arch Surg. 1983;118(8):919-21.
40. McAnulty JF, Ploeg RJ, Southard JH, Belzer FO. Successful five-day perfusion preservation of the canine kidney. Transplantation. 1989;47(1):37-41.
41. McAnulty JF, Vreugdenhil PK, Lindell S, Southard JH, Belzer FO. Successful 7-day perfusion preservation of the canine kidney. Transplant Proc. 1993;25(1 Pt 2):1642-4.
42. Merion RM, Oh HK, Port FK, Toledo-Pereyra LH, Turcotte JG. A prospective controlled trial of cold-storage versus machine-perfusion preservation in cadaveric renal transplantation. Transplantation. 1990;50(2):230-3.
43. Halloran P, Aprile M. A randomized prospective trial of cold storage versus pulsatile perfusion for cadaver kidney preservation. Transplantation. 1987;43(6):827-32.
44. Mozes MF, Finch WT, Reckard CR, Merkel FK, Cohen C. Comparison of Cold-Storage and Machine Perfusion in the Preservation of Cadaver Kidneys - a Prospective, Randomized Study. Transplant Proc. 1985;17(1):1474-7.
45. Heil JE, Canafax DM, Sutherland DE, Simmons RL, Dunning M, Najarian JS. A controlled comparison of kidney preservation by two methods: machine perfusion and cold storage. Transplant Proc. 1987;19(1 Pt 3):2046.
46. van der Vliet JA, Kievit JK, Hene RJ, Hilbrands LB, Kootstra G. Preservation of non-heart-beating donor kidneys: a clinical prospective randomised case-control study of machine perfusion versus cold storage. Transplant Proc. 2001;33(1-2):847.
47. Danielewicz R, Kwiatkowski A, Polak W, Kosieradzki M, Michalak G, Wegrowicz I, et al. An assessment of ischemic injury of the kidney for transplantation during machine pulsatile preservation. Transplant Proc. 1997;29(8):3580-1.
48. O'Callaghan JM, Morgan RD, Knight SR, Morris PJ. Systematic review and meta-analysis of hypothermic machine perfusion versus static cold storage of kidney allografts on transplant outcomes. Br J Surg. 2013;100(8):991-1001.
49. Watson CJ, Wells AC, Roberts RJ, Akoh JA, Friend PJ, Akyol M, et al. Cold machine perfusion versus static cold storage of kidneys donated after cardiac death: a UK multicenter randomized controlled trial. Am J Transplant. 2010;10(9):1991-9.
50. Taylor MJ, Baicu SC. Current state of hypothermic machine perfusion preservation of organs: The clinical perspective. Cryobiology. 2010;60(3 Suppl):S20-35.

51. Maathuis MH, Manekeller S, van der Plaats A, Leuvenink HG, t Hart NA, Lier AB, et al. Improved kidney graft function after preservation using a novel hypothermic machine perfusion device. Ann Surg. 2007;246(6):982-8; discussion 9-91.
52. Wszola M, Kwiatkowski A, Diuwe P, Domagala P, Gorski L, Kieszek R, et al. One-year results of a prospective, randomized trial comparing two machine perfusion devices used for kidney preservation. Transpl Int. 2013;26(11):1088-96.
53. Dittrich S, Groneberg DA, von Loeper J, Lippek F, Hegemann O, Grosse-Siestrup C, et al. Influence of cold storage on renal ischemia reperfusion injury after non-heart-beating donor explantation. Nephron Exp Nephrol. 2004;96(3):e97-102.
54. Guy AJ. Machine perfusion in kidney transplantation: clinical application & metabolomic analysis. College of Medical & Dental Sciences: University of Birmingham; 2015.
55. Boutilier RG. Mechanisms of cell survival in hypoxia and hypothermia. J Exp Biol. 2001;204(Pt 18):3171-81.
56. Fuller BJ, Lee CY. Hypothermic perfusion preservation: the future of organ preservation revisited? Cryobiology. 2007;54(2):129-45.
57. Freshney RI. Culture of animal cells : a manual of basic technique. 4th ed. New York: Wiley; 2000. xxvi, 577 p. p.
58. Brasile L, Stubenitsky BM, Booster MH, Haisch C, Kootstra G. NOS: the underlying mechanism preserving vascular integrity and during ex vivo warm kidney perfusion. Am J Transplant. 2003;3(6):674-9.
59. Brasile L, Stubenitsky BM, Haisch CE, Kon M, Kootstra G. Repair of damaged organs in vitro. Am J Transplant. 2005;5(2):300-6.
60. Baicu SC, Taylor MJ, Brockbank KG. Modulating biochemical perturbations during 72-hour machine perfusion of kidneys: role of preservation solution. Cryobiology. 2007;54(1):114-20.
61. van Breussegem A, van Pelt J, Wylin T, Heedfeld V, Zeegers M, Monbaliu D, et al. Presumed and actual concentrations of reduced glutathione in preservation solutions. Transplant Proc. 2011;43(9):3451-4.
62. Evans PJ, Tredger JM, Dunne JB, Halliwell B. Catalytic metal ions and the loss of reduced glutathione from University of Wisconsin preservation solution. Transplantation. 1996;62(8):1046-9.
63. Izamis ML, Tolboom H, Uygun B, Berthiaume F, Yarmush ML, Uygun K. Resuscitation of ischemic donor livers with normothermic machine perfusion: a metabolic flux analysis of treatment in rats. PLoS One. 2013;8(7):e69758.
64. Bruinsma BG, Yeh H, Ozer S, Martins PN, Farmer A, Wu W, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. Am J Transplant. 2014;14(6):1400-9.
65. Ferrigno A, Rizzo V, Boncompagni E, Bianchi A, Gringeri E, Neri D, et al. Machine perfusion at 20 degrees C reduces preservation damage to livers from non-heart beating donors. Cryobiology. 2011;62(2):152-8.
66. Vekemans K, Liu Q, Brassil J, Komuta M, Pirenne J, Monbaliu D. Influence of flow and addition of oxygen during porcine liver hypothermic machine perfusion. Transplant Proc. 2007;39(8):2647-51.
67. de Rougemont O, Breitenstein S, Leskosek B, Weber A, Graf R, Clavien PA, et al. One hour hypothermic oxygenated perfusion (HOPE) protects nonviable liver allografts donated after cardiac death. Ann Surg. 2009;250(5):674-83.

68. Vairetti M, Ferrigno A, Carlucci F, Tabucchi A, Rizzo V, Boncompagni E, et al. Subnormothermic machine perfusion protects steatotic livers against preservation injury: a potential for donor pool increase? Liver Transpl. 2009;15(1):20-9.
69. Baicu SC, Simmons PM, Campbell LH, Taylor MJ, Brockbank KG. Interstitial fluid analysis for assessment of organ function. Clin Transplant. 2004;18 Suppl 12:16-21.
70. Audard V, Matignon M, Dahan K, Lang P, Grimbert P. Renal transplantation from extended criteria cadaveric donors: problems and perspectives overview. Transpl Int. 2008;21(1):11-7.
71. Metzger RA, Delmonico FL, Feng S, Port FK, Wynn JJ, Merion RM. Expanded criteria donors for kidney transplantation. Am J Transplant. 2003;3 Suppl 4:114-25.
72. Ojo AO, Hanson JA, Meier-Kriesche H, Okechukwu CN, Wolfe RA, Leichtman AB, et al. Survival in recipients of marginal cadaveric donor kidneys compared with other recipients and wait-listed transplant candidates. J Am Soc Nephrol. 2001;12(3):589-97.
73. Port FK, Bragg-Gresham JL, Metzger RA, Dykstra DM, Gillespie BW, Young EW, et al. Donor characteristics associated with reduced graft survival: an approach to expanding the pool of kidney donors. Transplantation. 2002;74(9):1281-6.
74. Cockfield SM, Moore RB, Todd G, Solez K, Gourishankar S. The prognostic utility of deceased donor implantation biopsy in determining function and graft survival after kidney transplantation. Transplantation. 2010;89(5):559-66.
75. Jochmans I, Pirenne J. Graft quality assessment in kidney transplantation: not an exact science yet! Curr Opin Organ Transplant. 2011;16(2):174-9.
76. Louvar DW, Li N, Snyder J, Peng Y, Kasiske BL, Israni AK. "Nature versus nurture" study of deceased-donor pairs in kidney transplantation. J Am Soc Nephrol. 2009;20(6):1351-8.
77. Bohra R, Klepacki J, Klawitter J, Klawitter J, Thurman JM, Christians U. Proteomics and metabolomics in renal transplantation-quo vadis? Transpl Int. 2013;26(3):225-41.
78. Jochmans I, Moers C, Smits JM, Leuvenink HG, Treckmann J, Paul A, et al. The prognostic value of renal resistance during hypothermic machine perfusion of deceased donor kidneys. Am J Transplant. 2011;11(10):2214-20.
79. Sonnenday CJ, Cooper M, Kraus E, Gage F, Handley C, Montgomery RA. The hazards of basing acceptance of cadaveric renal allografts on pulsatile perfusion parameters alone. Transplantation. 2003;75(12):2029-33.
80. Mozes MF, Skolek RB, Korf BC. Use of perfusion parameters in predicting outcomes of machine-preserved kidneys. Transplant Proc. 2005;37(1):350-1.
81. Guarrera JV, Goldstein MJ, Samstein B, Henry S, Reverte C, Arrington B, et al. 'When good kidneys pump badly': outcomes of deceased donor renal allografts with poor pulsatile perfusion characteristics. Transpl Int. 2010;23(4):444-6.
82. Bhangoo RS, Hall IE, Reese PP, Parikh CR. Deceased-donor kidney perfusate and urine biomarkers for kidney allograft outcomes: a systematic review. Nephrol Dial Transplant. 2012;27(8):3305-14.
83. Hosgood SA, Nicholson HF, Nicholson ML. Oxygenated kidney preservation techniques. Transplantation. 2012;93(5):455-9.
84. t Hart NA, van der Plaats A, Faber A, Leuvenink HG, Olinga P, Wiersema-Buist J, et al. Oxygenation during hypothermic rat liver preservation: an in vitro slice study

to demonstrate beneficial or toxic oxygenation effects. Liver Transpl. 2005;11(11):1403-11.
85. Lim MW. The history of extracorporeal oxygenators. Anaesthesia. 2006;61(10):984-95.
86. Cabrales P, Carlos Briceno J. Delaying blood transfusion in experimental acute anemia with a perfluorocarbon emulsion. Anesthesiology. 2011;114(4):901-11.
87. Brasile L, DelVecchio P, Amyot K, Haisch C, Clarke J. Organ preservation without extreme hypothermia using an Oxygen supplemented perfusate. Artif Cells Blood Substit Immobil Biotechnol. 1994;22(4):1463-8.
88. Stubenitsky BM, Booster MH, Brasile L, Araneda D, Haisch CE, Kootstra G. Exsanguinous metabolic support perfusion--a new strategy to improve graft function after kidney transplantation. Transplantation. 2000;70(8):1254-8.
89. Thuillier R, Dutheil D, Trieu MT, Mallet V, Allain G, Rousselot M, et al. Supplementation with a new therapeutic oxygen carrier reduces chronic fibrosis and organ dysfunction in kidney static preservation. Am J Transplant. 2011;11(9):1845-60.
90. Jassem W, Roake J. The molecular and cellular basis of reperfusion injury following organ transplantation. Transplant Rev. 1998;12(1):14-33.
91. Land W, Schneeberger H, Schleibner S, Illner WD, Abendroth D, Rutili G, et al. The beneficial effect of human recombinant superoxide dismutase on acute and chronic rejection events in recipients of cadaveric renal transplants. Transplantation. 1994;57(2):211-7.
92. Pollak R, Andrisevic JH, Maddux MS, Gruber SA, Paller MS. A randomized double-blind trial of the use of human recombinant superoxide dismutase in renal transplantation. Transplantation. 1993;55(1):57-60.
93. Southard JH, van Gulik TM, Ametani MS, Vreugdenhil PK, Lindell SL, Pienaar BL, et al. Important components of the UW solution. Transplantation. 1990;49(2):251-7.
94. Land WG. The role of postischemic reperfusion injury and other nonantigen-dependent inflammatory pathways in transplantation. Transplantation. 2005;79(5):505-14.
95. Land W. The potential impact of the reperfusion injury on acute and chronic rejection events following organ transplantation. Transplant Proc. 1994;26(6):3169-71.
96. Friedl HP, Smith DJ, Till GO, Thomson PD, Louis DS, Ward PA. Ischemia-reperfusion in humans. Appearance of xanthine oxidase activity. Am J Pathol. 1990;136(3):491-5.
97. Akhtar MZ, Sutherland AI, Huang H, Ploeg RJ, Pugh CW. The role of hypoxia-inducible factors in organ donation and transplantation: the current perspective and future opportunities. Am J Transplant. 2014;14(7):1481-7.
98. Conde E, Alegre L, Blanco-Sanchez I, Saenz-Morales D, Aguado-Fraile E, Ponte B, et al. Hypoxia inducible factor 1-alpha (HIF-1 alpha) is induced during reperfusion after renal ischemia and is critical for proximal tubule cell survival. PLoS One. 2012;7(3):e33258.
99. Bernhardt WM, Gottmann U, Doyon F, Buchholz B, Campean V, Schodel J, et al. Donor treatment with a PHD-inhibitor activating HIFs prevents graft injury and prolongs survival in an allogenic kidney transplant model. Proc Natl Acad Sci U S A. 2009;106(50):21276-81.

100. Shen X, Wan C, Ramaswamy G, Mavalli M, Wang Y, Duvall CL, et al. Prolyl hydroxylase inhibitors increase neoangiogenesis and callus formation following femur fracture in mice. J Orthop Res. 2009;27(10):1298-305.
101. Schroppel B, Legendre C. Delayed kidney graft function: from mechanism to translation. Kidney Int. 2014;86(2):251-8.
102. Alfarouk KO, Verduzco D, Rauch C, Muddathir AK, Adil HH, Elhassan GO, et al. Glycolysis, tumor metabolism, cancer growth and dissemination. A new pH-based etiopathogenic perspective and therapeutic approach to an old cancer question. Oncoscience. 2014;1(12):777-802.
103. Morten KJ, Badder L, Knowles HJ. Differential regulation of HIF-mediated pathways increases mitochondrial metabolism and ATP production in hypoxic osteoclasts. J Pathol. 2013;229(5):755-64.
104. Ebert BL, Firth JD, Ratcliffe PJ. Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences. Journal of Biological Chemistry. 1995;270(49):29083-9.
105. Wishart DS. Metabolomics: the principles and potential applications to transplantation. Am J Transplant. 2005;5(12):2814-20.
106. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, et al. HMDB 3.0--The Human Metabolome Database in 2013. Nucleic Acids Res. 2013;41(Database issue):D801-7.
107. Goldsmith P, Fenton H, Morris-Stiff G, Ahmad N, Fisher J, Prasad KR. Metabonomics: a useful tool for the future surgeon. J Surg Res. 2010;160(1):122-32.
108. Bory C, Boulieu R, Chantin C, Mathieu M. Diagnosis of alcaptonuria: rapid analysis of homogentisic acid by HPLC. Clin Chim Acta. 1990;189(1):7-11.
109. Bales JR, Bell JD, Nicholson JK, Sadler PJ. 1H NMR studies of urine during fasting: excretion of ketone bodies and acetylcarnitine. Magn Reson Med. 1986;3(6):849-56.
110. Dona AC, Jimenez B, Schafer H, Humpfer E, Spraul M, Lewis MR, et al. Precision high-throughput proton NMR spectroscopy of human urine, serum, and plasma for large-scale metabolic phenotyping. Anal Chem. 2014;86(19):9887-94.
111. Griffiths WJ, Wang Y. Mass spectrometry: from proteomics to metabolomics and lipidomics. Chem Soc Rev. 2009;38(7):1882-96.
112. Ludwig C, Ward DG, Martin A, Viant MR, Ismail T, Johnson PJ, et al. Fast targeted multidimensional NMR metabolomics of colorectal cancer. Magn Reson Chem. 2009;47 Suppl 1:S68-73.
113. Smolinska A, Blanchet L, Buydens LM, Wijmenga SS. NMR and pattern recognition methods in metabolomics: from data acquisition to biomarker discovery: a review. Anal Chim Acta. 2012;750:82-97.
114. Claridge TDW. High-resolution NMR techniques in organic chemistry. 1st ed. Amsterdam ; New York: Pergamon; 1999. xiv, 382 p. p.
115. Devarajan P. Proteomics for biomarker discovery in acute kidney injury. Semin Nephrol. 2007;27(6):637-51.
116. Snoeijs MG, Pulinx B, van Dieijen-Visser MP, Buurman WA, van Heurn LW, Wodzig WK. Characterization of the perfusate proteome of human donor kidneys. Ann Clin Biochem. 2013;50(Pt 2):140-6.

117. Hollmen ME, Kyllonen LE, Inkinen KA, Lalla ML, Merenmies J, Salmela KT. Deceased donor neutrophil gelatinase-associated lipocalin and delayed graft function after kidney transplantation: a prospective study. Crit Care. 2011;15(3):R121.
118. Parikh CR, Jani A, Mishra J, Ma Q, Kelly C, Barasch J, et al. Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation. Am J Transplant. 2006;6(7):1639-45.
119. Malyszko J, Koc-Zorawska E, Malyszko JS, Mysliwiec M. Kidney injury molecule-1 correlates with kidney function in renal allograft recipients. Transplant Proc. 2010;42(10):3957-9.
120. Nijboer WN, Schuurs TA, Damman J, van Goor H, Vaidya VS, van der Heide JJ, et al. Kidney injury molecule-1 is an early noninvasive indicator for donor brain death-induced injury prior to kidney transplantation. Am J Transplant. 2009;9(8):1752-9.
121. van Timmeren MM, Vaidya VS, van Ree RM, Oterdoom LH, de Vries AP, Gans RO, et al. High urinary excretion of kidney injury molecule-1 is an independent predictor of graft loss in renal transplant recipients. Transplantation. 2007;84(12):1625-30.
122. Backman L, Appelkvist EL, Ringden O, Dallner G. Glutathione transferase in the urine: a marker for post-transplant tubular lesions. Kidney Int. 1988;33(2):571-7.
123. Przybylowski P, Koc-Zorawska E, Malyszko JS, Kozlowska S, Mysliwiec M, Malyszko J. Liver fatty-acid-binding protein in heart and kidney allograft recipients in relation to kidney function. Transplant Proc. 2011;43(8):3064-7.
124. Bone JM, Amara AB, Shenkin A, Hammad A, Sells RA, Alexander JL, et al. Calcineurin inhibitors and proximal renal tubular injury in renal transplant patients with proteinuria and chronic allograft nephropathy. Transplantation. 2005;79(1):119-22.
125. Bon D, Billault C, Thuillier R, Hebrard W, Boildieu N, Celhay O, et al. Analysis of perfusates during hypothermic machine perfusion by NMR spectroscopy: a potential tool for predicting kidney graft outcome. Transplantation. 2014;97(8):810-6.
126. Foxall PJ, Mellotte GJ, Bending MR, Lindon JC, Nicholson JK. NMR spectroscopy as a novel approach to the monitoring of renal transplant function. Kidney Int. 1993;43(1):234-45.
127. Hauet T, Baumert H, Gibelin H, Hameury F, Goujon JM, Carretier M, et al. Noninvasive monitoring of citrate, acetate, lactate, and renal medullary osmolyte excretion in urine as biomarkers of exposure to ischemic reperfusion injury. Cryobiology. 2000;41(4):280-91.
128. Serkova N, Fuller TF, Klawitter J, Freise CE, Niemann CU. H-NMR-based metabolic signatures of mild and severe ischemia/reperfusion injury in rat kidney transplants. Kidney Int. 2005;67(3):1142-51.
129. Le Moyec L, Pruna A, Eugene M, Bedrossian J, Idatte JM, Huneau JF, et al. Proton nuclear magnetic resonance spectroscopy of urine and plasma in renal transplantation follow-up. Nephron. 1993;65(3):433-9.
130. Serkova NJ, Christians U. Biomarkers for toxicodynamic monitoring of immunosuppressants: NMR-based quantitative metabonomics of the blood. Ther Drug Monit. 2005;27(6):733-7.

131. Rush D, Somorjai R, Deslauriers R, Shaw A, Jeffery J, Nickerson P. Subclinical rejection--a potential surrogate marker for chronic rejection--may be diagnosed by protocol biopsy or urine spectroscopy. Ann Transplant. 2000;5(2):44-9.
132. Wang JN, Zhou Y, Zhu TY, Wang X, Guo YL. Prediction of acute cellular renal allograft rejection by urinary metabolomics using MALDI-FTMS. J Proteome Res. 2008;7(8):3597-601.
133. Wang J, Zhou Y, Xu M, Rong R, Guo Y, Zhu T. Urinary metabolomics in monitoring acute tubular injury of renal allografts: a preliminary report. Transplant Proc. 2011;43(10):3738-42.
134. Hene RJ, van der Grond J, Boer WH, Mali WP, Koomans HA. Pre-transplantation assessment of renal viability with 31P magnetic resonance spectroscopy. Kidney Int. 1994;46(6):1694-9.
135. Schmitz V, Klawitter J, Bendrick-Peart J, Schoening W, Puhl G, Haschke M, et al. Metabolic profiles in urine reflect nephrotoxicity of sirolimus and cyclosporine following rat kidney transplantation. Nephron Exp Nephrol. 2009;111(4):e80-91.
136. Klawitter J, Bendrick-Peart J, Rudolph B, Beckey V, Klawitter J, Haschke M, et al. Urine metabolites reflect time-dependent effects of cyclosporine and sirolimus on rat kidney function. Chem Res Toxicol. 2009;22(1):118-28.
137. Klawitter J, Haschke M, Kahle C, Dingmann C, Klawitter J, Leibfritz D, et al. Toxicodynamic effects of ciclosporin are reflected by metabolite profiles in the urine of healthy individuals after a single dose. Br J Clin Pharmacol. 2010;70(2):241-51.
138. Klawitter J, Klawitter J, Kushner E, Jonscher K, Bendrick-Peart J, Leibfritz D, et al. Association of immunosuppressant-induced protein changes in the rat kidney with changes in urine metabolite patterns: a proteo-metabonomic study. J Proteome Res. 2010;9(2):865-75.
139. Waikar SS, Sabbisetti VS, Bonventre JV. Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. Kidney Int. 2010;78(5):486-94.
140. Pollak VE, Mattenheimer H. Enzyme activity in the nephron. Quantitative study by ultramicrochemical techniques. Arch Intern Med. 1962;109:473-84.
141. Mattenheimer H, Pollak VE, Muehrcke RC. Quantitative enzyme patterns in the nephron of the healthy human kidney. Nephron. 1970;7(2):144-54.
142. Salway JG. Metabolism at a glance. 3rd ed. Malden, Mass.: Blackwell Pub.; 2004. 125 p. p.
143. Dorman DE, Roberts JD. Nuclear magnetic resonance spectroscopy: 13C spectra of some common nucleotides. Proc Natl Acad Sci U S A. 1970;65(1):19-26.
144. Fan TW, Lane AN, Higashi RM, Farag MA, Gao H, Bousamra M, et al. Altered regulation of metabolic pathways in human lung cancer discerned by (13)C stable isotope-resolved metabolomics (SIRM). Mol Cancer. 2009;8:41.
145. Peltz M, He TT, Adams GAt, Chao RY, Meyer DM, Jessen ME. Characterizing lung metabolism with carbon-13 magnetic resonance spectroscopy in a small-animal model: evidence of gluconeogenesis during hypothermic storage. Transplantation. 2005;80(3):417-20.
146. Hochachka PW. Defense strategies against hypoxia and hypothermia. Science. 1986;231(4735):234-41.
147. Baverel G, Conjard A, Chauvin MF, Vercoutere B, Vittorelli A, Dubourg L, et al. Carbon 13 NMR spectroscopy: a powerful tool for studying renal metabolism. Biochimie. 2003;85(9):863-71.

148. Rolfe DF, Brown GC. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol Rev. 1997;77(3):731-58.
149. Donohoe PH, West TG, Boutilier RG. Respiratory, metabolic, and acid-base correlates of aerobic metabolic rate reduction in overwintering frogs. Am J Physiol. 1998;274(3 Pt 2):R704-10.
150. Storey KB, Storey JM. Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. Q Rev Biol. 1990;65(2):145-74.
151. Land SC, Hochachka PW. Protein turnover during metabolic arrest in turtle hepatocytes: role and energy dependence of proteolysis. Am J Physiol. 1994;266(4 Pt 1):C1028-36.
152. Land SC, Hochachka PW. A heme-protein-based oxygen-sensing mechanism controls the expression and suppression of multiple proteins in anoxia-tolerant turtle hepatocytes. Proc Natl Acad Sci U S A. 1995;92(16):7505-9.
153. Lutz PL, Nilsson GE. Contrasting strategies for anoxic brain survival--glycolysis up or down. J Exp Biol. 1997;200(Pt 2):411-9.
154. Bickler PE, Buck LT. Adaptations of vertebrate neurons to hypoxia and anoxia: maintaining critical Ca2+ concentrations. J Exp Biol. 1998;201(Pt 8):1141-52.
155. Donohoe PH, West TG, Boutilier RG. Factors affecting membrane permeability and ionic homeostasis in the cold-submerged frog. J Exp Biol. 2000;203(Pt 2):405-14.
156. Di Lisa F, Menabo R, Canton M, Petronilli V. The role of mitochondria in the salvage and the injury of the ischemic myocardium. Biochim Biophys Acta. 1998;1366(1-2):69-78.
157. Gnaiger E, Mendez G, Hand SC. High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia. Proc Natl Acad Sci U S A. 2000;97(20):11080-5.
158. Schumacker PT, Chandel N, Agusti AG. Oxygen conformance of cellular respiration in hepatocytes. Am J Physiol. 1993;265(4 Pt 1):L395-402.
159. Budinger GR, Chandel N, Shao ZH, Li CQ, Melmed A, Becker LB, et al. Cellular energy utilization and supply during hypoxia in embryonic cardiac myocytes. Am J Physiol. 1996;270(1 Pt 1):L44-53.
160. Heusch G. Hibernating myocardium. Physiol Rev. 1998;78(4):1055-85.
161. Bunn HF, Poyton RO. Oxygen sensing and molecular adaptation to hypoxia. Physiol Rev. 1996;76(3):839-85.
162. Haddad GG, Jiang C. O2-sensing mechanisms in excitable cells: role of plasma membrane K+ channels. Annual Review of Physiology. 1997;59:23-42.
163. Budinger GR, Duranteau J, Chandel NS, Schumacker PT. Hibernation during hypoxia in cardiomyocytes. Role of mitochondria as the O2 sensor. Journal of Biological Chemistry. 1998;273(6):3320-6.
164. Stefanovich P, Ezzell RM, Sheehan SJ, Tompkins RG, Yarmush ML, Toner M. Effects of hypothermia on the function, membrane integrity, and cytoskeletal structure of hepatocytes. Cryobiology. 1995;32(4):389-403.
165. Kruuv J, Glofcheski D, Cheng KH, Campbell SD, Al-Qysi HM, Nolan WT, et al. Factors influencing survival and growth of mammalian cells exposed to hypothermia. I. Effects of temperature and membrane lipid perturbers. J Cell Physiol. 1983;115(2):179-85.

166. Singer D, Bretschneider HJ. Metabolic reduction in hypothermia: pathophysiological problems and natural examples--Part 1. Thorac Cardiovasc Surg. 1990;38(4):205-11.
167. Plesnila N, Muller E, Guretzki S, Ringel F, Staub F, Baethmann A. Effect of hypothermia on the volume of rat glial cells. J Physiol. 2000;523 Pt 1:155-62.
168. Wang SQ, Huang YH, Liu KS, Zhou ZQ. Dependence of myocardial hypothermia tolerance on sources of activator calcium. Cryobiology. 1997;35(3):193-200.
169. Wang SQ, Zhou ZQ. Alpha-stat calibration of indo-1 fluorescence and measurement of intracellular free calcium in rat ventricular cells at different temperatures. Life Sci. 1999;65(9):871-7.
170. Hansen AJ. Effect of anoxia on ion distribution in the brain. Physiol Rev. 1985;65(1):101-48.
171. Giraud S, Favreau F, Chatauret N, Thuillier R, Maiga S, Hauet T. Contribution of large pig for renal ischemia-reperfusion and transplantation studies: the preclinical model. J Biomed Biotechnol. 2011;2011:532127.
172. Hannon JP, Bossone CA, Wade CE. Normal Physiological Values for Conscious Pigs Used in Biomedical-Research. Laboratory Animal Science. 1990;40(3):293-8.
173. Minor T, Paul A, Efferz P, Wohlschlaeger J, Rauen U, Gallinat A. Kidney transplantation after oxygenated machine perfusion preservation with Custodiol-N solution. Transpl Int. 2015;28(9):1102-8.
174. Gallinat A, Paul A, Efferz P, Luer B, Kaiser G, Wohlschlaeger J, et al. Hypothermic reconditioning of porcine kidney grafts by short-term preimplantation machine perfusion. Transplantation. 2012;93(8):787-93.
175. Jochmans I, Lerut E, Heedfeld V, Wylin T, Pirenne J, Monbaliu D. Reproducible model for kidney autotransplantation in pigs. Transplant Proc. 2009;41(8):3417-21.
176. Nicholson ML, Hosgood SA, Metcalfe MS, Waller JR, Brook NR. A comparison of renal preservation by cold storage and machine perfusion using a porcine autotransplant model. Transplantation. 2004;78(3):333-7.
177. La Manna G, Conte D, Cappuccilli ML, Nardo B, D'Addio F, Puviani L, et al. An in vivo autotransplant model of renal preservation: cold storage versus machine perfusion in the prevention of ischemia/reperfusion injury. Artif Organs. 2009;33(7):565-70.
178. Hwang TL, Shaka AJ. Water Suppression That Works. Excitation Sculpting Using Arbitrary Wave-Forms and Pulsed-Field Gradients. J Magn Reson. 1995;112,(2):275-9.
179. Ludwig C, Gunther UL. MetaboLab--advanced NMR data processing and analysis for metabolomics. BMC Bioinformatics. 2011;12:366.
180. Savorani F, Tomasi G, Engelsen SB. icoshift: A versatile tool for the rapid alignment of 1D NMR spectra. J Magn Reson. 2010;202(2):190-202.
181. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. Nmrpipe - a Multidimensional Spectral Processing System Based on Unix Pipes. Journal of Biomolecular Nmr. 1995;6(3):277-93.
182. Kazimierczuk K, Orekhov VY. Accelerated NMR Spectroscopy by Using Compressed Sensing. Angewandte Chemie-International Edition. 2011;50(24):5556-9.

183. Orekhov VY, Jaravine VA. Analysis of non-uniformly sampled spectra with multi-dimensional decomposition. Progress in Nuclear Magnetic Resonance Spectroscopy. 2011;59(3):271-92.
184. Smith SA, Levante TO, Meier BH, Ernst RR. Computer-Simulations in Magnetic-Resonance - an Object-Oriented Programming Approach. Journal of Magnetic Resonance Series A. 1994;106(1):75-105.
185. Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, et al. HMDB: the human metabolome database. Nucleic Acids Res. 2007;35:D521-D6.
186. Jofre R, Lopez-Gomez JM, Moreno F, Sanz-Guajardo D, Valderrabano F. Changes in quality of life after renal transplantation. Am J Kidney Dis. 1998;32(1):93-100.
187. Fiebiger W, Mitterbauer C, Oberbauer R. Health-related quality of life outcomes after kidney transplantation. Health Qual Life Outcomes. 2004;2:2.
188. NHS Blood and Transplant. Organ Donation and Transplantation Activity Report 2006/7 2007 [cited 2016 1st June]. Available from: https://nhsbtmediaservices.blob.core.windows.net/organ-donation-assets/pdfs/transplant_activity_uk_2006-2007.pdf.
189. Collaborative Transplant Study (CTS) [1st June 2016]. Available from: http://www.ctstransplant.org.
190. Kirk AD. Crossing the bridge: large animal models in translational transplantation research. Immunol Rev. 2003;196:176-96.
191. Sachs DH. The pig as a potential xenograft donor. Vet Immunol Immunopathol. 1994;43(1-3):185-91.
192. Hannon JP, Bossone CA, Wade CE. Normal physiological values for conscious pigs used in biomedical research. Laboratory Animal Science. 1990;40(3):293-8.
193. Swindle MM. The development of swine models in drug discovery and development. Future Med Chem. 2012;4(14):1771-2.
194. Merrifield CA, Lewis M, Claus SP, Beckonert OP, Dumas ME, Duncker S, et al. A metabolic system-wide characterisation of the pig: a model for human physiology. Mol Biosyst. 2011;7(9):2577-88.
195. Nielsen KL, Hartvigsen ML, Hedemann MS, Laerke HN, Hermansen K, Bach Knudsen KE. Similar metabolic responses in pigs and humans to breads with different contents and compositions of dietary fibers: a metabolomics study. Am J Clin Nutr. 2014;99(4):941-9.
196. Gilbert NF, Meyer PE, Tauriainen MP, Chao RY, Patel JB, Malloy CR, et al. Effects of hypothermia on myocardial substrate selection. Ann Thorac Surg. 2002;74(4):1208-12.
197. Peltz M, He TT, Adams GAt, Koshy S, Burgess SC, Chao RY, et al. Perfusion preservation maintains myocardial ATP levels and reduces apoptosis in an ex vivo rat heart transplantation model. Surgery. 2005;138(4):795-805.
198. Rosenbaum DH, Peltz M, DiMaio JM, Meyer DM, Wait MA, Merritt ME, et al. Perfusion preservation versus static preservation for cardiac transplantation: effects on myocardial function and metabolism. J Heart Lung Transplant. 2008;27(1):93-9.
199. Buchs JB, Lazeyras F, Ruttimann R, Nastasi A, Morel P. Oxygenated hypothermic pulsatile perfusion versus cold static storage for kidneys from non heart-beating donors tested by in-line ATP resynthesis to establish a strategy of preservation. Perfusion. 2011;26(2):159-65.

200. Minor T, Sitzia M, Dombrowski F. Kidney transplantation from non-heart-beating donors after oxygenated low-flow machine perfusion preservation with histidine-tryptophan-ketoglutarate solution. Transpl Int. 2005;17(11):707-12.
201. Thuillier R, Allain G, Celhay O, Hebrard W, Barrou B, Badet L, et al. Benefits of active oxygenation during hypothermic machine perfusion of kidneys in a preclinical model of deceased after cardiac death donors. J Surg Res. 2013;184(2):1174-81.
202. Treckmann J, Nagelschmidt M, Minor T, Saner F, Saad S, Paul A. Function and quality of kidneys after cold storage, machine perfusion, or retrograde oxygen persufflation: results from a porcine autotransplantation model. Cryobiology. 2009;59(1):19-23.
203. Hosgood SA, Yang B, Bagul A, Mohamed IH, Nicholson ML. A comparison of hypothermic machine perfusion versus static cold storage in an experimental model of renal ischemia reperfusion injury. Transplantation. 2010;89(7):830-7.
204. Pegg DE, Wusteman MC, Foreman J. Metabolism of normal and ischemically injured rabbit kidneys during perfusion for 48 hours at 10 C. Transplantation. 1981;32(5):437-43.
205. Schlegel A, Kron P, Graf R, Dutkowski P, Clavien PA. Warm vs. cold perfusion techniques to rescue rodent liver grafts. J Hepatol. 2014;61(6):1267-75.
206. Van Caenegem O, Beauloye C, Vercruysse J, Horman S, Bertrand L, Bethuyne N, et al. Hypothermic continuous machine perfusion improves metabolic preservation and functional recovery in heart grafts. Transpl Int. 2015;28(2):224-31.
207. Van Caenegem O, Beauloye C, Bertrand L, Horman S, Lepropre S, Sparavier G, et al. Hypothermic continuous machine perfusion enables preservation of energy charge and functional recovery of heart grafts in an ex vivo model of donation following circulatory death. Eur J Cardiothorac Surg. 2016;49(5):1348-53.
208. Lazeyras F, Buhler L, Vallee JP, Hergt M, Nastasi A, Ruttimann R, et al. Detection of ATP by "in line" 31P magnetic resonance spectroscopy during oxygenated hypothermic pulsatile perfusion of pigs' kidneys. MAGMA. 2012;25(5):391-9.
209. The COPE consortium for organ preservation in Europe. [cited 2016 20/09/2016]. COPE consortium for organ preservation in Europe.]. Available from: http://cope-eu.com/work programme/trials.html.
210. Hiller K, Hangebrauk J, Jager C, Spura J, Schreiber K, Schomburg D. MetaboliteDetector: comprehensive analysis tool for targeted and nontargeted GC/MS based metabolome analysis. Anal Chem. 2009;81(9):3429-39.
211. Chong M, Jayaraman A, Marin S, Selivanov V, de Atauri Carulla PR, Tennant DA, et al. Combined Analysis of NMR and MS Spectra (CANMS). Angew Chem Int Ed Engl. 2017.
212. Sellevold OF, Jynge P, Aarstad K. High performance liquid chromatography: a rapid isocratic method for determination of creatine compounds and adenine nucleotides in myocardial tissue. J Mol Cell Cardiol. 1986;18(5):517-27.
213. Hoyer DP, Gallinat A, Swoboda S, Wohlschlaeger J, Rauen U, Paul A, et al. Influence of oxygen concentration during hypothermic machine perfusion on porcine kidneys from donation after circulatory death. Transplantation. 2014;98(9):944-50.
214. Tuma Z, Kuncova J, Mares J, Matejovic M. Mitochondrial proteomes of porcine kidney cortex and medulla: foundation for translational proteomics. Clin Exp Nephrol. 2016;20(1):39-49.

215. Hosgood SA, Nicholson ML. Ex vivo normothermic perfusion of declined human kidneys after inadequate in situ perfusion. Am J Transplant. 2014;14(2):490-1.
216. Bagul A, Hosgood SA, Kaushik M, Kay MD, Waller HL, Nicholson ML. Experimental renal preservation by normothermic resuscitation perfusion with autologous blood. Br J Surg. 2008;95(1):111-8.

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APPENDICES

THE CLINICAL BENEFITS AND METABOLIC MECHANISMS OF *EX VIVO* MACHINE PERFUSION OF KIDNEYS PRIOR TO TRANSPLANTATION.

By

Jay Nath MBChB (Hons), MRCS

A thesis submitted to

The School of Medicine and Dentistry

The University of Birmingham

For the degree

DOCTOR OF PHILOSOPHY

Institute of Metabolism and Systems Research

The University of Birmingham

March 2017

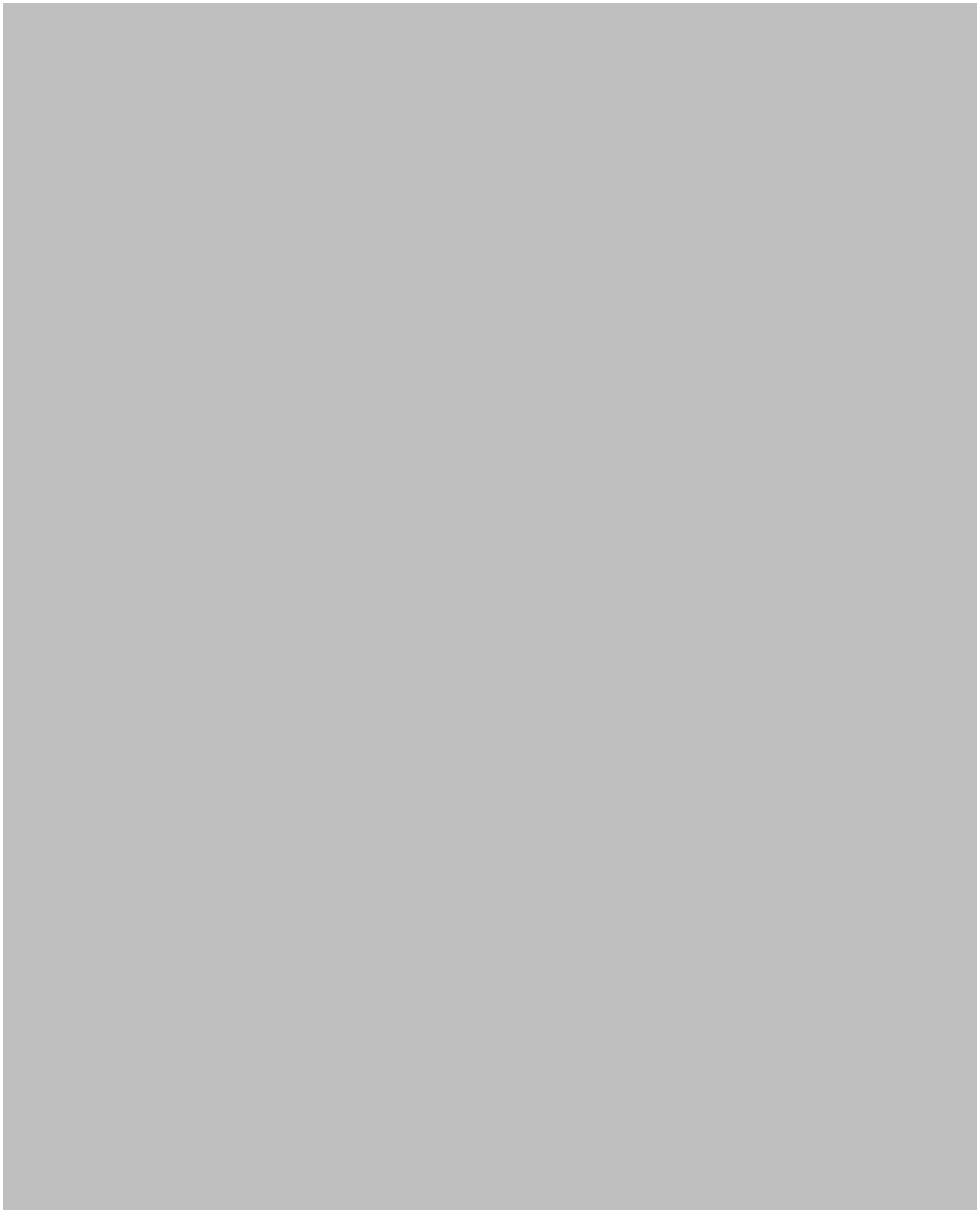
Table of Contents

Chapter 3
Nuclear Magnetic Resonance Strategies for Metabolic Analysis

Clement Heude, Jay Nath, John Bosco Carrigan, and Christian Ludwig

Abstract NMR spectroscopy is a powerful tool for metabolomic studies, offering highly reproducible and quantitative analyses. This burgeoning field of NMR metabolomics has been greatly aided by the development of modern spectrometers and software, allowing high-throughput analysis with near real-time feedback. Whilst one-dimensional proton (1D-^{1}H) NMR analysis is best described and remains most widely used, a plethora of alternative NMR techniques are now available that offer additional chemical and structural information and resolve many of the limitations of conventional 1D-^{1}H NMR such as spectral overlay. In this book chapter, we review the principal concepts of practical NMR spectroscopy, from common sample preparation protocols to the benefits and theoretical concepts underpinning the commonly used pulse sequences. Finally, as a case study to highlight the utility of NMR as a method for metabolomic investigation, we have detailed how NMR has been used to gain valuable insight into the metabolism occurring in kidneys prior to transplantation and the potential implications of this.

Keywords NMR • Metabolism • Metabolomics • Tracer • Kidney • Transplantation • HMP • Hypothermic • Perfusion



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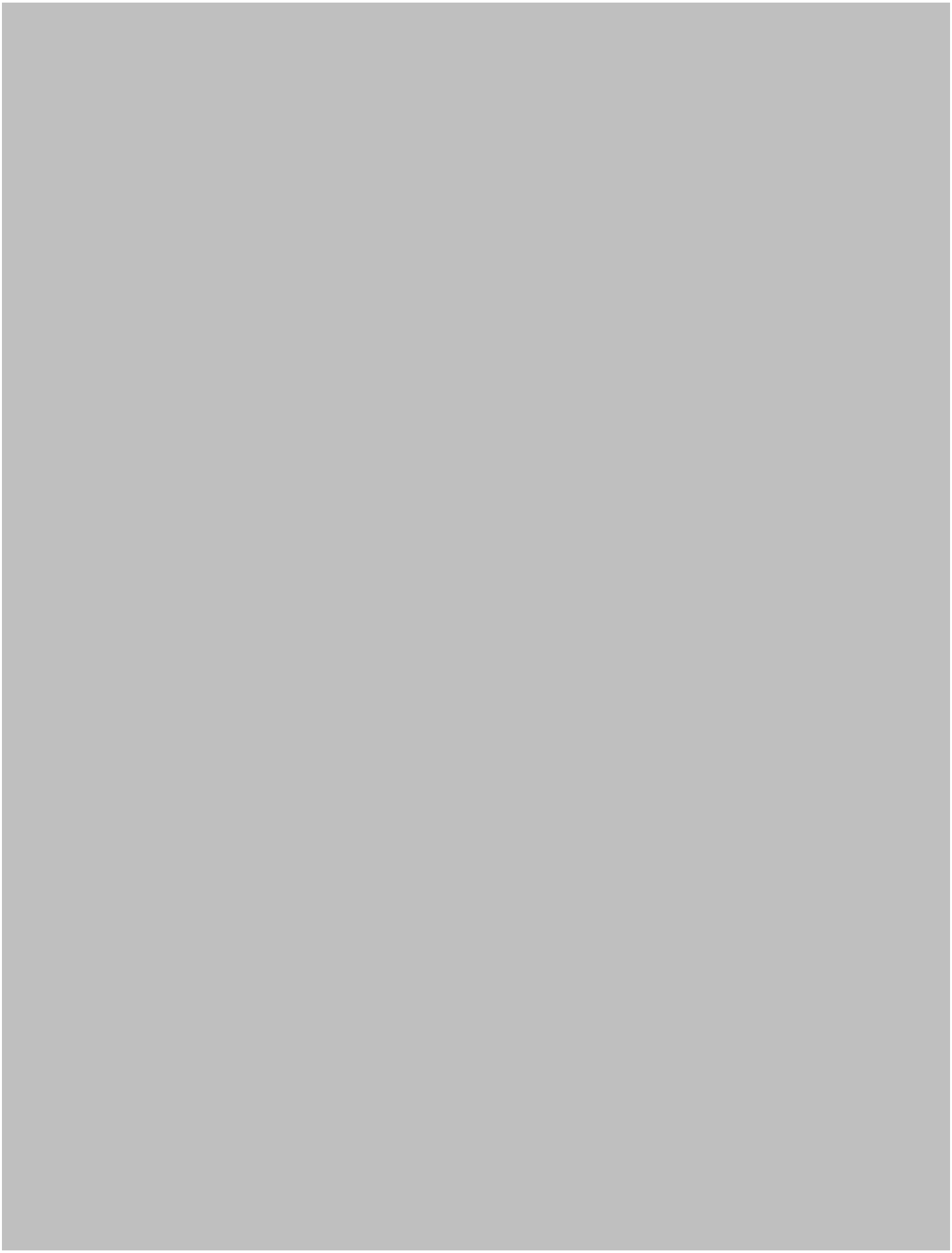

P61

Hypothermic machine perfusion during extended cold ischaemic times – an opportunity to improve pathways of care in cadaveric renal transplantation

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Introduction: The logistics of cadaveric renal transplantation are largely driven by Cold Ischaemic Time (CIT). However, to achieve successful outcomes in complex donor-recipient combinations, recipient and operative issues demand equal consideration. Extending CIT without detriment to graft function would therefore be of value. We have investigated the role of Hypothermic Machine Perfusion (HMP) as a tool by which such an extension of CIT might be obtained.

Methods: Cadaveric kidneys were allocated to a storage method depending on predicted time to theatre. Kidneys to be transplanted between 8am – 8pm in the dedicated transplant theatre remained in Static Cold Storage. If predicted operating time was out-of-hours, the kidney was transferred to HMP and transplanted at the earliest opportunity on the elective transplant list.

Results: 75 kidneys were transplanted from SCS; 68 from HMP. Median CIT was 24 hours in the HMP group compared to 13 hours in the SCS group (p=<.0001). 18 HMP kidneys suffered from DGF (26%) compared to 36 (48%) in the SCS group (p=0.01). There were no other significant differences in graft or post-operative complications.

Discussion: This study demonstrates that comparable outcomes can be achieved following longer CITs by utilising HMP storage rather than traditional SCS. This effect is likely to be multi-factorial; including improved recipient preparation, better peri-operative conditions and the inherent effects of HMP itself.

"Effect of cold ischaemia time on outcome after living donor renal transplantation"

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Background

The aim of this study was to determine whether modest prolongation of Cold ischaemia time CIT (up to 8 hours) impacts negatively on transplant outcome. This is clinically important, owing to the increasing number of cross centre kidney transplants performed.

Methods

Retrospective cohort study of 9,156 adult primary kidney transplants (January 2001-December 2014). Data was obtained from national (NHSBT and UK Renal Registry) databases. Patients were divided into 3 groups depending on CIT (0-2 hours, 2-4 hours and 4-8 hours) and outcomes compared.

Results

There was a higher rate of delayed graft function in the group with the longest CIT (5.0%, 4.4% and 7.2% p=0.008) However, there was no difference in five year graft survival (death censored) between groups (91.0%, 90.8% and 89.9% p=0.250). There was also no difference in five year patient survival (p=0.263) or 12 months creatinine (0.061) between groups.

Conclusion

Patients receiving kidneys transplants with modest prolongation of cold ischaemia time have excellent functional outcomes. The small increase in delayed graft function in this group is unlikely to alter clinical practice such as cross centre living donor kidney transplantation.

Appendix S1 Statistical methodology

Initially, univariable analyses were performed to compare across the three cold ischaemia time (CIT) groups. To account for the ordering of the CIT groups, continuous variables were analysed using Jonckheere–Terpstra tests, and binary or ordinal variables using Kendall's τ. Discrete variables that were non-ordinal (such as ethnicity) were assessed using χ^2 tests.

Multivariable analyses were then performed, in order to account for differences in demographics across the CIT groups. The binary and time-to-event outcomes were analysed using binary logistic and Cox proportional hazards regression respectively. In each case, CIT was entered into the model, with all potentially confounding factors considered for inclusion using a forwards stepwise approach. To maximize the amount of data available for analysis, a three-step process was used. First, this analysis was performed considering all factors. As there were a large number of patients with no available data for sensitization or wait time, these factors were excluded if they were not present in the final model, and the analysis rerun. The factors found to be significant in this model were then entered into a final analysis, in order to prevent the loss of patients owing to unavailable data in the non-significant factors. Continuous variables were tested with Hosmer–Lemeshow tests before the binary logistic regression analyses to assess the goodness of fit. Where poor fit was detected, the variables were either converted into categories, or $\log_{10}$-transformed, as applicable. For the Cox regression models, continuous variables were divided into categories, and all factors were tested graphically to ensure that the proportional hazards assumption was met.

Multivariable analysis of creatinine was performed using a general linear model. Owing to the level of skew in the distribution of creatinine, values were $\log_{10}$-transformed before analysis, in order to normalize the distribution to meet the assumptions of the test. All potentially confounding factors were then entered into the analysis, and a backwards stepwise approach was used to produce a parsimonious model. As mentioned previously, the factors in the parsimonious model were then entered into a model that did not consider the non-significant factors, in order to maximize the available sample size. The resulting coefficients were then antilogged, and converted to represent the percentage differences across the levels of the factors.

Adjusted rates for each outcome were then produced for each of the CIT groups. For the continuous factors in the model, the mean value was assumed. For categorical factors, the 'average' coefficients were generated by multiplying the proportions of patients within each category by the coefficients in the model. The models were then evaluated to give the estimated outcome for patients in the three CIT groups for the average patient; that is, with

average values of all of the factors included in the model. For the Cox regression models, the survival rates were read off from the adjusted survival plots produced by SPSS® version 22 (IBM, Armonk, New York, USA).

Table S1 Patient demographics

	No. of people* (n = 9156)	Missing data
Recipient		
Age (years)†	44 (33–54)	0 (0)
Sex (men)	5508 (60.2)	7 (0.1)
Ethnicity		88 (1.0)
White	7823 (86.3)	
Asian	738 (8.1)	
Black	353 (3.9)	
Other	154 (1.7)	
Diabetes	533 (5.8)	0 (0)
Dialysis at transplant		537 (5.9)
Haemodialysis	4316 (50.1)	
Peritoneal dialysis	1916 (22.2)	
Not on dialysis	2387 (27.7)	
Graft no.		0 (0)
1	7957 (86.9)	
2	1027 (11.2)	
≥ 3	172 (1.9)	
CMV-positive	3868 (45.1)	586 (6.4)
Blood group		6 (0.1)
A	3752 (41.0)	
AB	319 (3.5)	
B	1086 (11.9)	
O	3993 (43.6)	
Donor		
Age (years)†	48 (39–56)	5 (0.1)
Sex (men)	4180 (45.7)	6 (0.1)
Body mass index (kg/m^2)‡	26.5(4.0)	483 (5.3)
Ethnicity		73 (0.8)
White	7921 (87.2)	
Asian	659 (7.3)	
Black	327 (3.6)	
Other	176 (1.9)	
Blood group		10 (0.1)
A	2978 (32.6)	
B	796 (8.7)	
O	5372 (58.7)	

*With percentages in parentheses unless indicated otherwise; values are †median (i.q.r.) and ‡mean(s.d.). CMV, cytomegalovirus.

Table S2 Matching/transplant factors

	No. of people* (n = 9156)	Missing data
Wait time (days)†	299 (122–643)	2551 (27.9)
Cold ischaemia time (h)		0 (0)
< 2	2662 (29.1)	
2–4	4652 (50.8)	
4–8	1842 (20.1)	
Sensitization (% PRA)		4455 (48.7)
0	2739 (58.3)	
1–20	280 (6.0)	
21–80	1008 (21.4)	
81–100	674 (14.3)	
Year of transplant		0 (0)
2001–2005	1904 (20.8)	
2006–2008	2059 (22.5)	
2009–2011	2482 (27.1)	
2012–2014	2711 (29.6)	
HLA mismatch group		14 (0.2)
1	1064 (11.6)	
2	1300 (14.2)	
3	4186 (45.8)	
4	2592 (28.4)	
Related donor	5661 (61.9)	6 (0.1)
Antibody incompatibility		0 (0)
Not present	8196 (89.5)	
HLAi	425 (4.6)	
ABOi	475 (5.2)	
Both HLAi and ABOi	60 (0.7)	

*With percentages in parentheses unless indicated otherwise; values are †median (i.q.r.). PRA, panel reactive antibody; HLAi, HLA-incompatible; ABOi, ABO-incompatible.

Table S3 Outcomes

	No. of recipients* (n = 9156)	Missing data
Death	526 (5.8)	45 (0.5)
Graft failure	899 (9.9)	46 (0.5)
Rejection within 3 months	1330 (15.8)	722 (7.9)
Delayed graft function	419 (5.1)	1020 (11.1)
Creatinine at 12 months (μmol/l)†	124 (69–225)	1926 (21.0)‡

*With percentages in parentheses unless indicated otherwise; †values are geometric means and 95 per cent prediction intervals (the antilog of the logged mean ± 1.96 s.d., that is, the range in which 95 per cent of cases fall). ‡Comprising 152 patients with graft failure, 1650 with insufficient follow-up and 124 with no measurements recorded.

Table S4 Multivariable analysis of factors predictive of death-censored graft survival

	Hazard ratio	*P*
Cold ischaemia time (h)		0.049
< 2	1.00 (reference)	–
2–4	1.06 (0.91, 1.24)	0.471
4–8	1.31 (1.05, 1.62)	0.016
Dialysis at transplant		0.009
Haemodialysis	1.00 (reference)	–
Peritoneal dialysis	0.96 (0.81, 1.14)	0.647
Not on dialysis	0.75 (0.63, 0.91)	0.003
Donor ethnicity		< 0.001
White	1.00 (reference)	–
Asian	1.01 (0.75, 1.36)	0.947
Black	2.34 (1.75, 3.14)	< 0.001
Other	1.12 (0.64, 1.95)	0.688
Year of transplant		0.058
2001–2005	1.00 (reference)	–
2006–2008	0.89 (0.74, 1.07)	0.211
2009–2011	0.76 (0.61, 0.94)	0.014
2012–2014	0.74 (0.55, 0.98)	0.036
Antibody incompatibility		< 0.001
Not present	1.00 (reference)	–
HLAi	1.93 (1.45, 2.57)	< 0.001
ABOi	1.57 (1.11, 2.21)	0.011
Both HLAi and ABOi	2.57 (1.35, 4.89)	0.004
Recipient age (years)		< 0.001
< 30	1.00 (reference)	–
30–39	0.74 (0.61, 0.90)	0.002
40–49	0.53 (0.43, 0.65)	< 0.001
50–59	0.43 (0.34, 0.55)	< 0.001
≥ 60	0.55 (0.42, 0.72)	< 0.001
Graft no.		0.013
1	1.00 (reference)	–
2	1.24 (0.99, 1.55)	0.067
≥ 3	1.73 (1.14, 2.62)	0.009
HLA mismatch group		0.008
1	1.00 (reference)	–
2	1.34 (0.99, 1.81)	0.058
3	1.55 (1.20, 2.02)	0.001
4	1.39 (1.05, 1.85)	0.023
Donor age (years)		0.001
< 30	1.00 (reference)	–
30–39	0.99 (0.72, 1.34)	0.930
40–49	1.33 (1.00, 1.75)	0.047
50–59	1.22 (0.92, 1.63)	0.163
≥ 60	1.65 (1.21, 2.24)	0.002
Donor body mass index (kg/m^2)		0.006
≤ 25	1.00 (reference)	–
25–30	1.11 (0.95, 1.30)	0.201
> 30	1.36 (1.13, 1.65)	0.002

Values in parentheses are 95 per cent confidence intervals. Parsimonious model from a forwards stepwise Cox regression analysis. All factors in *Tables S1* and *S*2 (supporting information) were considered for inclusion. n = 8056 after exclusions owing to missing data. HLAi, HLA-incompatible; ABOi, ABO-incompatible.

Table S5 Multivariable analysis of factors predictive of delayed graft function

	Odds ratio	*P*
Cold ischaemia time (h)		< 0.001
< 2	1.00 (reference)	–
2–4	1.14 (0.88, 1.49)	0.314
4–8	2.16 (1.60, 2.92)	< 0.001
Year of transplant		< 0.001
2001–2005	1.00 (reference)	–
2006–2008	0.61 (0.44, 0.83)	0.002
2009–2011	0.44 (0.32, 0.60)	< 0.001
2012–2014	0.31 (0.22, 0.44)	< 0.001
Donor age (per decade)	1.21 (1.11, 1.33)	< 0.001
Recipient sex		0.007
F	1.00 (reference)	–
M	1.36 (1.09, 1.70)	0.007
Dialysis at transplant		< 0.001
Haemodialysis	1.00 (reference)	–
Peritoneal dialysis	0.43 (0.32, 0.58)	< 0.001
Not on dialysis	0.25 (0.18, 0.35)	< 0.001
Recipient CMV		0.008
Negative	1.00 (reference)	–
Positive	1.33 (1.08, 1.65)	0.008
Antibody incompatibility		< 0.001
Not present	1.00 (reference)	–
HLAi	4.66 (3.35, 6.50)	< 0.001
ABOi	4.90 (3.52, 6.83)	< 0.001
Both HLAi and ABOi	6.04 (2.99, 12.20)	< 0.001

Values in parentheses are 95 per cent confidence intervals. Parsimonious model from a forwards stepwise logistic regression analysis. All factors in *Tables S1* and *S*2 (supporting information) were considered for inclusion. $n = 7168$ after exclusions owing to missing data. CMV, cytomegalovirus; HLAi, HLA-incompatible; ABOi, ABO-incompatible.

Table S6 Multivariable analysis of factors predictive of patient survival

	Hazard ratio	*P*
Cold ischaemia time (h)		0.440
< 2	1.00 (reference)	–
2–4	1.08 (0.88, 1.33)	0.468
4–8	1.20 (0.91, 1.58)	0.205
Recipient age (years)		< 0.001
< 30	1.00 (reference)	–
30–39	1.28 (0.81, 2.03)	0.290
40–49	2.25 (1.48, 3.41)	< 0.001
50–59	4.15 (2.77, 6.21)	< 0.001
≥ 60	7.61 (5.02, 11.53)	< 0.001
Diabetes		< 0.001
No	1.00 (reference)	–
Yes	2.37 (1.82, 3.09)	< 0.001
Dialysis at transplant		< 0.001
Haemodialysis	1.00 (reference)	–
Peritoneal dialysis	0.70 (0.56, 0.88)	0.003
Not on dialysis	0.54 (0.42, 0.69)	< 0.001
Recipient CMV		0.014
Negative	1.00 (reference)	–
Positive	1.26 (1.05, 1.52)	0.014
Donor age (years)		0.004
< 30	1.00 (reference)	–
30–39	1.41 (0.86, 2.31)	0.167
40–49	1.69 (1.06, 2.69)	0.026
50–59	1.44 (0.90, 2.30)	0.124
≥ 60	2.14 (1.33, 3.45)	0.002
Antibody incompatibility		0.002
Not present	1.00 (reference)	–
HLAi	1.89 (1.28, 2.80)	0.001
ABOi	1.34 (0.86, 2.06)	0.192
Both HLAi and ABOi	2.58 (1.06, 6.28)	0.036

Values in parentheses are 95 per cent confidence intervals. Parsimonious model from a forwards stepwise Cox regression analysis. All factors in *Tables S1* and *S*2 (supporting information) were considered for inclusion. $n = 8019$ after exclusions owing to missing data. CMV, cytomegalovirus; HLAi, HLA-incompatible; ABOi, ABO-incompatible.

Table S7 Multivariable analysis of factors predictive of 12-month creatinine level

	Coefficient	*P*
Cold ischaemia time (h)		0.025
< 2	Reference	–
2–4	1.5 (–0.2, 3.2)	0.094
4–8	3.1 (0.8, 5.5)	0.007
Recipient age (per decades)	–3.0 (–3.6, –2.5)	< 0.001
Recipient sex		< 0.001
F	Reference	–
M	23.0 (21.2, 24.9)	< 0.001
Recipient ethnicity		< 0.001
White	Reference	–
Asian	–4.3 (–6.8, –1.7)	0.001
Black	14.0 (9.7, 18.4)	< 0.001
Other	–1.7 (–7.9, 4.9)	0.607
Diabetes		0.054
No	Reference	–
Yes	–3.2 (–6.3, 0.1)	0.054
Graft no.		0.065
1	Reference	–
2	–0.8 (–2.9, 1.3)	0.441
≥ 3	5.2 (0.4, 10.3)	0.035
Year of transplant		< 0.001
2001–2005	Reference	–
2006–2008	–8.2 (–10.2, –6.3)	< 0.001
2009–2011	–12.5 (–14.3, –10.6)	< 0.001
2012–2014	–12.7 (–14.8, –10.5)	< 0.001
Antibody incompatibility		< 0.001
Not present	Reference	–
HLAi	9.1 (5.6, 12.8)	< 0.001
ABOi	6.5 (3.1, 10.1)	< 0.001
Both HLAi and ABOi	13.2 (4.8, 22.3)	0.002
Donor sex		< 0.001
F	Reference	–
M	–4.7 (–6.1, –3.3)	< 0.001
Donor age (per decade)	7.6 (6.9, 8.3)	< 0.001
Log_{10} wait time (days)*	1.2 (0.1, 2.2)	0.034

Values in parentheses are 95 per cent confidence intervals. Parsimonious model from a backwards stepwise general linear model. All factors in *Tables S1* and *S*2 (supporting information) were considered for inclusion. $n = 5191$ after exclusions owing to missing data. The coefficients represent the percentage difference in creatinine between the stated category and the reference category, or for a 1-unit increase in continuous variables. *Wait time was log_{10} transformed, before analysis, to improve model fit. Hence, the coefficient represents the increase in creatinine for a tenfold increase in wait time. HLAi, HLA-incompatible; ABOi, ABO-incompatible.

Table S8 Multivariable analysis of rejection within 3 months

	Odds ratio	*P*
Cold ischaemia time (h)		0.004
< 2	1.00 (reference)	–
2–4	0.81 (0.71, 0.94)	0.004
4–8	1.00 (0.84, 1.20)	0.970
Recipient age (per decade)	0.86 (0.82, 0.90)	< 0.001
Recipient sex		< 0.001
F	1.00 (reference)	–
M	1.29 (1.13, 1.46)	< 0.001
Recipient ethnicity		0.006
White	1.00 (reference)	–
Asian	0.73 (0.57, 0.94)	0.014
Black	1.43 (1.06, 1.92)	0.019
Other	0.91 (0.54, 1.53)	0.730
Donor age (per decade)	1.10 (1.04, 1.16)	0.001
Year of transplant		< 0.001
2001–2005	1.00 (reference)	–
2006–2008	0.52 (0.44, 0.62)	< 0.001
2009–2011	0.43 (0.36, 0.51)	< 0.001
2012–2014	0.39 (0.32, 0.47)	< 0.001
HLA mismatch group		< 0.001
1	1.00 (reference)	–
2	1.83 (1.30, 2.57)	< 0.001
3	3.82 (2.86, 5.12)	< 0.001
4	4.69 (3.47, 6.33)	< 0.001
Antibody incompatibility		< 0.001
Not present	1.00 (reference)	–
HLAi	1.43 (1.09, 1.86)	0.009
ABOi	1.44 (1.11, 1.87)	0.006
Both HLAi and ABOi	2.29 (1.25, 4.20)	0.007

Values in parentheses are 95 per cent confidence intervals. Parsimonious model from a forwards stepwise logistic regression analysis. All factors in *Tables S1* and *S*2 (supporting information) were considered for inclusion. $n = 8334$ after exclusions owing to missing data. HLAi, HLA-incompatible; ABOi, ABO-incompatible.

Supplementary Digital Content

SDC1: 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(Maastricht Category III)	23 (88%)	18 (95%)	5 (71%)	0.17
DCD(Maastricht Category IV)	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
HCV +	0 (0%)	0 (0%)	0 (0%)	1.00
HIV+	0 (0%)	0 (0%)	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
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

CMV: cytomegalovirus, DBD: donation after brain-death, DCD: donation after cardiac-death, Maastricht Category: as defined in Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. Transplantation proceedings 1995; 27: 2893. SCD: standard criteria donor, ECD: extended criteria donor: defined as age >60 or 50-59 with 2 or more of hypertension, intracranial cause of death, creatinine >132 uM/L. HAV: hepatitis A virus, HBV: hepatitis B virus, HCV: hepatitis C virus, HIV; human immune-defiecieny virus, cAb: core antibody, sAb: surface antibody. HD: haemodialysis, PD: peritoneal dialysis. PRA: panel reactive antibodies. HMP: Hypothermic machine perfusion, CIT: cold ischaemic time. 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 $p < 0.05$

SDC2: Composition 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

SDC3: 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

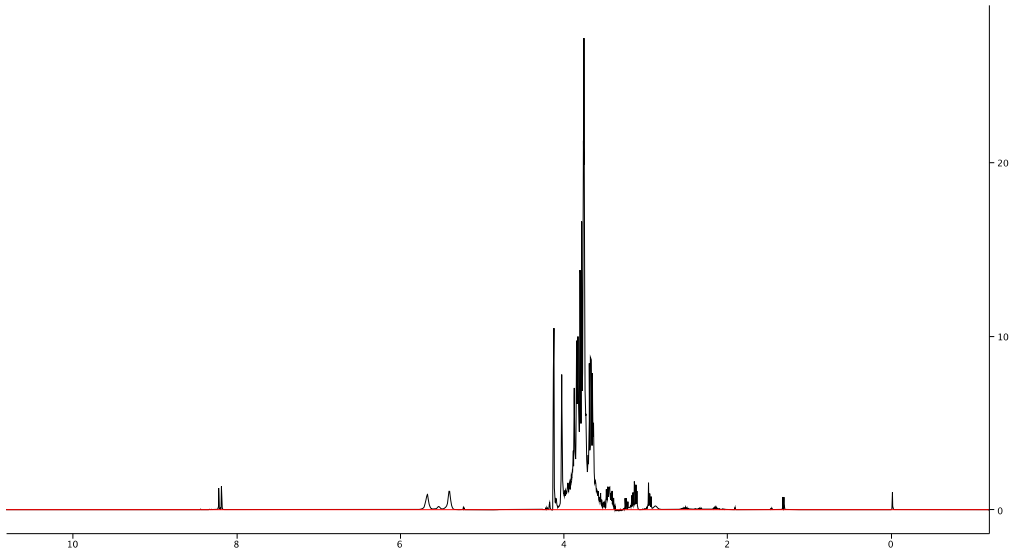
Due to small sample size, cutoffs are likely to have a wide degree of error

SDC4 : Chemical Shifts References Used for Metabolite Quantification

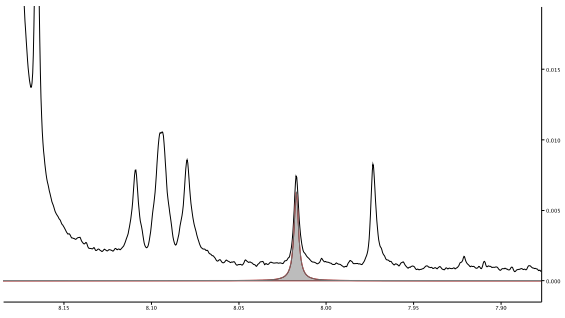
Metabolite	**NMR Chemical Shift Reference (ppm)**	**Metabolite**	**NMR Chemical Shift Reference (ppm)**
3-Hydroxybutyrate	1.204	Hippurate	7.82
3-Methylxanthine	8.02	Hypoxanthine	8.20
Acetate	1.91	Inosine	6.055
Adenine	8.11	Isoleucine	0.997
Alanine	1.46	Isopropranolol	1.162
Citrate	2.67	Lactate	1.40
Ethanol	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

SDC5: Example NMR Spectra

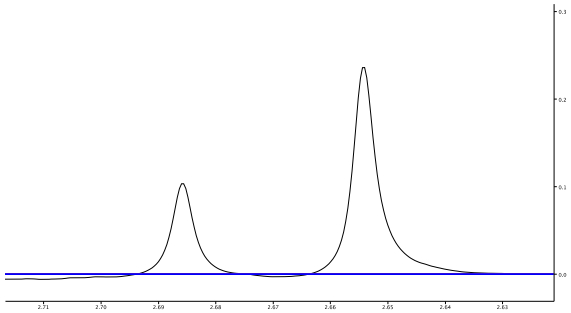
Whole Spectra



3-Methylxanthine



Citrate



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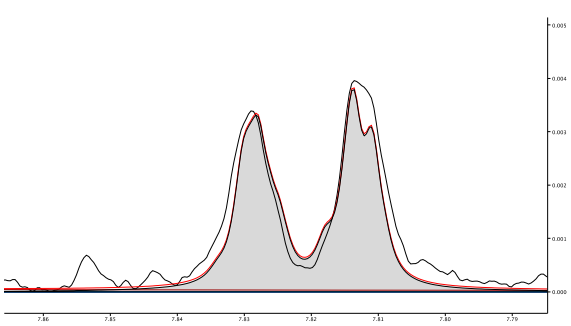
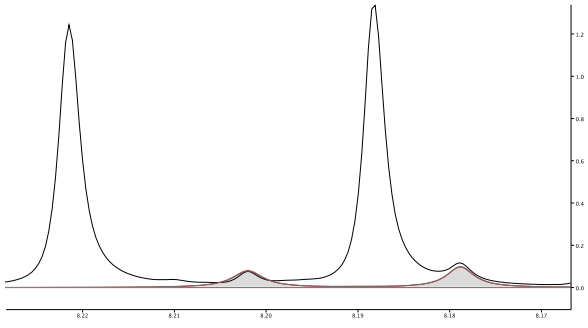


Hypoxanthine



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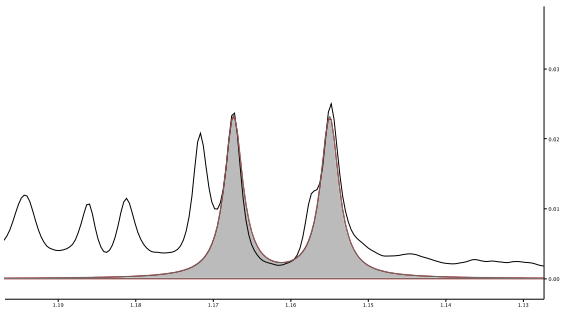

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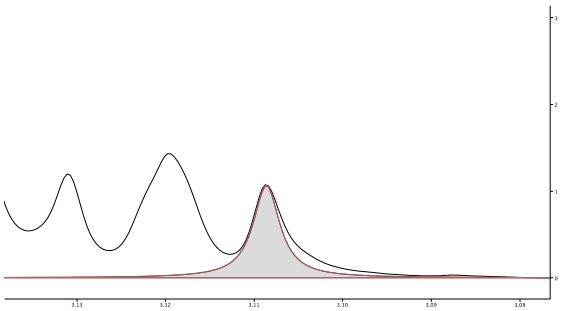
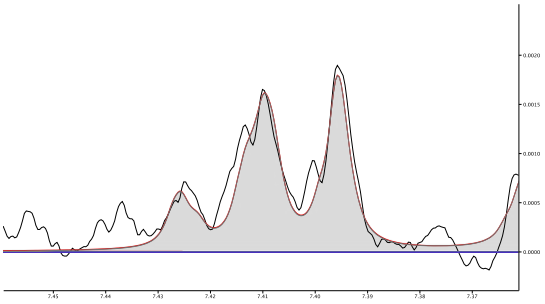


N-phenylacetylglycine



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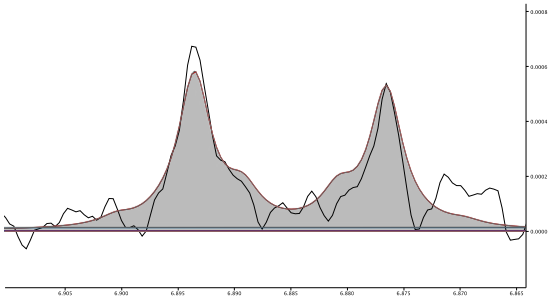
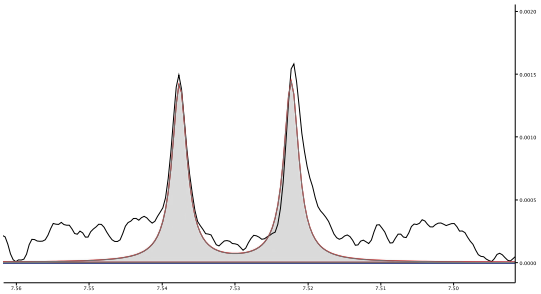
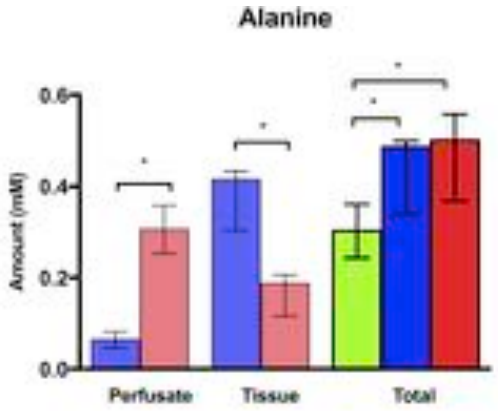
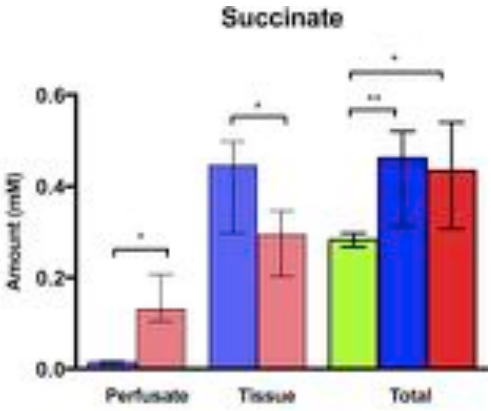


Uracil




Alanine
Amount (mM)
0.6
0.4
0.2
0.0
Perfusate
Tissue
Total


Succinate
Amount (mM)
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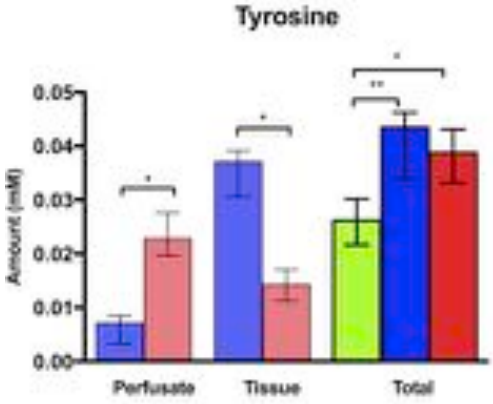
0.4
0.2
0.0
Perfusate
Tissue
Total


Tyrosine
Amount (mM)
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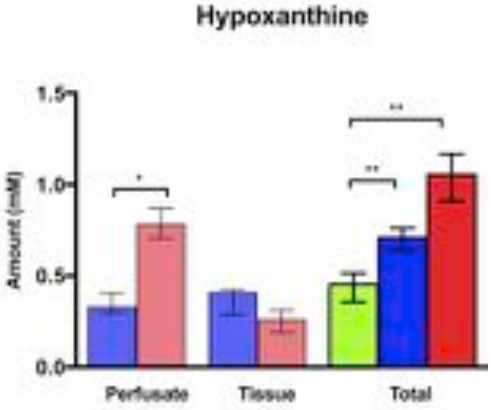
0.04
0.03
0.02
0.01
0.00
Perfusate
Tissue
Total


Hypoxanthine
Amount (mM)
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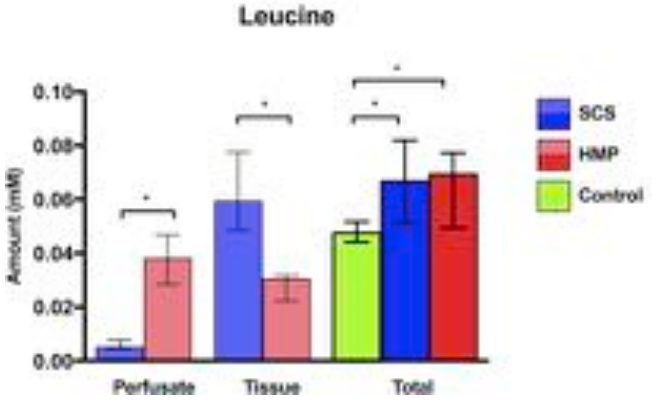
1.0
0.5
0.0
Perfusate
Tissue
Total


Leucine
Amount (mM)
0.10
0.08
0.06
0.04
0.02
0.00
Perfusate
Tissue
Total
SCS
HMP
Control

	Storage	Total storage fluid amount (mmol)	p-value[#]	Total kidney tissue amount (mmol)	p-value[#]
2-aminoadipate	SCS	-	0.031*	0.094 (0.063-0.112)	0.031*
	HMP	0.048 (0.037-0.062)		0.055 (0.050-0.059)	
Choline	SCS	0.039 (0.035-0.045)	0.031*	0.404 (0.329-0.423)	0.063
	HMP	0.102 (0.099-0.115)		0.267 (0.205-0.299)	
Creatinine	SCS	0.032 (0.008-0.067)	0.031*	0.564 (0.127-0.699)	0.156
	HMP	0.196 (0.038-0.386)		0.228 (0.130-0.328)	
Hippurate	SCS	0.002 (0.001-0.002)	0.031*	0.005 (0.003-0.007)	0.094
	HMP	0.006 (0.005-0.010)		0.001 (0.000-0.005)	
Isoleucine	SCS	0.004 (0.003-0.005)	0.031*	0.036 (0.032-0.041)	0.031*
	HMP	0.026 (0.023-0.027)		0.019 (0.014-0.020)	
TMNO	SCS	0.037 (0.031-0.047)	0.031*	0.152 (0.096-0.272)	0.219
	HMP	0.254 (0.165-0.266)		0.143 (0.099-0.218)	
Valine	SCS	0.006 (0.006-0.010)	0.031*	0.068 (0.057-0.083)	0.031*
	HMP	0.041 (0.038-0.053)		0.035 (0.025-0.036)	
β-Alanine	SCS	0.013 (0.010-0.021)	0.031*	0.101 (0.078-0.112)	0.031*
	HMP	0.085 (0.078-0.099)		0.038 (0.031-0.048)	
Taurine	SCS	0.196 (0.136-0.227)	0.031*	0.981 (0.929-1.056)	0.063
	HMP	0.693 (0.640-0.821)		0.505 (0.437-0.554)	
ADP	SCS	0.014 (0.013-0.015)	0.031*	0.013 (0.011-0.026)	0.438
	HMP	-		0.024 (0.019-0.031)	
AMP	SCS	0.003 (0.002-0.003)	0.031*	0.128 (0.105-0.169)	0.563
	HMP	-		0.140 (0.131-0.166)	
NAD+	SCS	-	-	0.025 (0.022-0.031)	0.094
	HMP	-		0.037 (0.033-0.049)	

S1 Table. Distribution of metabolite quantity between extracellular storage fluid and entire kidney tissue for 12 metabolites detected in comparable total amounts to controls.

Data reported as median (interquartile range), unless stated otherwise. Statistical test: [#]Wilcoxon paired signed rank test (two-tailed). *Significant at $p<0.05$.

A study to determine the mechanism and effect of machine perfusion on cadaveric kidneys unsuitable for transplantation.

STUDY TITLE:

A study to determine the mechanism and effect of machine perfusion on cadaveric kidneys unsuitable for transplantation.

Protocol Number:	1
Phase:	Not Applicable
R&D Ref:	RRK5323
Version Number:	1
Date:	**1st Januaury 2015**
Study Sponsor Name & Address	Dr Christopher Counsell Room 17, Education Centre, Queen Elizabeth Hospital Birmingham, Edgbaston, Birmingham, B15 2WB.

VERSION CONTROL LOG

Version number	Date	Summary of changes
1	1st January 2015	N/A

PROTOCOL SIGNATURE SHEET

This protocol has been approved by:

Name & Address: *<print – delete this text on completion>*	**Role:** **Sponsor Representative**
Signature: _____	**Date:** _____

Name & Address *<print name – delete this text on completion>*	**Role:**
Signature: _____	**Date:** _____

Principal Investigator Declaration:

I have read and understood the requirements and conditions of the study protocol. I am aware of my responsibilities as an Investigator under the guidelines of the Internal Conference on Harmonisation Good Clinical Practice (ICH GCP) standards, the Declaration of Helsinki, local regulations (as applicable) and the study protocol. I agree to conduct the study according to these guidelines and to appropriately direct and assist the study team assigned to me who will be involved in the study.

I agree to use the study material, including medication, only as specified in the protocol.
I understand that changes to the protocol must be made in the form of an amendment that must be approved by the Ethics Committee and Regulatory Authorities prior to its implementation.

I understand that non-compliance with the study protocol may lead to early termination of the study.

Local Investigator's Name & Address: *<print name – delete this text on completion>*	
Signature: _____	**Date:** _____

Return the original wet signature* page to the sponsor and retain a copy* with the study protocol within the Investigator's Site file.

* *Reference to wet signature means signature in ink, copy can be a scanned copy or photocopy of the signature page.*

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TABLE OF CONTENTS

Additional Information

- Appendix 1: Submission to NHSBT review board
- Appendix 2: Published paper Transplantation
- Appendix 3: Favourable letter previous REC application
- Appendix 4: ^{13}C safety information

LIST OF ABBREVIATIONS & DEFINITIONS

Abbreviation	Definition
MP	Machine Perfusion
DCD	Donation after Cardiac Death
ECD	Expanded Criteria Donor
SCD	Standard Criteria Donor
DGF	Delayed Graft Function
CS/SCS	Cold Storage/Static Cold Storage
HMP	Hypothermic Machine Perfusion
PNF	Primary Non-Function

1. Background Information

The aim of this research project is to investigate the use of Machine Perfusion (MP) on human kidneys that are not suitable for transplantation.

Kidney transplantation is the gold standard treatment for patients with end-stage renal failure, providing both survival benefit and improved quality of life when compared with dialysis.[1] In addition there are economic benefits. Renal transplantation provides a cost saving of over £25,000 per patient per year compared with other forms of renal replacement therapy.[2]

The rate of kidney transplantation in the UK has increased predominantly due to live donation, with the incidence of cadaveric donation remaining static over the last decade. Strategies to increase donor numbers have recently been introduced, including the use of more Donations after Cardiac Death (DCD) and utilisation of donors that were previously considered unsuitable. These strategies, whilst increasing numbers, have not concentrated on the quality of kidneys offered and one of the most recent changes to emerge is the acceptance of kidneys that might previously have been rejected. These kidneys are termed marginal, or Expanded Criteria donor (ECD) kidneys. Compared to Standard Criteria Donor (SCD) kidneys, these ECD kidneys are associated with higher risks of graft failure. This is also the case for DCD kidneys that have higher rates of delayed graft function (DGF).[3] The greater availability of marginal kidneys has led to an increased interest in methods to ensure and maintain quality and outcomes, not only for this subgroup but for all donated kidneys.

Traditionally, the standard storage method for kidneys in the UK is static Cold Storage (CS). After procurement, the organ is flushed with a cold preservation fluid and stored and transported on ice. The hypothermic conditions lower the metabolic rate and oxygen requirements of the kidney, which helps to preserve it until implantation.

The alternative to static cold storage is Machine Perfusion (MP),, Which is normally performed under hypothermic condition. Such devices contain a pump that provides a pulsatile recirculation of preservation fluid. Large international studies in recent years, have demonstrated the benefits of MP, including reduced rates of DGF and Primary Non-Function (PNF), as well as long term survival benefit in selected kidneys compared to static sold storage (see below). In addition to the direct benefits to the organ, the ability to prevent injury to the kidney during perfusion provides more time to biopsy and/or better prepare the recipient for transplantation. This is predicted to result in the acceptance of more kidneys and better assessment of those organs accepted.

Summary of Trial Findings

Both animal experiments and historical clinical studies have demonstrated that Hypothermic Machine Perfusion of cadaveric kidneys provides improved early graft function when compared with Static Cold Storage.[4,5,6,7,8,9]. Studies from the 1990's reported reduced rates of DGF and increased rates of immediate function of renal grafts[10,11,12,13]. MP was shown to be preferable for prolonged preservation times, both for kidneys donated after cardiac death and for ECD kidneys[14,15,16]. Daemen *et al* (1997) demonstrated reduced DGF for DCD kidneys when preserved by MP instead of CS[17] However, controversy still remained due to a lack of prospective studies and perceived costs associated with MP.

A European multicenter randomized controlled trial has provided important evidence[18]. The study involved 336 consecutive deceased donors from whom one kidney was randomized to CS and the other to HMP. It was found that HMP reduced the risk of DGF and graft failure, and improved graft survival in the first year following transplantation. This group has released 3-year outcomes which show these benefits continue, particularly for ECD kidneys[19].

In addition to improved outcomes, MP provides the ability to evaluate organs from ECDs and DCDs prior to transplantation. This may significantly increase the number of organs available.[20], Cost benefits of HMP have also been demonstrated[21].

Biochemical parameters and ischaemic injury markers have been identified in the perfusate of MP kidneys[16,22,23,24,25]. Findings from our group, following the initial ethical approval (RRK4459) have demonstrated that the metabolites present within the perfusate of human kidneys prior to transplantation correlates with post transplant graft function (Appendix 2). This work, utilising 1D NMR (Nuclear Magnetic Resonance) Spectroscopy has provided us with a metabolic 'fingerprint' of the perfusion fluid at various time points of perfusion. Characterisation of metabolic processes occurring during machine perfusion is at an early stage, but such metabolism appears to be fundamental to the success of the transplant graft.

Although we have gained significant understanding of the active metabolic processes during perfusion from this perfusate analysis study, and will continue to add to the number of samples obtained, experimental studies are needed in addition to this to gain a more complete understanding.

Perfusate 1D NMR Spectroscopy, as we have performed, provides a metabolic snapshot during perfusion in these hypoxic, hypothermic conditions, utilising the gold standard perfusion fluid (KPS-1). However these conditions, including the chemical composition of the perfusion fluid were developed on the understanding that little, if any metabolism occurs during organ preservation. However, as we have shown, metabolic support appears to be a key beneficial component of organ preservation and greater support during perfusion could prolong the duration that organs can safely be stored, and potentially convert severely damaged organs into ones that could be utilised.

We have a successful abattoir pig kidney perfusion model established in our research group and have performed multiple experimental studies using it. We have compared the metabolic products during machine perfusion of these abattoir pig kidneys with selected human kidneys and found that they were similar but not identical [26].

We have performed metabolic studies using the porcine model with alteration of the perfusion parameters and have found that we are able to activate specific metabolic pathways which we believe would convey a more favorable outcome.

The rationale for using human kidneys for further studies in addition to the pig abattoir organs is twofold. Firstly, the pre-retrieval conditions in the human setting are impossible to replicate in the pig model (e.g. a 70 year old donor who has been in intensive care for four weeks following an intra-cerebral bleed with hypertension and a recent chest infection). Secondly, it is necessary to determine whether our metabolic findings during perfusion of pig kidneys with altered perfusion parameters are corroborated in the human kidney.

In order to develop a greater understanding of the metabolic benefits of machine perfusion, we would like to utilise human organs not suitable for transplantation and modify the parameters of perfusion.

The evidence suggests that MP has a beneficial effect on the kidney. Metabolic support appears to be a key component of this. We aim to further our understanding of this mechanism by performing experimental studies on cadaveric kidneys not suitable for transplantation.

Compliance

The study will be conducted in compliance with the protocol, GCP and research governance guidelines.

Study Population

Participants are deceased donors. They are consented for organ donation prior to death and this is confirmed during the donation process by the national organ retrieval service. Confirmation is performed by a senior nurse in organ donation.

Only organs that have been declined for transplantation by all UK centres and for whom consent has been obtained for organ utilisation for research purposes will be included in these experimental studies.

References:

1. Wolfe RA, Ashby VB, Milford EL, Ojo AO, Ettenger RE, Agodoa LY, *et al*. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of first cadaveric transplant. *N Engl J Med* 1999;341:1725-30.
2. NHS Blood and Transplant. Organ donation: cost-effectiveness of transplantation.2011.www.uktransplant.org.uk/ukt/newsroom/fact_sheets/cost_effectiveness_of_transplantation.jsp.
3. Jochmans I, Pirenne J. Graft quality assessment in kidney transplantation: not an exact science yet! *Curr Opin Organ Transplant* 2011,16:174-179.
4. Matsuoka L, Shah T, Aswad S, Bunnapradist S, Cho Y, Mendez RG, Mendez R, Selby R. Pulsatile perfusion reduces the incidence of delayed graft function in expanded criteria donor kidney transplantation. *Am J Transplant*. 2006;6:1473-1478.
5. Nicholson ML, Hosgood SA, Metcalfe MS, Waller JR, Brook NR. A comparison of renal preservation by cold storage and machine perfusion using a porcine autotransplant model. *Transplantation*. 2004;78:333-337.
6. Schold JD, Kaplan B, Howard RJ, Reed AI, Foley DP, Meier-Kriesche. Are we frozen in time? Analysis of the utilization and efficacy of pulsatile perfusion in renal transplantation. *Am J Transplant*. 2005;5:1681-1688.
7. Stratta RJ, Moore PS, Farney AC, Rogers J, Hartmann EL, Reeves-Daniel A, Gautreaux MD, Iskander SS, Adams PL. Influence of pulsatile perfusion preservation on outcomes in kidney transplantation from expanded criteria donors. *J Am Coll Surg*. 2007;204:873-882.
8. Wight J, Chilcott J, Holmes M, Brewer N. The clinical and cost-effectiveness of pulsatile machine perfusion versus cold storage of kidneys for transplantation retrieved from heart-beating and non-heart-beating donors. *Health Technol Assess*. 2003;7:1-94.
9. Zhou YC, Cecka JM. Effect of HLA matching on renal transplant survival. In: Terasaki PI, Cecka JM, editors. *Clinical Transplants*. UCLA Tissue Typing Typing Laboratoty; Los Angeles: 1993.pp.499-510.
10. Barbar WH, Deierhoi MH, Phillips MG, Diethelm AG. Preservation by pulsatile perfusion improves early renal allograft function. *Transplant Proc*. 1988;20:865-868.
11. Halloran P, Aprile M. A randomized prospective trial of cold storage versus pulsatile perfusion for cadaveric kidney transplantation. *Transplantation*. 1987;43:827-832.
12. Koyama H, Cecka JM, Terasaki PI. A comparison of cadaveric kidney storage methods: pump perfusion and cold storage solutions. *Clinical Transplantation*. 1993;7:199-205.
13. Merion RM, Oh HK, Port FK, Toledo-Pereyra LH, Turcotte JG. A prospective controlled trial of cold-storage versus machine-perfusion preservation in cadaveric renal transplantation. *Transplantation.* 1990;50:230-233.
14. McAnulty JF, Ploeg RJ, Southard JH, Belzer FO. –Successful Five-Day Perfusion Preservation of the Canine Kidney. *Transplantation*. 1989;47:37-41.
15. Matsuno N, Sakurai E, Tamaki I, Uchiyama M, Kozaki K, Kozaki M. Effect of machine perfusion preservation on delayed graft function in non-heart-beating donors. *Transplantation*. 1994;57:293-294.
16. Polyak MMR, Arrington MO, Stubenbord WT, Boykin J, Brown T, Jean-Jacques MA, Estevez J, Kapur S, Kinkhabwala M. Influence of pulsatile perfusion on renal transplantation in the 1990s. *Transplantation.* 2000;69(2):249-258.
17. Daemen JHC, DeVries B, Oomen APA, DeMeester J, Koostra G. Effect of machine perfusion preservation on delayed graft function in non-heart beating donor kidneys-early results. *Transpl International*. 1997;10:317-322.
18. Moers *et al* (for the Machine Preservation Trial Study Group). Machine perfusion or cold storage in deceased-donor kidney transplantation. *New Engl J Med*. 2009;360:7-19.

19. Moers C, Pirenne J, Paul A, Ploeg R. Correspondence: Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 2012;366:770-771.
20. Baicu SC, Simmons PM, Campbell LH, Taylor MJ, Brockbank KG. Interstitial fluid analysis for assessment of organ function. *Clinical Transplantation.* 2004;18(Suppl 12):16-21.
21. Groen H, Moers C, Smits JM, Treckman J, van Gelder F, Rahmel A, Paul A, Pirenne J, Ploeg RJ, Buskens E. Cost-effectiveness of hypothermic machine perfusion versus static cold storage in kidney transplantation: first results of the prospective european RCT. *Transplantation.* Supplement. 2009;86(2S):93-94.
22. Kievet JK, Nederstigt AP, Oomen AP, Janssen MA, Schoot L, Koostra G. Release of alpha-glutatione S-transferase (alpha GST) and pi-glutathione S-transferase (pi GST) from ischaemic damaged kidneys into the machine perfusate-relevance to viability assessment. *Transplant Proceedings*. 1997;29:3591-3593.
23. Kievet JK, Oomen APA, Janssen MA, van Kreel BK, Heineman E, Koostra G. Viability assessment of non-heart-beating donor kidneys by alpha glutathione S-transferase in the machine perfusate. *Transplant Proceedings.* 1997;29:1381-1383.
24. Polyak MMR, Arrington MO, Stubenbord WT, Kapur S, Kinkhabwala M. Prostaglandin E1 influences pulsatile preservation characteristics and early graft function in the expanded criteria donor kidneys. *Journal of Surgical Research*. 1999;85:17-25.
25. Jochmans I, Monbaliu D, Pirenne J. Neutrophil gelatinase-associated lipocalin, a new biomarker candidate in perfusate of machine-perfused kidneys: a porcine pilot experiment. *Transplant Proceedings*. 2011 Nov;43(9):3486-9.
26. Nath J, Guy A, Smith TB, Cobbold M, Inston NG, Hodson J, Tennant DA, Ludwig C, Ready AR. Metabolomic analysis during kidney machine perfusion: The pig provides an appropriate model for human studies. *PloS One* .1. 2014; 9(12):e114818

2. Objectives

2.1. Primary Objectives

To determine if we can optimise the metabolic function of discarded human kidneys during machine perfusion by altering the parameters of perfusion (perfusate constituents, flow dynamics, thermal environment) and then monitor this by analysing kidney tissue, fluid and cells .

2.2. Secondary Objectives

To determine if the fluid and/or cells washed out of a kidney during perfusion can help us to assess the kidney and diagnose problems within the organ that can be addressed. The cellular response to machine perfusion (including immunological and endothelial) will be evaluated during standard and altered perfusion conditions .

3. Study Design

3.1. Methodology

This is an experimental study involving laboratory investigations. When a suitable organ donor is identified, potential recipients for the kidneys (and other organs) are determined and the kidneys are accepted for transplantation on the proviso that the retrieval and implanting surgeon have no concerns regarding the organ. However, if during the retrieval process there are concerns regarding the suitability of the organ (e.g. mechanical damage, suspicious mass, evidence of infection, anatomical abnormality) then this is relayed to the surgical team that had provisionally accepted the organ. If the surgical team then decline the kidney based upon this information for their particular patient, the organ is offered to all other UK transplant centres with a suitable recipient on their transplant waiting list. If the organ is rejected for transplantation by all UK centres, and the donor has given consent for organ utilisation for research purposes, the organ is then offered via the specialist nurse for organ donation at the donor centre to suitable centres for research. It is important to stress that organs are always retrieved with the intention of transplantation and are only used for research purposes (subject to consent) if they are deemed unsuitable for transplantation by all UK centres.

Upon the arrival of a research organ at our unit, machine perfusion will commence. The parameters of perfusion will be modified to gain further information regarding metabolism. Fluid will circulate throughout the kidney during its time on the machine as occurs in standard clinical practice. However the parameters of perfusion (temperature, perfusion pressures, and perfusion fluid) will be altered from standard practice. Studies will include perfusion fluid containing glucose labeled with a naturally occurring form of carbon (^{13}C). This is a non-harmful isotope, is not radioactive and has a natural abundance of 1.1% (Appendix 4).

Samples of perfusion fluid will be taken throughout the experiment for analysis. As the organ will not be transplanted, we will be able to prolong the duration the organ undergoes machine perfusion

(typically < 15 hours in clinical practice). This will help us to understand and support the long term metabolic effects of machine perfusion. At the end of perfusion, we will take tissue from both cortex and medulla of the perfused kidney. This will be primarily for cell extraction in order to perform tissue NMR (Nuclear Magnetic Resonance) analysis and will provide important information regarding the intracellular metabolism during perfusion, which will complement the extracellular indicators derived from the perfusion fluid. In addition to this, tissue samples will also be utilised to gain information regarding cellular and structural function, both immunological and endothelial. Primary cell lines may also be cultured from kidney tissue. At the experiment endpoint we will remove the filter from the machine, through which the fluid recirculates, and analyse the cells captured in the filter.

Laboratory analysis of the samples will be performed following storage and processing. The basis of the studies will be the molecular analysis of inflammatory markers. Cellular studies will be based on characterising the number and nature of the cells. The analysis is simple and currently standard practice in most laboratories. No identifiable donor details other than age and sex will be utilised in this study.

3.2. Endpoint

The end will be a time point (1 March 2018) or after the collection of samples from 50 donor perfusates.

3.3. Target Number of Patients

It is estimated that over the 3 year period, 50 kidneys will be accepted for research purposes at our centre.

3.4. Expected Duration of Study

3 years.

3.5. Primary and Secondary Outcome Measures

Primary: Characterisation of the active renal metabolic pathways during alterations of perfusion parameters.

Secondary: Determination of how alterations in machine perfusion conditions affect other cellular processes within the kidney.

4. Subject Enrolment

4.1. Inclusion Criteria

All deceased donor kidneys that have been declined for transplantation by all UK centres and have valid consent for research, which are offered and accepted to our unit for research purposes via the NHSBT allocation system. The procedure for donor consent is discussed in section 7.

4.2. Exclusion Criteria

Experimental studies will be performed on all kidneys accepted by our unit for research purposes.

4.3. Control Groups

Outcomes will be compared within the MP group rather than to a separate control group.

At the endpoint of each study, a tissue biopsy will be performed. Human discarded organs are clearly a non-uniform group and have severe ischaemic damage. Any experimental result will be corroborated on a second organ to minimise any organ dependent errors.

5. Study Specific Procedures

5.1. Clinical and Laboratory Assessments

We will take samples of the perfusion fluid for analysis at multiple timepoints during machine perfusion, (commonly T0, at 45minutes, and at 4, 18, 24 and 48 hours). Five milliliters of perfusion fluid will be taken at each time interval, except for the final sample which will include all remaining perfusion fluid from the machine (approximately 970mls). Tissue sampling will occur at the end point of perfusion and will include sample from both cortex and medulla. Up to 100g of tissue from each organ will be removed for sampling.

Samples in the laboratory (tissue and fluid) will be labeled and placed in a -80°C freezer and will not contain any patient identifiable data. Sample preparation for NMR analysis currently involves mixing the perfusate samples with phosphate buffer solution containing 2 mM TSP [(3-trimethylsilyl)propionic-(2,2,3,3-d4)-acid sodium salt] and deuterium oxide (D_2O) however this may alter slightly with different NMR protocols. Tissue samples will be mechanically homogenized and cell extraction performed to allow metabolic analysis of the intracellular environment.

Assessment of the samples will involve immunological and biochemical assays (e.g ELISA), with microscopic and histological analysis of structural integrity. However, the principle objective will be metabolomic analysis using NMR spectroscopy. DNA and genomic studies will not be performed.. The majority of these processes are standard practice in most laboratories

6. Data Management and Statistics

6.1. Statistical Analysis

This will be a laboratory based experimental study to determine metabolic pathway activity during perfusion. As such no complex statistical analysis will be performed.

6.2. Data Monitoring & Interim Analysis

Data will be prospectively collected and interim analysis is not planned as it will not affect outcomes and no patient intervention is dependent upon this assessment.

6.3. Determination of Sample Size

Over the study period, it is estimated that 30-50 kidneys per year will be offered to our department for research purposes, of which assuming a 50% acceptance rate due to organ suitability and researcher availability will allow a sample collection for 45-75. This will enable the accurate determination of metabolic pathway processes occurring during differing perfusion conditions.

6.4. Direct Access to Source Data/Documentation/Data Handling

Access to source data/documents by appropriate regulatory bodies will be permitted. Data will be stored on an excel spreadsheet on the departmental laptop which is password protected with full McAfee security accessed only by the chief investiagtor. It will be stored in a locked office in the renal department which requires swipe card access. Data will be encrypted.

7. Ethics

Consent for participation in this experimental research will be obtained either through patients pre-morbid declaration of consent (in the form of donor card or register), or by next of kin proxy consent acting on behalf of the donor. This is standard practice for cadaveric organ donation in England. All organs are obtained with the pre-operative intent of transplantation. Kidneys will be used in this study when such organs are later found to be non-transplantable, and the patient or relative has given specific consent for research purposes.

8. Finance

The financing of the project will be via the surgical research fund administered via the UHB charitable trust funds.

9. Publication Policy

It is predicted that data derived from this study will be disseminated via transplantation meetings and peer reviewed journals. Data will be treated according to GCP and Caldicott data protection with no patient identifiable data.

Appendix 1 Submission to NHSBT (Approved)

A study to determine the mechanism and effect of machine perfusion on cadaveric kidneys unsuitable for transplantation

Submission to NHSBT/ODT Research Approval Process

Mr. Jay Nath
Dr. Mark Cobbold
Mr. Andrew Ready

University Hospital Birmingham/ University of Birmingham

Abstract of the proposed investigation

Introduction

The use of Hypothermic Machine Perfusion (HMP) in the period between kidney retrieval and implantation is supported by robust clinical evidence with improved early graft outcome [1–9]. Flow dynamics during perfusion are likely to account for some of the benefits of HMP, with reduced intra-renal resistance (and therefore increased flow) a marker of good graft function [10–12]. However, the exact mechanism by which HMP improves outcome remains unclear and there is likely to be a metabolic component underlying these beneficial effects [13, 14]. Accordingly, improved metabolic support during perfusion becomes a target for graft optimisation. Experimental studies are needed to clarify these mechanisms and optimisation of preservation may lead to improved transplant outcomes, especially in marginal kidneys. However the metabolic activity in this ex vivo, hypoxic, hypothermic environment is poorly understood.

Metabolomic analysis using NMR spectroscopy permits identification and quantification of a large number of metabolites within a biological sample and is the subject of great interest. Easy access to perfusate during HMP and ability to perform serial measurements render this an attractive technique and may provide the option to improve the metabolic function of organs with obvious potential benefits. We have shown that perfusate analysis of human cadaveric kidneys is feasible and can be used to reliably predict post transplant graft function [15]. However, whilst perfusate analysis remains promising, only limited conclusions can be made regarding the nature of the renal intracellular metabolism without tissue sampling.

Aim

The aim of this study is to determine the metabolic function of human kidneys during a prolonged period of machine perfusion (up to 72 hours) and to try and optimise this metabolic function by altering the parameters of perfusion (perfusate constituents, flow dynamics, thermal environment).

Methods

Upon the arrival of a research organ at our unit, machine perfusion will commence.

However, the parameters of perfusion (temperature, perfusion pressures, and perfusion fluid) will be altered from standard practice. This will include altering the constituents of the perfusion fluid. Studies will include introducing metabolites labeled with a naturally occurring form of carbon (13C) . This is a non-harmful isotope, is not radioactive and has a natural abundance of 1.1%.

As the organ will not be transplanted, we will be able to prolong the duration the organ undergoes machine perfusion (typically < 15 hours in clinical practice). This will help us to understand and support the long term metabolic effects of machine perfusion. Samples of perfusion fluid will be taken throughout the experiment for analysis. Timepoints will include T0, 30 minutes and at 6, 12, 18, 24 and 48 hours. At the end of perfusion, we will take tissue from both cortex and medulla of the perfused kidney. Up to 100g of tissue from each organ will be removed for analysis. Laboratory analysis of the samples will be performed following storage and processing. The basis of the studies will be metabolomic analysis using NMR (Nuclear Magnetic Resonance) spectroscopy using both 1D and 2D tecniques.

Tissue samples will be mechanically homogenised and cell extraction performed to allow metabolic analysis of the intracellular environment. Sample preparation for NMR analysis involves mixing the perfusate samples with phosphate buffer solution containing 2 mM TSP [(3-trimethylsilyl)propionic-(2,2,3,3-d4)-acid sodium salt] and deuterium oxide (D2O).

In addition to the primary metabolic information, we will also perform further complementary studies on these valuable organs. These will include immunological and biochemical assays, as well as indicators of structural integrity, including microscopic and histological investigations.

Expected outcomes
1. Characterisation of metabolites and active metabolic pathways within a machine perfused human kidney during 72 hours of machine perfusion.

2. Determination of the effect of temperature, perfusion pressure, and oxygenation upon renal metabolism during machine perfusion.

3. Determination of the effect of HMP on cellular integrity using electron microscopy and immunohistochemistry.

References

1. Moers C, Smits JM, Maathuis MH, Treckmann J, van Gelder F, et al. (2009) Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med 360: 7-19.
2. Cannon RM, Brock GN, Garrison RN, Smith JW, Marvin MR, et al. (2013) To pump or not to pump: a comparison of machine perfusion vs cold storage for deceased donor kidney transplantation. J Am Coll Surg 216: 625-633; discussion 633-624.

3. Lodhi SA, Lamb KE, Uddin I, Meier-Kriesche HU (2012) Pulsatile pump decreases risk of delayed graft function in kidneys donated after cardiac death. Am J Transplant 12: 2774-2780.
4. Kwiatkowski A, Wszola M, Kosieradzki M, Danielewicz R, Ostrowski K, et al. (2007) Machine perfusion preservation improves renal allograft survival. Am J Transplant 7: 1942-1947.
5. Wight JP, Chilcott JB, Holmes MW, Brewer N (2003) Pulsatile machine perfusion vs. cold storage of kidneys for transplantation: a rapid and systematic review. Clin Transplant 17: 293-307.
6. Shah AP, Milgrom DP, Mangus RS, Powelson JA, Goggins WC, et al. (2008) Comparison of pulsatile perfusion and cold storage for paired kidney allografts. Transplantation 86: 1006-1009.
7. Matsuoka L, Shah T, Aswad S, Bunnapradist S, Cho Y, et al. (2006) Pulsatile perfusion reduces the incidence of delayed graft function in expanded criteria donor kidney transplantation. Am J Transplant 6: 1473-1478.
8. Suarez JF, Riera L, Franco E, Ruiz R, Roig M, et al. (1999) Preservation of kidneys from marginal donors with pulsatile perfusion machine. Transplant Proc 31: 2292-2293.
9. Sellers MT, Gallichio MH, Hudson SL, Young CJ, Bynon JS, et al. (2000) Improved outcomes in cadaveric renal allografts with pulsatile preservation. Clin Transplant 14: 543-549.
10. Polyak MM, Arrington BO, Stubenbord WT, Boykin J, Brown T, et al. (2000) The influence of pulsatile preservation on renal transplantation in the 1990s. Transplantation 69: 249-258.
11. Matsuno N, Kozaki K, Degawa H, Narumi Y, Suzuki N, et al. (2000) A useful predictor in machine perfusion preservation for kidney transplantation from non-heart-beating donors. Transplant Proc 32: 173-174.
12. Kozaki K, Sakurai E, Uchiyama M, Matsuno N, Kozaki M, et al. (2000) Usefulness of high-risk renal graft conditioning: functional improvement of high-risk grafts by addition of reagents to continuous hypothermic perfusion preservation solution. Transplant Proc 32: 164-166.
13. Fuller BJ, Lee CY (2007) Hypothermic perfusion preservation: the future of organ preservation revisited? Cryobiology 54: 129-145.
14. Henry SD, Nachber E, Tulipan J, Stone J, Bae C, et al. (2012) Hypothermic machine preservation reduces molecular markers of ischemia/reperfusion injury in human liver transplantation. Am J Transplant 12: 2477-2486.
15. Guy AJ, Nath J, Cobbold M, Ludwig C, Tennant DA, et al. (2014) Metabolomic Analysis of Perfusate During Hypothermic Machine Perfusion of Human Cadaveric Kidneys. Transplantation.

Lay summary

A non-scientific description of the study

Kidney transplantation is the best treatment for patients with renal failure, providing both survival benefit and an improved quality of life when compared with the alternative, regular dialysis. Most kidneys available for transplantation in the UK are from deceased patients who have agreed to organ donation. There are more patients waiting for a kidney transplant than there are organs available. This has lead to several measures to try to

increase the number of donated kidneys suitable for transplantation.
One of these measures is the use of kidneys that might otherwise have been rejected as they were deemed unsuitable. These kidneys are termed 'marginal' kidneys and may not work as well as kidneys which fulfil the usual criteria. If the quality of these 'marginal' kidneys can be improved or maintained, then more kidney transplantations could take place, improving outcomes for more patients with renal failure.
When kidneys are removed from deceased individuals who have agreed to donation, there is a variable delay before the kidney can be transplanted into an appropriate patient. During this time, the kidney is normally stored in a cool box. The cold environment helps to preserve the kidney before it is transplanted. However, this method of storage is not perfect and damage to the kidney can occur, especially with increased storage time. Marginal kidneys may be particularly prone to damage.
An alternative to ice box storage is machine perfusion. The perfusion machine again provides a cold environment, but also allows fluid to be circulated through the kidney whilst it is being stored. Research has been published suggesting that machine perfusion may be beneficial to the kidney and improve outcomes for the recipients of these kidneys. It has also been suggested that machine perfusion may be of particular benefit for marginal kidneys.
The mechanism by which machine perfusion produces these beneficial effects is probably multifactorial, but there is increasing evidence that supporting metabolism is a key component of this. By gaining further information about which metabolic pathways are active during machine perfusion from kidneys with such severe damage that they are deemed unsuitable for human transplantation, we aim to manipulate the parameters of perfusion to improve the organs metabolic function. We have defined the metabolic fingerprint of a kidney that has immediate function post transplantation and those which do not.
The aim of this study is to try and convert the unhealthy metabolic fingerprint of a severely damaged kidney into one with a 'good prognosis' metabolic fingerprint. This will complement our previous studies using the pig kidney model that have shown that metabolic manipulation is possible. Ultimately this knowledge will enable metabolic optimisation of kidneys prior to transplant in order to maximise the quality of donor organs.

Project duration and or number of organs/ samples required/ Extent of REC approval ie what counties within the UK does it cover/ Funding

Start date 02/03/2015
Completion date 01/03/2018
N=X 50
Areas covered by REC approval We are currenlty awaiting REC approval.
Funding £200,000

Support Required from ODT

Please include all support your study requires from NHSBT i.e. SN-OD involvement: consent/authorisation referral to researche; retrieval by the NORS teams; packing of the organ; access to donor data (requires require a separate application to Clinical Audit & Statistics dept) etc...

We require consent/authorisation and referral to researche via SN-OD, retrieval by NORS team and packing of organs.

Internal Use Only

University Hospitals Birmingham NHS
NHS Foundation Trust

A Study to Determine the Metabolic effects of Machine Perfusion on Cadaveric Kidneys for Transplantation

STUDY TITLE:

A Study to Determine the Metabolic effects of Machine Perfusion on Cadaveric Kidneys for Transplantation

Protocol Number:	1
Phase:	Not Applicable
Study Reference Code:	
Version Number:	1
Date:	1st June 2015
Study Sponsor Name & Address	Dr Christopher Counsell Room 17, Education Centre, Queen Elizabeth Hospital Birmingham, Edgbaston, Birmingham, B15 2WB.

VERSION CONTROL LOG

Version number	Date	Summary of changes
1	1st June 2015	N/A

PROTOCOL SIGNATURE SHEET

This protocol has been approved by:

Name & Address: *<print – delete this text on completion>*	**Role:** **Sponsor Representative**
Signature: _____	**Date:** _____

Name & Address *<print name – delete this text on completion>*	**Role:**
Signature: _____	**Date:** _____

Principal Investigator Declaration:

I have read and understood the requirements and conditions of the study protocol. I am aware of my responsibilities as an Investigator under the guidelines of the Internal Conference on Harmonisation Good Clinical Practice (ICH GCP) standards, the Declaration of Helsinki, local regulations (as applicable) and the study protocol. I agree to conduct the study according to these guidelines and to appropriately direct and assist the study team assigned to me who will be involved in the study.

I agree to use the study material, including medication, only as specified in the protocol.
I understand that changes to the protocol must be made in the form of an amendment that must be approved by the Ethics Committee and Regulatory Authorities prior to its implementation.

I understand that non-compliance with the study protocol may lead to early termination of the study.

Local Investigator's Name & Address: *<print name – delete this text on completion>*	
Signature: _____	**Date:** _____

Return the original wet signature* page to the sponsor and retain a copy* with the study protocol within the Investigator's Site file.

* *Reference to wet signature means signature in ink, copy can be a scanned copy or photocopy of the signature page.*

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS & DEFINITIONS

Abbreviation	Definition
MP	Machine Perfusion
DCD	Donation after Cardiac Death
ECD	Expanded Criteria Donor
SCD	Standard Criteria Donor
DGF	Delayed Graft Function
CS/SCS	Cold Storage/Static Cold Storage
HMP	Hypothermic Machine Perfusion
PNF	Primary Non-Function

1. Background Information

The aim of this research project is to investigate the metabolism of kidneys for transplant during the Machine Perfusion (MP) process.

Kidney transplantation is the gold standard treatment for patients with end-stage renal failure providing both survival benefit and improved quality of life when compared with dialysis.[1] In addition there are economic benefits. Renal transplantation provides a cost saving of over £25000 per patient per year compared with other forms of renal replacement therapy.[2]

The rate of kidney transplantation in the UK has increased predominantly due to live donation, with cadaveric donation remaining static over the last decade. Strategies to increase donor numbers have recently been introduced including the use of more donations after cardiac death (DCD) and identification of more possible donors that previously were considered unsuitable. These strategies whilst increasing numbers have not concentrated on the quality of kidneys offered and one of the changes to emerge is the acceptance of kidneys that might previously have been rejected. These kidneys are termed marginal or expanded criteria donor (ECD) kidneys. Compared to standard criteria donor (SCD) kidneys these ECD kidneys are associated with higher risks of graft failure. This is also the case for DCD kidneys that have higher rates of delayed graft function (DGF).[3] The greater availability of marginal kidneys has led to an increased interest in methods to ensure and maintain quality and outcomes not only for this subgroup but for all donated kidneys.

Traditionally the standard storage method for kidneys in the UK is static cold storage (CS). After procurement, the organ is flushed with cold preservation fluid and stored and transported on ice. The hypothermic conditions lower the metabolic rate and oxygen requirement of the kidney, which helps to preserve it until implantation.

The alternative to static cold storage is machine perfusion (MP), which is usually hypothermic (HMP). The kidney is stored in a box that provides a cold environment and pulsatile recirculation of preservation fluid. From large international studies in recent years evidence has been generated to support the benefits of MP in particular reduced DGF and primary non-function (PNF), and a long-term survival benefit for MP compared to CS kidneys (see below). In addition to the direct benefits to the organ the ability to prevent injury to the kidney during perfusion allows further time to allow biopsy and/or better prepare the recipient for transplantation. This is predicted to allow acceptance of more kidneys and better assessment of those organs accepted.

Summary of Trial Findings

Both animal experiments and historical clinical studies have demonstrated that Hypothermic Machine Perfusion of cadaveric kidneys provides improved early graft function when compared with Static Cold Storage.[4,5,6,7,8,9] Studies from the 1990's reported reduced rates of DGF and increased rates of immediate function of renal grafts[10,11,12,13] MP was shown to be preferable for prolonged

preservation times, for kidneys donated after cardiac death and for ECD kidneys.[14,15,16] Daemen *et al* demonstrated reduced DGF for DCD kidneys when preserved by MP instead of CS[17] However, controversy still remained due to a lack of prospective studies and perceived costs associated with MP.

A recent European multicenter randomized controlled trial has provided important evidence[18]. The study involved 336 consecutive deceased donors from whom one kidney was randomized to CS and the other to MP. HMP reduced the risk of DGF and graft failure and improved graft survival in the first year following transplantation. This group has released 3-year outcomes which show these benefits continue, particularly for ECD kidneys[19]

In addition to improved outcomes, MP provides the ability to evaluate organs from ECDs and DCDs prior to transplantation. This may significantly increase the number of organs available for transplantation.[20] Cost benefits have also been demonstrated.[21]

Summary of study to date

Biochemical parameters and ischaemic injury markers have been identified in the perfusate of MP kidneys.[16,22,23,24,25] Based on results gained following initial ethical approval (RRK4459), we have shown that the metabolites that are present in the perfusion fluid of the kidneys can be used to predict the outcome for that kidney, demonstrating that metabolism during perfusion plays a key role in the function post transplantation (Appendix 3). The intention of the current

study is to derive information regarding metabolic pathways during the perfusion process. This will be achieved through perfusing kidneys with clinically used perfusion fluid enriched with a safe, non radioactive, naturally occurring ^{13}C isotope and analyzing small volumes of perfusion fluid, urine samples and kidney tissue biopsy.

Risks of Machine Perfusion, perfusate sampling, urine sampling and tissue biopsy.
There is no evidence of any harmful effects of MP. If there is a machine fault during perfusion the kidney remains cooled as it would if in static cold storage.

There are no health and safety concerns involved in using fluid enriched with ^{13}C isotope and therefore poses no risk to patients (Appendix 4). This is a naturally occurring safe isotope and needless to say is not radioactive. Increasing the proportion of ^{13}C compared to ^{12}C form of glucose will allow informative metabolic studies through NMR analysis. However the total amount of glucose within the fluid (10mM/L) will not be altered and this increased proportion of ^{13}C glucose does not change the biological properties of the fluid with regards to organ perfusion. Therefore this is not a request to use a different perfusion fluid, merely a functionally identical fluid with an increased proportion of a naturally occurring heavy isotope. All fluid will be produced to clinical grade pharmaceutical standards by Organ Recovery Systems.

There are no risks from taking perfusion fluid samples during the perfusion process. There is a designated port for fluid samples and can therefore be

performed in an aseptic manor. The total amount of fluid removed during the study will be less than 5% of the circulating volume.

Urine samples will be obtained from urine collected postoperatively for other purposes and no additional samples needed. This poses no additional risk to patients.

The risk of tissue biopsy is minimal. The biopsy procedure and technique is the same as kidney biopsies that are performed for diagnostic purposes and are performed routinely pre-transplantation by many centres. The risk of any adverse event as a direct result of biopsy is minimal and this is further reduced by performing the biopsy under direct vision pre-operatively, whereby any potential adverse event (bleeding being the most common) can be rectified at operation.

Compliance

The study will be conducted in compliance with the protocol, GCP and research governance guidelines.

Study Population

Participants are deceased donors and transplant recipients. The study will only be performed where there is donor and recipient consent.

Donors are consented for organ donation prior to death and this is confirmed during the donation process by the national organ retrieval service. Only donors who have been consented for both organ donation and research purposes will be

included. Confirmation is performed by a senior nurse in organ donation and is nationally agreed practice.

Transplant recipients will be consented for inclusion in the study by a member of the research team.

References:

1. Wolfe RA, Ashby VB, Milford EL, Ojo AO, Ettenger RE, Agodoa LY, *et al*. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of first cadaveric transplant. *N Engl J Med* 1999;341:1725-30.
2. NHS Blood and Transplant. Organ donation: cost-effectiveness of transplantation.2011.www.uktransplant.org.uk/ukt/newsroom/fact_sheets/cost_effectiveness_of_transplantation.jsp.
3. Jochmans I, Pirenne J. Graft quality assessment in kidney transplantation: not an exact science yet! *Curr Opin Organ Transplant* 2011,16:174-179.
4. Matsuoka L, Shah T, Aswad S, Bunnapradist S, Cho Y, Mendez RG, Mendez R, Selby R. Pulsatile perfusion reduces the incidence of delayed graft function in expanded criteria donor kidney transplantation. *Am J Transplant*. 2006;6:1473-1478.
5. Nicholson ML, Hosgood SA, Metcalfe MS, Waller JR, Brook NR. A comparison of renal preservation by cold storage and machine perfusion using a porcine autotransplant model. *Transplantation*. 2004;78:333-337.
6. Schold JD, Kaplan B, Howard RJ, Reed AI, Foley DP, Meier-Kriesche. Are we frozen in time? Analysis of the utilization and efficacy of pulsatile perfusion in renal transplantation. *Am J Transplant*. 2005;5:1681-1688.
7. Stratta RJ, Moore PS, Farney AC, Rogers J, Hartmann EL, Reeves-Daniel A, Gautreaux MD, Iskander SS, Adams PL. Influence of pulsatile perfusion preservation on outcomes in kidney transplantation from expanded criteria donors. *J Am Coll Surg*. 2007;204:873-882.
8. Wight J, Chilcott J, Holmes M, Brewer N. The clinical and cost-effectiveness of pulsatile machine perfusion versus cold storage of kidneys for

transplantation retrieved from heart-beating and non-heart-beating donors. *Health Technol Assess*. 2003;7:1-94.
9. Zhou YC, Cecka JM. Effect of HLA matching on renal transplant survival. In: Terasaki PI, Cecka JM, editors. *Clinical Transplants*. UCLA Tissue Typing Typing Laboratoty; Los Angeles: 1993.pp.499-510.
10. Barbar WH, Deierhoi MH, Phillips MG, Diethelm AG. Preservation by pulsatile perfusion improves early renal allograft function. *Transplant Proc*. 1988;20:865-868.
11. Halloran P, Aprile M. A randomized prospective trial of cold storage versus pulsatile perfusion for cadaveric kidney transplantation. *Transplantation*. 1987;43:827-832.
12. Koyama H, Cecka JM, Terasaki PI. A comparison of cadaveric kidney storage methods: pump perfusion and cold storage solutions. *Clinical Transplantation*. 1993;7:199-205.
13. Merion RM, Oh HK, Port FK, Toledo-Pereyra LH, Turcotte JG. A prospective controlled trial of cold-storage versus machine-perfusion preservation in cadaveric renal transplantation. *Transplantation.* 1990;50:230-233.
14. McAnulty JF, Ploeg RJ, Southard JH, Belzer FO. –Successful Five-Day Perfusion Preservation of the Canine Kidney. *Transplantation*. 1989;47:37-41.
15. Matsuno N, Sakurai E, Tamaki I, Uchiyama M, Kozaki K, Kozaki M. Effect of machine perfusion preservation on delayed graft function in non-heart-beating donors. *Transplantation*. 1994;57:293-294.
16. Polyak MMR, Arrington MO, Stubenbord WT, Boykin J, Brown T, Jean-Jacques MA, Estevez J, Kapur S, Kinkhabwala M. Influence of pulsatile perfusion on renal transplantation in the 1990s. *Transplantation.* 2000;69(2):249-258.
17. Daemen JHC, DeVries B, Oomen APA, DeMeester J, Koostra G. Effect of machine perfusion preservation on delayed graft function in non-heart beating donor kidneys-early results. *Transpl International*. 1997;10:317-322.
18. Moers *et al* (for the Machine Preservation Trial Study Group). Machine perfusion or cold storage in deceased-donor kidney transplantation. *New Engl J Med*. 2009;360:7-19.
19. Moers C, Pirenne J, Paul A, Ploeg R. Correspondence: Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 2012;366:770-771.
20. Baicu SC, Simmons PM, Campbell LH, Taylor MJ, Brockbank KG. Interstitial fluid analysis for assessment of organ function. *Clinical Transplantation.* 2004;18(Suppl 12):16-21.
21. Groen H, Moers C, Smits JM, Treckman J, van Gelder F, Rahmel A, Paul A, Pirenne J, Ploeg RJ, Buskens E. Cost-effectiveness of hypothermic machine perfusion versus static cold storage in kidney transplantation: first results of the prospective european RCT. *Transplantation.* Supplement. 2009;86(2S):93-94.

22. Kievet JK, Nederstigt AP, Oomen AP, Janssen MA, Schoot L, Koostra G. Release of alpha-glutatione S-transferase (alpha GST) and pi-glutathione S-transferase (pi GST) from ischaemic damaged kidneys into the machine perfusate-relevance to viability assessment. *Transplant Proceedings*. 1997;29:3591-3593.
23. Kievet JK, Oomen APA, Janssen MA, van Kreel BK, Heineman E, Koostra G. Viability assessment of non-heart-beating donor kidneys by alpha glutathione S-transferase in the machine perfusate. *Transplant Proceedings.* 1997;29:1381-1383.
24. Polyak MMR, Arrington MO, Stubenbord WT, Kapur S, Kinkhabwala M. Prostaglandin E1 influences pulsatile preservation characteristics and early graft function in the expanded criteria donor kidneys. *Journal of Surgical Research*. 1999;85:17-25.
25. Jochmans I, Monbaliu D, Pirenne J. Neutrophil gelatinase-associated lipocalin, a new biomarker candidate in perfusate of machine-perfused kidneys: a porcine pilot experiment. *Transplant Proceedings*. 2011 Nov;43(9):3486-9.

2. Objectives

2.1. Primary Objectives

Can we detect the metabolic effects of machine perfusion on kidney transplants using fluid, tissue and cells washed out of the kidney?

The evidence suggests that MP has a beneficial metabolic effect on the kidney. The mechanism of this is poorly understood. We aim to improve understanding of this mechanism by detecting markers in the fluid and tissue that will be representative of processes occurring during perfusion.

2.2. Secondary Objectives

Does the fluid and/or cells washed out of a kidney before transplant help us to assess the kidney and diagnose problems with the kidney that can be identified and addressed?

Various parameters have been suggested as prognostic indicators of how a kidney will function post-MP. Few have proven to be reliable. We will aim to identify markers in the fluid which may fulfill this role.

3. Study Design

3.1. Diagrammatic Overview of Study Design

3.2. Methodology

This is a prospective observational trial involving laboratory investigations. Kidneys available for transplantation are offered through a national matching scheme. When transplant recipients are identified, they will be invited to

participate in the research study. Provided they agree to this and that the organs are suitable, when kidneys arrive at our unit, they will be placed on machine perfusion using ^{13}C enriched perfusion fluid. For patients that do not consent to inclusion, potential kidneys will be machine perfused using standard non enriched perfusion fluid if suitable. Kidneys will be perfused on clinical grounds based on an evidence-based algorithm agreed upon by the renal unit. There will be no randomization or blinding. There is no evidence to date to suggest any harmful effect of perfusing all kidneys but benefits appear limited if perfusion time is short (<12 hours).

Fluid will circulate throughout the kidney during its time on the machine as standard clinical practice. The fluid is designed specifically for use in the machine and to preserve organs (KPS-1®, Kidney Preservation solution, Organ Recovery Systems). The proportion of naturally occurring heavy carbon (^{13}C) incorporated into the glucose within the KPS-1® fluid may be increased in some studies from the naturally abundant level of 1.1% to over 50%. As discussed in background information (p12), this is non radioactive and non harmful and is naturally occurring. There are no health and safety concerns involved in using this (Appendix 4). Increasing the proportion of ^{13}C compared to the ^{12}C form of glucose will allow informative metabolic studies through NMR analysis. However the total amount of glucose within the fluid (10mM/L) will not be altered and this increased proportion of ^{13}C glucose does not change the biological properties of the fluid with regards to organ perfusion. Therefore this is not a request to use a different perfusion fluid, merely a functionally identical fluid

with an increased proportion of a naturally occurring heavy isotope. All fluid will be produced to clinical grade pharmaceutical standards by Organ Recovery Systems. We will take small samples (<5ml) of the fluid for analysis during the perfusion (at 30 mins, between 6-12 hours, 12-18 hours, 18 to 24 hours and at the end point). This does not affect the perfusion in any way. At the end of perfusion and prior to transplantation, we will take a tissue biopsy (14-18F biopsy gun) to be used for research analysis. This type of biopsy is standard practice to provide a baseline tissue histology in many centres.

A small amount of urine (about 10ml) of the urine that is collected routinely postoperatively will be used for research analysis.

Laboratory analysis of the samples will be performed following storage and processing. The basis of studies will be metabolite analysis using Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectroscopy (MS). Cellular studies likewise will be based on characterizing the number and nature of the cells. The analysis is simple and currently standard practice in most laboratories. Donor and recipient data to correlate with this is routinely collected by the transplant unit and includes donor details (age, mode of death, creatinine, history of hypertension) and recipient outcome (creatinine levels, graft function, graft loss and other complications). Donor details need to be collected to identify whether the donor is a SCD or ECD as this may influence outcome. Recipient outcome needs to be collected to compare with standard cold storage and historic groups.

3.3. Endpoint

The end will be a timepoint (1 June 2018) or collection samples from a further 75 donors.

3.4. Target Number of Patients

It is estimated that over the 3 year period 50 kidneys will be perfused per year of which 25 should allow sample collection. Thus we anticipate including 75 kidneys.

3.5. Expected Duration of Study

3 years.

3.6. Primary and Secondary Outcome Measures

Primary: Identification of markers in the perfusate, urine and tissue of MP kidneys representative of metabolic activity during MP.

Secondary: Identification of indicators which may diagnose problems with the MP kidney prior to transplantation or that may help to predict outcome.

4. Subject Enrolment

4.1. Inclusion Criteria

All deceased donor kidneys accepted by the unit for potential transplantation that also have valid consent for research purposes. The procedure for donor consent is discussed in section 11. Recipients of MP kidneys will also be

consented for inclusion in the study and therefore data collection will only be performed whereby there is donor and recipient consent. Recipients are provided with an information sheet (appendix 1 & 2).

4.2. Exclusion Criteria

Kidneys which are anatomically unsuitable for connection to the machine. Kidneys without valid consent for research.

Kidneys where a short cold ischaemic time is predicted (<12 hours)

Recipients whom do not wish to participate in the research.

4.3. Control Groups

Outcomes will be compared within the MP group rather than a separate control group as comparable fluid cannot be collected from cold stored kidneys. We will compare different types of donors i.e. SCD v ECD and outcomes dependent on MP times and levels of biomarkers in the perfusate. Functional outcome can be compared with historic and cold storage groups.

8. Study Specific Procedures

8.7. Clinical and Laboratory Assessments

We will take samples of the perfusion fluid for analysis during machine perfusion (at 30 mins, between 6-12 hours, 12-18 hours, 18 to 24 hours and at the end point). 5 milliliters of perfusion fluid will be taken at each time interval. The fluid will be decanted into separate centrifuge tubes and transported to the lab.

Samples will be taken to the laboratory at each time interval to avoid prolonged storage and possible damage to the samples.

Tissue sampling will be performed using a 14-18 gauge TruCut biopsy gun. A single cores of tissue will be sampled which is less than for standard biopsies.

A small amount of the urine collected routinely in the initial postoperative period (about 10ml) will be utilised for research analysis.

In the laboratory, fluid samples will be spun on the centrifuge at 2000rpm for 10 minutes. This will separate cells and fluid from within the sample. For tissue samples, cell extraction may be performed involving methanol and cholorform separation techniques. The fluid will be decanted from the sample, labeled with the donor number, date and time, and placed in a -80°C freezer. The cells, in the form of a pellet, will be mixed with 1ml of freezing solution (mix of 5ml DMSO/45ml FCS) in an aliquot, labeled as previously, and placed in a -80°C freezer.

Samples will be prepared for NMR analysis by mixing 150 µL of 400 mM (pH 7.0) phosphate buffer containing 2 mM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) and 8mM imidazole 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 DSS concentration of 500 µM.

Assessment of the samples will involve immunological assays (multiplex/ELISA) and also NMR spectroscopy. Tissue architecture will be elucidated from routine histology stains (H&E) and electron microscopy. DNA and genomic studies will not be performed. The basis of studies will be metabolic analysis using nuclear

magnetic resonance and mass spectroscopy. Cellular studies likewise will be based on characterizing the number and nature of the cells. The analysis is simple and currently standard practice in most laboratories

9. Data Management and Statistics

9.1. Statistical Analysis

Statistical analysis is based on statistical advice (Dr P Nightingale) on a similar study for biomarkers in urine of renal transplant donors. This included both multivariate and univariate analysis. The markers used have been assessed in other models. It is not assumed that distribution will be normal and therefore mann-whitney and kruskal-wallis analysis will be used. In addition the levels derived will be interrogated by ROC curve analysis to identify test values and statistics.

9.1.1. Data Monitoring & Interim Analysis

Data will be prospectively collected and interim analysis is not planned as it will not affect outcomes and no patient intervention is dependent upon this assessment.

9.1.2. Determination of Sample Size

Over the study period it is estimated that 50-75 kidneys will be perfused per year of which assuming a 50% consent rate will allow sample collection for 25-37. The assay used is highly sensitive and assumptions of results from similar assays suggest the test is sensitive to discriminate injury from non-injury. A

simple power calculation assuming levels of ~700pg/L with SD 300pg/L for injury and 300pg/L SD 100pg/L with an 80% power and p of 0.05 would require a sample size of 15 for each sample group. As data will be continuous for ROC analysis control vs MP will be compared.

9.2. Direct Access to Source Data/Documentation/Data Handling

Access to source data/documents by appropriate regulatory bodies will be permitted. Data will be stored on an excel spreadsheet on the departmental laptop which is password protected with full McAfee security accessed only by the lead researcher and PI. It will be stored in a locked office in the renal department which requires swipe card access. Data will be encrypted.

11. Ethics

Whilst the biobank cannot yet accept cadaveric tissue the ownership of the samples collected is open to interpretation. There has been debate about ownership and whether tissue and fluid taken from a donor and not yet implanted in the recipient is either donor or recipient property. Although possibly an over cautious approach, we will only include kidneys in this study whereby the donor has consented for research purposes and the recipient agrees to participate. We feel that this is a pragmatic approach and has been the approach involving other transplantation studies within QEHB and UoB.

12. Finance

The financing of the project will be via the surgical research fund administered via the UHB charitable trust funds.

13. Publication Policy

It is predicted that data derived from this study will be disseminated via transplantation meetings and peer reviewed journals. Data will be treated according to GCP and Caldicott data protection with no patient identifiable data.

University Hospitals Birmingham NHS
NHS Foundation Trust

CONSENT FORM FOR RESEARCH STUDY

Effects of Machine Perfusion on Cadaveric Kidneys for Transplantation

Name of researchers: Mr Nicholas Inston,,Mr Jay Nath, Mr Kamlesh Patel

Please initial to confirm

- I confirm that I have read and understood the information sheet version …….Date……..…….. **Effects of Machine Perfusion on Cadaveric Kidneys for 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 the 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 the researcher)	Date	Signature
Researcher	Date	Signature

Machine Perfusion in Kidney Transplantation

PARTICIPANT INFORMATION SHEET
KIDNEY PERFUSION STUDY

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

The following information describes a study which we are inviting you to take part in, the results of which will contribute towards a PhD qualification for the lead researcher.

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 have been reported in medical studies to be 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.

It has been standard practice to flush transplant kidneys with fluid using a specialised machine for over five years and is recommended in national guidelines.

We are undertaking this research to help us understand how the flushing process benefits the kidney at a metabolic level.
To better understand this, one of the components of the fluid used to flush the kidney (glucose) will be labelled or 'chemically dyed'. This labelled glucose is a safe, naturally occurring substance and is identical to the unlabelled version with regards to kidney biology.

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. The kidney will be perfused by the standard perfusion fluid. However in order to gain further information regarding metabolism in the organ, one of the components of the fluid (glucose) will be 'labelled' or 'chemically dyed' in order for us to identify what happens to this during perfusion. This is a safe, naturally occurring compound and is biologically identical to the unlabeled version with regards to kidney perfusion. At the end of the perfusion process, we will take a small biopsy of the kidney to identify what metabolites are present within the cells. This type of biopsy is performed routinely prior to transplantation by many centres. 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.

We hope that by better understanding what happens in the kidney during this flushing process, we will be able to improve their function. This could lead to an increase in the number of kidneys available for transplant in the future.

What would I have to do?

We need your permission to perfuse the kidney with labelled fluid, perform a research biopsy following perfusion and 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.

We need your permission to take part in the study. This will include using labelled fluid to flush through the transplant kidney, taking a small sample (biopsy) of the kidney at the end of flushing for research, taking a sample of your urine after your operation and recording the results of your blood tests and how well your kidney is working and any problems you might have. Medical records and data may be accessed by researchers, regulatory authorities or the NHS trust. There are no extra blood tests or extra appointments.

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.

Potential Harm or Benefit to Participants

Studies suggest that kidneys that are machine perfused work better than those stored in an ice box. No harmful effects of machine perfusion have been identified. There are no harmful effects of using labelled perfusion fluids. The risk associated with kidney biopsy is minimal and is is a test that performed routinely during and following kidney transplant in order to determine the cellular structures. Risks of biopsy are reduced by performing this procedure under vision in theatre. No extra tests are carried out following your operation.

There are no harmful effects of using labelled fluids during this flushing process. The risk associated with kidney biopsy is very small and is a test performed routinely for non-research purposes. We have had no serious problems with this type of biopsy in this hospital and estimate the risk of serious harm to be less than 1:1000. No extra tests are carried out following your operation.

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.

If you are interested in the results of the study a summary can be sent to you once all of the analyses are complete.

Funding

This study is funded by University Hospitals Birmingham Charities and Organ Recovery Systems and has been approved by the relevant ethics committee.

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.

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. At the end of the study, any personal data is kept securely for 12 months and then destroyed.

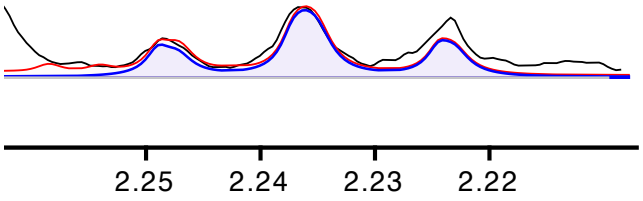
Thank you very much for reading this information and we hope you will be part of the study. Feel free to ask any questions.

If you would like general, impartial advice on taking part in research studies we would suggest contacting the PALS office on 0121 3712550.

Mr Inston (Consultant Surgeon)
Mr Nath (Research registrar)
Tel: [redacted]

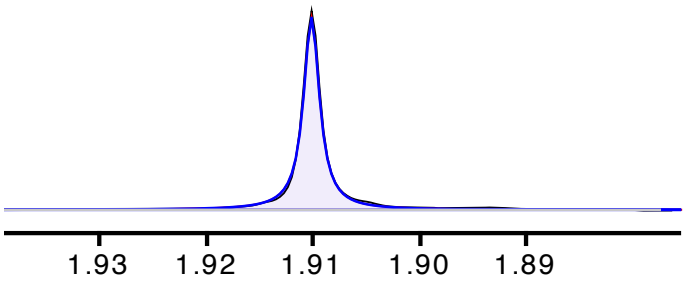
Spectral peak allocation for 26 metabolites detected in a porcine model of HMP and SCS kidney storage

2-aminoadipate



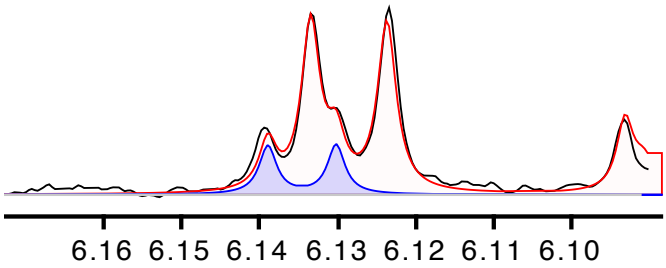
^{1}H [PPM]

Acetate



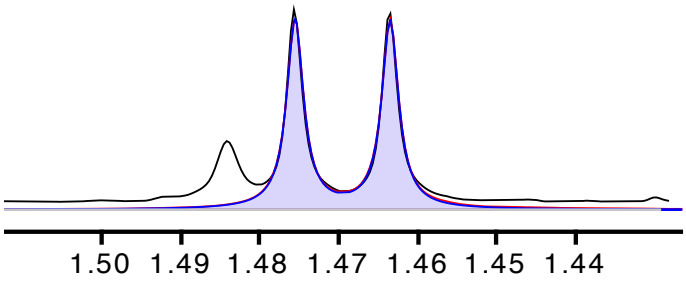
^{1}H [PPM]

ADP



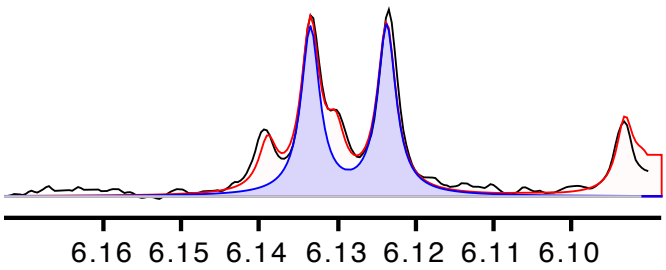
^{1}H [PPM]

Alanine



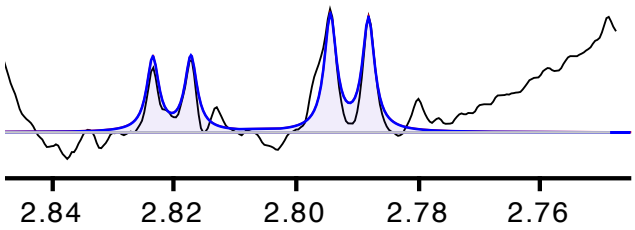
^{1}H [PPM]

AMP



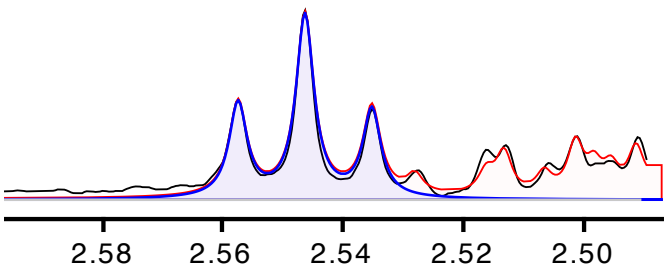
^{1}H [PPM]

Apartate



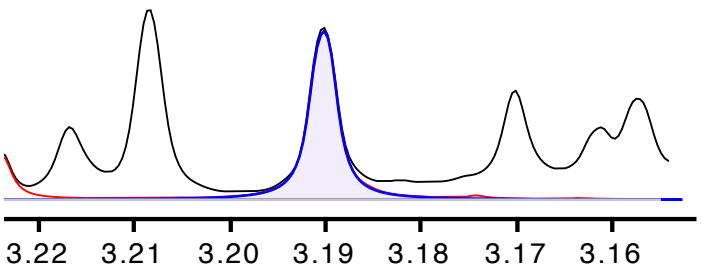
^{1}H [PPM]

β-Alanine

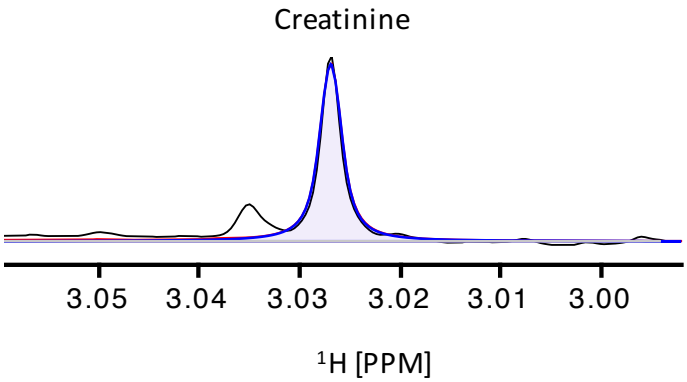


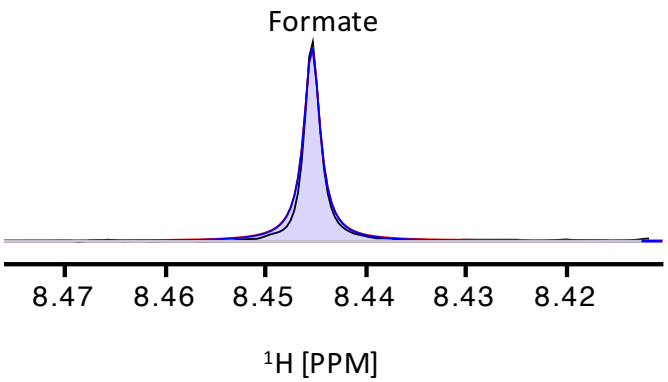
^{1}H [PPM]

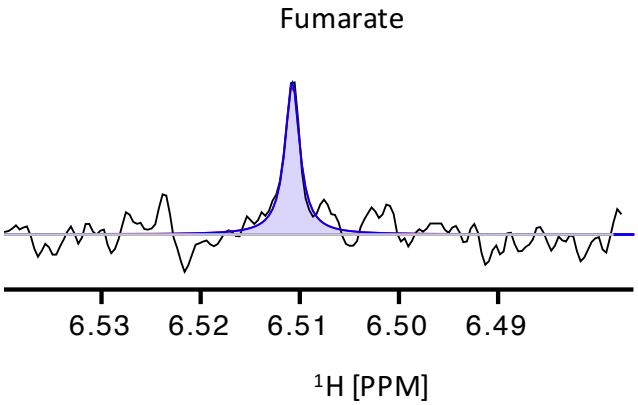
Choline

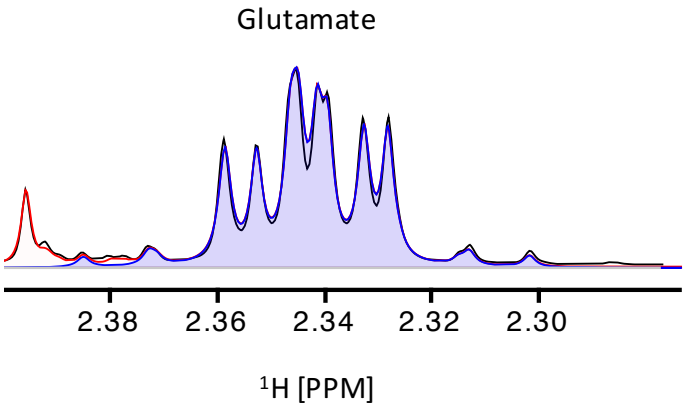


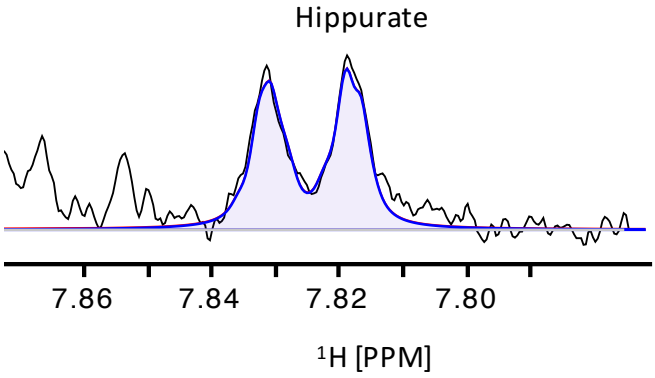
^{1}H [PPM]

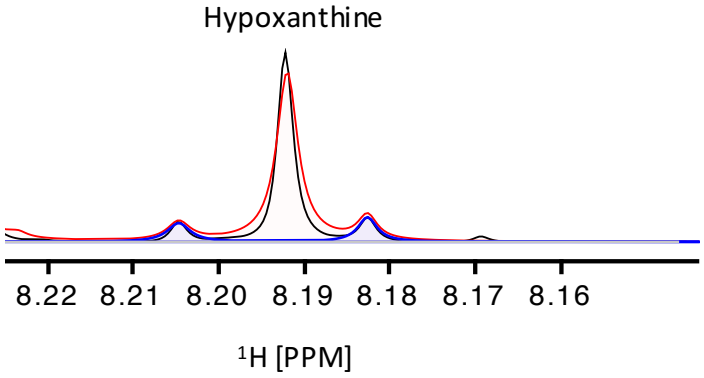

Creatinine
3.05
3.04
3.03
3.02
3.01
3.00
¹H [PPM]

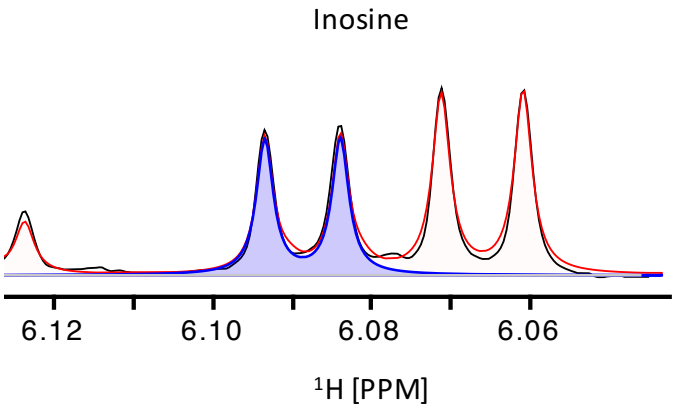

Formate
8.47
8.46
8.45
8.44
8.43
8.42
¹H [PPM]

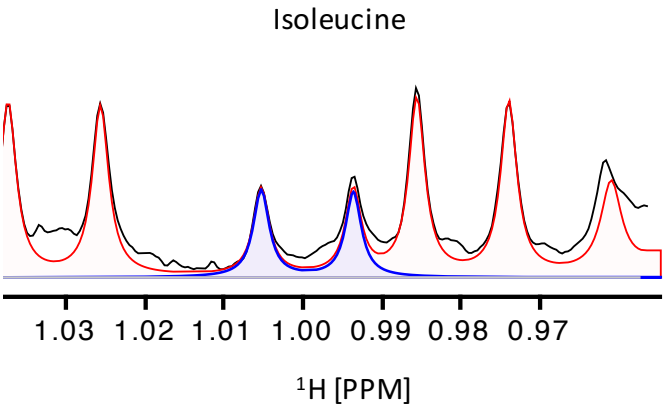

Fumarate
6.53
6.52
6.51
6.50
6.49
¹H [PPM]

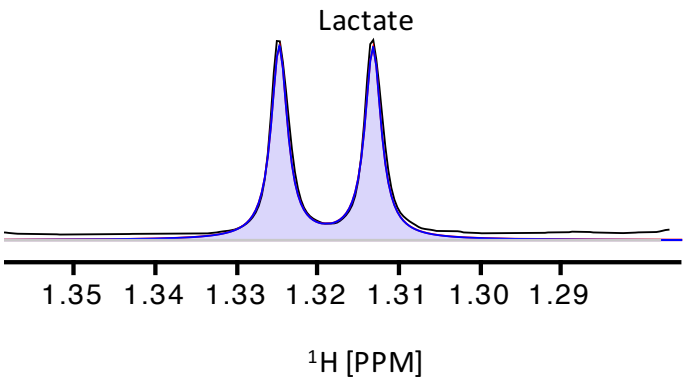

Glutamate
2.38
2.36
2.34
2.32
2.30
¹H [PPM]

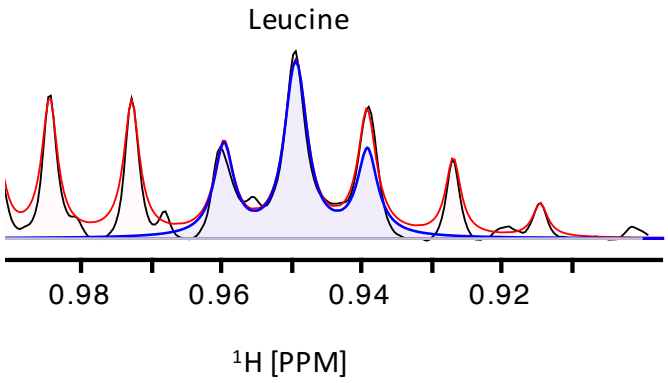

Hippurate
7.86
7.84
7.82
7.80
¹H [PPM]

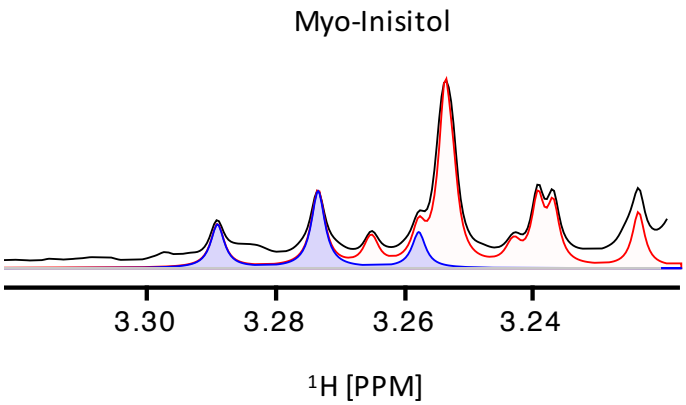

Hypoxanthine
8.22
8.21
8.20
8.19
8.18
8.17
8.16
¹H [PPM]

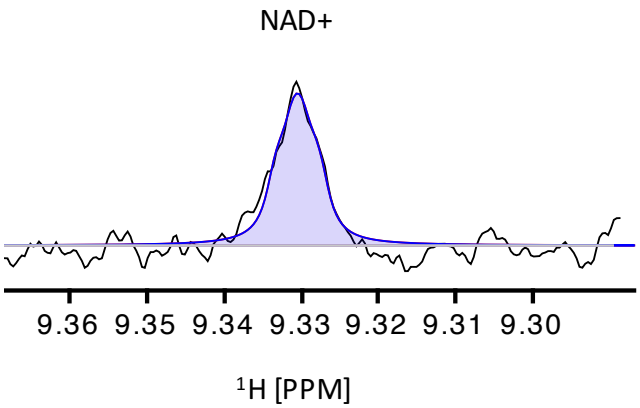

Inosine
6.12
6.10
6.08
6.06
¹H [PPM]

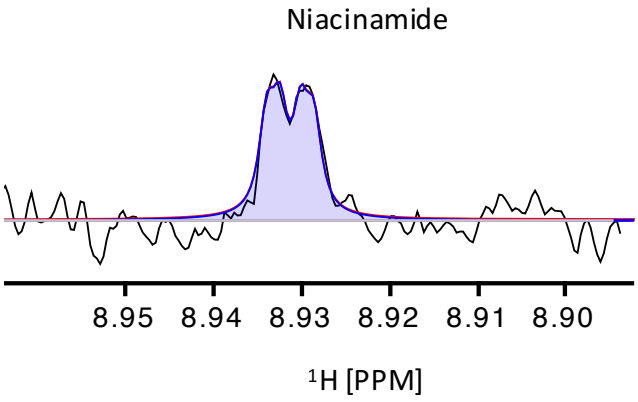

Isoleucine
1.03
1.02
1.01
1.00
0.99
0.98
0.97
¹H [PPM]

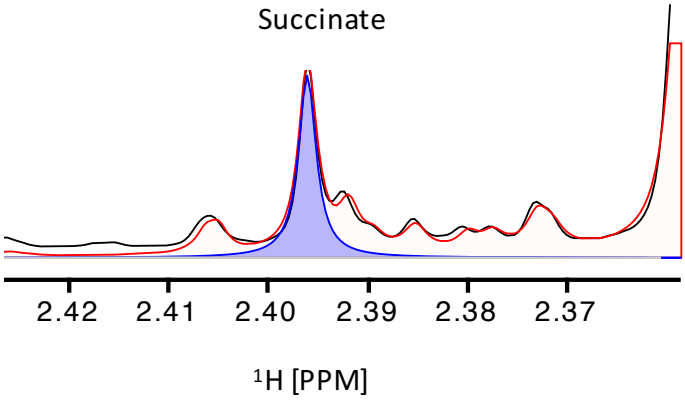

Lactate
1.35 1.34 1.33 1.32 1.31 1.30 1.29
1H [PPM]

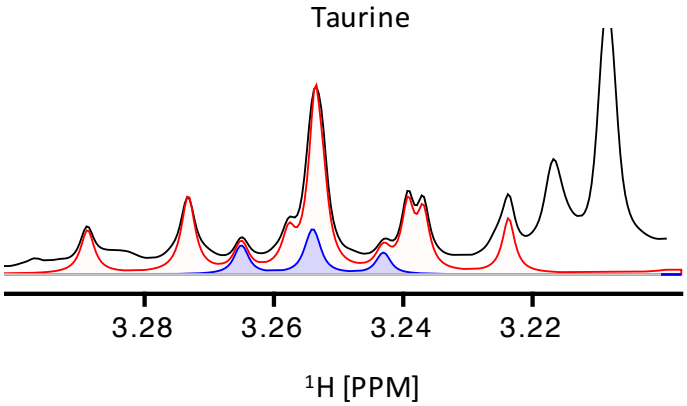

Leucine
0.98 0.96 0.94 0.92
1H [PPM]

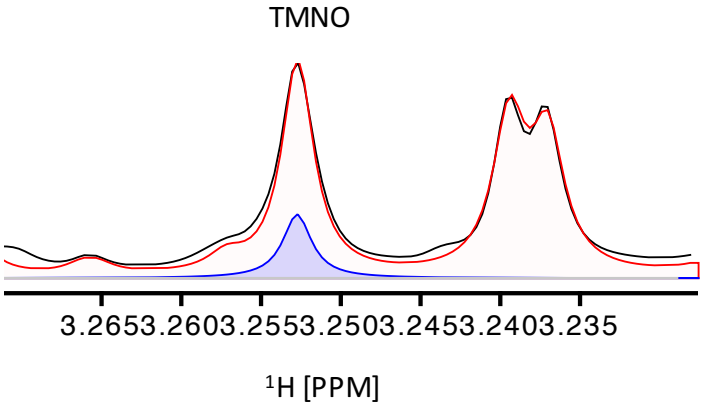

Myo-Inisitol
3.30 3.28 3.26 3.24
1H [PPM]

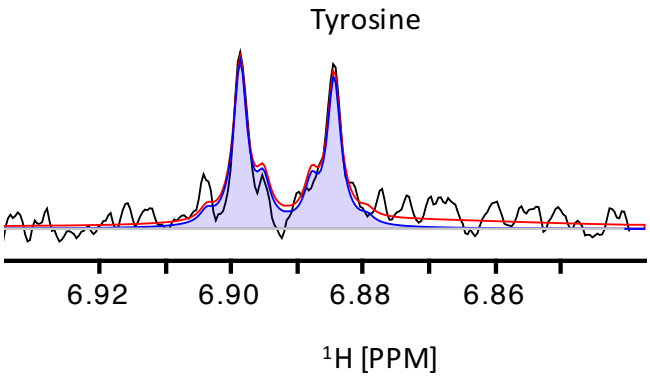

NAD+
9.36 9.35 9.34 9.33 9.32 9.31 9.30
1H [PPM]

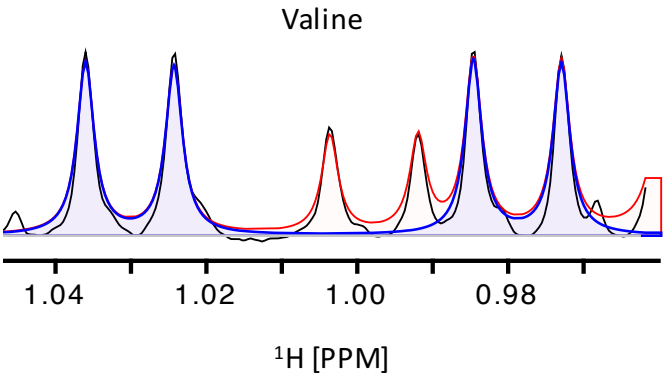

Niacinamide
8.95 8.94 8.93 8.92 8.91 8.90
1H [PPM]


Succinate
2.42 2.41 2.40 2.39 2.38 2.37
1H [PPM]


Taurine
3.28 3.26 3.24 3.22
1H [PPM]

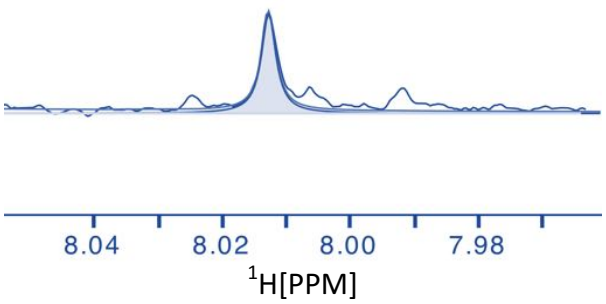

TMNO
3.2653.2603.2553.2503.2453.2403.235
1H [PPM]


Tyrosine
6.92
6.90
6.88
6.86
1H [PPM]

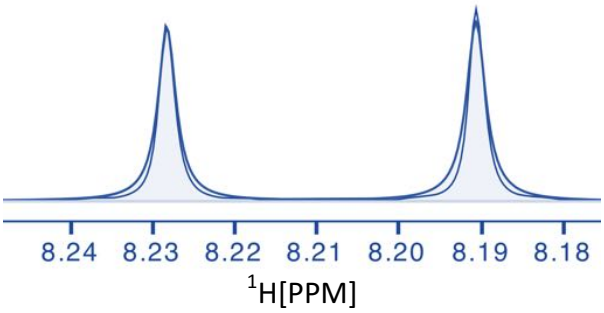

Valine
1.04
1.02
1.00
0.98
1H [PPM]

Spectral Peak allocation for 18 metabolites found in porcine kidney cortex tissue after storage by HMP

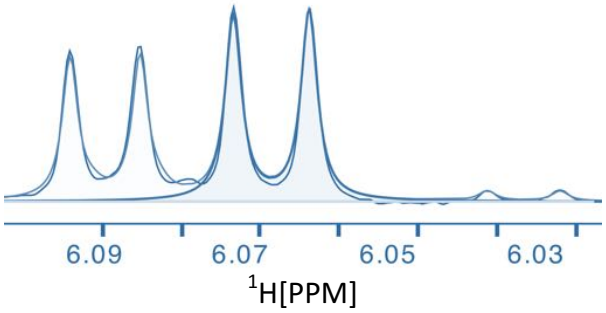
3-Methylxanthine



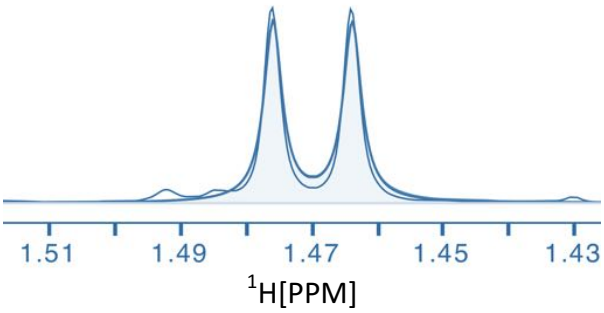
Adenine



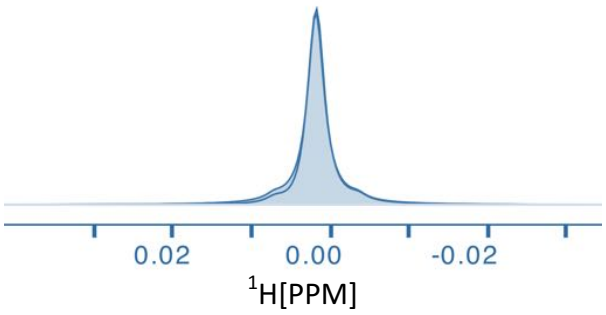
Adenosine



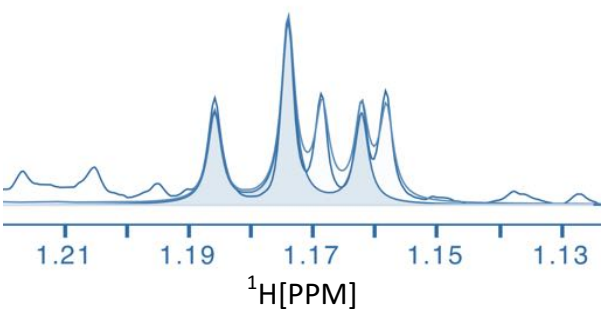
Alanine



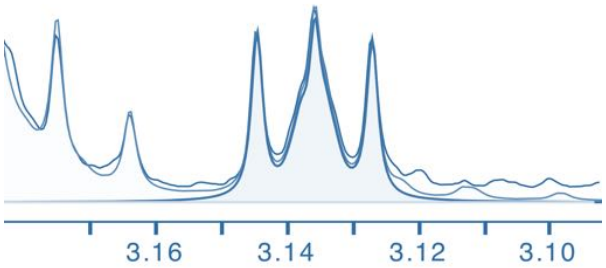
DSS-d6



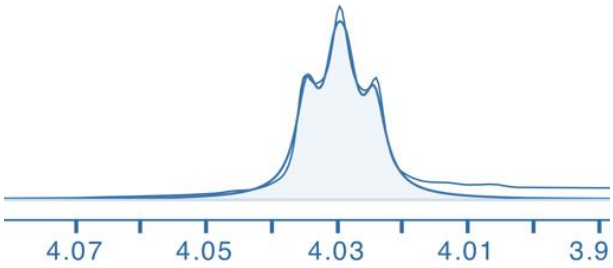
Ethanol



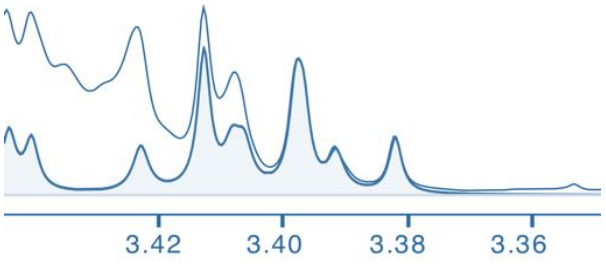
Ethanolamine



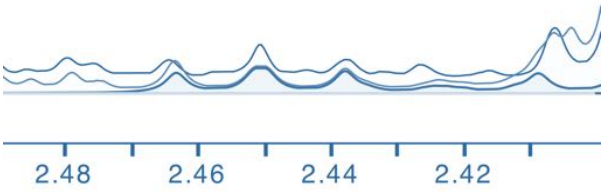
Gluconate



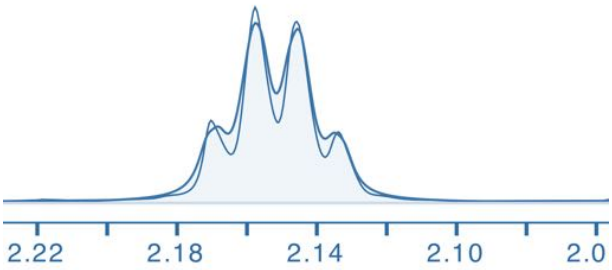
Glucose



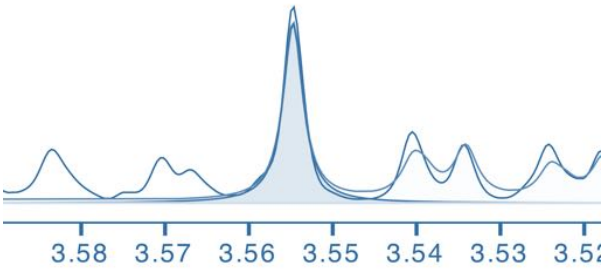
Glutamine



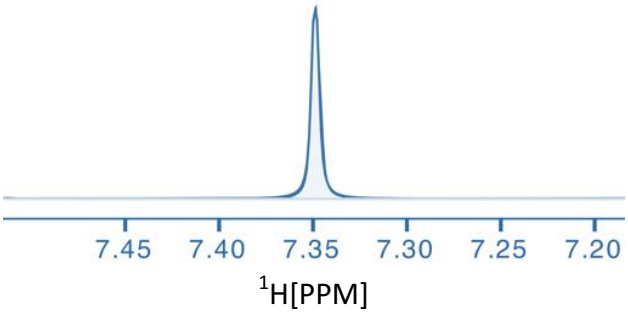
Glutathione (reduced)



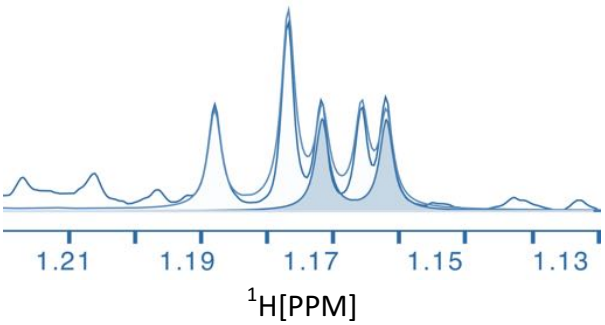
Glycine



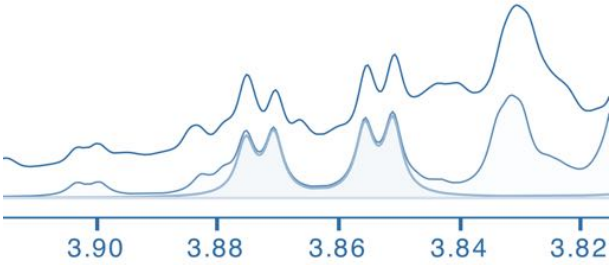
Imidazole



Isopropanol

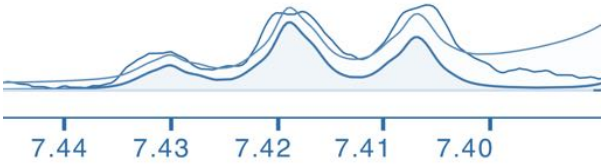


Mannitol



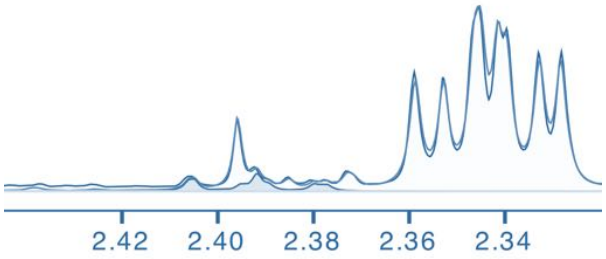
^{1}H[PPM]

Phenylalanine



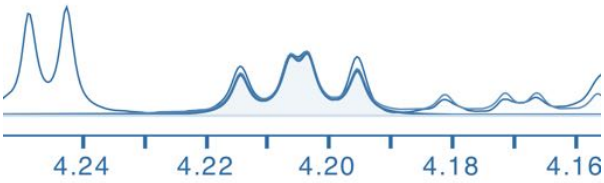
^{1}H[PPM]

Pyroglutamate



^{1}H[PPM]

Ribose



^{1}H[PPM]