Normothermic Kidney Preservation

Thesis submitted to the Examination Schools for the degree of

Doctor of Philosophy

Dr. Annemarie Weißenbacher

Green Templeton College

Nuffield Department of Surgical Sciences

Oxford Transplant Centre

University of Oxford

Trinity Term 2018





GREEN TEMPLETON COLLEGE | OXFORD

Gewidmet meinen lieben Eltern Veronika und Koloman Weißenbacher.

In Liebe und Dankbarkeit.

ABSTRACT

The thesis describes the development of a transportable normothermic kidney preservation device which is able to perfuse discarded human and porcine kidneys for up to 24 hours for the very first time. Recirculation of the urine to facilitate maintenance of perfusate volume and homeostasis is a novel approach. Utilisation of this perfusion device could enable viability assessment, decrease organ discard rate and improve transplantation logistics. Thirty-six clinically declined human kidneys were perfused, 19 from donors after brain stem death and 17 from donors after circulatory death: n=4 kidneys were perfused using Ringer's lactate to replace excreted urine volume, and n=32 using urine recirculation. In all cases, normothermic perfusion either maintained or slightly improved the histopathologically assessed tubular condition, achieved physiological arterial flow levels and effective urine production. Biomarkers, neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1) and liver fatty acid-binding protein (L-FABP) were detected and quantified in the perfusates with and without urine recirculation. NGAL could be promising for clinical use, as it was inversely correlated with median arterial flow. Only kidneys with urine recirculation were readily perfused up to 24 hours and exhibited physiological perfusate sodium levels, whilst kidneys without urine recirculation achieved reduced normothermic perfusion time and significantly higher perfusate sodium. Metabolomics analyses of perfusate samples detected urea and two sugars as the main difference between kidneys with and without urine recirculation. Proteomics analyses not only revealed that human kidneys have become metabolically active during normothermic perfusion, but they also detected that damage-associated molecular patterns known to contribute to ischaemia-reperfusion-injury, were significantly downregulated in biopsies from kidneys with urine recirculation compared to kidneys without. As a final part, porcine perfusion studies were performed with the aim to revisit the findings obtained during the human experiments and to render a valid comparison between urine recirculation and urine replacement in undamaged kidneys.

ACKNOWLEDGEMENTS

A very special word of thanks goes to Professor Peter J. Friend and Professor Constantin-C. Coussios who have not only been excellent supervisors, but have also become great mentors. They encouraged me and supported me with their wisdom and sage advice to develop this project within a fantastic environment, a composite of surgical and engineering science. My particular gratitude to the entire team of OrganOx® Limited, especially to Daniel Voyce, Nikolay Mikov, Chris Morris and Andrew Cook who were available and active around the clock in order to develop the perfusion system with me. I am very grateful to Maria Letizia Lo Faro and Professor Rutger J. Ploeg, who introduced me to the world of basic science and taught me the first steps. My unending gratitude to Professor Benedikt Kessler and his team of scientists, in particular Honglei Huang, who made the omics-work possible. My most sincere thanks to Maria Fernanda Soares, Professor Ian Roberts and the team of the Oxford Centre for Histopathology Research. Thank you to Tim James and staff of the Biochemistry Laboratory and Marianne McClure and staff of the Haematology Laboratory at the John Radcliffe Hospital, Oxford University Hospitals, NHS Foundation Trust. I am grateful also to the staff of the Oxford University Biomedical Services. A special thanks to Carlo D.L. Ceresa who assisted me in all porcine kidney recoveries. Many thanks to John Stone and James Fildes from The University of Manchester. Recognition must go to the Consultants, the transplant coordinators and clinical staff at the Oxford Transplant Centre who have made me feel so welcome. Thank you to NHS blood and transplant and the RINTAG community for their support. Last and of course not least, the greatest thanks must go to all the organ donors and their families consenting for research and making projects like mine possible.

DECLARATION

All work presented in this thesis is my own, unless otherwise stated. I was involved in the design and conduct of all studies, with the support of my supervisors. Kidney perfusions, human and porcine, were all performed by myself. Perfusate samples were taken and prepared by myself. Data was collated, analysed and interpreted by myself. Engineering work was conducted by the team of OrganOx® Limited. Assays undertaken as part of the metabolomics and proteomics studies were performed by laboratory specialists from the Kessler Lab, Target Discovery Institute, University of Oxford. Analyses conducted for the immune cells and cytokine studies were ran by members of the Fildes Lab, The University of Manchester.

SUPERVISORS

Professor Peter J. Friend

Professor of Transplantation

Director Oxford Transplant Centre

Nuffield Department of Surgical Sciences

University of Oxford

Professor Constantin-C. Coussios

Statuary Chair of Biomedical Engineering

Director Oxford Institute of Biomedical Engineering

Director Oxford Centre for Drug Delivery Devices

University of Oxford

GLOSSARY OF ABBREVIATIONS

AKI	acute kidney injury
ANOVA	analysis of variance
CID	collision-induced dissociation
CIT	cold ischemia time
CKD	chronic kidney disease
CNI	calcineurin inhibitor toxicity
CVA	cerebrovascular accident
DBD	donor after brain stem death
DCD	donor after circulatory death
DDA	data-dependent acquisition
DGF	delayed graft function
DTT	1,4-dithiothreitol
ECD	extended criteria donor
ESI	electron spray ionisation
ESRD	end stage renal disease
FDR	false discovery rate
GFR	glomerular filtration rate
GUI	graphical user interface
HLA	human leukocyte antigen
HMP	hypothermic machine perfusion
IAA	Iodoacetamide
IRI	ischaemia reperfusion injury
KDRI	kidney donor risk index
KEGG	Kyoto encyclopaedia of genes and genomes
LFQ	label-free quantification
MAP	mean arterial pressure
NIST	National Institute of Standards and Technology
NMP	normothermic machine perfusion
OPTN	organ procurement and transplantation network
PRA	panel reactive antibody
PSM	peptide spectrum matches
RIPA	Radioimmunoprecipitation assay buffer
RRT	renal replacement therapy
SCD	standard criteria donor
SCD	standard criteria donor
SCS	static cold storage
UNOS	United Network for Organ Sharing
WIT	warm ischemia time

TABLE OF CONTENTS

Chapter 1: Introduction 1

1.1 The success story of kidney transplantation 1

1.2 Organ preservation 6

1.3 Cold storage and ischaemia reperfusion injury 9

1.4 Indications for kidney transplantation 12

1.5 Donor organ shortage, declined kidneys and risk scores 14

1.6 Normothermic kidney perfusion – experimental and clinical data 21

1.6.1 Experimental investigations in large animal models 22

1.6.2 NMP of human kidneys and clinical trials 32

1.7 Aim of my DPhil 35

Chapter 2: Methods 36

2.1 Building a normothermic kidney perfusion device 36

2.1.1 Components of the perfusion circuit 39

2.1.1.1 The blood reservoir 40

2.1.1.2 The centrifugal blood pump 40

2.1.1.3 The membrane oxygenator 41

2.1.2 Haemodynamics 41

2.1.2.1 Pressure control 42

2.1.2.2 Flow measurement 43

2.1.2.3 The bypass system 44

2.1.3 Oxygen and gas control 45

2.1.4 Temperature setting 46

2.1.5 Urine measurement 47

2.1.5.1 Urine recirculation 48

2.1.6 Control system of the kidney device 49

2.2 Composition of perfusate 50

2.2.1 Discarded human kidneys 50

2.2.2 Porcine kidneys 51

2.3 Preparation of the kidney graft 53

2.3.1 Discarded human kidney 53

2.3.2 Porcine kidneys 55

2.4 Measurement of perfusion parameters 56

2.5 Perfusate and urine analysis 56

2.5.1 ELISAs human kidneys 56

2.5.1.1 NGAL, KIM-1, L-FABP 56

2.5.2 ELISAs porcine kidneys 58
2.5.2.1 NGAL and KIM-1 58
2.5.3 Anti-Xa assay 59
2.5.4 Analysis of urine protein and urine sodium 59
2.5.5 Flow cytometry 60
2.5.6 Luminex analysis 61
2.5.7 Metabolomics 61
2.5.7.1 Extraction of metabolites from perfusate samples 61
2.5.7.2 Metabolomic analysis using gas chromatography 62
2.6 Tissue analysis 63
2.6.1 Histology human kidneys 63
2.6.2 Histology porcine kidneys 64
2.6.3 Proteomics human kidneys 64
2.6.3.1 Protein extraction for proteomic analysis 64
2.6.3.2 Protein identification and quantitation by mass spectrometry 65
2.6.3.3 Perseus statistical analysis 66
2.6.4 Western Blot validation of differentially expressed proteins 67
2.7 Statistical analysis 68
Chapter 3: Perfusion of discarded human kidneys 69
3.1 Kidneys accepted for my investigations 69
3.1.1 How does a kidney become a kidney for research 70
3.2 Preparation of kidney grafts 72
3.3 Perfusion solution 72
3.3.1 Anticoagulation of the perfusion circuit 73
3.4 Management of fluid volume loss due to urine production 74
3.5 Donor characteristics 74
3.6 Comparison of donor and perfusion characteristics in different categories 78
3.6.1 Development stages of the perfusion device 78
3.6.1.1 Era one- kidneys perfused on a “hand-mad” NMP circuit 78
3.6.1.2 Era two – kidneys perfused on the prototype 80
3.6.1.3 Era three – kidneys with air and CO_2 combination for oxygenation 83
3.6.1.4 Summary: Development stages of the perfusion device 85
3.6.2 Length of cold ischaemia time 85
3.6.2.1 Cold ischaemia time up to 24 hours 86
3.6.2.2 Cold ischaemia time between 25 and 50 hours 88
3.6.2.3 Cold ischaemia time longer than 50 hours 89

3.6.2.4 Summary: Length of cold ischaemia time 90
3.6.3 Perfusion duration 91
3.6.3.1 Kidneys perfused up to 5 hours....91
3.6.3.2 Kidneys perfused up to 10 hours....92
3.6.3.3 Kidneys perfused up to 18 hours....93
3.6.3.4 Kidneys perfused up to 24 hours....93
3.6.3.5 Summary: Perfusion duration....94
3.6.4 Kidney perfusions without urine recirculation....94
3.7 Haemodynamic and metabolic function parameters – all kidneys....97
3.7.1 Arterial pressure....102
3.7.2 Arterial flow 102
3.7.3 Intrarenal resistance....103
3.7.4 PO_2 and PCO_2 levels 103
3.7.5 pH levels 103
3.7.6 Glucose measurements 107
3.7.7 Lactate levels during perfusion 107
3.7.7.1 Correlation of lactate, administered glucose and arterial flow....114
3.7.8 Urine production and sodium levels in perfusate and urine 116
3.7.9 Anti-Xa as an anticoagulant and a biomarker useful?....117
3.8 Histology results....119
Chapter 4: Biomarkers and metabolism in NMP kidneys 126
4.1 Enzyme-linked immunosorbent assays 127
4.1.1 Introduction of potential biomarkers: NGAL, KIM-1 and L-FABP....127
4.1.2 NGAL, KIM-1 and L-FABP measurements in the perfusate....128
4.1.3 NGAL, KIM-1 and L-FABP levels in kidneys with urine recirculation 129
4.1.3.1 NGAL and donor factors....132
4.1.3.2 NGAL and perfusion characteristics....132
4.1.3.3 KIM-1 and donor factors....133
4.1.3.4 KIM-1 and perfusion characteristics....133
4.1.3.5 L-FABP and donor factors....134
4.1.3.6 L-FABP and perfusion characteristics....134
4.1.4 NGAL, Kim-1 and L-FABP levels in kidneys without urine recirculation 134
4.2 Proteinuria....141
4.2.1 Results from NMP in discarded human kidneys without urine recirculation....141
4.3 Metabolomics....142
4.3.1 Amino acids and sugars the main proportion of the perfusate metabolome....143

4.3.2 Metabolomic profile in perfusions with and without urine recirculation 148
4.3.2.1 Comparison of time points in kidneys with urine recirculation.....149
4.3.2.2 No metabolite changes without urine recirculation.....153
4.3.2.3 More urea after one hour of NMP with urine recirculation.....153
4.3.2.4 Sugars represent the difference between open and closed circuit...154
4.4 Proteomics reveals molecular window of kidney allografts.....155
4.4.1 Tissue analysed to generate proteome profile.....155
4.4.2 Protein selection – the pathway to the NMP kidney proteome.....156
4.4.3 Enzymes involved in glucose and succinate metabolism 164
4.4.4 Significant changes in the proteome with and without urine recirculation.....166
4.4.4.1 Differences with and without urine recirculation after six hours 167
4.4.4.1.1 Upregulated proteins 167
4.4.4.1.2 Downregulated proteins.....171
4.4.5 Individual kidneys have individual proteomes.....177
4.4.5.1 Protein profiles with urine recirculation are the opposite to the ones without.....181
4.5 Validation of proteomics results with Western Blot.....183

Chapter 5: Immunological effects of normothermic perfusion on human kidneys 194

5.1 The importance of normothermic perfusion from an immunological point of view.....194
5.2 Measurement of immune cells in the normothermic perfusion circuit.....195
5.2.1 Kidney perfusions undergoing cell counting with flow cytometry 195
5.2.2 Perfusions with urine recirculation 196
5.2.3 Perfusions without urine recirculation 198
5.2.4 Comparison of perfusates with and without recirculated urine 199
5.2.5 Graphical representation of cells in normothermic perfusion.....199
5.3 Measurement of cytokines in the normothermic perfusion circuit.....203
5.3.1 Cytokine analysis with Luminex® 203
5.3.2 Perfusions with urine recirculation 204
5.3.2.1 Cytokines with urine recirculation stratified for 24 hours of CIT 204
5.3.3 Perfusions without urine recirculation 205
5.3.3.1 Cytokines in urine of kidneys without urine recirculation.....205
5.3.4 Comparison of perfusates with and without recirculated urine.....205
5.3.5 Illustration of cytokines in perfusions with and without urine recirculation 206

Chapter 6: Normothermic kidney perfusion in the porcine model 212
6.1 Aims 212
6.2 Porcine kidneys for NMP 212
6.3 Perfusion protocol 213
6.3.1 Composition of perfusate 213
6.3.1.1 Epoprostenol sodium (Flolan®) 214
6.3.1.2 Verapamil (Securon®) 215
6.3.1.3 Glyceryl trinitrate (GTN) 216
6.3.2 Control, monitoring and collection of haemodynamic parameters 217
6.3.3 Perfusate samples 218
6.3.4 Urine samples 218
6.3.5 Tissue samples 219
6.4 Haemodynamic and metabolic function parameters 219
6.4.1 Arterial flow 221
6.4.2 Intrarenal resistance 222
6.4.3 PO_2 and PCO_2 levels 223
6.4.4 pH levels 223
6.4.5 Glucose measurements 225
6.4.6 Lactate levels during perfusion 226
6.4.7 Urine excretion and sodium levels in perfusate and urine 227
6.4.8 Association of lactate, administered glucose, arterial flow and urine output 229
6.4.9 Proteinuria 229
6.4.9.1 Urine protein levels in porcine perfusions 230
6.5 Histology results 230
6.5.1 Kidneys with interstitial inflammation 232
6.5.2 Vacuolation 234
6.5.3 KIM-1 immunohistochemistry 235
6.6 Biomarker results: NGAL and KIM-1 236
6.6.1 NGAL 236
6.6.2 KIM-1 238
6.7 What did we learn from the porcine perfusions? 239
Chapter 7: Discussion 242
APPENDIX: References 268

LIST OF TABLES

Chapter 1: Introduction

Table 1.1: Studies investigating normothermic kidney perfusion in large animal models.....30
Table 1.2: Studies investigating normothermic kidney perfusion in human kidneys and clinical trials.....34

Chapter 3: Perfusion of discarded human kidneys

Table 3.1: Organ procurement parameters and reasons for discard of individual kidneys....75
Table 3.2: Donor characteristics 77
Table 3.3: Perfusion characteristics.....101
Table 3.4: Haemodynamic and metabolic function parameters per kidney 108
Table 3.5: Histology results and KIM-1 immunohistochemistry – tubular condition.....121

Chapter 4: Biomarkers and metabolism in NMP kidneys

Table 4.1: Observed perfusate biomarker concentrations 137
Table 4.2: Metabolites detected in 26 perfusate samples of normothermic kidney preservation 144
Table 4.3: Kidneys undergoing proteomics after normothermic perfusion 157

Chapter 5: Immunological effects of normothermic perfusion on human kidneys

Table 5.1: Flow cytometry results 197

Chapter 6: Normothermic kidney perfusion in the porcine model

Table 6.1: Perfusion characteristics.....220

LIST OF FIGURES

Chapter 1: Introduction

Figures 1.1A–1.1C: Long-term graft survival after first adult kidney only transplant in UK.....4
Figure 1.2: Adult patients on the kidney only transplant list at 31 March.....13
Figure 1.3: Number of deceased adult kidney only transplants between 1 April 2016 and 31 March 2017.....13
Figure 1.4: Global development of deceased organ donors between 2011 and 2015.....15
Figure 1.5: Age of deceased donors in the UK, 1 April 2007 – 31 March 2017.....16
Figure 1.6: UK kidney donor risk index of transplanted DBD donor kidneys 1 April 2007 – 31 March 2017.....18
Figure 1.7: Percentage of kidneys retrieved that are not transplanted from deceased organ donors in the UK, 1 April 2007 – 31 March 2017.....19

Chapter 2: Methods

Figures 2.1A–2.1D: Development of the automatically-regulated, closed loop (pre)clinical prototype in three steps.....37
Figure 2.2: Flow sensor by SONOTEC.....44
Figure 2.3: Bypass line and pinch valve.....45
Figure 2.4: Experiment - testing the temperature regulation.....47
Figure 2.5: Urine flow measurement with a liquid flow meter and urine waveform.....48
Figures 2.6A–2.6B: Control system of the kidney device.....49
Figures 2.7A–2.7B: Cannulated discarded human kidneys.....54

Chapter 3: Perfusion of discarded human kidneys

Figure 3.1: Arterial flow values over time for each individual kidney perfused on a manually-assembled perfusion circuit.....79
Figure 3.2: The manually assembled perfusion circuit.....80
Figure 3.3: Construction pathway from the era 1 perfusion circuit to the prototype.....81
Figure 3.4: Prototype device in operation mode.....82
Figure 3.5: Arterial flow values over time for each individual kidney perfused on the transportable prototype device.....83
Figure 3.6: Arterial flow values over time for each individual kidney perfused on the transportable prototype device with air/CO_2.....85
Figure 3.7: Arterial flow values over time for each individual kidney perfused after a CIT ≤ 24 hours.....87

Figure 3.8: Arterial flow values over time for each individual kidney perfused after maximum CIT of 50 hours 89
Figure 3.9: Arterial flow values over time for each individual kidney perfused after a duration of CIT longer than 50 hours 90
Figures 3.10A–3.10D: Mean ± SD arterial flows for all kidneys....95
Figures 3.11A–3.11D: Mean ± SD intrarenal resistance for all kidneys 98
Figures 3.12A–3.12D: Mean ± SD pH for all kidneys 105
Figures 3.13A–X: Examples of histology results, before perfusion start and at the end of perfusion, for kidneys with and without urine recirculation 122

Chapter 4: Biomarkers and metabolism in NMP kidneys

Figures 4.1A–H: NGAL, KIM-1 and L-FABP levels in kidneys with urine recirculation....130
Figures 4.2A–D: NGAL, KIM-1 and L-FABP levels in kidneys without urine recirculation...136
Figure 4.3: Workflow for metabolomics and proteomics....143
Figures 4.4A–C: Graphical illustration of metabolites and their detection over time in perfusate samples of kidney 36 147
Figure 4.5A: Volcano plot with significantly increased and decreased metabolites after six hours NMP with urine recirculation 149
Figure 4.5B: Volcano plot with significantly increased and decreased metabolites after twelve hours NMP with urine recirculation....151
Figure 4.5C: Volcano plot with significantly increased and decreased metabolites after 24 hours NMP with urine recirculation 152
Figure 4.5D: Volcano plot with significantly increased metabolites after one hour NMP with urine recirculation compared to urine replacement....153
Figure 4.5E: Volcano plot with significantly increased metabolites after six hours NMP with urine recirculation compared to urine replacement....154
Figure 4.6: Groups of 4538 identified proteins in n=19 NMP kidneys....158
Figures 4.7A–D: Volcano plots for the comparison of CIT duration....159
Figures 4.8A–C: Volcano plots for the comparison of donor types....161
Figures 4.9A–B: Volcano plots for the comparison of perfusion types 163
Figure 4.10: STRING database extract of upregulated proteins involved in glucose and succinate metabolism 166
Figure 4.11: STRING database extract of downregulated proteins after six hours NMP with urine recirculation compared to urine replacement....176
Figures 4.12A–G: Heat maps and scatter plots of seven NMP kidneys with urine recirculation....178

Figure 4.13: Comparison of up- and downregulated proteins in kidney perfusions with and without urine recirculation 182
Figures 4.14A–O: Western Blot analyses to validate proteomics results 186

Chapter 5: Immunological effects of normothermic perfusion on human kidneys

Figures 5.1A–B: Photographs H&E staining of kidney 30 199
Figures 5.2A–I: Cells in samples combined (with and without urine recirculation) 201
Figures 5.3A–I: Comparison of cells in NMP with and without urine recirculation 202
Figures 5.4A–B: Measured cytokines in perfusate samples with urine recirculation 207
Figures 5.5A–B: Measured cytokines in perfusate samples with urine replacement 208
Figures 5.6A–B: Measured cytokines in urine samples 209
Figures 5.7A–E: Comparison of selected, relevant, cytokines in perfusates with and without urine recirculation 210
Figures 5.8A–E: Comparison of selected, relevant, cytokines in perfusate and urine of kidney perfusions without urine recirculation 211

Chapter 6: Normothermic kidney perfusion in the porcine model

Figure 6.1: Arterial flow in ml/min in kidneys perfusions with/without urine recirculation 222
Figure 6.2: Intrarenal resistance of kidneys perfused with and without urine recirculation 223
Figure 6.3: pH of kidneys perfused with and without urine recirculation 225
Figure 6.4: Perfusate sodium of kidneys perfused with and without urine recirculation 228
Figures 6.5A–D: Examples of all histological findings for kidneys perfused with and without urine recirculation; photographs of H&E staining 232
Figures 6.6A–D: Photographs of H&E staining of kidney 7 with interstitial inflammation 233
Figures 6.7A–B: KIM-1 immunostaining of kidneys 7 and 4 235
Figure 6.8: NGAL levels in kidneys with interstitial inflammation 237

Chapter 7: Discussion

Figure 7.1: Development of preservation techniques 243

Chapter 1: Introduction

1.1 The success story of kidney transplantation

Emerich Ullmann (1861-1937), an Austro-Hungarian surgeon, is the acknowledged pioneer of kidney transplantation. In 1902, he performed the first canine kidney transplant in Vienna. He implanted a dog's kidney heterotopically into another dog's neck where the allograft lasted for five days. After an unsuccessful attempt to (xeno)transplant a porcine kidney into a human patient suffering from chronic kidney disease, he ceased his work in this field (1). At the same time, Alexis Carrel developed in Lyon the end-to-end vascular triangulation suture techniques that are still widely used for anastomosis in transplantation. For his successful procedures of vascular suturing and anastomosis technique, Carrel was awarded the Nobel Prize for Medicine in 1912 (2). In January 1906, the French surgeon Mathieu Jaboulay (1860-1913) transplanted a pig kidney into the left elbow of a woman suffering from nephrotic syndrome; the graft failed because of an early vascular thrombosis. The patient died three days later (3). The first "technically successful" human-to-human kidney transplant was performed in 1933 by Voronoy and colleagues. The kidney was AB0-incompatible (B to 0) and was rejected. The recipient died 48 hours later. Overall, Voronoy *et al.* reported six transplants but no significant long-term renal function occurred in any of them (4). In the 1940s, light was shed within the darkness of transplant rejection and enormous immunological advances were made. In 1944, Sir Peter Medawar detected that graft recipients would form antibodies against a graft, unless they had been exposed to similar foreign tissue early in life. His work demonstrated that the body's rejection of foreign tissue was indeed an immune response. In collaboration with Brent and Billingham, Medawar (called "The Holy

Trinity") proceeded to unravel many aspects of tissue immunology. He established the field of modern transplant immunology with his historic achievements. Their milestone paper on immune tolerance was published in 1951 and Medawar himself was awarded the Nobel Prize in medicine in 1960 (5–7).

The first successful renal transplants were performed between 1945 and 1953 in Europe and in the United States of America and it is quite difficult to state who did the first human ones. A group in Boston at the Peter Bent Brigham Hospital (Hufnagel, Landsteiner and Hume) transplanted a kidney to the antecubital vessels of a woman with acute renal failure. The transplant lasted only for four days but may have been the first functionally successful deceased donor transplant outside the abdomen (8,9). In 1950, Richard Lawler transplanted a kidney into a patient with polycystic kidney disease, after having removed one of the polycystic giant kidneys at the same time to get some space. The kidney was functioning for at least 53 days as reported (10). In the 1950s and early 1960s, the "French Transplantation Club" René Küss, Charles Dubost and Marceau Servell performed the first renal transplants in France. The French were the first to use the extraperitoneal approach which is commonly used today (11,12). A total of nine patients were transplanted in France during this time and all nine of them rejected their grafts. Having had this experience and the already established understanding of immune related rejection, Küss stated in his publication in 1951 that "in the present state of knowledge, the only rational basis for kidney replacement would be between monozygotic twins" (11). Before this concept made history a couple of years later, the Canadian Murray might have contributed the first long-term success in renal transplantation. In May 1952, he transplanted a 26-year-old woman with Bright's disease (historical classification of acute or chronic nephritis).

The patient stayed well for the next 21 years and the kidney has never been removed according to the literature (13,14).

A milestone, and maybe the most important one, in the history of renal transplantation occurred in December 1954. J Hartwell Harrison, a urologist, removed a kidney from a living donor in the Peter Bent Hospital in Boston and Joseph Edward Murray transplanted it into the pelvic location of the donor's identical twin brother. The kidney lasted for eight years when the recipient died of a myocardial infarction. This was the first long-term success in living donor renal transplantation taking the principle of genetic identity for whole organ transplantation into account. More kidney transplants between identical twins were carried out successfully and some of those kidney transplant recipients are still alive today (15). In July 1959, the first successful deceased donor renal transplantation in the UK was performed by F. Peter Raper in Leeds. The team in Leeds used cyclophosphamide as immunosuppression; the patient died eight months later (14,16). A year later, Sir Michael Woodruff performed the first living donor kidney transplantation between identical twins in the UK (17). The year 1960 became a memorable and important year in the history of clinical transplantation as the era of immunosuppression started. Willard Goodwin started using corticosteroids in Los Angeles and Roy Calne first used 6-mercaptopurine in dogs. In November 1960 the first successful non-twin sibling living transplant in the UK was carried out which was also the first use of 6-mercaptopurine in humans and the first use of prednisolone in the UK (14). The next big steps followed soon in 1962 when Roy Calne and Murray first used azathioprine as immunosuppressant (18). Thomas Starzl started a new era in transplantation by combining azathioprine and corticosteroids, as an effective "dual therapy" from the start of a transplant in 1963 (19). Roy Calne pioneered the use of cyclosporine in renal transplantation in the UK

in 1978 (20). From this time on the field progressed quickly and kidney transplant teams around the world have continued their research. Joseph E. Murray noted in his Nobel Lecture in 1990 (21) that by 1965, one-year survival rates for living donor kidney transplants were about 80% and for deceased transplants 65% (21). When one takes into account that there was a substantial death rate back then and no return to dialysis, it is interesting that the one-year graft survival rates are not that different in the modern age with 95% for living donor kidneys and 85% for deceased ones (14).

Outcomes have continued to improve to the current era. Figures 1.1A to 1.1C show the survival rates for adult kidney only transplants from donors after brain death, donors after circulatory death and living donor kidney transplant in the UK between January 2003 and December 2015.

Long-term graft survival after first adult kidney only transplant from donors after brain death, 1 January 2003 – 31 December 2015

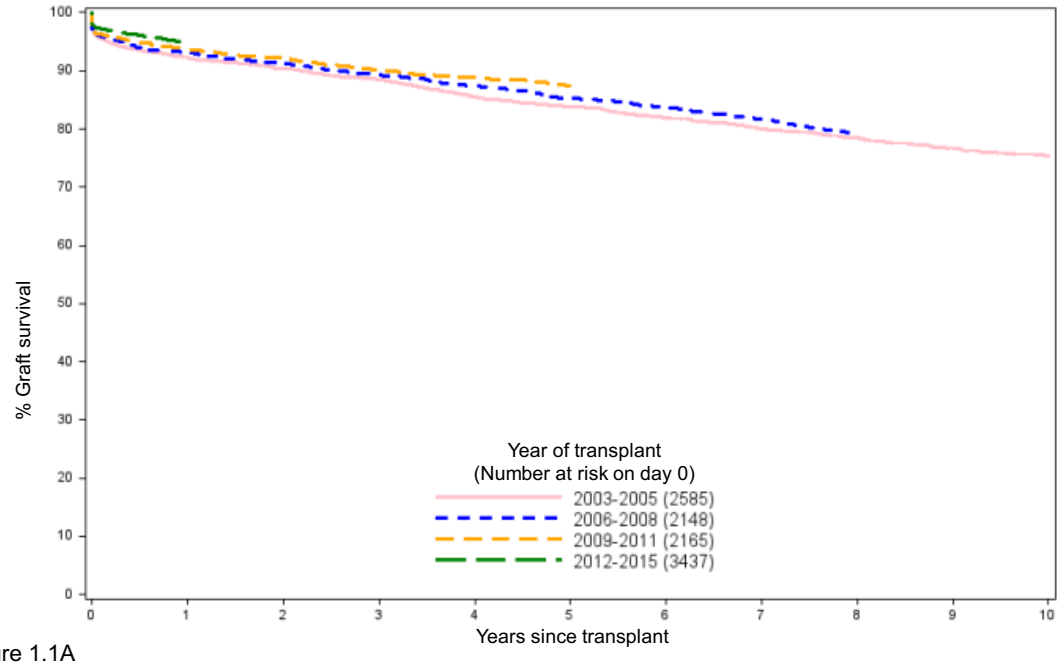


Figure 1.1A

Source: Transplant activity in the UK, 2016-2017, NHS Blood and Transplant

Long-term graft survival after first adult kidney only transplant from donors after circulatory death, 1 January 2003 – 31 December 2015

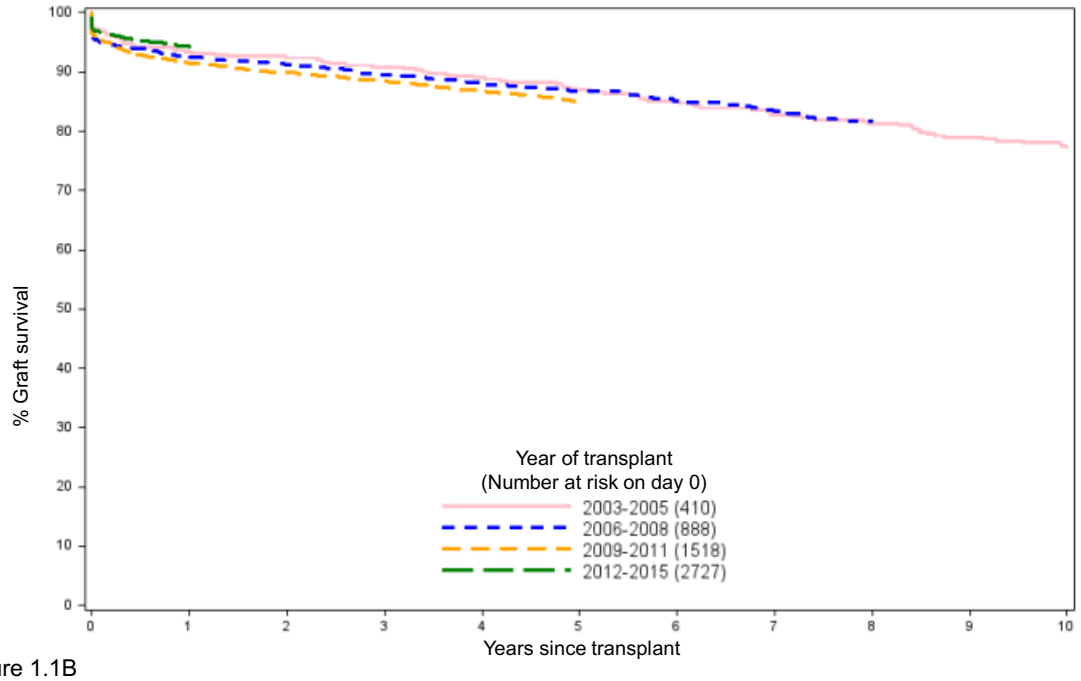


Figure 1.1B

Source: Transplant activity in the UK, 2016-2017, NHS Blood and Transplant

Long-term graft survival after first adult living donor kidney only transplant in the UK, 1 January 2003 – 31 December 2015

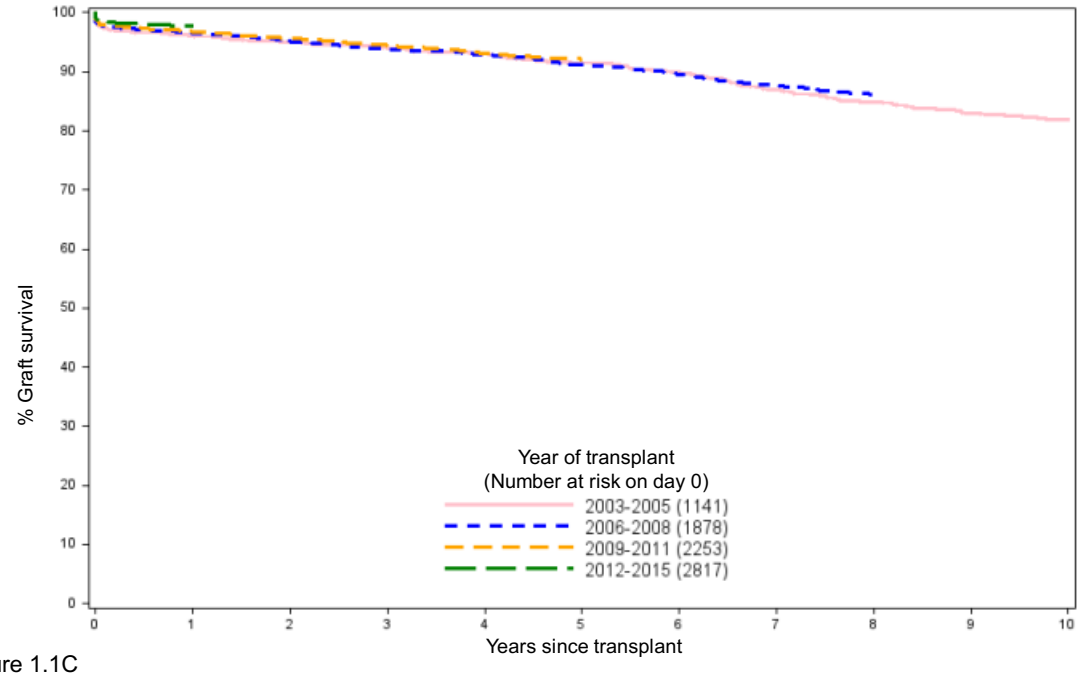


Figure 1.1C

Source: Transplant activity in the UK, 2016-2017, NHS Blood and Transplant

Current surgical procedures and techniques are remarkably similar to those developed during the pioneering period in the last century. Permanent function of the transplanted organ without immunosuppression remains, as it has always been, the 'holy grail', the target for researchers and clinicians alike. Immunosuppression, tissue matching and organ preservation have not yet reached the level that permits this yet (14).

1.2 Organ preservation

The interest in organ preservation and the importance of this topic is older than clinical solid organ transplantation itself. Long before Joseph Murray performed the first successful kidney transplantation between identical twins in 1954 (15) investigation of organ preservation had been carried out already. In 1812 César Julien Jean Le Gallois, a French medical doctor and physiologist, whose most important discovery was the demonstration of the metameric organisation of the spinal cord, stated: *"If one could replace the heart with some form of injection and at the same time continuously provide natural or artificially produced arterial blood for this injection – provided that such an artificial production is possible – life would succeed effortlessly maintaining each part of the body for an indefinite period of time. Consequently, after decapitation, one could maintain all brain functions in the mind itself. In this way one could not only maintain life in the head or in any other part isolated from the body of the animal, but also recall it here even after its complete annihilation. One could also call it back into the whole body and thus accomplish its true resurrection in the truest sense of the word"* (22,23).

It took longer than 100 years to realise Le Gallois' conception, as it was not possible to maintain an organ in a surviving condition for a number of reasons, including bacterial infection: antiseptic procedures had not been implemented in the

laboratories. In the mid 1930s, Alexis Carrel and Charles Lindbergh merged their skills and invented an ex vivo perfusion pump. Lindbergh, the aviator and mechanical expert, was the designer and inventor of the hand-blown, 18-inch high, glass perfusion pump. He collaborated with the Nobel Prize winning scientist Carrel who perfected the perfusion fluid he wanted to use as “artificial blood” to keep organs alive for several days. They described in their work “The culture of whole organs”, published in Science in June 1935 (24), reporting more than 20 experiments including thyroid, ovary, suprarenal, spleen, heart and kidney. They showed that the structural and functional changes the perfused organs experienced depended on the chemical composition and the physical conditions of the perfusate (24). In 1960, Lapchinsky noting their work on perfusion, published a slightly different approach for preservation of canine limbs and kidneys. He described the use of an artificial circulation for one hour after beginning of preservation, using hypothermic blood to cool the organ, and for one hour at the end of the preservation period, using heated blood to warm up the organ prior to transplantation. The preservation time in between the two one-hour periods of artificial perfusion took up to 28 hours in a refrigerator unit at 2ºC and 4ºC (25).

Following the initial efforts in the 19th and early 20th century, to perfuse organs with “blood-like” fluids and to keep them at normal body temperature at 37ºC, the preservation field moved more towards cold preservation. Hypothermic machine perfusion (HMP) was launched in the late 1960s by Belzer *et al.* Belzer’s group was the first to publish their experience with pulsatile hypothermic (8-12ºC) perfusion of canine kidneys for periods of 24 to 72 hours (26). Shortly thereafter, Belzer *et al.* were able to preserve human-cadaver kidneys for up to 50 hours using this technique and transplanted these successfully (27,28). However, HMP was soon replaced by static

cold storage (SCS) (29,30). The golden age of SCS preservation started with the development of “Collins” solution (31,32) and extended further with the introduction of the University of Wisconsin solution by the Belzer group in the 1980s (33).

To the present day, SCS has remained the clinical standard for solid organ preservation, its popularity mainly due to its simplicity, operational and economic feasibility (29). As long as transplantation was mostly confined to the use of standard criteria donor (SCD) grafts, kidney transplant outcomes improved incrementally with developments in immunosuppression and clinical care. However, prolonged SCS causes impaired renal graft function, especially in kidneys used from extended criteria donors (ECD) and organs from donors after circulatory death (DCD). Rates of delayed graft function (DGF) and primary non-function (PNF) are increased and accompanied by inferior long-term results (29,34–39). In recent years, there has been a resurgence of interest in HMP, perfusing the donor organ with cold preservation solution prior to transplantation. There is evidence that this form of preservation is superior to static cold storage, especially in the context of higher risk, ECD and DCD organs (40–42). More recently, with the addition of delivering oxygen in some experimental perfusion systems, it has been claimed that further improvement in outcomes are possible with HMP (43,44).

Currently, there are two prospective randomised controlled clinical trials ongoing to investigate oxygenated HMP in ECD and DCD kidney transplantation with the endpoint of 1-year graft survival (45). Both studies are run by COPE, the Consortium for Organ Preservation in Europe. The “POMP” study addresses oxygenated HMP re-conditioning of ECD kidneys after SCS (46). The “COMPARE” trial is designed to compare oxygenated HMP of Maastricht III DCD kidneys (50 years or older) with non-oxygenated HMP (46). Although HMP studies have shown a decrease in delayed graft

function and better survival (40–42), improved utilisation of marginal kidneys including from DCD has remained elusive.

1.3 Cold storage and ischaemia reperfusion injury

Organs retrieved for transplantation experience injury at three or four (in DCD) consecutive stages: 1) warm ischaemia prior to preservation (DCD), 2) cold preservation injury, 3) ischaemic rewarming during surgical implantation and 4) reperfusion injury (47). These consecutive events lead to a cumulative cellular injury that may not be compatible with recovery after transplantation. In kidneys retrieved from DCD donors, the deleterious effects of cold ischaemia are superimposed on the injury sustained during warm ischaemia before the organ retrieval even starts, which causes rapid depletion of ATP.

Standard clinical practice during organ retrieval involves flushing and cooling the kidneys in situ with preservation solution. University of Wisconsin solution (UW) and Custodiol Histidine-Tryptophane-Ketoglutarate (HTK) solution are the most commonly used solutions; for kidney-only retrieval in the United Kingdom hypertonic citrate ("Marshall's") perfusion fluid is used. Several litres of cold preservation solution are used both, in situ and after removing the kidneys from the donor, before packing for transport and storage. The kidneys are placed in sterile plastic bags containing preservation solution and stored in an ice-box until transplantation. The available preservation solutions differ in their chemical composition, but the function is essentially the same: to prevent cellular swelling and death caused by fluid shifts as the membrane ion-exchange pumps cease operating in the cold environment. Although cold preservation slows metabolism by 1.5- to 2-fold for every 10ºC drop in temperature, considerable metabolic activity still occurs at 4ºC. This leads to accumulation of metabolic products which act as substrates for metabolism that takes

place when the organ is re-perfused with oxygenated blood. The reperfusion of the ischaemic kidney releases oxygen free radicals and other molecules that cause tissue damage and trigger the acute inflammation that is characteristic of the ischaemia-reperfusion-injury (IRI) phenomenon (48,49). In addition, mitochondria play an important role in IRI by generating a pool of succinate under hypoxic conditions. During ischaemia, the electron transport chain ceases quickly and the electron donors and carrier pools, as NADH or coenzyme Q for example, are reduced. By oxidation of fumarate to succinate the mitochondria can compensate for this. When the allograft, or any other ischaemic area after a stroke or infarction, is undergoing reperfusion, the accumulated succinate is rapidly oxidised which maintains a reduced coenzyme Q pool, supporting a reverse electron transport and the production of reactive oxygen species (ROS) (50,51). These findings published by Chouchani *et al.* do not only describe a conserved metabolic response of tissue to hypoxia and reperfusion it also opens a new window of opportunity to control ROS production in vivo by inhibiting succinate accumulation due to ischaemia. Therefore it is a possible therapeutic target to decrease IRI (50) in a number of pathologies including solid organ transplantation. Research groups working on hypothermic oxygenated perfusion (HOPE) demonstrated that HOPE could prevent oxidative stress or release of damage associated molecular patterns (DAMPs) during perfusion via protection against the initial (mitochondrial) ROS release (43,52,53).

Delayed graft function (DGF) is one of the main consequences of IRI and influences the course of the transplanted kidney (49). Endothelial cells and the tubular cells are the first cells in the kidney to suffer from reduced oxygen supply. Superoxide radicals and an acidic milieu is formed by anaerobic glycolysis and ATP degradation. Na/K ATPase exchangers cannot function without ATP which leads to intracellular K+ and

extracellular Na+ retention, building an environment for inflammation and production of ROS (54). The returning (reperfusion) recipient blood brings white blood cells, and those release a host of cytokines, chemo-attractants and pro-coagulant factors. Furthermore, the restored blood flow reintroduces oxygen that damages the cell membrane with release of free radicals. During the early phase of ATP depletion, the superoxide dismutase, an important mitochondrial antioxidant that eliminates superoxide, is inactivated. As a consequence, the oxidants mediate mitochondrial damage and activation of the receptors of apoptotic pathway, with consequent death of tubular epithelial cells. All these changes, which are proportional to the duration of the ischaemic period, will result in cellular damage, which manifests as DGF, or less-reversible forms of organ injury (49,55,56).

The short-term clinical consequences of DGF are the need for several post-transplantation dialysis sessions, leading to increased morbidity, an increased length of hospitalisation and increased costs (57). Ojo *et al.* reported in 1997 already that DGF has major implications on renal allograft survival. Their analysis included nearly forty-thousand primary kidney transplants (DBD only at that time). The occurrence of DGF was an independent significant predictor for graft loss after five years and this deleterious impact was more severe than poor HLA-matching (58). As Ojo *et al.* stated in their publication 20 years ago, acute rejection of the graft is more common in DGF-grafts and it has a negative add-on effect on graft survival too (58). This is not just a historic finding. Even in the modern era of kidney transplantation DGF remains a significant risk factor for BPAR (biopsy proven acute rejection) (59). In DBD kidneys, DGF is associated with inferior graft survival but for the DCD cohort the long-term consequences of DGF are still a matter of debate (57). A study, published by Weber *et al.* in 2002 in the New England Journal of Medicine, concluded that despite the

significant higher DGF-rate in DCD kidneys, the long-term graft survival (10 years) was similar to DBD organs (35). More recently, another investigation on DGF and its impact on long-term outcome after DBD kidney transplantation strengthened previous findings and revealed DGF as a significant risk factor for patient death five years post-transplant regardless if patients recovered from DGF or not (60).

1.4 Indications for kidney transplantation

Kidney transplantation is the primary choice for renal replacement therapy (RRT) in most patients with end stage renal disease (ESRD). As of 2015, the most common causes of chronic kidney disease (CKD) are diabetes mellitus, hypertension and glomerulonephritis (61). The Global Burden of Disease study (61) shows that over a ten-year period from 2005 to 2015, the number of people dying from CKD rose by 31.7% worldwide; meaning that 1.2 million people are dying every year because of CKD. According to the KDIGO (Kidney Disease: Improving Global Outcomes) guidelines, CKD is defined as abnormalities of kidney structure of function, present for >3 months, with implications for health and CKD is classified based on cause, GFR (glomerular filtration rate) category, and albuminuria category (62).

At the end of March 2017, 5197 (61.5%) patients were active on the waiting list to receive a kidney transplant in the UK; total number of patients listed was 8453 (100%) (NHSBT data, annual kidney report (63)); these numbers include adult and paediatric patients and patients listed for multi-organ transplants including the kidney. At the same time, 3256 (38.5%) patients were temporarily suspended from the waiting list due to a condition making them unfit or unavailable for transplant (63); 261 actively listed patients died on the transplant list or within one year (1 April 2016 to 31 March

2017) of removal from the list (64). Figure 1.2 displays all adult patients (total, active, suspended) on the kidney waiting list for the period 31 March 2008 to 31 March 2017.

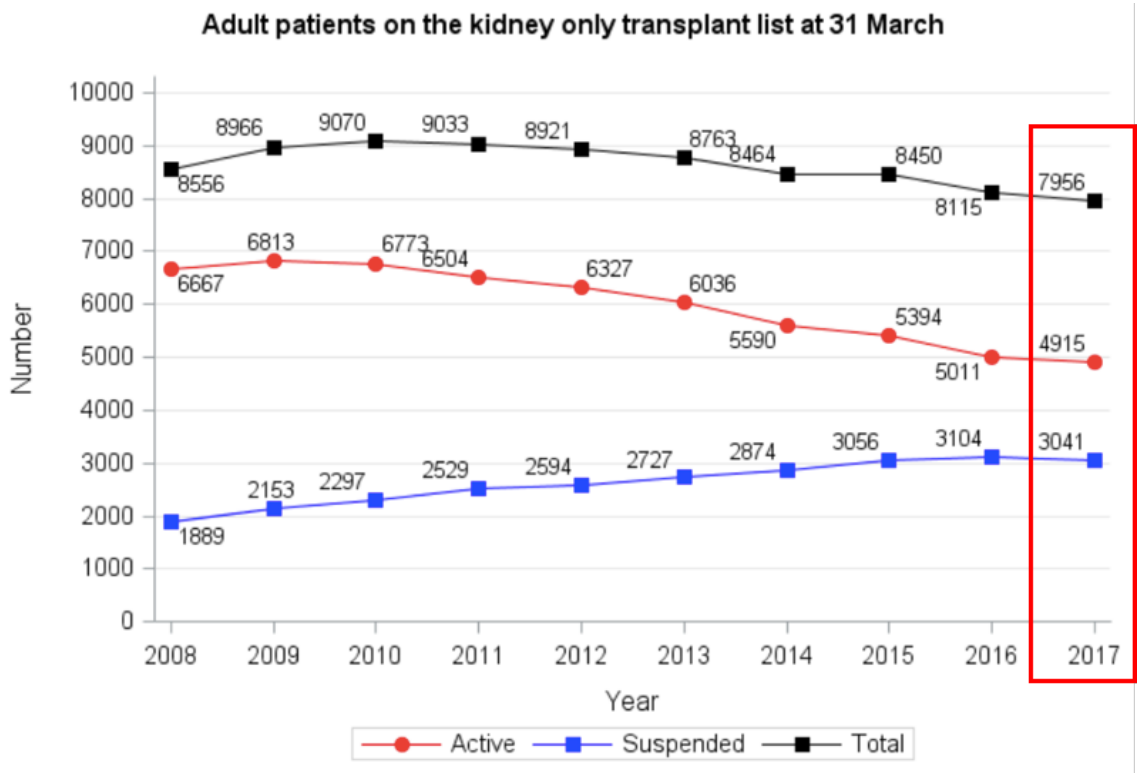


Figure 1.2: Modified figure 3.1 from the annual report on kidney transplantation (63).

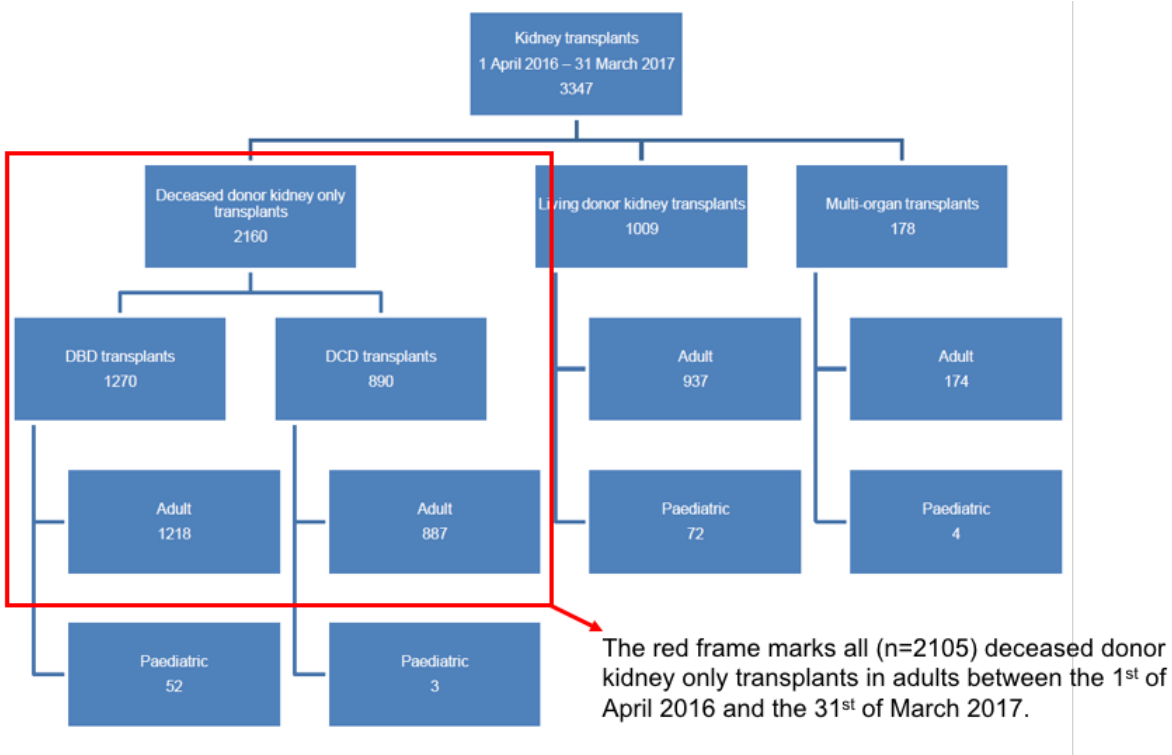


Figure 1.3: Modified figure 2.5 from the annual report on kidney transplantation (63).

Comparing figures 1.2 and 1.3 clearly points out the substantial unmet need of donor organs: end of March 2016, 5011 potential adult kidney transplant recipients were actively listed and 2105 were transplanted between March 2016 and April 2017.

1.5 Donor organ shortage, declined kidneys and risk scores

Solid organ transplantation has become victim of its own success. One-year graft survival for DBD and DCD kidneys is 95% and higher; kidney allograft survival after five years of transplantation is currently >90% for DBD kidney transplants and >85% for DCD kidney transplants in the United Kingdom; NHSBT data shown in figures 1.1A and 1.1B. During April 2016 and March 2017, 2160 deceased donor kidney transplants (figure 1.3) were carried out in the United Kingdom. However, more than 5000 patients were registered actively for a kidney on the waiting list at the same time as described above. This shortfall is typical for transplant services around the globe. Less than 10% of the global needs are met by the current number of solid organ transplants, as reported in the Global Activity in Organ Transplantation (GODT 2015; http://www.transplant-observatory.org) (65). The number of deceased organ donors has developed positively globally and increased about 21% between 2011 and 2015; shown in figure 1.4.

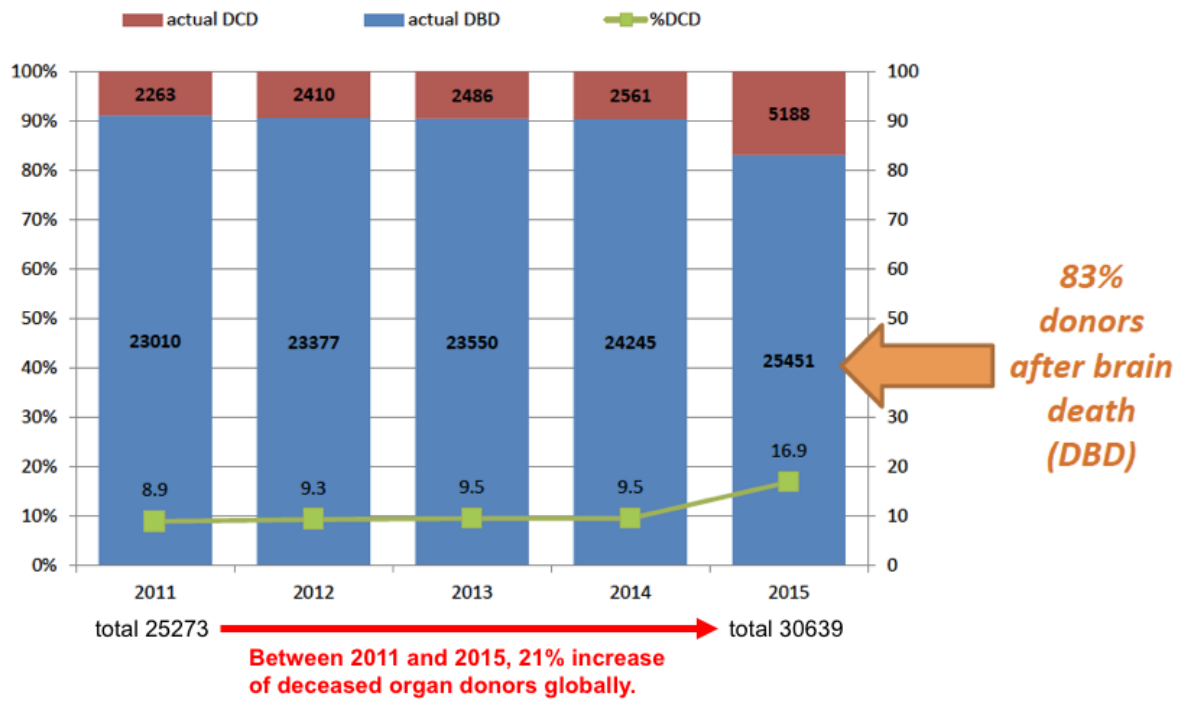


Figure 1.4: Global development of deceased organ donors between 2011 and 2015; modified figure from GODT 2015 (65).

Much progress has been made in the UK to increase the number of organ donors. The annual number of deceased donors has increased from 809 (2007-2008) to 1413 (2016-2017), an increase of 75% (figure 1.5).

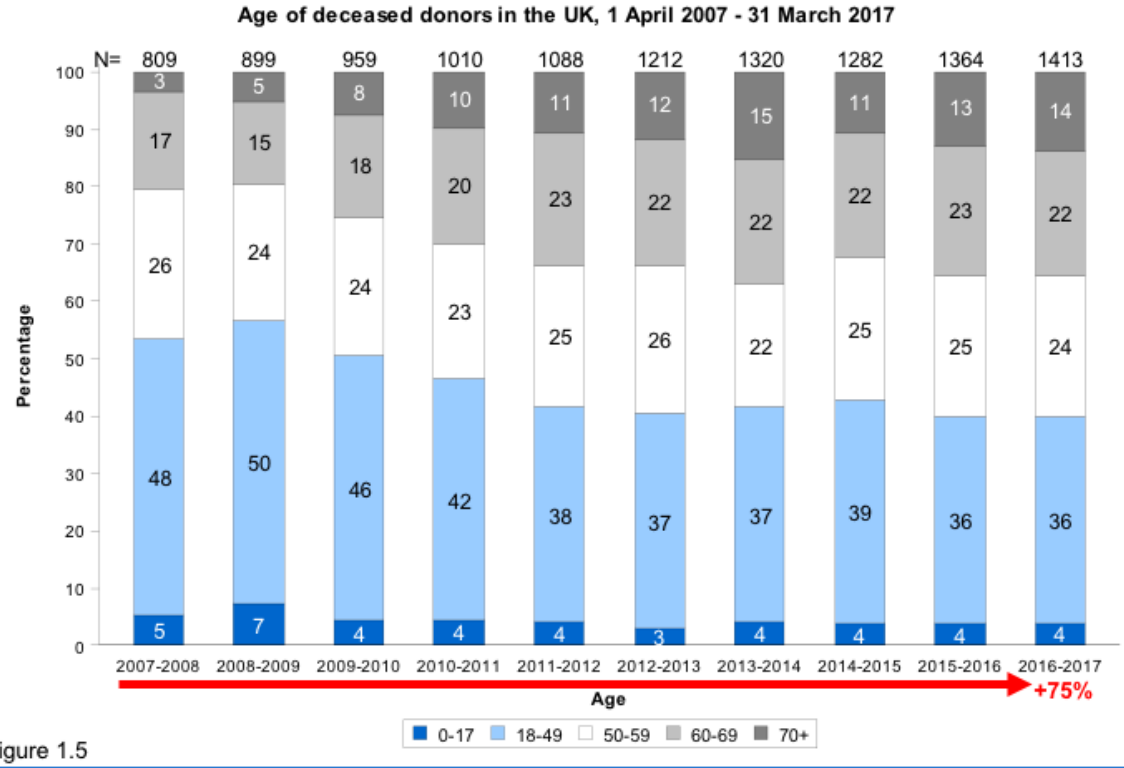


Figure 1.5

Source: Transplant activity in the UK, 2016-2017, NHS Blood and Transplant

The rate of increase in transplant numbers does not reflect the same change: this is because many of the additional donors are less well suited to the needs of patients. In addition, the demand for kidney transplantation is increasing, due to a 4% annual increase in the UK-population of patients requiring RRT (66). Many more patients are referred for transplantation as progressively improving results have broadened the applicability of transplantation, particularly in older patients and those with comorbidities. This has increased demand on the limited organ source. To address this disparity the transplant community has therefore increasingly turned to the use of older and higher risk donor organs, those which would not have been considered acceptable for transplant previously. Figure 1.5 demonstrates the age distribution of deceased donors between 2007 and 2017. Looking at the percentage of deceased donors age ≥50 (DBD and DCD), having in mind the definition for SCD and ECD, it is

pretty obvious that about 50% of the donors and their organs might be categorised as “higher risk”. The categorisation of DBD kidneys in either SCD or ECD is simple, but neither reflects the huge variety of donor kidney quality nor applies to DCD organs. As these extended/expanded criteria kidneys include higher risk organs from older donors, donors with additional comorbidities, including cardiovascular disease and diabetes, a more refined approach to assess the quality of deceased donor kidneys was developed by Watson *et al.*, the UK kidney donor risk index (UKKDRI) (67). The UKKDRI was developed by determining the factors influencing kidney transplant survival and is calculated as follows: exp{-0.245 x (donor age <40) + 0.396 x (donor age ≥60) + 0.265 x (history of hypertension) + 0.0253 x [donor weight(kg)-75]/10) + 0.00461 x (days in hospital) + 0.0465 x (adrenaline)}; UKKDRI <1.35 stands for standard risk and ≥1.35 for higher risk kidneys. A UKKDRI of 1.0 represents a donor between 40 and 59 years of age with 75 kg body weight, no history of hypertension, hospitalisation <1 day and not given any adrenaline (67). The survival rates for adult deceased donor first kidney only transplants in an adult recipient according to the UKKDRI are: 92.8 to 88.7% one-year graft survival and 83.1 to 76.1% five-year graft survival for UKKDRI <1.35; for kidneys with a UKKDRI ≥1.35 the one-year graft survival is 88.6% and the five-year graft survival 70% (67). The predicted transplant survival rates are based on a “baseline” recipient with age of 50 years, white, on dialysis, with glomerulonephritis, no HLA mismatches and a CIT of 17 hours (67).

The annual kidney report from NHSBT provides a figure showing the number of DBD kidneys transplanted according to the UKKDRI (63); figure 1.6.

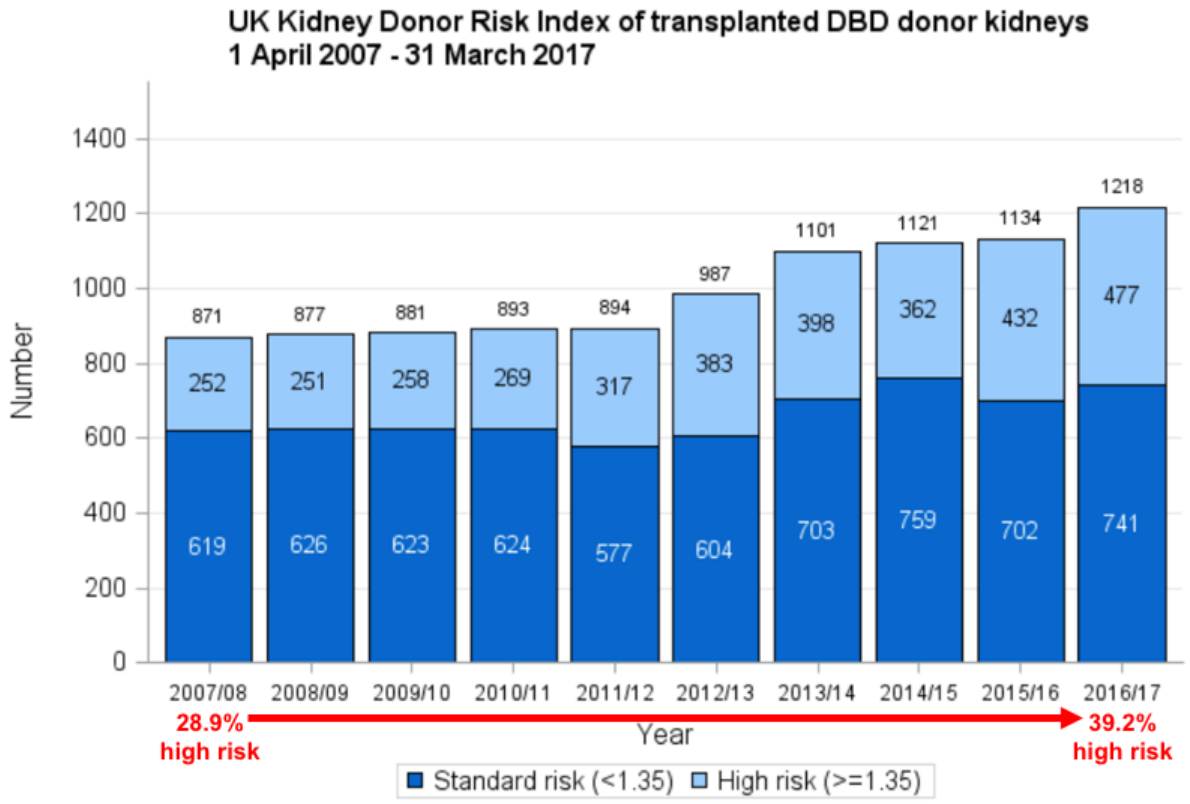


Figure 1.6: The proportion of high-risk kidneys according to the UKKDRI increased within the DBD donor kidney cohort about 10% in a period of ten years. Modified figure 5.10 from the annual report on kidney transplantation (63).

Unfortunately, many such higher risk organs are not transplanted, sometimes because of clear evidence of non-viability (obvious vascular and/or parenchymal damage, neoplasm, polycystic organ), but more commonly due to uncertainty as to the quality of the organ. Such organs are either not accepted for transplantation or retrieved and then declined after inspection. Figure 1.7 illustrates the percentage of kidneys retrieved from deceased organ donors in the United Kingdom between April 2007 and March 2017 but not transplanted; the median number of discarded human kidneys per year in the mentioned period was 212.7 (93.8 – 282.2); calculated from numbers given in figure 1.7.

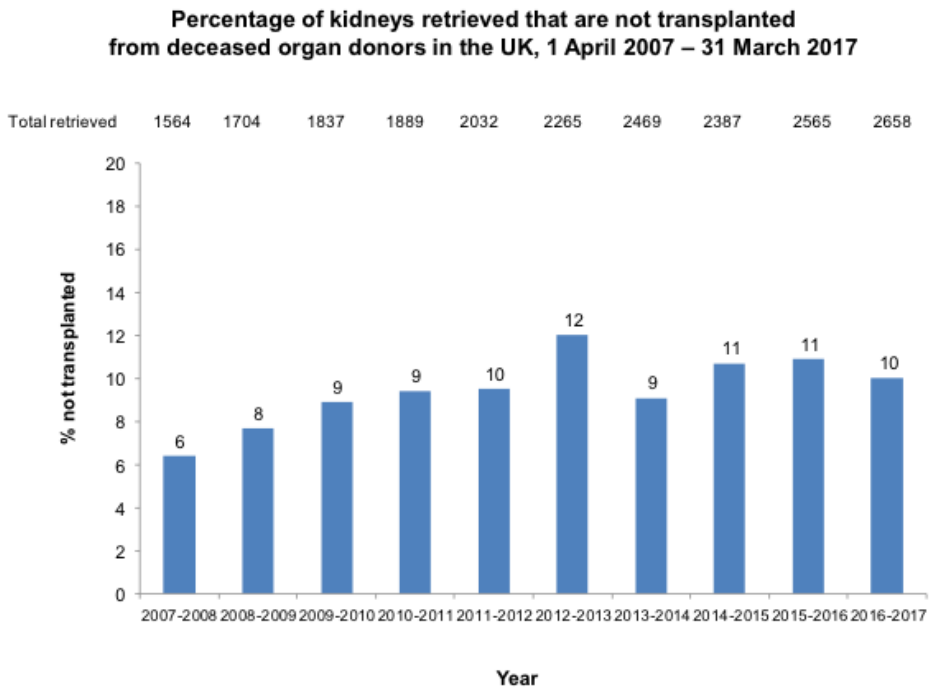


Figure 1.7

Source: Transplant activity in the UK, 2016-2017, NHS Blood and Transplant

Worldwide, the transplant community is seeking for tools to predict graft survival better and thereby to enable the use of as many organs as possible without compromising outcomes for patients. In the United States of America more kidneys from older donors are declined compared to kidneys accepted from the same donor population for transplant in Europe (68). Before the UKKDRI has been implemented in the UK, Rao and co-workers published the kidney donor risk index (KDRI) in 2009 based on US registry data (69). The KDRI is an estimate of the relative risk of post-transplant kidney graft failure (in an average, adult recipient) from a particular deceased donor compared to a reference donor (69). The reference donor (KDRI=1) chosen by Rao *et al.* in their original work, was age 40, non-African American race, serum creatinine 1 mg/dl, non-hypertensive, non-diabetic, cause of death other than cerebrovascular accident, height 170 cm, weight more than or equal to 80 kg, brain dead donor and

HCV negative. Donors with an index <1 have a lower risk of graft failure than the reference donor, kidneys from a donor with an index >1 have a higher risk of graft failure than the reference donor; a kidney from a deceased donor with a KDRI of 1.30 for example, has an estimated graft failure risk 30% greater than the reference donor (69). The Organ Procurement and Transplantation Network and the United Network for Organ Sharing (OPTN/UNOS) created the kidney donor profile index (KDPI) which is a numerical measure combining ten (same as for KDRI) donor factors, including clinical parameters and demographics. The KDPI should summarise the quality of deceased donor kidneys relative to other recovered kidneys into a single number. The KDPI is based on the KDRI for a deceased donor (70). The ten donor factors included in the score are: age, height, weight, ethnicity, history of hypertension, diabetes status, cause of death, serum creatinine, HCV status and DCD status. The KDPI is a mapping of the KDRI to measure relative risk in a chosen reference population. For KDPI, the reference population of donors is all donors in the United States of America from whom a kidney was recovered during the prior calendar year (70). As an example: A KDPI of 75% in a deceased donor kidney (SCD or ECD) retrieved in the year 2018 means that the estimated risk of kidney graft failure from this donor is higher than 75% of kidney donors recovered in the year 2017. The KDPI should be an improvement over the ECD/SCD dichotomy by using more donor factors, by being a continues score rather than a binary (yes/no) indicator and KDPI illuminates the fact that not all ECD are alike; some ECD kidneys have good estimated quality and vice versa some SCD kidneys have a lower estimated quality than some ECD allografts. The main intended use of the KDPI was implementation of the “longevity matching” concept into the kidney allocation system. Potential kidney recipients with longer estimated post-transplant life expectancy should receive priority for kidneys from a donor with a better

KDPI. The KDPI should also provide a measure of donor quality and help transplant professionals to evaluate the quality of the deceased donor organ offer better and to help making decisions (70). First analysis after the implementation of the KDPI/KDRI scores showed that some viable kidneys were inappropriately discarded. Kidneys from a SCD with a higher KDPI were more often discarded compared to the era without the scoring system using SCD/ECD criteria only. These kidneys also confer to long-term survival. The recipients of the SCD/high-KDPI kidneys showed a decreased mortality compared to those who remained on the waiting list until receiving a kidney from a donor with a lower KDPI (71). Watson *et al.* compared both scores, KDRI and UKKDRI, detected that both indices are highly significant prognostic indicators for the UK data and that the predictive ability of the two models is practically identical. Compared to the KDRI/KDPI system, the UKKDRI score is less complex though as it incorporates just five donor factors, about a third of factors included in KDRI, and has therefor the advantage of simplicity and provides a clinically useful tool (67). Despite the decision aid that these indices provide, the scoring systems are based on a "perfect" recipient with an optimal HLA match, and do not improve the utilisation of kidneys per se. Normothermic kidney preservation as a clinical reality could potentially manipulate/improve damage caused by cold and/or warm ischaemia time for example and consequently increase realisation of kidney transplants.

1.6 Normothermic kidney perfusion – experimental and clinical data

Normothermic organ perfusion is nothing new as history of transplantation, especially the experiments of Charles Lindbergh and Alexis Carrel who both had a daring quest to be immortal (authors of the book "The Immortalist"), taught us. At the end of the 20th century and beginning of the 21st century normothermic organ perfusion underwent a

revival. The main reasons for this were (i) the demand for viability assessment of high-risk organs, (ii) the need for extending the period of safe organ preservation to enable organ procurement over longer distances and better management of transplantation logistics and (iii) growing interest in the therapeutic potential of ex vivo reconditioning. An effective means of pre-transplant viability assessment would not only allow greater use of (higher risk) donors but also minimise the risk of non-function by identifying and excluding organs unfit for purpose before subjecting a patient to the risk of surgery and immunosuppression. The hypothesis behind the application of normothermic machine perfusion (NMP) as a reconditioning tool is to reverse the injury sustained by the organ before and during the process of retrieval, treating the organ in such a way as to minimise the immediate damage that occurs after transplantation – ischaemia reperfusion injury.

1.6.1 Experimental investigations in large animal models

In the 1980s the research group of Gauke Kootstra from the University of Groningen published their experience with experimental kidney preservation up to 144 hours in a model using mongrel dogs. With a combination of perfusion techniques they achieved successful six-day canine kidney preservation. In their early model van der Wijk *et al.* combined continuous hypothermic perfusion using a Gambro machine with albumin perfusate with a spell of ex vivo normothermic perfusion. The one to four-hour period of ex vivo perfusion was achieved by connecting the kidney to a donor dog; this was then followed by continued hypothermic perfusion. After a total preservation time of 144 hours the preserved canine kidneys were auto-transplanted after performing a simultaneous contralateral nephrectomy. The results of this study showed that short periods of ex vivo normothermic perfusion, three to four hours, resulted in successful

auto-transplantation, with near-normal creatinine levels within ten days after transplantation. This contrasted with no more than 30% survival in animals that received autotransplants after shorter periods (one to two hours) of ex vivo normothermic perfusion (72–74). Rijkmans *et al.* developed this combination of perfusion techniques further and used a heart-lung machine filled with heparinised blood from non-related dogs for an intermediate normothermic (38ºC) perfusion of three hours after three days of hypothermic perfusion. The preserved kidneys, hypothermic only or combined with normothermic, were auto-transplanted in subsequently nephrectomised dogs. The survival rate and kidney function parameters were better in the group with intermediate normothermic perfusion and the authors concluded that it was possible to prevent irreversible preservation damage by normothermic blood perfusion (75). Maessen *et al.* studied the beneficial effect of intermediate normothermic perfusion during cold storage of ischaemically injured kidneys by analysing renal nucleotide homeostasis during hypothermia in the dog. Five experimental groups of six mongrel dogs were investigated. Canine kidneys were stored on ice for 24 or 48 hours, with and without 30 minutes of warm ischaemia before preservation; in the fifth group the kidneys underwent 30 minutes of warm ischaemia, then a total of 48 hours cold storage, with an interval of normothermic ex vivo perfusion midway through cold storage. The abstract does not mention the duration of normothermic ex vivo perfusion (76). Tissue levels of adenine nucleotides, guanine nucleotides and purine degradation products were assessed in biopsies of the renal cortex during preservation. The spell of NMP significantly prevented the drop in the nucleotide content that was otherwise observed during the last 24 hours of cold storage in the control group (76,77).

About 20 years later, Arnaud *et al.* used a blood perfusion system to assess the renal function of eight isolated rabbit kidneys, in a life-supporting auto-transplant model. In the seven surviving rabbits, serum creatinine was measured after transplantation. The peak creatinine was 6.8 ± 3.2 mg/dl two days after transplantation. These kidneys, after perfusion with blood for two hours at 36ºC, achieved normal post-transplant function (78).

After these major contributions of the Kootstra group, leadership in this field moved to the Leicester (now Cambridge) research group of Michael Nicholson. In 2002, this group published their first experiences with normothermic autologous blood perfusion compared to hypothermic pulsatile perfusion. Metcalfe *et al.* perfused six porcine kidneys for 16 hours with a pulsatile organ perfusion system (POPS, Transmedics®). In comparison to the hypothermically perfused partner kidneys, with similar warm and cold ischaemia times, the ability of kidneys to concentrate creatinine and conserve sodium were significantly better in the POPS-preserved kidneys (79). Harper *et al.* investigated the effect of white cells in early renal reperfusion injury, again using an isolated haemoperfused porcine kidney model. Six porcine kidneys were perfused with normothermic autologous blood per group; in one perfusion group a leucocyte filter was included in the circuit. Their results showed that renal blood flow, oxygen consumption and acid-base homeostasis were all improved by perfusion with leucocyte-depleted blood and histologically confirmed tubular damage was ameliorated (80).

Arterial perfusion pressure is likely to be crucial in normothermic organ perfusion. Hosgood *et al.* tested three different arterial pressure values, 95, 75 or 55 mmHg, in a porcine ex vivo normothermic perfusion model. The kidneys perfused at a higher mean arterial pressure (MAP) showed improved renal function; pressures of 95 mmHg and

75 mmHg were associated with better acid-base balance and improved renal haemodynamics compared to lower pressure. The kidneys in the 95 mmHg group demonstrated an overall superior renal function (81). In a later study, they compared the effect of different arterial pressures during NMP on tubular and endothelial injury. Patel *et al.* showed that an arterial pressure of 75 mmHg resulted in less tubular damage and less endothelial injury during ex vivo reperfusion compared with kidneys perfused at 55 mmHg (82).

Another perfusion parameter often debated is the optimal perfusion temperature. Adams *et al.* analysed whether sub-normothermia of 32°C would influence the conditioning effect of ex vivo perfusion when compared with NMP at 37°C and SCS. Porcine kidneys were cold stored for 23 hours and then perfused with leukocyte-depleted blood at 32°C or 37°C for one hour. This total preservation of 24 hours was followed by three hours of reperfusion with whole autologous blood at 37°C (in order to test organ function). This assessment revealed that tubular and renal functions were better preserved at a temperature of 37°C compared to 32°C or SCS (83). Bagul *et al.*, also from the Leicester research group, investigated normothermic perfusion with autologous blood as a means to resuscitate controlled non-heart-beating donor kidneys perfusion in a porcine model. Four preservation groups were compared after a warm ischaemia time of ten minutes. Renal blood flow and renal function were optimised in the group which was treated with two hours of normothermic perfusion after a cold storage time of 16 hours. The worst outcome was seen in kidneys after a conventional cold storage time of 18 hours, in terms of measured serum creatinine and renal blood flow in this reperfusion model. In addition, this study demonstrated restoration of depleted ATP levels with normothermic perfusion, as well as reversal of some of the deleterious effects of cold storage (84).

Hosgood *et al.* assessed the feasibility of a short period of normothermic machine preservation (NMP) in a porcine auto-transplant model. The study included twelve pigs, which received auto-transplanted kidneys after (i) 30 minutes of warm ischaemia and 22 hours of hypothermic machine perfusion (HMP) or (ii) 30 minutes of warm ischaemia and 20 hours of HMP followed by two hours of NMP, using autologous blood. The comparison of the two treatment groups did not show significant differences in levels of renal function, but the creatinine levels fell below 250 µmol/ in all surviving animals in the NMP group compared with only two in the HMP group. Lipid peroxidation levels were significantly lower post-transplant in NMP group (85).

Also in the porcine reperfusion model, Hosgood *et al.* demonstrated improved metabolic function and less tubular injury in kidneys that had undergone a short period of NMP compared to static cold storage. They showed increased expression of heat shock protein 70 and IL-6, presumably upregulated due to normothermic reconditioning (86).

The next step from the same group, was to assess the effect of NMP in an experimental porcine model with increasing durations of warm ischaemia (15, 60, 90 and 120 minutes). All kidneys underwent three hours of NMP at 38ºC with a whole blood based perfusate after a SCS time of two hours. Creatinine clearance, urine output and fractional excretion of sodium deteriorated with increasing warm ischaemia time. It was possible to accurately assess the possible recovery from damage caused by warm ischaemia (87).

Up to this point, much of the experimental work on normothermic kidney preservation had been based on short-term, (maximum three hours) normothermic perfusion. The Toronto group of Markus Selzner focused in their large animal work on longer-term NMP. They published their first experiences together with a video illustrating the

retrieval, cannulation and perfusion techniques in a porcine model (88). An erythrocyte-based perfusion solution was used with a perfusion circuit based (as with Nicholson *et al.*) on components from cardiopulmonary bypass circuits. After NMP for ten hours, urine production was mainly dependent on the composition of the perfusion solution: the higher the oncotic pressure and osmolarity, the lower the urine production (88). They compared cold preserved and normothermically perfused porcine kidneys in a porcine DCD model. Renal heterotopic auto-transplantation was performed after either eight hours of SCS or NMP and the animals followed-up for ten days. Serum creatinine and urea values were comparable to baseline values in the normothermic group, whereas, in the SCS group, these parameters were significantly inferior to the basal levels (89). Kaths *et al.* used the porcine model of DCD auto-transplantation to compare eight hours of static cold storage (using HTK solution) to NMP. Animals that received NMP-treated kidneys had lower serum creatinine levels in the first seven days, significantly lower peak values of creatinine and an increased creatinine clearance compared to the SCS group (90). The Toronto group also demonstrated that continuous NMP was superior to brief normothermic perfusion following SCS in a porcine DCD auto-transplant model. The warm ischaemia time in this model was 30 minutes. Kidneys were allocated to four groups: (i) 16 hours SCS throughout, (ii) 15 hours SCS followed by one hour of NMP, (iii) eight hours SCS followed by eight hours NMP and (iv) 16 hours of NMP for the whole preservation period. The pigs were followed-up for eight days after transplantation. The grafts preserved normothermically for the whole period of 16 hours demonstrated the lowest serum creatinine peak, the best 24-hour creatinine clearance on day three after transplantation and histologically less apoptotic cells on day eight (91). These results were reproduced in another DCD auto-transplant model by the same group. They set out to examine the potential

recovery of kidneys undergoing varying durations NMP following injury by cold ischaemia, using different durations of NMP (one, eight and 16 hours) following a clinically relevant period of eight hours SCS. Again, they demonstrated the lowest serum creatinine levels in the eight and 16-hour NMP group. Tubular injury was most severe in the group with eight hours of SCS followed by one hour NMP (92).

Kaths *et al.* used the same model to test the feasibility of NMP as a means of graft assessment prior kidney transplantation. They investigated eight hours of NMP followed by auto-transplantation in a DBD and DCD (30 and 60 minutes warm ischaemia time). Perfusion parameters, renal resistance and acid-base homeostasis correlated with the post-transplantation renal function. As expected, lactate and aspartate aminotransferase levels were lowest in non-injured grafts compared to DCD kidneys. The investigators concluded that NMP allows the prediction of post-transplantation graft function and may therefore be a valuable means of deciding whether grafts are viable for transplantation (93).

Quintini's group from Cleveland Clinic has also developed a protocol for prolonged warm ex vivo perfusion in a DCD kidney model. They preserved porcine kidneys in three different settings: (i) normothermic whole blood; (ii) normothermic Steen Solution™ with whole blood and (iii) sub-normothermic Steen Solution™ alone at 21°C. In this setting, kidney grafts perfused sub-normothermically with Steen Solution™ alone showed the best results in terms of low vascular resistance and adequate histological results. In contrast to tubular and glomerular necrosis in the normothermic groups, histology in the sub-normothermic group revealed proteinaceous casts only (94). Because this study suggested that using an iso-oncotic albumin-based perfusate led to oliguria and the formation of proteinaceous casts, the investigators decided to lower the oncotic pressure of the perfusate by reducing the

albumin concentration. They then compared normothermic and hypothermic perfusion of pig kidneys followed by whole blood normothermic reperfusion (95). A porcine DCD model with a warm ischaemia time of 45 minutes was used. Five hours of cold ischaemia were followed by eight hours of HMP or NMP then two hours of normothermic reperfusion using Ringer's Lactate and autologous whole blood. The oxygenation was carried out with 95% oxygen and 5% carbon dioxide. During the reperfusion period there were no differences seen in oxygen consumption, urine production, creatinine clearance and proteinuria. The intrarenal resistance was similar in HMP and NMP after 30 minutes of reperfusion. Despite the change of the perfusate composition used in the previous study of Urcuyo *et al.* (94), NMP kidneys still developed increased vacuolisation after preservation and inferior tubular status after reperfusion, compared to the HMP group (95).

Table 1.1 summarises the most important experimental studies on normothermic kidney perfusion in large animal models in chronological order.

Table 1.1: Studies investigating normothermic kidney perfusion in large animal models

	Number of kidneys	Temperature	Perfusion time	Perfusate	Arterial pressure in mmHg	Renal blood flow	Urine output
van der Wijk *et al.* 1980 (72)	24	hypothermic 8°C, normothermic 38°C	hypothermic 96-140 hrs, normothermic 4 hrs	blood of donor dog (kidney connected to the dog)	hypothermic 20-60, normothermic n.a.	hypothermic: fixed fow 100 ml/min, normothermic 100 ml/min	1-3ml/hour during 4 hrs of normothermic
Rijkmans *et al.* 1984 (75)	11	hypothermic 8°C, normothermic 38°C	hypothermic 141 hrs, normothermic 3 hrs	non-related canine blood	n.a	n.a.	n.a.
Maessen *et al.* 1989 (77)	6	hypothermic 8°C, normothermic 38°C	3 hrs (after 30 min WIT and 48 hrs SCS)	non-related canine blood	n.a	n.a.	n.a.
Arnaud *et al.* 2000 (78)	8	36°C	2 hrs	rabbit blood	87.2±5.5	3.7±1 ml/min/gram	0.11±0.04 ml/min/gram
Arnaud *et al.* 2002 (96)	20 (7 control, 6 EC perfused, 7 CPA perfused)	36.2 ± 0.9°C, 35.1 ± 0.6°C, 35.6 ± 0.9°C	2 hrs	rabbit blood	89.1 ± 3.9, 87.6 ± 3.7, 86.3 ± 6.8	2.91 ± 0.57, 4.07 ± 0.75, 4.65 ± 1.64 ml/min/gram	0.068±0.05 ml/min/gram, 0.056±0.022 ml/min/gram, 0.208±0.08 ml/min/gram
Metcalfe *et al.* 2002 (79)	6	37°C	16 hrs + 2 hrs examination	porcine blood on POPS	50	n.a.	n.a.
Harper *et al.* 2006 (80)	12	37°C	6 hrs	6 whole porcine blood/ 6 leukocyte depleted porcine blood	53 / 55	73 ± 17 / 53 ± 130 ml/min/100gram	191 ± 75 / 70 ± 32 ml/h
Hosgood *et al.* 2006 (81)	18 (3 groups)	37.3 - 37.9°C	6 hrs	porcine blood	55, 75 and 95	up to 40, 80 and 140 ml/min/100gram	up to 63, 189 and 493 ml/h

Bagul *et al.* 2008 (84)	24 (6 2 hrs CS, 6 18 hrs CS, 6 18 hrs HMP, 6 16 hrs CS+ 2 hrs NP)	n.a.	2 hrs	porcine blood	75	76-90 ml/min/100gram	median 406 ml
Hosgood *et al.* 2011 (85)	12 (6 22 hrs HMP, 6 20 hrs HMP+ 2 hrs NP)	n.a.	2 hrs	porcine blood	n.a	n.a.	n.a.
Hosgood *et al.* 2013 (86)	6	38°C	1 hr	porcine, leukocyte depleted blood	70	83.2 ml/min/100gram	total 245 ± 76 ml
Patel *et al.* 2014 (82)	12	38°C	1 hr	porcine, leukocyte depleted blood	55 / 75	126.8 / 273.1 ml/min/100gram	total 200 ml / 41.5 ml
Hosgood *et al.* 2015 (87)	22 (6 15 min WIT, 6 60 min WIT, 6 90 min WIT, 4 120 min WIT)	38°C	3 hrs (after 2 hrs SCS)	porcine blood	85	10-70 ml/min/100gram	15 min 622 ± 40, 60 min 206 ± 142, 90 min 69 ± 17, 120 min 64 ± 14 ml
Kaths *et al.* 2015 (88)	6	37°C	10 hrs (after 3 hrs SCS)	porcine blood	60-80	170±57 ml/min	≈30ml at hour 1, ≈80 ml at hour 10
Kaths *et al.* 2016 (89)	5, heartbeating model	37°C	8 hrs	leukocyte depleted porcine blood	65	114±18 - 200 ml/min	30 ml cumulative (mean)
Kaths *et al.* 2017 (90)	5, DCD model	37°C	8 hrs	leukocyte depleted porcine blood	75	48±13 - 194±30 ml/min	80 ml cumulative (mean)
Kaths *et al.* 2017 (91)	20, DCD model (5 SCS, 15 NMP)	37°C	1 hr, 8 hrs, 16 hrs	leukocyte depleted porcine blood	65 - 75	142±25, 198±23, 215±30 ml/min	3 ± 1, 29 ± 25, 161±103 ml cumulative (mean)
Adams *et al.* 2017 (83)	12	6 32°C, 6 37°C	1 hr	leukocyte depleted porcine blood	75	90±23, 246±61 ml/min/100gram	total 151 ± 57, 245 ± 76 ml
Blum *et al.* 2017 (95)	10	5 4°C, 5 37°C	8 hrs	washed red porcine blood cells	85	n.a.	n.a.

1.6.2 NMP of human kidneys and clinical trials

Hosgood and Nicholson have developed a score for kidney quality assessment during NMP, based upon a large series of normothermic perfusions of kidneys retrieved with the intention of clinical transplantation, but then declined for reasons of clinical/macroscopic assessment (97–99). The score consists of three main parameters during NMP: (i) macroscopic assessment of the kidney; (ii) renal blood flow and (iii) total urine output. The score comprises five levels of kidney quality; 1 the best and 5 the worst quality. The scoring 1 to 5 is a composite of the main parameters which are subdivided:

- Macroscopic assessment of the kidney: excellent perfusion (1 point), moderate perfusion (2 points), poor perfusion (3 points)
- Renal blood flow (ml/min/100g): ≥ 50 (0 points), ≤ 50 (1 point)
- Total urine output (ml): ≥ 43 (0 points), ≤ 43 (1 point)

Nicholson and Hosgood proposed that kidneys with a score 1 to 3 can be transplanted successfully, kidneys with a score of 4 may be suitable for transplantation (as anuria per se should not be interpreted as a predictor of non-viability). Kidneys with a score of 5 are proposed to be too risky to be considered for transplantation (97–99). In addition to no improvement in perfusion parameters during the 60 minutes of NMP, kidneys scoring 5 showed a low level of tubular function compared to the kidneys with a score 1 to 4 (97–99).

So far, this is the only score available to evaluate a human kidney after ex vivo NMP, to decide whether the organ should be deemed suitable for transplantation.

Nicholson *et al.* in Leicester (and later Cambridge) performed the first NMP kidney transplant in man, and the first reported series of such cases. In line with a large body of experimental evidence generated by their group over many years and the data from NMP studies in discarded human organs, they used a 60 minutes period of ex vivo normothermic perfusion in 2011 (99,100). In their first clinical study, the Leicester group transplanted 18 kidneys from extended criteria donors after a period of ex vivo NMP. The duration of perfusion was 63 ± 16 minutes at about 35ºC. The rate of delayed graft function was significantly lower than in the compared SCS group of transplanted kidneys (99,101). Later, the (now Cambridge-based) group reported the transplantation of a pair of DCD kidneys declined for transplantation by all UK transplant centres due to inadequate in situ perfusion. During 60 minutes of NMP the patchy areas cleared in both kidneys and the decision to transplant these organs was made. Both recipients had immediate graft function (99,102). This favourable outcome is encouraging for protagonists of normothermic reconditioning and demonstrates the potential benefit of short periods of NMP prior to transplant.

Following this success in the early implementation of clinical NMP, Nicholson and Hosgood initiated a randomised controlled trial of ex vivo normothermic perfusion in DCD renal transplantation. The UK-based multicentre trial compares the efficacy of pre-transplant NMP, as previously used, with the conventional SCS technique. Four hundred patients receiving a kidney from controlled DCD donors will be recruited into the study. The primary outcome of this study is the rate of DGF; results are expected by 2020 (99,103).

Table 1.2 summarises investigations on human kidneys and clinical trials on normothermic kidney perfusion and preservation in chronological order.

Table 1.2: Studies investigating normothermic kidney perfusion in human kidneys and clinical trials

	Number of kidneys	Temperature	Perfusion time	Perfusate	Arterial pressure in mmHg	Renal blood flow	Urine output
Hosgood *et al.* 2011 (100)	1 DCD	34°C	35 min	red blood cell based	65–70	n.a.	n.a.
Nicholson *et al.* 2013 (101)	18 ECD	34.6 ± 1.1°C	63 ± 16 min	red blood cell based	62 ± 5	67 ± 29 ml/min/100gram	189 ± 119 ml
Hosgood *et al.* 2014 (104)	2 DCD	36 ± 0.9°C	60 min	red blood cell based	n.a.	33.4 and 67.3 ml/min/100gram	150 and 200 ml
Hosgood *et al.* 2014 (105)	1 ECD	35°C	60 min	red blood cell based	60	93.6 ml/min/100gram	60ml
Hosgood *et al.* 2015 (97)	22 (19 DCD)	near body temperature	60 min	red blood cell based	set pressure	30–90 ml/min/100gram (mean)	n.a.
Hosgood *et al.* 2016 (102)	2 DCD	35.2°C	60 min	red blood cell based	75	68.0 and 59.9 mL/min/100gram	560 ml and 430 ml
Hosgood *et al.* 2018 (98)	5 DCD (transplanted)	35.3 ± 0.2°C	60 min	red blood cell based	77 ± 5	40-70 ml/min/100gram	total 100–550 ml

1.7 Aim of my DPhil

Normothermic machine perfusion provides several potential advantages over both SCS and HMP. Firstly, the fact that the organ remains in a functioning state under physiological conditions potentially allows direct measurement of organ function that could more closely predict performance after transplantation. Secondly, the detrimental effects of cooling and hypoxia are potentially avoided, reducing the severity of ischaemia-reperfusion at the time of implantation. Thirdly, NMP offers the unproven possibility that injuries sustained before and/or during retrieval (eg hypoxia) may be reversed.

There is good evidence from large animal studies and preliminary clinical evidence (as discussed above) that NMP may be valuable in the assessment and preservation/reconditioning of damaged kidneys before transplantation. Although there appears to be significant benefit in the short periods of NMP studied by the Nicholson group, there is also evidence from the Selzner group that longer periods of NMP may be even more beneficial (ie normothermic preservation). This corroborates the evidence from clinical studies now published in NMP liver transplantation (106,107).

The aim of my DPhil studies is to evaluate for the first time the feasibility of longer-term transportable normothermic machine perfusion in human kidneys for periods of up to 24 hours.

Chapter 2: Methods

2.1 Building a normothermic kidney perfusion device

The normothermic kidney perfusion system was designed to support kidneys *ex vivo* for a prolonged preservation period using perfusion with an oxygenated suspension of packed red cells in a colloid, supplemented by nutrients, at normal body temperature. The overall circuit and controller algorithm design was carried out in collaboration with Professor Coussios and Professor Friend, whilst the component selection, component integration, and software implementation were performed by Dr Daniel Voyce and Mr Andrew Cook at OrganOx® Limited, whose contributions are gratefully acknowledged. A high-level description of key aspects of the device development is provided here for completeness.

The development of this automatically-regulated, closed loop (pre)clinical prototype device comprised three steps:

Step 1: Establishment of the underlying circuit design: Trial circuit designs were assembled manually from components in the laboratory of the Institute of Biomedical Engineering (IBME), in order to find the optimum circuit design. The design for the first iteration of the circuit is displayed in figure 2.1A, which was designed and produced with Chris Morris, a Clinical Perfusion Scientist working at OrganOx® Limited.

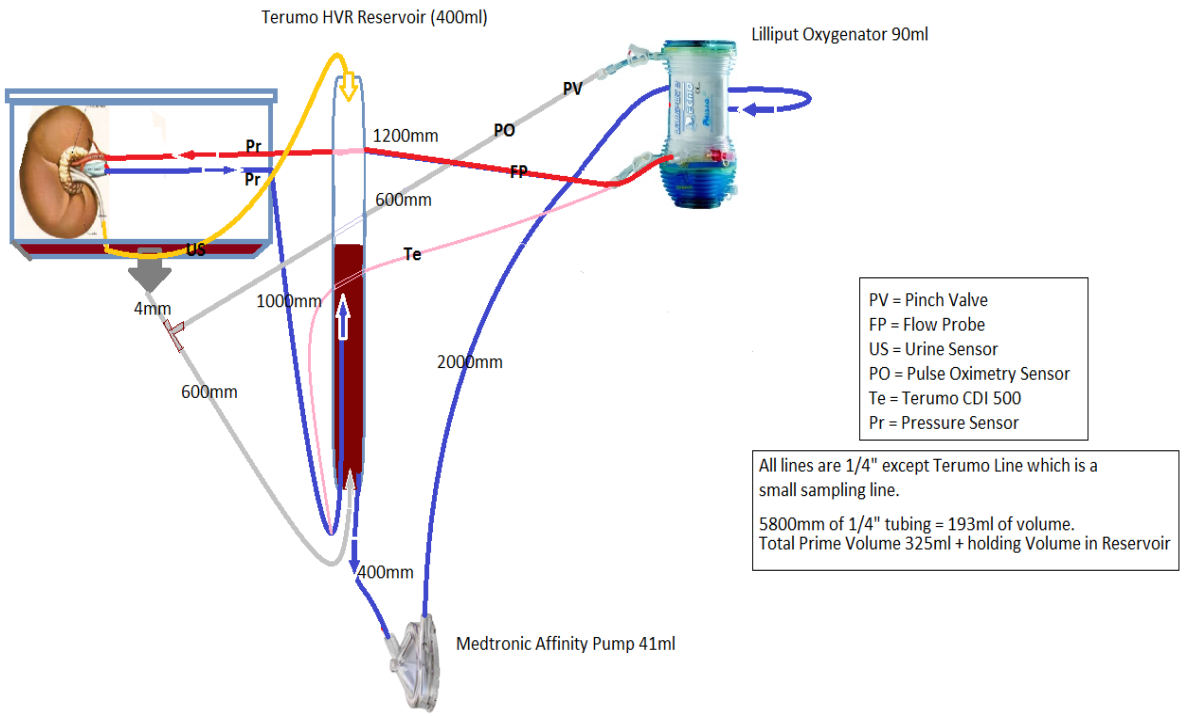


Figure 2.1A: Schematic representation of the underlying circuit design for the assembled, initial normothermic perfusion circuit.

Step 2: On the basis of the "bench-top" perfusion circuit, the design for a prototype device was built; shown in figure 2.1B.

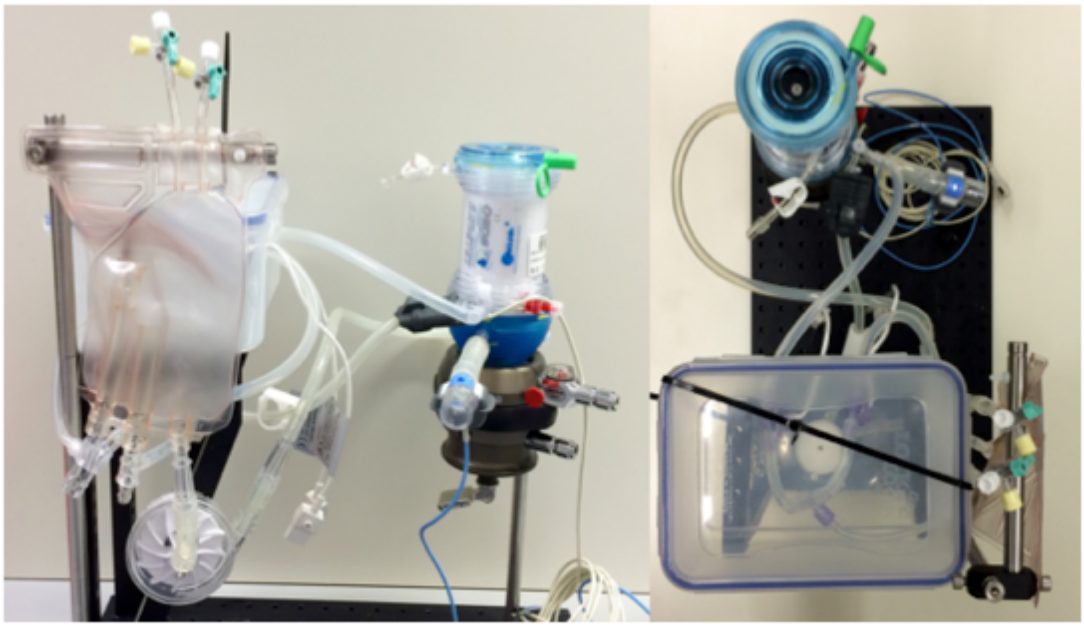


Figure 2.1B: Model for a portable prototype for the normothermic kidney perfusion device.

Step 3: The design model was used as the basis for an automated prototype. The prototype was built in collaboration with engineers from OrganOx® Limited and comprised a single use sterile fluid circuit and a plastic enclosure for the electronics and actuators. Figure 2.1C illustrates the schematic drawing of the prototype device, figure 2.1D displays the first actual prototype device.

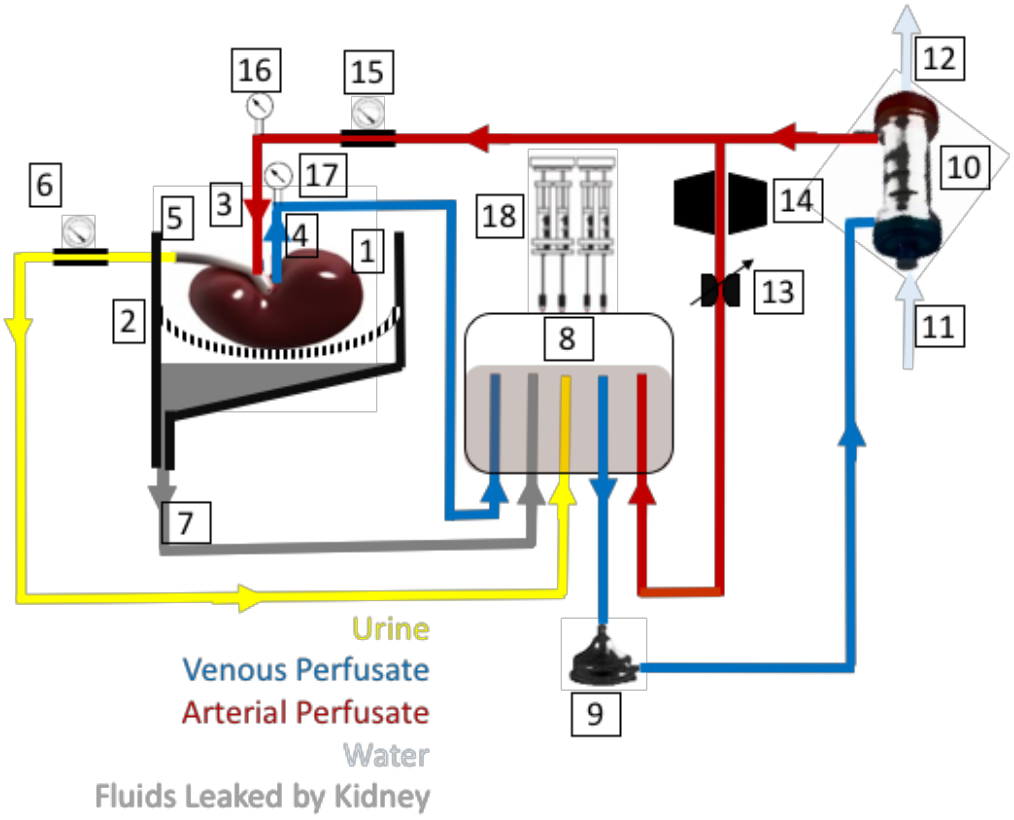


Figure 2.1C: Detailed diagram of the layout for the prototype.

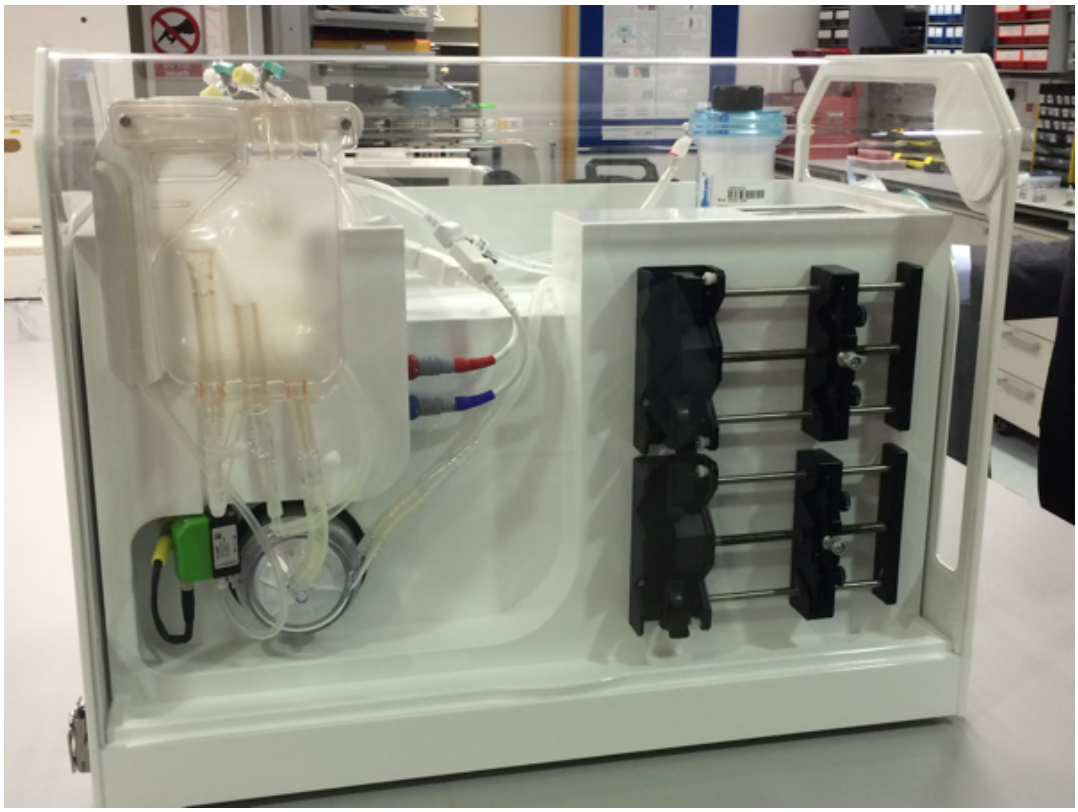


Figure 2.1D: First integrated portable prototype for normothermic kidney perfusion.

2.1.1 Components of the perfusion circuit

The components of the circuit were sourced from cardiopulmonary bypass suppliers and comprised: blood reservoir (Terumo® Capiox® venous reservoir); centrifugal blood pump (Medtronic Affinity™ CP centrifugal blood pump, Medtronic Limited, Watford, United Kingdom); membrane oxygenator/heat exchanger (Lilliput 2 ECMO, LivaNova PLC, former Sorin SpA); medical grade silicone tubing with internal diameters of 1/4 and 3/8 inches. A plastic container was modified to house the kidney during perfusion. Modifications included a drain to allow perfusate lost from the circuit due to bleeding to return to the reservoir under gravity.

Maintenance of the perfusate temperature at 37°C was achieved using a water-based heat-exchanger (integral to the oxygenator). Water heating (and potentially cooling) was achieved by means of a custom-built thermo-electric heater and cooler.

Pressure in the renal artery was measured in-line using single-use pressure sensors (PendoTECH single use pressure sensors™; PendoTECH, Princeton, New Jersey, USA). Haemodynamic control was based on arterial pressure, without any direct adjustment of arterial flow and maintained in the range 70-110 mmHg by adjustment of the centrifugal pump speed between 1300-1500 rpm. The flow through the kidney was measured by an external ultra-sonic flow sensor (SONOFLOW CO.55/080 by SONOTEC).

2.1.1.1 The blood reservoir

A CAPIOX® flexible venous reservoir with a capacity of 400 ml was used, produced by Terumo Cardiovascular Systems Corporation.

2.1.1.2 The centrifugal blood pump

The Affinity™ CP centrifugal blood pump from Medtronic Limited was chosen for the following reasons: 1) compact size with low priming volume of 40 ml, 2) low index of haemolysis with <0.1 grams haemoglobin released per 100 litres blood pumped at 5 l/min, 3) even blood flow, 4) low heat generation (only generates heat until it reaches temperature equilibrium of 4.7°C above blood temperature and 5) hydrophilic biosurface without heparin. All the above data is according to manufacturer's information.

2.1.1.3 The membrane oxygenator

The Lilliput 2 ECMO membrane oxygenator/heat exchanger system from LivaNova PLC (former Sorin Group) is designed and certified for long-duration use in neonatal and infant procedures. This system is equipped with a long-term usage (5 days), validated polymethylpentene membrane which guarantees optimal performance (pressure drop, O_2 and CO_2 transfer) for paediatric patients undergoing long-duration surgeries or extended cardiac-respiratory support. All the surfaces in contact with blood are P.h.i.s.i.o (phosphorylcholine inert surface) coated. The phosphorylcholine molecule is highly haemocompatible, safe, reduces activation of coagulation factors, reduces inflammatory reactions and cannot be removed by priming or blood during cardio pulmonary bypass. The priming volume of this oxygenator is 90 ml and the heat exchanger performance factor is 0.48 (at 2.3 l/min). All the above data is according to manufacturer's information.

2.1.2 Haemodynamics

The prototype design provides normothermic perfusion for 24 hours with a single arterial input. Venous blood returns to the reservoir under gravity: the relative heights of the organ and the fluid level in the reservoir create the venous pressure, and were set to avoid any venous back-pressure. In this respect, the kidney circuit design differs from the previous liver perfusion system (108) in which venous return is pumped.

The final iteration of the prototype device regulated the mean arterial pressure solely by controlling the speed of the blood pump. In this configuration the flow rate of the perfusate through the kidney was a function of the arterial pressure and the intrarenal resistance of the organ. A vasodilator, prostacyclin (Flolan®, epoprostenol sodium), is the only non-device related factor interfering with the renal blood flow.

2.1.2.1 Pressure control

The non-pulsatile mean arterial pressure in the renal artery is measured in-line using a single-use pressure sensor (PendoTECH single-use pressure sensors™; PendoTECH, Princeton, New Jersey, USA). The PendoTECH sensors comprise a pressure transducer housed within a polycarbonate housing. The sensors can be connected in-line via two ¼" hose barbs integrated into the plastic housing.

During the first step of the device development, the pressure was manually regulated by changing the speed of the centrifugal pump in the range from 1300 to 1500 rpm to achieve a mean arterial pressure between 70 and 110 mmHg. In the third step of the prototype development, the mean arterial pressure (MAP) was maintained automatically at a fixed set point of 90 mmHg for instance, under software control. A physiological MAP can be calculated by using the following formula: ((2 x diastolic) + systolic) divided by 3. As an example: an adult human being with a systolic blood pressure of 130 mmHg and a diastolic blood pressure of 80 mmHg would have a MAP of 97 mmHg.

To maintain a specified blood pressure at a certain level, a PID (proportional integral differential) closed-loop control system was developed. The pressure sensor, acting as a transducer, produces a signal that is amplified by the electronics and digitised by a microcontroller for the calculation of the PID error terms. The software sets the speed of the centrifugal pump to minimise the error from the setpoint and maintains the blood pressure within specified limits.

2.1.2.2 Flow measurement

For measuring the arterial flow, a SONOFLOW clamp-on sensor from SONOTEC ultrasonic solutions (SONOTEC Ultraschallsensorik, Halle an der Saale, Germany) was implemented in the device. This type of flow measurement was chosen as SONOFLOW clamp-on sensors are specifically made for non-invasive liquid flow measurements in tubes (109). The product has several advantages: highly accurate non-invasive measurements of liquids in plastic tubes such as PVC and silicone etc.; no direct contact or interference with the liquid (in our case the blood based perfusate) which makes the sensor particularly suitable for applications with the highest hygienic standards; the clamp-on design avoids any risk of contamination or leaking; measurement of pulsating (of interest for future work) and bi-directional flow is possible; the electronics are contained within the sensor, and therefore no external electronic board is required.

- Technical data (109): The underlying methodology is based on ultrasound, with four piezo elements arranged in an X-pattern, see figure 2.2. There is a measurement cycle of 20 ms. This system has a wide range of operation, with an upper value of 8000 ml/min (CO.55/080). The accuracy tested with water is stated as ± 16 ml/min in the range of 0 to 800 ml/min and ± 2% in the range of 800 to 8000 ml/min.

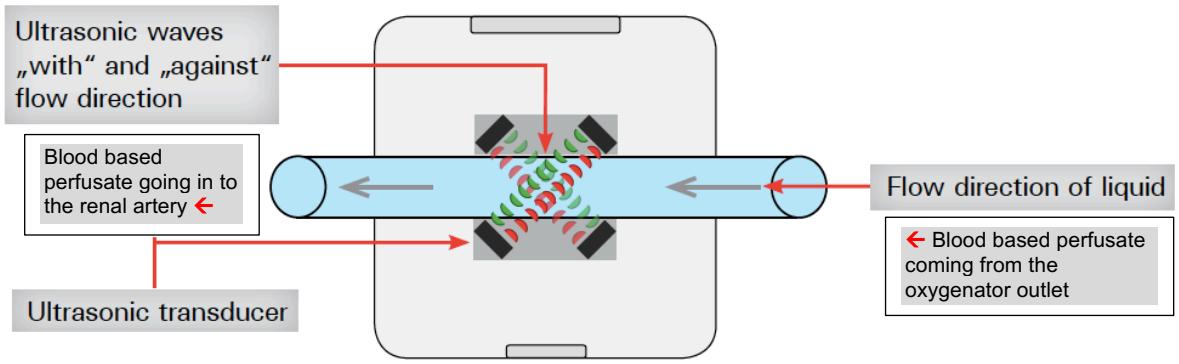


Figure 2.2: The modified picture of the flow sensor by SONOTEC (www.sonotec.eu) shows the actual size of the sensor, the principle of the ultrasound technology used for flow measurement (an ultrasonic pulse travelling in the flow direction of the fluid is accelerated, the one travelling against the flow is decelerated), the piezo elements arranged in an X-pattern and the ultrasonic transducer.

2.1.2.3 The bypass system

In the earlier iterations of this circuit (including the early stage of the pre-clinical prototype), a bypass line with a pinch valve was used, to divert blood from the arterial line directly to the reservoir. The objective was to adjust the arterial pressure by varying the proportion of bypassed blood, by changing the resistance in the system with the adjustment of the pinch valve; figure 2.3.

During the development of the device it became clear that the bypass line was not of significant benefit to overall haemodynamic control. The bypass was therefore removed in order to simplify the system and reduce overall tubing length which has an impact on haemolysis. As a result, in the final design renal blood flow was a function of the renal pressure and the intra renal resistance of the kidney being perfused.

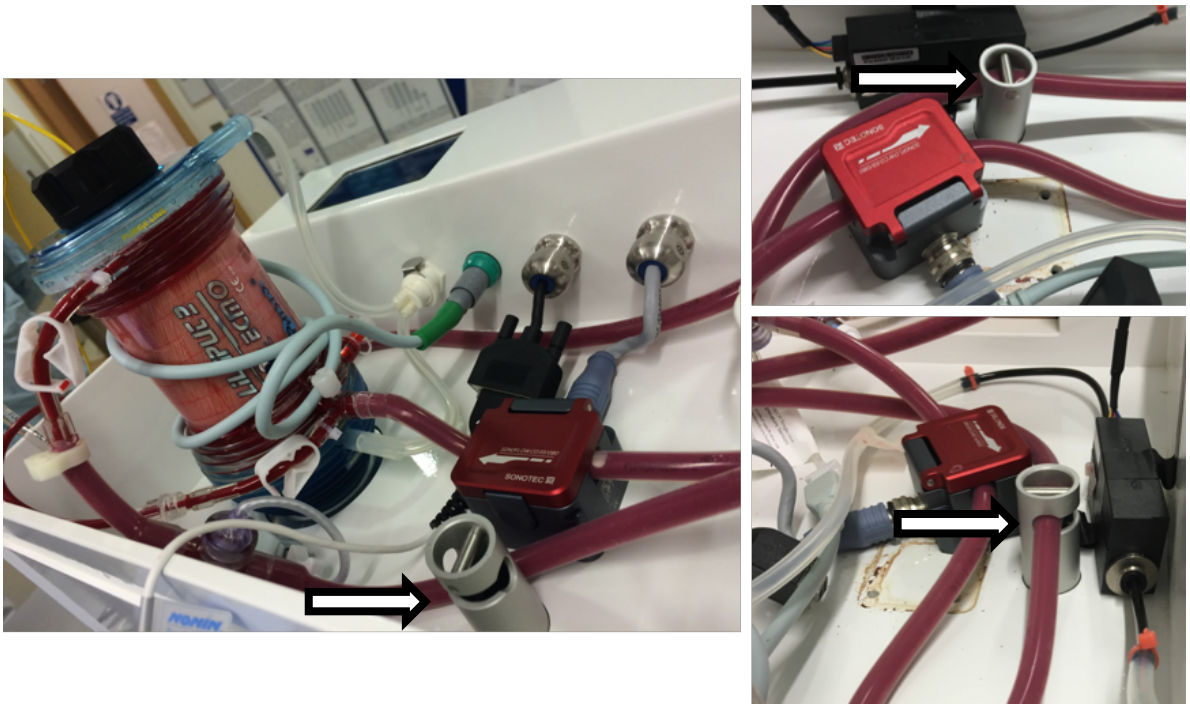


Figure 2.3: Bypass line and pinch valve, indicated by arrows.

2.1.3 Oxygenation and gas control

In the early stage of the device development, blood gases were maintained within physiological limits using a closed loop controller, comprising an oxygen concentrator and air pump that delivered oxygen flow rates of the order of 10 ml/min and air flow rates of the order of 100-150 ml/min. Proportional control valves were used to automatically regulate gas flow rates in order to maintain PO_2 between 10-20 kPa and PCO_2 between 2-6 kPa. It proved to be difficult to manage the blood gases accurately, mainly because the demand for oxygen was very low and difficult to regulate when produced by the oxygen concentrator (a method which works well in the much larger liver device). Therefore, an alternative method of gas control was tested, using an air pump and bottled CO_2. Oxygenating with air improved control because lower O_2 concentration (21%) and higher flow rates around 100 ml/min, reduced the time to fill the oxygenator with a given mixture of gases and produced more favourable transfer characteristics from the oxygenator.

Consumption of oxygen per gram kidney was not calculated. In our past experience (liver and kidney NMP), we found oxygen consumption very difficult to interpret in the context of normothermic perfusion, particularly when the flow rates of oxygen infused are so low.

2.1.4 Temperature setting

The temperature sensor we used is an in-line PendoTECH single use hose barb sensor (PendoTECH, Princeton, New Jersey, USA). The temperature range of the sensor is 0-70°C with an accuracy better than ± 0.2°C (typical better than 0.1°C) (110). Temperature regulation in the perfusion device was based on an implementation of a Proportional-Integral (PI) controller.

A thermo electric cooler device, we are using, works based on the Peltier effect which is the presence of heating or cooling at an electrified junction of two different conductors. This effect was discovered by the French physicist Jean Charles Athanase Peltier in 1834 (111). The “setpoint” temperature was set to 37°C but this could be adjusted by a few degrees, to allow for any offset differences if required. An experiment validating the performance of the temperature controller following introduction of cold and hot water into the circuit is shown in figure 2.4.

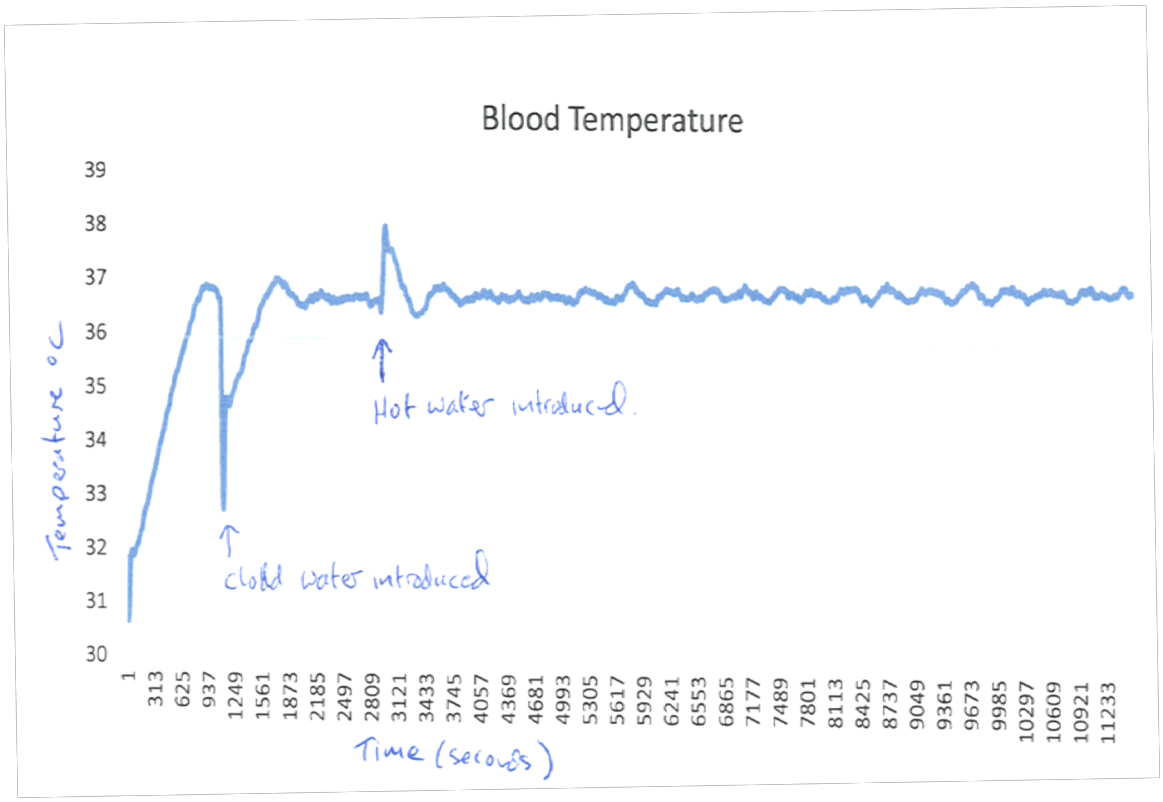


Figure 2.4: Testing the temperature regulation by adding cold and hot water to the circuit.

2.1.5 Urine measurement

Urine production was continuously monitored using an in-line flow sensor (LD20-0600L Liquid Flow Sensor, Sensirion AG, The Sensor Company, Stäfa, Switzerland). The in-line flow sensor was embedded, using a luer lock system, in the ureter outflow using a 6 or an 8 Fr tube and extension lines (type Heidelberger, B. Braun Melsungen AG, Germany). The sensor was shown to be accurate compared to manual urine volume measurement. In addition to measuring the urine flow-rate, we could see pulses of urine flow presumably caused by the physiological peristalsis of a functioning ureter; figure 2.5.

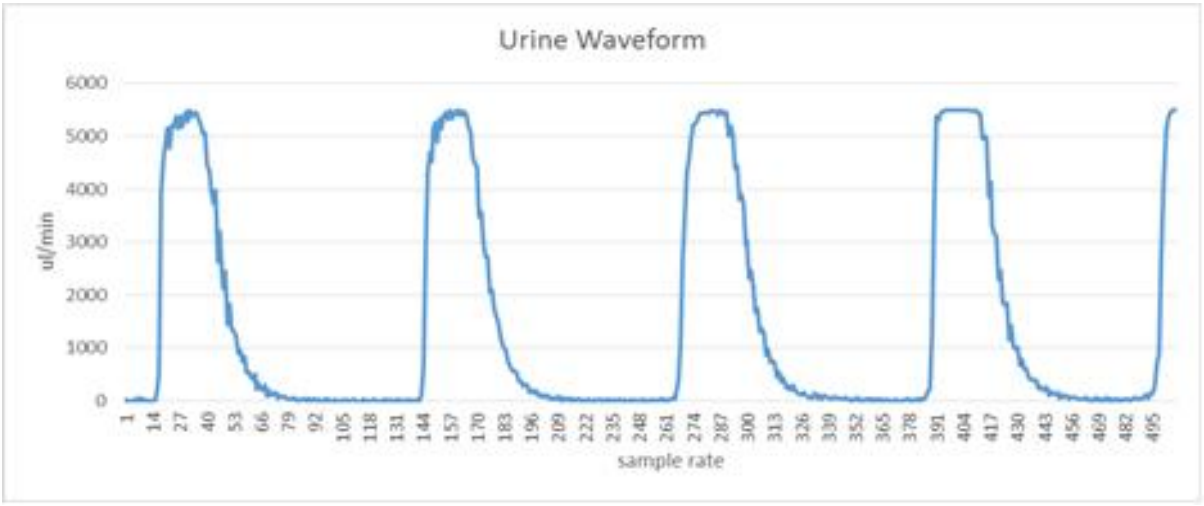


Figure 2.5: Urine flow measurement with a liquid flow meter and urine waveform.

The sensor measures dynamic flow rates, ranging from about 20 ml/h up to 1000 ml/h. Its performance, as given by the manufacturer, met our requirement to measure even a small volume of urine: Full scale flow rate is 20 ml/h (0.3 ml/min); sensor output limit is 25 ml/h (0.4 ml/min); accuracy below full scale is 5% of measured value or 0.25 % of full scale (whichever error is larger); the sensor is calibrated for water; the recommended maximum use duration is 7 days (112).

2.1.5.1 Urine recirculation

The produced urine was recirculated via a tube placed in the ureter, continued by an extension line (type Heidelberger, B. Braun AG, Melsungen, Germany) into the softshell reservoir. This was tested in order not only to maintain a constant circulating volume, but also to avoid the electrolyte imbalance that would result from large volume replacement with crystalloid fluids.

2.1.6 Control system of the kidney device

The kidney device can be split into three blocks: Kidney graphical user interface (GUI), kidney application (APP) and sensors/actuators. Each of these blocks have their own specific function and communicate with each other using a transmission control protocol (TCP). This communication is shown in figure 2.6A.



Figure 2.6A: Three blocks of the control system of the kidney perfusion device.

Sensors are used to acquire information about the current state of the device for example: blood temperature, arterial pressure, PO_2 level, etc. This information is then fed back to the kidney APP and used within it to maintain the desired conditions within the device. This is achieved using controls loops which send commands to the actuator to attempt to correct any differences between current and desired conditions. The kidney APP also feeds back sensor reading to the kidney GUI which can then be displayed to the user. Additional, purely for development, a settings menu can be accessed via the kidney APP, as shown in figure 2.6B, to allow manual control of the actuators if required.



Figure 2.6B shows the graphical user interface (GUI) I was working with; A = before perfusion start, B = during perfusion. The arrow indicates the button for fixing the blood pressure (BP). 90 mmHg were chosen for human and 100 mmHg for porcine kidneys.

2.2 Composition of perfusate

2.2.1 Discarded human kidneys

The perfusion solution was composed of 1 unit of packed red blood cells of the same blood group as the kidney, re-suspended in 250ml of 5% human albumin solution, giving a total perfusate volume of 500 ml with a physiological oncotic pressure. For all experiments expired or no longer usable units (e.g. interrupted freezer chain, incorrect labelling) of packed red blood cells were used. During priming of the circuit, before connection of the kidney, the perfusate was supplemented with bolus doses of: cefuroxime (750mg); calcium gluconate 10% (10ml to counteract the calcium binding of citrate). The pH was adjusted by titration with sodium bicarbonate 8.4% (5-15 ml), if necessary, to a physiological level of 7.3 as soon as the perfusion solution reached physiological body temperature. It is of utmost importance to evaluate pH at 37°C under a physiological PCO_2. As described by Danzl and colleagues, due to the direct effect of temperature on both the dissociation of hydrogen ions and gas pressures in

a watery solution, the arterial pressure of carbon dioxide falls, and the arterial pH increases when blood cools. The pH of neutrality is higher at 30°C than at 37°C as water dissociates less readily at a lower temperature than at a higher one. A pH of 7.4 and a PCO_2 of 5.3kPa at a normal body temperature are physiologically equivalent to a pH of 7.5 and a PCO2 of 4kPa at a body temperature of 30°C (113). No further sodium bicarbonate was given at any point during perfusion following kidney connection. After starting perfusion 10ml of 10% mannitol was added. During the perfusion, epoprostenol sodium (Flolan® 0.5mg; GSK Glaxo Smith Kline, Middlesex, United Kingdom) was infused at a constant rate of 4 µg/hour, to optimise the microcirculation. For glucose control, 2-hourly blood glucose measurements were carried out. A bolus injection of 2.5ml of a lipid free parenteral nutrition solution (TPN) (Nutriflex® special, B. Braun AG, Melsungen, Germany; containing 0.24mg/ml glucose) or a bolus injection of 11ml of glucose 5% (Baxter Healthcare Ltd; containing 0.05mg/ml glucose; overall 836.8kJ (200kcal) per litre) was administered once blood glucose levels dropped below 4mmol/l. The temperature of the perfusate was maintained at 37°C throughout the perfusion period.

Perfusions were carried out both with urine recirculation (as described above) and without recirculation (in which case urine losses were replaced with Ringer's lactate).

2.2.2 Porcine kidneys

White Landrace pigs (40-50kg) were used and treated in accordance with the United Kingdom Animal Protection Act 1986. Kidneys were retrieved using a standardised technique under general anaesthesia according to standard clinical practice for living donation. For anticoagulation 25.000 units of unfractionated heparin were used and administered intravenously 10 minutes prior to clamping of the first renal artery.

Retrieved kidneys were flushed and stored with Marshall's hypertonic citrate solution (Soltran® kidney perfusion fluid, Baxter healthcare Ltd, Deerfield, Illinois, USA) at ice temperature, according to standard clinical practice. After flushing the kidneys on the back bench with 500ml of Soltran® each, the pig was terminated and exsanguinated to collect the blood for organ perfusion. The blood collection was carried out with direct puncture of the abdominal aorta using the blood collection needle coming with the COMPOFLEX® blood bag (COMPOFLEX®, Fresenius Kabi, Bad Homburg vor der Höhe, Germany) to achieve the best and most sterile autologous blood for perfusion. One kidney was stored on ice for 2 hours before being connected to the circuit for normothermic perfusion. One kidney was stored on ice for 27 hours before the connection to the circuit. On an alternating basis, the kidney with the short/long CIT was perfused with/without recirculation of the urine.

The perfusate for the normothermic preservation of porcine kidneys consisted of 500ml of autologous whole blood. The priming of the circuit with the porcine blood took place with a giving set including a leukocyte filter disk; HAEMONETICS® RS1 leukocyte filter for salvaged blood (HAEMONETICS®, Braintree, Massachusetts, USA). During priming of the circuit, before connection of the kidney, the perfusate was supplemented with bolus doses of: unfractionated heparin (5000 units); cefuroxime (750mg); calcium gluconate 10% (10ml to counteract the calcium binding of citrate). The pH was adjusted by titration with sodium bicarbonate 8.4% (5-15 ml), if necessary, to a physiological level between 7.4-7.5 as soon as the perfusion solution reached physiological porcine body temperature. No further sodium bicarbonate was given at any point during perfusion following kidney connection. During the perfusion, epoprostenol sodium (Flolan® 0.5mg; GSK Glaxo Smith Kline, Middlesex, United Kingdom) was infused at a constant rate of 4μg/hour, to optimise the microcirculation.

In addition, 2.5mg verapamil (Securon® IV 2.5mg/ml, FAMAR health care services, Madrid s.a.u, Spain) were added directly into the artery after perfusion start and 10ml of glyceryl trinitrate 1mg/ml were administered via a syringe pump driver within the first hour of perfusion into the softshell reservoir (Glyceryl Trinitrate 1mg/ml solution for infusion, Hameln Pharmaceuticals Ltd, Gloucester, United Kingdom). For glucose control, 2-hourly blood glucose measurements were carried out. A bolus injection of 2.5ml of a lipid free parenteral nutrition solution (TPN) (Nutriflex® special, B. Braun AG, Melsungen, Germany; containing 0.24mg/ml glucose) was administered once blood glucose levels dropped below 4mmol/l. As with the human kidney studies, depending on the handling of the excreted urine, the perfusate comprised also urine in the urine recirculation group or Ringer's lactate as a volume replacement 1:1 for the excreted urine.

2.3 Preparation of the kidney graft

2.3.1 Discarded human kidneys

Back-table preparation and priming of the perfusion machine were carried out in parallel. If necessary, accessory arteries were reconstructed during the back-table preparation to provide a single inflow; figure 2.7A.



Figure 2.7A: Reconstruction of three renal arteries to allow a single arterial inflow.

Cannulation of the renal artery and the renal vein with 20-Fr and 10-Fr cannulae was carried out. The ureter was cannulated with a 6-Fr or 8-Fr tube in order to recirculate or to collect the urine. All cannulas were secured with ligatures. Thirty to 40 minutes were needed for graft preparation. Back-table preparation was performed with kidneys still immersed in ice cold preservation solution. Grafts were then flushed with approximately 200-300ml of crystalloid Ringer's lactate at room temperature, before connection to the NMP device.

Figure 2.7B shows a kidney fully cannulated ready to go on to the perfusion device.

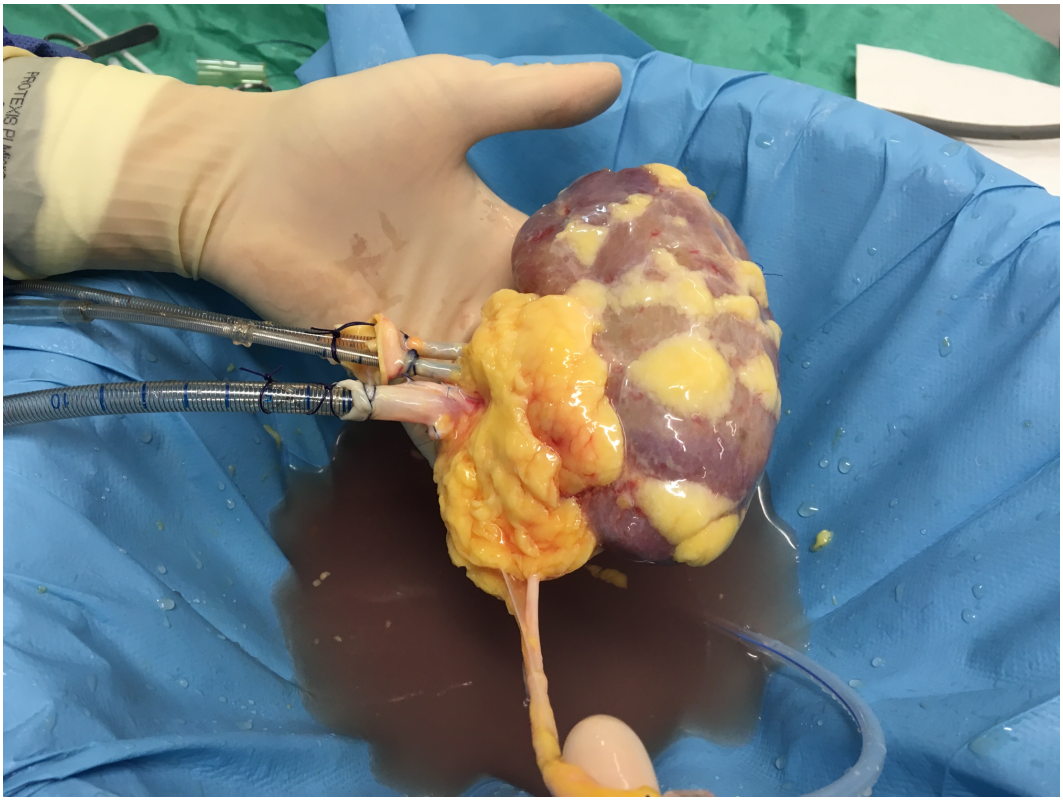


Figure 2.7B: A typical patchy perfused human kidney declined for clinical use, cannulated and ready to go on the perfusion device. This kidney had two arteries which were cannulated separately as there would not have been enough vessel length ahead of an artificial bifurcation to allow a single arterial cannulation. The two cannulae were connected with a Y-piece to make a single arterial inflow (direct single outflow from the oxygenator) feasible.

2.3.2 Porcine kidneys

Kidney grafts for the porcine perfusion experiments were prepared and cannulated in the exact same way as the human kidneys, directly after the organ retrieval; 10-Fr cannula for the artery, 20-Fr cannula for the vein, 8-Fr tube for the ureter; in a situation of two arteries or two veins, both vessels were cannulated separately and connected with a Y-piece to achieve a single in- or outflow.

2.4 Measurement of perfusion parameters

Blood gas analysis was performed by using an in-line blood gas analyser (CDI 500, Terumo Medical Corporation, Somerset, New Jersey, USA). Glucose, lactate and electrolyte measurements were performed off-line using a hand-held blood gas analyser (iSTAT, Abbott Point of Care Inc., Princeton, New Jersey, USA), which was also used to further confirm PO_2 and PCO_2, and pH arterial and venous measurements where needed; CG4+ and CHEM8+ cartridges were used. The CG4+ cartridge for the iSTAT measures lactate, pH, bicarbonate, base excess, oxygen saturation, the partial pressures of oxygen and carbon dioxide and total carbon dioxide. The CHEM8+ cartridge measures sodium, potassium, chloride, total carbon dioxide, anion gap, ionised calcium, glucose, urea, nitrogen, creatinine, lactate, haematocrit and haemoglobin. Both i-STAT cartridges are single-use and require 2 to 3 drops of blood each (114).

2.5 Perfusate and urine analysis

2.5.1 ELISAs human kidneys

2.5.1.1 NGAL, KIM-1, L-FABP

Perfusate samples were collected at 5 time points (at 1, 6, 12, 18 and 24 hours) during NMP and processed by centrifugation at 4000 rpm for 15 minutes at 4°C. The supernatant was aliquoted and stored at -80°C. Neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1) and liver-type fatty acid-binding protein (L-FABP) levels in the perfusate samples were measured by a quantitative sandwich enzyme immunoassay technique using NGAL and KIM-1 Quantikine ELISA kits (R&D systems, USA) and Human FABP1/L-FABP ELISA Kit (CMIC Co., Ltd., supplied by R&D systems) according to manufacturer's instructions. Briefly, after

preparation of the reagents, the assay buffer, standards (recombinant human NGAL, KIM-1 or L-FABP), positive controls and perfusate samples were added to the wells of a 96-well plate coated with a monoclonal antibody specific for human NGAL, KIM-1 or L-FABP, respectively. After incubation (2h at 4°C for NGAL, 2h at room temperature for KIM-1 and 1h at room temperature for L-FABP, according to manufacturer's instructions), wells were emptied by aspiration and washed 4 times with the wash buffer provided. After the last wash, secondary antibodies specific for NGAL, KIM-1 and L-FABP, conjugated to horseradish peroxidase (HRP), were added to each well. Plates were sealed and incubated (2h at 4°C for NGAL, 2h at room temperature for KIM-1 and 1h at room temperature for L-FABP). Following incubation each well was emptied and again washed 4 times. HRP substrates (hydrogen peroxide and the chromogen tetramethylbenzidine) were added to each well and incubated for 30 min at room temperature protected from light. After 30min the reaction was stopped by the addition of sulfuric acid and the plates were read at 450 nm (with wavelength correction at 540 nm) for NGAL and KIM-1 and at 492 nm (with background correction at 630 nm) for L-FABP, in a microplate reader (BMG Omega plate reader, BMG labtech, Germany). Biomarker levels in perfusate samples were extrapolated from the standard curves.

NGAL and KIM-1 ELISAs were performed for both, perfusate and urine samples, for kidneys perfused without recirculation of the urine (kidneys 30-33).

2.5.2 ELISAs porcine kidneys

2.5.2.1. NGAL and KIM-1

Perfusate samples were collected at 3 time points during perfusion: at the start with no kidney connected; after 2 hours of perfusion; at the end of perfusion. Samples were processed by centrifugation at 4000 rpm for 15 minutes at 4°C. The supernatant was aliquoted and stored at -80°C. Neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) levels in the perfusate samples were measured by a quantitative sandwich enzyme immunoassay technique using NGAL (DLDEVELOP, China, catalogue number: DL-NGAL-p) and KIM-1 ELISA kits (Fine Test, China, catalogue number: EP0102) according to manufacturer's instructions. Briefly, after preparation of the reagents, the assay buffer, standards (recombinant porcine NGAL or KIM-1) positive controls and perfusate samples were added to the wells of a 96-well plate coated with a monoclonal antibody specific for human NGAL or KIM-1. After incubation (2h at 37°C for NGAL, 90 minutes at 37°C for KIM-1, according to manufacturer's instructions), wells were emptied by aspiration and washed 3 (NGAL) or 2 times with the wash buffer provided. After the last wash, secondary antibodies specific for NGAL and KIM-1, conjugated to HRP- (horseradish peroxidase) streptavidin were added to each well. Plates were sealed and incubated (1h at 37°C for NGAL and KIM-1). Following incubation each well was emptied and again washed 5 (NGAL) or 3 times (KIM-1). HRP substrates (hydrogen peroxide and the chromogen tetramethylbenzidine) were added to each well and incubated for 15-25 minutes at 37° for NGAL and 30 minutes at 37° for KIM-1 protected from light. After 30min the reaction was stopped by the addition of sulfuric acid and the plates were read at 450 nm for NGAL and KIM-1 immediately, in a microplate reader (BMG Omega plate reader, BMG

Labtech GmbH, Ortenberg, Germany). Biomarker levels in perfusate samples were extrapolated from the standard curves.

2.5.3 Anti-Xa assay

The samples for the anti-Xa assay were collected in a citrate anticoagulant tube 1, 4, 6, 12, 18 and 24 hours after perfusion start and at the end of human kidney perfusions. The samples were processed by centrifugation at 4000 rpm for 15 minutes. The supernatant was aliquoted and stored at -20^{0}C. The assay was carried out at the Haematology Laboratory at the John Radcliffe Hospital, Oxford University Hospitals, NHS Foundation Trust. The Biophen Heparin (LRT) kit was used for measuring the concentration of the low molecular weight heparin (LMWH) enoxaparin sodium (Clexane®). Biophen Heparin (LRT) is a chromogenic anti-Xa method developed for measuring homogeneously unfractionated heparin and LMWH. It is a kinetics method based on the inhibition of a constant amount of factor Xa, by the tested LMWH in presence of endogenous antithrombin, and hydrolysis of a factor Xa specific chromogenic substrate, by the factor Xa in excess. Para-nitroanaline (pNA) is released from the substrate; the amount of pNA is then a relation of the residual factor Xa activity. The relationship between the concentration of LMWH and colour development, measured at 405nm, is inverse (115).

2.5.4 Analysis of urine protein and urine sodium

Urine protein and sodium analysis were performed for human urine samples of perfused kidneys without urine recirculation and for urine of normothermically perfused porcine kidneys both with and without urine recirculation. Samples were taken within the first 15-30 minutes after perfusion start and 2-hourly thereafter throughout the

perfusion, as far as there was urine production, centrifuged at 4000 rpm for 15 minutes, aliquoted (without the sediments) and stored at -80°C. The analyses were carried out in the Biochemistry Laboratory at the John Radcliffe Hospital, Oxford University Hospitals, NHS Foundation Trust.

2.5.5 Flow cytometry

Samples of perfusate, 4ml, were collected in an EDTA tube, 0.4ml dimethyl sulfoxide (DMSO) were added and well mixed; 2ml of this solution were transferred into a cryogenic storage vial, moved to a CoolCell® Cell Freezing Container and stored in a -80°C freezer. Immunophenotyping of the human perfusate samples was performed on a BD LSR II flow cytometer (Becton Dickinson, Oxford, United Kingdom). Leukocytes were identified and gated as CD45+ and their viability assessed using an efluor 506 viability dye (ebioscience, San Diego, California, USA). Following this, a panel of antibodies was utilized to characterize T helper cells (CD3ε+CD4α+), cytotoxic T cells (CD3ε+CD8α+), double-positive T cells (CD3ε+CD4α+CD8α+), double-negative T cells (CD3ε+CD4α-CD8α-), γδ T cells (γδ+), B cells (CD3ε-CD21+), classical monocytes (CD14+CD163-), nonclassical monocytes (CD14+CD163+), immature neutrophils (6D10+2B2-), mature neutrophils (6D10+2B2+), mature eosinophils/basophils (6D10-2B2+), and natural killer cells (CD335+). Cells were treated with red blood cell lysing solution (BD Biosciences, United Kingdom), washed, and resuspended in 0.3 ml of staining buffer. A 20-ml quantity of e123count beads (eBioscience, California, USA) were added, and samples were analysed for 3 minutes. All gating strategies and analysis were performed using FlowJo version 10.0.6.

2.5.6 Luminex analysis

Perfusate samples were collected at 5 time points (at 1, 6, 12, 18 and 24 hours) during NMP and processed by centrifugation at 4000 rpm for 15 minutes at 4°C and stored at -80°C until use. A commercially available human 13-plex magnetic bead panel (Merck Millipore, Billerica, Massachusetts, USA) was used, following the manufacturer's protocol. The plate was read using a Bio-Plex 200 system (Bio Rad, Hertfordshire, United Kingdom). Thirteen cytokines and chemokines were assessed: Interferon (IFN)-γ, Interleukin (IL)-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor (TNF)-α.

2.5.7 Metabolomics

2.5.7.1 Extraction of metabolites from perfusate samples

Two hundred microliters of perfusate collected at the beginning of perfusion, after 6 hours (kidneys with and without urine recirculation), after 12 and 24 hours of perfusion (kidneys with urine recirculation) were used for metabolites extraction. Two hundred µl methanol were added to perfusate and mixed for 5 minutes on a shaker at 1500 rpm. Five µl of myristic acid-14,14,14,d3 (1mg/ml, Cambridge Isotope Laboratories, United Kingdom) was spiked into perfusate samples as normalisation control. The samples were vortexed for 5 minutes then followed by adding of 1 ml of *tert*-butyl methyl ether (MTBE). The samples were mixed by shaking for 5 minutes then centrifuged for 20 minutes at 13,000 g at 4°C. The top layer organic phase (MTBE) were transferred into a glass vial and dried in a Speed Vac (Eppendorf, United Kingdom). Subsequently, 800 µl of methanol was added into the aqueous remain (bottom layer), the samples were vortexed for 5 minutes and centrifuged for 20

minutes at 13,000 g at 4°C. The supernatant (aqueous phase) was collected and added into the glass vial containing the organic phase to dry under vacuum. The dried samples were kept at -80°C until use.

Chemical derivatisation was performed essentially as described (116). In brief, samples were oxidated in 50 µl of methoxyamine hydrochloride in pyridine (20 µg/µl) and shaken (1200 rpm) for 90 minutes at 30°C. Seventy microliters of N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% chlorotrimethylsilane (TMCS) and 30 µl pyridine were added to the samples, followed by incubation for one hour at 60°C at a shaking speed of 1200 rpm. The samples were cooled down to temperature ambient and injected directly for GC-MS analysis.

2.5.7.2 Metabolomic analysis using gas chromatography mass spectrometry (GCxGC-MS)

Two µl of derivatised metabolites were injected into a GCxGC-qMS instrument (GP2010, Shimadzu, United Kingdom) with a split ratio of 1 in 20. The oven temperature was programmed to rise from 80°C to 330°C at a rate of 6°C per minute, and the total run time was 35 minutes. The metabolites were first separated by a non-polar column (30m x 0.25mm inner diameter), followed by a second polar column (6m x 0.25 mm inner diameter) controlled by a modulator that operated at six second cycles as described (117). In brief, metabolite effluents separated on the first column were accumulated for 6 seconds, then remobilised, focused and injected into the second column. Ions were generated within GC-MS by electron impact (EI) ionisation and collected at a mass range of 50*m/z* to 800*m/z* using an acquisition rate of 20,000 u/sec and 100 Hz. Metabolites were identified by matching collected fragment ions to the NIST (National Institute of Standards and Technology) library using GC solution

software (Ver.2.32). The identification confidence score cut off was set to 80, and a panel of collected standards were used to verify their identification (such as palmitic acid, stearic acid). Metabolites quantitation was performed using Chrome square software 2.1.6 (Shimadzu, Japan). Each metabolite quantity was normalised to spiked myristic acid for comparison.

2.6 Tissue analysis

2.6.1 Histology human kidneys

Biopsies were taken at six different time points (zero biopsy at start of back table preparation, 1, 6, 12, 18 and 24 hours after perfusion start/end of perfusion). Core needle biopsy specimens, 18 gauge and 33mm long were cut in half. One part was snap frozen and stored in a cryo vial at -80°C until further analysis, the second half was fixed in Millonigs solution and processed for paraffin embedding. Three µm-thickness sections were stained by haematoxylin and eosin. Light microscopy observations were carried out on a Nikon Eclipse 50i microscope (Nikon Corporation, Japan). Histological assessment was based on modified Remuzzi scores (118) and used the following parameters: number of glomeruli; percentage of globally sclerosed glomeruli; percentage of chronic damage (interstitial fibrosis and tubular atrophy in the cortex); arteriolar hyalinosis (0 – absent; 1 – present); intimal elastosis (0 – absent; 1 – less than or equal to the thickness of the media; 2 – greater than the thickness of the media); presence of acute tubular injury (0 – absent; 1 – loss of brush borders / vacuolation of tubular epithelial cells; 2 – cell detachment / cellular casts; 3 – coagulation necrosis). Immunohistochemistry for KIM-1 was performed (R&D Systems, Human TIM-1/KIM-1/HAVCR Antibody, monoclonal Mouse IgG2B Clone #219211) following heat induced antigen retrieval, incubation with primary and

secondary antibodies, staining with diaminobenzidine and haematoxylin counterstaining. Positivity was characterised by brown staining in the apical borders in epithelial cells from proximal tubuli or from the outer stripe of the renal medulla. Percentage of stained tubules and their location was noted.

The analysing pathologist was blinded for the duration of cold ischaemia time, the timing of the kidney biopsy and the type of perfusion (with or without urine recirculation).

2.6.2 Histology porcine kidneys

Biopsies were taken at three different time points (zero biopsy at start of back table preparation, 2 and 24 hours after perfusion start/end of perfusion). The process of taking the biopsy, the embedding, the staining, the analysis and the assessment of the biopsy specimens were carried out exactly as described under 2.6.1.

2.6.3 Proteomics human kidneys

2.6.3.1 Protein extraction for proteomic analysis

Biopsy samples were stored at -80°C until use. Two hundred µl RIPA (radioimmunoprecipitation assay buffer) lysis buffer (ThermoFisher Scientific, United Kingdom) with protease and phosphatase inhibitor cocktail (Cat. No. 78441B, ThermoFisher Scientific, United Kingdom) were added to tissue. Tissues were homogenized for 4 times at 6,500 Hz for 40 seconds in a beads-beater (Stretton, United Kingdom). The samples were centrifuged at 10,000g for 5 minutes at 4°C to remove insoluble tissue debris. The protein concentration in the homogenates was determined by BCA (bicinchoninic adic) assay (ThermoFisher Scientific, United Kingdom) and 50 µg of total proteins was added to a 30 kDa filter (Merck Millipore,

United Kingdom). Proteins were reduced by 20 mM DTT (1,4-dithiothreitol) (Sigma, United Kingdom) at 37°C for 1 hour, and then alkylated with 100 mM IAA (iodoacetamide) (Sigma, United Kingdom) for 45 minutes in the dark at room temperature. Samples were centrifuged for 20 minutes at 14,000 g to remove DTT and IAA and followed by buffer exchange with 8 M urea twice and 50 mM ammonia bicarbonate for 3 times. One hundred µl of trypsin were added at a trypsin/protein ratio of 1:50 for digestion at 37°C overnight. Digested peptides were collected by upside down spin and membrane filters washed twice with 0.5 M NaCl and water respectively. The peptides were cleaned by a SepPak C18 cartridge (Waters, United Kingdom), dried by miVac centrifugal vacuum sample concentrators (Genovac, SP Scientific, United Kingdom), and resuspended in buffer A (2% acetonitrile 0.1% formic acid) for LC-MS/MS analysis (119).

2.6.3.2 Protein identification and quantitation by mass spectrometry

LC-MS/MS analysis was carried out by Dionex Ultimate 3000 nano-ultra performance liquid chromatography tandem mass spectrometry (Orbitrap Fusion Lumos, ThermoFisher Scientific, United Kingdom) using a 75 µm-inner diameter x 50 cm C18 EASY-Spray column (2 µm particle size, ThermoFisher Scientific, United Kingdom). Peptides were separated with a 60 minutes gradient of 0% solvent A to 40% solvent B (solvent A: 99.9% H_2O, 0.1% formic acid, 5% DMSO; solvent B: 94.9% ACN, 0.1% formic acid, 5% DMSO) at 250 nl/min. MS data were acquired in electron spray ionisation (ESI) positive mode. The MS survey was set with a resolution of 120,000 FWHM with a recording window between 400 and 1,500 m/z. MS/MS spectra were acquired in the linear ion trap after collision-induced dissociation (CID) fragmentation at a collison energy of 35% and an automatic gain control target of

4.0E3 for up to 250ms, employing a maximal duty cycle of 3 seconds. Selected precursor masses were excluded for 30 seconds. A maximum of 20 MS/MS scans were triggered in data-dependent acquisition (DDA) mode (120).

Raw LC-MS/MS data were uploaded to Andromeda/Maxquant version 1.5.2.8 for MS/MS spectra matching against complete human data set (UPR_HumanSapiens, retrieved at 15/02/2017, 92,954 entries) with trypsin specificity (121). Two missed cleavages, NQ deamination and methionine oxidation were selected as variable modification, carbamidomethylation of cysteine was selected as fixed modification. For identification, the FDR (false discovery rate) at the peptide spectrum matches (PSM) and protein level were set to 0.01. Unmodified unique+razor peptides were chosen for label-free quantification (LFQ) with minute ratio count set as 2. Match between run was selected to increased number of proteins with quantitative value. PANTHER Classification system was used to perform gene ontology enrichment analysis (over-represented or under-represented). KEGG (Kyoto Encyclopaedia of Genes and Genomes) was used for canonical pathway assignment.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier.

2.6.3.3 Perseus statistical analysis

Quantitative MS data were uploaded to Perseus for data visualisation and statistical analysis. LFQ values were first log2 transformed, then plotted in histogram to check normality of the data. Multi scatter plot was used to assess the reproducibility of samples. Hierarchical clustering was used to group samples based on abundance of

identified proteins after Z-score normalisation. Two-sample test was applied to detected differentially expressed proteins between comparisons. Statistical FDR set as 0.05, s0 as 0.1, volcano plot was used to visualise differentially expressed proteins.

2.6.4 Western blot validation of differentially expressed proteins

Twenty µg of homogenised renal cortex proteins were denatured at 95°C for 5 minutes in Laemmli buffer and loaded onto 4–12% pre-cast SDS-PAGE gels (Bio-Rad, USA). Proteins separated by SDS-PAGE were transferred to hydrophobic PVDF membranes (Merck Millipore, USA) in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 400 mA for 2 hours. PVDF membranes were blocked for 1 hour in TBST buffer (25 mM Tris, pH=7.5, 0.15 M NaCl, 0.05% Tween 20) containing 2% fat free milk. Membranes were incubated overnight at 4°C with rabbit anti-human CA1 (1:500 dilution, Protein tech, United Kingdom), BLVRB (1:500 dilution, Cell signalling, United Kingdom), Glut1 (1:1000 dilution, Cell signalling, United Kingdom), HO-1 (1:1000 dilution, Cell signalling, United Kingdom), ICAM-1 (1:1000 dilution, Cell signalling, United Kingdom), SLC4A1 (1:1000 dilution, Cell signalling, United Kingdom), mouse anti-human HSP70 (1:5000 dilution, ThermoFisher scientific, United Kingdom) and rabbit anti-human β-actin (1:1000 dilution, Abcam, United Kingdom). PVDF membranes were washed for 30 minutes with 5 changes of TBST and then incubated at room temperature for 1 hour in blocking buffer containing a 1:10,000 dilution of Dye-800-conjugated anti-mouse or -rabbit secondary antibody (Li-Cor, USA), and visualisation was performed with Odyssey CLx (Li-Cor, USA). Detected signals were quantified and normalized to the β-actin signal on the same blot using Image Studio Lite (version 5.2, USA). Difference in mean was calculated with a Mann-Whitney U

test (Prism v5.0, GraphPad Software, USA). Significance was set at a p-value less than .05 and all graphs report results as mean ± standard deviation of the mean.

2.7 Statistical analysis

For statistical evaluation of the data (excluding ELISA, metabolomics and proteomics), unpaired t tests (parametric) and Mann-Whitney tests (nonparametric) were performed using GraphPad Prism 7. Correlation analysis was performed using IBM® SPSS® Statistics Version 23. A p-value of less than 0.050 was considered significant.

Chapter 3: Perfusion of discarded human kidneys

3.1 Kidneys accepted for my investigations

Thirty-six kidney grafts were accepted for research between July 2015 and February 2018 coordinated with work on the development of the perfusion device. All organs were initially retrieved for the purpose of clinical transplantation but discarded during post-retrieval assessment at the donor hospital or at the transplant centre. These kidneys were made available as part of the NHSBT organ research allocation system. Donor organs were only offered if research consent had been given by the family of the donor.

After transport to Oxford, perfusions were performed at the Institute of Biomedical Engineering (IBME), University of Oxford. The setup for normothermic perfusion of these kidney grafts was progressively modified during the course of this study, as a result of engineering progress towards the objective of a stand-alone, transportable fully automated normothermic kidney perfusion device. Haemodynamic and biochemical perfusion parameters were analysed. Perfusate samples and biopsies were obtained at regular time points during perfusion.

The study was evaluated and approved by the national ethics review committee of the United Kingdom (REC reference 12/EE/0273 IRAS project ID 106793). The NHS Blood and Transplant (NHSBT) study reference number for accepting discarded human kidneys for research was 37 *“Normothermic Perfusion of Discarded Kidneys”*. NHSBT ODT (organ donation and transplantation) progress reports for my study 37 were delivered on a regular basis.

At the beginning of my DPhil, the organ for research offering system was telephone-call based; this was changed to a text-message based offering system in early 2017. In late 2017, the text-message based offering system was combined with offers being sent out by e-mail.

3.1.1 How does a kidney become a kidney for research

The Kidney Advisory Group of NHSBT designed the policy for deceased donor kidney allocation. To optimise the utilisation rate of donor kidneys, a "Kidney Fast Track" scheme was introduced for DBD kidneys on the 1st of December 2012 and for DCD kidneys on the 1st of March 2013 (122). When a kidney is not accepted via the Fast Track System, it is offered out for research; as long as consent has been provided for research purposes.

Kidneys from DBD donors will be offered through the Kidney Fast Track Scheme under a number of circumstances: the organ is deemed not to be usable by a SNOD or a member of the retrieving or transplanting team; five kidney transplant centres decline a kidney-only offer for either donor history or organ quality reasons; the organ has accrued six hours of CIT and has not yet been accepted for transplantation. In the case of kidneys that are first accepted as part of a multi-organ transplant, kidneys are not fast-tracked until the organ has accrued 12 hours of CIT (122).

DCD-kidneys are fast-tracked when: a kidney is deemed not transplantable; three kidney transplant centres have declined the kidney for either donor or organ quality purposes; the organ has accrued three hours of CIT and has not yet been accepted for transplantation; if the kidney has been accepted for transplantation but subsequently declined by the accepting centre after treatment withdrawal but before organ retrieval. In case of kidneys that are first offered and accepted as part of a multi-

organ transplant, the kidney should not be fast-tracked until the organ has accrued 6 hours of CIT (122).

Here are five examples of kidneys offered for research which were declined for the most common reasons I have experienced during my research time: poor perfusion; anatomy; prolonged cold ischaemia time; arteriosclerosis; biopsy findings. The donor type (DBD or DCD) was never stated in the organ offer. Donor number and Hospital name are removed in the text for donor confidentiality and data safety reasons.

1) Right kidney available for research, Age: 58, ABO: O+, Organ location: Oxford, Perfusion time: 02:42, Primary reason declined for transplant: Kidney is not well perfused; kidney is firm; kidney is not improving; atherosclerotic patch. Restrictions on consent: None.
2) Both kidneys available for research, Age: 39, ABO: A+, Organ location: LK/Addenbrookes & RK/Birmingham, Perfusion time: 01:40, Primary reason declined for transplant: Poor perfusion. Restrictions on consent: None.
3) Both kidneys available for research, Age: 70, ABO: A+, Organ location: Leeds, Perfusion time: 14:49, Primary reason declined for transplant: Untransplantable, atherosclerosis of renal arteries and poor perfusion to both. Restrictions on consent: None.
4) Left kidney available for research, Age: 41, ABO: A+, Organ location: Manchester Royal Infirmary, Perfusion time: 03:24, Primary reason declined for transplant: Cold ischaemic time + biopsy. Restrictions on consent: Animal/DNA.

5) Right kidney available for research, Age: 65, ABO: A+, Organ location: Addenbrookes, Cambridge, Perfusion time: 13:38, Primary reason declined for transplant: Infarcted areas & Remuzzi score 5. Restrictions on consent: None.

3.2 Preparation of kidney grafts (2.3 in Chapter 2)

Organs were either preserved with Marshall's hypertonic citrate solution (Soltran®) or UW solution (Belzer UW CSS, Bridge to Life) packed and shipped according to standard clinical practice. Upon arrival at the research laboratory, kidneys were immediately assessed and prepared for connection to the NMP circuit. Back table preparation and priming of the perfusion machine were carried out in parallel. If necessary, accessory arteries were reconstructed during the back-table preparation to provide a single inflow. For some grafts a Y-connector was used to maintain a single arterial inflow in situations where two arteries were cannulated separately: this was done when two arteries were too distant or too short for surgical reconstruction into a single inflow. The urine cannula was connected to the inline flow sensor. All cannulas were secured with ligatures.

3.3 Perfusion solution (2.2.1 in Chapter 2)

While preparing the kidney for cannulation, the perfusion circuit was assembled and primed with one unit of packed red blood cells of the same blood group as the kidney, re-suspended in 250ml of 5% human albumin solution, giving a total perfusate volume of 500 ml. During priming of the circuit, before connection of the kidney, the perfusate was supplemented with bolus doses of: cefuroxime and calcium gluconate 10%. The pH was adjusted by titration with sodium bicarbonate 8.4% to the physiological level of 7.3. Immediately after perfusion start 10ml of 10% mannitol was added from kidney

10 onwards. Prior to the perfusion of kidney 10 up to 60 ml of 10% mannitol were added. During the perfusion, epoprostenol sodium was infused. A bolus injection of 2.5ml of a lipid free parenteral nutrition solution (TPN) or a bolus injection of 11ml of glucose 5%was administered once blood glucose levels dropped below 4mmol/l. The temperature of the perfusate was maintained at 37˚C throughout the perfusion period. The perfusate osmolality at perfusion start was 281±5 mosm/kg.

3.3.1 Anticoagulation of the perfusion circuit

The low molecular weight heparin (LMWH) enoxaparin sodium (Clexane® prefilled syringes 8000 IU (80mg)/0.8ml solution for injection; Sanofi S.A., Paris, France) was used to anticoagulate the circuit (123). The three reasons for using LMWH were (i) the indication of enoxaparin sodium for prevention of thrombus formation in extra corporeal circulation during haemodialysis, (ii) LMWH is cleared by the renal route and could therefore offer (iii) the possibility to measure anti-factor Xa activity over time and to use it as a biomarker. There are several publications and clinical experience to adjust the dose of LMWH in patients with impaired renal function to avoid accumulation of LMWH and bleeding complications as a result (124). Ten percent of the administered dose are cleared by the kidneys as active fragments (123). Despite the primary metabolisation in the liver (which is not existent on my perfusion circuit), by desulfation and depolymerisation, I wanted to analyse if anti-Xa levels over time differ between various normothermically perfused kidneys.

Factor X is an enzyme of the coagulation cascade which is activated by hydrolysis into factor Xa and cleaves prothrombin resulting in active thrombin. The anti-Xa assay is a functional test that facilitates the measurement of antithrombin catalysed inhibition of

factor Xa by direct inhibition of factor Xa by LMWH (125). The anti-Xa assay was carried out as a standard assay by the clinical haematology lab (2.5.3 Chapter 2).

3.4 Management of fluid volume loss due to urine production

The rate of urine production was continuously measured in all kidneys using a liquid flow sensor (Sensirion AG, LD20 Liquid Flow Sensor). In kidneys without urine recirculation (n=4), perfusate volume was maintained by immediately replacing every 20-30 ml of excreted urine with an equivalent volume of Ringer's lactate, as described by previous investigators (91,101). In all other kidneys, urine was recirculated into the circuit in order to maintain a constant circulating volume and to avoid the electrolyte imbalance that would result from replacement with crystalloid fluids. The result of urine recirculation in this situation has not been published previously, although the concept of returning urine to the perfusion system was described by Brasile *et al.* in their description of their exsanguinous metabolic support (EMS) system (126).

3.5 Donor characteristics

Table 3.1 summarises organ retrieval characteristics and the reasons why the 36 investigated kidneys had been declined for clinical use. For logistical reasons, discarded human kidneys offered for research typically have a longer cold ischaemia time (CIT) than those transplanted. Five kidneys (11, 15, 18, 19 and 22) had an extraordinary long CIT >100 hours for logistical reasons.

Table 3.1: Organ procurement parameters and reasons for discard of individual kidneys

	Age in years	Sex	BMI in kg/m2	Donor Type	WIT in min	CIT in hours+min	Hypertension	Reason for Discard
Kidney 1	58	female	22.2	DBD	n.a.	70 + 37	no	patchy perfusion
Kidney 2	72	male	24.5	DCD	28	41 + 35	yes	patchy perfusion
Kidney 3	73	male	27	DBD	n.a.	18 + 35	yes	arteriosclerosis
Kidney 4	70	male	21.5	DCD	18	14 + 59	no	patchy perfusion
Kidney 5	61	male	24	DBD	n.a.	19 + 4	no	esophageal cancer
Kidney 6	70	male	29	DCD	29	31 + 59	no	patchy perfusion
Kidney 7	58	male	27.7	DBD	n.a.	10 + 26	no	huge cyst lower pole
Kidney 8	73	male	35.8	DBD	n.a.	20 + 39	yes	patchy perfusion
Kidney 9	51	female	22	DCD	18	11 + 23	no	renal neoplasia
Kidney 10	73	female	22.8	DBD	n.a.	17 + 6	yes	vascular damage, anatomy
Kidney 11	70	male	37.7	DCD	20	100 + 8	yes	organ size, anatomy
Kidney 12	48	male	21.1	DCD	13	51 + 10	no	patchy perfusion, long CIT
Kidney 13	44	female	19.7	DCD	15	36 + 7	no	patchy perfusion, long CIT
Kidney 14	75	female	33.6	DBD	n.a.	8 + 8	yes	poor perfusion
Kidney 15	44	female	26.4	DCD	33	104 + 3	no	patchy perfusion, cortical necrosis
Kidney 16	69	female	23.8	DBD	n.a.	14 + 45	no	patchy perfusion
Kidney 17	49	male	25.7	DCD	12	25 + 18	no	patchy perfusion
Kidney 18	59	male	24.3	DCD	11	101+ 39	no	patchy perfusion, anatomy
Kidney 19	59	male	24.3	DCD	11	106 + 7	no	patchy perfusion
Kidney 20	67	female	27.9	DBD	n.a.	18 + 31	no	patchy perfusion
Kidney 21	38	male	20.9	DCD	10	29 + 9	yes	suspicion of pancreas cancer
Kidney 22	68	male	23.2	DBD	n.a.	108 + 5	no	patchy perfusion
Kidney 23	73	female	27.8	DBD	n.a.	19	yes	poor perfusion, anatomy

continued Table 3.1:

	Age in years	Sex	BMI in kg/m2	Donor Type	WIT in min	CIT in hours+min	Hypertension	Reason for Discard
Kidney 24	59	male	35.1	DCD	15	21 + 16	yes	arteriosclerosis
Kidney 25	60	male	35.1	DCD	14	42 + 17	yes	poor perfusion
Kidney 26	50	male	22.3	DBD	n.a.	86 + 30	no	clear cell renal cancer
Kidney 27	44	male	41.4	DCD	12	46 + 59	yes	biopsy findings
Kidney 28	66	female	31.2	DBD	n.a.	15 + 9	no	patchy perfusion
Kidney 29	70	female	24.3	DBD	n.a.	17 + 30	yes	stenosis of renal artery
Kidney 30	74	female	24.8	DCD	11	22	yes	lesion on partner kidney (monomorphic cell infiltration)
Kidney 31	71	female	28.1	DBD	n.a.	46 + 47	yes	anatomy, long CIT
Kidney 32	78	female	25.4	DBD	n.a.	18 + 22	yes	vascular damage
Kidney 33	71	female	29.1	DBD	n.a.	21 + 4	no	organ size
Kidney 34	47	female	39.1	DBD	n.a.	12 + 41	no	vascular damage, patchy perfusion
Kidney 35	62	female	23.5	DCD	9	19 + 52	no	suspicion of cancer
Kidney 36	76	female	24.5	DBD	n.a.	15 + 26	yes	long CIT

DCD = donation after circulatory death, DBD = donation after brain death, CIT = cold ischemia time, WIT = warm ischemia time

The donor demographics are summarised in table 3.2. Overall, 36 discarded human kidneys were normothermically perfused. Organs coming from DCD donors were significantly younger than organs declined from DBD donors; median DCD age 59 years vs. median DBD age 70 years, p=0.006. The DBD cohort included significantly more female donors compared to the DCD cohort. All other donor demographics were similar. Perfusion characteristics are shown in table 3.3. Sixteen DBD and 16 DCD kidneys were perfused with urine recirculation. A further four kidneys, three from DBD and one from a DCD donor, were perfused without urine recirculation which was associated with a significantly shorter time on the perfusion device compared to the 32 kidneys with urine recirculation; p<0.02. Some perfusions were stopped for reasons of problems with oxygenation and temperature control. In cases in which these problems have occurred, only haemodynamic parameters and tissue/perfusate samples were considered for my analyses from the period before the problems have occurred.

Table 3.2: Donor characteristics	**DBD (n=19)**	**DCD (n=17)**
Donor age in years (median (min-max))	70 (47-78)	59 (38-74)
Donor girth in cm (mean, SD)	96.5 ± 14.2	98.3 ± 15.8
Donor BMI kg/m2 (mean, SD)	27.4 ± 4.7	26.9 ± 6.5
Serum crea at admission in µmol/l (mean, SD)	79.5 ± 31.9	87.2 ± 31.6
Serum crea at retrieval in µmol/l (mean, SD)	79.3 ± 42.4	69 ± 35
Serum urea at admisison in mmol/l (mean, SD)	5.6 ± 2	5.4 ± 2.1
Serum urea at retrieval in mmol/l (mean, SD)	5.7 ± 3.2	7.7 ± 4.4
Estimated GFR in ml/min/1.73m2* (mean, SD)	82.3 ± 32.9	103.6 ± 60.8
Urine output last hour in ml (mean, SD)	130.5 ± 127.1	92.1 ± 56
Urine output last 24 hours in ml (mean, SD)	2742 ± 1501	2666 ± 2006
CIT in hours (mean, SD)	29.4 ± 28.1	47.4 ± 33.6
WIT in minutes (mean, SD)	n.a.	16.4 ± 7.2

GFR = gomerular filtration rate; *calculated using the MRDR (modification of diet in renal disease) formula
DCD = donation after circulatory death
CVA = cerebro vascular accident
CIT = cold ischaemia time
WIT = warm ischaemia time
n.a. = not applicable

3.6 Comparison of donor and perfusion characteristics in different categories

3.6.1 Development stages of the perfusion device

One of the central aspects of my research was contributing to the development of a normothermic perfusion device that is transportable and automated regulated. This development process was based on the perfusion of discarded human kidneys. The progress which had been made with the device yielded in kidneys perfused with a different setup as an inevitable consequence.

The following sections describe the perfusion characteristics of the “historical” kidneys perfused on a manually assembled circuit, the perfusion results gained from a device which we call the prototype device and our latest achievement, the refinement of gas supply for the kidney on the circuit and its impact.

3.6.1.1 Era one - kidneys perfused on a “hand-made” NMP circuit

Nine discarded human kidneys were perfused in order to reach the goal to create a clinically-relevant normothermic perfusion device. These results formed the basis of the design of the subsequent perfusion device tested later. Five kidneys were from DBD donors and four from DCD donors. The median (min-max) donor age was 70 (51-73) years. The mean ± SD donor creatinine at retrieval was 69.1± 27.8 µmol/l, the mean ± SD donor urea at retrieval was 6.1 ± 2.9 mmol/l and the mean ± SD donor estimated glomerular filtration rate (eGFR) was 87.3 ± 30.5 ml/min/1.73m^2. The eGFR was calculated using the MDRD (modification of diet in renal disease) formula. The last mean ± SD 24-hour urine output reported for the donors was 3266 ± 1394 ml. The mean ± SD CIT for the nine kidneys was 26.6 ± 19.3 hours. Warm ischaemia time in the four DCD kidneys was 23.3 ± 6.1 minutes. The median (min–max) perfusion time of this cohort was 16.2 (5.1 – 24.3) hours on the circuit. The mean ± SD median arterial

pressure, which was set by adjusting the pump speed at this time, was 71.8 ± 13 mmHg. The mean ± SD median flow was 331.1 ± 41.1 ml/min; minimum flow rate was 273.3 ± 52.9 ml/min; maximum perfusion flow measured was 370 ± 35.4 ml/min.

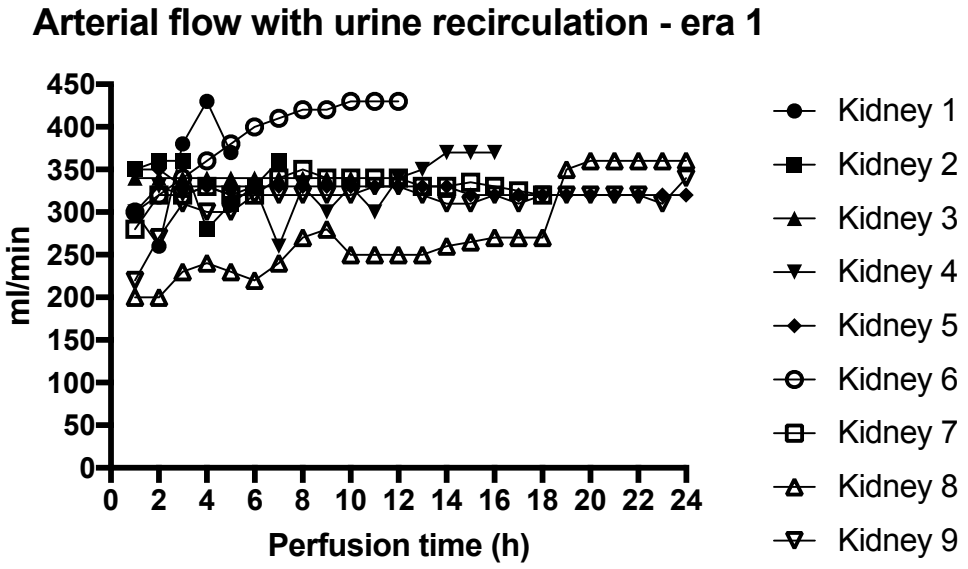


Figure 3.1: Arterial flow values over time for each individual kidney perfused on a manually assembled perfusion circuit.

As described in Chapter 2, my initial approach was to use an oxygen/air combination (as used in the preceding liver perfusion system). This involved an oxygen concentrator and an air pump, with ratios adjusted to maintain physiological partial pressures of O_2 and CO_2. During priming of the circuit, pH and PCO_2 were adjusted to bring them within the normal physiological range (using additives described in Chapter 2). The mean ± SD median PO_2 level with this setup was 21.1 ± 6.8 kPa. The mean ± SD median PCO_2 level was 4.7 ± 1.7 kPa. The mean ± SD median pH of the perfusate was 7.35 ± 0.1. The total median (min – max) urine output was 190 (25 – 1380) ml; the hourly median (min – max) urine output was 20 (3.3 – 86.3) ml. The mean ± SD total parenteral nutrition (TPN, Nutriflex® special, B. Braun AG, Melsungen, Germany) administered was 38.4 ± 27.5 ml during the preservation period; this amount of TPN

is equal to 9.2 ± 6.6 mg of glucose as 1 ml of TPN contains 0.24 mg glucose. Figure 3.2 shows the manually assembled perfusion circuit.

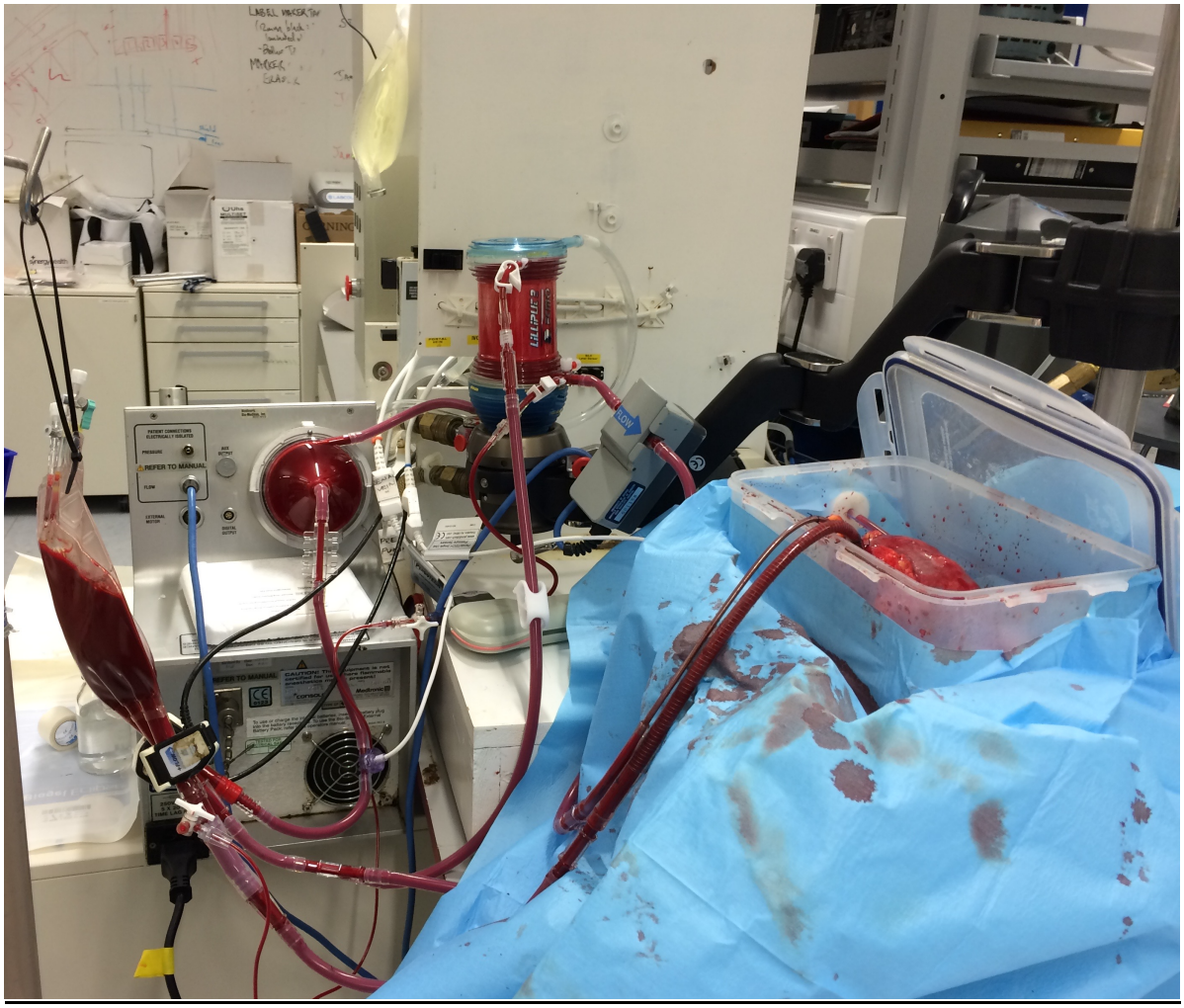


Figure 3.2: Manually assembled perfusion circuit used for the first nine normothermically perfused discarded human kidneys.

3.6.1.2 Era two - kidneys perfused on the prototype

The lesson learned in the developmental phase (above) were implemented in a prototype device. These comprised automation of the blood gas and temperature control and incorporation in a purpose-built container, as might be suitable for a transportable device; figure 3.3.

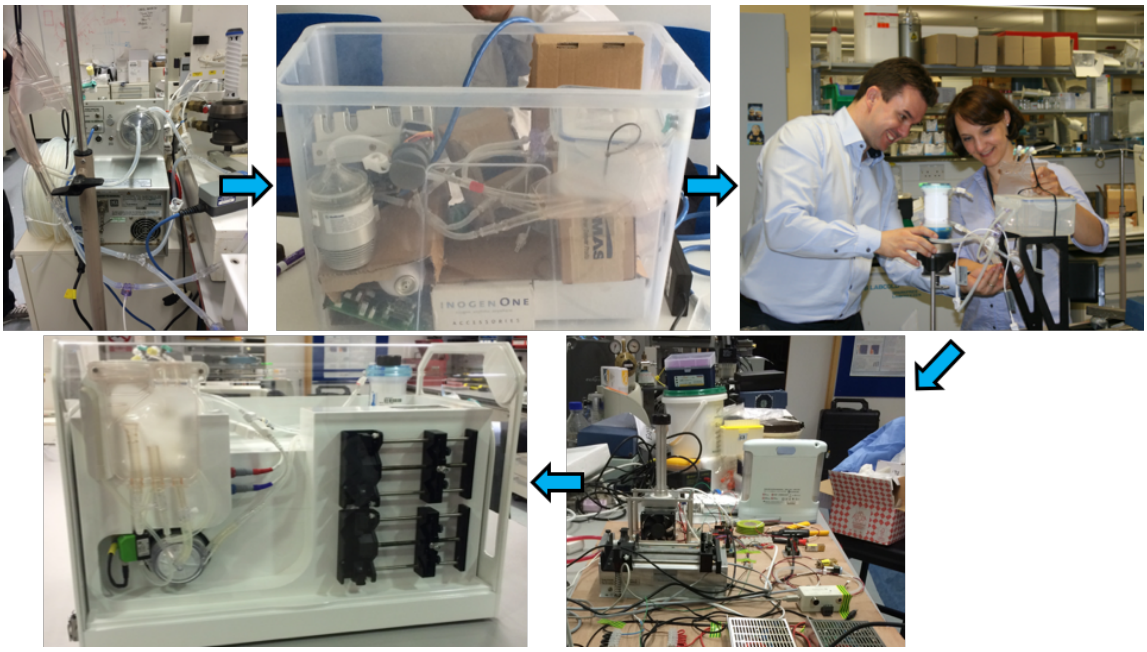


Figure 3.3: The construction pathway from the era 1 perfusion circuit to the prototype, a transportable device, for kidneys perfused in era 2 and 3.

Twelve kidneys were perfused consecutively; four from DBD donors and eight from DCD donors. The median (min – max) donor age was 59 (38 – 75) years. The mean ± SD donor creatinine at retrieval was 74 ± 36.7 µmol/l, the mean ± SD donor urea at retrieval was 6.6 ± 5.2 mmol/l and the mean ± SD donor eGFR was 92.1 ± 56.4 ml/min/1.73m^2. The eGFR was calculated using the MDRD formula. The last mean ± SD 24-hour urine output reported for the donors was 2841 ± 2174 ml. The mean ± SD CIT for the twelve kidneys was 51 ± 39.9 hours. Warm ischaemia time in the eight DCD kidneys was 15.6 ± 7.7 minutes. The median (min – max) perfusion time of this cohort was 13.7 (1.5 – 24.4) hours on the circuit. The mean ± SD median arterial pressure, which was set by adjusting the pump speed for the first six and could be fixed for the latter six, was 82.1 ± 13.5 mmHg. The mean ± SD median flow was 286.4 ± 105 ml/min; minimum flow rate was 194.6 ± 113.5 ml/min; maximum perfusion flow measured was 406.4 ± 137.3 ml/min. An Inogen oxygen concentrator (Inogen, Goleta, California, USA) and an air pump were used to maintain sufficient gas supply. The

mean ± SD median PO_2 level with Inogen and air pump was 14.4 ± 7 kPa but the mean ± SD maximum PO_2 level was 25.4 ± 21.4 kPa which demonstrates episodes of over-oxygenation. The mean ± SD median PCO_2 level was 4.5 ± 1 kPa. The mean ± SD median pH of the perfusate was 7.4 ± 0.2. The total median (min – max) urine output was 406 (30 – 1677) ml; the hourly median (min – max) urine output was 28.1 (11 – 111.8) ml. The mean ± SD TPN administered was 20.9 ± 29.6 ml during the preservation period; this amount of TPN is equal to 5 ± 7.1 mg of glucose as 1ml of TPN contains 0.24 mg glucose. Figure 3.4 displays the device in operation mode; figure 3.5 shows arterial flows of kidneys perfused in era 2.

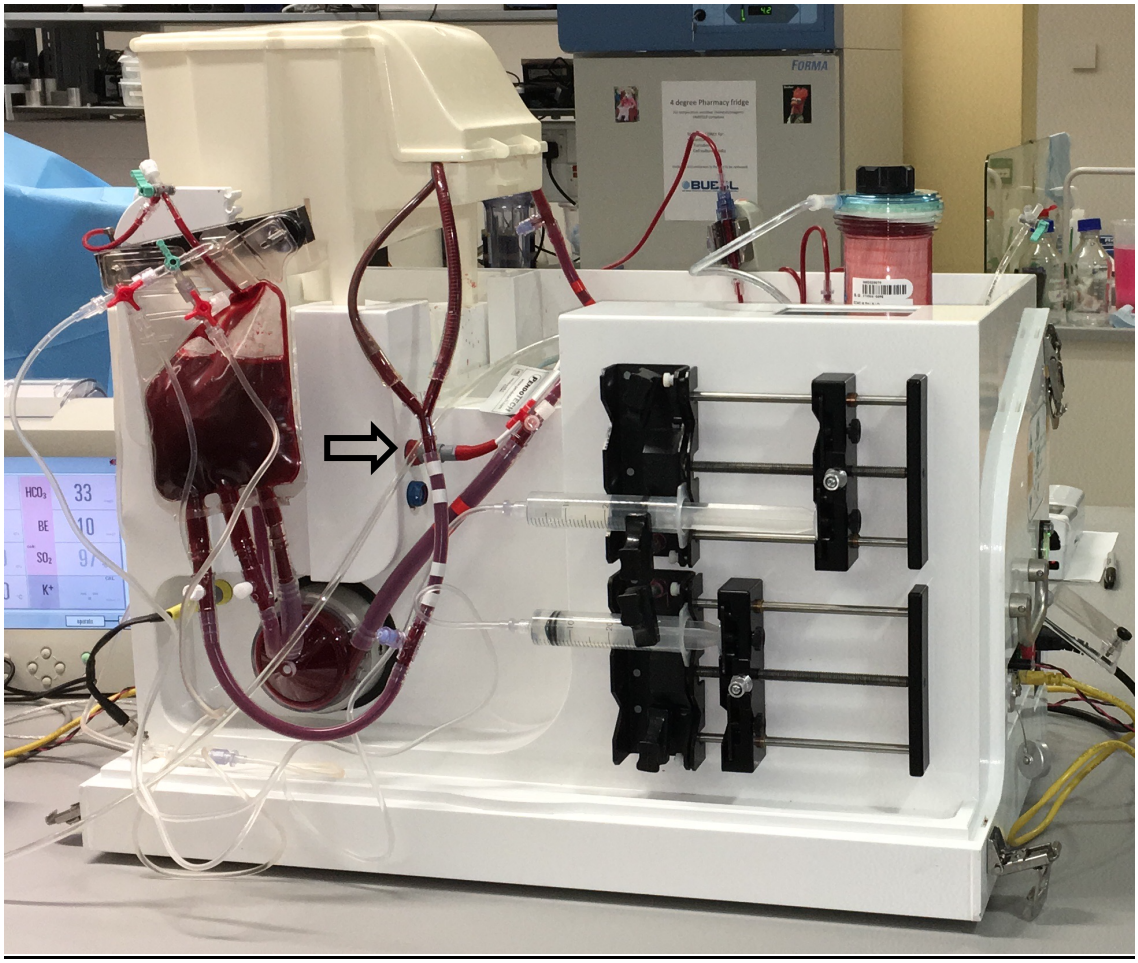


Figure 3.4: The photograph illustrates the prototype in operation mode. In this particular perfusion, the arrow indicates a Y-piece connecting two venous cannulas allowing single, gravity driven, venous outflow back into the soft-shell reservoir.

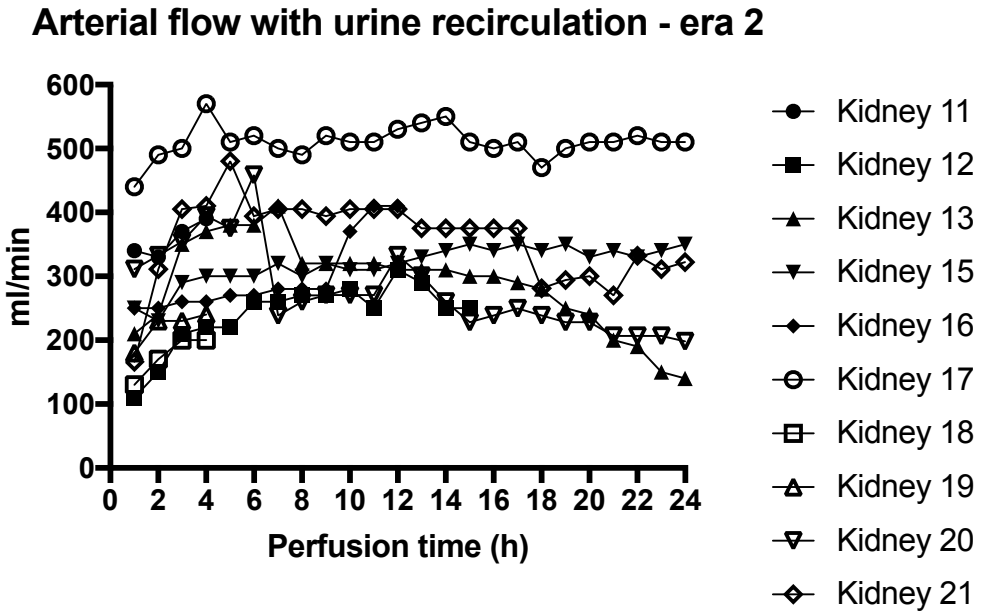


Figure 3.5: Arterial flow values over time for each individual kidney (n=10/12) perfused on the transportable prototype device. Arterial flows of kidney 10 are not presented as this kidney was used for the first test run on the device. Measured values of kidney 14 are not illustrated in the graph due to problems with the automated software control.

3.6.1.3 Era three - kidneys with air and CO_2 combination for oxygenation

As described above, I found the oxygen/air combination very difficult to control accurately. In particular, there was a tendency to periods of over-oxygenation, which we believe to be detrimental due to the formation of oxygen free radicals and induction of haemolysis (127–132). We believed that the problem was due to the capacity of the oxygen concentrator and the extremely low oxygen flow rates required. As we believed that accurate oxygen control is important, much effort was then invested to find the best setting to deliver the correct amount of gas to the kidney aiming for stable physiological partial O_2 and CO_2 pressures. With the perfusion experience and the analysis of the oxygen values described in the previous two paragraphs, the originally used closed loop controller, comprising the oxygen concentrator and the air compressor (2.1.3 in Chapter 2) was revisited and a more refined gas control was performed by using the same air pump for oxygenation and bottled CO_2 to keep

physiological levels between 4 and 6 kPa. The need for exogenous CO_2 was because an airflow that was adequate to achieve a physiological PO_2 resulted in a very low PCO_2 (ie CO_2 was blown off in the oxygenator).

Eleven kidneys were perfused; seven from DBD donors, four from DCD donors. The median (min – max) donor age was 62 (44 – 76) years. The mean ± SD donor creatinine at retrieval was 81 ± 55.2 µmol/l, the mean ± SD donor urea at retrieval was 7 ± 4.3 mmol/l and the mean ± SD donor eGFR was 104.6 ± 59.9 ml/min/1.73m^2. The eGFR was calculated using the MDRD formula. The last mean ± SD 24-hour urine output reported for the donors was 1947 ± 1284 ml. The mean ± SD CIT for the eleven kidneys was 36.8 ± 32.3 hours. Warm ischaemia time in the four DCD kidneys was 12.5 ± 2.6 minutes. The median (min – max) perfusion time of this cohort was 24 (0.75 – 24.2) hours on the circuit. The mean ± SD median arterial pressure, which was fixed at 90 mmHg, was 90.3 ± 4 mmHg. The mean ± SD median flow was 426 ± 201.9 ml/min; minimum flow rate was 151 ± 69.6 ml/min; maximum perfusion flow measured was 545.7 ± 219.5 ml/min. The mean ± SD median PO_2 level was 13.3 ± 1.3 kPa with a mean ± SD maximum PO_2 level of 18 ± 4.7 kPa and a mean ± SD minimum PO_2 level of 10.6 ± 1.7 kPa representing more stable method of oxygenation. The mean ± SD median PCO_2 level was 4.8 ± 0.5 kPa. The mean ± SD median pH of the perfusate was 7.35 ± 0.1. The total median (min – max) urine output was 828 (0 – 11325) ml; the hourly median (min – max) urine output was 46.9 (1.67 – 471.9) ml. The mean ± SD TPN or 5% glucose (Baxter Healthcare Ltd, Deerfield, Illinois, USA) administered was 38.1 ± 37.1 ml during the preservation period; this amount of TPN is equal to 3.6 ± 3.5 mg of glucose as 1ml of TPN contains 0.24 mg glucose and 1.2 ± 1.12 mg of glucose as 1ml of 5% glucose contains 0.05 mg glucose.

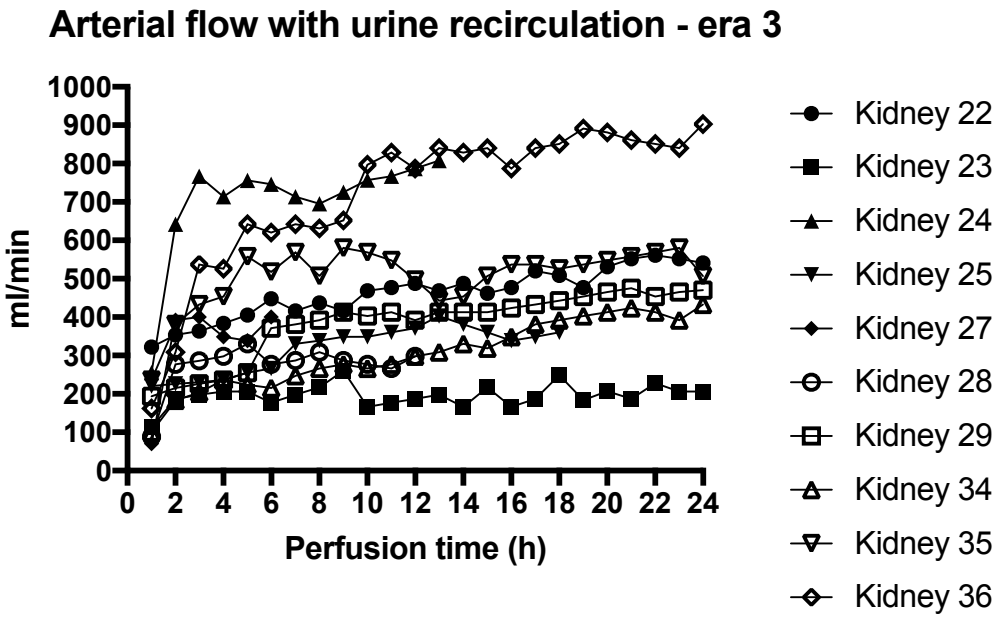


Figure 3.6: Arterial flow values over time for each individual kidney (n=10/11) perfused on the transportable prototype device with air/CO_2 used for blood gas control. Arterial flows of kidney 24 are not presented as this kidney could not be maintained on the circuit due to a severe volume of blood loss coming from a deep biopsy lesion.

3.6.1.4 Summary: Development stages of the perfusion device

Comparing the three eras of kidney perfusions, donor characteristics were comparable; donor age, p=0.35; eGFR, p=0.31; last donor urine, p=0.54. The flow rates achieved during the development process were increasing but not significantly; p=0.07 and hourly urine production was similar, p=0.45. What was achieved from era 1 to era 3 was a significantly more stable oxygenation using CO_2 and air within a physiological range; p=0.01 for median PO_2 and p=0.009 for maximum PO_2. All comparisons were carried out using an ordinary one-way ANOVA comparing the means of era 2 and 3 with the means of era 1.

3.6.2 Length of cold ischaemia time

The use of human kidneys which had been discarded from the national clinical programme, brought an inevitably very prolonged cold ischaemia time in many cases.

I was concerned that this might have a detrimental effect on the interpretation of the results. The duration of cold ischaemia is a factor well known and studied for its impact on kidney function after transplantation. A CIT longer than a certain time, mostly beyond 24 hours but depending on the transplant centre and on donor and recipient characteristics, is one of the factors why organs are declined and finally discarded. During my research of the behaviour of discarded human kidneys on a normothermic perfusion device and its development, I accepted and perfused kidneys with a huge variety of CIT. For a better understanding of the perfusion results and to obtain comparable groups, I classified the kidneys in three different CIT groups; up to 24 hours, between 25 and 50 hours and beyond 50 hours including 5 kidneys with a CIT longer than 100 hours. These groups include only kidneys in which urine was recirculated into the perfusate (n=32). The reasons for perfusing kidneys perfused after a cold storage time longer than four days were mainly related to the development of the device: in particular there were instances where I was testing automation and monitoring innovations and I needed to carry out the perfusions when the relevant engineering expertise was available.

3.6.2.1 Cold ischaemia time up to 24 hours

In seventeen kidneys normothermic perfusion was started within a time frame of 24 hours of CIT. Thirteen kidneys were from DBD donors, four were DCD organs. Median (min – max) donor age was 69 (47 – 76) years. The mean ± SD donor creatinine at retrieval was 76.7 ± 47 µmol/l, the mean ± SD donor urea at retrieval was 6 ± 3.1 mmol/l and the mean ± SD donor eGFR was 87.8 ± 51.6 ml/min/1.73m^2. The eGFR was calculated using the MDRD formula. The last mean ± SD 24-hour urine output reported for the donors was 2530 ± 1531 ml. The mean ± SD CIT for the seventeen

kidneys was 15.7 ± 3.8 hours. Warm ischaemia time in the four DCD kidneys was 15 ± 4.2 minutes. The median (min – max) perfusion time of this cohort was 24 (1.5 – 24.3) hours on the circuit. The mean ± SD median arterial pressure was 83.7 ± 13.8 mmHg. The mean ± SD median flow was 352.6 ± 179.6 ml/min; minimum flow rate was 198.2 ± 84.9 ml/min; maximum perfusion flow measured was 476.7.7 ± 198.9 ml/min. The mean ± SD median PO_2 level was 16.3 ± 7.3 kPa. The mean ± SD median PCO_2 level was 4.6 ± 1.3 kPa. The mean ± SD median pH of the perfusate was 7.36 ± 0.1. The total median (min – max) urine output was 828 (30 – 11325) ml; the hourly median (min – max) urine output was 41 (5.8 – 471.9) ml. The mean ± SD TPN or 5% glucose administered was 43.6 ± 32.8 ml.

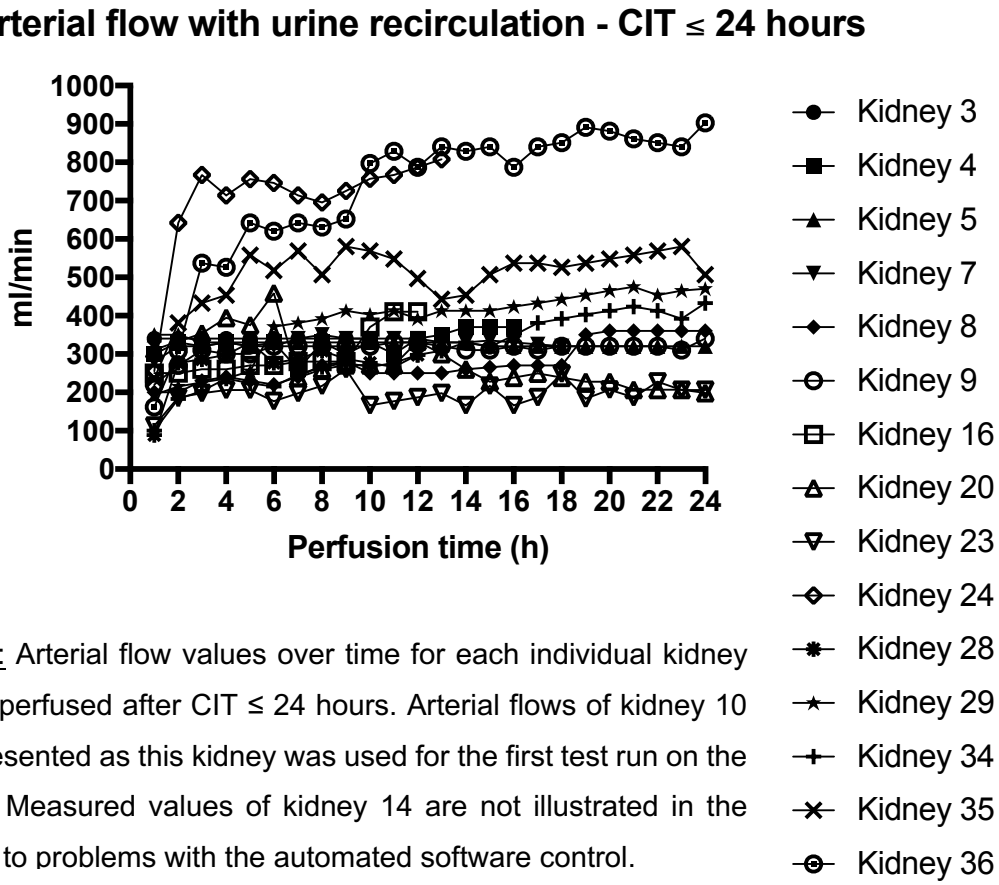


Figure 3.7: Arterial flow values over time for each individual kidney (n=15/17) perfused after CIT ≤ 24 hours. Arterial flows of kidney 10 are not presented as this kidney was used for the first test run on the prototype. Measured values of kidney 14 are not illustrated in the graph due to problems with the automated software control.

3.6.2.2 Cold ischaemia time between 25 and 50 hours

Seven kidneys were perfused with a CIT longer than 24 hours but shorter than 50 hours. All kidneys were from DCD donors. Median (min – max) donor age was 49 (38 – 72) years. The mean ± SD donor creatinine at retrieval was 80.6 ± 39 μmol/l, the mean ± SD donor urea at retrieval was 9.7 ± 5.5 mmol/l and the mean ± SD donor eGFR was 101.1 ± 44 ml/min/1.73m^2. The eGFR was calculated using the MDRD formula. The last mean ± SD 24-hour urine output reported for the donors was 2914 ± 866.4 ml. The mean ± SD CIT for the seven kidneys was 35.7 ± 7.7 hours. Warm ischaemia time was 17.1 ± 7.9 minutes. The median (min – max) perfusion time of this cohort was 18.1 (6.3 – 24.4) hours on the circuit. The mean ± SD median arterial pressure was 75.2 ± 11.5 mmHg. The mean ± SD median flow was 368.5 ± 71.9 ml/min; minimum flow rate was 214.8 ± 142.8 ml/min; maximum perfusion flow measured was 452.3 ± 91 ml/min. The mean ± SD median PO_2 level was 18.2 ± 7.1 kPa. The mean ± SD median PCO_2 level was 5.2 ± 0.5 kPa. The mean ± SD median pH of the perfusate was 7.45 ± 0.3. The total median (min – max) urine output was 286 (10 – 630) ml; the hourly median (min – max) urine output was 15.9 (1.7 – 26.3) ml. The mean ± SD TPN or 5% glucose administered was 28.1 ± 33.1 ml.

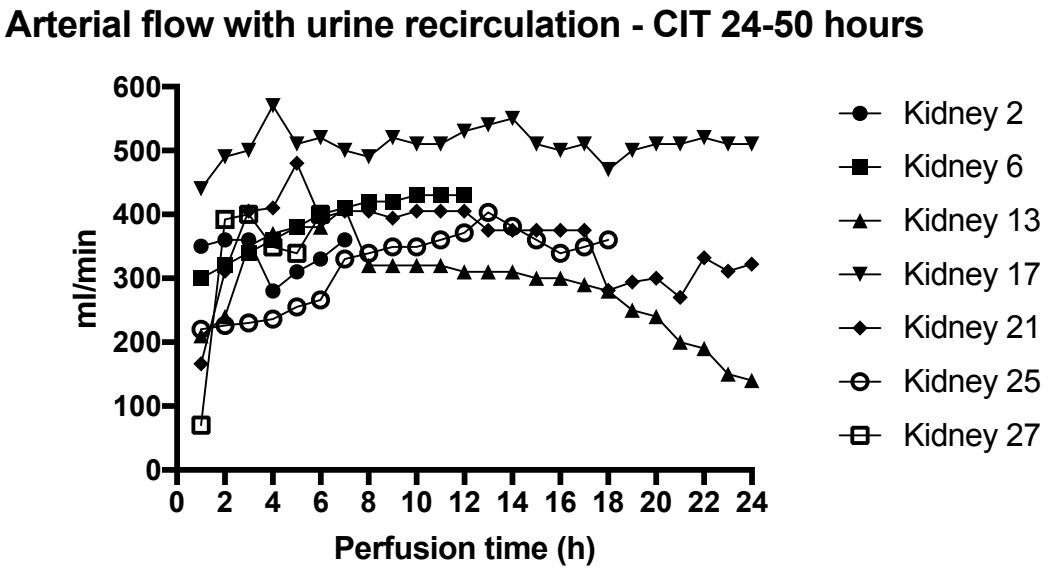


Figure 3.8: Arterial flow values over time for each individual kidney (n=7) perfused after a maximum CIT of 50 hours.

3.6.2.3 Cold ischaemia time longer than 50 hours

Normothermic perfusion was started after more than 50 hours of static cold storage for eight kidneys. Three kidneys were from DBD donors, five from DCD donors. The median (min – max) donor age was 58.5 (44 – 70). The mean ± SD donor creatinine at retrieval was 62.8 ± 30 µmol/l, the mean ± SD donor urea at retrieval was 4.1 ± 2 mmol/l and the mean ± SD donor eGFR was 105.1 ± 58.1 ml/min/1.73m^2. The eGFR was calculated using the MDRD formula. The last mean ± SD 24-hour urine output reported for the donors was 2538 ± 2684 ml. The mean ± SD CIT for the eight kidneys was 90.8 ± 20.5 hours. Warm ischaemia time was 17.6 ± 9.4 minutes. The median (min – max) perfusion time of this cohort was 4.7 (0.75 – 24) hours on the circuit. The mean ± SD median arterial pressure was 83.7 ± 12.2 mmHg. The mean ± SD median flow was 309.9 ± 106 ml/min; minimum flow rate was 205.7 ± 70 ml/min; maximum perfusion flow measured was 362 ± 137.2 ml/min. The mean ± SD median PO_2 level was 13.5 ± 2.3 kPa. The mean ± SD median PCO_2 level was 4.3 ± 1.1 kPa. The mean

± SD median pH of the perfusate was 7.38 ± 0.1. The total median (min – max) urine output was 261.5 (10 – 1677) ml; the hourly median (min – max) urine output was 47.4 (0 – 111.8) ml. The mean ± SD TPN or 5% glucose administered was 10 ± 13 ml.

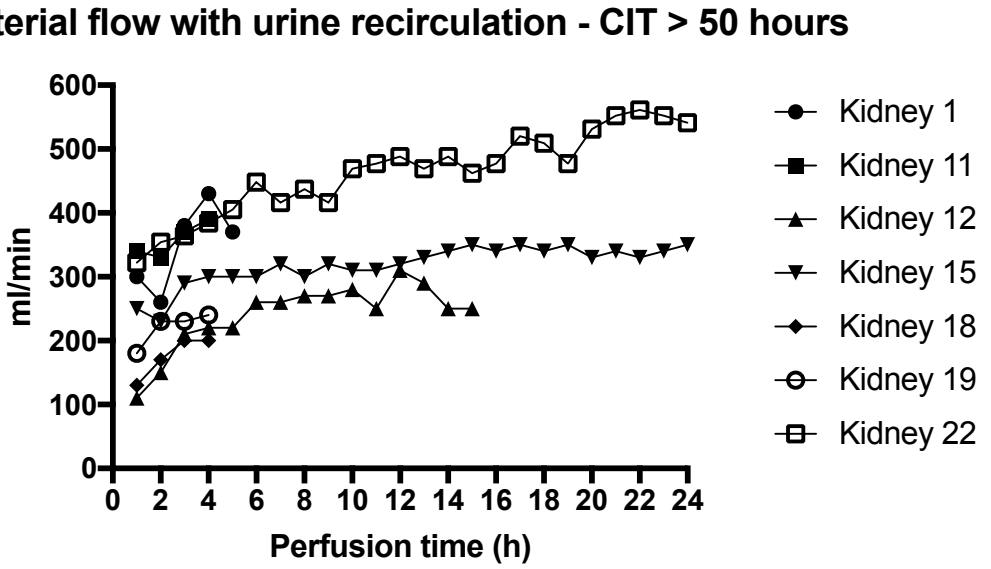


Figure 3.9: Arterial flow values over time for each individual kidney (n=7/8) perfused after a duration of CIT longer than 50 hours. Arterial flows of kidney 24 are not presented as this kidney could not be maintained on the circuit due to a severe volume of blood loss coming from a deep biopsy lesion.

3.6.2.4 Summary: Length of cold ischaemia time

Donors were significantly older in the group of kidneys with CIT less than 24 hours, p=0.017. All other donor demographics were similar in the groups; donor eGFR, p=0.70 and last donor urine, p=0.54. The kidneys with the shortest CIT produced the highest hourly volume of urine, p=0.045. The median arterial flow rate was 330ml/min amongst all perfused organs (with urine recirculation) and there was no significant difference between the CIT groups; p=0.43. All comparisons were done using an ordinary one-way ANOVA comparing the means of group 1 with means of groups 2 and 3.

3.6.3 Perfusion duration

Beside the aim to create a normothermic perfusion system which is able to maintain or improve the parenchymal quality of a human kidney, our aspiration was also to enable long-term normothermic kidney preservation - for up to 24 hours – without detriment. A number of perfusions were terminated before the 24-hour target. The main reasons for terminating perfusions early were: (i) problems with the early iterations of the automation systems; (ii) specific experimental needs for machine testing (oxygenation, heating) which required a “planned” deterioration of a kidney. Predefined reasons for termination of any kidney perfusions were one or more of: (i) arterial flow 50ml/min or less; (ii) pH <7.0 or >7.7 measured at a PCO_2 level of 5; (iii) sudden cessation of urine production (although this was always in association with reduced arterial flow). It is notable that only one perfusion with urine recirculation was terminated early due to one of mentioned conditions above.

3.6.3.1 Kidneys perfused up to 5 hours

Seven kidneys were perfused for five hours or less; these perfusions were used for circuit development (eg testing the bypass design) and included almost all the very prolonged CIT organs (greater than 100 hours). Four kidneys were from DBD donors, three from DCD. The median (min – max) donor age was 58 (50 – 75). The mean ± SD donor creatinine at retrieval was 69 ± 29.6 µmol/l, the mean ± SD donor urea at retrieval was 4.7 ± 2 mmol/l and the mean ± SD donor eGFR was 77.4 ± 30.5 ml/min/1.73m^2. The eGFR was calculated using the MDRD formula. The last mean ± SD 24-hour urine output reported for the donors was 1930 ± 1138 ml. The mean ± SD CIT for the seven kidneys was 70 ± 41 hours. Warm ischaemia time was 14 ± 5.2 minutes. The median (min – max) perfusion time of this cohort was 4.2 (0.75 – 5.3)

hours on the circuit. The mean ± SD median arterial pressure was 87.8 ± 15.4 mmHg. The mean ± SD median flow was 236 ± 98 ml/min; minimum flow rate was 207.5 ± 71.8 ml/min; maximum perfusion flow measured was 315 ± 112.1 ml/min. The mean ± SD median PO_2 level was 13.8 ± 2.3 kPa. The mean ± SD median PCO_2 level was 4 ± 1.4 kPa. The mean ± SD median pH of the perfusate was 7.34 ± 0.1. The total median (min – max) urine output was 30 (0 – 432) ml; the hourly median (min – max) urine output (n=6 kidneys) was 47.4 (30 – 108) ml.

3.6.3.2 Kidneys perfused up to 10 hours

Seven kidneys, four from DBD and three from DCD donors, were perfused for up to 10 hours. The median (min – max) donor age was 72 (44 – 78). The mean ± SD donor creatinine at retrieval was 75.1 ± 13.8 µmol/l, the mean ± SD donor urea at retrieval was 6.6 ± 1.7 mmol/l and the mean ± SD donor eGFR was 80.4 ± 25 ml/min/1.73m^2. The eGFR was calculated using the MDRD formula. The last mean ± SD 24-hour urine output reported for the donors was 3033 ± 1296 ml. The mean ± SD CIT for the seven kidneys was 30.8 ± 13.6 hours. Warm ischaemia time was 17 ± 9.5 minutes. The median (min – max) perfusion time of this cohort was 7.8 (6.1 – 10.2) hours on the circuit. The mean ± SD median arterial pressure was 86 ± 8.4 mmHg. The mean ± SD median flow was 301.4 ± 132.6 ml/min; minimum flow rate was 152.6 ± 123 ml/min; maximum perfusion flow measured was 393.1 ± 150.3 ml/min. The mean ± SD median PO_2 level was 15.3 ± 2.6 kPa. The mean ± SD median PCO_2 level was 4.7 ± 1.5 kPa. The mean ± SD median pH of the perfusate was 7.34 ± 0.2. The total median (min – max) urine output was 190 (10 – 920) ml; the hourly median (min – max) urine output was 19 (1.7 – 102) ml.

3.6.3.3 Kidneys perfused up to 18 hours

Eight kidneys underwent normothermic perfusion for up to 18 hours. Three were from DBD and five from DCD donors. The median (min – max) donor age was 63 (48 – 70). The mean ± SD donor creatinine at retrieval was 83 ± 60.9 µmol/l, the mean ± SD donor urea at retrieval was 8.4 ± 4.7 mmol/l and the mean ± SD donor eGFR was 121.3 ± 78.3 ml/min/1.73m^2. The eGFR was calculated using the MDRD formula. The last mean ± SD 24-hour urine output reported for the donors was 1895 ± 1310 ml. The mean ± SD CIT for the seven kidneys was 25.3 ± 14.9 hours. Warm ischaemia time was 17.8 ± 6.5 minutes. The median (min – max) perfusion time of this cohort was 14 (12.3 – 18.6) hours on the circuit. The mean ± SD median arterial pressure was 80 ± 12.7 mmHg. The mean ± SD median flow was 364.6 ± 149.7 ml/min; minimum flow rate was 204.5 ± 92.4 ml/min; maximum perfusion flow measured was 461.5 ± 197.2 ml/min. The mean ± SD median PO_2 level was 17.5 ± 8.2 kPa. The mean ± SD median PCO_2 level was 5.1 ± 0.7 kPa. The mean ± SD median pH of the perfusate was 7.42 ± 0.1. The total median (min – max) urine output was 323 (40 – 1677) ml; the hourly median (min – max) urine output was 18 (3.3 – 111.8) ml.

3.6.3.4 Kidneys perfused up to 24 hours

Fourteen kidneys were perfused for 24 hours. Eight kidneys were from DBD donors, six were DCD kidneys. The median (min – max) donor age was 61.5 (38 – 76). The mean ± SD donor creatinine at retrieval was 72.4 ± 41.3 µmol/l, the mean ± SD donor urea at retrieval was 6.3 ± 4.5 mmol/l and the mean ± SD donor eGFR was 89.4 ± 40.3 ml/min/1.73m^2. The eGFR was calculated using the MDRD formula. The last mean ± SD 24-hour urine output reported for the donors was 3651 ± 2083 ml. The mean ± SD CIT for the fourteen kidneys was 32.6 ± 31.8 hours. Warm ischaemia time

was 16.2 ± 8.9 minutes. The median (min – max) perfusion time of this cohort was 24.1 (24 – 24.4) hours on the circuit. The mean ± SD median arterial pressure was 81.2 ± 13.5 mmHg. The mean ± SD median flow was 378.1 ± 157.2 ml/min; minimum flow rate was 197.2 ± 102.2 ml/min; maximum perfusion flow measured was 485.7 ± 165.8 ml/min. The mean ± SD median PO_2 level was 15.8 ± 6.7 kPa. The mean ± SD median PCO_2 level was 4.8 ± 1 kPa. The mean ± SD median pH of the perfusate was 7.42 ± 0.2. The total median (min – max) urine output was 807 (140 – 11325) ml; the hourly median (min – max) urine output was 33.6 (5.8 – 471.9) ml.

3.6.3.5 Summary: Perfusion duration

The duration of CIT was significantly longer in the 5-hours perfusion group compared to the other kidney perfusion duration groups, p=0.02. The donor age was in the same range in all groups, p=0.3. Donor kidney function of the discarded human kidneys, eGFR and last urine output were not different between the groups, p=0.27 and p=0.5. Notably, the results of two main perfusion characteristics, median flow and hourly urine production, were statistically similar in every group, p=0.25 and 0.4. All comparisons were done using an ordinary one-way ANOVA comparing the means of group 1 (up to 5 hours perfusion) with means of groups 2, 3 and 4.

3.6.4 Kidney perfusions without urine recirculation

Four kidneys were normothermically perfused without recirculating the urine. One kidney was from a DCD donor. The median (min – max) donor age was 72.5 (71 – 78). The mean ± SD donor creatinine at retrieval was 72 ± 13 µmol/l, the mean ± SD donor urea at retrieval was 6.2 ± 1.3 mmol/l and the mean ± SD donor eGFR was 71 ± 15.5 ml/min/1.73m^2. The eGFR was calculated using the MDRD formula. The last

mean ± SD 24-hour urine output reported for the donors was 3314 ± 1516 ml. The mean ± SD CIT for the four kidneys was 27 ± 13.2 hours. Warm ischaemia time was 11 minutes. The perfusion characteristics are shown in table 3.3.

Mean ± SD arterial flows for all kidneys (with and without urine recirculation) are displayed in figures 3.10A-D and juxtaposed in tables 3.3 and 3.4.

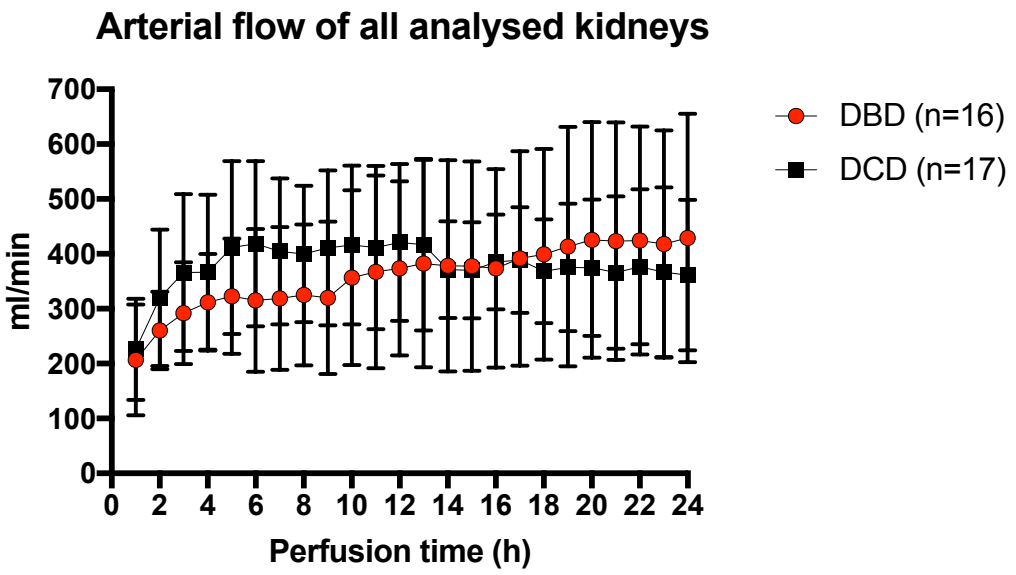


Figure 3.10A: Arterial flows for all analysed kidneys (n=33/36), per donor type, are shown per hour of perfusion, 1 to 24, as longitudinal mean ± SD values over the course of the perfusion. Three kidneys are not included in this graph: kidneys 10, 14 and 26 (3 DBD kidneys); for reasons explained in table 3.4. Kidneys (n) per hour DBD: 16 1-5 hours, 14 6-8 hours, 13 9 hours, 12 10 hours, 11 11-12 hours, 9 13-18 hours, 8 19-24 hours. Kidneys (n) per hour DCD: 17 1-4hours, 14 5-6hours, 12 7 hours, 11 8-12 hours, 10 13 hours, 9 14-15 hours, 8 16 hours, 7 17-18 hours, 6 19-24 hours.

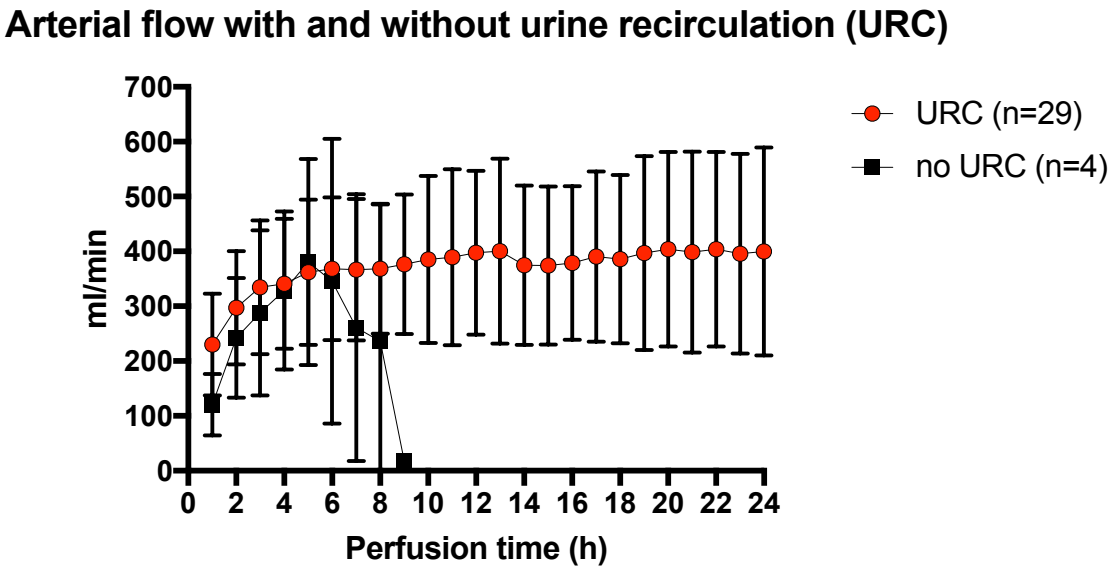


Figure 3.10B: Graphical comparison of arterial flows in kidneys perfused with and without urine recirculation (URC). Flow levels given as longitudinal mean ± SD values over the course of the perfusion. Kidneys (n) per hour URC: 29 1-4 hours, 26 5 hours, 25 6 hours, 24 7 hours, 23 8-10 hours, 22 11-12 hours, 19 13 hours, 18 14-15 hours, 17 16 hours, 16 17-18 hours, 14 19-24 hours; kidneys 10, 14 and 26 not included. (Kidneys (n) per hour without URC: 4 1-6 hours, 2 7-8 hours, 1 9 hours.

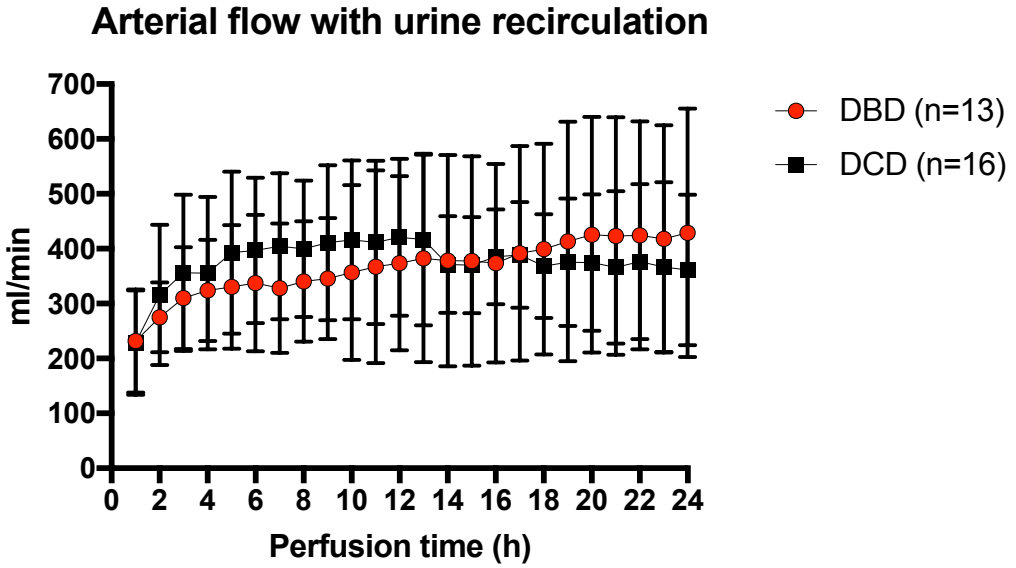


Figure 3.10C: Graphical comparison of arterial flows in kidneys with urine recirculation (URC), per donor type. Flow levels given as longitudinal mean ± SD values over the course of the perfusion. Kidneys (n) per hour URC DBD: 13 1-5 hours, 12 6-10 hours, 11 11-12 hours, 9 13-18 hours, 8 19-24 hours; kidneys 10, 14 and 26 not included. Kidneys (n) per hour URC DCD: 16 1-4 hours, 13 5-6hours, 12 7 hours, 11 8-12 hours, 10 13 hours, 9 14-15 hours, 8 16 hours, 7 17-18 hours, 6 19-24 hours.

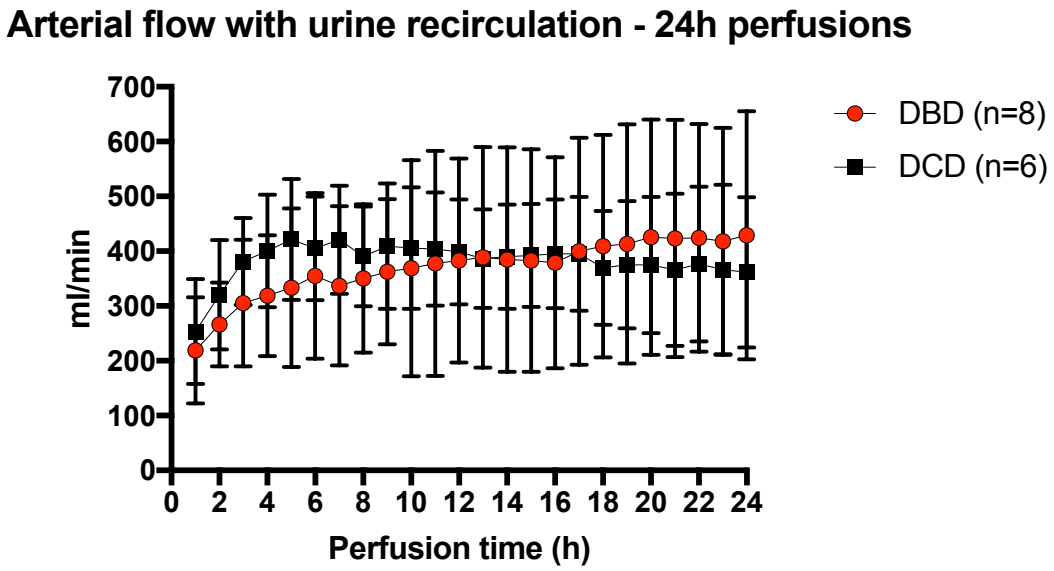


Figure 3.10D: Graphical comparison of arterial flows in kidneys with urine recirculation, per donor type, perfused for 24 hours. Flow levels given as longitudinal mean ± SD values over the course of the perfusion. Kidneys (n) per hour DBD: 8 1-24 hours. Kidneys (n) per hour DCD: 6 1-24 hours.

The hypothesis underpinning the detailed and repeated presentation of arterial flow for different groups of kidneys is that arterial flow could be a crucial marker for assessment of (discarded) human kidneys perfused on an NMP device. Analysis of mean arterial flows from different donor types demonstrates that, independently of CIT, era of device development and version of blood gas control, the pattern of arterial flow remains the same throughout the experiments; DBD and DCD kidneys reached similar arterial flow levels after approximately twelve hours of NMP.

3.7 Haemodynamic and metabolic function parameters – all kidneys

Perfusion parameters for the overall cohort, with or without urine recirculation, and the mean ± SD values for DBD and DCD kidneys are shown in table 3.3. Physiological flows could be achieved in both donor categories in the urine recirculation group (n=32) and in two of the kidneys without urine recirculation (n=4). The haemodynamic

parameters are graphically shown in figures 3.10A-D (arterial flow) and 3.11A-D (intrarenal resistance).

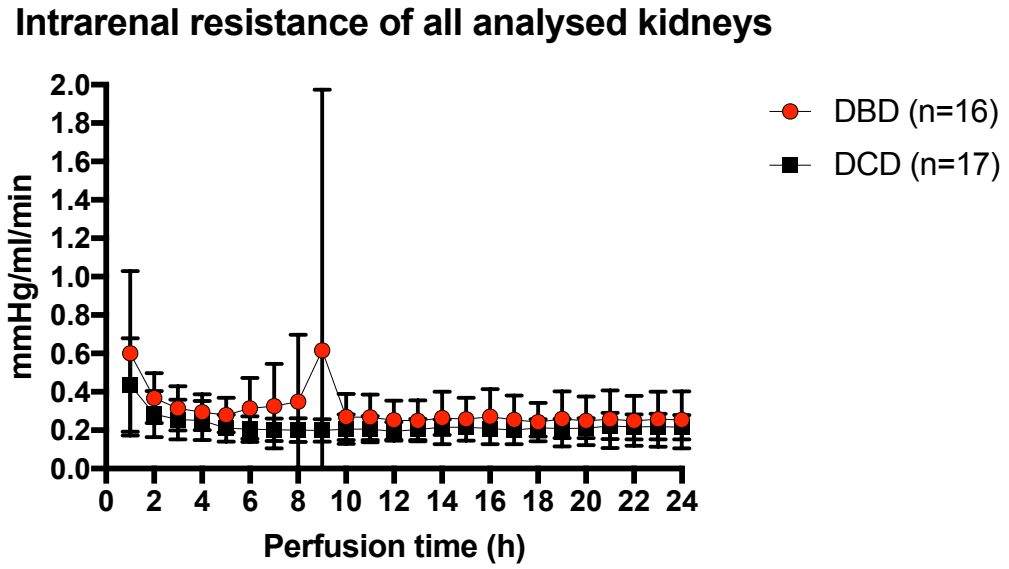


Figure 3.11A: Intrarenal resistance (IRR) for all analysed kidneys (n=33/36), per donor type, is shown per hour of perfusion, 1 to 24, as longitudinal mean ± SD values over the course of the perfusion. Three kidneys are not included in this analysis: kidneys 10, 14 and 26 (3 DBD kidneys); for reasons explained in table 3.4. Kidneys (n) per hour DBD: 16 1-5 hours, 14 6-8 hours, 13 9 hours, 12 10 hours, 11 11-12 hours, 9 13-18 hours, 8 19-24 hours. Kidneys (n) per hour DCD: 17 1-4hours, 14 5-6hours, 12 7 hours, 11 8-12 hours, 10 13 hours, 9 14-15 hours, 8 16 hours, 7 17-18 hours, 6 19-24 hours.

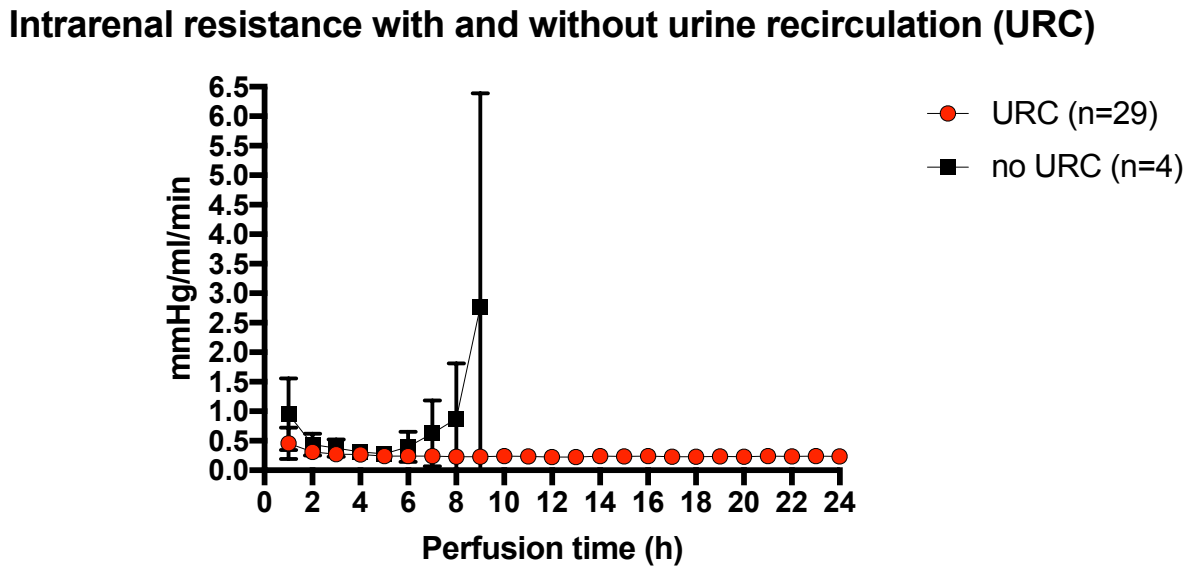


Figure 3.11B: Graphical comparison of IRR in kidneys perfused with and without urine recirculation (URC). IRR given as longitudinal mean ± SD values over the course of the perfusion. Kidneys (n) per hour URC: 29 1-4 hours, 26 5 hours, 25 6 hours, 24 7 hours, 23 8-10 hours, 22 11-12 hours, 19 13 hours, 18 14-15 hours, 17 16 hours, 16 17-18 hours, 14 19-24 hours; kidneys 10, 14 and 26 not included. Kidneys (n) per hour without URC: 4 1-6 hours, 2 7-8 hours, 1 9 hours.

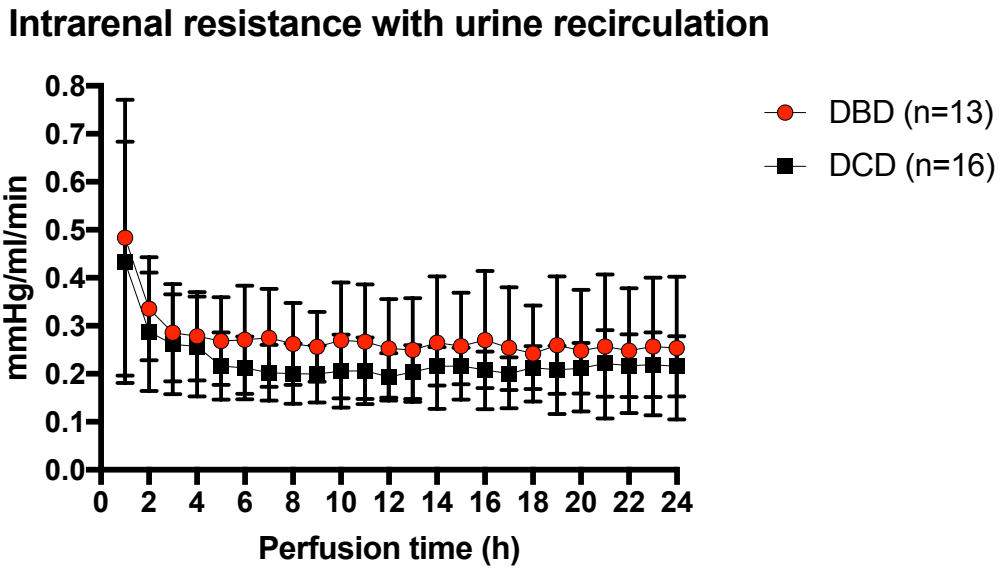


Figure 3.11C: Graphical comparison of IRR in kidneys with URC, per donor type. IRR given as longitudinal mean ± SD values over the course of the perfusion. Kidneys (n) per hour URC DBD: 13 1-5 hours, 12 6-10 hours, 11 11-12 hours, 9 13-18 hours, 8 19-24 hours; kidneys 10, 14 and 26 not included. Kidneys (n) per hour URC DCD: 16 1-4 hours, 13 5-6hours, 12 7 hours, 11 8-12 hours, 10 13 hours, 9 14-15 hours, 8 16 hours, 7 17-18 hours, 6 19-24 hours.

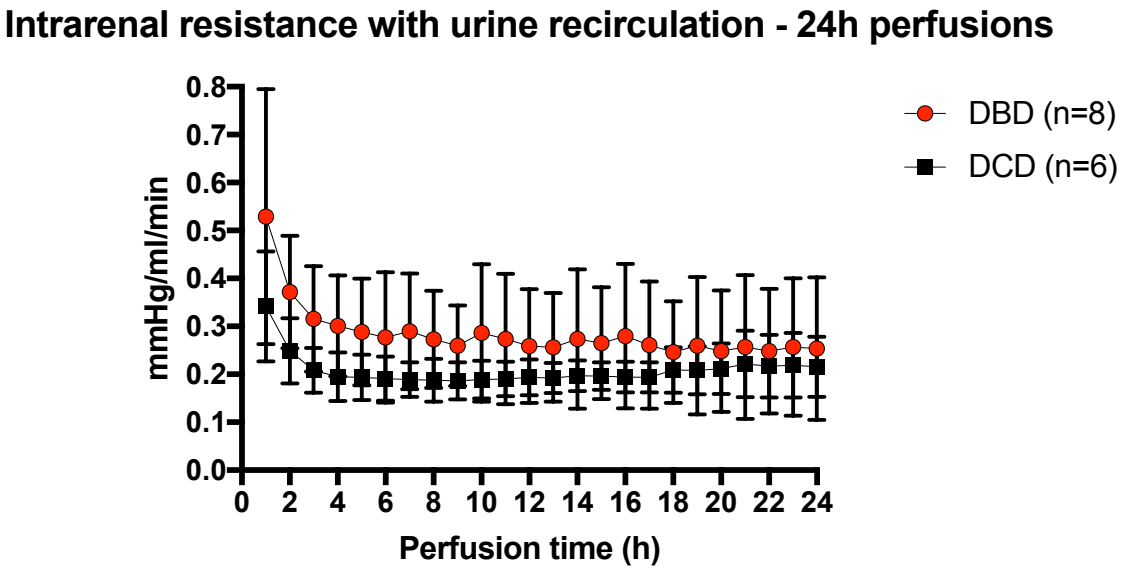


Figure 3.11D: Graphical comparison of IRR in kidneys with URC, per donor type, perfused for 24 hours. IRR given as longitudinal mean ± SD values over the course of the perfusion. Kidneys (n) per hour DBD: 8 1-24 hours. Kidneys (n) per hour DCD: 6 1-24 hours.

Table 3.3: Perfusion characteristics	Kidneys with urine recirculation			Kidneys without urine recirculation			*p-value**
	Overall (n=32)	**DBD (n=16)**	**DCD (n=16)**	**Overall (n=4)**	**DBD (n=3)**	**DCD (n=1)**	
Arterial pressure in mmHg (mean, SD)	81.7 ± 13	78.82 ± 13.23	76.5 ± 14.6	90.4 ± 0.8	90.7 ± 0.7	89.5	*0.2*
Arterial flow in ml/min (mean, SD)	346.3 ± 143.3	331.1 ± 129.6	413 ± 104.7	277.5 ± 181.9	208 ± 143.6	486	*0.59*
IRR in mmHg/ml/min (mean, SD)	0.25 ± 0.1	0.27 ± 0.11	0.24 ± 0.1	0.48 ± 0.33	0.58 ± 0.33	0.18	*0.25*
pH (mean, SD)	7.39 ± 0.16	7.32 ± 0.07	7.43 ± 0.03	7.41 ± 0.22	7.35 ± 0.23	7.6	*0.86*
PO_2 in kPa (mean, SD)	16.1 ± 6.4	17 ± 5.8	15.4 ± 4.8	14.4 ± 0.5	14.2 ± 0.3	15	*0.74*
PCO_2 in kPa (mean, SD)	4.7 ± 1.1	5 ± 0.7	5.2 ± 0.4	5.1 ± 0.3	5.2 ± 0.3	4.8	*0.36*
Overall urine output in ml (mean, SD)	855 ± 1970	3151 ± 5464	819.3 ± 474.3	523 ± 370	548 ± 449	445	*0.79*
Urine output in ml/hour (mean, SD)	49.5 ± 83.4	131.3 ± 227.6	34.14 ± 19.8	67.3 ± 41	65 ± 50	74.17	*0.25*
Amount of glucose in mg/perfusion duration (mean, SD)	4.2 ± 3.2	5 ± 3.8	3.4 ± 2.4	0.9 ± 0.5	1 ± 0.5	0.55	***0.03***
Time on circuit in hours (mean, SD)	15.8 ± 8.6	16.2 ± 9.2	15.4 ± 8.2	7.5 ± 1.6	7.9 ± 1.7	6.2	***0.02***

DBD = donation after brain death

DCD = donation after circulatory death

* comparing values for the 32 kidneys with urine recirculation vs. the values for the 4 kidneys without urine recirculation

3.7.1 Arterial pressure

The arterial pressure was regulated by changing the pump speed. The initial aim was to test a mean arterial pressure between 70 and 100mmHg. Later in the project this was fixed at 90 mmHg at a later stage for perfusions on the pre-clinical prototype: I calculated that this was the closest that could be achieved to a normal physiological situation, allowing that the circuit was using non-pulsatile perfusion. Detailed pressure values per kidney are shown in table 3.4.

3.7.2 Arterial flow

Arterial flows for all kidneys are shown in figures 3.10A-D and table 3.4. Overall, the best arterial flow was achieved in kidneys 24 and 36 with median (min – max) flows of 714.6 ml/min (153.5 – 934.3 ml/min) and 787 ml/min (153 – 903 ml/min). Kidney 24 was a 59-year old DCD kidney with a CIT of 21 hours perfused for 13 hours (one of the first kidneys on the prototype) This perfusion was terminated because of a software fault (disrupting both temperature and oxygen regulation). Kidney 36 was a 76-year old DBD kidney with a CIT of 15.5 hours perfused for 24 hours (declined for long CIT). Both were perfusions with urine recirculation. The lowest flows were seen in kidneys 18 and 23 with median (min – max) flows of 190 ml/min (130 – 200 ml/min) and 187 ml/min (113 – 260 ml/min). Kidney 18 was 59-year old DCD kidney with a CIT of 101.5 hours undergoing a planned perfusion of 4 hours to test the bypass line (which was abandoned at a later stage). Kidney 23 was a 73-year old DBD kidney with a CIT of 19 hours perfused for 24 hours. All of those kidneys underwent perfusions with recirculation of urine. In kidney 32, a perfusion with urine replenishment, the arterial flow decreased drastically to 142 ml/min (previously 309 ml/min) after four hours on

the prototype and replenishment of 700ml of urine with Ringer's lactate. From hour 7 onwards, the flow in kidney 32 was < 50ml/min. Figures 3.10A-D.

3.7.3 Intrarenal resistance

Intrarenal resistance (IRR) was calculated by dividing pressure at a specific time point by the flow at the same time point. The changes of IRR over time are shown in figures 3.11A-D and table 3.4. As with arterial flow, IRR was lowest in kidneys 24 and 36 compared to the other perfused kidneys; kidney 24 median (min-max) IRR was 0.12 mmHg/ml/min (0.50 – 0.21) and kidney 36 had an IRR of 0.11 mmHg/ml/min (0.56 – 0.1). The highest IRR median (min – max) levels could be observed in kidney 32, 0.91 mmHg/ml/min (5.29 – 0.31). Figures 3.11A-D.

3.7.4 PO_2 and PCO_2 levels

Mean arterial PO_2 and PCO_2 levels for each kidney are displayed in table 3.4. Arterial PO_2 levels were measured using a Terumo® CDI 500 device (which provides on-line blood gas analysis) and i-STAT CG4+ which was used on-demand, with cartridges. Venous PO_2 levels were measured with i-STAT CG4+ cartridges only. The differences between arterial and venous oxygenation were significant in the perfused kidneys both with and without urine recirculation; artery 15.6 ± 7.3 kPa vs. vein 7.9 ± 2.2 kPa, $p<0.0001$.

3.7.5 pH levels

The course of pH for all perfused kidneys is shown in figures 3.12A-D and displayed in table 3.4. In all perfusions 10-15ml of sodium bicarbonate 8.4% was given to reach

a physiological pH, above 7.2 at 37°C, before starting perfusion. No further sodium bicarbonate was given at any point during perfusion.

One kidney stands out in the cohort of kidneys with urine recirculation: Kidney 21 became severely alkalotic, pH >7.8 due to an excessive, malfunctioning, air flow which led to remarkably low PCO_2 levels of 2 and less (table 3.4). In addition, it did not receive TPN despite reaching a hypoglycaemic state due to a malfunctioning syringe pump.

Notable derangement of pH was seen in the four kidneys without urine recirculation. Kidney 30 became very alkalotic with a pH >7.7 after four hours of perfusion and replenishment of 405 ml with Ringer's lactate. The PCO_2 levels were physiological throughout (4.6±0.6). Urine production stopped after 4hours and 30 minutes; the perfusion was terminated after 6 hours. Kidney 31 produced only 50 ml of urine within the first hour which was replaced with Ringer's lactate but became acidotic with a pH of 7.1 from hour 5 onwards combined with a declining flow which ended in termination of the perfusion after 7 hours with an arterial flow <50ml/min and a pH of 7.0. Kidney 32 developed a slightly acidotic pH of 7.15 (PCO_2 4.8±1.1) after hour 6 in combination with a steadily decreasing arterial flow and tailing off of urine production from the same time. The perfusion was stopped after 9 hours and 30 min with an arterial flow of 17ml/min. Kidney 33 had a good arterial flow throughout the 8 hours of normothermic perfusion. The perfusion was stopped as the kidney became very alkalotic with pH >7.8 (PCO_2 5±07) and stopped producing urine after 6 hours and 30 min; 550 ml of urine were produced/replenished by the end of hour 5 after start of the perfusion.

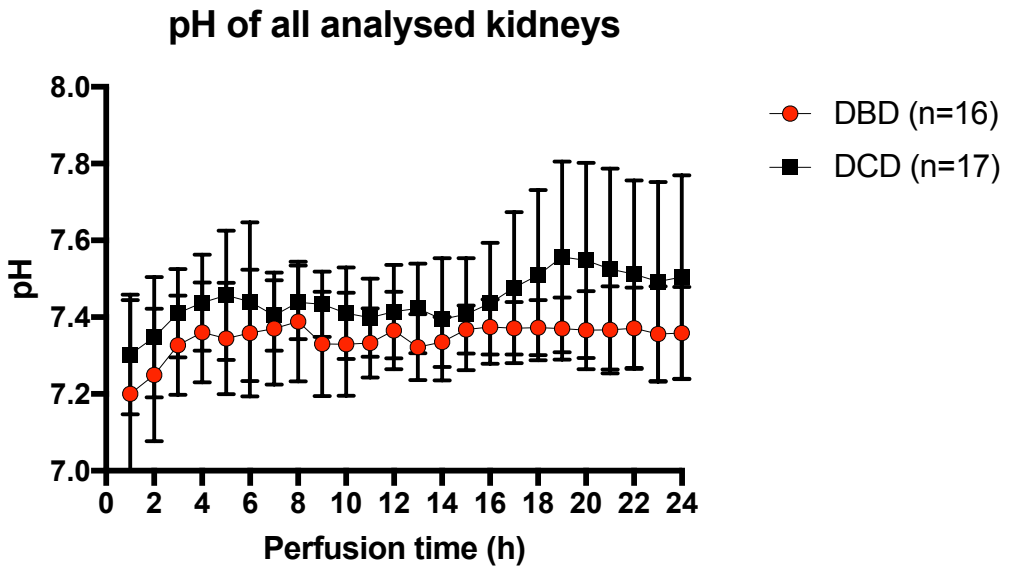


Figure 3.12A: pH for all analysed kidneys (n=33) is shown per hour of perfusion, 1 to 24, as longitudinal mean ± SD values over the course of the perfusion. Three kidneys are not included in this analysis: kidneys 10, 14 and 26 (3 DBD kidneys); for reasons explained in table 3.4. Kidneys (n) per hour DBD: 16 1-5 hours, 14 6-8 hours, 13 9 hours, 12 10 hours, 11 11-12 hours, 9 13-18 hours, 8 19-24 hours. Kidneys (n) per hour DCD: 17 1-4hours, 14 5-6hours, 12 7 hours, 11 8-12 hours, 10 13 hours, 9 14-15 hours, 8 16 hours, 7 17-18 hours, 6 19-24 hours.

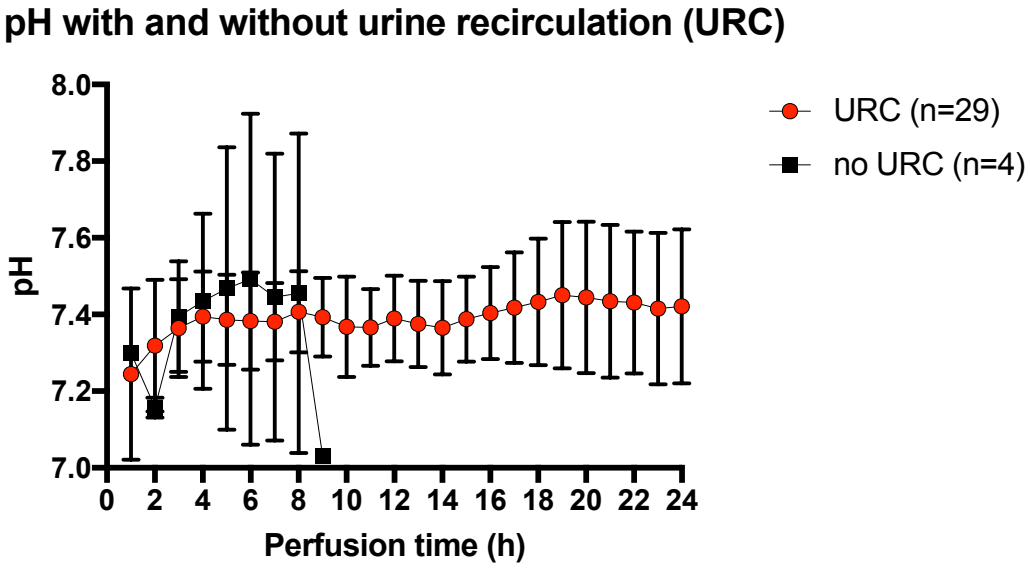


Figure 3.12B: Graphical comparison of pH in kidneys perfused with and without urine recirculation (URC). pH given as longitudinal mean ± SD values over the course of the perfusion. Kidneys (n) per hour URC: 29 1-4 hours, 26 5 hours, 25 6 hours, 24 7 hours, 23 8-10 hours, 22 11-12 hours, 19 13 hours, 18 14-15 hours, 17 16 hours, 16 17-18 hours, 14 19-

24 hours; kidneys 10, 14 and 26 not included. Kidneys (n) per hour without URC: 4 1-6 hours, 2 7-8 hours, 1 9 hours.

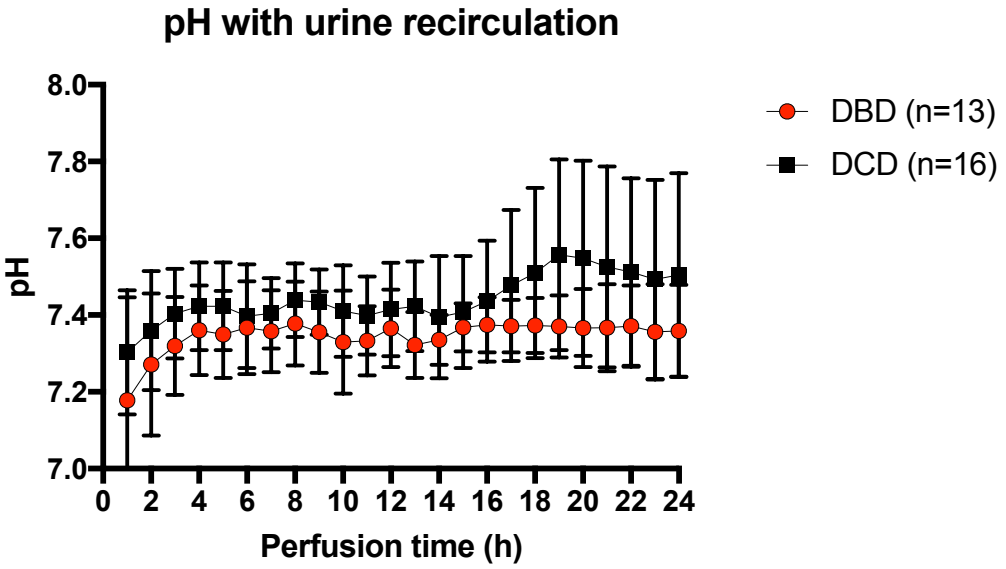


Figure 3.12C: Graphical comparison of pH in kidneys with URC, per donor type. pH given as longitudinal mean ± SD values over the course of the perfusion. Kidneys (n) per hour URC DBD: 13 1-5 hours, 12 6-10 hours, 11 11-12 hours, 9 13-18 hours, 8 19-24 hours; kidneys 10, 14 and 26 not included. Kidneys (n) per hour URC DCD: 16 1-4 hours, 13 5-6hours, 12 7 hours, 11 8-12 hours, 10 13 hours, 9 14-15 hours, 8 16 hours, 7 17-18 hours, 6 19-24 hours.

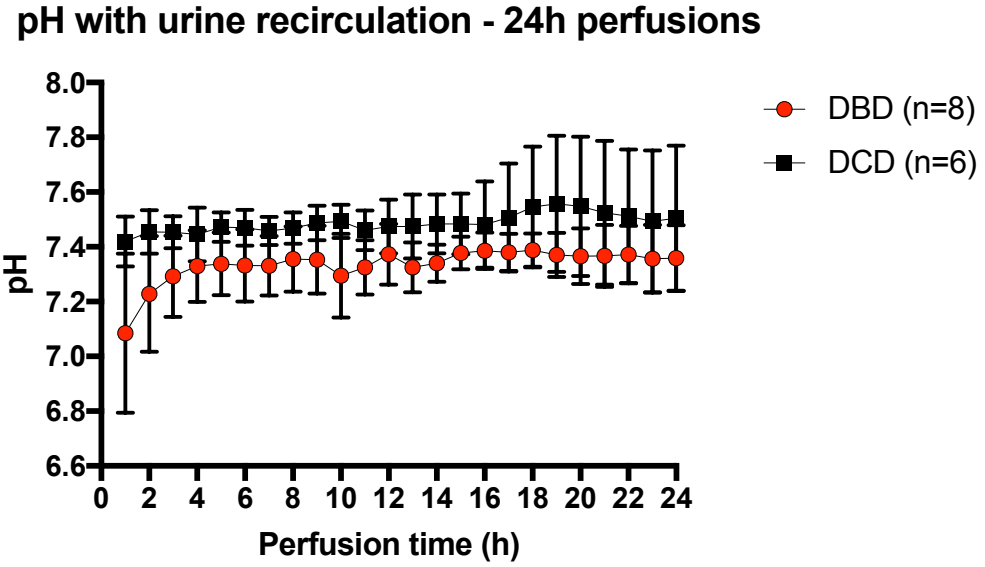


Figure 3.12D: Graphical comparison of pH in kidneys with URC, per donor type, perfused for 24 hours. pH given as longitudinal mean ± SD values over the course of the perfusion. Kidneys (n) per hour DBD: 8 1-24 hours. Kidneys (n) per hour DCD: 6 1-24 hours.

3.7.6 Glucose measurements

The perfusions included administration of nutrition starting when the perfusate glucose dropped below 4mmol/l, and thereafter throughout the 24-hours perfusion period. Either lipid free total parenteral nutrition solution Nutriflex® special (B. Braun AG, Melsungen, Germany) which contains glucose (glucose monohydrate 396g; 0.24mg glucose/ml); overall 5190kJ (1240kcal) per litre solution, or glucose 5% (Baxter Healthcare Ltd; containing 0.05mg/ml glucose; overall 836.8kJ (200kcal) per litre), depending on availability, was used. The overall amount of glucose given throughout the perfusions is shown in tables 3.3 and 3.4 (n=29 for glucose).

Evaluating glucose consumption in an isolated kidney is not simple, because of the conflicting influences of glucose consumption and gluconeogenesis, glycolysis, glucose filtration and glucose reabsorption, all taking place at the same time (133–135).

3.7.7 Lactate levels during perfusion

The lactate levels are shown in table 3.4 (n=28 for lactate). The median (min – max) lactate levels after 1 hour, after 6 hours and at the end of perfusion were 11.91 mmol/l (5.58 – 15.72), 14.34 mmol/l (2.55 – 20) and 14.2 mmol/l (2.9 – 20) in the urine recirculation group (n=24). The median lactate levels for the urine replenishment group (n=4) were 12.75 mmol/l (9.87 – 12.9) after 1 hour, 17.43 mmol/l (4.51 – 20) and 18.2 mmol/l (4.51 – 19.65) at the end of perfusion. Allowing that the urine replenishment group received Ringer's lactate, the differences in lactate levels between the urine recirculation and replenishment groups were not remarkable.

Table 3.4: Haemodynamic and metabolic function parameters per kidney	**Kidney 1**	**Kidney 2**	**Kidney 3**	**Kidney 4**	**Kidney 5**	**Kidney 6**
Arterial pressure in mmHg (mean, SD)*	81 ± 8.7	70 ± 2.9	75.5 ± 3.5	93.4 ± 7.8	67.2 ± 7.2	60 ± 2.4
Arterial flow in ml/min (mean, SD)*	326 ± 76	336 ± 31	340 ± 10.6	321.4 ± 34.4	327.5 ± 8.5	396.7 ± 38.3
IRR in ml/min/mmHg (mean, SD)*	0.27 ± 0.09	0.21 ± 0.02	0.22 ± 0.01	0.29 ± 0.03	0.21 ± 0.02	0.16 ± 0.02
pH (mean, SD)*	7.33 ± 0.14	7.27 ± 0.09	7.35 ± 0.07	7.26 ± 0.18	7.34 ± 0.12	7.5 ± 0.05
arterial PO_2 in kPa (mean, SD)*	26.5 ± 5.5	18.3 ± 7.7	16.5 ± 0.7	21.6 ± 12.2	21.2 ± 6.1	20.3 ± 9
venous PO_2 in kPa (mean, SD)*	14.3 ± 4.7	8.8 ± 3.9	9 ± 0.6	13.6 ± 5.3	9.2 ± 3.2	9 .6 ± 1.2
arterial PCO_2 in kPa (mean, SD)*	2.9 ± 1.3	5 ± 1.5	1.5 ± 0.8	6.2 ± 0.8	4.9 ± 1.6	6 ± 0.63
Lactate level in mmol/l (mean, SD)*	not available	15.19 ± 4.47	14.81 ± 2.48	13.36 ± 1.39	17.71 ± 2.7	16.91 ± 3.53
Total glucose given in mg**	3.1	5	2.4	5.2	10.8	6
Total urine output in ml	150	25	190	1380	984	40
Total urine output in ml/hour	30	3.6	19	86.3	41	3.3
Urine recirculation yes/no	yes	yes	yes	yes	yes	yes
Time on the device (hours+min)	5 + 5	7 + 45	10 + 10	16 + 11	24 + 19	12 + 15

* time-averaged longitudinal mean value compiled from hourly measurements over the course of the perfusion

**circulating perfusate volume of 500ml

continued Table 3.4:	**Kidney 7**	**Kidney 8**	**Kidney 9**	**Kidney 10**	**Kidney 11**	**Kidney 12**
Arterial pressure in mmHg (mean, SD)*	64.3 ± 2.3	73.7 ± 14.8	56.5 ± 2.5	first test run	63.8 ± 5.6	74.8 ± 3.8
Arterial flow in ml/min (mean, SD)*	327.7 ± 17.9	269.4 ± 57	311.7 ± 23.5	of the	346 ± 35.1	240 ± 52.5
IRR in ml/min/mmHg (mean, SD)*	0.19 ± 0.01	0.27 ± 0.09	0.18 ± 0.03	prototype	0.19 ± 0.03	0.3 ±0.11
pH (mean, SD)*	7.4 ± 0.1	7.24 ± 0.04	7.44 ± 0.08		7.4 ± 0.09	7.35 ± 0.1
arterial PO_2 in kPa (mean, SD)*	28.8 ± 15.6	26.1 ± 12.7	26.5 ± 13.5	↑	13.8 ± 4	18 ± 7.7
venous PO_2 in kPa (mean, SD)*	12 ± 3.7	9.2 ± 2.9	10.1 ± 3	not available	6.5 ± 2.3	10.2 ± 2.1
arterial PCO_2 in kPa (mean, SD)*	4.6 ± 0.6	5.9 ± 0.4	5.6 ± 1	↓	5.4 ± 1.2	4.7 ± 1.3
Lactate level in mmol/l (mean, SD)*	5.12 ± 3.19	18.77 ± 2	13.6 ± 4.2	not available	not available	18.39 ± 3.6
Total glucose given in mg**	5	7.2	4.8	0	0.5	7.2
Total urine output in ml	360	140	1078	150	426.3	1677
Total urine output in ml/hour	20	5.8	44.9	30	106.6	111.8
Urine recirculation yes/no	yes	yes	yes	yes	yes	yes
Time on the device (hours+min)	18 + 36	24+4	24+12	5 + 20	4 + 10	15

* time-averaged longitudinal mean value compiled from hourly measurements over the course of the perfusion

**circulating perfusate volume of 500ml

continued Table 3.4:	**Kidney 13**	**Kidney 14**	**Kidney 15**	**Kidney 16**	**Kidney 17**	**Kidney 18**
Arterial pressure in mmHg (mean, SD)*	67.1 ± 7.9	device	81.7 ± 1.5	79.8 ± 8.5	77.8 ± 1.6	90 ± 0.7
Arterial flow in ml/min (mean, SD)*	287.1 ± 71.8	malfunction	318.3 ± 30.6	289.1 ± 51.9	509.6 ± 25.2	178 ± 29.5
IRR in ml/min/mmHg (mean, SD)*	0.24 ± 0.04	software	0.26 ± 0.03	0.28 ± 0.04	0.15 ± 0.01	0.51 ± 0.1
pH (mean, SD)*	7.48 ± 0.05	problem	7.45 ± 0.09	7.43 ± 0.06	7.44 ± 0.06	7.39 ± 0.05
arterial PO_2 in kPa (mean, SD)*	33.7 ± 21	↑	12.2 ± 2.6	12.1 ± 1.6	13.5 ± 0.7	12.8 ± 1
venous PO_2 in kPa (mean, SD)*	20.4 ± 15.1	not available	7.2 ± 1.1	6.9 ± 2.4	7.3 ± 1.1	8.6 ± 1.7
arterial PCO_2 in kPa (mean, SD)*	5.3 ± 2	↓	4.8 ± 1.8	5.3 ± 0.3	5 ± 0.3	4.6 ± 0.6
Lactate level in mmol/l (mean, SD)*	9.52 ± 4.53	not available	16.14 ± 2.3	7.77 ± 1.95	10.92 ± 0.97	not available
Total glucose given in mg**	2	0	5.28	0.85	4.5	0.6
Total urine output in ml	400	30	264	150	610	259
Total urine output in ml/hour	16.7	20	11	12.5	25.4	64.8
Urine recirculation yes/no	yes	yes	yes	yes	yes	yes
Time on the device (hours+min)	24 + 8	1 + 30	24	12 + 27	24 + 19	4 + 16

* time-averaged longitudinal mean value compiled from hourly measurements over the course of the perfusion

**circulating perfusate volume of 500ml

continued Table 3.4:	**Kidney 19**	**Kidney 20**	**Kidney 21**	**Kidney 22**	**Kidney 23**	**Kidney 24**
Arterial pressure in mmHg (mean, SD)*	101.5 ± 1.7	97 ± 5.3	78.9 ± 6.2	89.7 ± 2.6	99.5 ± 2.3	84.6 ± 1.1
Arterial flow in ml/min (mean, SD)*	220 ± 27.1	277.7 ± 67.3	357.1 ± 65.7	498.8 ± 92.9	189 ± 31.1	650 ± 191.6
IRR in ml/min/mmHg (mean, SD)*	0.47 ± 0.06	0.37 ± 0.09	0.24 ± 0.08	0.19 ± 0.05	0.53 ± 0.11	0.15 ± 0.08
pH (mean, SD)*	7.41 ± 0.09	7.26 ± 0.22	7.72 ± 0.23	7.38 ± 0.09	7.23 ± 0.2	7.39 ± 0.12
arterial PO_2 in kPa (mean, SD)*	11.7 ± 0.4	11.4 ± 5.6	14.6 ± 2.5	12.5 ± 1.5	12.9 ± 1.7	15 ± 1.6
venous PO_2 in kPa (mean, SD)*	8.4 ± 1.3	7.3 ± 1	7.8 ± 1.7	7.6 ± 1.4	7.5 ± 2.2	7.7 ± 1.9
arterial CO_2 in kPa (mean, SD)*	4.6 ± 0.5	4 ± 2	1.1 ± 0.5	5.5 ± 2.6	4.5 ± 0.9	4.3 ± 0.9
Lactate level in mmol/l (mean, SD)*	not available	not available	11.52 ± 5.8	7.78 ± 2.3	13.31 ± 6.1	13.4 ± 1.62
Total glucose given in mg**	0.6	12	0	4.32	1.2	2.4
Total urine output in ml	432	412	630	155	1029	828
Total urine output in ml/hour	108	17.2	26.3	6.5	42.9	63.7
Urine recirculation yes/no	yes	yes	yes	yes	yes	yes
Time on the device (hours+min)	4 + 4	24	24 + 25	24	24 + 10	13 + 1

* time-averaged longitudinal mean value compiled from hourly measurements over the course of the perfusion

**circulating perfusate volume of 500ml

continued Table 3.4:	**Kidney 25**	**Kidney 26**	**Kidney 27**	**Kidney 28**	**Kidney 29**	**Kidney 30**
Arterial pressure in mmHg (mean, SD)*	82.5 ± 7.8	perfusion	91.3 ± 6.4	90.3 ± 1.3	90.4 ± 2.1	88.9 ± 1.7
Arterial flow in ml/min (mean, SD)*	294.5 ± 74	could not be	325 ± 127.7	271 ± 54.6	383.9 ± 88.3	474.1 ± 149.7
IRR in ml/min/mmHg (mean, SD)*	0.3 ± 0.09	maintained	0.39 ± 0.36	0.36 ± 0.18	0.25 ± 0.08	0.2 ± 0.09
pH (mean, SD)*	7.21 ± 0.1		7.24 ± 0.17	7.33 ± 0.06	7.4 ± 0.04	7.66 ± 0.2
arterial PO_2 in kPa (mean, SD)*	14.7 ± 0.7	↑	12.6 ± 2	12 ± 1.5	13.8 ± 0.6	15 ± 3
venous PO_2 in kPa (mean, SD)*	7.6 ± 0.8	not available	6.4 ± 1.1	7 ± 2.4	8 ± 0.9	7.4 ± 0.9
arterial PCO_2 in kPa (mean, SD)*	5.1 ± 0.4	↓	5.9 ± 0.7	4.6 ± 0.7	5.1 ± 0.9	4.6 ± 0.6
Lactate level in mmol/l (mean, SD)*	13.27 ± 1.32		12.19 ± 3	18.52 ± 2.16	5.65 ± 3.8	9.62 ± 4
Total glucose given in mg**	3	↑	1.2	0.75	3.2	0.55
Total urine output in ml	286	not available	10	105	1285	445
Total urine output in ml/hour	15.9	↓	1.7	8.8	53.5	74.2
Urine recirculation yes/no	yes		yes	yes	yes	no
Time on the device (hours+min)	18 + 3	0 + 45	6 + 20	12 + 35	24 + 5	6 + 10

* time-averaged longitudinal mean value compiled from hourly measurements over the course of the perfusion

**circulating perfusate volume of 500ml

continued Table 3.4:	**Kidney 31**	**Kidney 32**	**Kidney 33**	**Kidney 34**	**Kidney 35**	**Kidney 36**
Arterial pressure in mmHg (mean, SD)*	92.4 ± 2.9	92.2 ± 2.8	89.8 ± 0.5	91.64 ± 2.3	89.2 ± 2.2	90.3 ± 2.1
Arterial flow in ml/min (mean, SD)*	148.2 ± 46.3	123.5 ± 79.16	339.6 ± 83.4	240.7 ± 120.9	468.8 ± 82.8	664.9 ± 228.1
IRR in ml/min/mmHg (mean, SD)*	0.76 ± 0.5	1.2 ± 1.02	0.3 ± 0.12	0.5 ± 0.3	0.19 ± 0.05	0.17 ± 0.13
pH (mean, SD)*	7.18 ± 0.08	7.2 ± 0.1	7.6 ± 0.2	7.33 ± 0.1	7.39 ± 0.04	7.43 ± 0.02
arterial PO_2 in kPa (mean, SD)*	13.6 ± 1.1	14.6 ± 2	14 ± 3.2	13.5 ± 2.9	15.4 ± 3.6	12.45 ± 0.8
venous PO_2 in kPa (mean, SD)*	8.3 ± 1.5	7.8 ± 1.8	6.4 ± 1.3	6.7 ± 1.3	7.1 ± 1.6	7.6 ± 1.3
arterial PCO_2 in kPa (mean, SD)*	5.4 ± 0.9	4.8 ± 1.1	5 ± 0.7	4.5 ± 0.9	4.7 ± 0.6	4.6 ± 0.4
Lactate level in mmol/l (mean, SD)*	16.18 ± 2.14	14.28 ± 4.5	16.34 ± 2.9	18.84 ± 2.2	9.19 ± 2	7.04 ± 1.46
Total glucose given in mg**	1	1.5	0.5	5	3	4.55
Total urine output in ml	50	920	675	11325	1325	1223
Total urine output in ml/hour	8.3	102.2	84.4	471.9	55.2	51
Urine recirculation yes/no	no	no	no	yes	yes	yes
Time on the device (hours+min)	6 + 5	9 + 25	8 + 10	24+5	24	24

* time-averaged longitudinal mean value compiled from hourly measurements over the course of the perfusion

**circulating perfusate volume of 500ml

3.7.7.1 Correlation between lactate, administered glucose and arterial flow

One of the reasons for correlating lactate, glucose and flow values in the following paragraph is based on observations made during kidney perfusions and was triggered by the use of an elementary formula – glucose measured at perfusion start in mmol/l plus glucose given over time in mmol/l minus glucose measured at the end of perfusion in mmol/l – to calculate “glucose consumption”. This analysis evidenced that some kidneys had a glucose level at the end of perfusion which would have required far more nutritional supplement (5% glucose or TPN) than they had actually received. “Glucose consumption” is set in quotation marks as the glucose metabolism in the kidney and the glucose handling by the kidney is too sophisticated (3.7.6 glucose measurements) to generate a result describing the renal glucose metabolism by using a simple computation formula as mentioned above. Moreover, the main reason for performing these calculations is published evidence that glucose and lactate are mutually implicated in renal metabolism. Anaerobic glycolysis in the inner medulla produces lactate which can be/ will be used for gluconeogenesis in the outer medulla and the cortex (proximal tubule) (136–138). A more recent publication demonstrated that glucose is essential in feeding the Krebs-cycle via circulating lactate (139). Hui *et al.* describe in their experimental setting in mice that they have detected strongly labelled lactate in the kidney after infusion of ^{13}C-labelled glucose, although intermediates of the Krebs-cycle are more labelled after lactate infusion. An explanation for this pattern in the kidney is the heterogeneity in the kidney; the presence of glycolytic cells producing lactate from glucose and oxidative cells using lactate to make Krebs-cycle intermediates (139).

There was a correlation between glucose administration and perfusate lactate: Kidneys (n=24, with urine recirculation) with lactate levels higher than the median of 14.2 mmol/l at the end of perfusion received more glucose than kidneys with an end-lactate lower than 14.2 mmol/l; p=0.036, 95% CI -0.053 - -0.001. The amount of glucose administered (3.9 ± 1.2 mg) during perfusion correlates significantly with the final lactate level (12.9 ± 6.5 mmol/l) at the end of perfusion; p=0.026, correlation coefficient (Spearman's rho) = 0.5.

There was a correlation between arterial flow and lactate level (in both urine recirculation and replenishment groups). The (longitudinal) median arterial flow throughout the perfusion was higher in kidneys with a lactate level less than the median of 14.87 mmol/l (n=28) or less than the median value of 14.2 mmol/l (n=24) at the end of perfusion; p=0.004, 95% CI -260.9 - -63.9 (including n=4 without urine recirculation) and p=0.016, 95% CI -251.9 - -40.1 (n=24). The median arterial flow (377.6 ± 153 ml/min) in the urine recirculation group correlates significantly with the final lactate measurement (12.9 ± 6.5 mmol/l), the higher the flow the lower the lactate; p=0.04, correlation coefficient (Spearman's rho) = -0.444. Furthermore, the median arterial flow (377.6 ± 153 ml/min) in the urine recirculation group correlates significantly with the lactate level measured 6 hours after perfusion start (12.9 ± 5.3 mmol/l); p=0.29, correlation coefficient (Spearman's rho) = -0.475.The delta-value between the lactate at the beginning and after 6 hours of normothermic perfusion (0.77 ± 4.98 mmol/l) also correlates significantly with the median arterial flow; p=0.016, correlation coefficient (Spearman's rho) = -0.519. Lactate levels did not correlate with the total or hourly urine output; neither the initial ones nor the 6-hour or the final measurements. We concluded that the interpretation of lactate levels in isolated kidney perfusion is

complex, reflecting factors other than cellular hypoxia alone, and that lactate should not be used in isolation to ascertain organ viability.

3.7.8 Urine production and sodium levels in perfusate and urine

All except one kidney produced urine. The average hourly urine production of kidneys (n=32) with urine recirculation was 28.1 ml (0 ml – 471.9 ml). Median hourly urine production of kidneys without urine recirculation (n=4) was 79.3 ml (8.3 ml – 102.2 ml). Kidney 26 accounted for zero urine production. This was a kidney with a biopsy lesion in the parenchyma of about 4cm^3 close to the hilum. Despite the benchwork, there was massive arterial blood loss from this site and this was too high to keep the perfusion running. The perfusion was stopped after 45 minutes. Kidney 34 was polyuric with very high volumes (>400 ml/hour) of urine production until hour 20. After this time, urine production decreased to about 70 ml/hour. Urine production is shown in tables 3.3 and 3.4.

Perfusate sodium levels of kidneys with urine recirculation (n=24) were in the physiological range throughout perfusion; 141.9 ± 4.2 mmol/l. Perfusate sodium levels in kidneys without urine recirculation (n=4) were significantly higher; 162.6 ± 14.8 mmol/l, $p<0.0001$, 95% CI -25.5 - -15.9.

Urine sodium levels of all but two kidneys, one with and one without urine recirculation, were low with sodium <100 mmol/l. In kidney 15 (histologically proven cortical necrosis) and 34 (polyuric during perfusion, histologically proven acute on chronic hypertensive nephropathy) the urine sodium levels were similar to the perfusate values, 142±3.9 mmol/l, potentially indicating tubular injury with lack of sodium reabsorption capacity. It could thus be concluded that urine sodium level is a useful biomarker of tubular injury.

3.7.9 Anti-Xa as an anticoagulant and a biomarker useful?

Anti-Xa results are available from 18/32 (56.3%) perfusions with urine recirculation and from 3/4 (75%) perfusions without urine recirculation. The reason for the small number of anti-Xa levels were (i) costs, (ii) logistics and (iii) haemolysis, which is more likely to occur in expired blood and in some of the perfusions, caused by over oxygenation in the early stages of device development. One and 6-hour anti-Xa measurement were used for comparing the perfusion groups as no later measurements were available for the perfusions without urine recirculation.

In the urine recirculation group, mean ± SD anti-Xa levels were 0.35 ± 0.15 IU/ml after one hour of perfusion, 0.33 ± 0.07 IU/ml after four hours of perfusion and 0.26 ± 0.14 IU/ml after six hours of NMP. In kidney perfusions without urine recirculation the anti-Xa levels measured at the same time-points were 0.27 ± 0.6 IU/ml, 0.16 ± 0.13 IU/ml and 0.08 ± 0.12 IU/ml respectively. The differences between the 1-hour anti-Xa level and the level measured after four hours of NMP are not significantly different, neither in the urine recirculation group ($p=0.38$) nor in the one without ($p=0.3$). Anti-Xa levels were significantly lower after six hours of NMP, compared to 1-hour measurements, on the completely closed circuit ($p=0.04$). The anti-Xa levels were also lower after six hours of NMP in the group without urine recirculation, but not significant ($p=0.2$).

Comparing anti-Xa levels between the perfusion groups reveals similar the same anti-Xa levels after one hour of NMP; 0.35 ± 0.15 IU/ml vs. 0.27 ± 0.06 IU/ml, $p=0.23$. Interestingly, anti-Xa levels measured after four and six hours of perfusion are significantly lower in the open perfusion circuit, 0.16 ± 0.13 IU/ml and 0.08 ± 0.12 IU/ml, compared to the closed, 0.33 ± 0.07 IU/ml and 0.26 ± 0.14 IU/ml, $p=0.02$ and 0.04. This finding demonstrates most likely the renal excretion of active and inactive compounds of the administered LMWH (123) rather than representing quality of kidney

function. Using anti-Xa as a biomarker could be possible, requires intra-kidney analysis comparing several time-points within an entire perfusion and perfect conditions, including fresh packed red blood cells, as free haemoglobin levels <1g/l are needed to receive a useful result. Following 24-hour perfusions should demonstrate the differences of anti-Xa levels between kidneys; enoxaparin dose and its half-life (about 5 hours (123)) were the same for all perfusions; percentages given are calculated from 1-hour anti-Xa level:

(i) Kidney 8, DBD, 73 years, 20 hours CIT, median arterial flow 250ml/min, anti-Xa after 6 hours 45% and 16.1% after 24 hours

(ii) Kidney 17, DCD, 49 years, 25 hours CIT, median arterial flow 510 ml/min, anti-Xa after 6 hours 75% and 47.5% after 24 hours

(iii) Kidney 22, DBD, 68 years, 108 hours CIT, median arterial flow 514 ml/min, anti-Xa after 6 hours 18.2% and 8.2% after 24 hours

(iv) Kidney 29, DBD, 70 years, 17 hours CIT, median arterial flow 413 ml/min, anti-Xa after 6 hours 85% and 60% after 24 hours

(v) Kidney 36, DBD, 76 years, 15 hours CIT, median arterial flow 787 ml/min, anti-Xa 75% after 6 hours and 50% after 24 hours

Anti-Xa levels were also particularly helpful to investigate haemolysis in our circuit. As mentioned earlier, especially during the “instable” oxygenation periods with peak PO_2 levels beyond 40 kPa (era 1), free haemoglobin reached >4g/l and it was not possible to receive an anti-Xa result of such haemolysed perfusates. I would like to point out here that perfusate samples at this stage of haemolysis were NOT considered for any other biomarker analyses as described in Chapter 4. Getting anti-Xa results until the end, 24 hours, of kidney NMP was also a success in regards to low levels of free

haemoglobin as the analyser for the anti-Xa test flags samples that are over 0.6 g/l (still giving a result though), but would not give a result (only the statement “too haemolysed”) when free haemoglobin levels are more than 1.2-1.5 g/l; information received from Marianne McClure from the Haematology Laboratory at the John Radcliffe Hospital, Oxford University Hospitals, NHS Foundation Trust.

3.8 Histology results

Tissue samples were obtained from 31/36 (86.1%) kidneys before start of normothermic perfusion and at 1, 6, 12, 18 and 24 hours after perfusion start, whilst a sample was always obtained at the end of perfusion (2.6.1 in Chapter 2). The presence of acute tubular injury before perfusion start and at the end of perfusion was evaluated based on following criteria: 0 – absent; 1 – loss of brush borders/vacuolation of tubular epithelial cells; 2 – cell detachment/cellular casts; 3 – coagulation necrosis. The baseline tubular condition could be described in all zero kidney biopsies. The tubular condition before perfusion start summarised: 3/31 (9.7%) 0; 24/31 (77.4%) 1; 3/31 (9.7%) 2; 1/31 (3.2%) 3. The baseline tubular condition appeared unchanged after normothermic perfusion in 27/31 (87.1%) kidneys. The tubular condition at the end of perfusion summarised: 4/31 (12.9%) 0; 25/31 (80.7%) 1; 1/31 (3.2%) 2; 1/31 (3.2%) 3.

Kidneys 5, 9 and 22 showed improvement, kidney 8 showed deterioration of baseline acute tubular injury, from a score of 0 to 1. In this kidney, widespread tubular epithelium vacuolation was observed in the 24-hour sample. Vacuolation is a non-specific finding that might be attributed to osmotically active perfusate compounds, for example mannitol (this might be the cause in our case), and to sustained ischaemic damage. In this particular case, KIM-1 immuno-expression was observed in 20% of

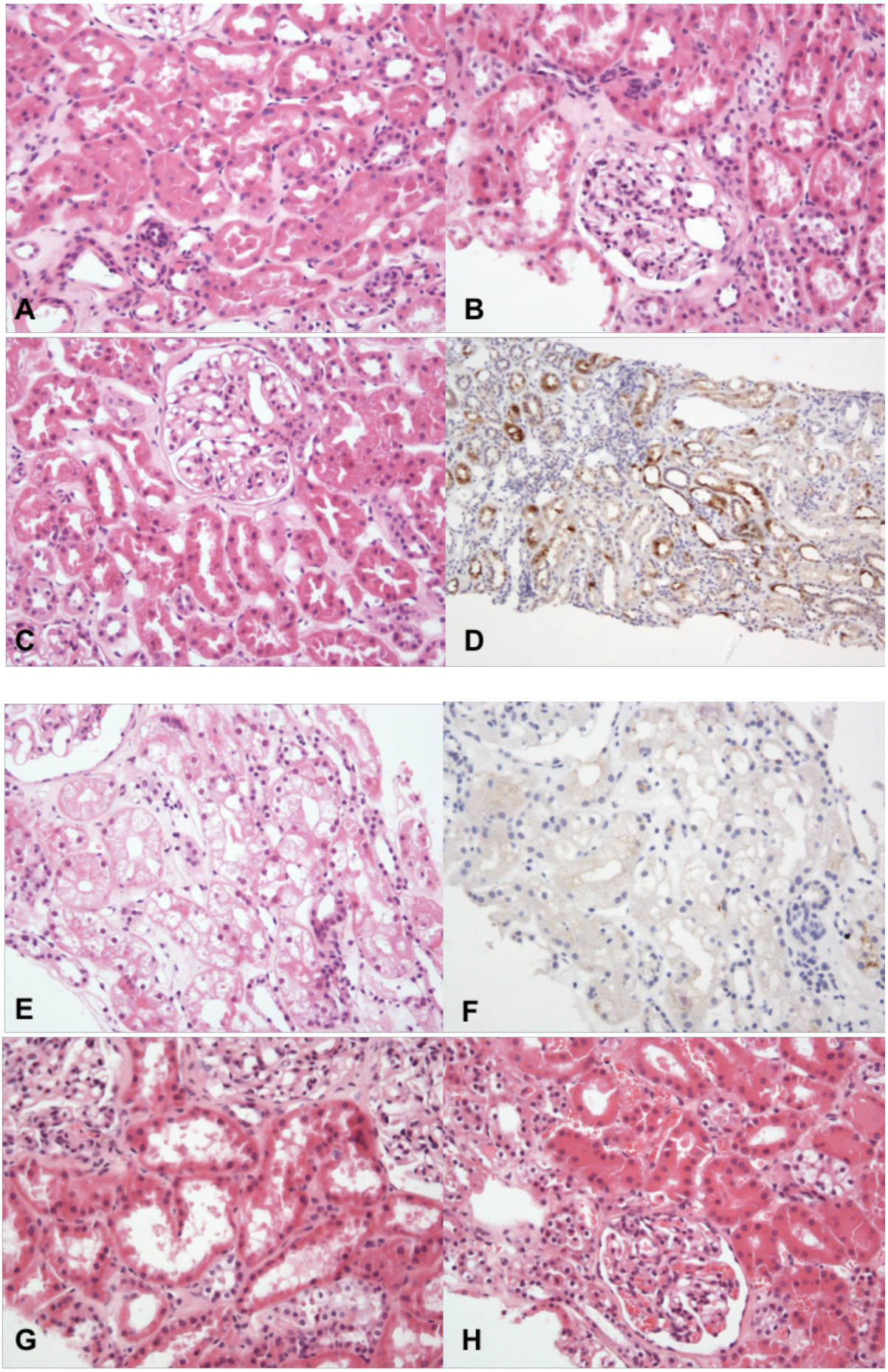
the tubules on the zero biopsy and decreased to <5% positivity on the 24-hour biopsy. We could see vacuolation in two more kidneys, 7 and 9. In these kidneys the tubular condition stayed the same or improved over time. Nevertheless, KIM-1 was not positive at all or decreased over time. These results suggest that the observed vacuolation is more likely to be attributed to an osmotic, rather than an ischaemic effect. Interestingly, in the five cases in which KIM-1 positivity increased over time, an "event" could be found. It is too early, and the numbers are too small, to claim that this is an explanation for the KIM-1 positivity, but it is an observation: Kidney 6 was the first kidney in which experiments were carried out on the bypass line; changing the flow via the bypass and the arterial line over a couple of hours. Kidneys 15, 25 and 28 experienced major heating problems with alternating periods of hypothermia and overheating. In kidney 31 the KIM-1 expression increased over time without any device malfunction or perfusion instabilities. It was an elderly DBD kidney which was declined for anatomical reasons (cut lower polar artery) and long ischaemia time.
No haemosiderin deposits could be detected at any time in the haematoxylin and eosin stainings.

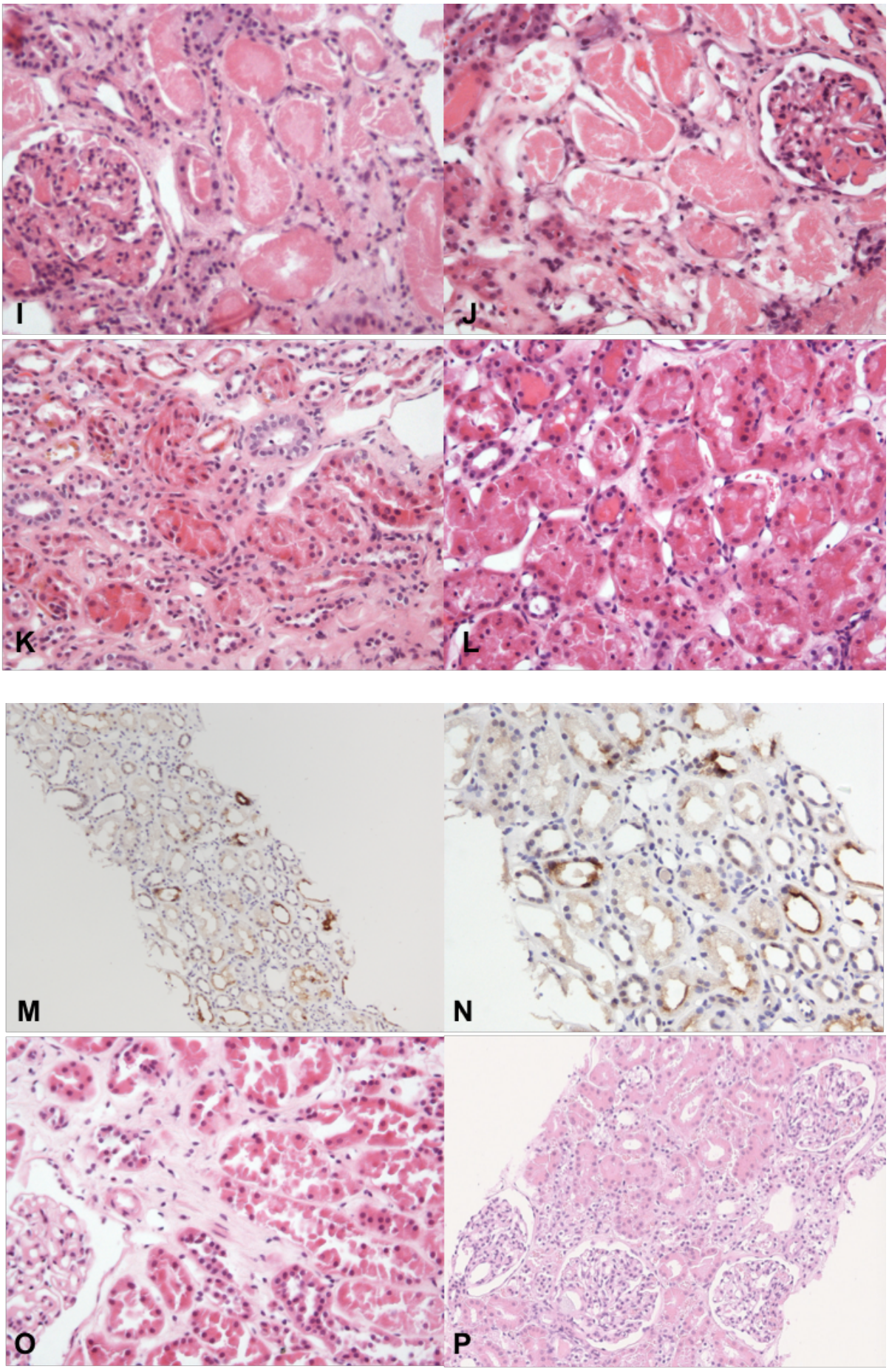
The tubular conditions and KIM-1 immunohistochemistry results for 31 kidneys are displayed in table 3.5. Examples of histology results (before perfusion start and at the end of perfusion) for kidneys with urine recirculation and without urine recirculation are illustrated in figures 3.13A-X.

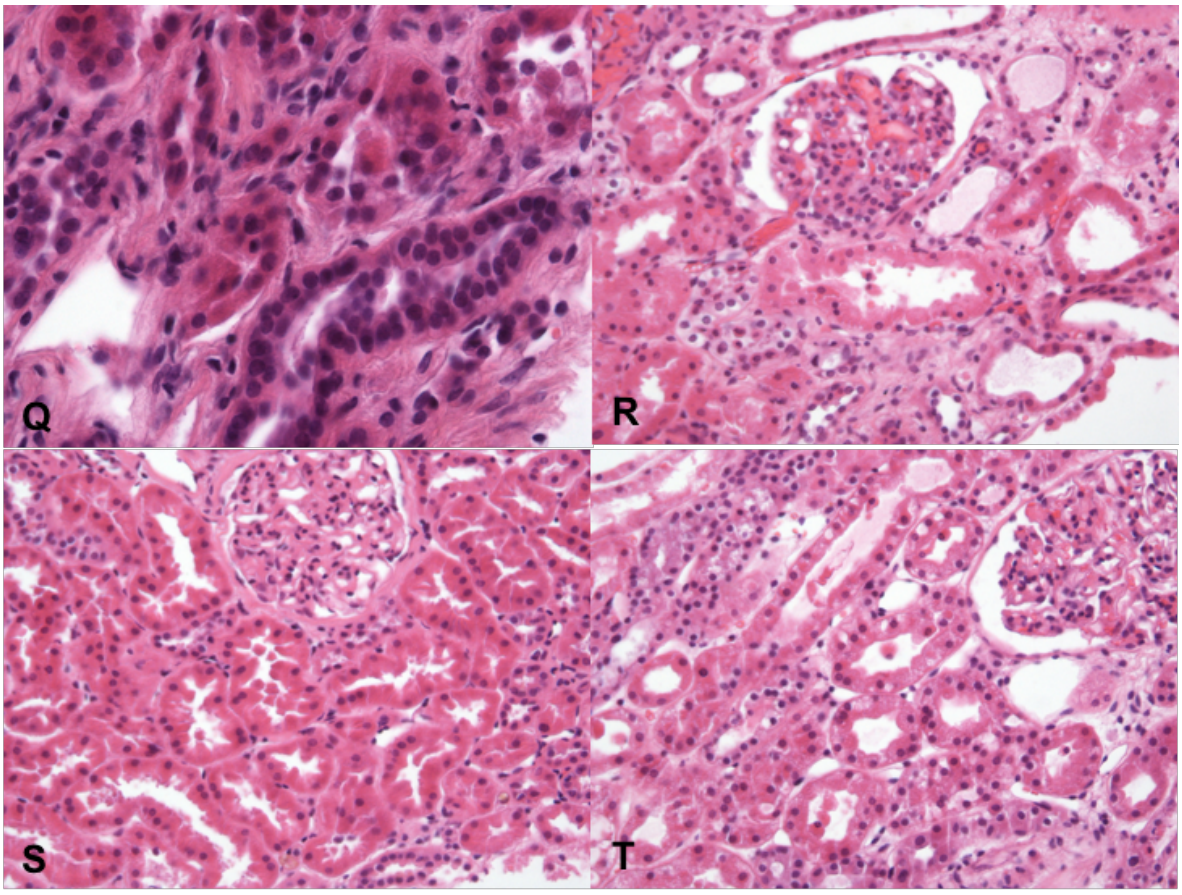
Table 3.5: Histology results and KIM-1 immunohistochemistry - tubular condition

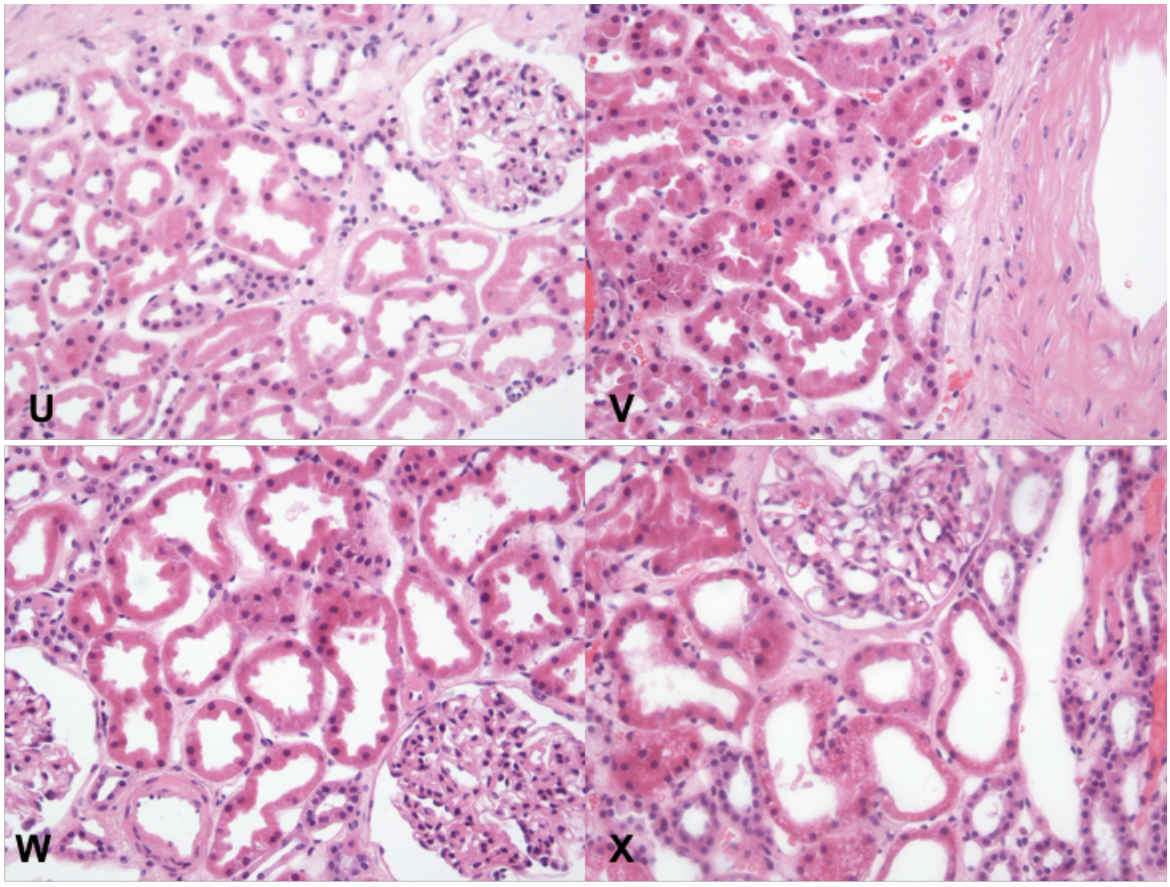
Kidney	Baseline tubular condition	Tubular condition end of perfusion	KIM-1 staining baseline	KIM-1 staining end of perfusion
1	no biopsy taken			
2	1	1	positive, 5%	positive, <5%
3	1	1	positive, 5%	positive, 5%
4	1	1	positive, 5%	positive, 5%
5	2	0	positive, 5%	positive, <5%
6	1	1	positive, 10%	positive, 20%
7	1	1	positive, 5%	positive, <5%
8	0	1 (vacuolation)	positive, 20%	positive, <5%
9	1	0	negative	negative
10	no biopsy taken			
11	no biopsy taken			
12	0	0	positive, 5%	positive, 5%
13	1	1	negative	negative
14	no biopsy taken			
15	3	3	positive, 20%	positive, <5%
16	1	1	positive, 5%	positive, 10%
17	0	0	positive, 20%	positive, 5%
18	1	1	positive, 30%	positive, 10%
19	1	1	positive, 20%	positive, 10%
20	1	1	positive, 20%	positive, 20%
21	1	1	positive, 30%	positive, 20%
22	2	1	positive, <5%	positive, 5%
23	1	1	positive, 10%	positive, 5%
24	1	1	positive, 5%	positive, <5%
25	1	1	positive, 10%	positive, 20%
26	no biopsy taken			
27	1	1	negative	negative
28	1	1	positive, 20%	positive, 40%
29	1	1	positive, <5%	negative
30	1	1	negative	negative
31	1	1	positive, 10%	positive, 20%
32	1	1	negative	negative
33	1	1	positive, 5%	positive, 5%
34	2	2	positive, 20%	positive, 5-10%
35	1	1	positive, 5%	positive, 5%
36	1	1	positive, <5%	positive, <5%

Presence of acute tubular injury: 0 – absent; 1 – loss of brush borders / vacuolation of tubular epithelial cells; 2 – cell detachment / cellular casts; 3 – coagulation necrosis


A
B
C
D
E
F
G
H


I
J
K
L
M
N
O
P


Q
R
S
T


U
V
W
X

Figures 3.13A-X:

A: Zero biopsy kidney 5; B: Biopsy after 24 hours of NMP kidney 5

C: Zero biopsy kidney 8; D: KIM-1 immunostaining of zero biopsy kidney 8

E: Biopsy after 24 hours of NMP kidney 8; F: KIM-1 immunostaining after 24 hours of NMP kidney 8

G: Zero biopsy kidney 9; H: Biopsy after 24 hours of NMP kidney 9

I: Zero biopsy kidney 15; J: Biopsy after 24 hours of NMP kidney 15

K: Zero biopsy kidney 17; L: Biopsy after 24 hours of NMP kidney 17

M: KIM-1 immunostaining of zero biopsy kidney 19; N: KIM-1 immunostaining after 4 hours of NMP kidney 19

O: Zero biopsy kidney 22; P: Biopsy after 24 hours of NMP kidney 22

Q: Zero biopsy kidney 34; R: Biopsy after 24 hours of NMP kidney 34

S: Zero biopsy kidney 35; T: Biopsy after 24 hours of NMP kidney 35

U: Zero biopsy kidney 32; V: Biopsy after 8 hours of NMP kidney 32

W: Zero biopsy kidney 33; X: Biopsy after 8 hours of NMP kidney 33

Chapter 4: Biomarkers and metabolism in NMP kidneys

According to the Oxford English dictionary, a biomarker is defined as “a naturally occurring molecule, gene, or characteristic by which a particular pathological or physiological process, disease, etc. can be identified” (140). We can distinguish between diagnostic, prognostic, predictive and therapeutic biomarkers (141). The aim of my studies was to analyse the perfusion solution and tissue samples of normothermically perfused discarded human kidneys for biomarkers and parameters to learn about the parenchyma and functional quality as well as the active or inactive metabolism in a human kidney under nearly-normal physiological conditions. The reason for primarily analysing the perfusate was the fact that I was operating with a fully closed perfusion circuit using urine recirculation. In a small number of kidneys, the excreted urine was analysed for its protein content. The research plan for perfusate analysis included enzyme-linked immunosorbent assays (ELISA) and metabolomics, “the scientific study of the set of metabolites present within an organism, cell, or tissue” (142,143). In addition, to learn about markers and processes in the tissue, proteomics “the study of proteomes and their functions” (144) were performed.

The chapter is divided into three parts: (i) perfusate ELISA results for three of the most recognised and reliable markers of kidney injury and results of protein measurement in the urine; (ii) perfusate metabolomics results; (iii) tissue proteomics results.

The ELISA results were performed by myself, initially under the guidance of Maria Letizia Lo Faro and Olga Boubriak in the IBME and the laboratory of Professor Rutger J. Ploeg, whose contributions are gratefully acknowledged. The tissue proteomics, perfusate metabolomics results and Western Blots were performed in the laboratory

of Professor Benedikt Kessler with Honglei Huang, whose support, guidance and assistance have been invaluable.

4.1 Enzyme-linked immunosorbent assays

4.1.1 Introduction of potential biomarkers: NGAL, KIM-1 and L-FABP

Neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1) and liver fatty acid-binding protein (L-FABP) levels in the perfusate samples were measured. NGAL, lipocalin-2, is in humans encoded by the LCN2 gene, involved in innate immunity, expressed in neutrophils and in low levels in the kidney, the prostate and the epithelia of the respiratory and intestinal tract (145). NGAL is in clinical use as a biomarker of kidney injury (AKI), as NGAL is secreted in high levels into both, blood and urine, within two hours of injury (146). KIM-1, known as hepatitis A virus cellular receptor 1 (HAVcr-1) or as T-cell immunoglobulin and mucin domain 1 (TIM-1) protein, is a protein upregulated in injured kidneys by various types of insults (147). KIM-1 is a type 1 transmembrane protein with an immunoglobulin and mucin domain and is not measurable in normal proximal tubule cells. KIM-1 is clearly upregulated with injury or cell dedifferentiation as it is the case in renal cell carcinoma (148). Fatty acid-binding protein 1 is primarily expressed in the liver and known as L-FABP. It is involved, amongst others, in binding, transport and metabolism of long chain fatty acids and other hydrophobic molecules (149,150). Elevated levels of urinary and serum L-FABP have been published as markers in progressive end-stage renal failure and ischaemic damage of the kidney after renal transplantation or cardiac bypass surgery (abstract (151)).

The following description of urinary biomarkers of kidney toxicity in a preclinical model is given by Bonventre *et al.* (152). (i) NGAL: nephrotoxic or ischaemic acute kidney

injury (AKI), proximal tubule and distal tubule; (ii) KIM-1: nephrotoxic or ischaemic AKI, proximal tubule; (iii) L-FABP: nephrotoxic or ischaemic AKI or unilateral ureteral obstruction, proximal tubule.

Levels of all three biomarkers may predict adverse outcome; death or renal replacement therapy in the clinical setting. Most of the literature regarding biomarkers used for assessing the renal function has evolved from urine analysis, but studies using the mentioned biomarkers measured in serum are on the rise (152–155).

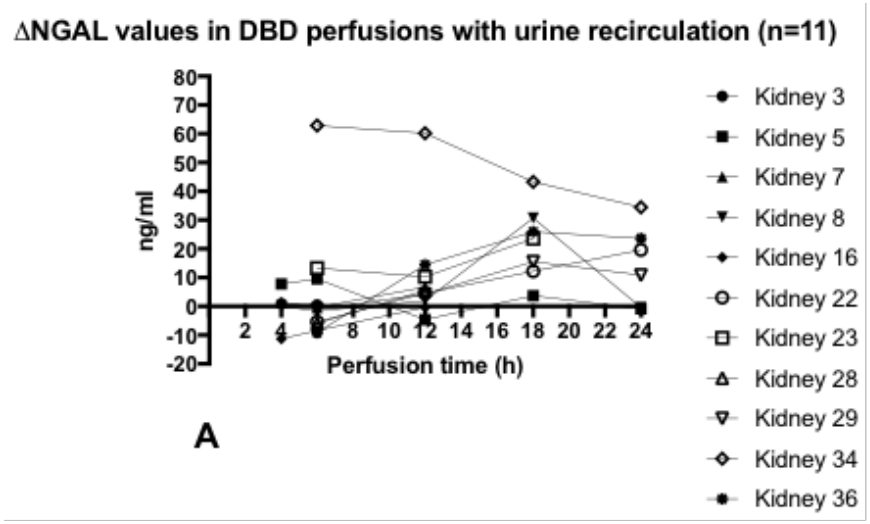
4.1.2 NGAL, KIM-1 and L-FABP measurements in the perfusate

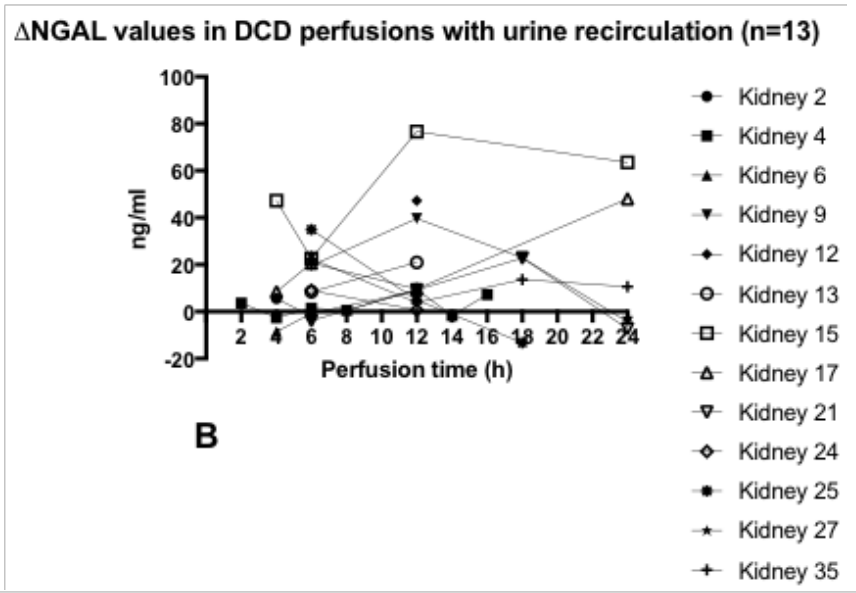
Perfusate samples were collected at 5 time points (at 1, 6, 12, 18 and 24 hours), where available, during NMP and processed by centrifugation at 4000 rpm for 15 minutes at 4°C. The supernatant was aliquoted and snap-frozen and stored at -80^{0}C until the ELISA was performed (2.5.1.1 Chapter 2). Analysis of perfusate was performed for 28 kidneys, 24 with urine recirculation and 4 without urine recirculation. Only kidneys with urine recirculation (n=24) were considered for analysis with stratified perfusion results and correlation with donor characteristics. The stratification was done for: median CIT (min-max) 20.3 (10.4-108.1) hours, CIT longer/shorter than 24 hours, median WIT (min-max) 15 (9-33) minutes for DCD kidneys, median urine output (min-max) 505 (10-11325) ml and median arterial flow (min-max) 330 (187-787) ml/min. A descriptive analysis was done for all measured biomarkers in both groups, with and without urine recirculation. Table 4.1 shows the first and the last measurement, in absolute numbers, for NGAL, KIM-1 (urinary and serum antibody*) and L-FABP, as well as the delta value (difference between first and last biomarker level), the overall mean ± SD for each single kidney (n=28). *Urinary and serum KIM-1 antibody ELISA kits were used for the following: When I started the biomarker analysis for KIM-1 in late 2016,

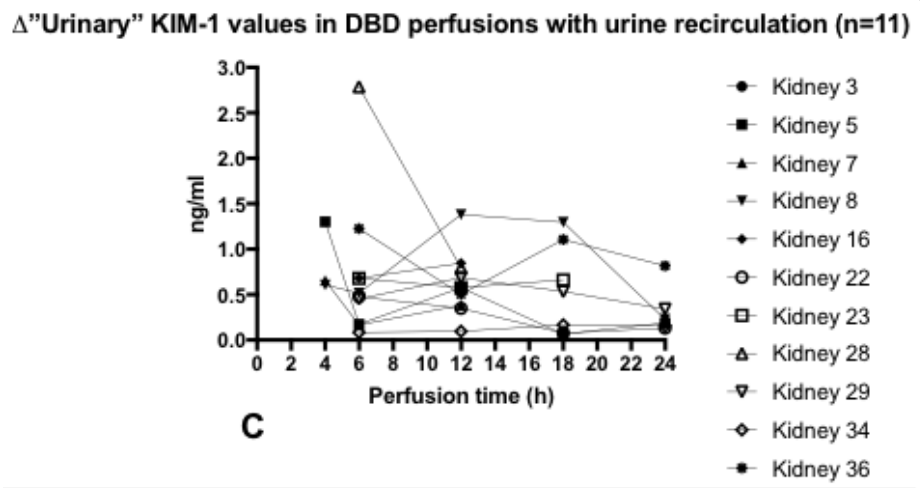
only the urinary type was available; beginning of 2018, a serum ELISA kit was also available, therefore I tested all samples with the urinary and the serum antibody-kit to receive comparable results.

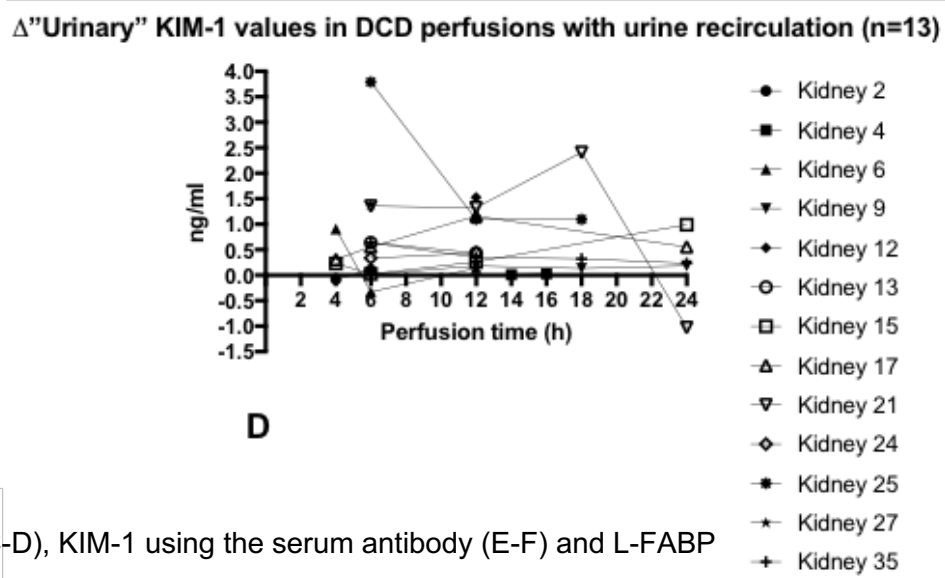
4.1.3 NGAL, KIM-1 and L-FABP levels in kidneys with urine recirculation

To show differences between the kidneys, the delta concentrations of all biomarkers were calculated (value of last measurement minus value of first measurement) and the different profiles are shown in figures 4.1A-H. For better readability, the biomarker profiles for DBD (n=11) and DCD (n=13) kidneys are shown in separate figures. There was no perfusate sample left for testing “serum” KIM-1 in kidney 2 (reason for n=12 in figure 4.1F).

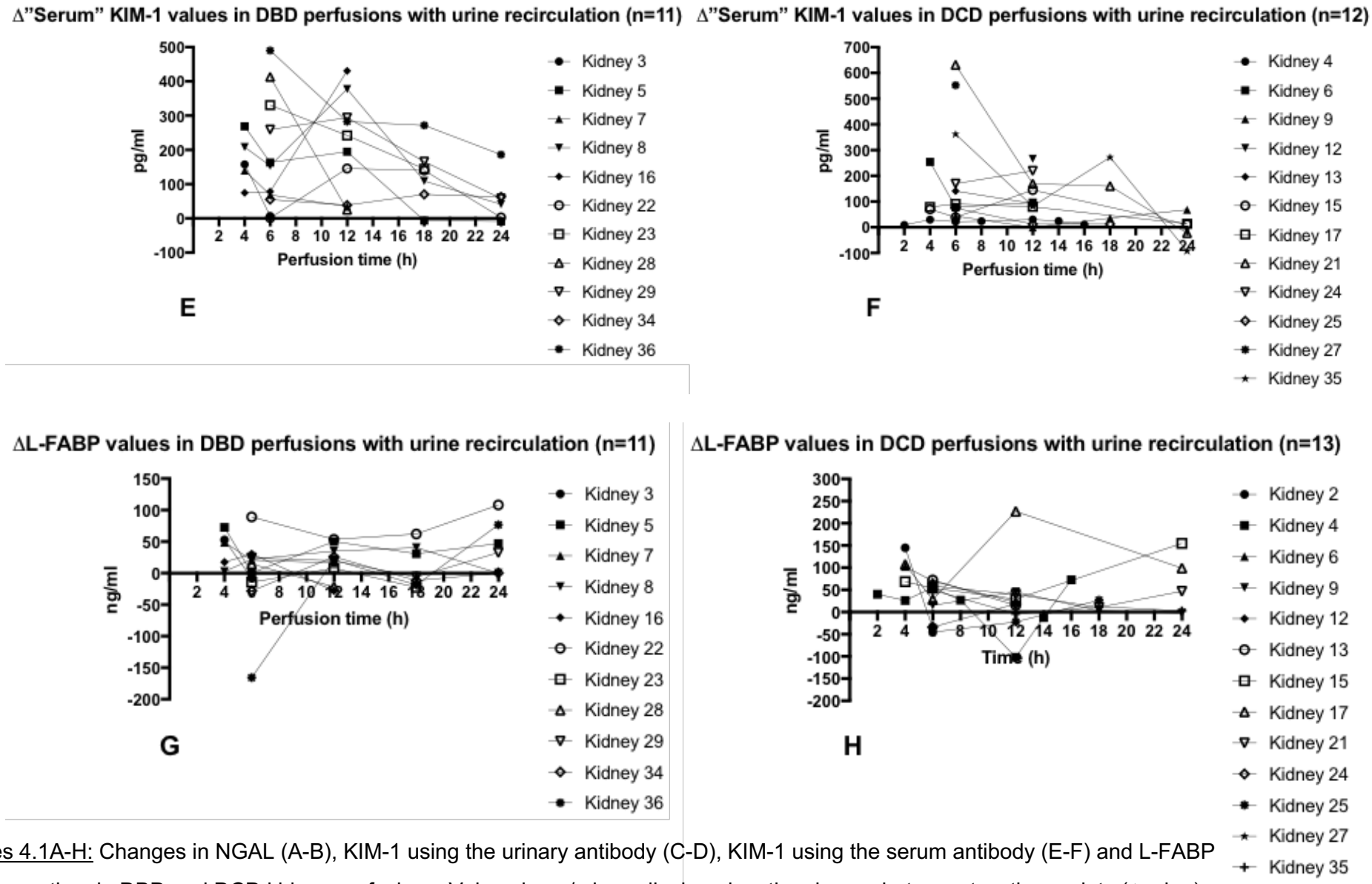








Figures 4.1A-H: Changes in NGAL (A-B), KIM-1 using the urinary antibody (C-D), KIM-1 using the serum antibody (E-F) and L-FABP (G-H) over time in DBD and DCD kidney perfusions. Values in ng/ml are displayed as the change between two time points (Δvalue).



Figures 4.1A-H: Changes in NGAL (A-B), KIM-1 using the urinary antibody (C-D), KIM-1 using the serum antibody (E-F) and L-FABP (G-H) over time in DBD and DCD kidney perfusions. Values in ng/ml are displayed as the change between two time points (Δvalue).

4.1.3.1 NGAL and donor factors

Donor serum creatinine (75.8 ± 43.7 µmol/l), donor serum urea (7 ± 4.2 mmol/l) and calculated donor eGFR (101.5 ± 55.2 ml/min/1.73m^2) did not correlate with NGAL levels; neither absolute measured NGAL levels nor the delta values. The NGAL levels were comparable within the groups of perfused DBD (42.6 ± 49.6 ng/ml) and DCD (51.9 ± 36.4 ng/ml) kidneys; p=0.3. Donor age (60.2 ± 11.7 years) correlated negatively with the changes in NGAL levels (delta NGAL; 12.6 ± 16.9 ng/ml); p=0.003, correlation coefficient (Spearman's rho) = -0.573.

4.1.3.2 NGAL and perfusion characteristics

There were no differences in NGAL levels in kidney perfusions with short or long CIT; 50.5 ± 34.1 ng/ml long CIT vs. 44.7 ± 50.5 ng/ml short CIT, p=0.7. Levels of NGAL did not differ between DCD kidneys with WIT shorter or longer than 15 minutes; 48.4 ± 43 ng/ml vs. 55.9 ± 30.6, p=0.7.

The median NGAL values were significantly lower in the group of kidneys with a median flow more than 330 ml/min; 29.4 ± 6.2 ng/ml vs. 73.2 ± 15.9 ng/ml, p=0.0088. The same could be seen for the changes of NGAL over time. Delta NGAL values were significantly lower in the higher flow group; 5.9 ± 1.6 ng/ml vs. 21.9 ± 7.2 ng/ml, p=0.019.

The higher the total urine production was (1062.5 ± 2245.6 ml), the higher were the changes of NGAL, delta NGAL values, (12.59 ± 16.95 ng/ml) p=0.022, correlation coefficient (Spearman's rho) = 0.465.

4.1.3.3 KIM-1 and donor factors

Donor serum creatinine (75.8 ± 43.7 µmol/l), donor serum urea (7 ± 4.2 mmol/l) and calculated donor eGFR (101.5 ± 55.2 ml/min/1.73m^2) did not correlate with KIM-1 levels measured either with the urinary or the serum kit; neither absolute measured KIM-1 levels nor the delta values. The "urinary" (in ng/ml) and the "serum" (in pg/ml) KIM-1 levels were comparable within the groups of perfused DBD (1.5 ± 0.3 ng/m and 614.2 ± 99.7 pg/ml) and DCD (1.9 ± 0.6 ng/ml and 617 ± 89.3 pg/ml) kidneys; p=0.7 and 0.9. Donor age did not impact any of the KIM-1 levels.

4.1.3.4 KIM-1 and perfusion characteristics

"Serum" KIM-1 levels (615.5 ± 315.5 pg/ml) correlated significantly with CIT (30.7 ± 25.9 hours) p=0.024, correlation coefficient (Spearman's rho) = 0.47.

The lowest levels of "serum" KIM-1 were significantly higher in kidneys in the longer CIT group; 472.5 ± 84.8 pg/ml vs. 189.7 ± 55.3 pg/ml, p=0.0098. The changes of "serum" KIM-1 over time was comparable between the long and the short CIT group; delta "serum" KIM-1 160.3 ± 44.7 pg/ml vs. 137 ± 25.4 pg/ml, p=0.65.

The lowest "urinary" KIM-1 levels were significantly higher in the CIT ≥ 24 hours group; 1.22 ± 0.41 ng/ml vs. 0.4 ± 0.12 ng/ml, p=0.039. The same could be seen for the "serum" KIM-1 levels; 526.8 ± 92.5 pg/ml vs. 195.2 ± 48.9 pg/ml, p=0.0023. Interestingly, the change of "urinary" KIM-1 over time is significantly less in the group with a longer WIT; delta "urinary" KIM-1 0.22 ± 0.07 ng/ml vs. 1.04 ± 0.20 ng/ml, p=0.0016. Kidneys with more than the average total urine production of 505 ml had significantly lower "serum" KIM-1 levels; 215 ± 40.13 pg/ml vs. 444.8 ± 101.7 pg/ml, p=0.041. There was no association between median arterial flow and "urinary" or "serum" KIM-1 levels.

4.1.3.5 L-FABP and donor factors

In concordance with NGAL and KIM-1, donor serum creatinine (75.8 ± 43.7 µmol/l), donor serum urea (7 ± 4.2 mmol/l) and calculated donor eGFR (101.5 ± 55.2 ml/min/1.73m^2) did not correlate with L-FABP levels and were comparable between DBD (165.5 ± 74 ng/ml) and DCD (372.2 ± 96.3 ng/ml) kidneys; p=0.1. Donor age did not significantly correlate with L-FABP levels.

4.1.3.6 L-FABP and perfusion characteristics

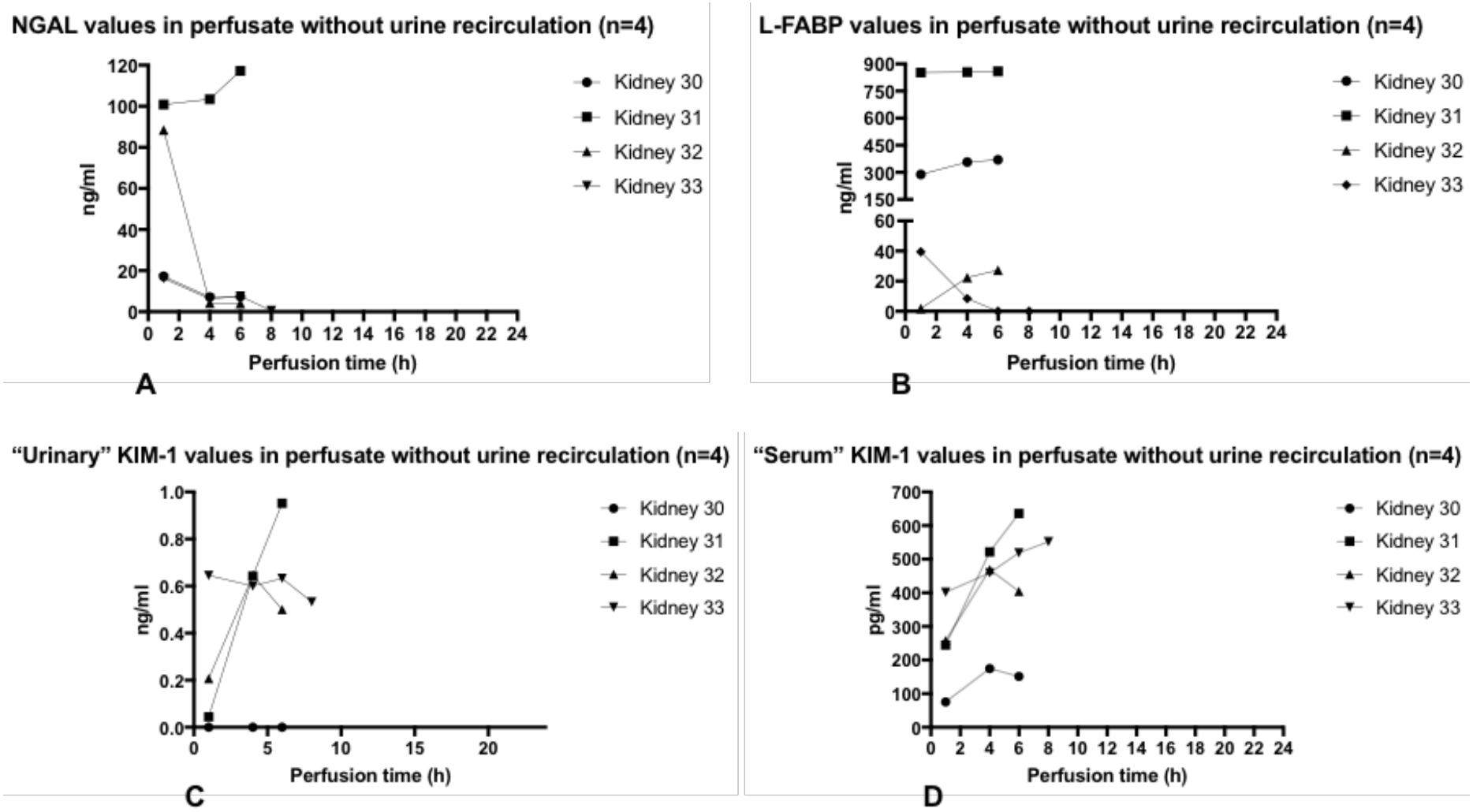
The duration of CIT was the only perfusion factor L-FABP levels were depending on significantly. The change of L-FABP over time was significantly different between the short and the long CIT group. The biomarker levels increased less in the shorter CIT group; delta L-FABP 42.1 ± 5.9 ng/ml vs. 147 ± 55.8 ng/ml, p=0.036. The maximum values of L-FABP were significantly higher in the CIT ≥ 24 hours group; 541 ± 149.8 ng/ml vs. 215.6 ± 62.31 ng/ml, p=0.036.

There were no associations between urine output, median arterial flow and L-FABP levels throughout the perfusions.

4.1.4 NGAL, KIM-1 and L-FABP levels in kidneys without urine recirculation

In view of the small case number of kidneys without urine recirculation (n=4), compared to the perfusates available for 24 kidneys with urine recirculation, I did not perform any statistical correlation analysis. In table 4.1, biomarker perfusate levels (first/last measurement and delta value) are displayed. Biomarker profiles, illustrating the absolute measured numbers, are shown in figures 4.2A-D.

It is worth noting that the absolute biomarker levels across the two groups, with and without urine recirculation, should not be directly compared, due to the replenishment of excreted urine with Ringer's lactate solution in one group and the difference in setup of the perfusion circuit; fully-closed vs. open.



Figures 4.2A-D: Changes in NGAL (A), L-FABP (B), KIM-1 using the urinary antibody (C), KIM-1 using the serum antibody (D) in kidney perfusions without urine recirculation. Values in ng/ml are displayed as the absolute number measured at the specific time point.

Table 4.1: Observed perfusate biomarker concentrations	First time point*	Last time point**	Delta	Mean ± SD****	NMP hours
NGAL in ng/mL with urine recirculation					
Kidney 2	15.647	19.226	3.580	18.69 ± 2.81	7
Kidney 3	6.697	8.256	1.559	7.54 ± 0.79	10
Kidney 4	7.621	25.751	18.129	14.10 ± 6.51	16
Kidney 5	12.627	28.159	15.532	24.28 ± 6.67	24
Kidney 6	31.986	31.582	-0.404	27.42 ± 5.05	12
Kidney 7	7.275	15.012	7.737	9.83 ± 3.51	18
Kidney 8	41.773	72.426	30.653	51.86 ± 16.10	24
Kidney 9	34.470	112.231	77.762	82.19 ± 36.37	24
Kidney 12	67.610	114.838	47.229	91.22 ± 33.40	15
Kidney 13	17.743	47.163	29.420	30.37 ± 15.15	24
Kidney 15	53.984	264.146	210.162	148.80 ± 83.45	24
Kidney 16	70.150	50.058	-20.092	57.38 ± 9.43	12
Kidney 17	18.418	105.254	86.836	51.05 ± 34.04	24
Kidney 21	28.429	56.620	28.191	35.86 ± 14.35	24
Kidney 22	32.026	43.402	11.376	33.33 ± 7.12	24
Kidney 23	8.608	55.746	47.138	29.62 ± 19.92	24
Kidney 24	28.265	37.899	9.634	34.45 ± 5.37	13
Kidney 25	67.061	93.766	26.705	92.47 ± 17.80	18
Kidney 27	19.018	43.008	23.989	31.01 ± 16.96	6
Kidney 28	60.207	59.819	-0.388	58.10 ± 3.33	12
Kidney 29	8.867	38.417	29.550	18.90 ± 13.51	24
Kidney 34	59.108	259.944	200.836	169.73 ± 80.50	24
Kidney 35	10.414	61.077	50.664	38.31 ± 19.20	24
Kidney 36	10.713	65.629	54.916	27.22 ± 26.20	24
NGAL in ng/mL without urine recirculation					
Kidney 30	17.273	7.444	-9.828	10.63 ± 5.75	6
Kidney 31	100.879	117.238	16.359	107.19 ± 8.79	6
Kidney 32	88.464	4.082	-84.382	32.25 ± 48.68	9
Kidney 33	16.238	0.590	-15.648	7.64 ± 6.47	8

continued Table 4.1:	First time point*	Last time point**	Delta	Mean ± SD****	NMP hours
KIM-1 in ng/mL with urine recirculation (urinary antibody)					
Kidney 2	0.125	0.150	0.024	0.11 ± 0.06	7
Kidney 3	<detection limit***	0.093	0.093	0.11 ± 0.02	10
Kidney 4	<detection limit***	0.089	0.089	0.06 ± 0.02	16
Kidney 5	0.727	3.033	2.305	2.27 ± 0.85	24
Kidney 6	1.618	2.342	0.724	2.17 ± 0.39	12
Kidney 7	0.371	1.576	1.204	1.04 ± 0.50	18
Kidney 8	0.941	4.971	4.029	2.95 ± 1.69	24
Kidney 9	1.319	1.834	0.515	1.53 ± 0.22	24
Kidney 12	0.456	1.983	1.527	1.22 ± 1.08	15
Kidney 13	1.342	2.418	1.076	1.91 ± 0.54	24
Kidney 15	1.235	2.744	1.509	1.74 ± 0.59	24
Kidney 16	<detection limit***	1.705	1.705	0.91 ± 0.77	12
Kidney 17	0.503	3.085	2.582	1.66 ± 1.11	24
Kidney 21	0.895	4.967	4.072	3.54 ± 2.04	24
Kidney 22	1.110	2.146	1.036	1.76 ± 0.42	24
Kidney 23	0.293	2.210	1.917	1.26 ± 0.82	24
Kidney 24	0.330	1.104	0.774	0.70 ± 0.39	13
Kidney 25	4.597	10.578	5.981	8.26 ± 2.60	18
Kidney 27	0.347	1.739	1.391	1.04 ± 0.98	6
Kidney 28	1.231	4.809	3.578	3.35 ± 1.88	12
Kidney 29	<detection limit***	1.987	1.987	1.03 ± 0.83	24
Kidney 34	0.079	0.583	0.503	0.30 ± 0.20	24
Kidney 35	<detection limit***	1.277	1.277	0.68 ± 0.51	24
Kidney 36	<detection limit***	3.321	3.321	1.62 ± 1.31	24
KIM-1 in ng/mL without urine recirculation (urinary antibody)					
Kidney 30	<detection limit***	<detection limit***	<detection limit***	n.a ± n.a.	6
Kidney 31	0.045	0.952	0.907	0.55 ± 0.46	6
Kidney 32	0.207	0.501	0.294	0.45 ± 0.22	9
Kidney 33	0.645	0.533	-0.112	0.60 ± 0.05	8

continued Table 4.1:	First time point*	Last time point**	Delta	Mean ± SD****	NMP hours
KIM-1 in pg/mL with urine recirculation (serum antibody)					
Kidney 3	96.280	261.460	165.180	203.91 ± 93.29	10
Kidney 4	29.330	177.420	148.090	103.11 ± 56.69	16
Kidney 5	61.600	673.230	611.630	488.26 ± 252.50	24
Kidney 6	593.790	1023.330	429.540	848.10 ± 184.01	12
Kidney 7	252.800	496.340	243.540	400.91 ± 107.72	18
Kidney 8	107.320	997.630	890.310	615.57 ± 370.21	24
Kidney 9	201.010	348.870	147.860	263.95 ± 55.20	24
Kidney 12	379.910	646.170	266.260	513.04 ± 188.27	15
Kidney 13	220.190	453.050	232.860	344.72 ± 117.27	24
Kidney 15	534.680	800.360	265.680	674.44 ± 116.38	24
Kidney 16	86.960	670.480	583.520	289.67 ± 261.43	12
Kidney 17	321.590	584.890	263.300	474.00 ± 112.48	24
Kidney 21	421.110	1360.220	939.110	1087.69 ± 395.22	24
Kidney 22	757.510	1047.430	289.920	902.52 ± 143.95	24
Kidney 23	305.534	1023.231	717.697	710.81 ± 313.86	24
Kidney 24	348.457	737.820	389.363	534.80 ± 195.22	13
Kidney 25	1132.676	1231.339	98.663	1197.52 ± 44.38	18
Kidney 27	379.582	932.095	552.513	655.84 ± 390.69	6
Kidney 28	731.107	1169.700	438.593	1015.03 ± 246.21	12
Kidney 29	170.457	947.962	777.505	632.44 ± 327.37	24
Kidney 34	211.753	438.780	227.027	319.86 ± 89.38	24
Kidney 35	73.880	698.750	624.870	503.56 ± 278.28	24
Kidney 36	55.580	1286.300	1230.720	763.14 ± 484.59	24
KIM-1 in pg/mL without urine recirculation (serum antibody)					
Kidney 30	75.252	151.538	76.286	133.84 ± 52.05	6
Kidney 31	244.505	635.902	391.397	467.40 ± 201.29	6
Kidney 32	258.338	404.400	146.062	377.34 ± 108.05	9
Kidney 33	401.552	552.293	150.741	483.03 ± 66.55	8

continued Table 4.1:	First time point*	Last time point**	Delta	Mean ± SD****	NMP hours
L-FABP in ng/mL with urine recirculation					
Kidney 2	279.831	388.527	108.696	364.29 ± 75.32	7
Kidney 3	21.459	65.937	44.478	53.86 ± 28.36	10
Kidney 4	221.110	328.057	106.947	291.57 ± 50.36	16
Kidney 5	47.235	248.146	200.911	151.20 ± 70.75	24
Kidney 6	134.653	329.556	194.903	252.22 ± 87.67	12
Kidney 7	95.422	181.380	85.957	146.58 ± 37.32	18
Kidney 8	7.257	107.006	99.749	54.86 ± 45.59	24
Kidney 9	432.014	493.669	61.655	472.18 ± 30.30	24
Kidney 12	736.604	1342.553	605.949	1039.58 ± 428.47	15
Kidney 13	369.259	456.755	87.496	422.54 ± 46.76	24
Kidney 15	361.790	668.887	307.097	491.93 ± 114.63	24
Kidney 16	150.395	170.385	19.990	171.76 ± 19.85	12
Kidney 17	924.510	1385.781	461.271	1137.77 ± 191.32	24
Kidney 21	124.462	280.359	155.898	208.77 ± 58.53	24
Kidney 22	128.821	441.897	313.077	278.82 ± 118.16	24
Kidney 23	18.971	<detection limit***	-18.970	8.94 ± 8.39	24
Kidney 24	14.992	<detection limit***	-14.990	5.00 ± 8.66	13
Kidney 25	47.906	7.035	-40.871	14.36 ± 22.55	18
Kidney 27	31.087	0.706	-30.382	15.90 ± 21.48	6
Kidney 28	862.786	855.552	-7.234	865.20 ± 11.05	12
Kidney 29	<detection limit***	59.661	59.661	26.68 ± 22.45	24
Kidney 34	70.150	56.044	-14.106	58.29 ± 11.31	24
Kidney 35	88.415	149.124	60.709	131.18 ± 24.68	24
Kidney 36	154.805	68.411	-86.394	46.73 ± 66.78	24
L-FABP in ng/mL without urine recirculation					
Kidney 30	289.151	369.445	80.294	338.58 ± 43.25	6
Kidney 31	852.478	859.350	6.872	855.67 ± 3.46	6
Kidney 32	1.791	27.290	25.499	17.16 ± 13.53	9
Kidney 33	39.587	<detection limit***	-39.587	12.02 ± 18.81	8

*1 hour after perfusion start, **end of perfusion (NMP hours), ***minimum detectable dose for assay ranged from 0.003-0.046 ng/ml (urinary KIM-1) and <6.25 ng/ml (L-FABP), ****time-averaged longitudinal mean value compiled from all measurements over the course of the perfusion; n.a. = not applicable

4.2 Proteinuria

Two mechanisms can lead to proteinuria: (i) abnormal transglomerular passage of proteins due to increased permeability of the glomerular capillary wall and (ii) the impaired reabsorption of protein by the epithelial cells of the proximal tubuli.

The analysis for proteinuria was carried out in the Biochemistry Laboratory at the John Radcliffe Hospital, Oxford University Hospitals, NHS Foundation Trust (2.5.4 Chapter 2).

4.2.1 Results from NMP in discarded human kidneys without urine recirculation

In the course of my research on discarded human kidneys, we analysed protein levels in urine samples of three kidneys undergoing NMP without recirculating the urine. Initial urine protein levels, measured within 30 minutes and one hour after perfusion start, were 23.300 mg/l in a 74-year old kidney from a donor after circulatory death (DCD) with 22 hours CIT; 17612 mg/l in a 78-year old kidney from a donor after brain death (DBD) with 18 hours CIT; 7335 mg/l in a 71-year old DBD kidney after 21 hours CIT. All three kidneys showed decreasing urine protein levels over six hours NMP. Urine protein levels after two hours were 5870 ± 3897 mg/l, after four hours 3209 ± 574.3 mg/l and after six hours 2710 ± 1002 mg/l.

These results raised the question if the decrease in proteinuria is really due to the effect of NMP without damaging the glomeruli and therefore a quality marker for the kidney or if the declining amount of protein is a consequence of the protein loss in the perfusate as a result of urine excretion in an open perfusion circuit and leaking glomeruli.

To investigate proteinuria further, we analysed urine protein levels in paired porcine kidney perfusions, with and without urine recirculation (6.4.9 Chapter 6).

4.3 Metabolomics

So far, metabolic processes occurring during normothermic preservation of the human kidney have not been studied and analysed yet. Metabolomics have been applied to analyse effects of ischaemia reperfusion injury, mainly in the experimental model (156). In clinical transplantation, examinations of the metabolomic profile of hypothermic machine perfusion perfusates have been carried out to identify discriminators between the profile of human cadaveric kidneys with initial and delayed graft function (157,158).

For my DPhil studies, I was particularly interested if we can detect changes of the metabolome during normothermic preservation of discarded human kidneys up to 24 hours. Furthermore, I was interested if we can investigate differences between kidney perfusions performed with either urine recirculation (a fully closed circuit) or replacement of the excreted urine (an open perfusion circuit). This interest is based on the observation that I was not able to perfuse discarded human kidneys without urine recirculation for longer than 9 hours, let alone to reach 24 hours of NMP (table 3.4, chapter 3, kidneys 30-33).

Perfusates of eight human kidney perfusions were analysed according to the metabolomics-protocol described under 2.5.7 Chapter 2. The omics-workflow is illustrated in figure 4.3, my own illustration based on the figure published by Huang *et al.* (119). Five kidneys with urine recirculation were analysed; perfusate samples taken after one, six, twelve and 24 hours of NMP. Three kidneys without urine recirculation were analysed; perfusate samples taken after one and six hours of perfusion.

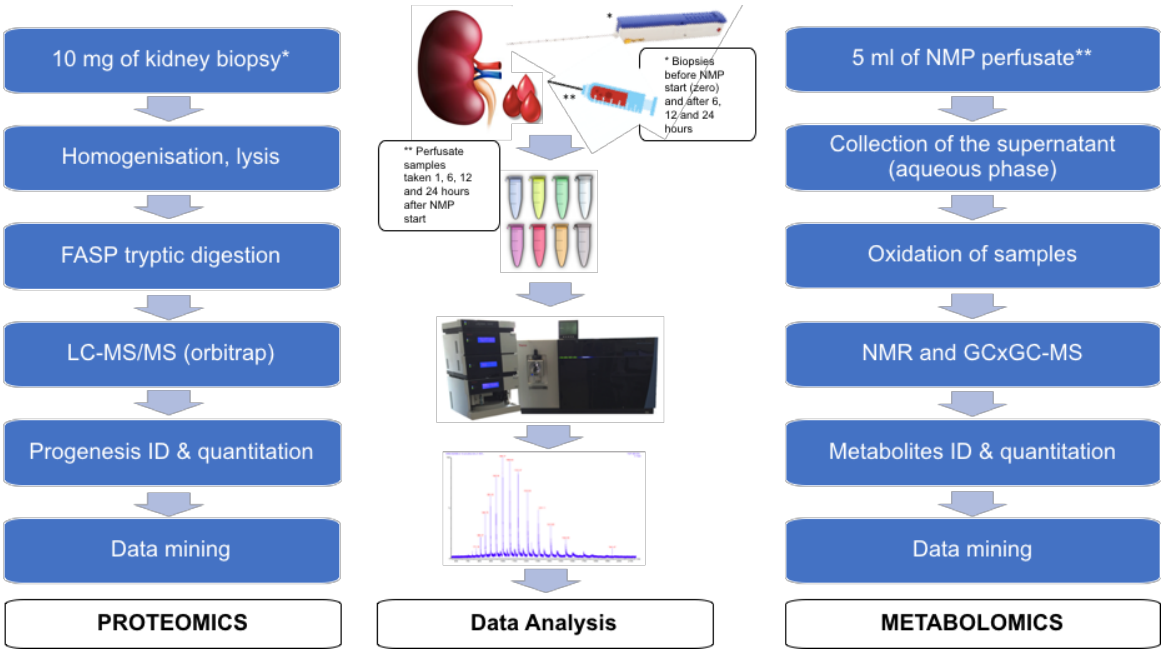


Figure 4.3 illustrates the workflow for metabolomics and proteomics from taking the biopsy or the perfusate sample to the state of data analysis.

4.3.1 Amino acids and sugars the main proportion of the perfusate metabolome

Overall, 68 metabolites comprising six metabolite groups were detected in 26 perfusate samples; 20 with urine recirculation and six without urine recirculation. Table 4.2 shows all metabolites (n=68) detected in all perfusate samples (n=26) assigned to their metabolite group: amino acid, organic acid, sugar, fatty acid, purine or carbamide. Amino acids (n=24) and sugars (n=18) stand out as the main involved metabolites. As a source of the metabolites we have to consider the human kidney, the blood based (red packed cells) perfusate and the supplements, especially added nutrition: TPN, (containing amino acids), and 5% glucose. Only three of the analysed kidneys (with urine recirculation) received TPN during NMP.

Table 4.2: Metabolites detected in 26 perfusate samples of normothermic kidney preservation*

Metabolite group	Name of metabolite
Amino acid	Alanine, phenyl-, trimethylsilyl ester
Amino acid	L-Isoleucine-2TMS
Amino acid	L-Lysine-4TMS
Amino acid	L-Phenylalanine-2TMS
Amino acid	L-Proline, 5-oxo-1-(trimethylsilyl)-, trimethylsilyl ester
Amino acid	L-Serine-3TMS
Amino acid	L-Threonine-3TMS
Amino acid	L-Tryptophan, N,1-bis(trimethylsilyl)-, trimethylsilyl ester
Amino acid	L-Methionine-2TMS
Amino acid	L-Ornithine, N2,N5,N5-tris(trimethylsilyl)-, trimethylsilyl ester
Amino acid	Tryptophan-2TMS
Amino acid	Tyrosine, O-trimethylsilyl-, trimethylsilyl ester
Amino acid	Valine-2TMS
Amino acid	l-Isoleucine, trimethylsilyl ester
Amino acid	l-Leucine, trimethylsilyl ester
Amino acid	l-Methionine, trimethylsilyl ester
Amino acid	l-Proline, trimethylsilyl ester
Amino acid	l-Threonine, O-(trimethylsilyl)-, trimethylsilyl ester
Amino acid	l-Tryptophan, trimethylsilyl ester
Amino acid	N-Acetyltyrosine, di-TMS
Amino acid	N,O-Bis-(trimethylsilyl)alanine
Amino acid	L-Aspartic acid-3TMS
Amino acid	Glutamic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester, L-
Amino acid	Glycine, N,N-bis(trimethylsilyl)-, trimethylsilyl ester
Organic acid	2,3,4-Trihydroxybutyric acid tetrakis(trimethylsilyl) derivative
Organic acid	2-Hydroxybutyric acid-2TMS ; Butanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester
Organic acid	3-Hydroxybutyric acid-2TMS
Organic acid	Butanedioic acid, bis(trimethylsilyl) ester
Organic acid	D-Gluconic acid, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, trimethylsilyl ester
Organic acid	Decanoic acid, 1,2,3-propanetriyl ester

* detected in 5 kidney perfusions with urine recirculation (n=20 samples) and in 3 kidney perfusions without urine recirculation (n=6 samples)

continued Table 4.2:

Metabolite group	Name of metabolite
Organic acid	Dodecanoic acid, trimethylsilyl ester
Organic acid	Indol-3-acetic acid-2TMS ; 1H-Indole-3-acetic acid, 1-(trimethylsilyl)-, trimethylsilyl ester
Organic acid	L-(+)-Lactic acid, trimethylsilyl ether, trimethylsilyl ester
Organic acid	Citric acid
Organic acid	Phosphoric acid-3TMS ; Phosphoric acid, tris(trimethylsilyl) ester
Organic acid	Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester
Organic acid	Acetic acid, [(trimethylsilyl)oxy]-, trimethylsilyl ester
Organic acid	2-Pyrrolidone-5-carboxylic acid, trimethylsilyl ester
Sugar	alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)
Sugar	beta.-D-Allopyranose, pentakis(trimethylsilyl) ether
Sugar	beta.-D-Galactofuranose, 1,2,3,5,6-pentakis-O-(trimethylsilyl)
Sugar	1,5-Anhydro-D-sorbitol, tetrakis(trimethylsilyl) ether
Sugar	2-.alpha.-Mannobiose, octakis(trimethylsilyl) ether (isomer 1)
Sugar	Arabinitol, pentakis-O-(trimethylsilyl)-
Sugar	D-(+)-Arabitol, pentakis(trimethylsilyl) ether
Sugar	D-(+)-Cellobiose, octakis(trimethylsilyl) ether (isomer 1)
Sugar	D-(+)-Cellobiose, octakis(trimethylsilyl) ether, methyloxime (isomer 1)
Sugar	D-(+)-Cellobiose, octakis(trimethylsilyl) ether, methyloxime (isomer 2)
Sugar	D-(-)-Erythrose, tris(trimethylsilyl) ether, methyloxime (syn)
Sugar	D-Glucose-5TMS-Methyloxime
Sugar	D-Lactose, octakis(trimethylsilyl) ether, methyloxime (isomer 2)
Sugar	D-Psicofuranose, pentakis(trimethylsilyl) ether (isomer 2)
Sugar	Glycerol tricaprylate
Sugar	Glycerol, tris(trimethylsilyl) ether
Sugar	Maltose, octakis(trimethylsilyl) ether, methyloxime (isomer 2)
Sugar	meso-Erythritol, tetrakis(trimethylsilyl) ether

* detected in 5 kidney perfusions with urine recirculation (n=20 samples) and in 3 kidney perfusions without urine recirculation (n=6 samples)

continued Table 4.2:

Metabolite group	Name of metabolite
Fatty acid	Myristic acid-D3-TMS
Fatty acid	Oleic acid, trimethylsilyl ester
Fatty acid	Palmitic acid-TMS
Fatty acid	Stearic acid-TMS
Fatty acid	Pentanedioic acid, 2-(methoxyimino)-, bis(trimethylsilyl) ester
Fatty acid	Pentanoic acid, 4-methyl-2-oxo-, bis(trimethylsilyl) deriv.
Fatty acid	n-Tridecanoic acid, trimethylsilyl ester
Fatty acid	Cholesterol trimethylsilyl ether
Fatty acid	2-Monopalmitoylglycerol trimethylsilyl ether
Purine	7-(Trimethylsilyl)-2,6-bis[(trimethylsilyl)oxy]-7H-purine
Purine	9H-Purine, 9-(trimethylsilyl)-6-[(trimethylsilyl)oxy]-
Carbamide	Urea-2TMS ; Urea, N,N'-bis(trimethylsilyl)

* detected in 5 kidney perfusions with urine recirculation (n=20 samples) and in 3 kidney perfusions without urine recirculation (n=6 samples)

For visualisation purposes, figures 4.4A-C display examples for the graphical illustration of metabolites and their detection over time in perfusate samples of kidney 36 (76-year old DBD, 15 hours CIT, with urine recirculation). Information for metabolite description was gathered from the human metabolome database http://www.hmdb.ca/metabolites (159).

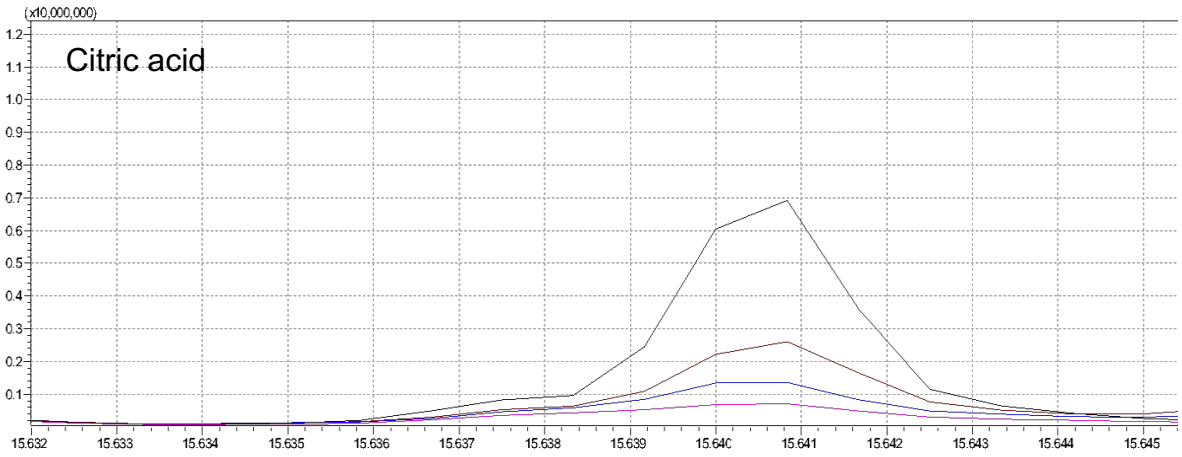


Figure 4.4A shows the profile of the metabolite citric acid in kidney 36 over time. The black line represents hour 1, pink hour 6, blue hour 12 and brown hour 24. Citric acid is a weak acid and produced in the tricarboxylic acid cycle (Krebs cycle). Citrate is formed by the condensation of oxaloacetate with acetyl CoA catalysed by citrate synthase. Oxidation of acetyl CoA is the “heart” of the Krebs cycle to release stored energy in an aerobic organism (160).

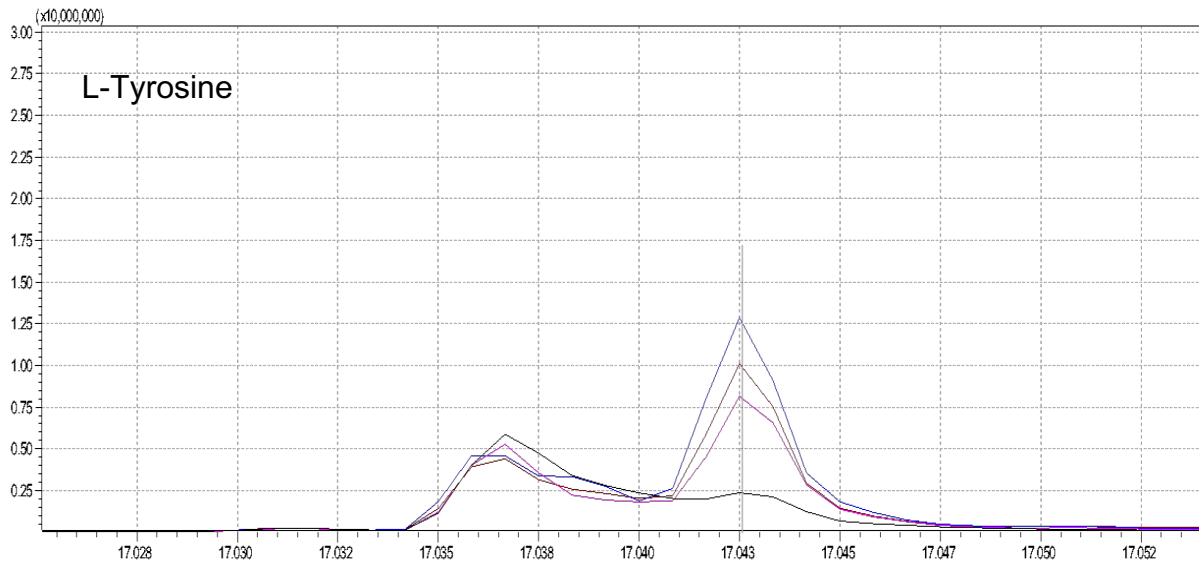


Figure 4.4B illustrates the profile of L-tyrosine over time in perfusate samples of kidney 36 (black 1 hour, pink 6 hours, blue 12 hours and brown 24 hours). L-tyrosine is an essential amino acid. The kidney plays a major role in the uptake of phenylalanine and can convert phenylalanine to tyrosine by hydroxylation (161). This conversion is significantly impaired in chronic kidney failure (162). It needs to be noted that kidney 36 did not receive TPN which would contain amino acids, it received solely 5% glucose for nutrition.

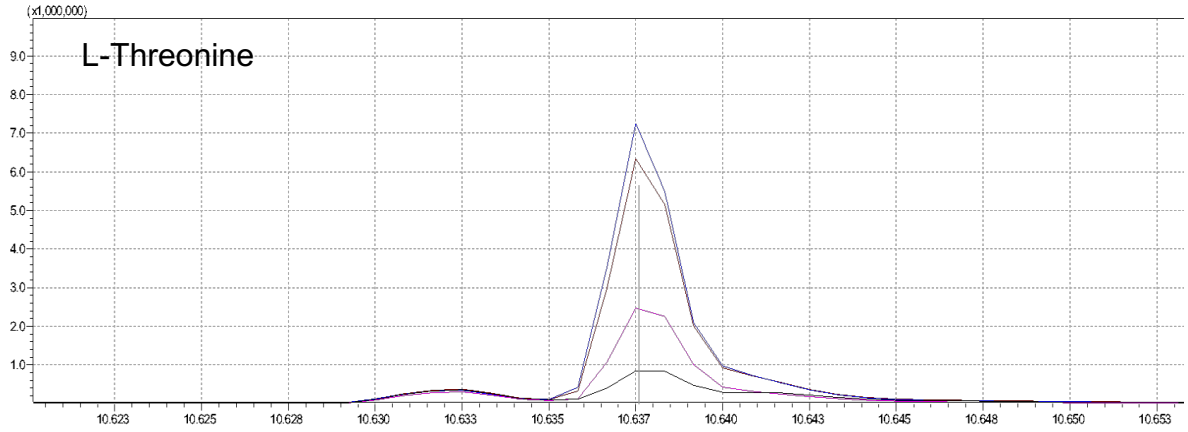


Figure 4.4C shows the profile of the essential amino acid L-threonine in perfusate samples of kidney 36 (black 1 hour, pink 6 hours, blue 12 hours and brown 24 hours). The literature for L-threonine regarding renal metabolism is scarce. It is published that in fasted humans, the kidney can release threonine (163). L-threonine could be from red blood cells as the perfusate is based on one unit of packed red cells. From research on stored blood and its metabolomic profile it is known that there is a progressive increase for the majority of amino acids, including threonine and tyrosine, especially after day 14 of storage (164).

4.3.2 Metabolomic profile in perfusions with and without urine recirculation

For each metabolite, four technical replicates were available per time point and per sample. These replicates were averaged as a first step. To start the comparison between perfusates with and without urine recirculation, I compared the metabolite abundance between the different timepoints in both groups as a second step; with and without urine recirculation. In a third step, we compared differences in metabolites between one-hour perfusate samples of both groups and six-hour perfusate samples of perfusions with and without urine recirculation. The metabolites which we discovered to be significantly changed are described in the following paragraphs.

4.3.2.1 Comparison of time points in kidneys with urine recirculation

As longitudinal measurements are one of the advantages of long-term normothermic perfusion, I wanted to analyse if the metabolite-profile changes significantly over time during perfusing a discarded human kidney. To gain a potential difference between the perfusion time points, statistical analysis (Student t-test) was applied to the averaged values of the metabolite replicates. A comparison of hours six, twelve and 24 after perfusion start with the metabolite results after the first hour of NMP was carried out. The comparison is illustrated as a volcano plot (x-axis data in log2 format) and significantly increased/decreased metabolites are colour-coded red/blue.

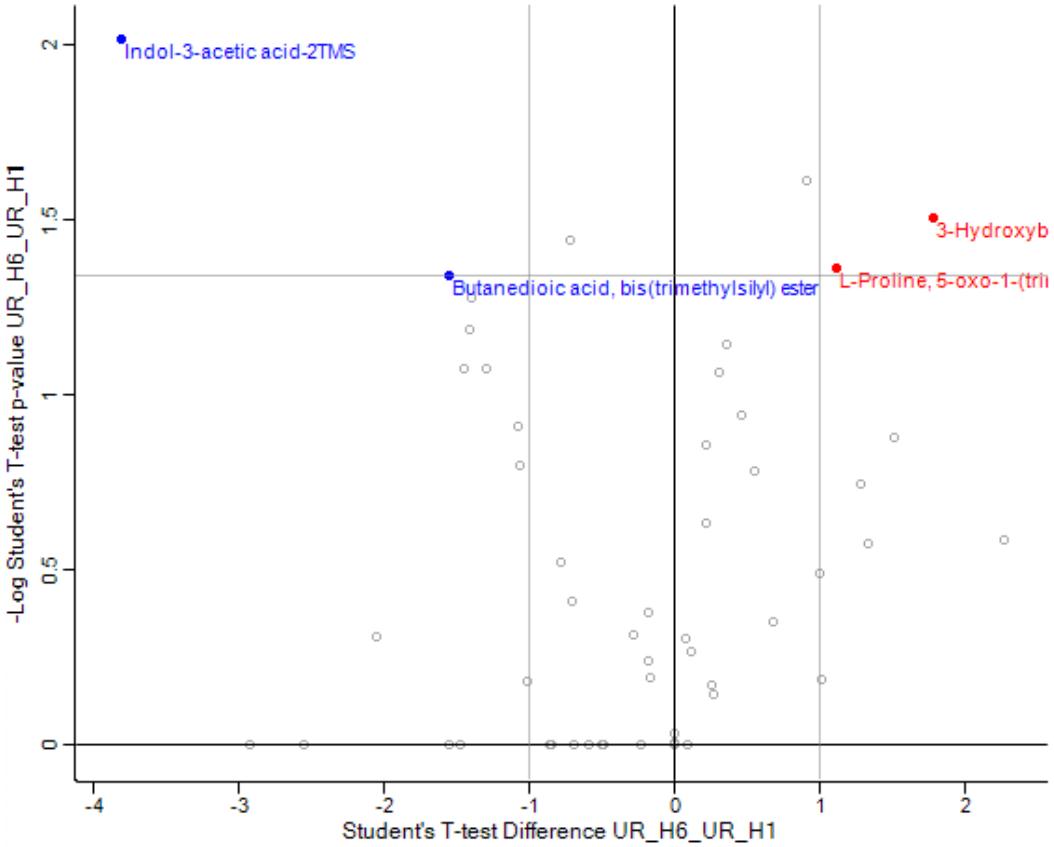


Figure 4.5A displays the volcano plot with the significantly ($p<0.050$) increased (in red) and decreased (in blue) metabolites after six hours of NMP.

The significantly increased metabolites are an organic acid and an amino acid: 3-Hydroxybutyric acid-2TMS is a keton body providing acetoacetyl-CoA and acetyl-CoA for synthesis of cholesterol, fatty acids, and complex lipids; L-Proline, 5-oxo-1-(trimethylsilyl)-, trimethylsilyl ester (Pyroglutamic acid) is an uncommon amino acid derivative in which the free amino group of glutamic acid cyclizes to form a lactam. It is formed nonenzymatically from glutamate, glutamine and gamma-glutamylated peptides, but it can also be produced by the action of gamma-glutamylcyclotransferase (165).

The significantly decreased metabolites at hour six are organic acids only: Butanedioic acid, bis(trimethylsilyl) ester (Succinic acid) is a dicarboxylic acid. The anion, succinate, is a component of the citric acid or Krebs cycle and is capable of donating electrons to the electron transfer chain; Indol-3-acetic acid-2TMS;1H-Indole-3-acetic acid, 1-(trimethylsilyl)-, trimethylsilyl ester (Indoleacetic acid) is a breakdown product of tryptophan metabolism (166).

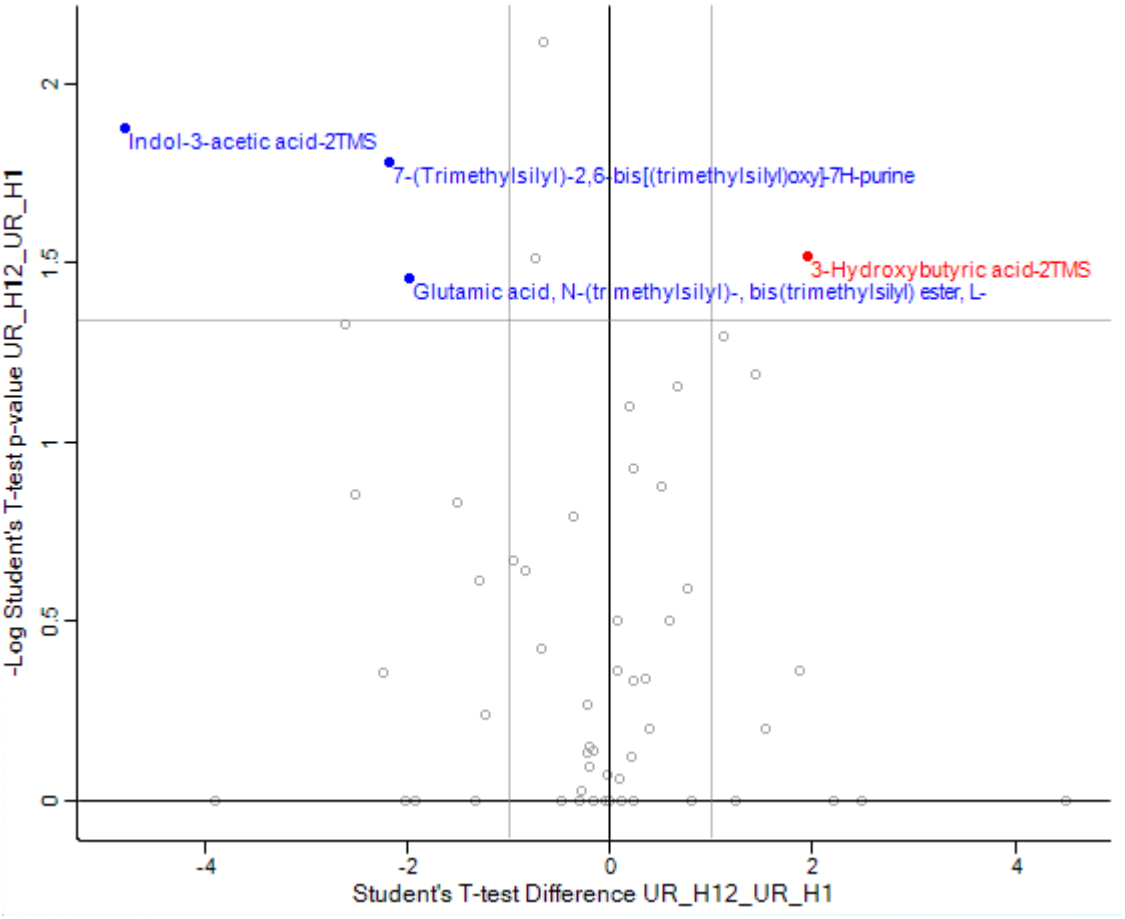


Figure 4.5B displays the volcano plot with the significantly ($p<0.050$) increased (in red) and decreased (in blue) metabolites after twelve hours of NMP.

The significantly more abundant metabolite is the organic acid 3-Hydroxybutyric acid-2TMS, which has been higher since hour six.

After twelve hours of NMP with urine recirculation, the organic acid Indoleacetic acid is still significantly less abundant (as at hour six already); furthermore, the purine 7-(Trimethylsilyl)-2,6-bis[(trimethylsilyl)oxy]-7H-purine and the amino acid Glutamic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester, L-(Glutamic acid), a non-essential amino acid, naturally occurring in the L-form, are significantly less abundant.

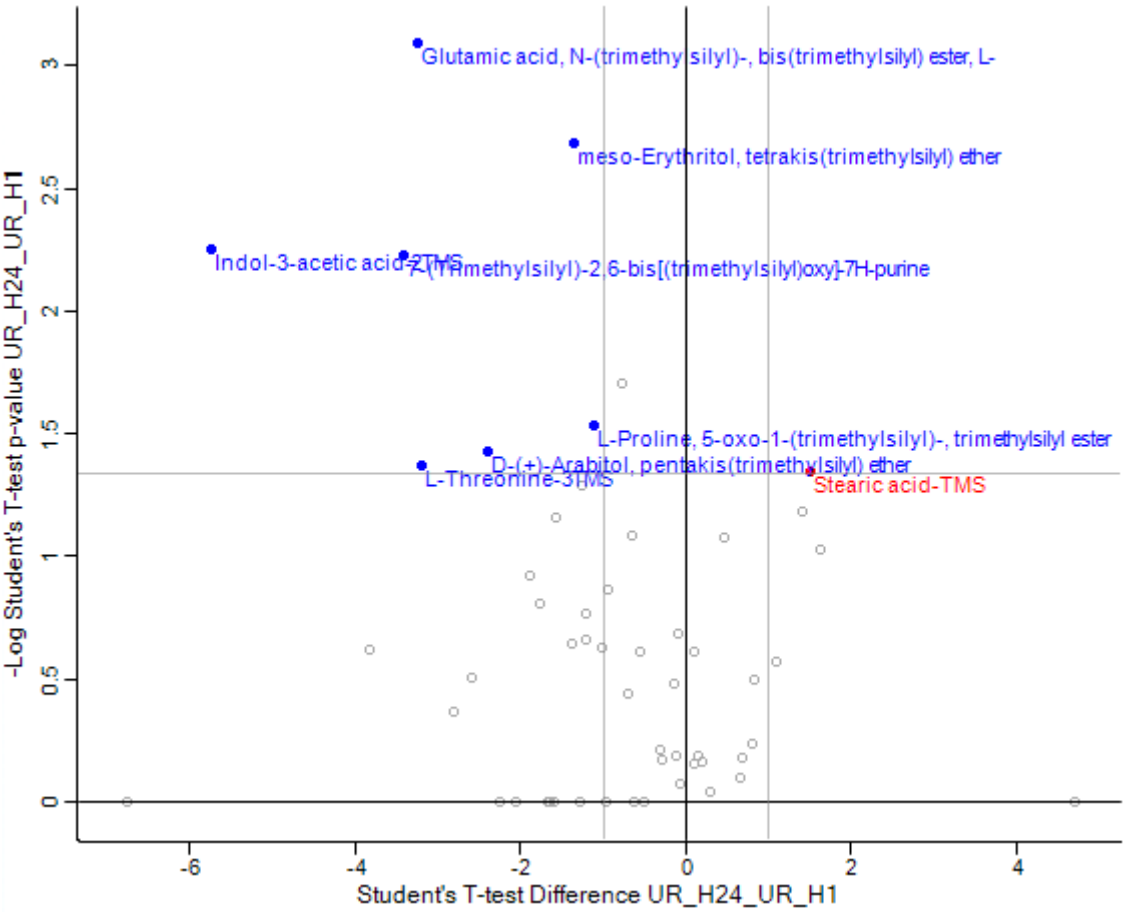


Figure 4.5C displays the volcano plot with the significantly ($p<0.050$) upregulated (in red) and downregulated (in blue) metabolites after 24 hours of NMP.

After 24 hours of NMP the fatty acid Stearic acid-TMS is significantly more abundant. It is a useful type of saturated fatty acids; it is produced from carbohydrates via the fatty acid synthesis machinery wherein acetyl-CoA contributes two carbon building blocks (167). In addition to the metabolites less abundant at hour twelve, sugars and amino acids are significantly less abundant after 24 hours of NMP. The sugars are D-(+)-Arabitol, and meso-Erythritol (Erythritol). Erythritol is a four-carbon sugar, twice as sweet as sucrose and can be used as a coronary vasodilator. The essential amino acid L-Threonine-3TMS, occurring naturally in the active L-form, is significantly less abundant as well.

4.3.2.2 No metabolite changes over the course of NMP without urine recirculation

None of the measured metabolites were significantly more or less abundant at any timepoint in the kidneys without urine recirculation.

4.3.2.3 More urea after one hour of NMP in perfusates with urine recirculation

After one hour of NMP, three metabolites were significantly more abundant in the group with urine recirculation compared to the one without (figure 4.5D). None of the metabolites were significantly decreased. The significantly increased metabolites were the sugar 2.alpha.-Mannobiose, the amino acid Glutamic acid and the carbamide Urea-2TM. The latter is not very surprising, as urea would be excreted via urine. The amount of urea "coming with" the kidney stayed about the same (1-4mg/dl) throughout the perfusion in a completely closed circuit.

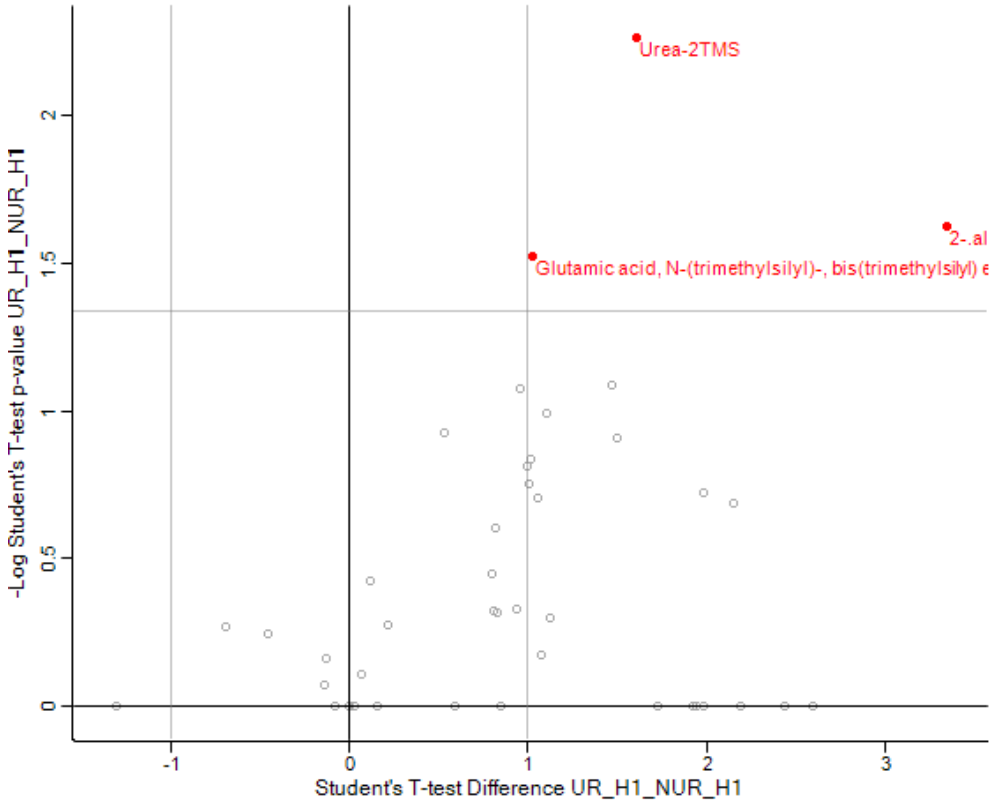


Figure 4.5D displays the volcano plot with the significantly ($p<0.050$) more abundant (in red) metabolites after one hour NMP with urine recirculation compared to perfusates of NMP with urine replacement.

4.3.2.4 Sugars represent the difference between open and closed circuit after 6 hours

Two metabolites are significantly increased in the group with urine recirculation after six hours of NMP compared to the group without. Figure 4.5E shows the significant metabolites in the volcano plot. The significantly more abundant metabolites in the group with urine recirculation were two sugars, meso-Erythritol and D-Glucose-5TMS-methyloxime.

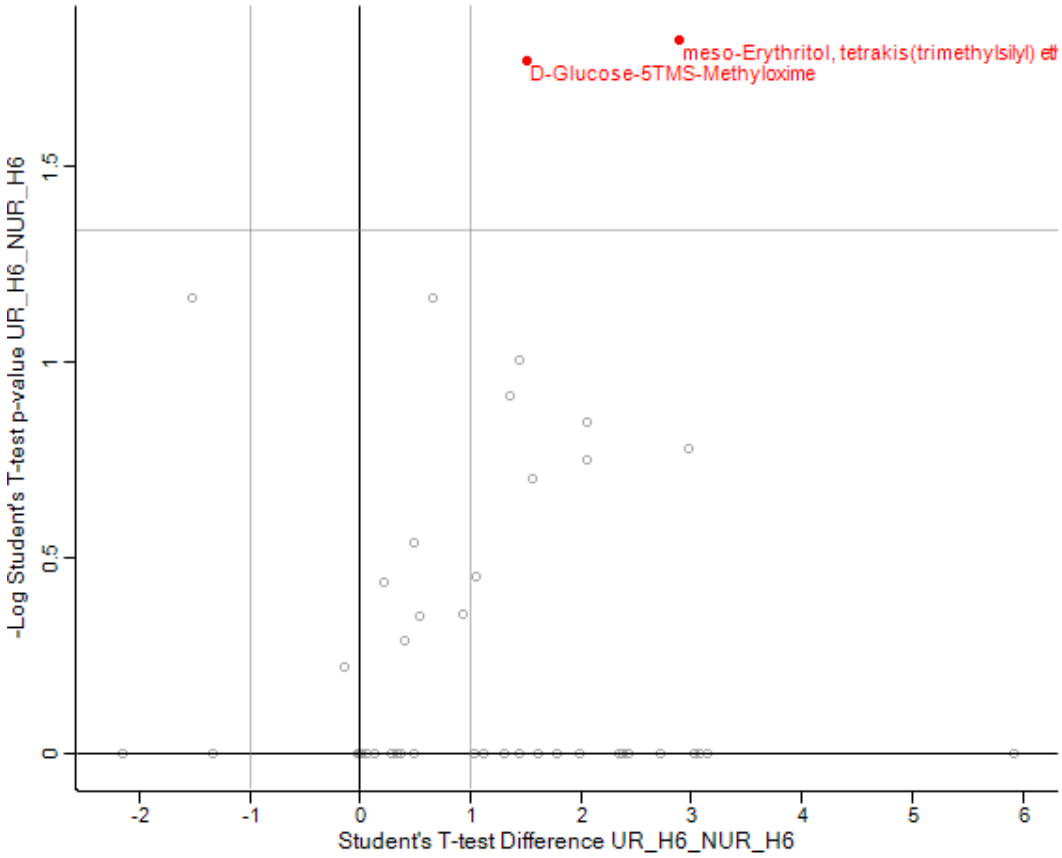


Figure 4.5E displays the volcano plot with the significantly ($p<0.050$) more abundant (in red) metabolites after six hours NMP with urine recirculation compared to perfusates of NMP with urine replacement.

At present it is unclear, if these significantly differential abundant metabolites are responsible for the deterioration of kidneys perfused without urine recirculation or if they contribute to it at all. The perfusion components are very heterogenous, both the discarded human kidneys and the expired red packed cells, and it is therefore not possible to draw a strong conclusion.

4.4 Proteomics reveals molecular window of kidney allografts

The proteome in higher organisms, as the homo sapiens, is very complex and dynamic. Certain proteins are only expressed in a specific biological context and others are modified post-translationally as a result of an event. The complete proteome in humans consists of 73.112 proteins, 20.373 reviewed and 52.739 unreviewed ones; Proteome ID UP000005640, 9606 – Homo sapiens, UniProt, May 2018 (168).

Proteomic profiling by mass spectrometry can rapidly identify significant changes to proteins in tissues and has been widely used in medical research and in the field of transplantation with great promise (169,170). The purpose of applying proteomics to tissue samples was to identify biomarkers of donor organ quality, and in our specific study to determine differences between and/or to detect common features of normothermically perfused discarded human kidneys. We aimed to look for a "signature proteomic profile" indicating kidneys which are biologically distinct from others. Furthermore, it was of particular interest to compare kidney tissue from perfusions with urine recirculation with biopsies taken from kidneys with urine replacement.

4.4.1 Tissue analysed to generate proteome profiles

Overall, biopsies from 19 kidneys were prepared for proteomics. Protein was extracted from 16 kidneys with urine recirculation and from three kidneys without. For logistical reasons, not all of the kidney biopsies taken during the perfusion (2.6.3 Chapter 2) underwent the preparation for the protein-analysis. The omics-workflow is illustrated in figure 4.3.

We wanted to have at least three timepoints per kidney to study upregulation and downregulation of proteins over time. For each kidney analysed with urine recirculation

(n=16, eight DBD and eight DCD kidneys, at least twelve hours of NMP), the zero biopsy and tissue taken after six, twelve or 24 hours (where available) underwent protein extraction (2.6.3.1 Chapter 2). Biopsies taken before perfusion start, after one and after six hours of NMP were processed from three kidneys perfused in the setting without urine recirculation. Table 4.3 shows the kidneys having undergone proteomics analysis.

4.4.2 Protein selection – the pathway to the NMP kidney proteome

The proteins were identified and quantified by mass spectrometry (MS). Quantitative MS data were uploaded to Perseus (171) for data visualisation and statistical analysis (2.6.3.3 Chapter 2). Two-fold up- or downregulated proteins were selected. The threshold level of ± 2-fold change, between the zero-biopsy and the tissue samples taken at the two later timepoints was set and subjected to statistical analysis using ANOVA. Proteins were determined to be significantly up- or downregulated when p value was <0.050. Figure 4.6 explains how I achieved to get significant different proteins for further analyses.

Table 4.3: Kidneys undergoing proteomics after normothermic perfusion

Kidneys with urine recirculation					Kidneys without urine recirculation				
Overall (n=16)	**DBD (n=8)**	**DCD (n=8)**	**CIT > 24 hours**	**> median arterial flow**	**Overall (n=3)**	**DBD (n=2)**	**DCD (n=1)**	**CIT > 24 hours**	**> median arterial flow**
Kidney 4		X		X	Kidney 30		X		X
Kidney 5	X			X	Kidney 32	X			
Kidney 6		X	X	X	Kidney 33	X			X
Kidney 7	X			X					
Kidney 8	X								
Kidney 9		X							
Kidney 12		X	X						
Kidney 13		X	X						
Kidney 15		X	X						
Kidney 17		X	X	X					
Kidney 20	X								
Kidney 21		X	X	X					
Kidney 22	X		X	X					
Kidney 29	X			X					
Kidney 34	X								
Kidney 36	X			X					

DBD = donation after brain death

DCD = donation after circulatory death

CIT = cold ischaemia time

median arterial flow = 330 ml/min in all kidneys perfused with urine recirculation (n=32)

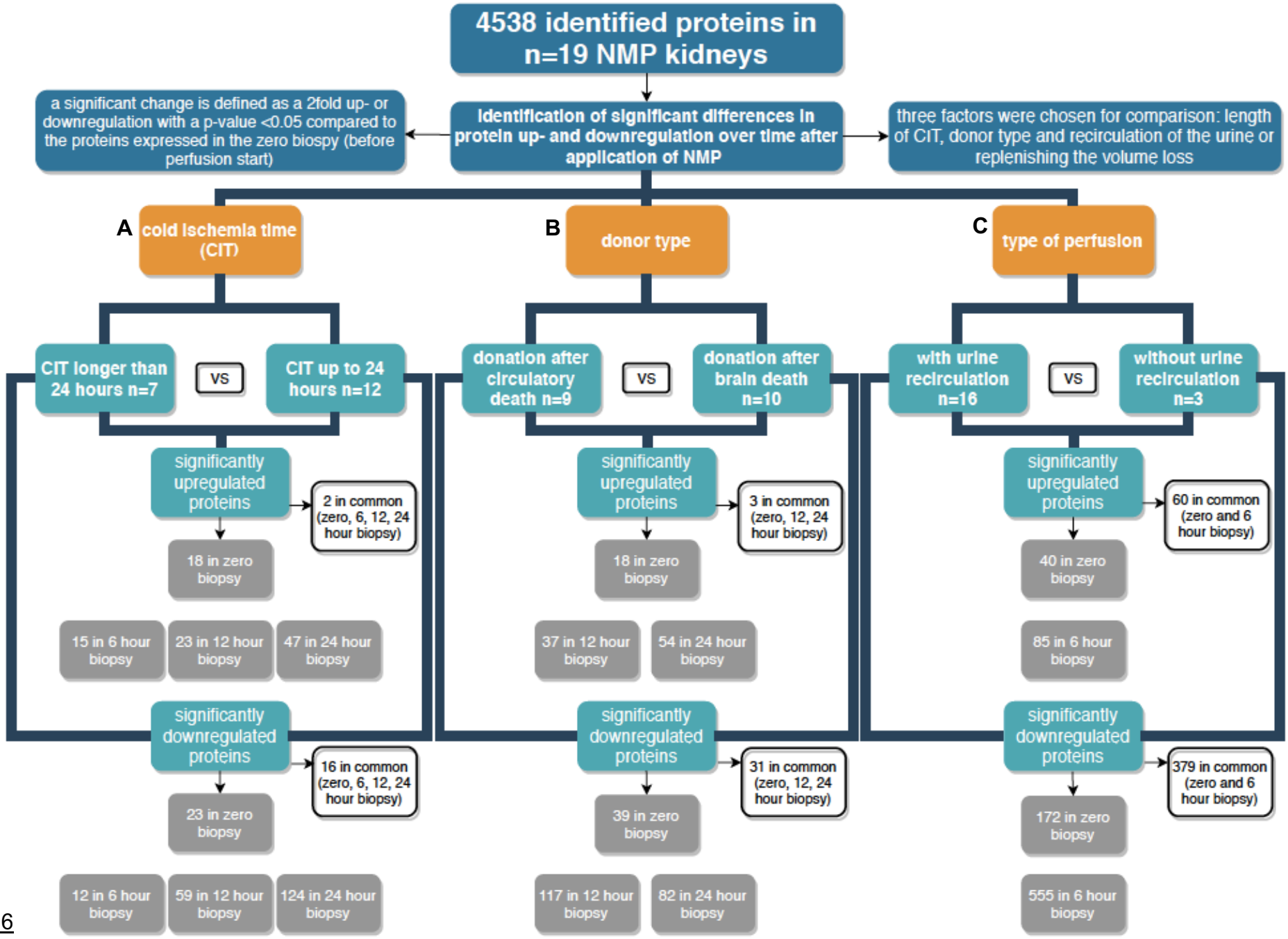
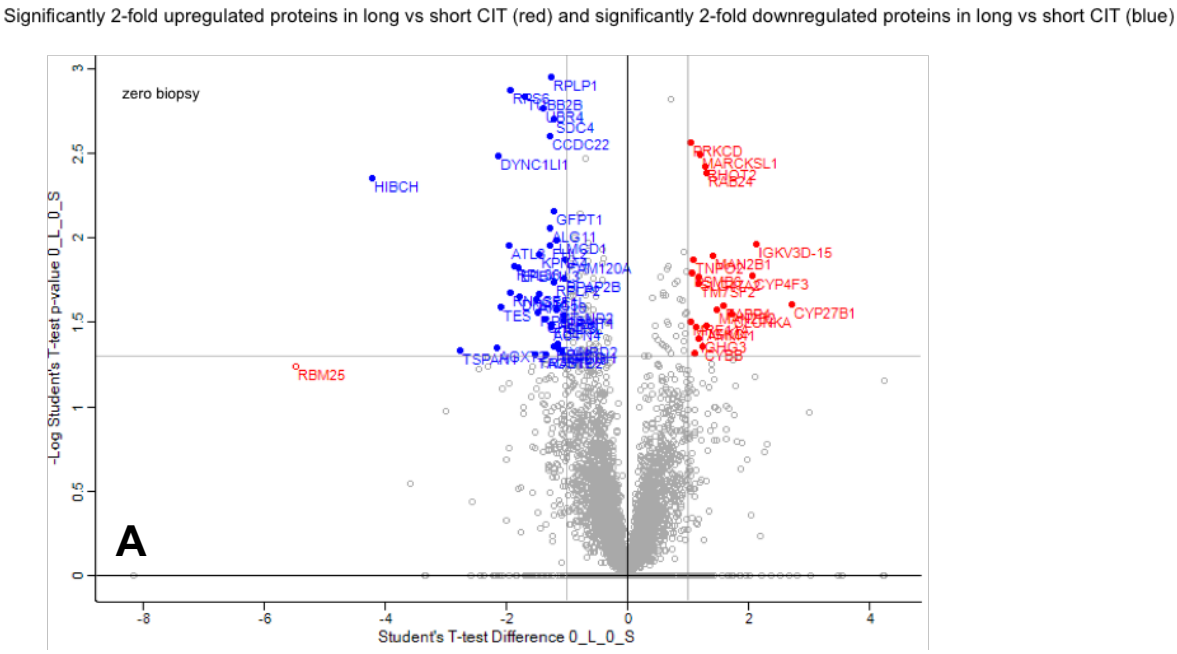
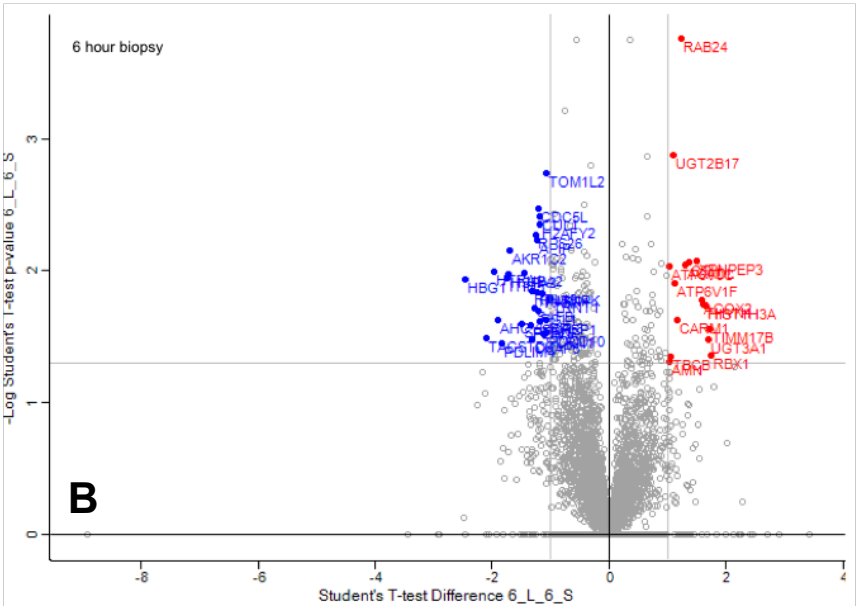

4538 identified proteins in n=19 NMP kidneys
a significant change is defined as a 2fold up- or downregulation with a p-value <0.05 compared to the proteins expressed in the zero biospy (before perfusion start)
Identification of significant differences in protein up- and downregulation over time after application of NMP
three factors were chosen for comparison: length of CIT, donor type and recirculation of the urine or replenishing the volume loss
A
cold ischemia time (CIT)
B
donor type
C
type of perfusion
CIT longer than 24 hours n=7
VS
CIT up to 24 hours n=12
donation after circulatory death n=9
VS
donation after brain death n=10
with urine recirculation n=16
VS
without urine recirculation n=3
significantly upregulated proteins
2 in common (zero, 6, 12, 24 hour biopsy)
18 in zero biopsy
15 in 6 hour biopsy
23 in 12 hour biopsy
47 in 24 hour biopsy
significantly upregulated proteins
3 in common (zero, 12, 24 hour biopsy)
18 in zero biopsy
37 in 12 hour biopsy
54 in 24 hour biopsy
significantly upregulated proteins
60 in common (zero and 6 hour biopsy)
40 in zero biopsy
85 in 6 hour biopsy
significantly downregulated proteins
16 in common (zero, 6, 12, 24 hour biopsy)
23 in zero biopsy
12 in 6 hour biopsy
59 in 12 hour biopsy
124 in 24 hour biopsy
significantly downregulated proteins
31 in common (zero, 12, 24 hour biopsy)
39 in zero biopsy
117 in 12 hour biopsy
82 in 24 hour biopsy
significantly downregulated proteins
379 in common (zero and 6 hour biopsy)
172 in zero biopsy
555 in 6 hour biopsy

Figure 4.6

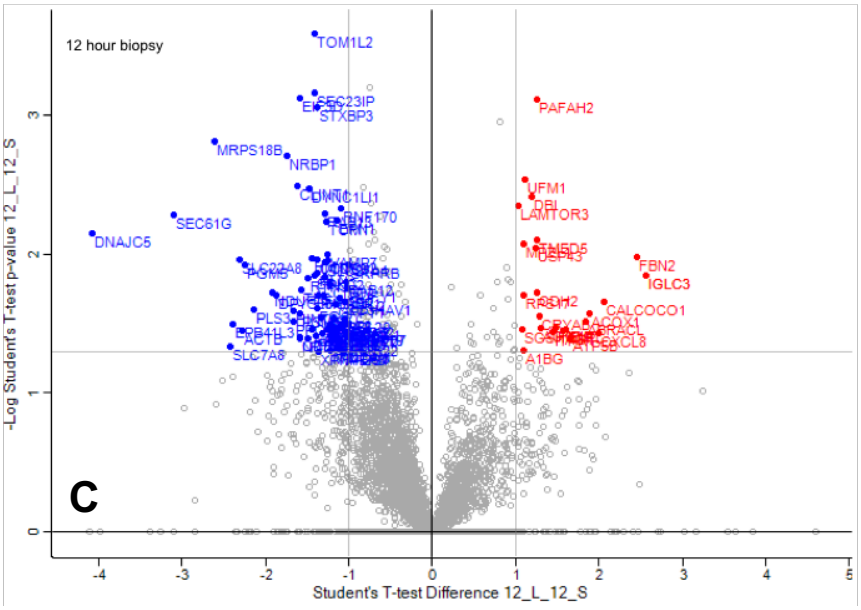
The proteome of my analysed NMP kidneys, with and without urine recirculation, consists of 4538 up- and downregulated proteins. To learn more about the perfused kidneys and to work towards a clinical application for proteomics regarding kidney NMP, I analysed and compared the proteins in three, what I found clinically sensible groups; the path is shown in figure 4.6. The first group was a comparison between protein up- and downregulation in kidneys with a CIT up to and longer than 24 hours as CIT is a well-established risk factor for the outcome after solid organ transplantation; section A in figure 4.6. There were significant differences in both directions, up and downregulation of proteins, over 24 hours of NMP. For each biopsy-time-point a Student t-test was performed between the long and short CIT group in comparison to the zero biopsy. Some proteins were consistently up- or downregulated throughout the duration of NMP and are described as “in common” in figure 4.6. The best way to visualize different protein expressions in two groups is to produce a volcano plot (x-axis data in all displayed volcano plots in log2 format).

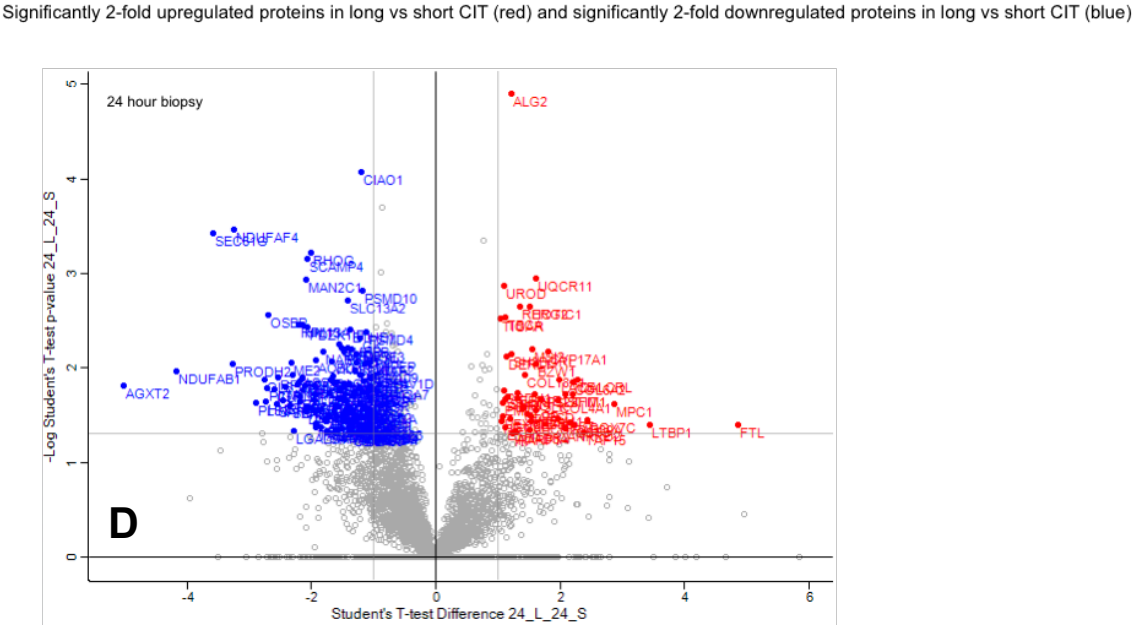


Significantly 2-fold upregulated proteins in long vs short CIT (red) and significantly 2-fold downregulated proteins in long vs short CIT (blue)



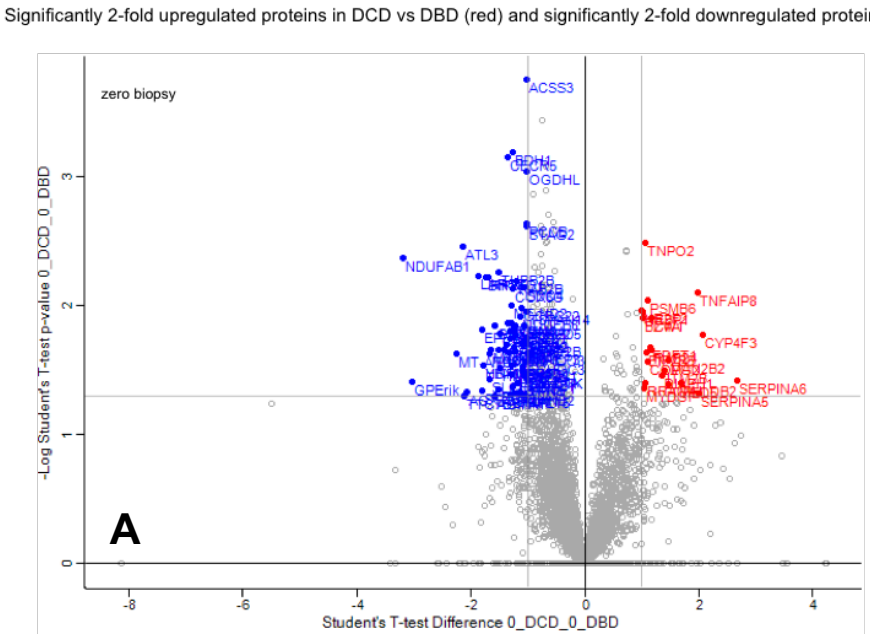
Significantly 2-fold upregulated proteins in long vs short CIT (red) and significantly 2-fold downregulated proteins in long vs short CIT (blue)

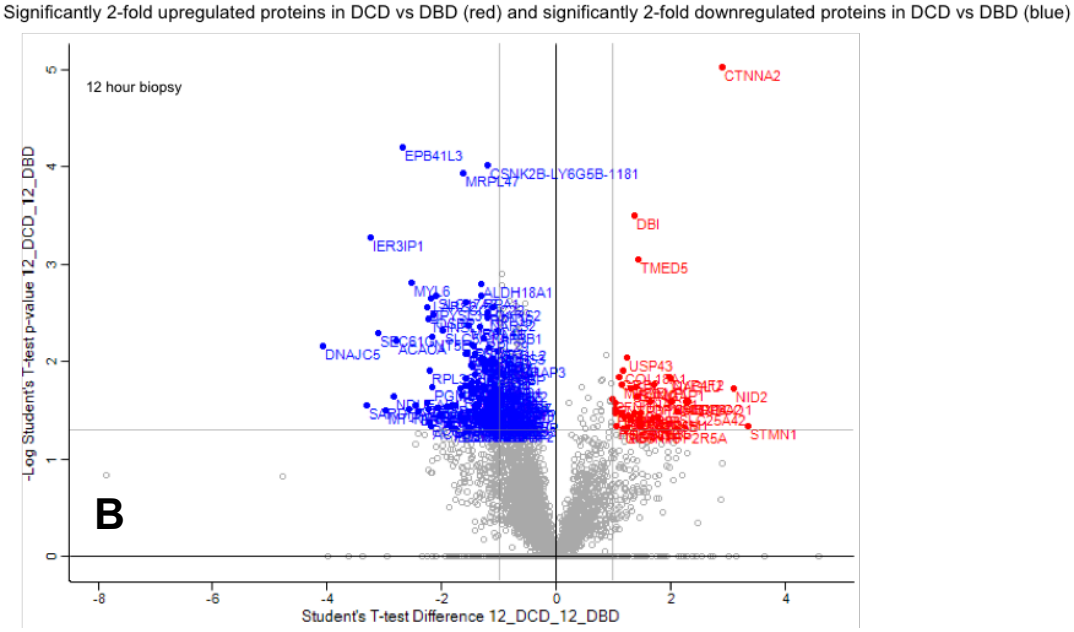


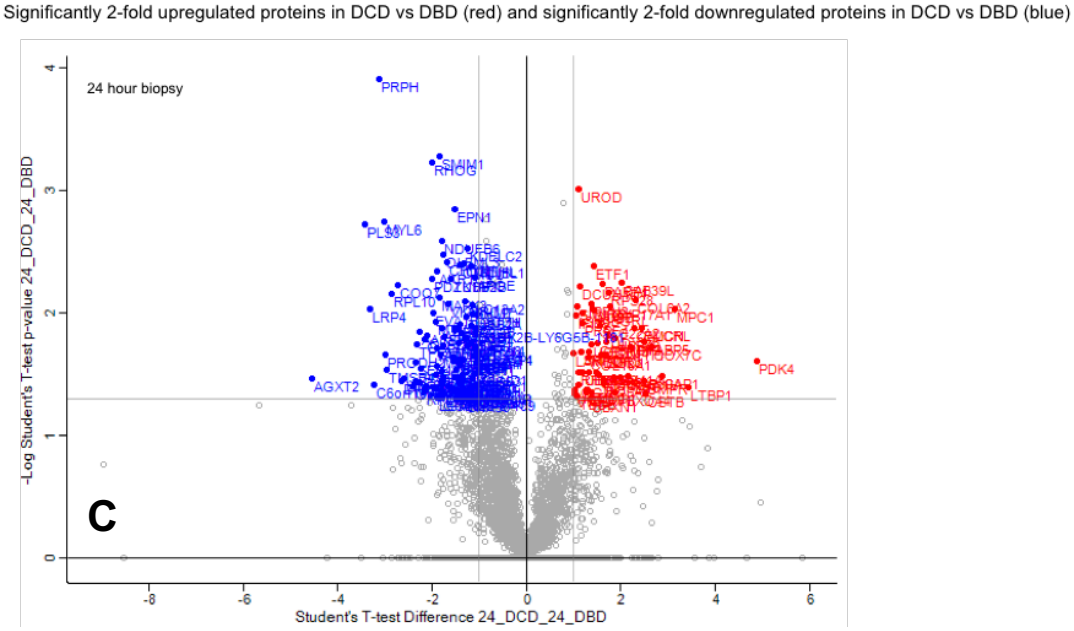


Figures 4.7A-D illustrate the volcano plots for the comparison of CIT duration. A=zero biopsy; B=6-hour biopsy; C=12-hour biopsy; D=24-hour biopsy.

The second group represents the comparison of protein expression in donor types over time; section B in figure 4.6. Proteins, up- and downregulated, were compared between the group of kidneys from donors after circulatory death (DCD) and donors after brain death (DBD).



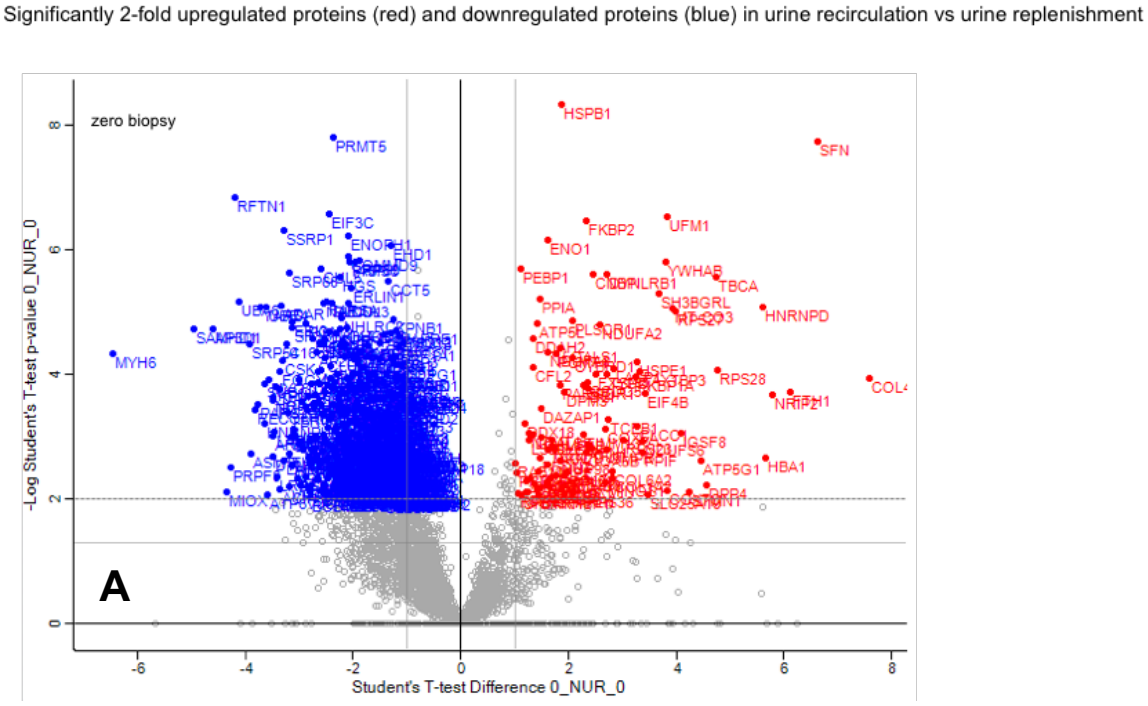


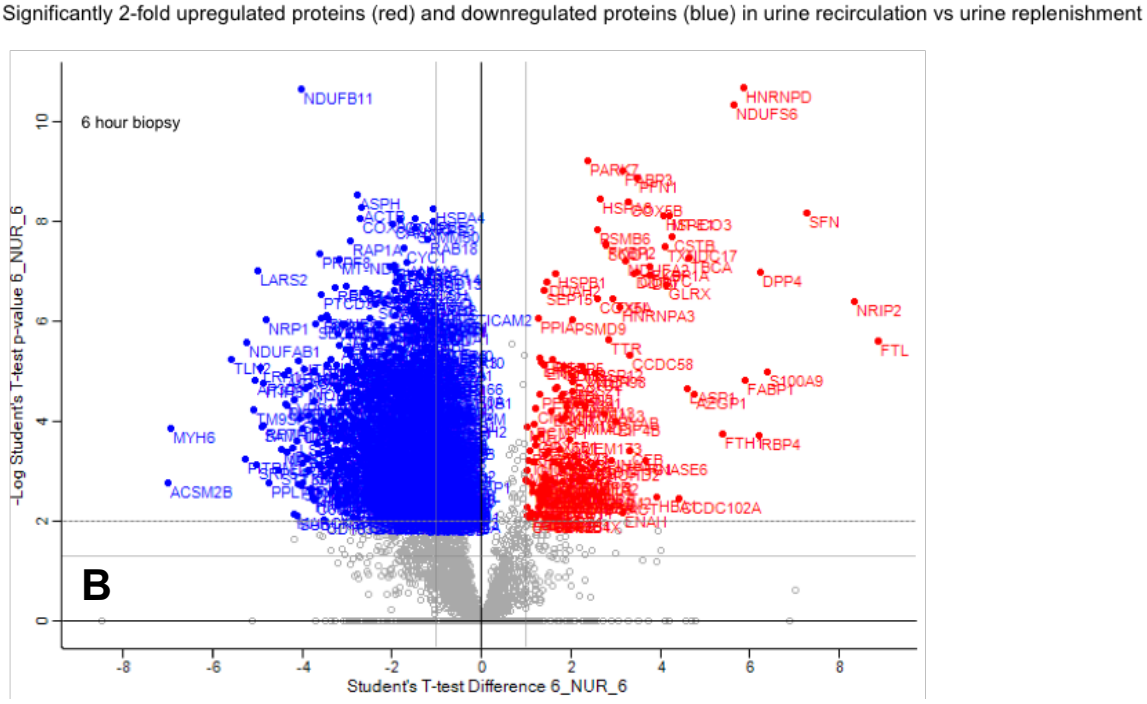


Figures 4.8A-C show the volcano plots for the comparison of donor types. A=zero biopsy; B=12-hour biopsy; C=24-hour biopsy.

The third group I compared for significant protein up- and downregulations were the different perfusion types; with and without urine recirculation, part C in figure 4.6. I was particularly interested in this comparison and analysed the up- and downregulated proteins further as the method itself, to recirculate the urine in a human setting of

kidney NMP, is new and I was not able to perfuse a discarded human kidney without urine recirculation as long as a kidney with urine recirculation.





Figures 4.9A-B display the volcano plots for the comparison of perfusion types. A=zero biopsy; B=6-hour biopsy.

4.4.3. Enzymes involved in glucose and succinate metabolism

Glucose metabolism in the kidney has aroused my interest whilst analysing my human kidney perfusion data. The results showed clearly a correlation of glucose and lactate results (3.7.7.1 in Chapter 3). After having received the proteomics analyses results, the comparisons of long and short CIT and DCD vs. DBD kidneys in particular, as illustrated in figures 4.7A-D and 4.8A-C, I searched specifically for enzymes involved in glucose metabolism to discover potential differences in their up- and/or downregulation. Ross *et al.* provide an excellent review including all aspects of glycolysis, glucose oxidation and gluconeogenesis in the kidney (136). According to this publication I studied hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) as key enzymes of glycolysis. Glucose-6-phosphatase (G6P), fructose-1,6-bisphosphatase (FBP), phosphoenolpyruvate carboxykinase (PCK) and pyruvate carboxylase (PC), the key enzymes of gluconeogenesis. In addition, I was interested in malate dehydrogenase (MDH) and aspartate transaminase (GOT). Both enzymes are important for the conversion of oxalacetate to malate (by MDH) or to aspartate (by GOT) for oxaloacetate being able to cross the mitochondrial membrane; presence/activation of both enzymes are therefore crucial for gluconeogenesis and an “indirect” marker for mitochondrial activity (136).

The comparison of biopsy samples from kidneys (n=16) with a long CIT (≥24 hours) with kidney tissue normothermically perfused after a short CIT (≤24 hours) revealed that all mentioned enzymes stayed 2-fold, or less, downregulated throughout the time of perfusion (not significantly) in the longer CIT cohort. It has to be noted that the long CIT group of biopsy samples comprises seven kidneys (six DCD and one DBD) whereof four have had a CIT > 50 hours.

When stratifying the proteomics analysis for donor type, DCD (n=8) vs. DBD (n=8) kidneys, I could detect an interesting development of all mentioned enzymes involved in glucose metabolism, including mitochondrial MDH and GOT. All stated enzymes above are downregulated in the DCD zero biopsies compared to the DBD zero biopsies; FBP isoenzyme 1 was significantly ($p<0.050$) downregulated in DCD kidneys compared to DBD ones. After twelve and 24 hours of NMP, all but one enzyme were more upregulated in DCD tissue compared to DBD kidney biopsies. The mitochondrial version of PCK, PCK2, was significantly downregulated ($p<0.050$) after 12 hours of NMP in DCD kidney biopsies. Interestingly, both the cytosolic (PCK1) and the mitochondrial (PCK2) enzyme were more upregulated after 24 hours of NMP compared to DBD tissue.

Studies on ischaemic tissue and reperfusion revealed a strong influence of succinate metabolism, succinate accumulation precisely, on the consequences caused by IRI (50,51). Chouchani *et al.* described in an ischaemic model of the heart that succinate accumulation is a metabolic signature of ischaemia and is responsible for mitochondrial ROS production during reperfusion. The succinate accumulation is caused by reverse function of succinate dehydrogenase (SDH) driven by fumarate overflow (50). In DCD kidneys, SDH (subunits A and B) were significantly upregulated in the zero-biopsy compared to DBD kidneys. After twelve and 24 hours of NMP the same proteins were significantly upregulated in DCD biopsies. Fumarate hydratase (FH), an enzyme which catalyses the hydration/dehydration of fumarate to malate reversibly, is also significantly upregulated in the zero biopsy of DCD kidneys compared to DBD tissue and vice versa after 12 hours of NMP. Fumarate hydratase is not part of the malate/aspartate shuttle, which is partially reversed during ischaemia

(50), but as the enzyme is functioning in reverse and part of the Krebs cycle in mitochondria, it could be that this enzyme has a significant impact on IRI in terms of being a fumarate source for SDH in DCD kidneys. All mentioned proteins are displayed in figure 4.10. At this stage of my research I am not able to provide sufficient data for delivering hard evidence, due to the small number of kidneys analysed, but FH/SDH expression in DCD and DBD kidneys could be an interesting aspect for future studies.

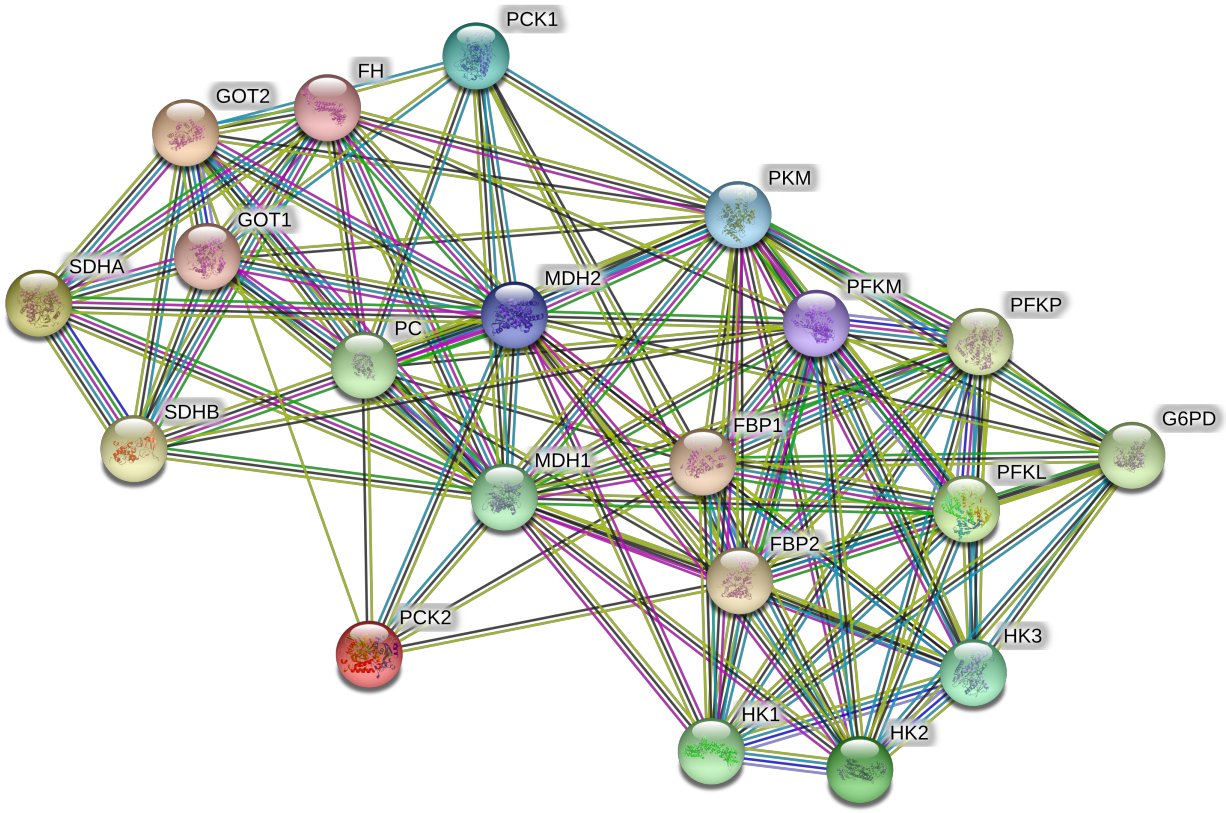


Figure 4.10 is extracted from the STRING database and displays all detected upregulated (≥ 2-fold and/or significant) proteins involved in glucose and the succinate metabolism. The connection-lines between the nodes (representing the proteins) indicate the interaction evidence (172).

4.4.4 Significant changes in the proteome with and without urine recirculation

Analysing the kidney biopsies from 16 perfusions with and three perfusions without urine recirculation (shown in Figure 4.6 part C) revealed 40 significantly upregulated proteins in the zero biopsies and 85 significantly upregulated proteins in the biopsy

taken six hours after perfusion start. Sixty upregulated proteins were the same in both, the zero and the six-hour biopsy. Interestingly, the analysis showed more significantly downregulated proteins over time in the kidneys with urine recirculation. In the zero biopsy samples, 172 proteins were significantly downregulated and even more, 555 in the six-hour biopsy. The zero and the six-hour samples had the downregulation of 379 proteins in common.

Two-fold significant changed proteins were looked up for their context and meaning in the STRING Database Version 10.5 (© STRING CONSORTIUM 2018, https://string-db.org, comprising 9,643.763 proteins of 2031 organisms (172)) and in the UniProt database (The Universal Protein Resource, produced by the UniProt consortium; of www.uniprot.org (168)).

I am describing the different up- and downregulated proteins during six hours of NMP with and without urine recirculation in the following paragraphs.

4.4.4.1 Differences between urine recirculation and urine replenishment after six hours

4.4.4.1.1 Upregulated proteins

From all of the 85 significantly upregulated proteins (figure 4.6) which were different after six hours of NMP between the kidneys perfused with a closed vs. an open circuit, I looked closer into some proteins as they might have a clinical impact or could be responsible for the fact that kidneys perfused with a completely closed circuit could be perfused significantly longer than the others. A selection of proteins might always be biased, but the idea behind performing proteomics was to learn about the (heterogenous) kidney tissue and its changes in a nearly-physiological environment over time rather than providing an absolute score for decision making if an organ should be used in future or not. It would have been desirable to provide such score,

however, the omics-technique itself and the logistics behind are too laborious and time-consuming at the current stage. It is a precious tool which we can use to learn retrospectively about the organ by correlating the findings (perfusion parameters, clinical parameters after transplant etc.) with donor characteristics available at the time of the organ offer rather than applying proteomics in real-time whilst the organ is on the NMP device.

A selection of proteins (n=24) significantly more expressed in the kidneys perfused with urine recirculation after six hours of perfusion are described according to the information published and available via the webpages of the STRING and UniProt databases (168,172).

- ATP5B ATP synthase, H+ transporting, mitochondrial F_1 complex
- ATP5G1 ATP synthase, H+ transporting, mitochondrial F_0 complex
- ATP5G2 ATP synthase, H+ transporting, mitochondrial F_0 complex
- ATP5G3 ATP synthase, H+ transporting, mitochondrial F_0 complex

  All of them produce ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain (168,172).
- AK1 Adenylate kinase 1; for reversible transfer of the terminal phosphate group between ATP and AMP; displays nucleoside diphosphate kinase activity; important role in cellular energy homeostasis and in adenine nucleotide metabolism (168,172).
- MINOS1 Mitochondrial inner membrane organizing system 1; Part of the MICOS complex, a large protein complex of the mitochondrial inner membrane which is crucial for maintenance of crista junctions, inner membrane architecture, and contact to the outer membrane (168,172).

- COL4A1 Collagen, type IV, alpha 1; type IV collagen is the major structural component of glomerular basement membranes (GBM), forming a 'chicken-wire' meshwork together with laminins, proteoglycans and nidogen (an essential component of the basement membrane) (168,172).
- SERPINA3 Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3; can inhibit neutrophil cathepsin G and mast cell chymase, which could convert angiotensin-1 to the active angiotensin-2 (168,172).
- COMMD3 COMM domain containing 3; modulates sodium transport in epithelial cells by regulation of apical cell surface expression of sodium channel subunits; may down-regulate activation of NF-kappa-B (168,172).
- BAG3 BCL2-associated athanogene 3; inhibits the chaperone activity of HSP70/HSC70; shows anti-apoptotic activity (168,172).
- HSPA6 Heat shock 70kDa protein 6; with other chaperones, Hsp70s stabilise pre-existent proteins against aggregation and mediate the folding of newly translated polypeptides in the cytosol and within organelles (168,172).
- SOD1 Superoxide dismutase 1, soluble
- SOD2 Superoxide dismutase 2, mitochondrial

  Both destroy radicals which are normally produced within the cells and which are toxic to biological systems (168,172).
- CCS Copper chaperone for superoxide dismutase; delivers copper to copper zinc superoxide dismutase (SOD1) (168,172).
- GLRX Glutaredoxin (thioltransferase); can act as a glutathione-disulfide oxidoreductase in the presence of NADPH and glutathione reductase (168,172).

- GLRX5 Glutaredoxin 5; required for normal iron homeostasis and for normal regulation of haemoglobin synthesis; might protect cells against apoptosis due to reactive oxygen species and oxidative stress (168,172).
- GGCT Gamma-glutamylcyclotransferase; catalyses the formation of 5-oxoproline from gamma- glutamyl dipeptides and could have a significant role in glutathione homeostasis (168,172).
- GSTA1 Glutathione S-transferase alpha 1; conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic compounds that tend to accept electrons (168,172).
- TXN Thioredoxin; participates in numerous redox reactions through the reversible oxidation of its active centre dithiol to a disulfide and catalyses dithiol-disulfide exchange reactions; plays a role in the reversible S-nitrosylation of cysteine residues, contributes to the response to intracellular nitric oxide (168,172).
- TXNDC17 Thioredoxin domain containing 17; disulfide reductase; could participate in redox reactions through the reversible oxidation of its active centre dithiol to a disulfide and catalyses dithiol-disulfide exchange reactions; modulates TNF-alpha signaling and NF-kappa-B activation; has peroxidase activity and could contribute to the elimination of cellular hydrogen peroxide (168,172).
- DDAH1 Dimethylarginine dimethylaminohydrolase 1; hydrolyzses N(G),N(G)-dimethyl-L-arginine and N(G)-monomethyl-L-arginine; both act as inhibitors of NOS, plays therefore a role in the regulation of nitric oxide generation (168,172).

- UBE2V1 Ubiquitin-conjugating enzyme E2 variant 1; has no ubiquitin ligase activity alone; plays a role in activating NF-kappa-B mediated by IL1B, TNF, TRAF6 and TRAF2; mediates transcriptional activation of target genes; plays a role in the control of progress through the cell cycle, differentiation and in error-free DNA repair (168,172).
- S100A9 S100 calcium binding protein A9; S100A9 is a calcium- and zinc-binding protein important for the regulation of inflammatory processes and immune response; it can induce neutrophil chemotaxis, adhesion and can increase the bactericidal activity of neutrophils by promoting phagocytosis (168,172).
- ITPA Inosine triphosphatase (nucleoside triphosphate pyrophosphatase); pyrophosphatase that hydrolyses the non-canonical purine nucleotides inosine triphosphate, deoxyinosine triphosphate and 2'-deoxy-N-6-hydroxylaminopurine triphosphate and xanthosine 5'-triphosphate to their respective monophosphate derivatives (168,172).

4.4.4.1.2 Downregulated proteins

The following paragraph lists a selection of proteins (n=27) significantly downregulated in kidney tissue after six hours of NMP including urine recirculation compared to perfusions without. Interestingly, some of the damage-associated molecular patterns (DAMPs) known to be contributing to IRI are significantly downregulated. DAMPs are molecules able to initiate innate immune responses and maintain a non-infectious inflammatory response. The term "DAMP" was first described by Seong and Matzinger in 2004 in the context of hydrophobicity (173), describing the reaction of the hydrophobic portion of a biological molecule as a universal damage-associated

molecular pattern initiating repair, remodelling and immunity when exposed to a stimulus (173). Protein DAMPs include heat-shock proteins, high-mobility group box 1 (HMGB1) and proteins generated following injury in the extracellular matrix. Other substrates described as DAMPs include DNA anywhere else than the nucleus or mitochondria and RNA which can trigger responses via toll like receptors; S100 proteins; purine metabolites; mono and polysaccharides (174). Furthermore, some of the mitochondrial succinate dehydrogenase proteins are significantly downregulated which could play a significant role regarding succinate accumulation and ROS production (50). Figure 4.11 shows a selection of significant downregulated proteins graphically.

- HMGB1 High-mobility group box 1; promotes host inflammatory response to sterile and infectious signals and is involved in the coordination and integration of innate and adaptive immune responses (168,172).
- ICAM1 Intercellular adhesion molecule 1; ICAM proteins are ligands for the leukocyte adhesion protein LFA-1 (integrin alpha-L/beta-2; lymphocyte function-associated antigen 1) (168,172).
- NFKB1 Nuclear factor NF-kappa-B p105 subunit; a pleiotropic transcription factor which is present in almost all cell types; is the endpoint of a series of signal transduction events related to many biological processes like inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis (168,172).
- MAPK1 Mitogen-activated protein kinase 1; serine/threonine kinase acting as an essential component of the MAP kinase signal transduction route (168,172).

- HSPA1B Heat shock 70kDa protein 1B; plays an important role in the protein quality control system, ensuring correct folding of proteins, the re-folding of misfolded proteins and controlling the targeting of proteins for subsequent degradation; achieved through cycles of ATP binding, ATP hydrolysis and ADP release (168,172).
- HSPA4 Heat shock 70kDA protein 4
- HSPA12B Heat shock 70kDA protein 12B
- ANXA1 Annexin A1; plays pivotal roles in the innate immune response as effector of glucocorticoid-mediated responses and regulator of the inflammatory process (168,172).
- FH Fumarate hydratase; catalyses the reversible hydration of fumarate to malate; involved in the Krebs Cycle (tricarboxylic acid cycle or citric acid cycle) (168,172).
- SDHB Succinate dehydrogenase complex, subunit B, iron-sulfur protein;
- SDHC Succinate dehydrogenase complex, subunit C, integral membrane protein;
- SDHD Succinate dehydrogenase complex, subunit D, integral membrane protein:

  All three are involved in complex II of the mitochondrial electron transport chain and are responsible for transferring electrons from succinate to ubiquinone (coenzyme Q) (168,172).
- S100A8 S100 calcium binding protein A8; is a calcium- and zinc-binding protein playing an important role in the regulation of inflammatory and immune processes and responses (168,172).

- ACE Angiotensin I converting-enzyme (peptidyl-dipeptidase A) 1; converts angiotensin I to angiotensin II by release of the terminal His-Leu resulting in an increase of the vasoconstrictor activity of angiotensin; furthermore able to inactivate bradykinin, a potent vasodilator (168,172).
- ACSS1 acyl-CoA synthetase short-chain family member 1; important for maintaining normal body temperature during fasting and for energy homeostasis; converts acetate to acetyl-CoA so that it can be used for oxidation through the tricarboxylic cycle to produce ATP (168,172).
- PDHA1 Pyruvate dehydrogenase (lipoamide) alpha 1; primary link between glycolysis and the Krebs cycle through catalysation of the irreversible conversion of pyruvate into acetyl-CoA (175).
- HIBADH 3-hydroxyisobutyrate dehydrogenase; mitochondrial enzyme that participates in valine, leucin and isoleucine degradation (176).
- ROMO1 Reactive oxygen species modulator 1; induces production of reactive oxygen species which are of necessity for cell proliferation; could play a role in inducing oxidative DNA damage and replicative senescence (168,172).
- STAT1 Signal transducer and activator of transcription 1, 91kDa; signal transducer and transcription activator mediating cellular responses to interferons and cytokines and growth factors; following type I IFN (IFN-alpha and IFN-beta) binding to cell surface receptors, signalling via protein kinases leads to activation of JAK kinases and to tyrosine phosphorylation of STAT1 and STAT2 (168,172).
- G6PD Glucose-6-phosphate dehydrogenase; catalyses the rate-limiting step of the oxidative pentose-phosphate pathway, representing a pathway for the dissimilation of carbohydrates beside glycolysis; main function is to provide

reducing power (NADPH) and pentose phosphates for fatty acid and nucleic acid synthesis (168,172).

- G6PC Glucose-6-phosphatase, catalytic subunit; hydrolyses glucose-6-phosphate to glucose in the endoplasmic reticulum; forms with the glucose-6-phosphate transporter the complex responsible for glucose production through glycogenolysis and gluconeogenesis; it is the key enzyme in homeostatic regulation of blood glucose levels (168,172).
- SLC2A9 Solute carrier family 2 (facilitated glucose transporter), member 9; transports urate and fructose; might play a role in the urate reabsorption by proximal tubules; transports glucose at a low rate (168,172).
- SLC5A12 Solute carrier family 5 (sodium/glucose cotransporter), member 12; Acts as an electroneutral and low-affinity sodium-dependent sodium-coupled transporter; catalyses the transport across the plasma membrane of many monocarboxylates such as lactate, pyruvate, nicotinate, propionate, butyrate and beta-D-hydroxybutyrate; maybe responsible for the first step of reabsorption of monocarboxylates from the lumen of the proximal tubule (168,172).
- SLC12A7 Solute carrier family 12 (potassium/chloride transporters), member 7; when activated through cell swelling it mediates electroneutral potassium-chloride cotransport; could be required for basolateral chloride extrusion in the kidney and contributes to renal acidification (168,172).
- SLC17A3 Solute carrier family 17 (sodium phosphate), member 3; isoform 2 functions as urate efflux transporter on the apical side of renal proximal tubule and is likely to act as an exit path for organic anionic drugs as well as urate in

vivo; could be involved in the active transport of phosphate into cells via sodium cotransport (168,172).

- SLC22A12 Solute carrier family 22 (organic anion/urate transporter), member 12; required for efficient urate re-absorption in the kidney, regulates blood urate levels; mediates saturable urate uptake by facilitating the exchange of urate against organic anions (168,172).
- F9 Coagulation factor IX; is a vitamin K-dependent plasma protein participating in the intrinsic pathway of blood coagulation by converting factor X to its active form in the presence of calcium ions, phospholipids, and factor VIIIa (168,172).

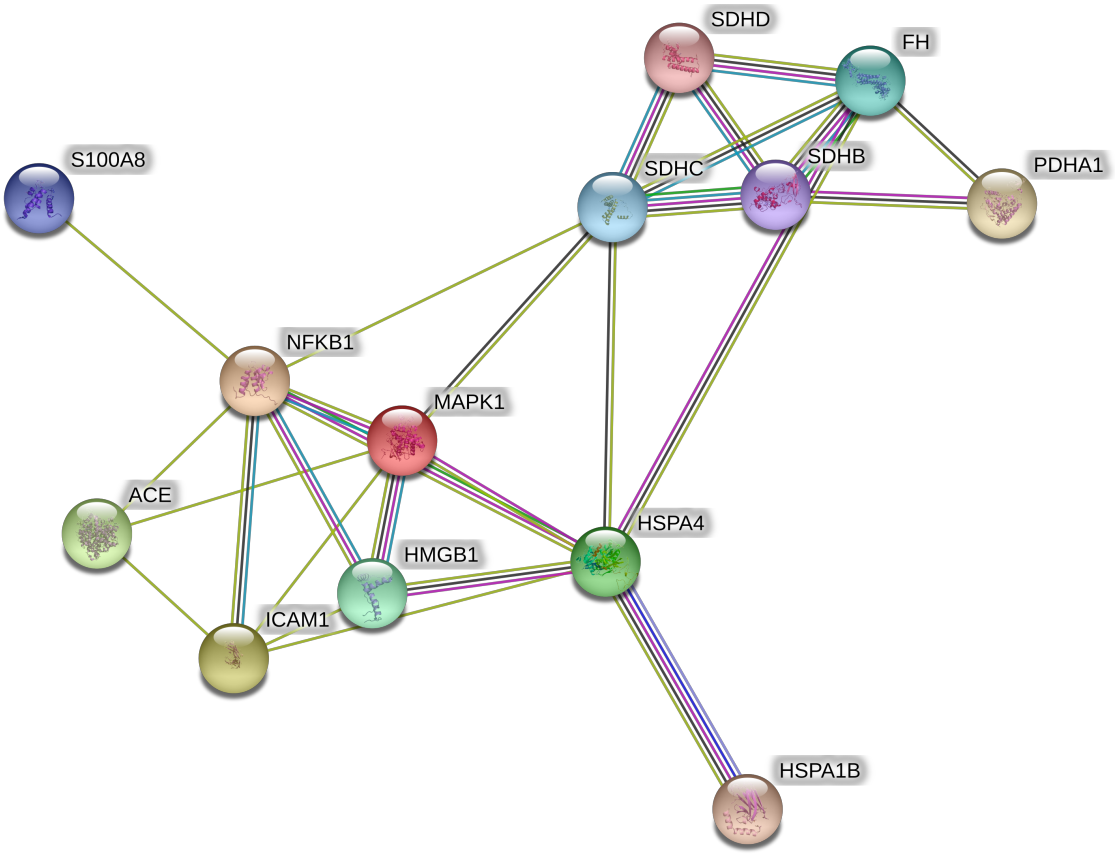


Figure 4.11 is extracted from the STRING database and displays ≥ 2-fold and/or significantly downregulated DAMPs and ACE proteins which are different between perfusions with urine recirculation or replacing the excreted urine with Ringer's lactate after six hours of kidney NMP. The connection-lines between the nodes (representing the proteins) indicate the interaction evidence (172).

4.4.5 Individual kidneys have individual proteomes

Proteins which were compared in the previous paragraphs encompassed the whole cohort of kidney biopsies undergoing proteomics analysis. For reasons of diversity in donor characteristics and length of ischaemia times, I wanted to investigate each kidney's protein profile. To start with, a heatmap and a scatter plot of protein alterations were created for each single kidney. Data illustration by using heat maps is an efficient method of visualizing complex data sets organised as matrices (177). The typical matrix of a heat map is created by arranging the proteomics data in a way that each column contains the data from a single sample (one kidney biopsy in my analysis) and each row corresponds to a single protein. A heat map reorders the rows and columns so that both, rows and columns, with similar profiles are closer to each other. This configuration makes the detected protein profiles more visible to the eye. In addition, each entry in the data matrix is displayed as a colour (red and green in my results), making the graphically view of the patterns clearly discernible (177). Generating a scatter plot is another method to communicate proteomics results by visualisation. X-Y scatter plots can illustrate striking differences between baseline data and changes over time; in my case changes in protein expression during NMP time compared to the zero biopsy (178). Proteins increased ≥ 2-fold compared to the zero (baseline) biopsy are displayed in the upper right quadrant and proteins decreased ≥ 2-fold compared to the biopsy taken before start of NMP can be seen in the left lower quadrant.

Figures 4.12A-G show heat maps and scatter plots of seven NMP kidneys with urine recirculation comprising examples of all variants of perfused discarded human kidneys. Proteins plotted in red are ≥ 2-fold upregulated compared to baseline;

proteins plotted in green/blue are ≥ 2-fold downregulated compared to the baseline results.

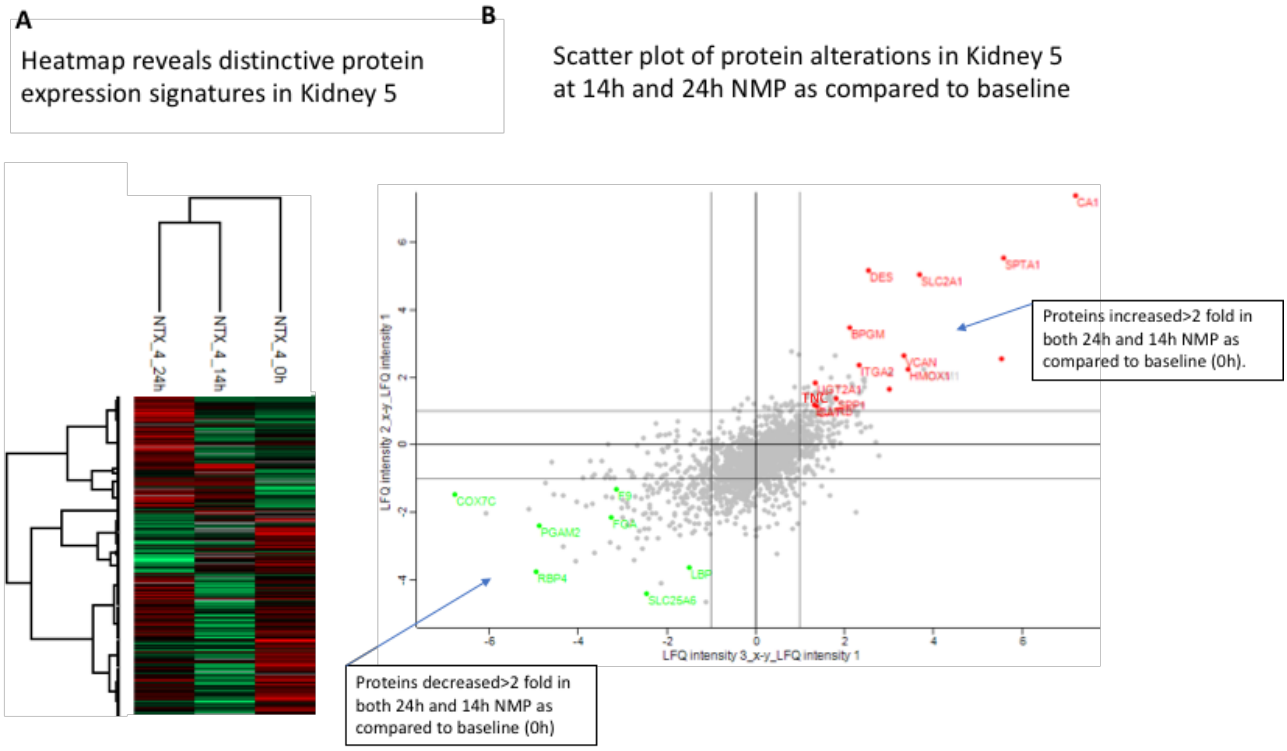


Figure 4.12A: Kidney 5 (NTX_4), 61-year old DBD, 19 hours CIT



Figure 4.12B: Kidney 7 (NTX_6), 58-year old DBD, 10 hours CIT.

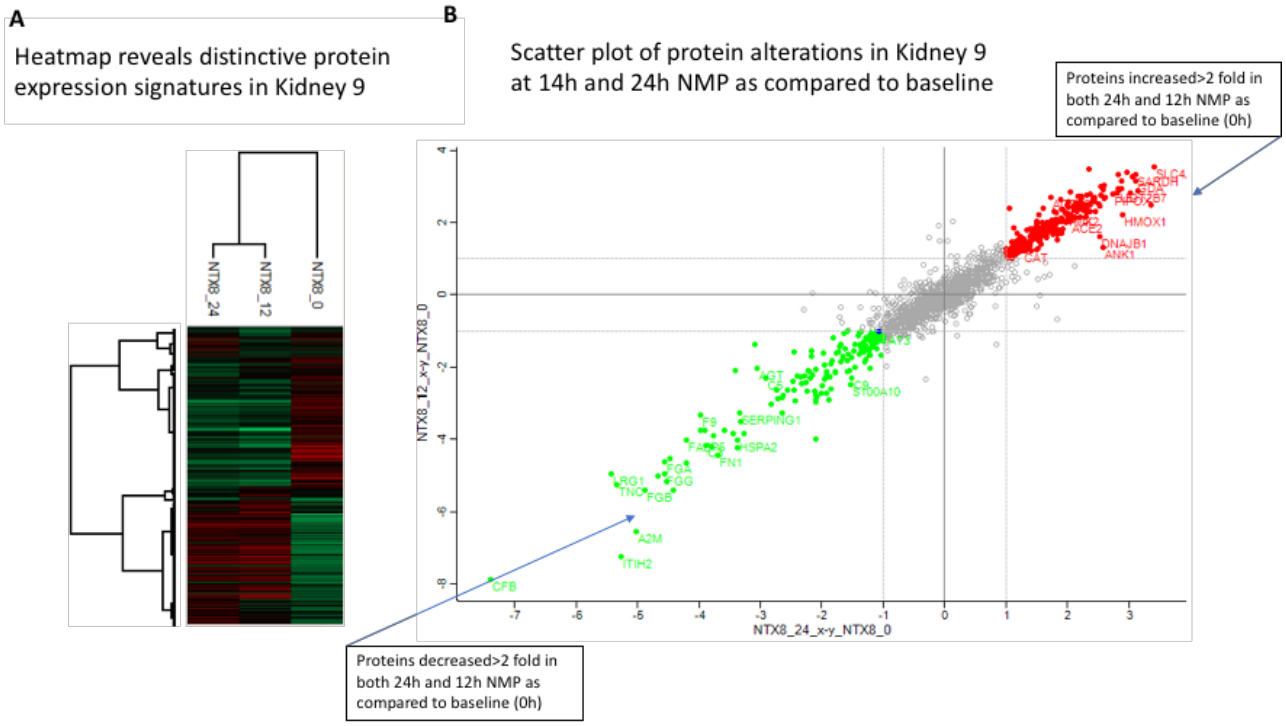


Figure 4.12C: Kidney 9 (NTX_8), 51-year old DCD, 11 hours CIT.

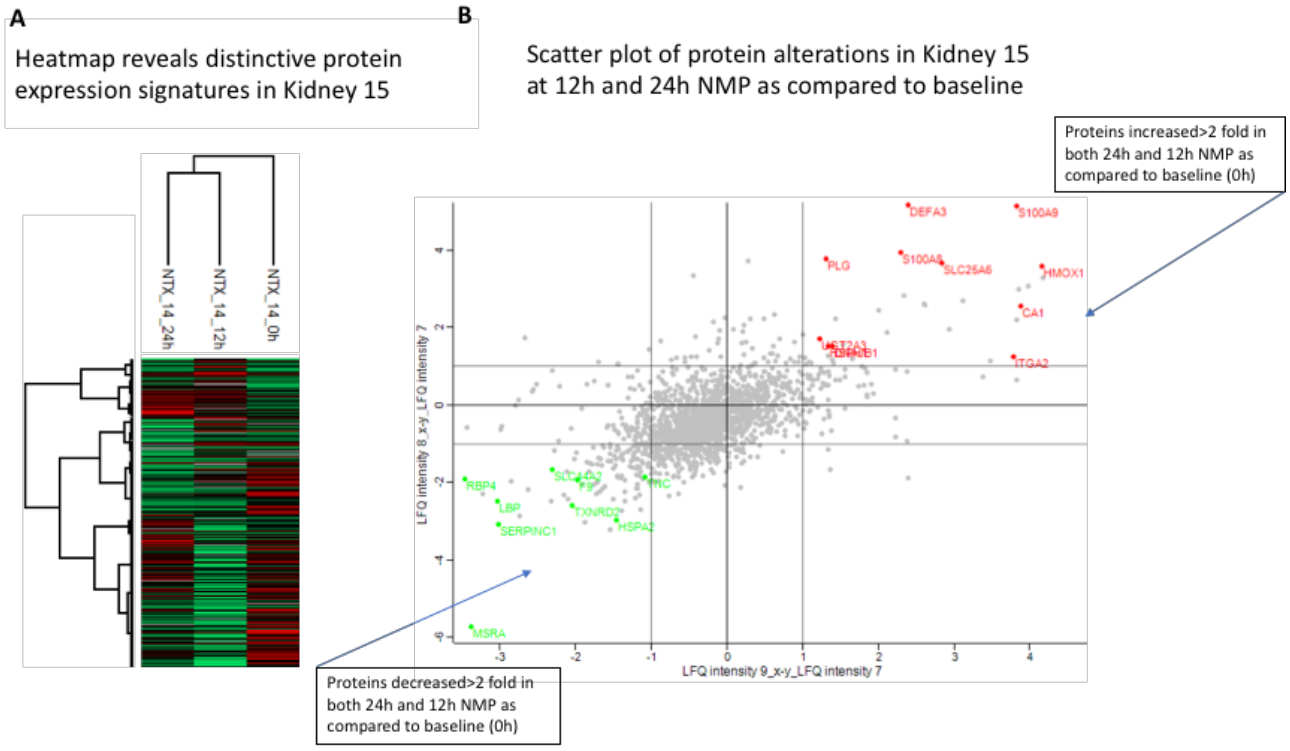


Figure 4.12D: Kidney 15 (NTX_14), 44-year old DCD, 104 hours CIT with histologically verified cortical necrosis.

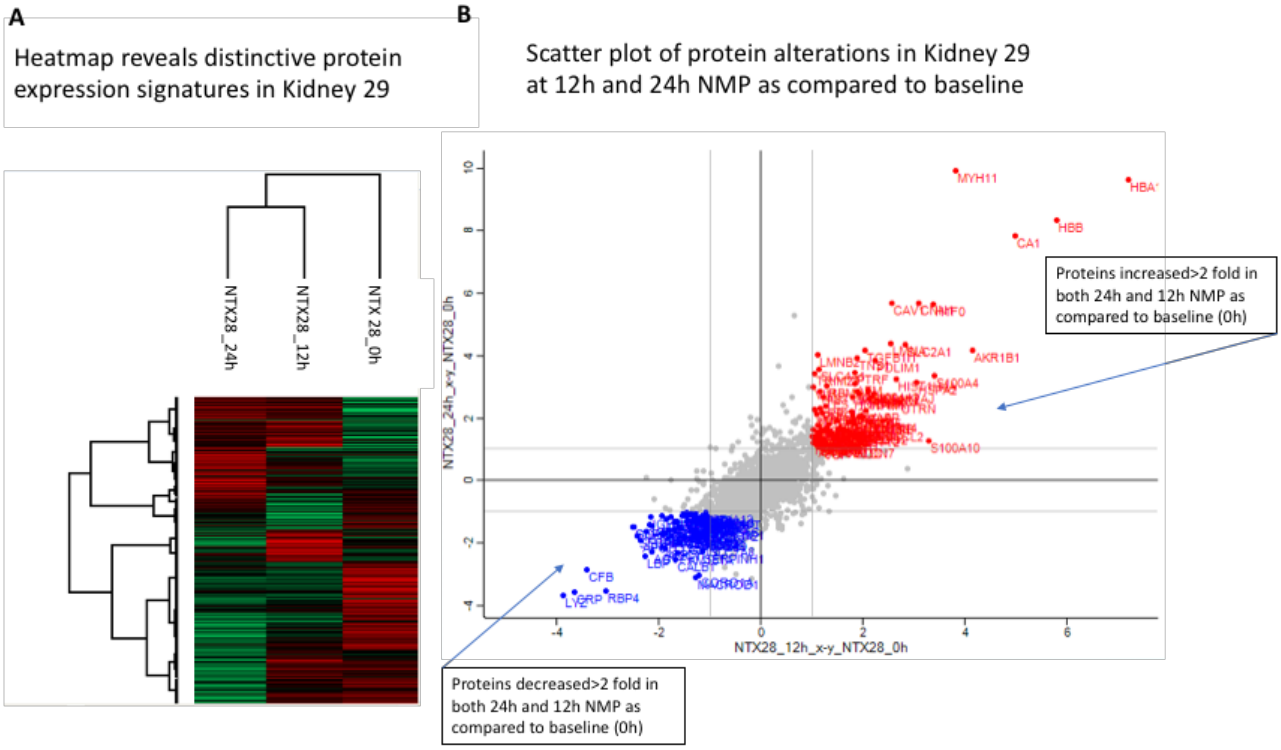


Figure 4.12E: Kidney 29 (NTX_28), 70-year old DBD, 17 hours CIT.

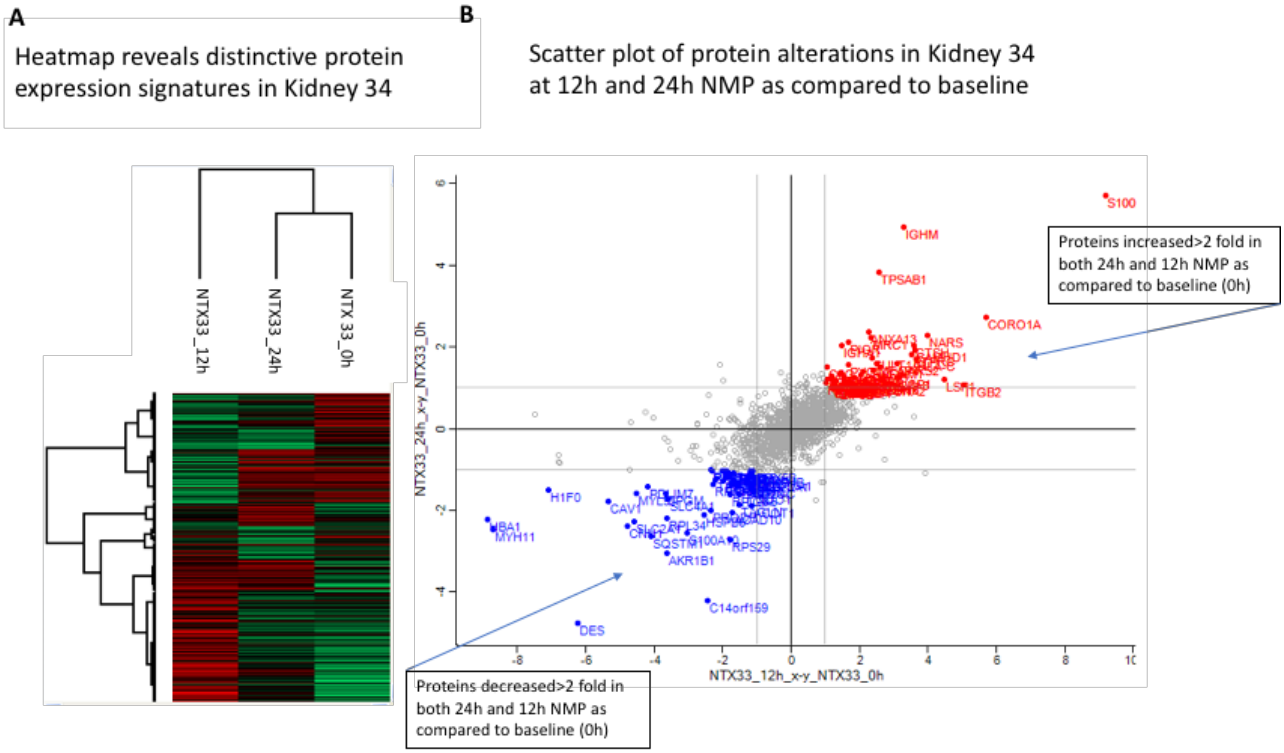


Figure 4.12F: Kidney 34 (NTX_33), 47-year old DBD, 12 hours CIT.

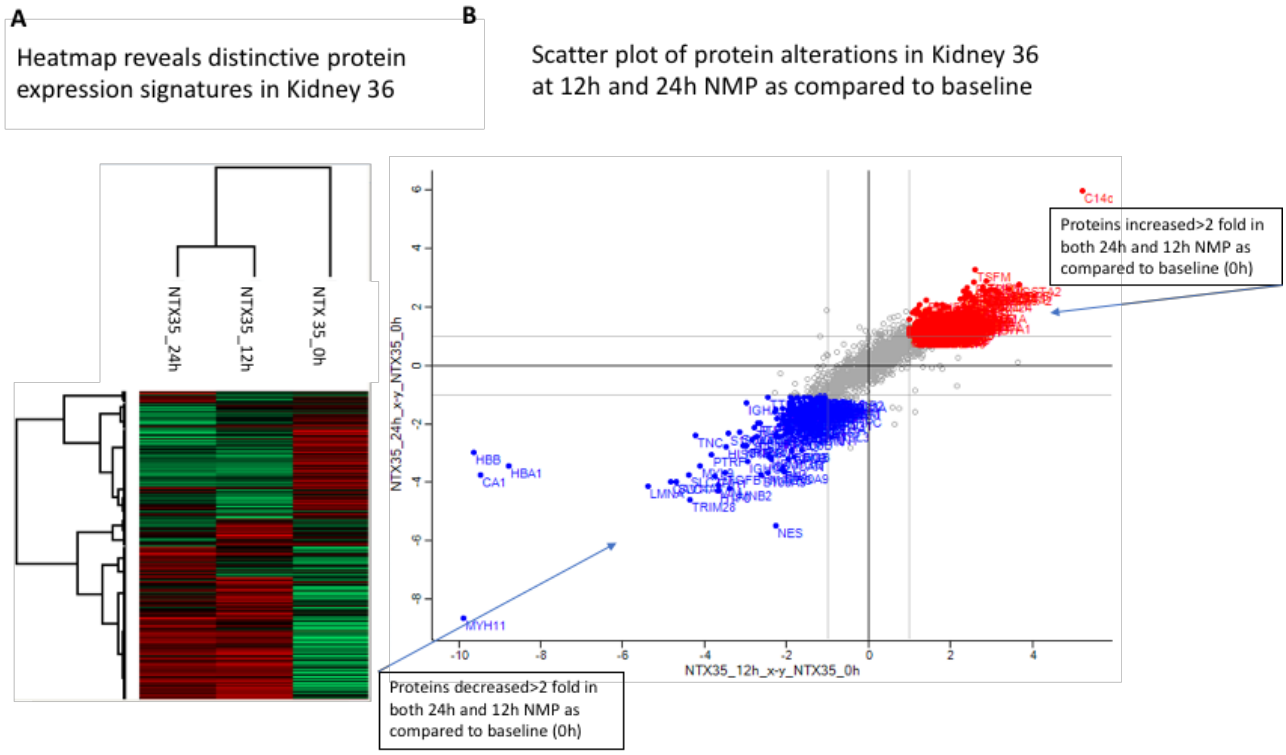


Figure 4.12G: Kidney 36 (NTX_35), 76-year old DBD, 17 hours CIT.

Kidney 7 was only 18 hours on the normothermic perfusion device, all others were perfused for 24 hours. Kidney results shown in Figures 4.12A-D: (scatter plot results in red and green) are kidneys perfused with the oxygen/air combination, whereas proteomics results for kidneys perfused with the air/CO_2 combination (2.1.3 Chapter 2), are displayed in Figures 4.12E-G; scatter plot results in red and blue.

4.4.5.1 Protein profiles with urine recirculation are the opposite to the ones without

To investigate which proteins, up- and/or downregulated, have individual kidneys with and without urine recirculation in common, I analysed all proteins per kidney and decided to study those proteins further which were at least up- or downregulated in 75% (12/16) of kidneys with urine recirculation and in 67% (2/3) of kidneys without urine recirculation. These are arbitrary numbers, but I wanted to investigate the majority of proteins the kidneys had in common within the group. More importantly, I

would like to illustrate the difference in protein expression on the tissue levels which might be caused by solely recirculating the urine or not in figure 4.13. The upregulations/downregulations in the individual kidneys were investigated as ≥ 2-fold changed proteins. For individual kidneys no Student t-tests were applied, therefore there is no p-value available.

A: upregulated without	B: downregulated with	C: upregulated with	D: downregulated without
AGT	AGT	AFM	AFM
CFB	CFB	CA1	CA1
GC	GC	HBA1	HBA1
GPT	GPT	HBB	HBB
LBP	LBP	HBD	HBD
ITIH4	ITIH4	SLC2A1	SLC2A1
ORM1	ORM1	SLC4A1	SLC4A1
PLG	PLG	SPTA1	SPTA1
RBP4	RBP4	SPTB	SPTB
SERPINA1	SERPINA1		
SERPINA3	SERPINA3		
SERPINC1	SERPINC1		
SOD3	SOD3		
TF	TF		
1) Columns A and D represent kidneys perfused without urine recirculation			
2) Columns B and C represent kdineys perfused with urine recirculation			
3) Proteins AGT, CFB, LBP, ORM1 and PLG were upregulated in 3/3 kidneys without urine recirculation			
4) Proteins HBB, CA1, SLC4A1 and HBA1 were downregulated in 3/3 kidneys without urine recirculation			

Figure 4.13: Comparison of up- and downregulated proteins in kidney perfusions with and without urine recirculation.

The protein for the gene angiotensinogen (AGT) (179) which is upregulated in the kidneys without urine recirculation and downregulated in kidneys with urine recirculation has raised great interest due to its function and the fact that kidneys without urine recirculation developed increasing intrarenal resistance during NMP which was accompanied by a significant deterioration of the arterial flow from hour six onwards. Angiotensinogen is part of the renin-angiotensin system regulating blood pressure, fluid balance and salts in the human body. Angiotensinogen is converted to angiotensin I which is converted to angiotensin II, a vasoconstrictor (180). In addition

to this finding, the comparison of the entire biopsy-cohort of kidneys with and without urine recirculation revealed significant differences in proteins involved in the renin-angiotensin system: Angiotensin I converting enzyme (ACE) is significantly downregulated in kidneys with urine recirculation; Serpin peptidase inhibitor (SERPINA3) can inhibit indirectly the conversion of angiotensin-1 to the active angiotensin-2 and is significantly upregulated in kidneys with urine recirculation after six hours (168,172).

The second protein which could play a role in the different behaviour of kidneys perfused with an open or a closed circuit, is carbonic anhydrase (CA1) which is downregulated in kidneys without urine recirculation. In kidneys perfused with urine recirculation I was able to maintain physiological pH values throughout the perfusion period without giving any supplements. In kidneys without urine recirculation the pH changed significantly over time. This could be lack of presence/activity of CA1. The most important function of the enzyme is to maintain acid-base balance in blood and tissue in the human being (168,172,181).

4.5 Validation of proteomics results with Western Blot

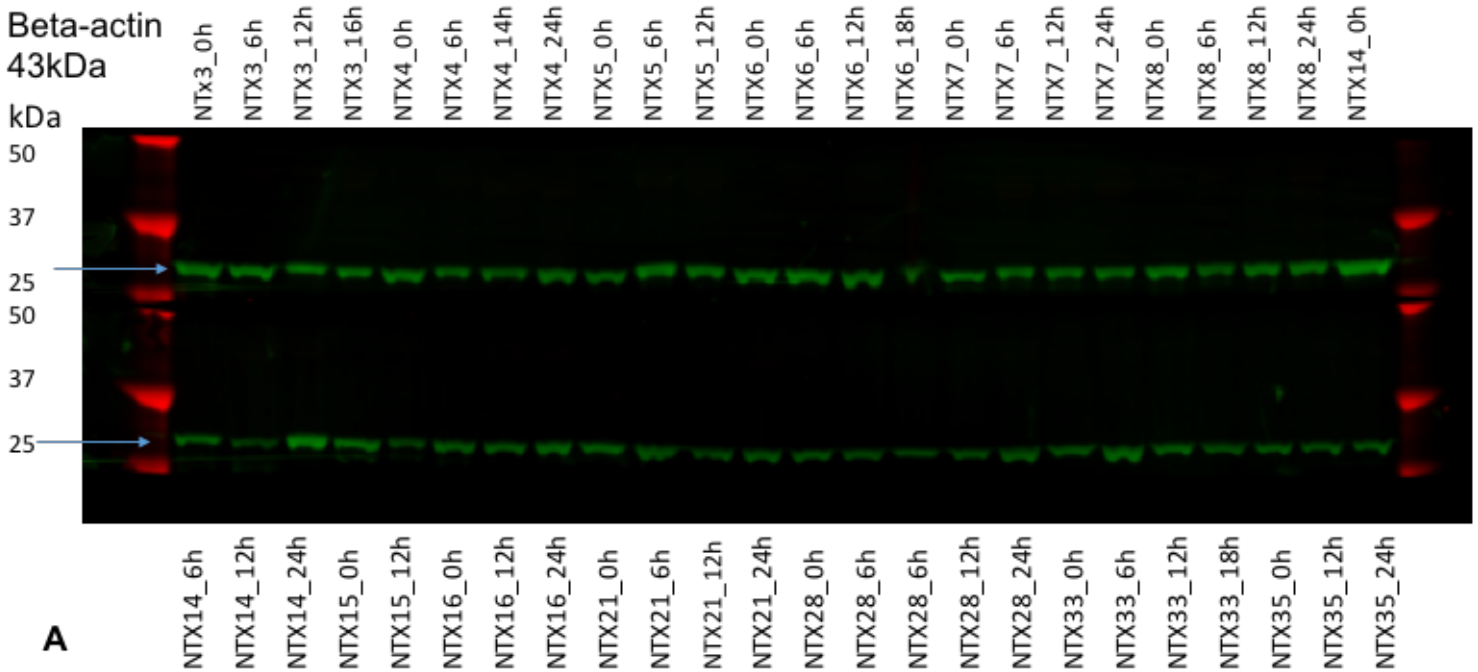
It is not enough to have some results about proteins and their up- and/or downregulation in biopsy samples, we need to confirm their presence directly in biopsy samples. In normothermically perfused kidneys with urine recirculation, proteins differentially expressed included antioxidants, stress proteins, anion exchangers, enzymes like anhydrases and carboxykinases. We selected seven of upregulated proteins in the group of kidneys with urine recirculation and validated the existence of the protein by performing Western Blots in the tissue.

The proteins selected were:

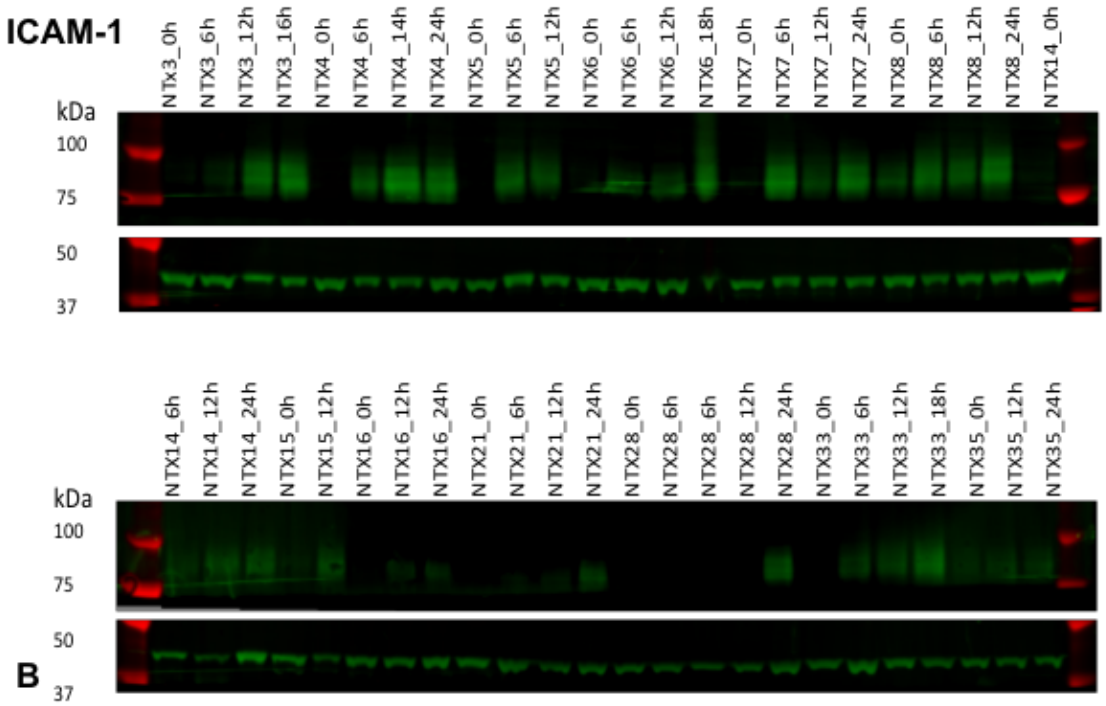
1) Intercellular Adhesion Molecule 1 (ICAM-1): ICAM-1 facilitates a variety of both afferent and efferent immune responses that require intercellular contact and collaboration, including T helper responses, T-dependent B cell responses, antigen-induced T cell proliferation, cell-mediated cytotoxicity, and the binding of leukocytes to vascular endothelium prior to extravasation into inflamed tissue (182); figures 4.14B-C.
2) Band 3 anion transport protein (SLC4A1): An anion exchanger 1, chloride/bicarbonate exchanger; to maintain the correct acid levels (pH) in the body. Mediates chloride-bicarbonate exchange in the kidney and is required for normal acidification of the urine (168,172); figures 4.14D-E.
3) Haem oxygenase 1 (HMOX1): Haem oxygenase cleaves the haem ring at the alpha methene bridge to form biliverdin. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. Under physiological conditions, the activity of haem oxygenase is highest in the spleen, where senescent erythrocytes are sequestrated and destroyed. Exhibits cytoprotective effects since excess of free haem sensitises cells to undergo apoptosis (168,172); figures 4.14F-G.
4) Flavin reductase (NADPH), gene BLVRB: Broad specificity oxidoreductase that catalyses the NADPH-dependent reduction of a variety of flavins, such as riboflavin, FAD (flavin adenine dinucleotide) or FMN (flavin mononucleotide), biliverdins, methaemoglobin and PQQ (pyrroloquinoline quinone). Converts biliverdin to bilirubin; converted back into biliverdin through ROS – this cycle allows for the neutralisation of ROS; recently found to be a regulator of glucose metabolism (168,172); figures 4.14H-I.

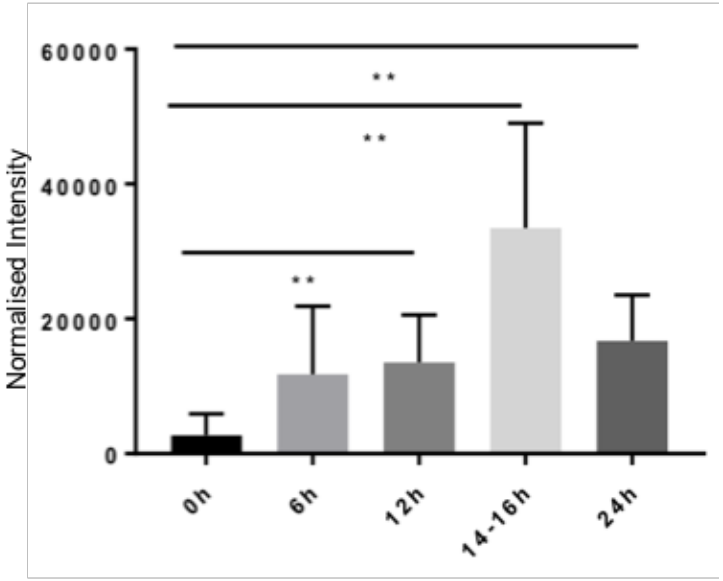
5) Carbonic anhydrase (CA1): Reversible hydration of carbon dioxide; most important function is to maintain acid-base balance (181); figures 4.14J-K
6) Phosphoenolpyruvate carboxykinase (PCK1): Catalyses the conversion of oxaloacetate (OAA) to phosphoenolpyruvate (PEP), the rate-limiting step in the metabolic pathway that produces glucose from lactate and other precursors derived from the citric acid cycle (168,172,183); figures 4.14L-M
7) Heat shock 70 kDa protein 1B (HSPA1B): Molecular chaperone involved in a huge variety of cellular processes: protection of the proteome from stress, folding and transporting newly synthesized polypeptides, activating proteolysis of misfolded proteins, formation and dissociation of protein complexes. Heat shock protein 70 plays an important role in the protein quality control system which is achieved through cycles of ATP binding, ATP hydrolysis and ADP release, mediated by co-chaperons (168); figures 4.14N-O.

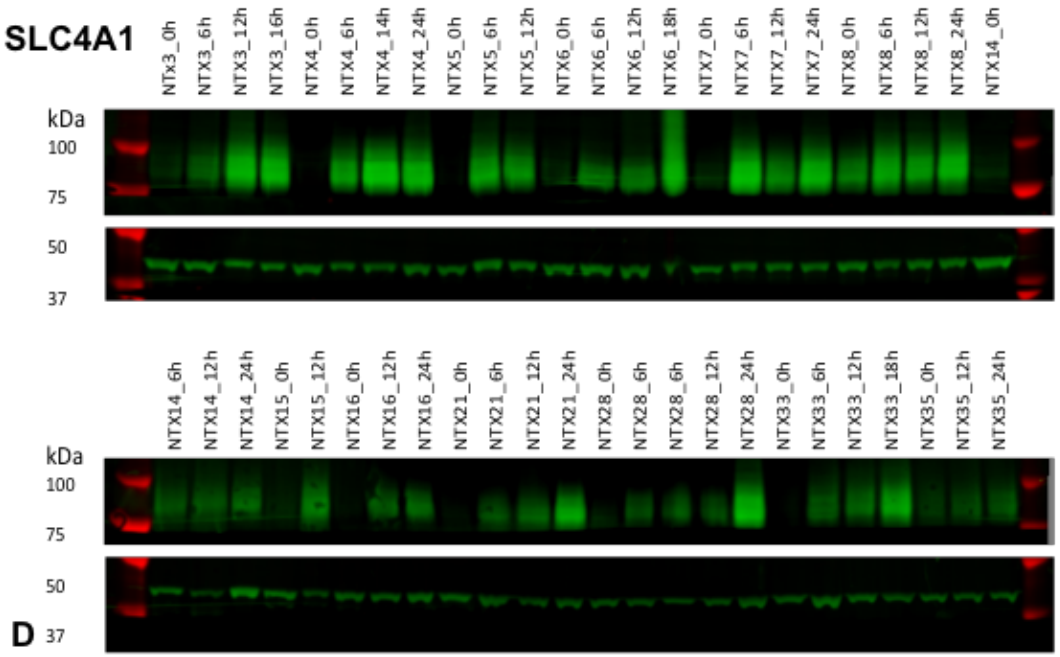
Western Blot analyses are displayed in figures 4.14A-O. Protein levels were normalised to β-actin abundance (blot shown in figure 4.14A). Data for comparing the time points is presented as mean ± SEM; *p-value <0.050; **p-value < 0.01; **p-value < 0.001; ***p-value <0.0001.

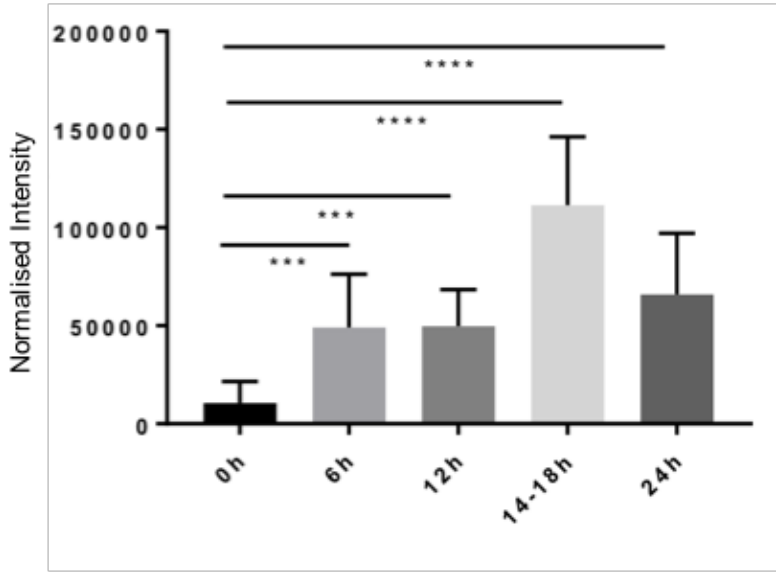


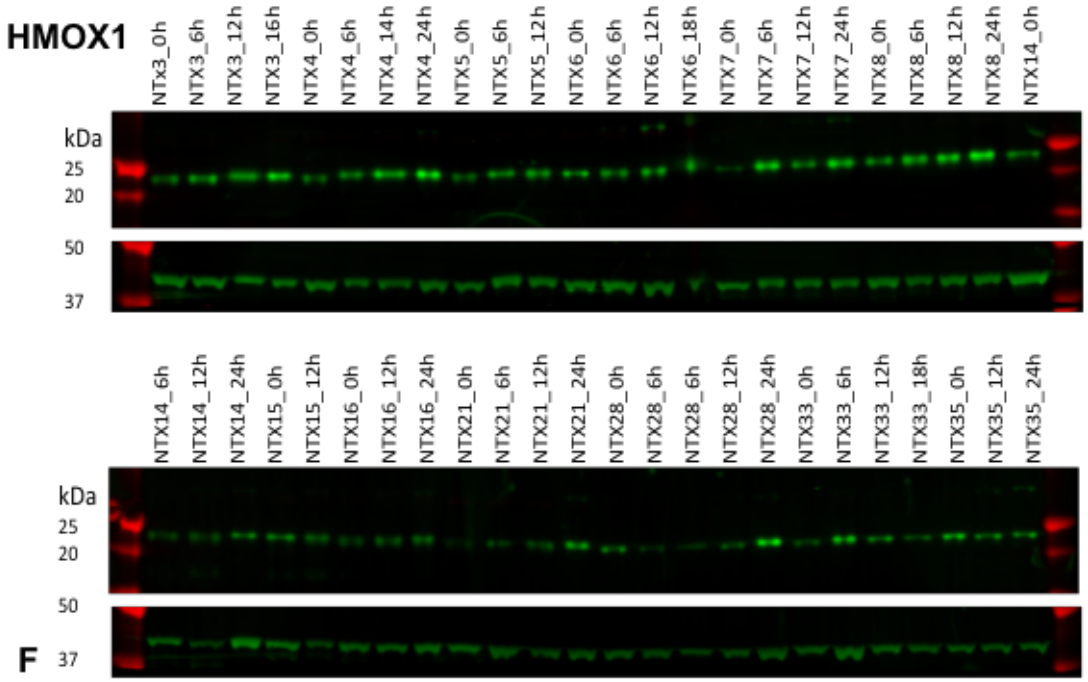
Figures 4.14A-O: NTx3=Kidney 4, NTx4=Kidney 5, NTx5=Kidney 6, NTx6=Kidney 7, NTx7=Kidney 8, NTx8=Kidney 9, NTx14=Kidney 15, NTx15=Kidney 16, NTx16=Kidney 17, NTx21=Kidney 22, NTx28=Kidney 29, NTx33=Kidney 34, NTx35=Kidney 36.

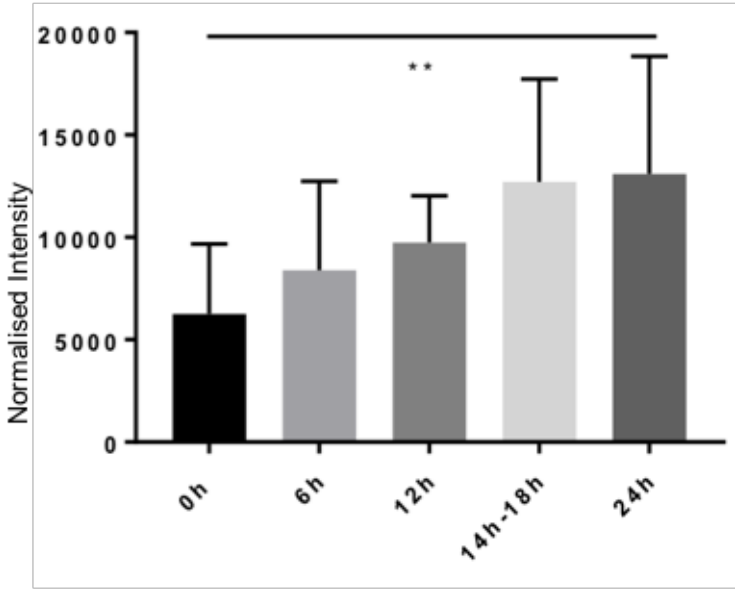

ICAM-1
NTx3_0h
NTX3_6h
NTX3_12h
NTX3_16h
NTX4_0h
NTX4_6h
NTX4_14h
NTX4_24h
NTX5_0h
NTX5_6h
NTX5_12h
NTX6_0h
NTX6_6h
NTX6_12h
NTX6_18h
NTX7_0h
NTX7_6h
NTX7_12h
NTX7_24h
NTX8_0h
NTX8_6h
NTX8_12h
NTX8_24h
NTX14_0h
kDa
100
75
50
37
NTX14_6h
NTX14_12h
NTX14_24h
NTX15_0h
NTX15_12h
NTX16_0h
NTX16_12h
NTX16_24h
NTX21_0h
NTX21_6h
NTX21_12h
NTX21_24h
NTX28_0h
NTX28_6h
NTX28_12h
NTX28_24h
NTX33_0h
NTX33_6h
NTX33_12h
NTX33_18h
NTX35_0h
NTX35_12h
NTX35_24h
kDa
100
75
50
37
B

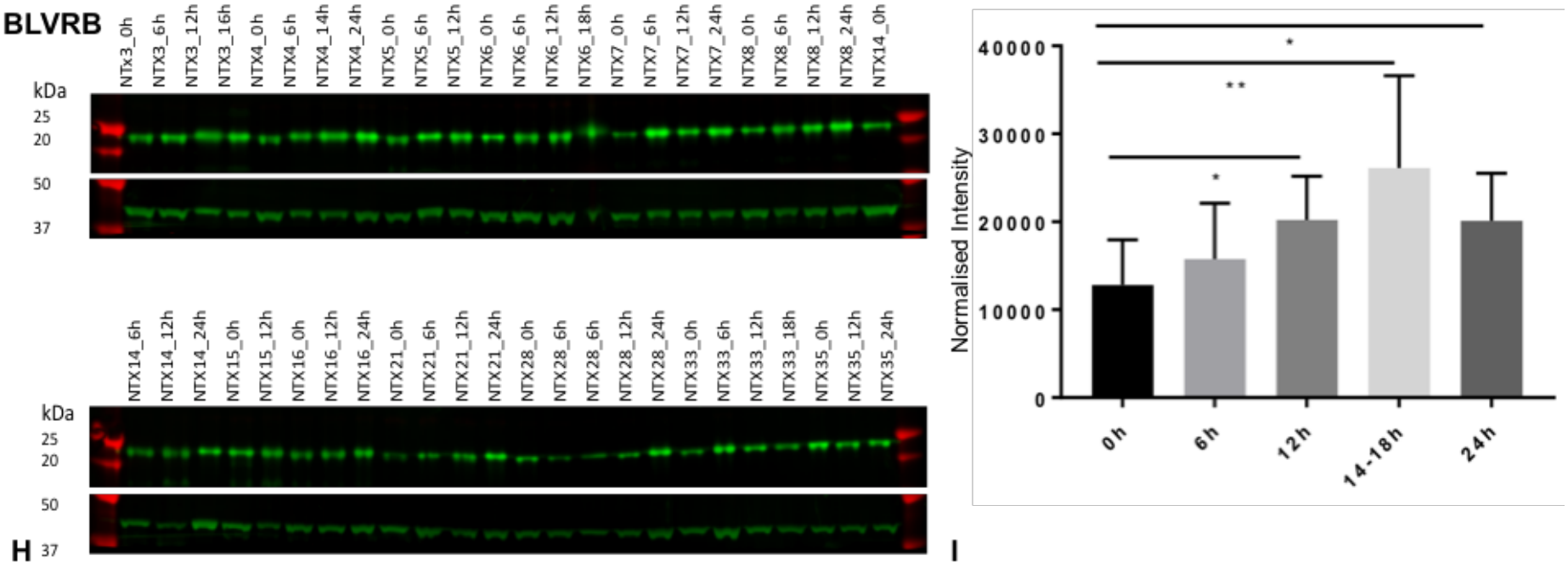

Normalised Intensity
60000
40000
20000
0
**
**
**
0h
6h
12h
14-16h
24h
C

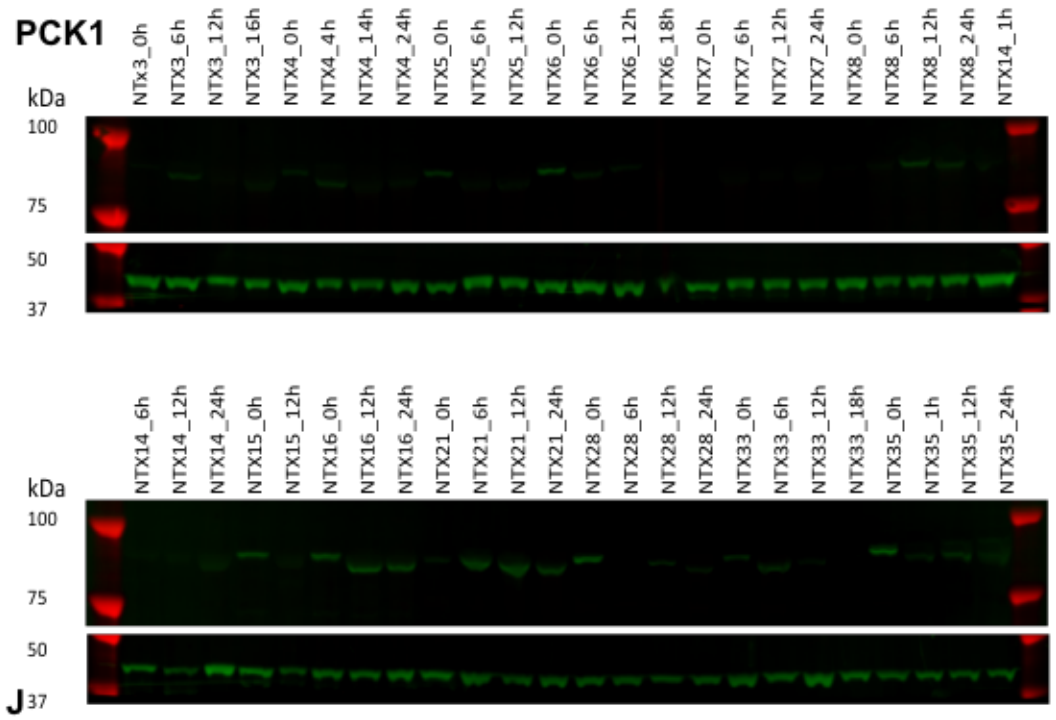

SLC4A1
NTx3_0h
NTX3_6h
NTX3_12h
NTX3_16h
NTX4_0h
NTX4_6h
NTX4_14h
NTX4_24h
NTX5_0h
NTX5_6h
NTX5_12h
NTX6_0h
NTX6_6h
NTX6_12h
NTX6_18h
NTX7_0h
NTX7_6h
NTX7_12h
NTX7_24h
NTX8_0h
NTX8_6h
NTX8_12h
NTX8_24h
NTX14_0h
kDa
100
75
50
37
NTX14_6h
NTX14_12h
NTX14_24h
NTX15_0h
NTX15_12h
NTX16_0h
NTX16_12h
NTX16_24h
NTX21_0h
NTX21_6h
NTX21_12h
NTX21_24h
NTX28_0h
NTX28_6h
NTX28_6h
NTX28_12h
NTX28_24h
NTX33_0h
NTX33_6h
NTX33_12h
NTX33_18h
NTX35_0h
NTX35_12h
NTX35_24h
kDa
100
75
50
37
D

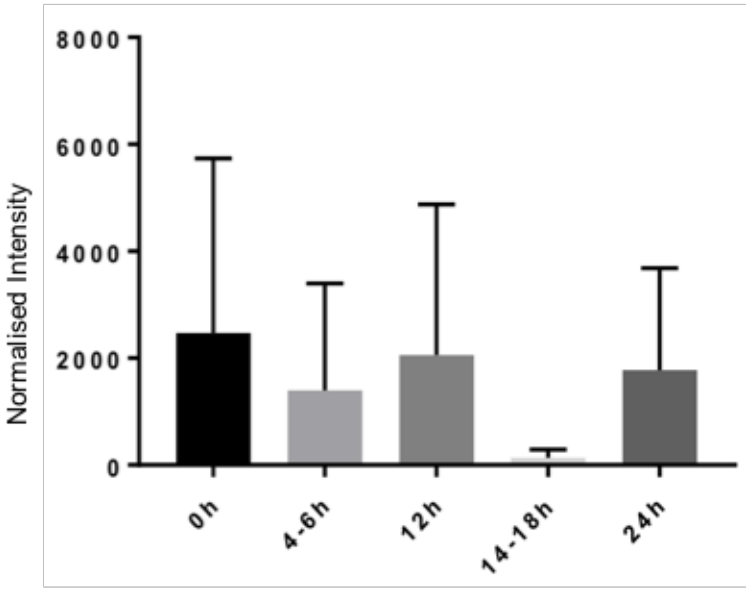

Normalised Intensity
200000
150000
100000
50000
0
****
****
***
***
0h
6h
12h
14-18h
24h
E

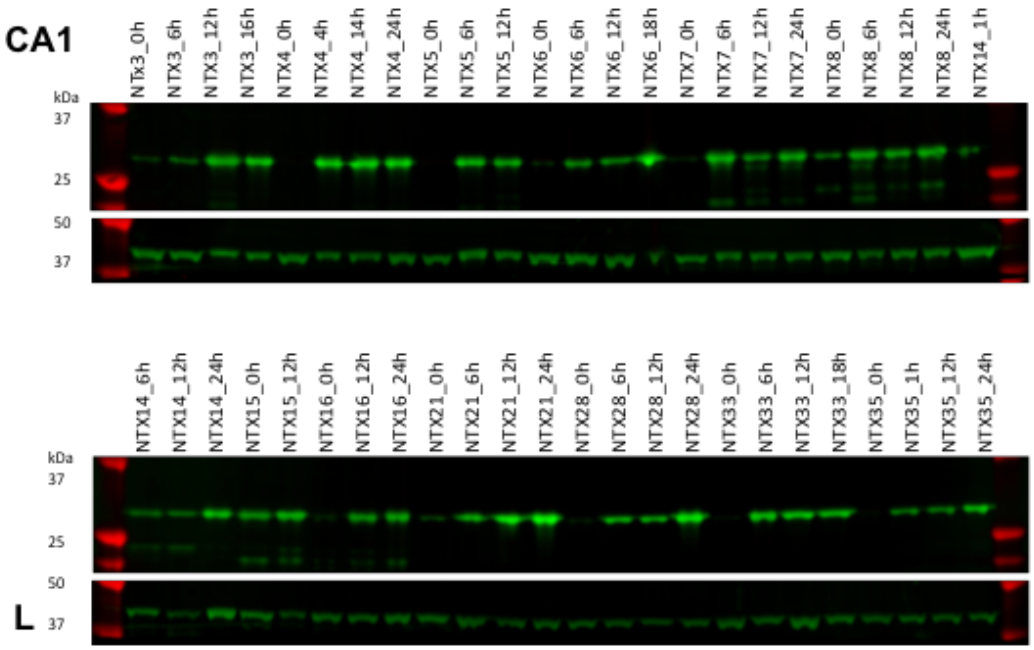

HMOX1
NTx3_0h
NTX3_6h
NTX3_12h
NTX3_16h
NTX4_0h
NTX4_6h
NTX4_14h
NTX4_24h
NTX5_0h
NTX5_6h
NTX5_12h
NTX6_0h
NTX6_6h
NTX6_12h
NTX6_18h
NTX7_0h
NTX7_6h
NTX7_12h
NTX7_24h
NTX8_0h
NTX8_6h
NTX8_12h
NTX8_24h
NTX14_0h
kDa
25
20
50
37
NTX14_6h
NTX14_12h
NTX14_24h
NTX15_0h
NTX15_12h
NTX16_0h
NTX16_12h
NTX16_24h
NTX21_0h
NTX21_6h
NTX21_12h
NTX21_24h
NTX28_0h
NTX28_6h
NTX28_12h
NTX28_24h
NTX33_0h
NTX33_6h
NTX33_12h
NTX33_18h
NTX35_0h
NTX35_12h
NTX35_24h
kDa
25
20
50
37
F

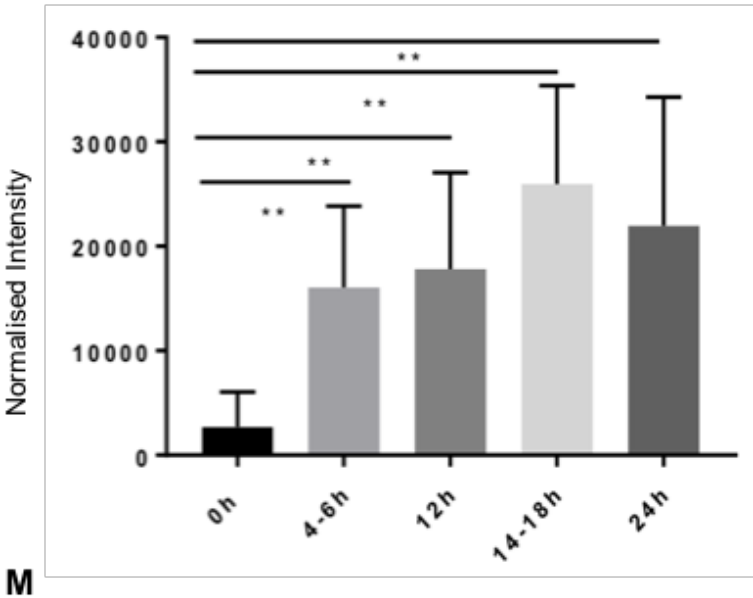

Normalised Intensity
20000
15000
10000
5000
0
**
0h
6h
12h
14h-18h
24h
G

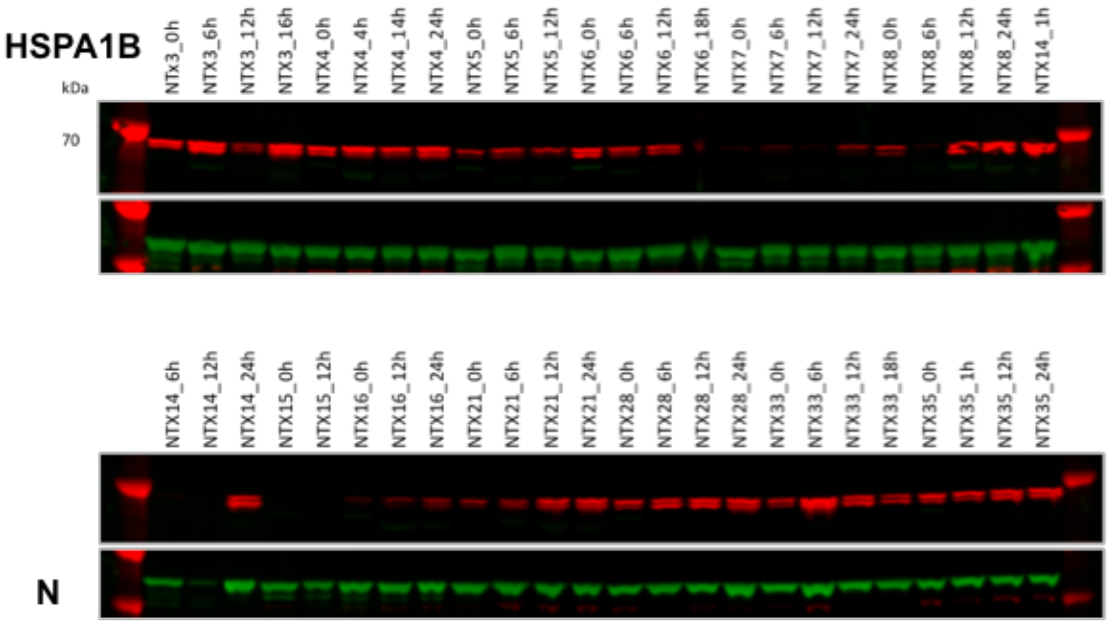

BLVRB
NTx3_0h
NTX3_6h
NTX3_12h
NTX3_16h
NTX4_0h
NTX4_6h
NTX4_14h
NTX4_24h
NTX5_0h
NTX5_6h
NTX5_12h
NTX6_0h
NTX6_6h
NTX6_12h
NTX6_18h
NTX7_0h
NTX7_6h
NTX7_12h
NTX7_24h
NTX8_0h
NTX8_6h
NTX8_12h
NTX8_24h
NTX14_0h
kDa
25
20
50
37
NTX14_6h
NTX14_12h
NTX14_24h
NTX15_0h
NTX15_12h
NTX16_0h
NTX16_12h
NTX16_24h
NTX21_0h
NTX21_6h
NTX21_12h
NTX21_24h
NTX28_0h
NTX28_6h
NTX28_12h
NTX28_24h
NTX33_0h
NTX33_6h
NTX33_12h
NTX33_18h
NTX35_0h
NTX35_12h
NTX35_24h
kDa
25
20
50
37
H
Normalised Intensity
40000
30000
20000
10000
0
*
**
*
0h
6h
12h
14-18h
24h
I


PCK1
kDa
100
75
50
37
NTx3_0h
NTX3_6h
NTX3_12h
NTX3_16h
NTX4_0h
NTX4_4h
NTX4_14h
NTX4_24h
NTX5_0h
NTX5_6h
NTX5_12h
NTX6_0h
NTX6_6h
NTX6_12h
NTX6_18h
NTX7_0h
NTX7_6h
NTX7_12h
NTX7_24h
NTX8_0h
NTX8_6h
NTX8_12h
NTX8_24h
NTX14_1h
kDa
100
75
50
37
NTX14_6h
NTX14_12h
NTX14_24h
NTX15_0h
NTX15_12h
NTX16_0h
NTX16_12h
NTX16_24h
NTX21_0h
NTX21_6h
NTX21_12h
NTX21_24h
NTX28_0h
NTX28_6h
NTX28_12h
NTX28_24h
NTX33_0h
NTX33_6h
NTX33_12h
NTX33_18h
NTX35_0h
NTX35_1h
NTX35_12h
NTX35_24h
J

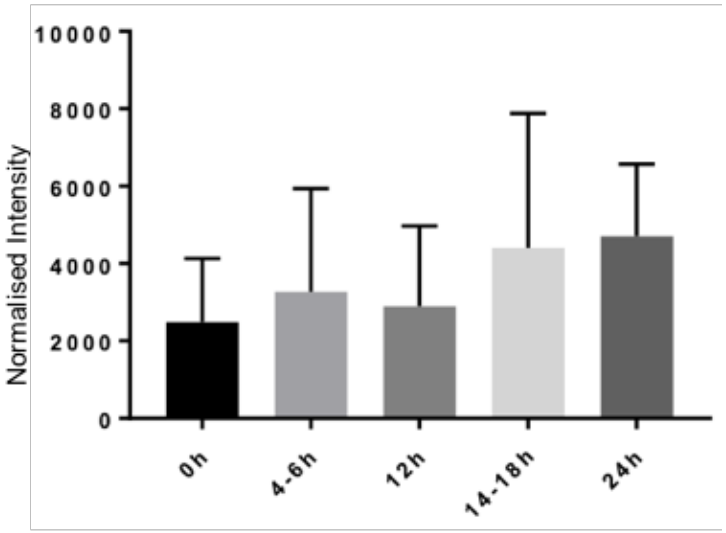

Normalised Intensity
8000
6000
4000
2000
0
0h
4-6h
12h
14-18h
24h
K


CA1
NTx3_0h
NTX3_6h
NTX3_12h
NTX3_16h
NTX4_0h
NTX4_4h
NTX4_14h
NTX4_24h
NTX5_0h
NTX5_6h
NTX5_12h
NTX6_0h
NTX6_6h
NTX6_12h
NTX6_18h
NTX7_0h
NTX7_6h
NTX7_12h
NTX7_24h
NTX8_0h
NTX8_6h
NTX8_12h
NTX8_24h
NTX14_1h
kDa
37
25
50
37
NTX14_6h
NTX14_12h
NTX14_24h
NTX15_0h
NTX15_12h
NTX16_0h
NTX16_12h
NTX16_24h
NTX21_0h
NTX21_6h
NTX21_12h
NTX21_24h
NTX28_0h
NTX28_6h
NTX28_12h
NTX28_24h
NTX33_0h
NTX33_6h
NTX33_12h
NTX33_18h
NTX35_0h
NTX35_1h
NTX35_12h
NTX35_24h
kDa
37
25
50
37
L


Normalised Intensity
40000
30000
20000
10000
0
**
**
**
**
0h
4-6h
12h
14-18h
24h
M


HSPA1B
kDa
70
NTx3_0h
NTX3_6h
NTX3_12h
NTX3_16h
NTX4_0h
NTX4_4h
NTX4_14h
NTX4_24h
NTX5_0h
NTX5_6h
NTX5_12h
NTX6_0h
NTX6_6h
NTX6_12h
NTX6_18h
NTX7_0h
NTX7_6h
NTX7_12h
NTX7_24h
NTX8_0h
NTX8_6h
NTX8_12h
NTX8_24h
NTX14_1h
NTX14_6h
NTX14_12h
NTX14_24h
NTX15_0h
NTX15_12h
NTX16_0h
NTX16_12h
NTX16_24h
NTX21_0h
NTX21_6h
NTX21_12h
NTX21_24h
NTX28_0h
NTX28_6h
NTX28_12h
NTX28_24h
NTX33_0h
NTX33_6h
NTX33_12h
NTX33_18h
NTX35_0h
NTX35_1h
NTX35_12h
NTX35_24h

N


Normalised Intensity
10000
8000
6000
4000
2000
0
0h
4-6h
12h
14-18h
24h

O

Chapter 5: Immunological effects of normothermic perfusion on human kidneys

5.1 The importance of normothermic perfusion from an immunological point of view

The human kidney is equipped with a complex resident immune system. Besides hosting tissue-resident macrophages (184), the kidney harbours lymphocytes including innate lymphoid cells, natural killer cells, natural killer T cells and γδ T cells (185). The immune population of the kidney remains in a state of homeostatic balance unless stimulated, for example by the insult of brain death in an organ donor, at which point a state of immunological activation may be induced. The response to a severe injury such as brain death, includes both local and systemic inflammation comprising a cytokine storm and cellular infiltrates (186). The result of this, with current preservation strategies, is that the kidney is transplanted unchanged in a state of immunological activation into a recipient in whom the immune response will occur immediately after transplantation via alloantigens, presented by donor-derived antigen presenting cells to recipient T cells (187,188). The importance of donor-derived leukocytes in the allorecognition and rejection process has been investigated and explained previously. Lechler and Batchelor demonstrated that dendritic cells were the principle “passenger leukocytes”, responsible for activating recipient T cells. Transplanted rat kidneys were “parked” in an intermediate recipient to deplete passenger leukocytes prior to the implantation in the final recipient, demonstrating that “clearance” of passenger leukocytes prolonged allograft survival (189,190).

This strategy of parking the kidney in an intermediate host is clearly not feasible in the clinical setting. However, instead of using an intermediate recipient, it is perhaps possible that preconditioning of the donor kidney by means of normothermic perfusion could be used to achieve the same end. This ex vivo setting might provide, therefore,

not only a different method of organ preservation, but also a means to assess the organ either prior to transplantation or to study its function and immunological characteristics in isolation.

The aim of the studies described in this chapter was to analyse the immune effector systems in the perfusate in a normothermic human kidney perfusion model. This included analysis of cellular subsets, cytokines and chemokines and changes in these over time.

The perfusate samples for the cell and cytokine analyses were collected and prepared by myself. The sample analyses were run by John Stone in the laboratory of James Fildes at The University of Manchester. The data interpretation was done by myself in consultation with the Fildes' team. The support and guidance of both were essential and greatly appreciated.

5.2 Measurement of immune cells in the normothermic perfusion circuit

5.2.1 Kidney perfusions undergoing cell counting with flow cytometry

Perfusates of twelve consecutive normothermic human kidney perfusions were collected according to the protocol mentioned under 2.5.5 Chapter 2.

Eight perfusions were with urine recirculation including four DBD and four DCD kidneys. Median (min-max) age of the DBD kidneys was 68 (47-76) years with a median CIT of 15 (12-17) hours. DCD kidneys were about ten years younger with a median age of 59.5 (44-62) years and a median cold storage time approximately twice as long as in DBD kidneys with 31.5 (19-46) hours.

Four kidney perfusions were performed without urine recirculation; three DBD and one DCD kidneys. The DCD organ was from a 74-year old donor and perfusion was started

after 22 hours of CIT. Median (min-max) age of DBD kidneys was 71 (71-78) years with a median CIT of 21 (18-46) hours. The excreted urine was replaced 1:1 with Ringer's lactate.

The perfusate samples for cell analysis were drawn at following time points: within the first 30 minutes after perfusion start, after one, six, twelve and 24 hours, where applicable, of NMP.

The cellular subsets measured over time were: total leukocytes; total T cells; CD4+ T cells; CD8+ T cells; B cells; classical, intermediate and non-classical monocytes; eosinophils, neutrophils, mast cells, NK cells, NK T cells, macrophages; FoxP3+ T cells.

5.2.2 Perfusions with urine recirculation

The median (min-max) perfusion time of the eight kidneys with urine recirculation was 21 (6.3-24.1) hours. The numbers of cells in the perfusate 30 minutes, one and six hours after start of NMP and their distribution are shown in table 5.1. The only parameter which changed significantly over time was the total number of leukocytes. This increased significantly within the first hour of perfusion ($p<0.001$). After hour one, the total number of leukocytes decreased slightly and then remained stable throughout the duration of NMP ($p=0.63$).

Table 5.1: Flow cytometry results*	**Kidneys with urine recirculation (n=8)**			**Kidneys without urine recirculation (n=4)**			***p-value****	***p-value***	***p-value***
	30 minutes**	**1 hour**	**6 hours**	**30 minutes**	**1 hour**	**6 hours**	***30 minutes***	***1 hour***	***6 hours***
Total leukocytes (mean, SD)	212382 ± 135908	306730 ± 132277	292742 ± 155986	132875 ± 68890	193359 ± 121078	231336 ± 226842	*0.30*	*0.18*	*0.59*
Total T Cells (mean, SD)	44322 ± 24387	65638 ± 27682	75114± 46080	25532 ± 15717	36998 ± 27799	43304 ± 40270	*0.20*	*0.12*	*0.27*
CD4 T cells (mean, SD)	17640 ± 12830	27250 ± 22979	34975 ± 27815	7730 ± 5366	9755 ± 6382	19802 ± 24614	*0.18*	*0.17*	*0.38*
CD8 T cells (mean, SD)	22036 ± 11777	32582 ± 12330	33567 ± 18560	14586 ± 10751	21471 ± 20100	18085 ± 13478	*0.31*	*0.26*	*0.17*
% FoxP3+ T cells (mean, SD)	2.7 ± 2.6	1.2 ± 1	1.9 ± 1.8	3.3 ± 1.6	3 ± 2	4.2 ± 3.6	*0.69*	*0.06*	*0.16*
NK T cells (mean, SD)	4641 ± 3021	5788 ± 4220	10024 ± 7519	6253 ± 3241	7406 ± 3060	7939 ± 3051	*0.41*	*0.51*	*0.61*
B cells (mean, SD)	19374 ± 14557	22839 ± 14305	32075 ± 21821	34466 ± 38197	33025 ± 38022	108920 ± 180341	*0.33*	*0.50*	*0.24*
Monocytes									
Classical (mean, SD)	9158 ± 8499	15286 ± 9791	8735 ± 7249	6789 ± 1762	14195 ± 10512	6073 ± 2675	*0.60*	*0.86*	*0.50*
Intermediate (mean, SD)	4712 ± 3657	8949 ± 6846	1904 ± 1967	4318 ± 2095	12209 ± 7170	1277 ± 915	*0.85*	*0.46*	*0.56*
Non-classical (mean, SD)	3534 ± 1748	8379 ± 4963	4197 ± 2843	1976 ± 1614	3023 ± 1536	1134 ± 733	*0.17*	*0.07*	*0.06*
Eosinophils (mean, SD)	7999 ± 15917	4370 ± 8524	11999 ± 21539	478 ± 429	425 ± 478	729 ± 339	*0.38*	*0.39*	*0.33*
Neutrophils (mean, SD)	890 ± 2162	441 ± 318	390 ± 577	165 ± 215	287 ± 230	1331 ± 1384	*0.53*	*0.41*	*0.12*
Mast Cells (mean, SD)	41 ± 116	68 ± 94	0	52 ± 105	0	0	*0.87*	*0.19*	*n.a.*
NK cells (mean, SD)	34784 ± 40916	41443 ± 31866	31449 ± 26835	9599 ± 6818	17083 ± 12134	4450 ± 3137	*0.26*	*0.18*	*0.08*
Macrophages (mean, SD)	5394 ± 5048	8618 ± 8236	4944 ± 3058	5356 ±3027	24350 ± 22587	8774 ± 8512	*0.99*	*0.10*	*0.27*

* number of cells in cells/ml; overall perfusate volume = 500ml

** time after start of NMP

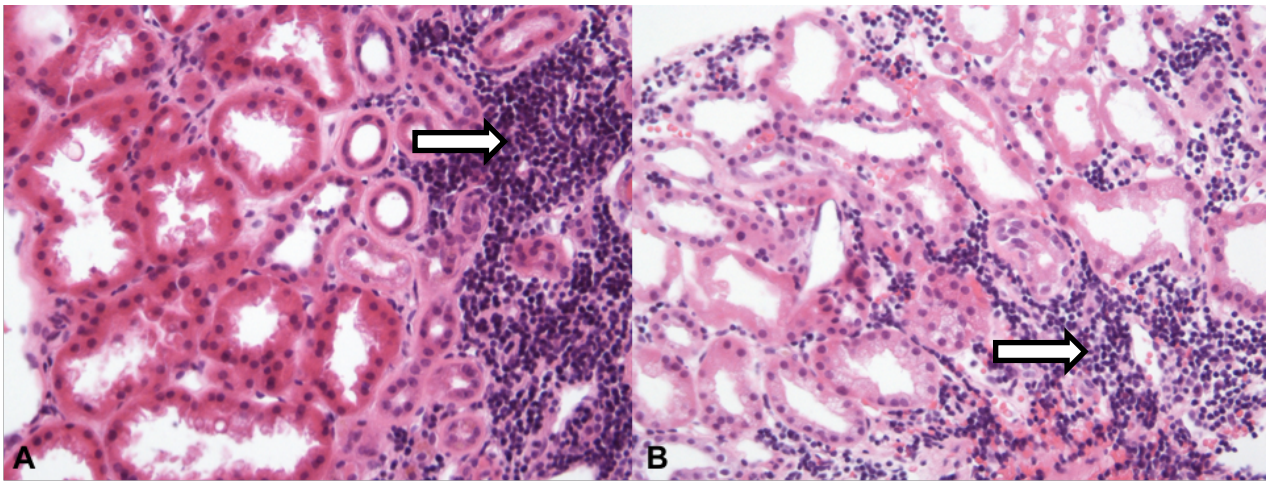
*** p-value result of comparison with and without urine recirculation

n.a. = not applicable

5.2.3 Perfusions without urine recirculation

The median (min-max) perfusion time of the four kidneys without urine recirculation was 7.2 (6.1-9.4) hours. The number of cells in the perfusate 30 minutes, one and six hours after start of NMP and the distribution is shown in table 5.1. Similar to kidneys without urine recirculation, the total number of leukocytes increased significantly within the first hour of NMP ($p<0.001$) in the perfusate. In contrast to the kidney perfusates with urine recirculation, leukocytes increased until hour six after perfusion start, although not significantly ($p=0.18$).

Also, in contrast to kidneys with urine recirculation, the only other cells which changed significantly over time in the perfusate were B cells. These increased significantly between the first measurement after 30 minutes and the last measurement after six hours of NMP ($p=0.002$). Notably, one kidney (kidney 30, table 3.1) in this group was declined for transplant due to a lesion on the partner kidney. Histology of this kidney revealed a monomorphic cell infiltration highly suspicious of haematological malignancy (eg lymphoma). This might have had some bearing on the significant increase of B cells in the perfusate, although we do not have immunohistochemistry results which could prove/identify the cell infiltration as B cells in the tissue (figures 5.1A-B). The B cell results of this single case stands out according to the number of cells revealed by flow cytometry: 90722 cells/ml after 30 minutes (kidneys 31-33 25845, 9280 and 12020 cells/ml), 379431 cells/ml after six hours (kidneys 31-33 18832, 17843 and 19578 cells/ml). Kidneys perfused with urine recirculation had also B cell results (median, min-max) less than detected in the perfusate of kidney 30: 17692 (2337-39076) cells/ml after 30 minutes; 31089 (5979-63390) cells/ml after six hours of NMP with urine recirculation.



Figures 5.1A-B: Photograph A of the performed H&E staining shows the monomorphic cell infiltrate (arrows) in the zero biopsy of kidney 30. Photograph B shows the monomorphic cell infiltrate after six hours of normothermic perfusion without urine recirculation in kidney 30.

5.2.4 Comparison of perfusates with and without recirculated urine

There were no significant differences in the number of cells and cell types between the two versions of perfusion setup. Table 5.1 gives an overview showing the number of cells at three different timepoints. Hour six was chosen as the latest time point for comparison, as the perfusions in the group without urine recirculation did not function in large enough numbers beyond this point, in contrast to kidneys with a fully-closed circuit.

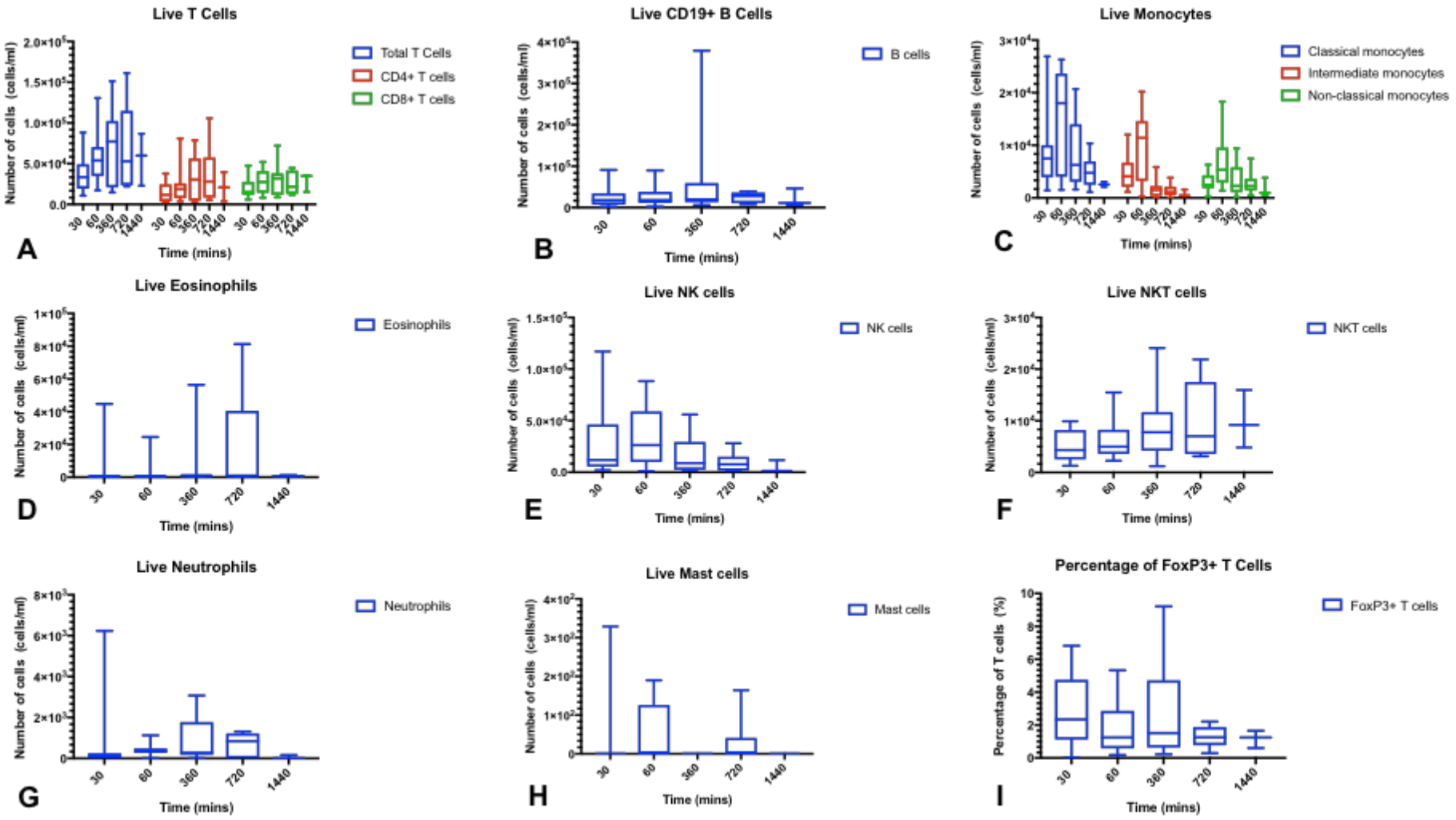
Cell numbers are expressed as cells/ml and the overall perfusate volume in the circuit was 500ml. It needs to be considered that, despite the same perfusate volume in both groups, the composition of the perfusate changed over time as the excreted urine was replaced.

5.2.5 Graphical representation of cells in normothermic perfusion

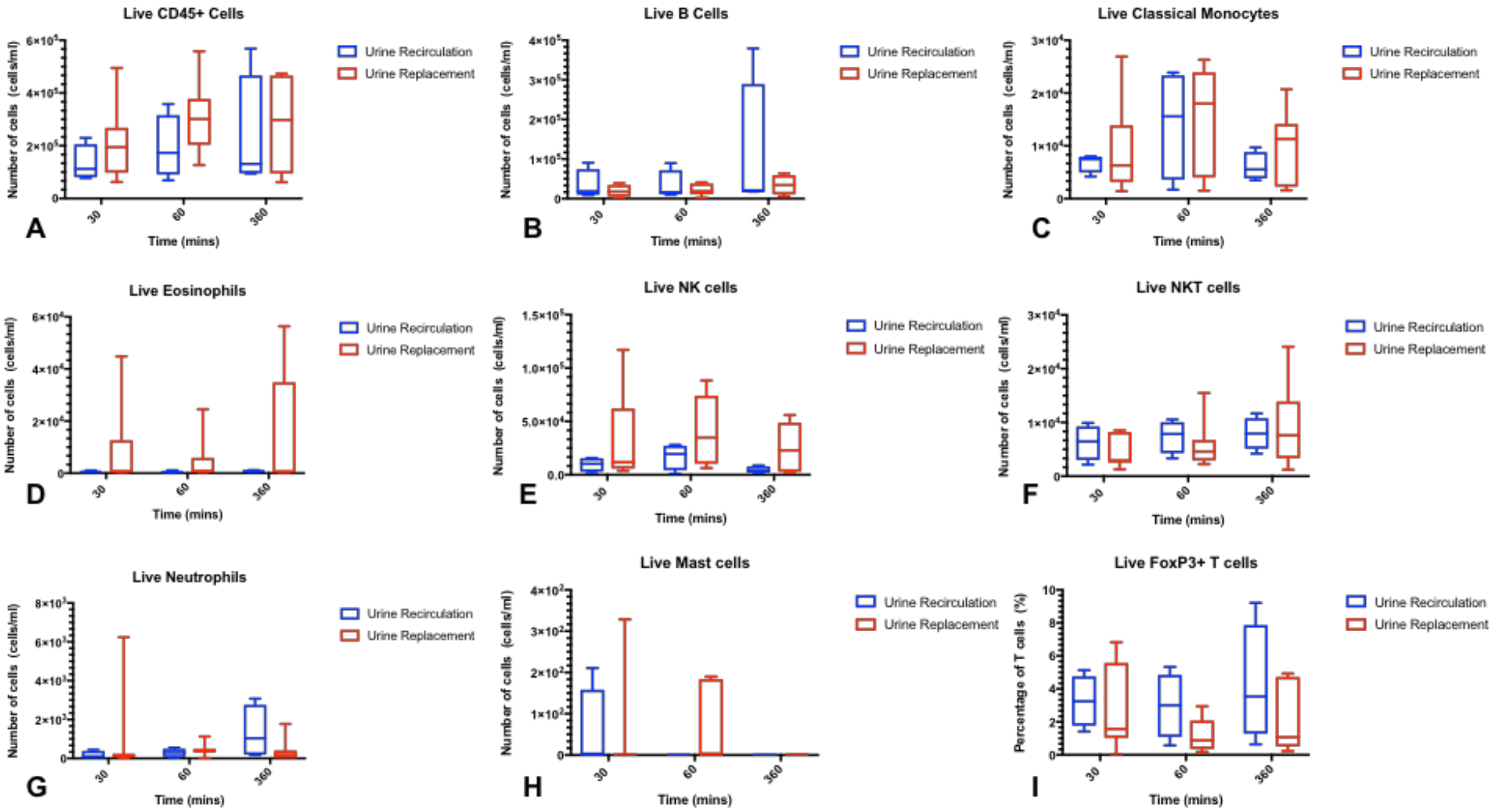
Figures 5.2A-I show cells in all samples combined (n=12 kidney perfusions) at the different timepoints. Cell types and their change over time displayed are live T cells (total, CD4+, CD8+) in figure 5.2A; live CD19+ B cells in figure 5.2B; live monocytes

(classical, intermediate and non-classical) in figure 5.2C; live eosinophils in figure 5.2D; live NK cells in figure 5.2E; live NK T cells in figure 5.2F; live neutrophils in figure 5.2G; live mast cells in figure 5.2H and percentage of FoxP3+ T cells in figure 5.2I.

Figures 5.3A-I illustrate the comparison of the cells mentioned above in perfusions with urine recirculation and perfusions with Ringer's lactate as 1:1 replacement for the amount of excreted urine.



Figures 5.2A-I: Cells in all samples combined (n=12 kidney perfusions); eight with and four without urine recirculation.



Figures 5.3A-I: Comparison of cells in the perfusate (cells/ml) in NMP kidney perfusions with (n=8) and without (n=4) urine recirculation.

5.3 Measurement of cytokines in the normothermic perfusion circuit

5.3.1 Cytokine analysis with Luminex®

Perfusates of 27 consecutive normothermic human kidney perfusions were collected according to the protocol mentioned under 2.5.6 Chapter 2.

Twenty-three perfusions were with urine recirculation including eleven DBD and twelve DCD kidneys. Median (min-max) age of the DBD kidneys was 69 (47-76) years with a median CIT of 75 (10-108) hours. DCD kidneys were from younger donors with a median age of 54 (38-72) years and a median cold storage time of 33.5 (14-104) hours.

Four kidney perfusions were performed without urine recirculation; three DBD and one DCD. The DCD organ was from a 74-year old donor and perfusion was started after 22 hours of CIT. Median (min-max) age of DBD kidneys was 71 (71-78) years with a median CIT of 21 (18-46) hours. The excreted urine was replaced 1:1 with Ringer's lactate.

The perfusate samples for cell analysis were drawn at following time points: after one, six, twelve, 18 and 24 hours, where applicable, of NMP start.

The cytokines measured over time were: Granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN), interleukin (IL) 10, IL-12p40, IL-12p70, IL-1RA, IL-1a, IL-1b, IL-2, IL-4, IL-6, IL-8 and tumour necrosis factor (TNF).

Some of the mentioned cytokines, are such with principal anti-inflammatory activities; IL-1RA, IL-4, IL-6 and IL-10. The function and the definition of an anti-inflammatory cytokine is the ability to inhibit the synthesis of IL-1, TNF and other proinflammatory cytokines (191,192)

5.3.2 Perfusions with urine recirculation

The median (min-max) perfusion time of the 23 kidneys with urine recirculation was 24 (6.3-24.4) hours. There were no differences at any time in any of the cytokines or in expression between DBD and DCD kidneys. However, within the DBD perfusion cohort three cytokines changed significantly over time. IL-6 increased significantly with the major proportion of increase between hour one and hour six (p=0.004). Interleukin 1a and IL-2 decreased significantly over time (p=0.004 and p=0.003 respectively). The decline of both cytokines occurred between perfusion start and hour twelve.

The analyses of the DCD-kidney perfusates also showed significant changes in three cytokines. Interleukin 2 decreased between perfusion start and hour twelve (p=0.002). Interleukin 8 increased up to hour twelve (p<0.001). Tumour necrosis factor increased in the first six hours of perfusion (p<0.001), followed by a steady decrease until hour twelve or 24 (p=0.06).

5.3.2.1 Cytokines in perfusions with urine recirculation stratified for 24 hours of CIT

To detect if cold ischaemia time influences cytokine expression, I stratified kidneys perfused (with urine recirculation) according to CIT shorter and longer than 24 hours. Fourteen out of 23 kidneys had an ischaemia time shorter than 24 hours. Direct comparison at each timepoint did not show any difference in cytokine expression between the CIT groups. However, within the group of kidneys with NMP less than 24 hours of CIT, IL-6 increased significantly during the first six hours of NMP (p=0.001). Interleukin 2 decreased significantly until hour twelve (p=0.002).

This contrasted with kidneys with a CIT longer than 24 hours, in which a different set of cytokines changed. Interleukin 8 increased within the first twelve hours of NMP (p=0.002). Tumour necrosis factor increased between perfusion start and hour six

(p=0.003), but thereafter showed a decrease until hour twelve and 24, although not statistically significant (p=0.07 and p=0.06 respectively).

5.3.3 Perfusions without urine recirculation

The median (min-max) perfusion time of the four kidneys was 7.2 (6.1-9.4) hours. The perfusate analysis of the three DBD kidneys revealed three significantly changing cytokines. Similar to kidneys with urine recirculation, IL-8 increased significantly between perfusion start and six hours (p=0.004). The GM-CSF decreased significantly over time (p<0.001). Tumour necrosis factor showed an increase in the DBD kidney perfusions (p=0.04). The cytokines of the only DCD kidney perfusion in this group showed no significant changes during six hours of NMP.

5.3.3.1 Cytokines in urine of kidneys without urine recirculation

Urine was sampled in three (75%) of these perfusions. The second kidney, a DBD kidney, excreted only 50 ml of urine in the first 30 minutes of NMP and was therefore not considered for the analysis. Interleukin 2 and IL-10, both decreased in the excreted urine over time, between hour one and hour six (p<0.001 for IL-2 and for IL-10). Tumour necrosis factor also increased, but not significantly.

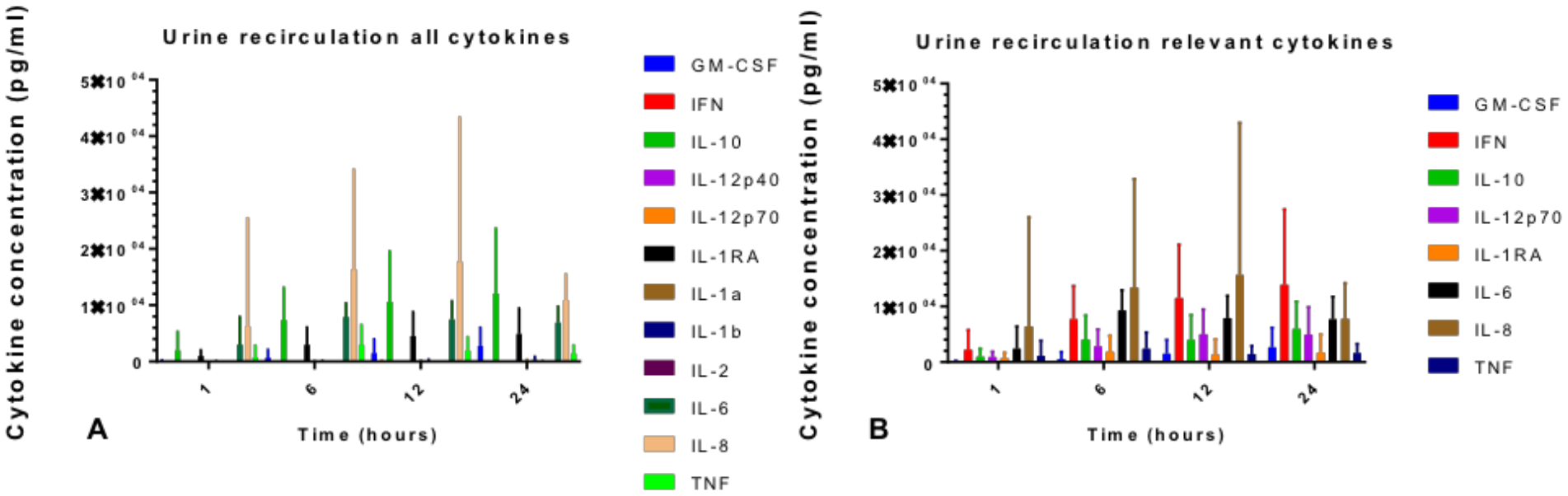
5.3.4 Comparison of perfusates with and without recirculated urine

Cytokine expression after one and six hours of normothermic perfusion was similar in both groups. There were no significant differences at any time. As for the cell analysis already, hour six was chosen as the last time point for comparison, as there were no 24-hour perfusions achieved in the group without urine recirculation. It should be noted that, although the perfusate volume remained the same in both groups, the

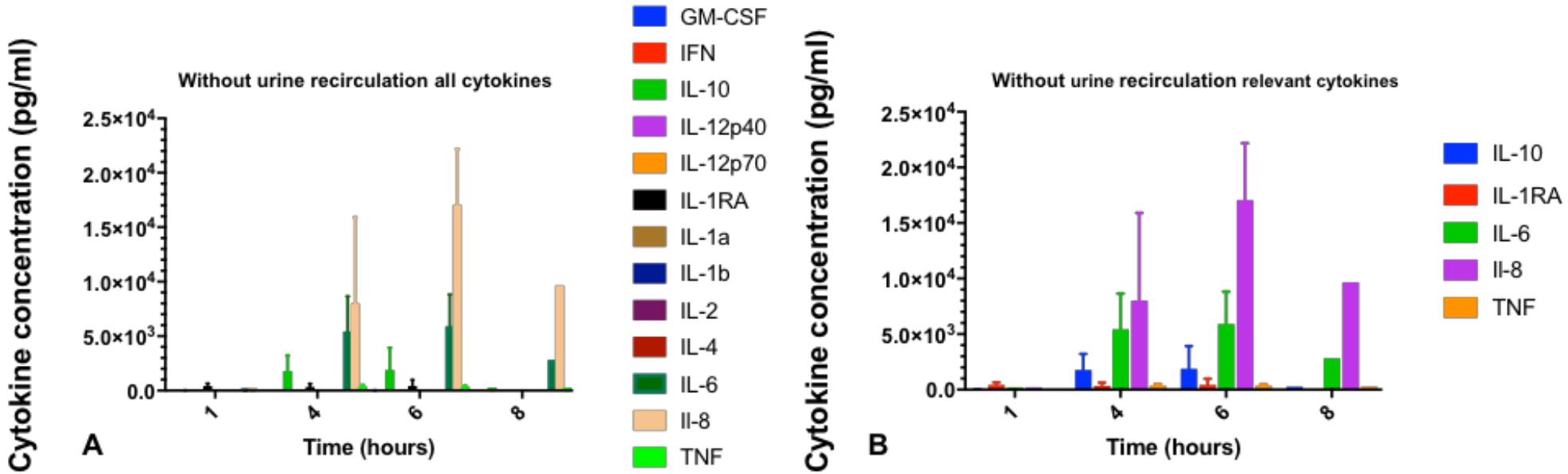
composition of the perfusate changed as the excreted urine was replaced with Ringer's lactate. The loss of cytokines in the urine replacement (as opposed to recirculation) group may have had some bearing on these results. Also, the number of kidneys perfused with the urine replacement version of the circuit was significantly smaller, 4 vs 23 kidney perfusions.

5.3.5 Illustration of cytokines in perfusions with and without urine recirculation

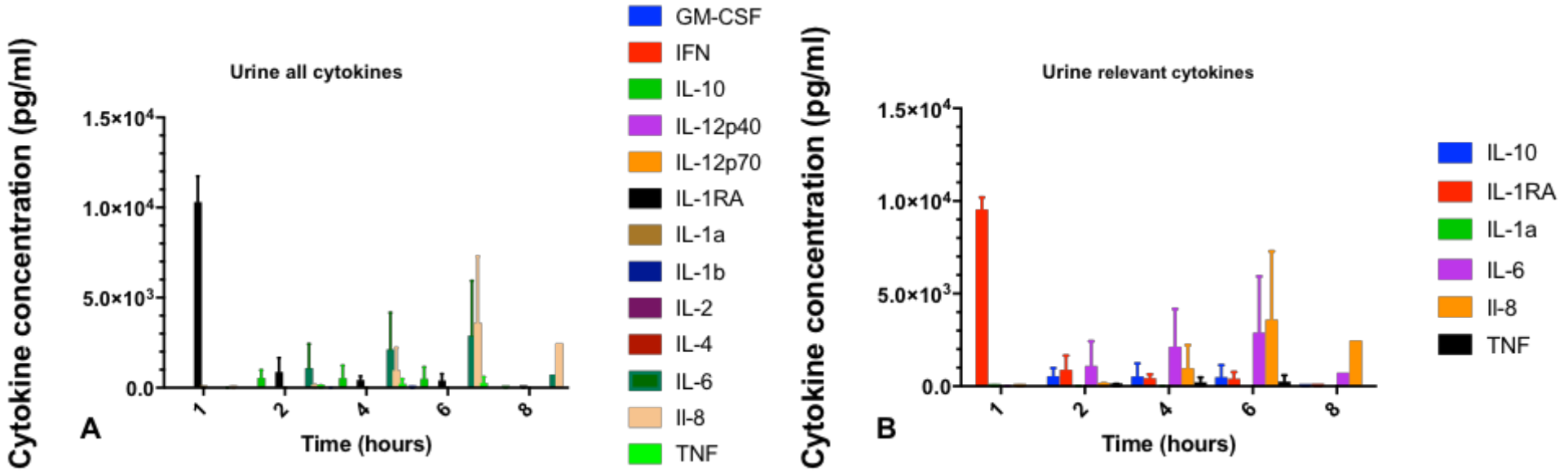
Figures 5.4A-B show cytokines in perfusions with urine recirculation; figures 5.5A-B without. Cytokines are measured in pg/ml; overall circulating volume in the circuit is 500ml. In addition, figures 5.6A-B show cytokines excreted in the urine over time. Figures 5.7A-E compare single cytokines between urine recirculation and urine replacement; IL-1RA, IL-6, IL-8, IL-10 and TNF-α. Figures 5.8A-E display single cytokine comparisons between perfusate and urine in the kidney perfusions without urine recirculation.



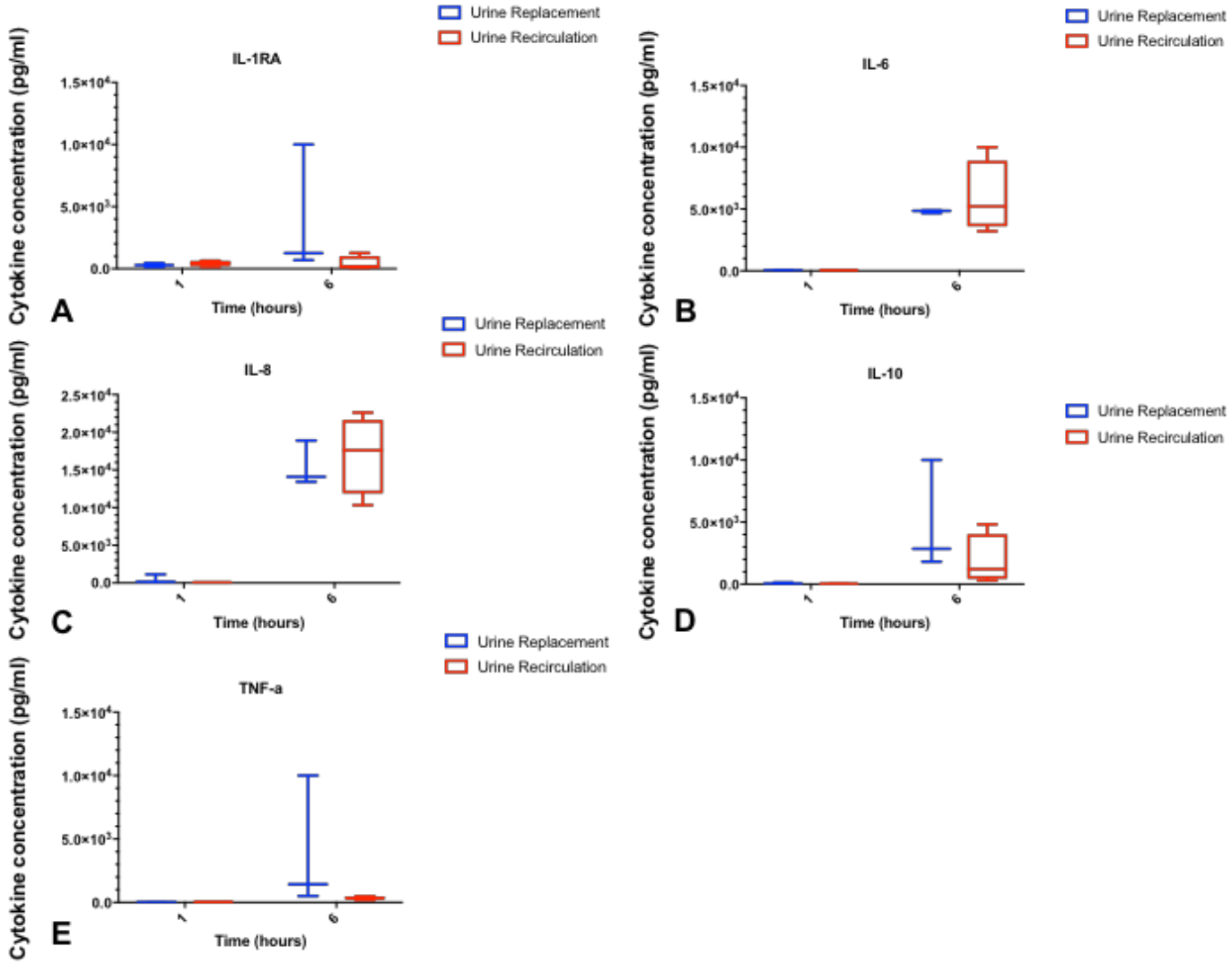
Figures 5.4A-B: Measured cytokines in perfusate samples with urine recirculation.



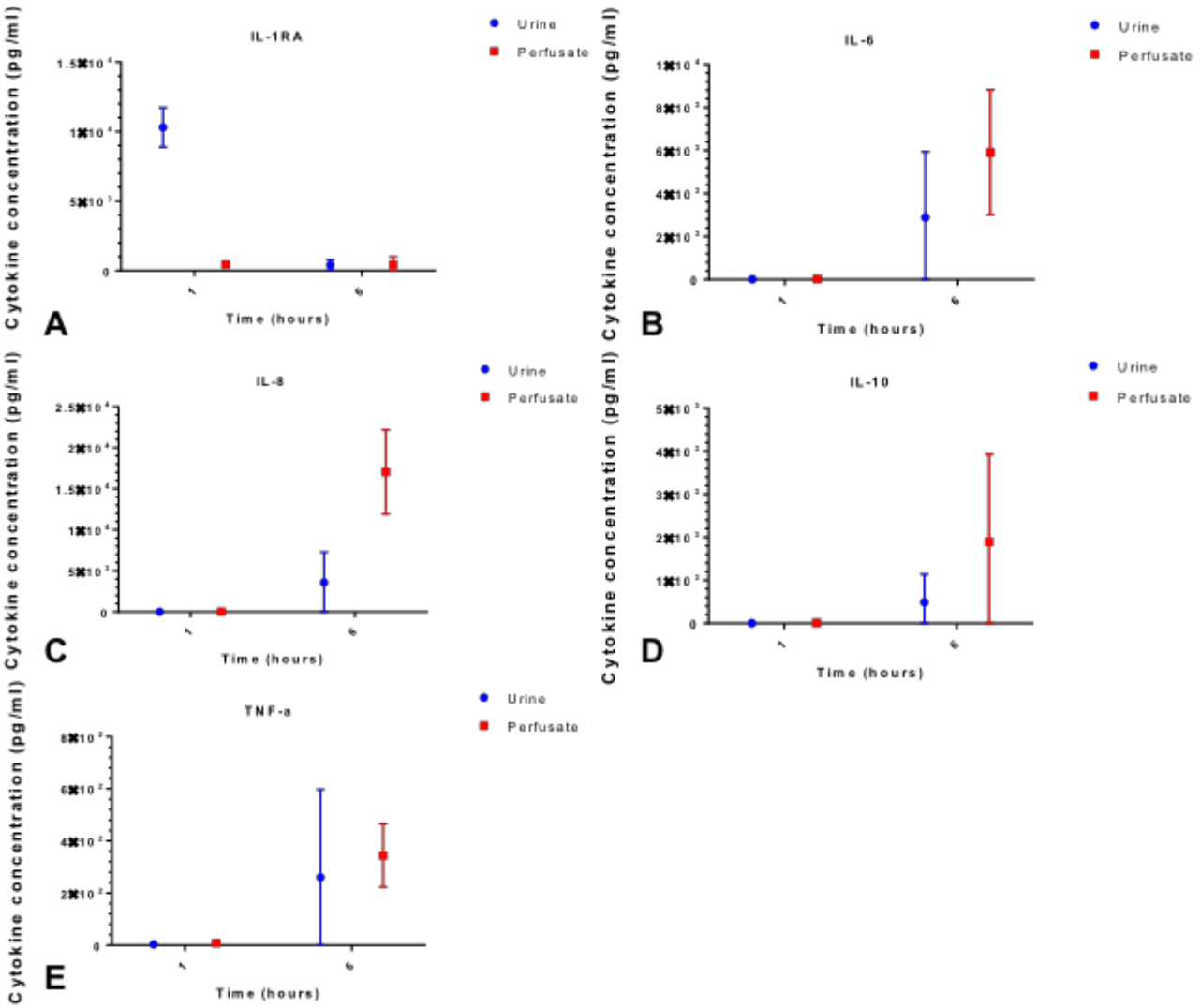
Figures 5.5A-B: Measured cytokines in perfusate samples with urine replacement.



Figures 5.6A-B: Measured cytokines in urine sample



Figures 5.7A-E: Comparison of selected, relevant, cytokines in perfusates with and without urine recirculation.



Figures 5.8A-E: Comparison of selected, relevant, cytokines in perfusate and urine of kidney perfusions without urine recirculation

Chapter 6: Normothermic kidney perfusion in the porcine model

6.1 Aims

After having gained experience with normothermic perfusion (NMP) of a heterogenous group of discarded human kidneys I decided to investigate NMP up to 24 hours on porcine kidneys for the following two reasons:

(i) This would enable me to study the effects of normothermic perfusion on normal, undamaged kidneys, without the variation inevitable in discarded human organs in terms of both underlying disease and preservation injury.

(ii) This would allow me to compare the two kidneys from a single donor – ie to make a valid comparison between urine recirculation and urine replacement.

6.2 Porcine kidneys for NMP

In accordance with the United Kingdom Animal Protection Act 1986, kidney retrieval was performed on five white female landrace pigs with a measured weight of 40-50kg (2.3.2 Chapter 2) between April and June 2018. The organ retrieval took place at the Oxford University Biomedical Services (BMS), University of Oxford. Ten recovered kidney grafts underwent normothermic perfusion at the Institute of Biomedical Engineering (IBME), University of Oxford, using the preclinical prototype device. Haemodynamic and biochemical perfusion parameters were recorded and analysed. Perfusate samples and biopsies were obtained at regular time points during perfusion. Please see Chapter 2 for full description.

6.3 Perfusion protocol

The two perfusions resulting from each donor animal were planned consecutively, with the second kidney being stored on ice until completion of the first perfusion. This, therefore, resulted in an NMP start after either two or 27 hours of cold ischaemia time (CIT).

The reasons for the specific durations of two and 27 hours of CIT are as follows: It takes two hours from the time of clamping the renal arteries in the donor (in the Biomedical Services building) to starting perfusion of the first kidney (in the Institute of Biomedical Engineering building). As there was only one perfusion system available, and, allowing that we had specified a 24-hour period of perfusion, and that it takes about one hour to stop the first perfusion and to prepare for the next, the start for the second kidney had to be planned to happen after 27 hours of CIT.

6.3.1 Composition of perfusate (2.2.2 in Chapter 2)

Cannulation of the porcine kidneys was performed immediately after the bilateral nephrectomy and flushing of the kidneys with Soltran® at the premises of the BMS. The perfusion circuit was assembled and primed with 500ml autologous whole blood using a leukocyte filter (HAEMONETICS® RS1). Before connecting the kidney, whilst waiting for the system to reach 38°C (the physiological temperature of a pig), the perfusate was supplemented with bolus doses of 5000 units of unfractionated heparin, cefuroxime and calcium gluconate. Sodium bicarbonate was not needed in any of the cases as all the perfusates reached a pH of 7.4-7.5 before the kidney was connected and perfusion had been started. This was in marked contrast to the human kidney perfusions. The reason for the higher blood pH lies in the fact that the pig has a higher arterial pH compared to humans, between 7.43 and 7.48 (information sent by e-mail

from the Royal Veterinary College). For glucose control, blood glucose measurements were carried out within 30 minutes after perfusion start and 2-hourly thereafter. A bolus injection of 2.5ml of a lipid free parenteral nutrition solution (TPN) was administered once blood glucose level dropped below 4mmol/l. To optimise the microcirculation and to overcome the tendency of vasospasm of porcine renal arteries (personal experience from previous work with a porcine model), a combination of three vasodilators was used: (i) the prostacyclin epoprostenol sodium in the same dosage as used for discarded human kidneys; (ii) the calcium channel blocker verapamil as a bolus of 2.5mg, administered 5 minutes after perfusion start intra-arterially; (iii) glyceryl trinitrate (GTN) 10mg as a continuous infusion within the first hour after perfusion start. In kidney perfusions without urine recirculation, the perfusate included also Ringer's lactate as a volume replenishment 1:1 for the excreted urine.

The selection, mode of administration (infusion or bolus) and dose of the agents used to improve arterial flow were based on:

(i) Agents known to be effective in optimising microcirculation (epoprostenol)
(ii) Agents known to be effective in causing vasodilatation (verapamil and GTN)
(iii) Agents that would be clinically approved
(iv) Consideration of the half-life and clearance of the agents in a kidney perfusion circuit

6.3.1.1 Epoprostenol sodium (Flolan®)

Epoprostenol sodium is a naturally occurring prostaglandin produced by the intima of blood vessels. This is a potent vasodilator and also inhibits platelet aggregation, inflammation, and vascular smooth muscle proliferation. Epoprostenol binds to G-

protein (guanine nucleotide-binding protein) coupled prostanoid IP (prostacyclin) receptors and this prostanoid IP receptor binding leads to increased levels of intracellular cyclic adenosine monophosphate resulting in vasodilation, platelet inhibition and antiproliferative effects (193,194).

At normal physiological pH and temperature, epoprostenol, which is rapidly distributed from blood to tissue after intravenous administration, breaks down spontaneously. The non-enzymatic spontaneous degradation by hydrolysis results in the metabolite 6-keto-PGF1α. The second metabolite 6,15-diketo-13,14-dihydro-PGF1α is enzymatically formed by 15-hydroxyprostaglandin-dehydrogenase and Δ13-prostaglandin-reductase. The half-life of epoprostenol is between two and six minutes in human whole blood which is estimated from in vitro rates of degradation. The reason for providing in vitro data is the lack of a chemical assay sufficiently sensitive and specific enough to assess the in vivo human pharmacokinetics of epoprostenol. Studies of administering radiolabelled epoprostenol to humans showed a recovery of 82% of the substance within a week in urine (195). Overall, 14 additional metabolites have been isolated from urine which is an indicator for extensive metabolisation of epoprostenol in the human (195–202). All metabolites have pharmacological activity orders of magnitude less than epoprostenol or are inactive (200,203). The very short half-life of the drug demands a continuous infusion. Most importantly, due to the less active and inactive metabolites, urine recirculation does not cause accumulation and toxicity.

6.3.1.2 Verapamil (Securon®)

Verapamil blocks voltage-dependent calcium channels. Calcium channels are present in the smooth muscle of blood vessels. Calcium channel blockers dilate blood vessels

by relaxing the tone of smooth muscle cells. Verapamil hydrochloride is primarily metabolised in the liver and eliminated bi-exponentially with a rapid early distribution phase (half-life about four minutes) and a slower terminal elimination phase (half-life two to five hours). Approximately 70% of the administered dose is excreted in the urine with about 3% to 4% as an unchanged drug in a healthy subject (204). Impaired renal function has little effect on verapamil pharmacokinetics, as demonstrated in studies in patients with end-stage renal failure and with healthy kidneys (205). Verapamil seemed to be the perfect drug to administer as a bolus in our setting (with urine recirculation) as the elimination is predominantly extrarenal, the bioavailability is significantly increased when there is hepatic cirrhosis in humans (equivalent to a missing liver on a “kidney only” circuit). Moreover, the plasma half-life which is three to six hours in humans is known to be increased up to 12 hours when the drug is administered long term due to the saturation of hepatic enzymes, which are not available in our perfusion circuit and as a consequence we can expect an even longer half-life (206). There are no reported negative effects on renal function (204).

6.3.1.3 Glyceryl trinitrate (GTN)

The vasodilating effect of GTN is mediated by its metabolite nitric oxide (NO), which is also known under its synonym endothelial-derived relaxing factor. The activation of soluble guanylate cyclase is induced by NO. This activation upregulates intracellular cyclic guanosine 3’,5’-monophosphate (cGMP). Subsequently, the decrease in cytoplasmic Ca^{2+} concentration leads to vasodilation via relaxation of smooth vascular muscle cells (207).

Different and diverse metabolic pathways are involved in NO production from GTN and moreover, the exact mechanism of GTN biotransformation and its key factors

have not been clarified (207). However, there is evidence that the vasodilating action of GTN is induced by the metabolic conversion of GTN to NO in vascular smooth muscle (208–210). Following five biotransformation pathways have been hypothetically established for GTN metabolism: (i) nonenzymatic pathway mediated by cellular thiol (sulfhydryl) compounds; (ii) nonenzymatic pathway mediated by haemoglobin and myoblobin; (iii) enzymatic pathway mediated by gluthatione S-transferase; (iv) enzymatic pathway mediated by cytochrome P450-related enzymes and (v) membrane-bound microsomal enzymatic pathway which is unidentified (207). The pharmacokinetics of GTN are complex, dependent on its route of administration, influenced by factors as arteriovenous concentration gradient, hydrolysis in the blood, rapid denitration in the liver and other tissues (lungs, kidneys, erythrocytes, vasculature), and by complicated metabolic pathways. The biotransformation of GTN results in less active metabolites (mostly glyceryl dinitrates, further metabolised to glyceryl mononitrates) which are mainly eliminated via the renal route by secretion (207,211). Important for the application in our perfusion set-up: GTN and its less active metabolites are not toxic for the kidneys and there is no liver on board to be involved in a “first-pass” effect (211).

6.3.2 Control, monitoring and collection of haemodynamic parameters

I used the final iteration of the perfusion circuit (which I have referred to as the prototype device) for all porcine perfusions. Haemodynamic control was based on arterial pressure, without any direct adjustment of arterial flow and automatically maintained in the range of 90-100 mmHg, according to a higher physiological mean arterial pressure in the pig, via continuous feedback adjustment of the centrifugal pump speed between 1300 and 1500 rpm. The flow through the kidney was measured

with an external ultrasonic flow sensor (Sonoflow CO.55/080; SonoTec, Halle, Germany). Blood gases were automatically maintained within physiological limits by using a closed-loop controller, consisting of an air pump (ie O_2 concentration 21%; flow rates approximately 100ml/min) and bottled CO_2 (2.1.3 Chapter 2).

Proportional control valves were used to automatically regulate gas flow rates to maintain PO_2 between 10 and 16 kPa and PCO_2 between 4 and 6 kPa. As described in Chapter 2, urine production was continuously monitored using an in-line flow sensor (LD20-0600L Liquid Flow Sensor by Sensirion AG, The Sensor Company, Stäfa, Switzerland).

Also, as previously described, arterial blood gas analysis comprised an in-line blood gas analyser (CDI 500, Terumo Medical Corporation, Somerset, NJ). All haemodynamic perfusion parameter and the amount of urine output were logged by the perfusion device automatically.

6.3.3 Perfusate samples

Perfusate samples were taken at three different time points: (i) after priming of the circuit with the autologous whole pig blood, using the leukocyte filter, before any supplements were added; (ii) after two hours of normothermic perfusion and (iii) at the end of perfusion. Detailed description under 2.5.2 in Chapter 2.

6.3.4 Urine samples

Urine was collected within the first 15 to 30 minutes after perfusion start and 2-hourly thereafter, as long as there was urine production; under 2.5.4 in Chapter 2.

6.3.5 Tissue samples

Core needle biopsies were taken before connecting the kidney with the perfusion circuit, after two hours of NMP and at the end of perfusion/after 24 hours of perfusion. Detailed description under 2.6.2 in Chapter 2.

6.4 Haemodynamic and metabolic function parameters

Perfusion parameters for all porcine kidneys (n=10), with or without urine recirculation, are displayed in table 6.1. Physiological mean arterial pressures and flows were achieved in both perfusion categories within the first hour of perfusion and remained stable throughout the whole perfusion period in 4/5 (80%) kidneys perfused with urine recirculation. In contrast, this was not achieved in any of the kidneys with urine replacement. The haemodynamic parameters are shown graphically in figure 6.1 (arterial flow) and figure 6.2 (intra-renal resistance).

Table 6.1: Perfusion characteristics	**Kidneys with urine recirculation (n=5)**	**Kidneys without urine recirculation (n=5)**	***p-value***
Arterial pressure in mmHg (mean, SD)	101.3 ± 3.3	101.8 ± 2.4	*0.27*
Arterial flow in ml/min (mean, SD)	318.1 ± 97	220.5 ± 118.3	***<0.0001***
IRR in mmHg/ml/min (mean, SD)	0.32 ± 0.13	0.67 ± 0.47	***<0.0001***
pH (mean, SD)*	7.57 ± 0.11	7.48 ± 0.23	***0.02***
arterial pO2 in kPa (mean, SD)	12.5 ± 1.1	13.8 ± 2	***0.003***
venous pO2 in kPa (mean, SD)	5.7 ± 0.6	6.4 ± 1.4	***0.004***
arterial pCO2 in kPa (mean, SD)	5.2 ± 0.5	4.7 ± 0.9	***<0.0001***
Overall urine output in ml (mean, SD)	2586 ± 1438	2540 ± 1618	*0.99*
Urine output in ml/hour (mean, SD)	150 ± 21.3	548 ± 450.8	***0.008***
Amount of glucose in mg/perfusion duration (mean, SD)	2.85 ± 3.2	0.7 ± 0.8	*0.38*
Time on circuit in hours (mean, SD)	17.3 ± 9.2	5.3 ± 1.3	***0.02***

*including the first kidney pair with CPDA-1 (citrate phosphate dextrose adenine) in the blood bag

6.4.1 Arterial flow

Significantly higher arterial flow levels could be achieved in the urine recirculation group; median (min-max) arterial flow of 319 ml/min (70-465 ml/min) in the perfusions recirculating the urine vs. 226 ml/min (17-565 ml/min) in perfusions with replacement of urine, $p<0.0001$. This effect was seen in 4/5 (80%) of the individual kidney pairs. Only in pair two were the arterial flow rates (mean ± SD) comparable with 381.8 ± 57.03 ml/min with vs. 346.5 ± 171.2 ml/min in the perfusion without urine recirculation, $p=0.84$.

The duration of CIT before start of NMP (two or 27 hours) had no impact on arterial flow, neither in the overall cohort (n=10) nor in the groups with or without urine recirculation. Arterial flow (mean ± SD) in kidneys with CIT of two hours was 280.6 ± 125.1 ml/min compared to 259.6 ± 111 ml/min in kidneys with CIT of 27 hours, $p=0.37$. In the urine recirculation group, arterial flow in the short CIT group was 317.4 ± 90.56 ml/min compared to 318.9 ± 106.4 ml/min in the long CIT group, $p=0.68$. In perfusions replenishing the urine with Ringer's lactate, the arterial flow was 225.3 ± 148.6 ml/min after two hours of CIT compared to 217.2 ± 94.01 ml/min after 27 hours of CIT, $p=0.41$. In one kidney, in the group with urine recirculation, the perfusion had to be stopped due to deterioration in the arterial flow <50 m/min after hour seven. In all but one kidney, 4/5 (80%), in the group without urine recirculation, major deterioration of the flow <50 ml/min was the reason to stop the perfusion after six to nine hours of NMP.

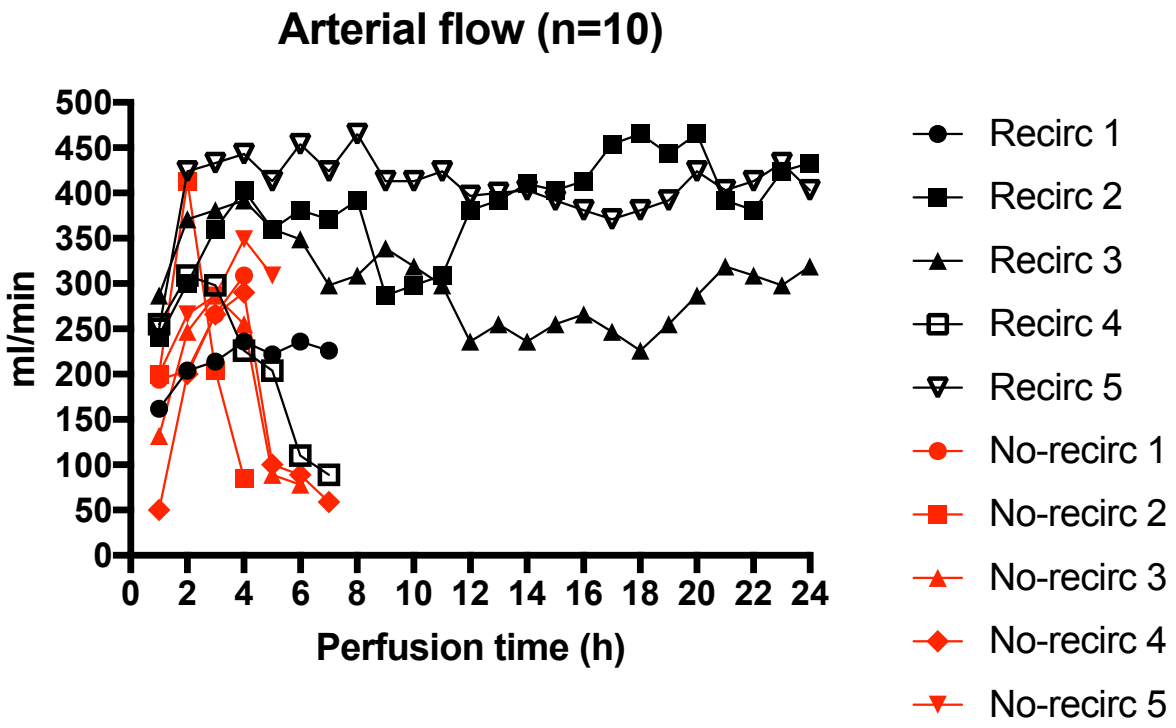


Figure 6.1: Arterial flow in ml/min in kidney perfusions with (black) and without (red) urine recirculation. Recirc and No-recirc with the same number are a kidney pair.

6.4.2 Intrarenal resistance

Intrarenal resistance (IRR) was expressed in mmHg/ml/min and calculated by dividing pressure in mmHg by the flow in ml/min at the same time point. The changes of IRR over time are shown in figure 6.2. IRR was significantly lower in kidney perfusions with urine recirculation; median (min-max) IRR of 0.26 mmHg/ml/min (0.21-1.24 mmHg/ml/min in the perfusions with recirculation vs. 0.49 mmHg/ml/min (0.24-2 ml/min) in perfusions with replacement, $p<0.0001$. As seen with arterial flow, the duration of CIT did not impact on IRR (mean ± SD); 0.41 ± 0.32 mmHg/ml/min after two hours CIT vs. 0.40 ± 0.25 mmHg/ml/min following 27 hours of CIT, $p=0.88$.

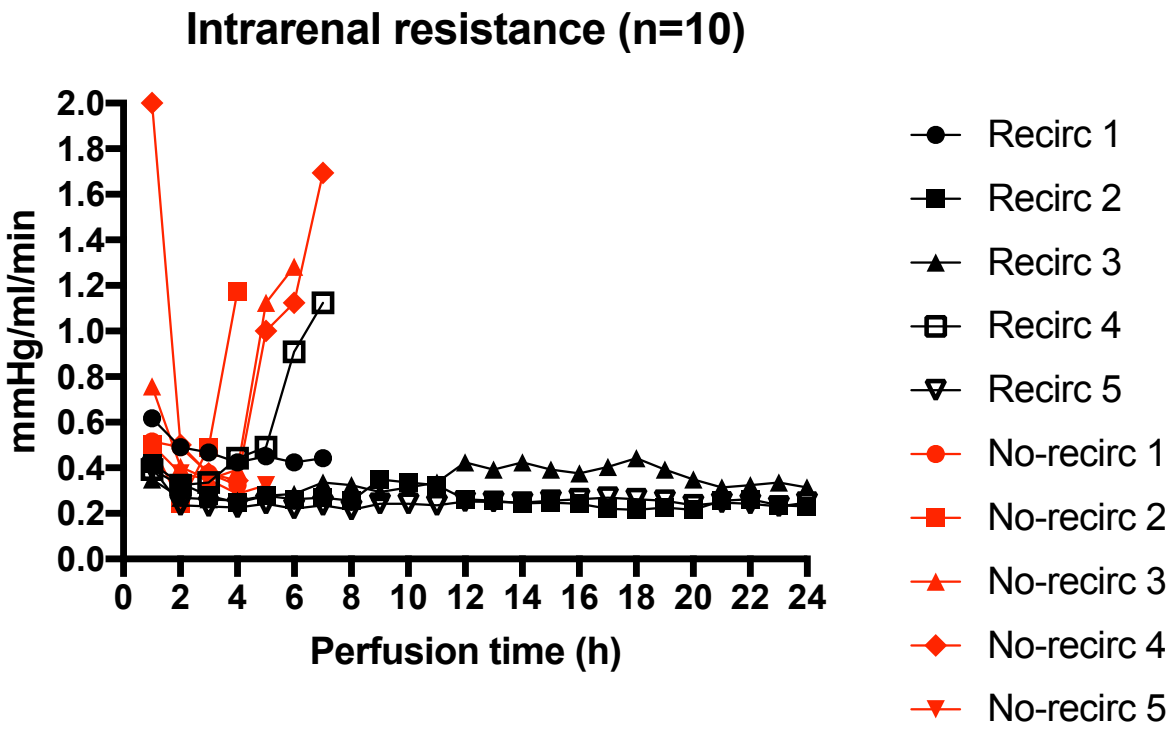


Figure 6.2: Intrarenal resistance of kidneys perfused with (black) and without (red) urine recirculation. Recirc and No-recirc with the same number are a kidney pair.

6.4.3 PO_2 and PCO_2 levels

The differences between arterial and venous oxygenation (mean ± SD) were significant in the overall cohort of all perfused kidneys, with and without urine recirculation; artery 12.5 ± 1.1 kPa vs. vein 5.7 ± 0.6 kPa in the urine recirculation group, $p<0.0001$; artery 13.8 ± 2 kPa vs. vein 6.4 ± 1.4 kPa with replacement of the excreted urine, $p<0.0001$. The PCO_2 levels were held between 4 and 6 kPa. Data comparison shown in table 6.1. Oxygen consumption per gram kidney was not calculated (2.1.3 Chapter 2).

6.4.4 pH levels

The course of pH for all perfused kidneys is shown in figure 6.3. No sodium bicarbonate 8.4% was given at any point, before or during perfusion, as all perfusates

had a starting pH (mean ± SD) of 7.43 ± 0.08 at normothermic temperature, before the kidney was connected. There was no significant difference in the pH of the autologous blood stored for 27 hours, compared to the short preservation perfusions using blood stored for two hours.; 7.39 ± 0.08 vs. 7.46 ± 0.07, p=0.22. Comparison of pH within each individual kidney pair, analysed by urine recirculation/replacement and short/long CIT, did not show major differences. Notably, in the first kidney-pair, a pH >7.7 was observed at hour five in the kidney with urine recirculation and two hours of CIT, and at hour four in the kidney without urine recirculation and 27 hours of CIT. Both kidneys started perfusion with a pH >7.45.

One reason for this elevating pH over time might be the metabolism of citrate, which is part of the anticoagulant CPDA-1 (citrate phosphate dextrose adenine) in the COMPOFLEX® blood bag; citrate is used to prevent coagulation by chelating calcium. Each blood bag contains 63ml CPDA-1 including 1.7 gram sodium citrate dihydrate (26.3 gram/1000ml). Each citrate molecule metabolised in a functioning kidney generates three molecules of bicarbonate causing metabolic alkalosis of the perfusate (212–215). To justify this suspicion that the citrate metabolism is the substrate leading to an alkalotic perfusion solution, I removed the 63 ml CPDA-1 from the blood bags I used for subsequent experiments and re-primed the blood bags with 10.000 units of unfractionated heparin and 20 ml of saline. Comparison of the pH levels of the first two kidney perfusions with citrate and the subsequent eight kidney perfusions without CPDA-1, showed a significant difference; pH 7.72 ± 0.16 vs. 7.46 ± 0.17 respectively, $p<0.0001$. All pH levels were measured at physiological PCO_2 levels (between 4 and 6 kPa).

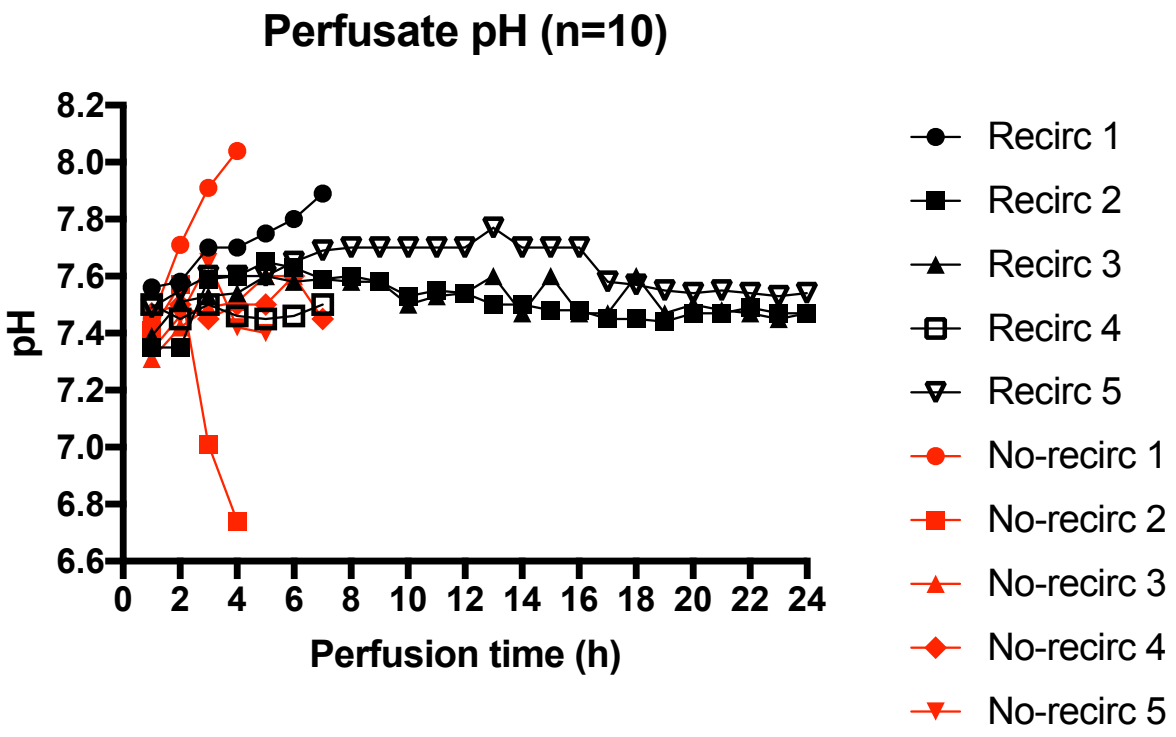


Figure 6.3: pH values of kidneys perfused with (black) and without (red) urine recirculation. Recirc and No-recirc with the same number are a kidney pair. Pair 1 had CPDA-1 (citrate phosphate dextrose adenine) in the blood collection bag.

6.4.5 Glucose measurements

As described in Chapter 2, all perfusions were supplemented with nutrition, depending on the measured glucose levels. Lipid free total parenteral nutrition solution Nutriflex® special (B. Braun AG, Melsungen, Germany) which contains glucose (glucose monohydrate 396g; 0.24mg glucose/ml); overall 5190kJ (1240kcal) per litre solution was used. The overall amount of glucose given during the period of perfusion was 2.85 ± 3.15 mg in the urine recirculation group and 0.69 ± 0.79 mg in the perfusions with urine replenishment.

As described in Chapter 3 for human kidneys, evaluating the glucose consumption of an isolated kidney is highly complex and not readily achievable as kidneys not only consume glucose, but also generate glucose by the processes of gluconeogenesis

and glycolysis. In addition, glucose filtration and reabsorption within the nephron further complicates any such calculation.

6.4.6 Lactate levels during perfusion

The native kidney is the second most important lactate-consuming organ after the liver and plays a major role in lactate metabolism. Due to the complexity of lactate metabolism in the kidney, it cannot be just used as a marker of “cell hypoxia” or “anaerobic metabolism”. Lactate is moreover heavily involved in intrarenal bioenergetics: (i) lactate production from glucose correlates with the glomerular filtration rate; (ii) lactate correlates with urine flow rates and (iii) sodium reabsorption; (iv) lactate consumption does not correlate with renal function (138,216).

The median (min – max) lactate levels after one hour, after four hours and at the end of perfusion were 4.34 mmol/l (0.53 – 5.53), 1.22 mmol/l (0.94 – 3.63) and 2.51 mmol/l (1.17 – 3.83) in the urine recirculation group (n=5). The median lactate levels for the urine replenishment group (n=5) were 7.32 mmol/l (1.79 – 11.57) after one hour, 8.22 mmol/l (1.2 – 20) and 6.99 mmol/l (1.2 – 20) at the end of perfusion. The lactate levels were numerically higher in the urine replacement group, but not significantly; at one hour p=0.09; at four hours p=0.06; at end of perfusion p=0.09.

When lactate levels are compared (mean ± SD) within each kidney pair, the perfusates with urine recirculation consistently showed significantly lower lactate levels. In all but one pair (the first perfused kidney pair), this difference was statistically significant. This finding was independent of the duration of CIT. Listing the urine recirculation kidney first in each case, the numerical data are as follows: first pair: 4.35 ± 1.5 vs. 5.99 ± 4.12, p=0.69; second pair: 1.13 ± 0.11 vs. 11.64 ± 9, p=0.04; third pair: 2.55 ± 1.28 vs.

6.9 ± 1.6, p=0.0002; fourth pair: 1.87 ± 0.64 vs. 9.07 ± 3.98, p=0.0012; fifth pair: 1.39 ± 0.74 vs. 6.49 ± 3.2, p=0.0032.

6.4.7 Urine excretion and sodium levels in perfusate and urine

All kidneys produced urine. The average hourly urine production of all the kidneys with urine recirculation was 143ml (123ml – 175ml). Median hourly urine production of kidneys without urine recirculation was 350ml (293ml – 1350ml). Total (mean ± SD) urine production is shown in table 6.1.

The initial sodium levels in the autologous whole, leukocyte filtered, porcine blood was higher in the first two perfusions (with CAPD-1 in the blood bag as anticoagulant) than in all following perfusions; 164.6 ± 0.6 mmol/l sodium in blood for perfusions one and two compared to 143.6 ± 3.5 mmol/l sodium in blood for perfusions three to ten. There were no differences in the initial sodium levels between the blood cold stored for about two or 27 hours. The course of sodium serum levels for each single kidney is displayed in figure 6.4.

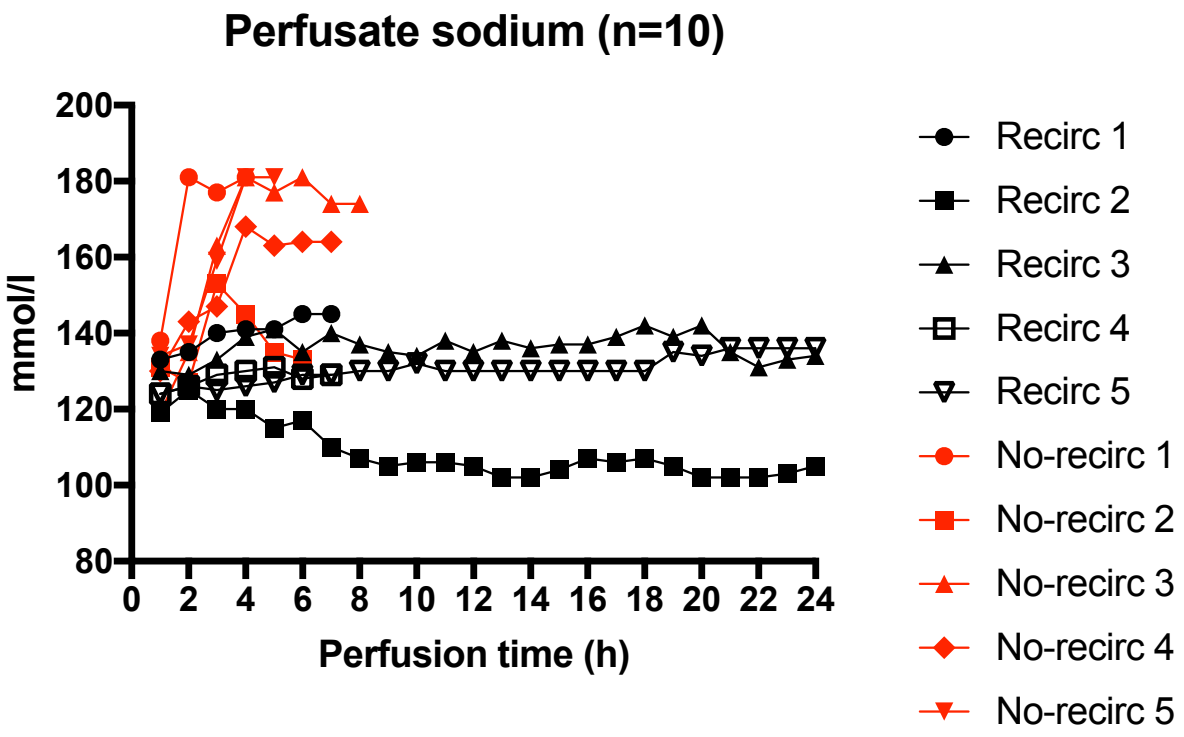


Figure 6.4: Perfusate sodium levels of kidneys perfused with (black) and without (red) urine recirculation. Recirc and No-recirc with the same number are a kidney pair.

Perfusate sodium levels of kidneys with urine recirculation were lower throughout the duration of perfusion compared to in kidneys without urine recirculation 129.9 ± 12.3 mmol/l versus 158.7 ± 19.4 mmol/l, 95% CI 22.44 – 35.21 ($p<0.0001$). The first measurements for this analysis were taken within the first 30 minutes after perfusion start.

Cold ischaemia time had an impact on initial urine sodium levels. Urine samples taken within 15 and 30 minutes after perfusion start revealed higher sodium levels after 27 hours CIT, 138.2 ± 9.2 mmol/l, compared to those after CIT of two hours, 101.6 ± 8.3 mmol/l urine sodium; 95% CI 23.81 – 49.35 ($p=0.008$). Similar to perfusate sodium levels, urine sodium levels were significantly higher in the group without urine recirculation; 94.9 ± 8 mmol/l versus 117.6 ± 17.6 mmol/l, 95% CI 10.12 – 35.15 ($p<0.001$).

6.4.8 Association of lactate, administered glucose, arterial flow and urine output

In the urine recirculation group (n=5), kidneys with lactate levels higher than the median of 2.51 mmol/l at the end of perfusion received less glucose than kidneys with an end lactate lower than 2.51 mmol/l; 1.28 ± 1.8 mg vs. 3.9 ± 3.76 mg. In the group with urine replenishment (n=5), kidneys with lactate levels higher than the median of 6.99 mmol/l at the end of perfusion received more glucose than kidneys with an end lactate lower than 6.99 mmol/l; 1.22 ± 0.94 mg vs. 0.33 ± 0.58 mg.

The median arterial flow throughout the perfusion was higher in kidneys with a lactate level less than the median of 2.51 mmol/l at the end of perfusion in the urine recirculation group; 339.7 ± 109.3 ml/min vs. 267 ± 43.8 ml/min. In the group with replacement of urine, the flow was higher in kidneys with end-lactate levels higher than the median of 6.99 mmol/l compared to the ones with lower end-lactate levels; 277.5 ± 191.6 ml/min vs. 237.3 ± 92.2 ml/min.

The hourly urine volume was higher in kidneys with a lactate level less than the median of 2.51 mmol/l at the end of perfusion in the urine recirculation group; 161.3 ± 17.9 ml/h vs. 133 ± 14.1 ml/h. In the group with replacement of the excreted urine volume, urine production was greater in kidneys with end-lactate levels higher than the median of 6.99 mmol/l compared to the ones with lower end-lactate levels; 821.5 ± 747.4 ml/h vs. 365.7 ± 50.4 ml/h.

With the overall low number of perfusion cases (n=10), only descriptive analysis can be performed without drawing any statistical conclusions.

6.4.9 Proteinuria

My studies on discarded human kidneys revealed a decreasing amount of urinary protein over time in three kidneys without urine recirculation (4.2.1 Chapter 4). To

investigate if this reduction is the positive effect of NMP on glomeruli and/or a better protein reabsorption capacity of the proximal tubuli or just due to a loss of protein by using an open circuit without urine recirculation, I analysed urine protein levels in paired porcine NMP perfusions.

6.4.9.1 Urine protein levels in porcine perfusions

Initial urine protein levels from urine samples taken within the first 30 minutes after perfusion start were significantly lower in the short CIT group (1100 ± 243.5 mg/l after two hours of cold storage) compared to the long CIT group (3467 ± 338.3 mg/l after 27 hours CIT), p=0.0006.

In the eight analysed perfusions, urinary protein diminished over time. In the urine recirculation group, the values after one hour were 1344 ± 834.9 mg/l and from hour six onwards 362.2 ± 165.1 mg/l urine protein.

In the group with replacement of the excreted urine, the one-hour values were 1528 ± 1435 mg/l and from hour four onwards 242.9 ± 126.8 mg/l urinary protein.

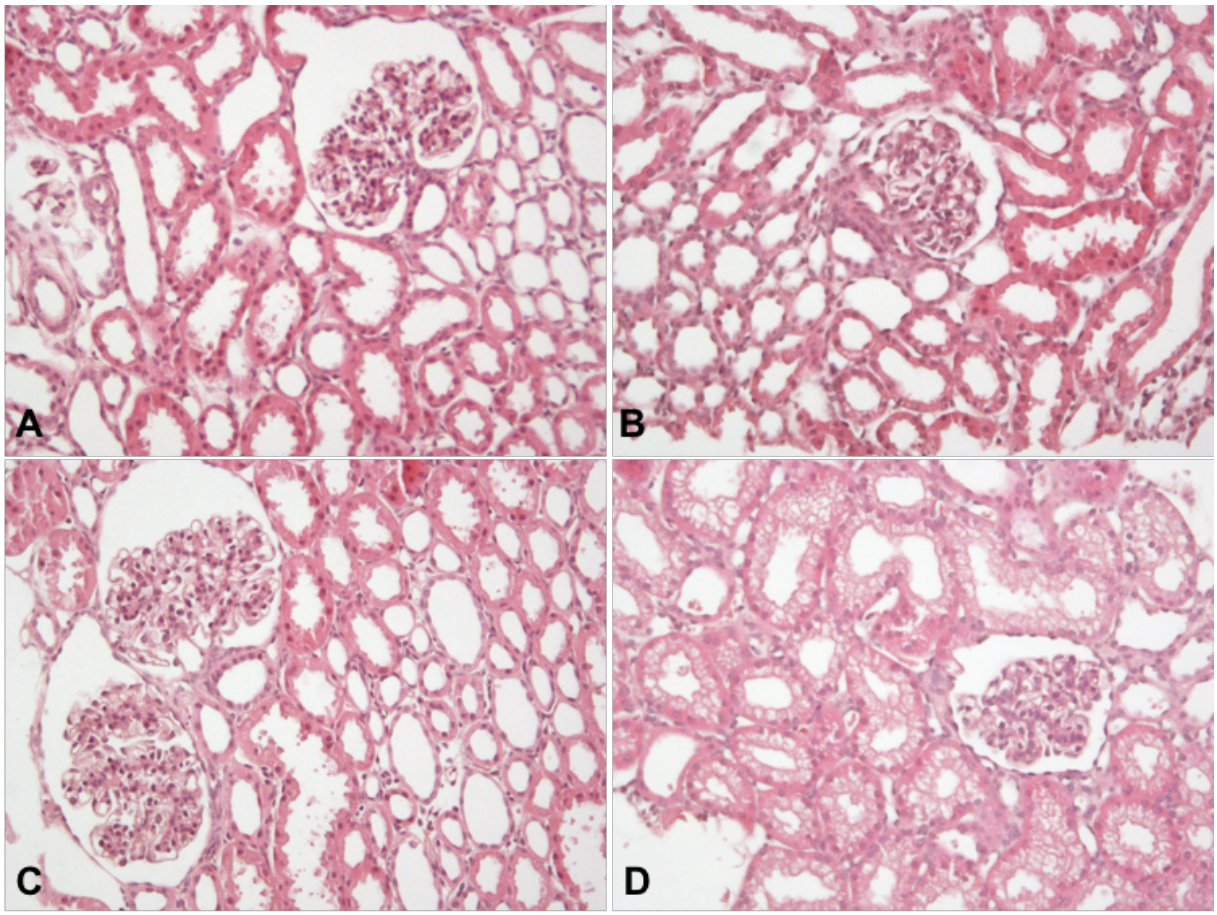
Only in the urine recirculation group we can suspect that the decreasing proteinuria is due to an effect of NMP which is not damaging the glomeruli as we are operating in a fully closed perfusion system in which protein cannot get lost via urine excretion.

6.5 Histology results

Tissue samples were obtained from all kidneys before start of normothermic perfusion, at two hours and anytime at the end of a perfusion/ after 24 hours (2.6.2 in Chapter 2). The presence of acute tubular injury before perfusion start and at the end of perfusion was adapted to the scoring for human kidneys and evaluated based on following criteria: 0 – absent; 1 – loss of brush borders/vacuolation of tubular epithelial

cells; 2 – cell detachment/cellular casts; 3 – coagulative necrosis. The baseline tubular condition could be described in all zero kidney biopsies. The tubular condition before perfusion start was slightly inferior in the zero biopsies taken after 27 hours of CIT compared to the ones with only two hours of cold storage (scores 0 to 1), but still only minimal with a score of 1 as the highest. The baseline tubular condition appeared unchanged after normothermic perfusion in 6/10 (60%) kidneys but was scored as 1 throughout. The change in the tubular condition was vacuolation in 4/10 (40%) kidneys, which is described in paragraph 6.5.2.

Figures 6.5A-D show examples of all histological findings for kidneys perfused with and without urine recirculation.

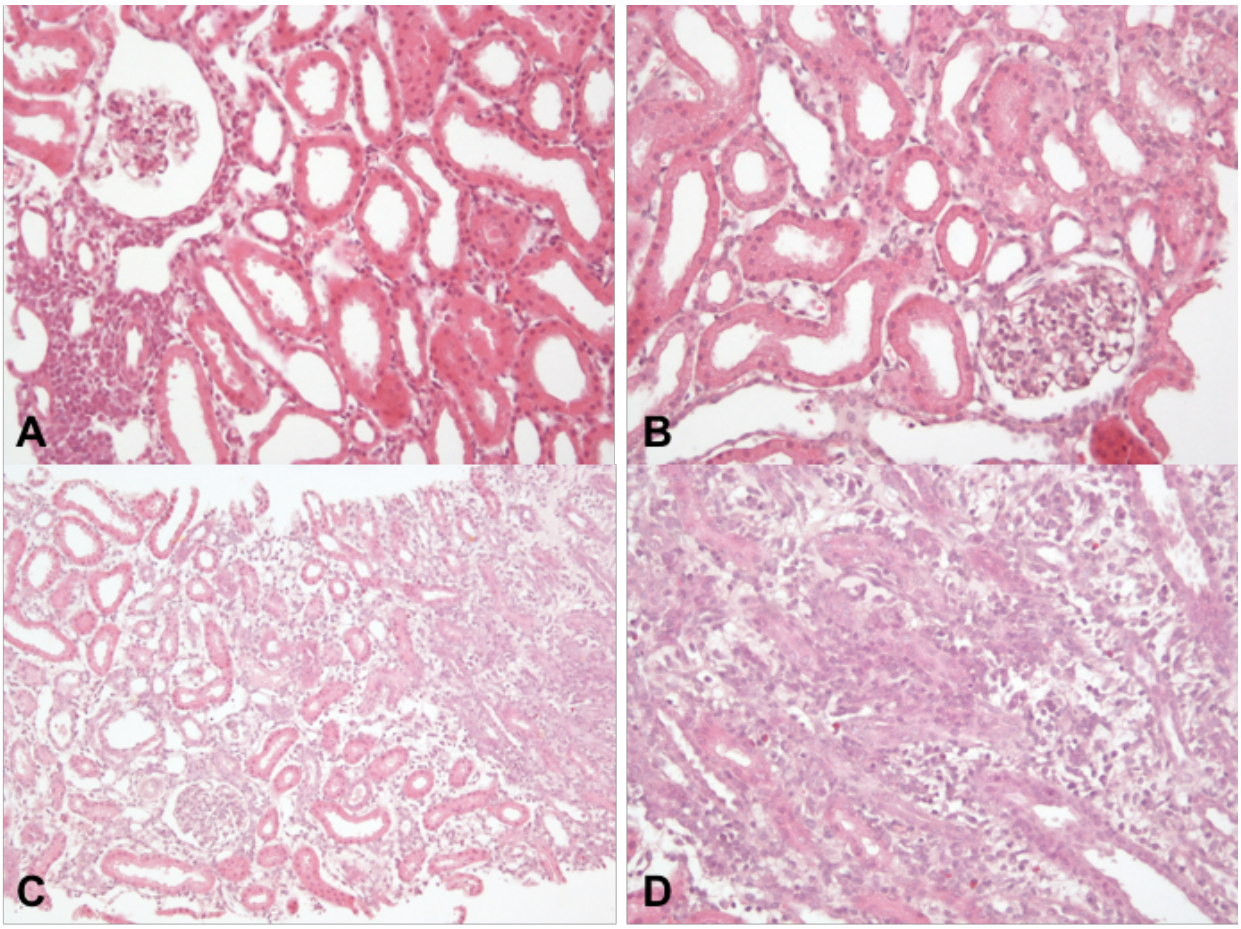


Figures 6.5A-D: Photographs of H&E staining. A: Kidney 3, zero biopsy after 2 hours CIT; B: Kidney 3, after 4 hours of NMP without urine recirculation; C: Kidney 4, zero biopsy after 27 hours CIT; D: Kidney 4, vacuolation after 24 hours NMP with urine recirculation with mannitol (Soltran) in the circuit.

6.5.1 Kidneys with interstitial inflammation

The fourth pair of kidneys did not perfuse as well as the other pairs and it was not possible to reach 24 hours of NMP in kidney eight (perfusion with urine recirculation). Retrospectively, the retrieval process was already different in terms of the status of the pig. The female landrace pig had several episodes of shivering during the surgical procedure and was remarkably hypovolaemic with a collapsed inferior vena cava, an empty bladder and quite dry peritoneal lining. Before proceeding with the bilateral nephrectomy, the hypovolaemic state of the animal was corrected by the anaesthetist.

Time zero biopsies of both kidneys showed a severe interstitial nephritis more marked in kidney seven than in kidney eight; patchy multifocal plasma cell rich mononuclear infiltrates. In kidney seven, the interstitial inflammation intensified during NMP (multifocal plasma cell rich mononuclear infiltrate, with eosinophils), figures 6.6A-D, whereas it showed improvement in kidney eight. Nevertheless, even kidney eight deteriorated with respect to arterial flow before reaching 24 hours of NMP.



Figures 6.6A-D: Photograph A shows the zero biopsy of kidney 7 with the interstitial inflammation, described as patchy multifocal plasma cell rich mononuclear infiltrate. B illustrates the biopsy after 2 hours of NMP without urine recirculation; C (100x magnification) and D (200x magnification) at the end of perfusion (7 hours) showing the multifocal plasma cell rich mononuclear infiltrate with eosinophils.

6.5.2 Vacuolation

We made an unexpected observation in two kidney pairs: whilst preparing to cannulate the kidneys, I primed the cannulae with Soltran® after flushing the kidney with the same preservation solution. In the other kidney pairs Ringer's lactate was used for priming the cannulas. The amount of fluid I used for the overall priming process was approximately 200 to 300ml. In all four kidneys, which were connected to the perfusion circuit with Soltran®-primed cannulas, the tubular condition showed widespread tubular epithelium vacuolation in the end-of-perfusion biopsies; including 24-hour perfusion tissue samples. This vacuolation was clearly more severe in kidneys with urine recirculation and appeared very similar to the vacuolation we detected in discarded human kidney perfusions with mannitol as a supplement in the perfusion solution (3.8 Chapter 3). Vacuolation is a non-specific histological finding that might be attributed to osmotically active perfusate compounds, for example mannitol, or to sustained ischaemic damage (217). In the human kidneys, in order to discriminate between these two options, we performed KIM-1 immunohistochemistry. In this particular case, KIM-1 positivity decreased over time and suggested that it was the osmotic agent causing vacuolation.

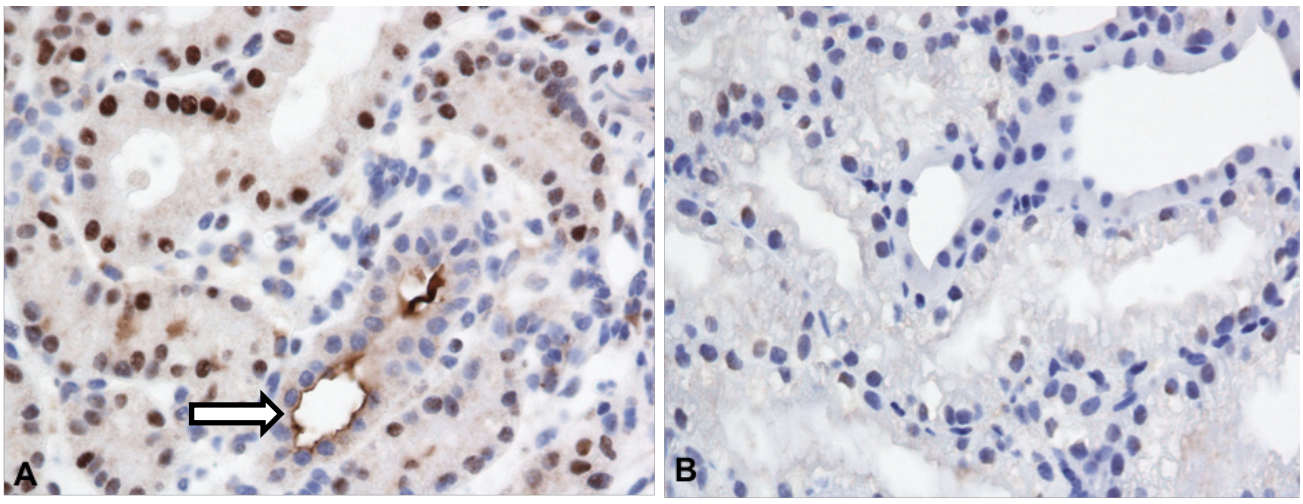
Mannitol was not used in any of the porcine perfusions. However, Soltran® (218) contains 3.38% mannitol per litre (33.8 mg/ml). When I fill a porcine kidney with about 200-300ml of Soltran® during the priming process, there are about six to ten gram of mannitol in the circuit, an amount exceeding the one given in the early human kidney perfusions (3.3 Chapter 3). The potential problem with mannitol in a closed circuit, as a system with urine recirculation constitutes, is the fact that mannitol is not metabolised (219,220). Mannitol is excreted via the urine, completely unchanged, stays in the perfusion circuit and will pass through the kidney countless times potentially

exacerbating the effect leading to tubular vacuolation. This may be the reason that we did not see this severe vacuolation in the end-of-perfusions biopsies in the perfusions with replacement of the excreted urine.

6.5.3 KIM-1 immunohistochemistry

According to our pathologist it is possible to stain porcine biopsy samples with the same KIM-1 antibody we used for discarded human kidney biopsies as there is a high chance of cross reactivity for such a specific protein. For this reason, we worked on immunohistochemistry for KIM-1 on my porcine samples together to analyse a potential KIM-1 expression, especially in those kidneys developing severe vacuolation. I am fully aware that this is not the perfect setting, to use a human antibody on porcine samples, but as we had KIM-1immunohistochemistry with this particular antibody already established, we gave it a try.

KIM-1 immunoexpression became only positive in kidney 7 over time, which is the kidney with the persisting interstitial nephritis; figures 6.7A-B. In all other biopsy-samples no KIM-1 positivity could be detected at any time.



Figures 6.7A-B: Photograph A shows the KIM-1 positivity (arrow) in kidney 7 at end of perfusion without urine recirculation; photograph B displays KIM-1 negativity in the 24-hour biopsy of kidney 4, with urine recirculation, showing vacuolation.

6.6 Biomarker results: NGAL and KIM-1

All perfused kidneys were analysed for NGAL (neutrophil gelatinase associated lipocalin) and KIM-1 (kidney injury molecule 1) perfusate levels. This was done by performing an ELISA, according to manufacturer's instructions (2.5.2 Chapter 2). Selected time-points for analysis were: (i) before connection of the kidney to the circuit and perfusate without any supplements, (ii) two hours after NMP start and (iii) at the end of NMP.

6.6.1 NGAL

An ELISA kit from DLdevelop (www.dldevelop.com) was used with a detection range of 1.56-100 ng/ml and a minimum detectable level of less than 0.64 ng/ml NGAL.

All but one pair of kidney perfusions were negative for any NGAL expression. The fourth pair, kidneys seven and eight, were the only perfusions in which NGAL (an acute tubular injury marker) could be detected. NGAL-levels for kidney seven stayed at about the same level (approx. 20 ng/ml) throughout the perfusion process. NGAL-levels decreased over time in kidney eight; the changes are graphically shown in figure 6.8. The values shown are the absolute numbers of the detected NGAL.

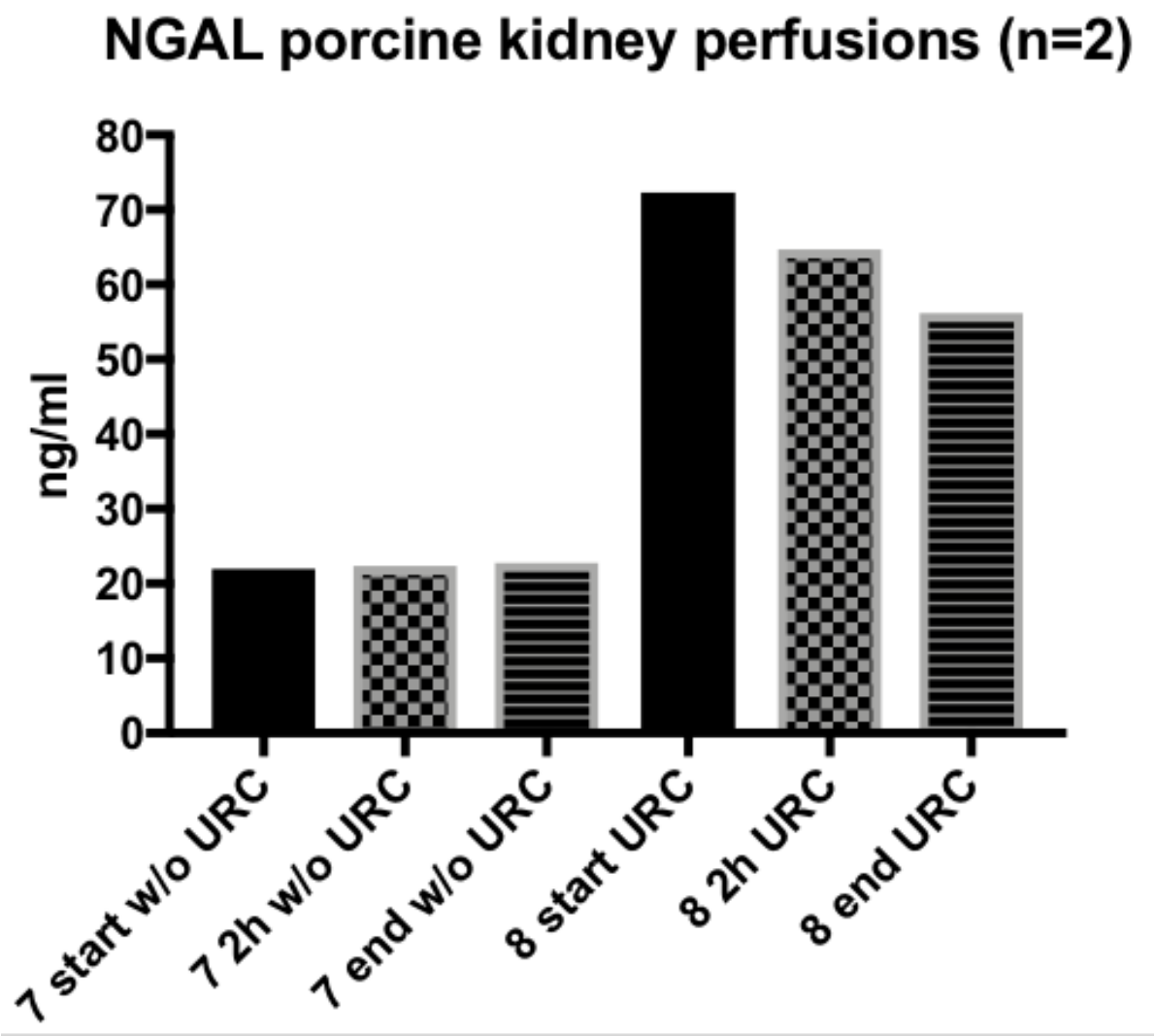


Figure 6.8: NGAL levels in kidneys with interstitial inflammation/nephritis in the zero biopsy and deterioration of arterial flow in both, in kidney 7 without urine recirculation and kidney 8 with urine recirculation. Start = NGAL level in the leukocyte filtered whole autologous pig blood, before perfusion start; 2h = 2 hours after start of normothermic perfusion (NMP); end = 7 hours after NMP start in kidney 7; 7 hours and 25 minutes after NMP start in kidney 8
w/o URC = without urine recirculation; URC = urine recirculation; ELISA detection range: 1.56 – 100 ng/ml; ELISA sensitivity: <0.64 ng/ml.

6.6.2 KIM-1

An ELISA kit from FineTest (www.fn-test.com) was used with a detection range of 0.156-10 ng/ml and a minimum detectable dose of less than 0.094 ng/ml.

The KIM-1 start levels (mean ± SD) were comparable between the two CIT groups, but marginally lower in the 2-hour CIT cohort; 109.1 ± 3.8 ng/ml vs. 111.9 ± 5.1 ng/ml after 27 hours CIT, p=0.22.

In all but kidney seven, KIM-1 levels decreased over time of NMP. Two-hour KIM-1 levels were clearly lower in the group with urine recirculation compared to the perfusions without; 65.7 ± 27.7 ng/ml versus 107.6 ± 5 ng/ml respectively, p=0.01. The same applies to the difference between start- and two-hour NMP values; delta 44.5 ± 25.4 ng/ml in the urine recirculation group vs. delta 3.2 ± 6.4 ng/ml without; p=0.02.

The perfusion-end levels of KIM-1 did not differ significantly between the urine recirculation group and the group with urine replacement; 76.8 ± 43.4 ng/ml with urine recirculation vs. 91.9 ± 33.3 ng/ml without, p=0.6. The difference between start- and end-perfusion value of KIM-1 was bigger in the urine recirculation group, but not significantly; delta 33.8 ± 44.5 with urine recirculation compared to delta 18.9 ± 33.6 ng/ml without, p=0.22.

The length of CIT did not impact the two-hour KIM-1 levels; 88.8 ± 23 ng/ml after two hours CIT vs. 84.5 ± 36.8 ng/ml after 27 hours CIT, p=0.7. The end-perfusion KIM-1 level was lower in the short CIT group, but not significantly; 65.6 ± 47 ng/ml after two hours CIT compared to 103.1 ± 7.8 ng/ml after 27 hours CIT, p=0.15.

6.7 What did we learn from the porcine perfusions?

I was able to demonstrate that normothermic machine perfusion with our kidney perfusion prototype effectively preserves the parenchymal quality of pristine porcine kidneys for 24 hours.

I had noted haematuria/ blood stained urine in the prior perfusions of discarded human kidneys and the porcine perfusions provided an opportunity to investigate whether this was a function of the perfusion circuit or a result of prior damage to the perfused organs. In none of the perfused porcine kidneys did haematuria/ blood stained urine appear at any time.

The porcine perfusions allowed me to validate the circuit design, in particular the innovation of urine recirculation: although the data from the human kidney perfusions had been encouraging, it was valuable to test the concept in a more consistent model – discarded human kidneys are very heterogeneous. The porcine perfusions confirmed the potential benefits of this design approach, particularly with respect to electrolyte and acid-base balance. I also obtained strong evidence that this technique is of value in enabling perfusions of at least 24 hours.

According to our findings, urine recirculation seems to be important if you would like to use normothermic preservation for longer than six hours (as an example) in the meaning of using this kind of perfusion for organ reconditioning purposes, for which short term perfusions (one hour for example) might not be enough. As soon as the kidney is excreting urine, the oncotic pressure and the electrolyte balance of the perfusion solution will change for two reasons:

(i) All donor kidneys will have proteinuria at the beginning of NMP. The amount of protein loss via urine within the first hour is likely to be dependent on the

duration of CIT, donor type, donor age and multiple more donor characteristic. One of the most important donor characteristics predicting graft survival of kidneys is the presence of proteinuria as a sign of glomerular damage. If we want to investigate if glomeruli are damaged, can be preserved or not with normothermic preservation, we need a fully closed system to know that the decrease of proteinuria is due to preserved and/or less leaking glomeruli and not caused by an overall loss of protein via urine output. When excreted urine is only substituted by Ringer's lactate, which has physiological osmolality and is published as the optimal solution to use by research groups leading the field of porcine NMP, the oncotic pressure of the perfusate will still change as the protein is lost via the excreted urine and not replaced. Moreover, a vicious circle will occur as a low oncotic pressure will change the perfusate homeostasis, protein will still be lost with the urine, oncotic pressure is declining more and so on.

(ii) An electrolyte imbalance will occur in any case fairly soon after starting replenishment of the excreted urine with Ringer's lactate as it includes 130 mmol sodium per litre and has a lactate content of 28 mmol/l. This was one of the most significant findings in our analysis that the perfusate sodium levels, and the urine sodium levels, were significantly higher in the perfusion group without urine recirculation. In addition, changes of lactate levels throughout the long-term perfusion period will become even more complicated to interpret in a metabolically active kidney.

If the electrolyte imbalance or the change in the oncotic pressure of the perfusate is instrumental for the significant lower perfusion duration we experienced, is still an unanswered question and will need further investigations.

In terms of applicability of the perfusion setup in a clinical trial with the aim of transplanting the normothermically perfused kidneys, our analysis revealed osmotic active inert compounds/sugars as mannitol and infusion solutions with a high content of those, as an absolute contraindication if we want to use the fully closed circuit with urine recirculation. The inert osmotic active compound will be excreted in the urine and circulate through the kidney and the urinary system endlessly, causing polyuria and tubular vacuolation. Tubular vacuolation is described as an unspecific, reversible finding, but for preservation purposes we cannot take the risk to cause vacuolation as we do not know the long-term impact of it on a cellular/mitochondrial level.

Chapter 7: Discussion

The journey of my DPhil brought me from thinking of how to assemble essential components to preserve a kidney at physiological body temperature to the development and construction of a normothermic kidney perfusion and preservation device in a very close collaboration with experts in biomedical engineering. We were working together, in an extremely productive and inspiring team, to get a prototype ready for long-term normothermic kidney perfusion and its clinical establishment with the future goal of initiating a clinical pilot trial. The journey did not end with the completion of a normothermic preservation device prototype, but merely marked the beginning. My main focus was on investigating a normothermically perfused kidney and to understand the science behind the effects of preserving an organ at 37°C. When working on a novel preservation technique, the guiding principle has to be “primum non nocere”, the Latin phrase that means "first, do no harm", which is one of the fundamental bioethical precepts (221). According to this, the first objective was to assure that the quality of the kidney parenchyma was maintained after up to 24 hours of normothermic machine perfusion (NMP) or, in best case, did improve in terms of less acute injury having been caused by previous events like brain death, warm ischaemia during the organ recovery process in organs from circulatory-death donors and, not to be forgotten the time of cold ischaemia preceding start of normothermic perfusion. My DPhil path led me, as a transplant surgeon, to experience the field of basic science research which was of paramount importance to analyse kidney tissue and the perfusate we used and how both changed over time.

The idea of perfusing organs with a perfusate composed similarly to human blood at 37°C is not novel itself. Normothermic organ preservation is more the revival of a very old technique used to keep organs alive and is currently undergoing a revamp. Long before the first attempts of clinical organ transplantation and even longer before the icebox became the standard of care for organ storage, preservation had been in the focus of science already. In the early 19th century, Le Gallois has philosophised already whether it would be possible to replace the human heart with a form of injection using artificial arterial blood and thus effortlessly maintaining life in each body part for an indefinite period of time (22).

Historically, from the announcement of Le Gallois' hypothesis onwards, the 21st century transition of preservation techniques has developed in the reverse sequence to its 20th century trajectory (figure 7.1).

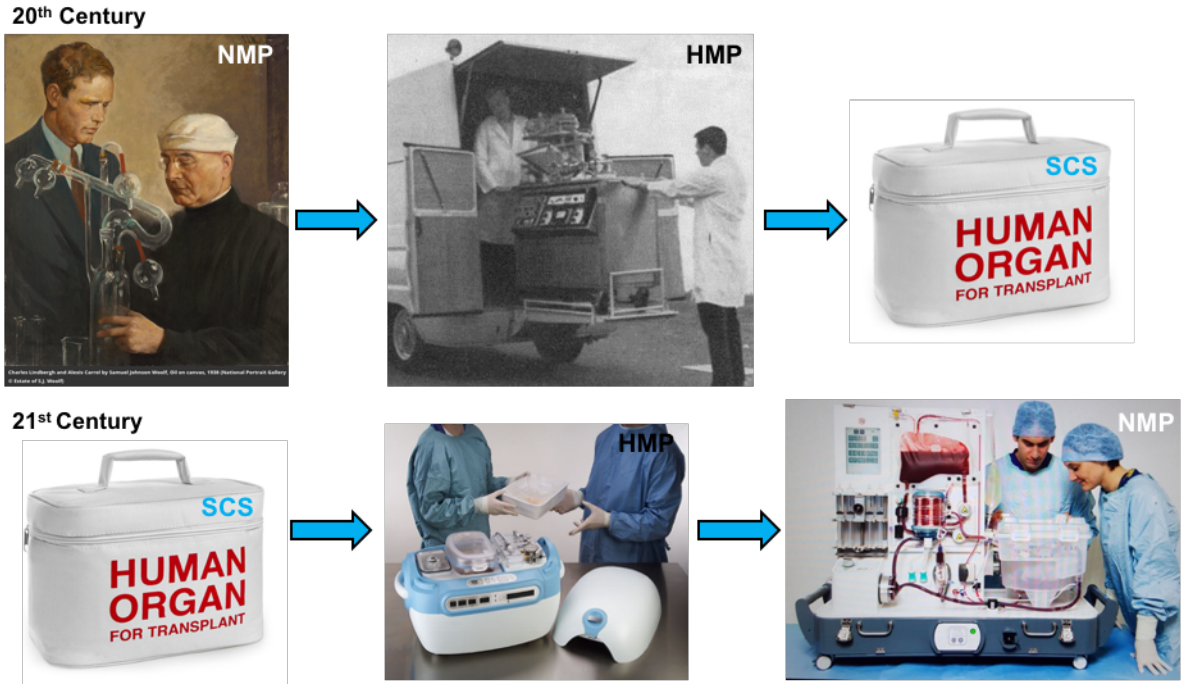


Figure 7.1 shows the development of preservation techniques "from complex to simple" in the 20th century: NMP→HMP→SCS and its reverse transition "from simple to complex" in the 21st century: SCS→HMP→NMP. Sources used for creation of figure 7.1: (222–224)

During the 20th century, whilst solid organ transplantation was becoming a clinical reality, efforts were made to evolve the complex technology of ex vivo NMP, first applied by Carrel *et al.* (24) to a technology offering the advantages of simplicity, permanent availability and cost-effectiveness. Normothermic preservation from the 1930s was replaced by Belzer's hypothermic machine perfusion (HMP) technology in the early 1960s (27), before Collins and co-workers (31) published a method to transport kidneys on ice using a preservation solution later in the 1960s. They stated that *"kidneys could be shipped in a small box containing ordinary ice for periods of up to 30 hours with almost no detectable damage"* (31). The era of static cold storage (SCS) was initiated and the icebox rose to become the iconic storage tool, indispensably connected and globally associated with organ transplantation.

The implementation of SCS enabled satisfactory results after solid organ transplantation in general. However, SCS is acceptable for high quality, but not for marginal organs; we need to address its limitations quickly in the urgent quest to utilise higher risk organs to fulfil the increasing demand for organs to transplant. With the cooling of the organs to 4°C we can slow down the metabolism, but besides the loss of cell membrane functions due to the cooling process, cellular metabolism does not stop completely. Under lack of oxygen, anaerobic metabolism continues leading to accumulation of metabolites which contribute later to the occurrence of ischaemia-reperfusion injury after reperfusing the organ in the recipient (54).

Given these facts, the progress in preservation technology during the 21st century started to reconsider past technologies, in order to move the field forward. There is increasing reliance upon extended criteria donors. This comprises kidneys with a UKKDRI >1.35, all DCD kidneys and DBD kidneys older than 60 years or those from deceased donors older than 50 years with two out of three criteria (arterial

hypertension, donor serum creatinine >1.5mg/dl, cerebrovascular accident as a reason for brain death). Such organs experience more severe ischaemia reperfusion injury (IRI). The need to transplant these higher-risk organs in larger numbers has, to a greater or lesser extent, revitalised research on alternative preservation methods and brought machine perfusion back to the fore. A landmark study for HMP in deceased-donor kidney transplantation was published by Moers *et al.* (41) in 2009. Hypothermic machine perfusion reduced the risk of delayed graft function (DGF) and improved the one-year graft survival and function in ECD kidneys, when compared to SCS (40,41). A more recent publication revealed that HMP is beneficial, with a significantly lower DGF rate, in kidneys with a CIT of ≤10 hours but this positive effect diminishes with prolonged CIT (225). However, cold ischaemia time (CIT) still remains one of the most powerful, independent and relevant risk factors for DGF after transplantation of deceased donor kidneys preserved with HMP (225). The duration of CIT and its impact are precisely the target where NMP could, potentially, make a major impact. Normothermic preservation entails several concepts: the organ is continuously perfused with oxygenated blood at body temperature during storage (+/- transport) (i) enabling normal cellular metabolism with recovery of cellular energy status; (ii) allowing repair of reversible injury; (iii) facilitating functional testing of the organ before transplantation through the measurement of perfusion and biochemical parameters during preservation. Furthermore, transplant logistics will become more demanding in future as more organs are transplanted into more complicated, surgically and immunologically, recipients within more stringent working regulations. For these reasons, not only are transplant professionals and researchers interested in NMP, but also the hospital management, as the application of NMP might actually be cheaper and more effective because of relocating transplants from night to daytime working

hours. These implications of NMP in clinical routine are of considerable current interest. In future, we might envisage an impact on emerging issues in transplantation: repair of injured organs; modulation of the immune presentation of the donor kidney, an issue that still poses a significant barrier to successful transplantation, by exploiting other novel technologies. Machine-based normothermic perfusion could be effective in the treatment and repair of damaged kidneys before transplantation as it offers a near-physiological environment. In addition, it could be a platform to study the immunogenicity of an organ and to treat it accordingly to the recipient's sensitisation status. Approaches like this will require the application of biological molecules, stem cells or genes. In relation to IRI, NMP might serve as an optimal system to prevent this damaging cascade of molecular events with targeted, drug-loaded nanoparticles for example (226).

The clinical and experimental experience gained so far, has shown highly promising results assuring that NMP is a real alternative to SCS. The evidence from transplanted organs, that kidney preservation by NMP is superior to SCS is primarily based upon clinical and experimental studies from Hosgood and Nicholson from the University of Cambridge (both former University of Leicester) who are clearly the frontrunners in the field of renal NMP (100,102,105,226,227). This team has developed incrementally over the last decade the concept of a period of normothermic perfusion in kidney transplantation at the end of a period of cold ischaemia. Hosgood and Nicholson apply one hour of NMP to kidneys after SCS, thereby restoring function and improving early graft function of extended criteria donor kidneys. Currently, a large multi-centre trial in the United Kingdom, led by the Cambridge team, is investigating the impact of normothermic perfusion on delayed graft function in a trial of DCD kidneys (228). A

review by Hosgood and Nicholson, published beginning of 2018 (227), exemplifies their contribution to the field of kidney NMP on the experimental and clinical sector. They illustrated their key publications on the development and application of normothermic perfusion, covering both therapeutic interventions and organ assessment. In the experimental setting, they could demonstrate that the depletion of leukocytes from blood in the perfusate improved post-ischaemic renal function indicating the importance of white cells in renal IRI (80). Yates *et al.*, compared the effects of reperfusion with leukocyte and platelet depleted blood and whole blood on renal blood flow. Reperfusion with leukocyte and platelet-free blood increased the renal blood flow significantly as well as creatinine clearance and renal oxygen consumption (229). These publications provide the basis for the perfusate composition I have used for my porcine perfusion studies, as described in Chapter 6. The clinical publications from this group comprise the first clinical applications world-wide of short-term NMP in humans receiving a deceased donor kidney transplant; these include a case report regarding the successful resuscitation and use of a pair of DCD kidneys declined by all transplant centres in the UK (101,102,104).

We set ourselves the challenge to create a device enabling long-term kidney NMP of at least 24 hours whilst maintaining or improving organ quality. The hypothesis behind this was that if we can maintain the cells of the kidney in a metabolically active state, then normothermic perfusion may be highly effective as a means to preserve organs for much longer than at present, without the progressive detriment of cold ischaemia. In other words, normothermia may be able to enhance preservation as well as providing organ reconditioning and viability assessment (as shown by the Nicholson group).

The novelty of my DPhil project lies in the longer duration of discarded human kidney perfusion using a newly-developed automated fully closed perfusion device applying urine recirculation to maintain volume ionic homeostasis of the perfusate.

When I have started my DPhil work in 2015, there were no publications about clinical experiences with long-term kidney NMP in humans; this remains the situation. The group of Selzner *et al.* at the University of Toronto have been working extensively on NMP in a DBD and a DCD porcine auto-transplantation model and demonstrated that longer periods of up to 16-hours of NMP are superior to both SCS alone and short-duration NMP; this is in terms of both tubular injury and post-transplant organ function (91,92,230). Regarding longer periods of NMP, our own group's recent phase I and phase III studies in human liver transplantation have provided evidence of the superiority of prolonged NMP over SCS for both conventional and extended criteria donor organs (106,107). As part of the EU-funded Consortium for Organ Preservation in Europe (COPE), a multi-centre liver transplant trial showed clear advantages of this more physiological preservation technique. The most important findings were of significantly lower levels of peak AST (a validated surrogate parameter for graft and patient survival, and the primary endpoint of the trial), despite a 50% reduction in the proportion of livers that were discarded after retrieval, and a 54% increase in the mean preservation time (107).

By 2018, there were still no published studies of clinical kidney transplantation after NMP for longer than two hours. However, our own research group has recently published our experience with 24-hour NMP of discarded human kidneys which were the essential piece of my DPhil work (231).

A longer perfusion duration, up to 24 hours, requires components used in contemporary ECMO (extracorporeal membrane oxygenation) devices (232,233) as beyond six hours of perfusion, problems with oxygenator membranes and haemolysis will occur in regular cardiopulmonary bypass oxygenators (234,235). One of our assumptions was that the longer you keep an organ in an artificial milieu, the more you have to invest in the perfusate and the haemodynamics. The key perfusion setting in the final prototype device was a mean arterial pressure (MAP) of 90 mmHg in a non-pulsatile environment for human kidneys. Care maintenance of arterial pressure is probably important, not only to avoid pressure-related kidney damage, but also to avoid the reflex vasoconstriction that has limited perfusion systems in the past. The composition of the perfusate logically need to have similar characteristics to human blood (in terms of oxygen delivery, viscosity and ionic composition/osmolarity) and the need for nutrition is an open question. I decided to use discarded human kidneys for the first part of my perfusion studies for a number of reasons.

The proof of principle had already been established by the Leicester-Cambridge and the Toronto-group. I recognised that to achieve 24-hour preservation and to make this clinically feasible, it was safest to develop this in the closest model to the clinical context. The consensus within our research group was that this should be in human kidneys, although these organs came with added complexity: (i) kidneys for research are available on a very unpredictable basis, (ii) the organs have been turned down by all UK transplant centres, (iii) such discarded human kidneys are damaged by definition, even more severely by the time they have arrived for research and (iv) the cohort is inevitably very heterogenous. As a consequence of these I decided that measuring kidney function in a conventional way was not appropriate because of the anticipated damage of the organ, but that haemodynamic and tissue/perfusate studies

would be appropriate. Once we had designed the circuit around the perfusion characteristics of human organs, I then validated this in an “undamaged” model; that’s why the final stage rather than the early stage of my DPhil focused on porcine perfusion studies.

The particularly novel part of my perfusion setup and the very obvious difference to circuits used by other research groups working on kidney NMP is the recirculation of urine. In order to manage the problems of perfusate composition that would occur in a 24-hour perfusion of a diuresing kidney, we tested a closed perfusion circuit with urine recirculation. Before we started practicing this, we considered the following: (i) the kidney would practically not function as an excretory organ if we have a fully closed circuit with recirculation, (ii) urine might cause crenation (if urine is hypertonic) or haemolysis (if urine is hypotonic) due to osmotic imbalances caused, (iii) adding antidiuretic hormone (ADH)/ arginine vasopressin (AVP) would be an option to stop diuresis, although with the downside of potentially increasing the vascular resistance and reducing the possibility to analyse urine, (iv) classical, clinically used, clearance studies are not possible in a recirculating system, (vi) biomarkers might accumulate and therefore be more difficult to interpret, but changes over time could still be calculated by using delta values (rates of change) and (vii) perfusate homeostasis and oncotic pressure should (theoretically) be the same throughout the perfusion period.
Besides these considerations, we calculated the metabolic effects of urine recirculation regarding the accumulation of metabolic waste products. Allowing for the relative mass of the kidney alone (versus the body mass) and relative volume of the circuit (versus the human circulation), there is no theoretical concern about waste product accumulation (ie the need for “dialysis” of the perfusate) for periods of up to

several months. We performed a hypothetical calculation to demonstrate that the lack of excretory function as a result of urine recirculation is most unlikely to have any significant adverse physiological consequences within the clinically relevant time frame (ie, several days). A 75-kg human with 5 litres of circulating volume in a stable state of renal function will develop clinical symptoms of renal failure after an anephric period of three days (as demonstrated by the interval between haemodialysis sessions). A single kidney has an approximate mass of 150-180 grams with a circulating volume of 500ml: at a 500th of the mass and one-tenth of the volume, renal replacement should be required 50 times less frequently than for an anephric patient, on the order of once every 150 days; therefore, the amount of accumulating urea would be trivial and acceptable in a system perfusing an isolated kidney (231).

The result of recycling urine into the perfusate has never been described in a normothermic perfusion device. Brasile *et al.* mentioned the possibility of doing so in their patent application for the EMS system (126). This was not, technically, a normothermic model, described at 32°C which is a “sub-normothermic” environment Also, it was only a theoretical description; results with this approach of handling the excreted urine have never previously been published.

Overall, I perfused 36 discarded human kidneys up to 24 hours (14 of them at the full duration) in a stepwise fashion as the achieved time of NMP was depending mainly on the development stage of the perfusion device. As described in Chapter 3, only one kidney seemed to deteriorate whilst undergoing NMP. Four kidneys were perfused without urine recirculation after I had gained experience with kidney perfusions using the fully-closed circuit. I was not able to keep the NMP going for 24 hours in the circuits without urine recirculation. The volume lost via urine excretion was replaced 1:1 with

Ringer's lactate, as performed by previous investigators and described as the ideal fluid to replace urine. With these experiments, I wanted to provide a "standard" comparison group to the kidneys perfused with urine recirculation, but it proved not possible to keep either a physiological pH or a desirable arterial blood flow after four to six hours of perfusion. The most obvious difference to the kidneys perfused with urine recirculation were the higher perfusate sodium levels, which appeared after about two hours of NMP, and the differences in perfusate pH. Ringer's lactate may have been responsible for the higher sodium serum levels as it contains 130 mmol/l, but in contrast, the lactate levels were not significantly different despite the fact that Ringer's infusion solution contains 28 mmol/l lactate. In fact, lactate levels were slightly lower in the kidneys without urine recirculation compared to kidneys with urine recirculation (lactate levels for all kidneys are displayed in table 3.4; kidneys 30-33 are without urine recirculation). For short perfusion durations of this sort (open circuit, without urine recirculation) the urine output can be replenished easily and in an uncomplicated way with Ringer's lactate: this was demonstrated by Hosgood and Nicholson in several publications. However, for longer-term perfusions, the disruption of perfusate electrolyte content and pH is potentially problematic.

The effect of urine replacement on the homeostasis and acid-base balance of the perfusate is no surprise, given the circulating volume of 500ml and the fact that this will have undergone a "turn over" of 100% after five hours if a kidney excretes 100ml of urine per hour. In addition to creating a steady perfusion environment with urine recirculation, there are other practical advantages of a fully-closed circuit: this is convenient in terms of logistics around the perfusion as it can be designed more easily as an automated perfusion system.

In comparison to our experience with normothermic liver preservation where lactate clearance (and potentially glucose metabolism) could be used as a parameter for the objective assessment of organ function during the perfusion period already, lactate metabolism of the kidney is not so straightforward in its interpretation, especially not in a very heterogeneous cohort of discarded human kidneys. In humans, lactate metabolism plays a major role in the native kidney and the renal cortex appears to be the most important lactate-consuming organ after the liver (216). Bartlett *et al.* showed years ago that the renal dysfunction impacts lactate and glucose metabolism. It is most likely that lactate levels are related to active glucose metabolism, glycolysis, and gluconeogenesis in the kidney. The analysis of lactate and glucose-level changes over time in 24 perfused kidneys with urine recirculation revealed that kidneys with higher lactate levels received more glucose, and that the lactate level at the end of perfusion correlates significantly with the overall amount of glucose administered during NMP. As described in the literature, the kidney contributes to glucose homeostasis via different pathways: gluconeogenesis, glucose filtration, glucose reabsorption and glucose consumption (133–135,138,216). Also, there seems to be a cortico-medullary glucose-lactate recycling system: the medulla consumes glucose by active glycolysis and generates lactate. The cortex has the ability to take up the lactate released by the medulla and uses it for oxidation and gluconeogenesis (138). Bartlett's research group also demonstrated that lactate production correlates with the urine flow rate and sodium resorption (138). I analysed and correlated lactate levels (first and last measurement, six-hour levels, delta start-end lactate values) with the hourly and the total volume of excreted urine and could not find any significant direct or indirect correlation. Regarding sodium perfusate levels, there appeared to be no correlation with the amount of excreted urine. Interestingly, median arterial flow inversely

correlated significantly with the median and final lactate measurements in my kidney perfusions; the higher the median arterial flow the lower the measured lactate levels and vice versa. Albeit the numbers in my DPhil study are small, I am thinking of future clinical implications and would recommend analysing glucose “consumption” and the change of lactate over time together and make a viability assessment depending on the arterial flow. I am fully aware that final judgments concerning kidney function in this context are very difficult to make for the reason that I did not investigate the creatinine clearance of the kidneys whilst on the circuit and, also, the interpretation of my findings are difficult as they are limited by the fact that none of the kidneys were transplanted. Therefore, it was not possible to corroborate the data we collected with an actual measured kidney function.

As the field is evolving, biomarkers which are already (more or less) established in the clinical area of acute kidney injury arouse considerable interest in the kidney NMP context, especially NGAL and KIM-1. In most of the kidneys with urine recirculation, the absolute levels of NGAL, KIM-1, and L-FABP increased over time, which aligns with a biomarker analysis in the clinical trial published by Parikh *et al.* demonstrating an increase of all biomarkers during perfusion (236). Their study investigated biomarkers in an HMP setting as did Hoogland *et al.* (236,237). The study from the research group in the Netherlands concluded that the diagnostic accuracy of the perfusate biomarkers to predict viability of DCD kidneys varied from “poor” to “fair” and decisions to discard or to transplant an organ should not be based on perfusate biomarker levels. Parikh *et al.* concluded their investigations very similarly and stated that most recipients of transplanted kidneys with isolated "undesirable" biomarker levels during HMP experienced acceptable 6-months kidney function (236,237).

Although a kidney on an HMP device is not producing any urine, our NMP prototype and the HMP device can be compared as both tools demonstrate a closed perfusion circuit with exactly the same perfusate throughout the entire time of hypo- or normothermic perfusion. The interpretation and the informative value of detected biomarkers might be more difficult in my setting with urine recirculation compared to kidney perfusion setups where the urine is excreted and replaced. However, most of the biomarker levels increased over time in both groups, with and without urine recirculation. Hosgood and Nicholson analysed the value of urine NGAL and KIM-1 after 60 minutes of NMP and could identify an association between higher levels of urine NGAL, not KIM-1, with raised donor creatinine before organ retrieval (238). In my kidney perfusion cohort with urine recirculation I could not detect any correlation between any of the three analysed biomarkers with donor characteristics; either with kidney function parameters or with the donor type. Cold ischaemia time seemed to have the highest impact on KIM-1 and L-FABP and, rather as expected, the longer the CIT the higher the biomarker levels. Perfusate KIM-1 levels were lower the higher the urine production was. NGAL, on the other hand, showed more to be a parameter being reversely proportional with the median arterial flow; the higher the NGAL levels, the lower the median arterial flow. As NGAL is the parameter best established in clinical use for acute kidney injury, this would be, in my opinion, the biomarker of most interest, together with perfusion characteristics in any clinical trial of kidney NMP. As reported in the literature, NGAL might have the potential to become a stand-alone troponin-like biomarker in plasma and urine for the prediction of kidney injury and for monitoring and prognosis of acute kidney injury (239,240).

Another, highly relevant biomarker for kidney function is proteinuria. Proteinuria is a frequent complication in almost all chronic kidney diseases and besides hypertension

the most important risk factor for progression of native kidney disease (241,242). The most common cause of proteinuria is diabetes mellitus. The following three mechanisms cause proteinuria: (i) glomerular disease, (ii) increased quantity of proteins in the serum (overflow proteinuria), (iii) low reabsorption of protein in the proximal tubule (Fanconi syndrome). After transplantation, proteinuria is usually mild and predominantly tubular in origin (242,243). The problem is though, that proteinuria is a risk factor for graft loss and patient death and even a mild proteinuria (0.1-0.2g/day) is associated with impaired graft and patient survival (242). Due to these facts, it is important to investigate urine protein in a kidney which is normothermically perfused for longer than one hour, as it is important to use the opportunity to assess such an important risk factor when there is the possibility to investigate it. For logistical reasons I did not have enough urine samples and not a complete data set of the human kidneys I perfused with urine recirculation. I analysed urine protein in three consecutive human kidneys without urine recirculation and the results, especially in the urine analysed within the first 30 minutes after NMP start, were astonishing as the protein content in the urine was nearly 18000 mg/l in an elderly DBD kidney with a CIT of 18 hours (which is an SCS time we would still consider for clinical transplantation). In all three kidneys analysed, the initial protein was higher after longer CIT and the amount of protein declined over time. However, in an open circuit, it is not possible to say if this pattern is due to recovery of the nephron or just the result of protein loss via the urine over time; 50% of the perfusate comprises 5% albumin which was chosen to provide physiological oncotic pressure in the circuit. There is little evidence in the kidney NMP literature regarding urine protein. Blum *et al.* from Cleveland clinic, described in their comparison of hypothermic and normothermic perfusion in porcine DCD kidneys that proteinuria during simulated reperfusion of both cold and warm

preserved kidney groups was similar. They calculated the urine protein/creatinine ratio and did not give the exact amount of protein in the urine (95). Hamar *et al.* from Toronto mentioned proteinuria in their latest publication about normothermic ex vivo kidney perfusion studies in a porcine DCD model. They described proteinuria around 1g/litre during the entire time of perfusion without showing any trend (230).

Proteinuria was not the only reason to move to the porcine model, but after a substantial number of human kidney perfusions and the awareness of the very heterogeneous nature of this cohort, there was a need to study a more controlled series of kidney perfusions. The international leader in porcine kidney NMP is currently Markus Selzner and his research group from the University of Toronto. His group dedicated a tremendous amount of work to porcine kidney NMP, studied different durations of NMP in DBD and DCD auto-transplant models and could describe a clear positive difference to cold stored porcine kidneys in terms of improved functional and histological results (91,93,230). By perfusing pristine porcine kidneys with our novel NMP technology, I was able to validate that we do not harm the kidney parenchyma.

As described in Chapter 6, I was able to show that porcine kidney perfusions are feasible for up to 24 hours with urine recirculation. As observed in the human kidney cohort, I was not able to perfuse kidneys without urine recirculation for 24 hours and the kidneys deteriorated on the perfusion circuit showing the exact same pattern as the human kidneys. In addition to this, I could confirm and strengthen some findings and results yielded in the human cohort:

(i) the correlation between lactate levels, glucose administration, and arterial flow were the same in the porcine kidneys – with and without urine recirculation – as in the human kidney perfusions; the higher the lactate

levels, the more glucose was administered in mg and the higher the arterial flow the lower the lactate levels

(ii) in the porcine cohort urine production per hour shows a correlation with the final lactate levels, as described by Bartlett *et al.* (138)

(iii) the small number of porcine kidney perfusion calls for caution and results of nonparametric t-tests must not be overinterpreted, but there was a correlation of perfusate KIM-1 levels after two hours of NMP with the type of circuit (open or closed); all KIM-1 levels were significantly lower in the five kidneys with urine recirculation

(iv) the amount of protein in the urine produced within the first 30 minutes of perfusion was significantly lower in the short CIT group; this is an argument in favour of a transportable NMP device

(v) proteinuria showed a clear decrease over time in the urine drawn from the circuit before the urine was returned into the soft-shell reservoir (urine recirculation) as a sign of preservation of glomeruli and tubuli and maybe even improving its status after two or 27 hours of CIT; the level of proteinuria also decreased in the urine with volume replenishment, but the difference in the change was bigger which is a clear sign for the protein loss in an open circuit with an inevitable change of oncotic pressure of the perfusate over time as long as the volume is replaced with protein-free fluid only

(vi) the use of mannitol, or other supplements with osmotic active sugars, might, in fact, be contraindicated for kidney perfusions in a closed circuit; mannitol is an inert substance excreted via the urine and causes tubular vacuolation by staying in the perfusate and keeping its diuretic effect throughout

Opinions regarding the role of mannitol and its possible protective effects with regards to IRI have experienced ups and downs over time. Purported effects after reperfusion of a transplanted kidney include higher renal blood flow, less cell swelling and scavenging of free radicals. One of the more recent publications in living-donor renal transplantation stated that mannitol is effective and leads to better posttransplant recovery of kidney function if it is administered 15 minutes before clamping the renal artery of the donor (244). However, there is no certainty as to whether this is an appropriate supplement for a closed kidney perfusion circuit.

Mild tubular vacuolation due to osmotically active substances is recognised and described as reversible in the literature, but nevertheless, as urine recirculation is a novel application, as is kidney perfusion for 24 hours, we have to assume that there might be a negative effect on the kidney. Osmotic nephrosis due to mannitol has been previously described in several animal models and in humans. The earliest description of tubular vacuolisation was published by Hamburger *et al*. (who performed the first kidney transplantation in France in 1952) who investigated the histology of mannitol-induced acute renal failure in rabbits (245). Mannitol is a six-carbon alcohol of the sugar mannose (xylitol and sorbitol are similar) and is used widely as an osmotic diuretic agent. It is freely filtered in the glomerulus with minimal tubular resorption (7%), thereby increasing the osmolality of the glomerular filtrate; 90% of infused mannitol is excreted in the urine within 24 hours (246). It has been reported that high doses of mannitol (maximum 200gram/day) can cause nephrotoxicity or acute renal failure in some patients. A typical histological pattern for mannitol-toxicity is the isometric tubular vacuolisation, which I saw in some of my early human kidney perfusions and in porcine kidney perfusions with urine recirculation when the cannulas were primed with Soltran®. Mannitol and similar agents can enter tubular cells via

pinocytosis and form vacuoles that fuse with each other and with lysosomes containing hydrolytic enzymes. In kidneys with chronic diseases, including diabetes mellitus, the impairment of lysosomal degradation and digestion predisposes to mannitol-induced acute renal failure (220). Early and mild vacuolar changes are reversible, but more overt damage can end in permanent injury to the renal tubules. The tubular effects of mannitol are also related to its indirect influence on sodium transport. The presence of mannitol in the tubular fluid reduces water reabsorption, this results in a decreased sodium concentration in the tubular fluid; the cellular water content falls and the concentration gradient against sodium increases. The outward transport of sodium diminishes, the passive backflux of sodium into the tubule increases and the sodium reabsorption is reduced and stops when the limiting gradient for sodium transport is reached. Besides the pinocytosis, swollen and vacuolised tubular cells which can lead to a substantial but reversible reduction in proximal luminal diameter, are a consequent effect of the impaired sodium transport (247).

Another important reason to make mannitol and other osmotic active sugar compounds a “forbidden” substance for kidney NMP using a closed circuit is the fact that tubular vacuolisation is a histological feature of acute calcineurin inhibitor (CNI) toxicity (248). Even if there were no substantial negative long-term effects of vacuolisation caused by mannitol, it would have a significant impact on the interpretation of biopsies performed after transplantation of an NMP kidney; the majority of renal transplant recipients receive CNI-based maintenance immunosuppressive therapy – the appearance of vacuolisation could cause inappropriate changes of the immunosuppressants leading to an immunological imbalance and concomitant difficulties posttransplant.

The pig kidney perfusions were particularly helpful to validate and replicate some findings revealed by NMP of discarded human organs but could not provide us an answer where exactly the difference lies between perfusions with and without urine recirculation and most importantly, what the pathomechanisms are behind the deterioration of kidney perfusions using an open circuit design. To shed some light on these questions, further investigations on human samples were initiated. We performed proteomics on the tissue; metabolomics and immune cell as well as cytokine analyses on perfusate samples.

Proteomics on biopsy samples showed possibly very relevant results: that proteins for maintaining acid-base balance, antioxidants, anhydrases and stress proteins were highly upregulated in kidney tissue with urine recirculation whereas, in contrast, these were downregulated in kidney biopsies from kidneys without urine recirculation. One such protein was angiotensinogen (AGT). The effect of angiotensinogen, which is mainly synthesised in the liver but can also be produced in the kidney, could play a role in the increasing intrarenal resistance over time in kidneys without urine recirculation and could have an additional impact on hypernatremia (beyond the effect of sodium added by replacing excreted urine with Ringer's lactate). Increased AGT formation in the kidney and its secretion into the proximal tubular lumen leads to the local formation of angiotensin II which elicits renal vasoconstriction and increased tubular sodium reabsorption in the proximal and distal nephron segment (249). Besides the unstable arterial flow accompanied by increasing resistance of the kidneys perfused without urine recirculation, acid-base homeostasis was deviating from a physiological pH during NMP. Another potentially relevant proteomics findings in this regard could be the downregulation of carbonic anhydrase (CA1) in kidneys without urine recirculation; this was upregulated in the kidneys with urine recirculation.

This metalloenzyme (the active site of most carbonic anhydrases contains a zinc ion) catalyses the reversible conversion of carbon dioxide and water and the dissociated ions of carbonic acid (e.g. bicarbonate in our perfusion circuit) and its most important functions in humans and animals is to maintain acid-base balance in blood and to help transport carbon dioxide out of tissues (168,172,181).

In the first two porcine perfusions, we experienced particularly high pH-levels, indicating a metabolic alkalosis, possibly due to the conversion of citrate coming in the blood collection bags and also, as a further source, the citrate in the Soltran® preservation solution that was used in these kidneys (described in Chapter 6). At this stage, before having knowledge of upregulated CA1 in the human kidney biopsies, we tried to decrease the pH by adding acidic substances to the circuit largely without success; these experiments were done after I had taken perfusate samples and biopsies described in the results of Chapter 6. With knowledge of the proteomics results, revealing the upregulation of CA1, I was curious to see if I could decrease the pH by administering acetazolamide (Diamox), a carbonic anhydrase inhibitor (250). I tested acetazolamide in one of my last 24-hour pig kidney perfusions, after having taken all the samples needed for my porcine perfusion series, to test if it is possible to interfere with the pH-level and to demonstrate that my kidneys on an NMP device are indeed metabolically active. I could create anecdotal evidence that acetazolamide works: bicarbonate levels in the perfusate dropped conclusively within 30 minutes of administration of the drug. The pH decreased during four hours of perfusion from 7.6 to 7.37 at physiological pCO_2 levels around 5 kPa. I am fully aware of the fact that this is currently a somewhat anecdotal observation and that more evidence is needed. However, it demonstrates the value of translational work and how important such basic-science can be for future clinical work.

Another interesting proteomics finding was that NMP activates glucose metabolism in a human kidney, confirming literature reports that the calculation of glucose consumption is complex in the kidney, as the organ contributes itself to the glucose balance. We demonstrated upregulation of the cytosolic enzyme phosphoenolpyruvate carboxykinase 1 (PCK1). This enzyme is the main controller for the regulation of gluconeogenesis in humans and catalyses the formation of phosphoenolpyruvate from oxaloacetate with the release of carbon dioxide and guanosine diphosphate (GDP) (183). This enzyme was upregulated in half of the NMP kidneys perfused with urine recirculation and in one of the three kidneys analysed without urine recirculation and confirmed with Western Blot analysis.

Metabolomics results showed marked differences in the urine recirculation vs. replacement groups. I tested these at specific time points (one and six hours after NMP start). Urea was significantly more expressed in perfusates of kidneys with urine recirculation compared to the open circuit. This makes sense of course as urea is not excreted via the urine in this group, but the absolute levels of urea in the perfusate were between 1 and 4 mg/dl only throughout the perfusion. After six hours NMP, Erythritol, a metabolite which contributes to vasodilation is significantly increased in kidneys with urine recirculation compared to the other group. Interesting is that there were not only significantly changed metabolites over time when comparing the groups of perfusions with and without urine recirculation. Also, the composition of metabolites of one perfusion type was different to the other. The amino acids Glutamic acid, L-Proline and L-Tryptophan, the sugar Erythritol and of course urea-2TMS were, as mentioned above, “typical” for the perfusates with urine recirculation. L-Threonine-3TMS could be detected in perfusates of both perfusion types, whereas the sugar .beta.-D-Galactofuranose and the amino acid L-Aspartic acid-3TMS for example were

part of the metabolite-profile of kidneys perfused on the open circuit. The number of analysed perfusates and kidneys is small, but the preliminary results present a good basis for further investigations within the scope of a clinical trial which will provide the opportunity to correlate the findings with clinical results of kidney function after transplant.

The last set of experiments we performed on perfusate samples were to analyse the composition of immune cells in the perfusate and the profile of cytokines expressed in the perfusion solution over time. Again, with the main focus of detectable differences between perfusates containing recirculating urine or not. Immune regulation, inflammation and the innate immune system, are key components of the host's response to damage or danger (251). However, after solid organ transplantation it is of utmost importance to control both the innate and the adaptive immune systems to enable the transplanted allograft to survive and function (251). I was interested to consider whether normothermic machine perfusion might function as the "intermediate recipient" to wash out the immune cells before implanting the kidney into the final host as described by Lechler *et al.* (189,190) regarding allorecognition.

Stone *et al.* from the Fildes' laboratory at the University in Manchester, with whom we collaborated for the flow cytometry and Luminex® analyses, was able to demonstrate that there was an immune cell efflux from the porcine donor kidney into the NMP circuit within the first hour of perfusion (252). Their flow cytometry results showed that there was an increased efflux of major populations of donor T cells throughout the perfusion and consistent populations of other cells including monocytes, natural killer cells, and macrophages (252). In both of my perfusion models, with and without urine recirculation, the number of leukocytes increased significantly in the perfusate within the first hour of NMP. No other cell type changed significantly over time in either group.

A result in line with the report of Stone *et al.*, we did detect a major B cell population after six hours of perfusion in kidneys without urine recirculation; however, this could be due to the fact that one of the perfused kidneys was suspected of having a B-cell lymphoproliferative disease. Furthermore, the Manchester group demonstrated in one of their previous publications an inflammatory cytokine storm after initiation of NMP (252). Cytokine profiles in my perfusions were comparable between the groups; most importantly urine recirculation did not lead to a more inflammatory environment. Indeed, the opposite was the case: in the perfusates of kidneys with urine recirculation, IL-6, a cytokine with anti-inflammatory potential (192) increased significantly over time up to hour six and the inflammatory cytokines IL-1a and IL-2 decreased during the same period; this pattern was not detected in kidneys without urine recirculation. What the two perfusion variants had in common though, was an increase of IL-8 over time. Interleukin-8 has been described as a universal biomarker used in a variety of medical sub-specialities (urology, nephrology, haematology, microbiology, oncology etc.) as a marker to find a diagnosis or as a predictor of prognosis (253). It plays an important role in inflammatory pathways leading to kidney allograft rejection (254); lower mRNA expression of IL-8 was associated with interstitial fibrosis in kidney transplant patient (255); IL-8 showed upregulation in patients with chronic kidney disease and after kidney transplantation compared to healthy control groups (255); in clinical liver transplantation prolonged elevation of IL-8 in plasma correlated with poor early graft function (256) and a lung-transplant study showed the same correlation with elevated IL-8 levels after two hours of reperfusion as the essential measurement (257).

Our results, gained from an ex vivo NMP setting cannot be correlated with clinical study results, but IL-8 offers obviously an important target to invest more research, particularly in forthcoming clinical use of the NMP device.

The downside of performing the cell and cytokine analysis solely in the perfusate and not in the tissue or from any other components in the circuit, is the missing answer to the question of where the immune cells are going to. I am not able to provide such data due to the lack of tissue analysis, but proteomics-results revealed some interesting results indicating a lower immunogenicity of organs perfused normothermically by significant downregulation of proteins involved in IRI and the innate immune system. Downregulated proteins (significantly lower in urine recirculation tissue) including, for example, NF-kappa-B, MAP-kinases, the high-mobility group box 1, CD14, CD74 and cell adhesion molecule 1, could be of interest for future research. Furthermore, some proteins were 2-fold downregulated, without significance, after 12 and 24 hours of NMP with urine recirculation which could give some insight into the inflammatory environment in which the kidney is residing. Parts of fibrinogen, alpha, beta and gamma chains, were downregulated: these are known to facilitate immune response via innate and T-cell mediated pathways. Another acute phase protein, inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), involved in inflammatory responses to trauma was downregulated, as well as the lipopolysaccharide-binding protein (LBP) which also plays a role in the innate immune response. This is just a selection of proteins and making such a selection brings a natural bias. Nevertheless, I would like to conclude that overall, perfusing discarding human kidneys on our NMP prototype with recirculation of the urine, did not cause a harmful inflammatory environment.

I believe that I was able to show a very relevant and positive impact of the normothermic preservation technology on human kidneys. It was a heterogenous group of organs (clinically-discarded, CIT beyond ≥ 50 hours, poorly perfused etc.)

which was perfused with a perfusion device which was itself under development during the early perfusion studies (Chapter 3). The fact that I could achieve near-physiological arterial flow, that parenchyma quality was histologically preserved, that biomarkers changed over time in relation to CIT and arterial flow and that omics-technology revealed active metabolism in this cohort of absolutely non-standardised kidneys, highlights the importance of NMP. There are still many questions, but this technology offers the potential for clinical benefit, and I feel that my results and the efforts of our working-group have laid the foundation for a clinical trial. The first logical step would be to perform a safety and feasibility trial using the newly developed prototype. Recirculating urine will help to maintain perfusate homeostasis and hence enable long-term kidney NMP using the transportable device.

REFERENCES

1. Druml W, Druml C. Emerich Ullmann (1861-1937): not only a pioneer of kidney transplantation. J Nephrol. 2004 Jun;17(3):461–6.
2. Alexis Carrel - Nobel Lecture: Suture of Blood-Vessels and Transplantation of Organs [Internet]. [cited 2018 Aug 15]. Available from: https://www.nobelprize.org/nobel_prizes/medicine/laureates/1912/carrel-lecture.html
3. Reemtsma K. Xenotransplantation: A Historical Perspective. ILAR J. 1995 Jan 1;37(1):9–12.
4. Matevossian E, Kern H, Hüser N, Doll D, Snopok Y, Nährig J, et al. Surgeon Yurii Voronoy (1895–1961) – a pioneer in the history of clinical transplantation: in Memoriam at the 75th Anniversary of the First Human Kidney Transplantation. Transpl Int. 2009 Dec 1;22(12):1132–9.
5. Billingham RE, Medawar PB. The Technique of Free Skin Grafting in Mammals. J Exp Biol. 1951 Sep 1;28(3):385–402.
6. Billingham RE, Brent L, Medawar PB. Actively Acquired Tolerance â□™ of Foreign Cells. Nature. 1953;172:603–6.
7. Ono SJ. The Birth of Transplantation Immunology: The Billingham—Medawar Experiments at Birmingham University and University College London. J Exp Biol. 2004 Nov 1;207(23):4013–4.
8. Starzl TE. The Early Days of Transplantation. JAMA J Am Med Assoc. 1994 Dec 7;272(21):1705.
9. Hume DM, Merrill JP, Miller BF, Thorn GW. Experiences with renal homotransplantation in the human: report of nine cases. J Clin Invest. 1955 Feb;34(2):327–82.
10. Lawler RH, West JW, McNULTY PH, Clancy EJ, Murphy RP. HOMOTRANSPLANTATION OF THE KIDNEY IN THE HUMAN. J Am Med Assoc. 1950 Nov 4;144(10):844–5.
11. Kuss R, Teinturier J, Milliez P. [Some attempts at kidney transplantation in man]. Memoires Acad Chir Fr. 1951 Jul 4;77(22-24):755–64.
12. Cooper DK. The forgotten French: The ‘heroic’ era of kidney transplantation. J Med Biogr. 2017 Nov 1;25(4):234–9.
13. Murray G, Holden R. Transplantation of kidneys, experimentally and in human cases. Am J Surg. 1954 Apr;87(4):508–15.
14. Renal Medicine: History Of [Internet]. [cited 2018 Aug 15]. Available from: http://www.renalmed.co.uk/history-of/renal-transplant
15. Murray JE, Merrill JP, Harrison JH. Kidney transplantation between seven pairs of identical twins. Ann Surg. 1958 Sep;148(3):343–59.
16. [cited 2018 Aug 15]. Available from: https://bts.org.uk/wp-content/uploads/2016/09/BTS_EarlyHistoryRenalFailure-1.pdf
17. Woodruff MF, Robson JS, Ross JA, Nolan B, Lambie AT. Transplantation of a kidney from an identical twin. Lancet Lond Engl. 1961 Jun 10;1(7189):1245–9.
18. Calne RY, Alexandre GP, Murray JE. A study of the effects of drugs in prolonging survival of homologous renal transplants in dogs. Ann N Y Acad Sci. 1962 Oct 24;99:743–61.
19. STARZL TE, MARCHIORO TL, WADDELL WR. THE REVERSAL OF REJECTION IN HUMAN RENAL HOMOGRAFTS WITH SUBSEQUENT DEVELOPMENT OF HOMOGRAFT TOLERANCE. Surg Gynecol Obstet. 1963 Oct;117:385–95.
20. Calne RY, Thiru S, McMaster P, Craddock GN, White DJ, Evans DJ, et al.

Cyclosporin A in patients receiving renal allografts from cadaver donors. 1978. J Am Soc Nephrol. 1998 Sep 1;9(9):1751–6.
21. Joseph E. Murray - Nobel Lecture: The First Successful Organ Transplants in Man [Internet]. NobelPrize.org. [cited 2018 Aug 15]. Available from: https://www.nobelprize.org/prizes/medicine/1990/murray/25012-joseph-e-murray-nobel-lecture-1990/
22. Legallois C (1770-1814) A du texte. Expériences sur le principe de la vie, notamment sur celui des mouvemens du coeur, et sur le siège de ce principe ; suivies du Rapport fait à l'Institut sur celles relatives aux mouvemens du coeur, par M. Le Gallois,... [Internet]. 1812 [cited 2018 Aug 15]. Available from: https://gallica.bnf.fr/ark:/12148/bpt6k1511249w
23. Hewitt RL, Creech O. History of the pump oxygenator. Arch Surg Chic Ill 1960. 1966 Oct;93(4):680–96.
24. Carrel A, Lindbergh CA. THE CULTURE OF WHOLE ORGANS. Science. 1935 Jun 21;81(2112):621–3.
25. Lapchinsky AG. Recent results of experimental transplantation of preserved limbs and kidneys and possible use of this technique in clinical practice. Ann N Y Acad Sci. 1960 May 31;87:539–71.
26. Belzer FO, Ashby BS, Dunphy JE. 24-hour and 72-hour preservation of canine kidneys. Lancet Lond Engl. 1967 Sep 9;2(7515):536–8.
27. Belzer FO, Ashby BS, Gulyassy PF, Powell M. Successful seventeen-hour preservation and transplantation of human-cadaver kidney. N Engl J Med. 1968 Mar 14;278(11):608–10.
28. Belzer FO, Kountz SL. Preservation and transplantation of human cadaver kidneys: a two-year experience. Ann Surg. 1970 Sep;172(3):394–404.
29. Kaths JM, Paul A, Robinson LA, Selzner M. Ex vivo machine perfusion for renal graft preservation. Transplant Rev Orlando Fla. 2018 Jan;32(1):1–9.
30. Cameron AM, Barandiaran Cornejo JF. Organ preservation review: history of organ preservation. Curr Opin Organ Transplant. 2015 Apr;20(2):146–51.
31. Collins GM, Bravo-Shugarman M, Terasaki PI. Kidney preservation for transportation. Initial perfusion and 30 hours' ice storage. Lancet Lond Engl. 1969 Dec 6;2(7632):1219–22.
32. Collins GM, Halasz NA. Letter: Composition of intracellular flush solutions for hypothermic kidney storage. Lancet Lond Engl. 1975 Jan 25;1(7900):220.
33. Kalayoglu M, Sollinger HW, Stratta RJ, D'Alessandro AM, Hoffmann RM, Pirsch JD, et al. Extended preservation of the liver for clinical transplantation. Lancet Lond Engl. 1988 Mar 19;1(8586):617–9.
34. Gill J, Rose C, Lesage J, Joffres Y, Gill J, O'Connor K. Use and Outcomes of Kidneys from Donation after Circulatory Death Donors in the United States. J Am Soc Nephrol JASN. 2017 Dec;28(12):3647–57.
35. Weber M, Dindo D, Demartines N, Ambühl PM, Clavien P-A. Kidney transplantation from donors without a heartbeat. N Engl J Med. 2002 Jul 25;347(4):248–55.
36. Cho YW, Terasaki PI, Cecka JM, Gjertson DW. Transplantation of kidneys from donors whose hearts have stopped beating. N Engl J Med. 1998 Jan 22;338(4):221–5.
37. Summers DM, Johnson RJ, Allen J, Fuggle SV, Collett D, Watson CJ, et al. Analysis of factors that affect outcome after transplantation of kidneys donated after cardiac death in the UK: a cohort study. Lancet Lond Engl. 2010 Oct 16;376(9749):1303–11.

38. van Heurn LWE, Talbot D, Nicholson ML, Akhtar MZ, Sanchez-Fructuoso AI, Weekers L, et al. Recommendations for donation after circulatory death kidney transplantation in Europe. Transpl Int Off J Eur Soc Organ Transplant. 2016 Jul;29(7):780–9.
39. Hamed MO, Chen Y, Pasea L, Watson CJ, Torpey N, Bradley JA, et al. Early graft loss after kidney transplantation: risk factors and consequences. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2015 Jun;15(6):1632–43.
40. Treckmann J, Moers C, Smits JM, Gallinat A, Maathuis M-HJ, van Kasterop-Kutz M, et al. Machine perfusion versus cold storage for preservation of kidneys from expanded criteria donors after brain death. Transpl Int Off J Eur Soc Organ Transplant. 2011 Jun;24(6):548–54.
41. Moers C, Smits JM, Maathuis M-HJ, Treckmann J, van Gelder F, Napieralski BP, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 2009 Jan 1;360(1):7–19.
42. Patel K, Nath J, Hodson J, Inston N, Ready A. Outcomes of donation after circulatory death kidneys undergoing hypothermic machine perfusion following static cold storage: A UK population-based cohort study. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2018 Jun;18(6):1408–14.
43. Kron P, Schlegel A, de Rougemont O, Oberkofler CE, Clavien P-A, Dutkowski P. Short, Cool, and Well Oxygenated - HOPE for Kidney Transplantation in a Rodent Model. Ann Surg. 2016 Nov;264(5):815–22.
44. Schopp I, Reissberg E, Lüer B, Efferz P, Minor T. Controlled Rewarming after Hypothermia: Adding a New Principle to Renal Preservation. Clin Transl Sci. 2015 Oct;8(5):475–8.
45. Jochmans I, Akhtar MZ, Nasralla D, Kocabayoglu P, Boffa C, Kaisar M, et al. Past, Present, and Future of Dynamic Kidney and Liver Preservation and Resuscitation. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2016;16(9):2545–55.
46. COPE - Trials [Internet]. [cited 2018 Aug 15]. Available from: http://cope-eu.com/work%20programme/trials.html
47. St Peter SD, Imber CJ, Lopez I, Hughes D, Friend PJ. Extended preservation of non-heart-beating donor livers with normothermic machine perfusion. Br J Surg. 2002 May;89(5):609–16.
48. Guibert EE, Petrenko AY, Balaban CL, Somov AY, Rodriguez JV, Fuller BJ. Organ Preservation: Current Concepts and New Strategies for the Next Decade. Transfus Med Hemotherapy Off Organ Dtsch Ges Transfusionsmedizin Immunhamatologie. 2011;38(2):125–42.
49. Ponticelli C. Ischaemia-reperfusion injury: a major protagonist in kidney transplantation. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc. 2014 Jun;29(6):1134–40.
50. Chouchani ET, Pell VR, Gaude E, Aksentijević D, Sundier SY, Robb EL, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature. 2014 Nov 20;515(7527):431–5.
51. Martin JL, Gruszczyk AV, Beach TE, Murphy MP, Saeb-Parsy K. Mitochondrial mechanisms and therapeutics in ischaemia reperfusion injury. Pediatr Nephrol Berl Ger. 2018 Jun 2;
52. Schlegel A, Dutkowski P. Role of hypothermic machine perfusion in liver transplantation. Transpl Int Off J Eur Soc Organ Transplant. 2015 Jun;28(6):677–89.
53. Schlegel A, Muller X, Dutkowski P. Hypothermic liver perfusion. Curr Opin Organ Transplant. 2017 Dec;22(6):563–70.

54. Collard CD, Gelman S. Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury. Anesthesiology. 2001 Jun;94(6):1133–8.
55. Maenpaa CJ, Shames BD, Van Why SK, Johnson CP, Nilakantan V. Oxidant-mediated apoptosis in proximal tubular epithelial cells following ATP depletion and recovery. Free Radic Biol Med. 2008 Feb 15;44(4):518–26.
56. Halloran PF, Hunsicker LG. Delayed graft function: state of the art, November 10-11, 2000. Summit meeting, Scottsdale, Arizona, USA. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2001 Jul;1(2):115–20.
57. Legendre C, Canaud G, Martinez F. Factors influencing long-term outcome after kidney transplantation. Transpl Int Off J Eur Soc Organ Transplant. 2014 Jan;27(1):19–27.
58. Ojo AO, Wolfe RA, Held PJ, Port FK, Schmouder RL. Delayed graft function: risk factors and implications for renal allograft survival. Transplantation. 1997 Apr 15;63(7):968–74.
59. Wu WK, Famure O, Li Y, Kim SJ. Delayed graft function and the risk of acute rejection in the modern era of kidney transplantation. Kidney Int. 2015 Oct;88(4):851–8.
60. Lee J, Song SH, Lee JY, Kim DG, Lee JG, Kim BS, et al. The recovery status from delayed graft function can predict long-term outcome after deceased donor kidney transplantation. Sci Rep. 2017 Oct 20;7(1):13725.
61. GBD 2015 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet Lond Engl. 2016 08;388(10053):1545–602.
62. Guidelines [Internet]. KDIGO. [cited 2018 Aug 16]. Available from: https://kdigo.org/guidelines/
63. [cited 2018 Aug 20]. Available from: https://nhsbtdbe.blob.core.windows.net/umbraco-assets-corp/4607/kidney-annual-report-2016-17.pdf
64. [cited 2018 Aug 20]. Available from: https://nhsbtdbe.blob.core.windows.net/umbraco-assets-corp/4493/section_2_overview_odt.pdf
65. Organ Donation and Transplantation Activities, 2015 Report [Internet]. GODT. [cited 2018 Aug 21]. Available from: http://www.transplant-observatory.org/organ-donation-transplantation-activities-2015-report-2/
66. [cited 2018 Aug 21]. Available from: https://www.renalreg.org/wp-content/uploads/2017/09/19th-Annual-Report_web_book.pdf
67. Watson CJE, Johnson RJ, Birch R, Collett D, Bradley JA. A simplified donor risk index for predicting outcome after deceased donor kidney transplantation. Transplantation. 2012 Feb 15;93(3):314–8.
68. Cecka JM, Cohen B, Rosendale J, Smith M. Could more effective use of kidneys recovered from older deceased donors result in more kidney transplants for older patients? Transplantation. 2006 Apr 15;81(7):966–70.
69. Rao PS, Schaubel DE, Guidinger MK, Andreoni KA, Wolfe RA, Merion RM, et al. A comprehensive risk quantification score for deceased donor kidneys: the kidney donor risk index. Transplantation. 2009 Jul 27;88(2):231–6.
70. [cited 2018 Aug 21]. Available from: https://optn.transplant.hrsa.gov/media/1512/guide_to_calculating_interpreting_kdpi.pdf
71. Bae S, Massie AB, Luo X, Anjum S, Desai NM, Segev DL. Changes in

Discard Rate After the Introduction of the Kidney Donor Profile Index (KDPI). Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2016;16(7):2202–7.
72. van der Wijk J, Slooff MJ, Rijkmans BG, Kootstra G. Successful 96- and 144-hour experimental kidney preservation: a combination of standard machine preservation and newly developed normothermic ex vivo perfusion. Cryobiology. 1980 Oct;17(5):473–7.
73. van der Wijk J, Rijkmans BG, Kootstra G. Six-day kidney preservation in a canine model. Influence of a one-to-four-hour ex vivo perfusion interval. Transplantation. 1983 May;35(5):408–11.
74. Rijkmans BG, Van der Wijk J, Donker AJ, Slooff MJ, Kootstra G. Functional studies in 6 days successful preserved canine kidneys. J Urol. 1982 Jan;127(1):163–6.
75. Rijkmans BG, Buurman WA, Kootstra G. Six-day canine kidney preservation. Hypothermic perfusion combined with isolated blood perfusion. Transplantation. 1984 Feb;37(2):130–4.
76. Maessen JG, van der Vusse GJ, Vork M, Kootstra G. The beneficial effect of intermediate normothermic perfusion during cold storage of ischemically injured kidneys. A study of renal nucleotide homeostasis during hypothermia in the dog. Transplantation. 1989 Mar;47(3):409–14.
77. Maessen JG, van der Vusse GJ, Vork M, Kootstra G. Intermediate normothermic perfusion during cold storage of ischemically injured kidneys. Transplant Proc. 1989 Feb;21(1 Pt 2):1252–3.
78. Arnaud FG, Khirabadi BS, Fahy GM. Normothermic blood perfusion of isolated rabbit kidneys. II. In vitro evaluation of renal function followed by orthotopic transplantation. ASAIO J Am Soc Artif Intern Organs 1992. 2000 Dec;46(6):707–18.
79. Metcalfe MS, Waller JR, Hosgood SA, Shaw M, Hassanein W, Nicholson ML. A paired study comparing the efficacy of renal preservation by normothermic autologous blood perfusion and hypothermic pulsatile perfusion. Transplant Proc. 2002 Aug;34(5):1473–4.
80. Harper S, Hosgood S, Kay M, Nicholson M. Leucocyte depletion improves renal function during reperfusion using an experimental isolated haemoperfused organ preservation system. Br J Surg. 2006 May;93(5):623–9.
81. Hosgood S, Harper S, Kay M, Bagul A, Waller H, Nicholson ML. Effects of arterial pressure in an experimental isolated haemoperfused porcine kidney preservation system. Br J Surg. 2006 Jul;93(7):879–84.
82. Patel M, Hosgood S, Nicholson ML. The effects of arterial pressure during normothermic kidney perfusion. J Surg Res. 2014 Oct;191(2):463–8.
83. Adams TD, Patel M, Hosgood SA, Nicholson ML. Lowering Perfusate Temperature From 37°C to 32°C Diminishes Function in a Porcine Model of Ex Vivo Kidney Perfusion. Transplant Direct. 2017 Mar;3(3):e140.
84. 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 Jan;95(1):111–8.
85. Hosgood SA, Barlow AD, Yates PJ, Snoeijs MGJ, van Heurn ELW, Nicholson ML. A pilot study assessing the feasibility of a short period of normothermic preservation in an experimental model of non heart beating donor kidneys. J Surg Res. 2011 Nov;171(1):283–90.
86. Hosgood SA, Patel M, Nicholson ML. The conditioning effect of ex vivo normothermic perfusion in an experimental kidney model. J Surg Res. 2013 Jun 1;182(1):153–60.

87. Hosgood SA, Shah K, Patel M, Nicholson ML. The effect of prolonged of warm ischaemic injury on renal function in an experimental ex vivo normothermic perfusion system. J Transl Med. 2015 Jun 30;13:207.
88. Kaths JM, Spetzler VN, Goldaracena N, Echeverri J, Louis KS, Foltys DB, et al. Normothermic Ex Vivo Kidney Perfusion for the Preservation of Kidney Grafts prior to Transplantation. J Vis Exp JoVE. 2015 Jul 15;(101):e52909.
89. Kaths JM, Echeverri J, Goldaracena N, Louis KS, Chun Y-M, Linares I, et al. Eight-Hour Continuous Normothermic Ex Vivo Kidney Perfusion Is a Safe Preservation Technique for Kidney Transplantation: A New Opportunity for the Storage, Assessment, and Repair of Kidney Grafts. Transplantation. 2016 Sep;100(9):1862–70.
90. Kaths JM, Echeverri J, Chun YM, Cen JY, Goldaracena N, Linares I, et al. Continuous Normothermic Ex Vivo Kidney Perfusion Improves Graft Function in Donation After Circulatory Death Pig Kidney Transplantation. Transplantation. 2017 Apr;101(4):754–63.
91. Kaths JM, Cen JY, Chun YM, Echeverri J, Linares I, Ganesh S, et al. Continuous Normothermic Ex Vivo Kidney Perfusion Is Superior to Brief Normothermic Perfusion Following Static Cold Storage in Donation After Circulatory Death Pig Kidney Transplantation. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2017;17(4):957–69.
92. Kaths JM, Echeverri J, Linares I, Cen JY, Ganesh S, Hamar M, et al. Normothermic Ex Vivo Kidney Perfusion Following Static Cold Storage-Brief, Intermediate, or Prolonged Perfusion for Optimal Renal Graft Reconditioning? Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2017 Oct;17(10):2580–90.
93. Kaths JM, Hamar M, Echeverri J, Linares I, Urbanellis P, Cen JY, et al. Normothermic ex vivo kidney perfusion for graft quality assessment prior to transplantation. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2018 Mar;18(3):580–9.
94. Urcuyo D, Blum MF, Liu Q, Nassar A, Buccini LD, Diago Uso T, et al. Development of a prolonged warm ex vivo perfusion model for kidneys donated after cardiac death. Int J Artif Organs. 2017 Jun 9;40(6):265–71.
95. Blum MF, Liu Q, Soliman B, Dreher P, Okamoto T, Poggio ED, et al. Comparison of normothermic and hypothermic perfusion in porcine kidneys donated after cardiac death. J Surg Res. 2017 Aug;216:35–45.
96. Arnaud FG, Khirabadi BS, Fahy GM. Normothermic blood perfusion of isolated rabbit kidneys. III. In vitro physiology of kidneys after perfusion with Euro-Collins solution or 7.5 M cryoprotectant (VS4). Transpl Int Off J Eur Soc Organ Transplant. 2002 Jun;15(6):278–89.
97. Hosgood SA, Barlow AD, Dormer J, Nicholson ML. The use of ex-vivo normothermic perfusion for the resuscitation and assessment of human kidneys discarded because of inadequate in situ perfusion. J Transl Med. 2015 Oct 16;13:329.
98. Hosgood SA, Thompson E, Moore T, Wilson CH, Nicholson ML. Normothermic machine perfusion for the assessment and transplantation of declined human kidneys from donation after circulatory death donors. Br J Surg. 2018 Mar;105(4):388–94.
99. Weissenbacher A, Hunter J. Normothermic machine perfusion of the kidney. Curr Opin Organ Transplant. 2017 Dec;22(6):571–6.
100. Hosgood SA, Nicholson ML. First in man renal transplantation after ex vivo

normothermic perfusion. Transplantation. 2011 Oct 15;92(7):735–8.
101. Nicholson ML, Hosgood SA. Renal transplantation after ex vivo normothermic perfusion: the first clinical study. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2013 May;13(5):1246–52.
102. Hosgood SA, Saeb-Parsy K, Hamed MO, Nicholson ML. Successful Transplantation of Human Kidneys Deemed Untransplantable but Resuscitated by Ex Vivo Normothermic Machine Perfusion. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2016;16(11):3282–5.
103. Hosgood SA, Saeb-Parsy K, Wilson C, Callaghan C, Collett D, Nicholson ML. Protocol of a randomised controlled, open-label trial of ex vivo normothermic perfusion versus static cold storage in donation after circulatory death renal transplantation. BMJ Open. 2017 23;7(1):e012237.
104. Hosgood SA, Nicholson ML. Ex vivo normothermic perfusion of declined human kidneys after inadequate in situ perfusion. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2014 Feb;14(2):490–1.
105. Hosgood SA, Nicholson ML. The first clinical case of intermediate ex vivo normothermic perfusion in renal transplantation. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2014 Jul;14(7):1690–2.
106. Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MTPR, et al. Liver Transplantation After Ex Vivo Normothermic Machine Preservation: A Phase 1 (First-in-Man) Clinical Trial. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2016;16(6):1779–87.
107. Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. A randomized trial of normothermic preservation in liver transplantation. Nature. 2018 May;557(7703):50–6.
108. Technology [Internet]. OrganOx. [cited 2018 Aug 25]. Available from: http://www.organox.com/metra/
109. GmbH SUH. SONOFLOW Clamp-on Sensor, SONOTEC Ultraschallsensorik Halle GmbH [Internet]. [cited 2018 Aug 25]. Available from: https://www.sonotec.eu/products/non-invasive-fluid-monitoring/flow-rate-measurement/sonoflow-clamp-on-sensor/
110. Temperature Sensors & Monitors [Internet]. PendoTECH. [cited 2018 Aug 25]. Available from: http://www.pendotech.com/pendotech-products/sensors-and-monitors/temperature-sensors-monitors/
111. Goupil C, Seifert W, Zabrocki K, Müller E, Snyder GJ, Goupil C, et al. Thermodynamics of Thermoelectric Phenomena and Applications. Entropy. 2011 Aug 15;13(8):1481–517.
112. LD20 - Liquid Flow Sensor | Sensirion [Internet]. [cited 2018 Aug 25]. Available from: https://www.sensirion.com/en/flow-sensors/liquid-flow-meters/page/ld20-disposable-flow-sensors/
113. Danzl DF, Pozos RS. Accidental hypothermia. N Engl J Med. 1994 Dec 29;331(26):1756–60.
114. [cited 2018 Aug 25]. Available from: https://www.nice.org.uk/advice/mib38/resources/i-stat-cg4-and-chem8-cartridges-for-pointofcare-testing-in-the-emergency-department-pdf-63499112313541
115. [cited 2018 Aug 25]. Available from: https://www.coachrom.com/fileadmin/docs/hbm/en/221011.pdf
116. Yu Z, Huang H, Reim A, Charles PD, Northage A, Jackson D, et al. Optimizing 2D gas chromatography mass spectrometry for robust tissue, serum and urine metabolite profiling. Talanta. 2017 Apr 1;165:685–91.

117. Beens J, Adahchour M, Vreuls RJ, van Altena K, Brinkman UA. Simple, non-moving modulation interface for comprehensive two-dimensional gas chromatography. J Chromatogr A. 2001 Jun 1;919(1):127–32.
118. Remuzzi G, Cravedi P, Perna A, Dimitrov BD, Turturro M, Locatelli G, et al. Long-term outcome of renal transplantation from older donors. N Engl J Med. 2006 Jan 26;354(4):343–52.
119. Huang H, van Dullemen LFA, Akhtar MZ, Faro M-LL, Yu Z, Valli A, et al. Proteo-metabolomics reveals compensation between ischemic and non-injured contralateral kidneys after reperfusion. Sci Rep. 2018 Jun 4;8(1):8539.
120. Davis S, Charles PD, He L, Mowlds P, Kessler BM, Fischer R. Expanding Proteome Coverage with CHarge Ordered Parallel Ion aNalysis (CHOPIN) Combined with Broad Specificity Proteolysis. J Proteome Res. 2017 03;16(3):1288–99.
121. Tyanova S, Temu T, Cox J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat Protoc. 2016 Dec;11(12):2301–19.
122. [cited 2018 Aug 25]. Available from: http://odt.nhs.uk/pdf/kidney_allocation_policy.pdf
123. Clexane pre-filled syringes - Summary of Product Characteristics (SmPC) - (eMC) [Internet]. [cited 2018 Aug 25]. Available from: https://www.medicines.org.uk/emc/product/4499/smpc#PHARMACOKINETIC_PROPS
124. Hetzel GR, Sucker C. The heparins: all a nephrologist should know. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc. 2005 Oct;20(10):2036–42.
125. Newall F. Anti-factor Xa (anti-Xa) assay. Methods Mol Biol Clifton NJ. 2013;992:265–72.
126. Brasile L. System for exsanguinous metabolic support of an organ or tissue [Internet]. US20100316705A1, 2010. Available from: https://patents.google.com/patent/US20100316705/en
127. Kamler M, Wendt D, Pizanis N, Milekhin V, Schade U, Jakob H. Deleterious effects of oxygen during extracorporeal circulation for the microcirculation in vivo. Eur J Cardio-Thorac Surg Off J Eur Assoc Cardio-Thorac Surg. 2004 Sep;26(3):564–70.
128. Chartrand C, Langevin L, Forest F, Stanley P. [Hyperoxemia in extracorporeal circulation]. Can J Surg J Can Chir. 1975 Jan;18(1):84–7.
129. Zwemer CF, Shoemaker JL, Hazard SW, Davis RE, Bartoletti AG, Phillips CL. Hyperoxic reperfusion exacerbates postischemic renal dysfunction. Surgery. 2000 Nov;128(5):815–21.
130. Watson CJE, Kosmoliaptsis V, Randle LV, Gimson AE, Brais R, Klinck JR, et al. Normothermic Perfusion in the Assessment and Preservation of Declined Livers Before Transplantation: Hyperoxia and Vasoplegia-Important Lessons From the First 12 Cases. Transplantation. 2017;101(5):1084–98.
131. Onodera K, Omoi N-O, Fukui K, Hayasaka T, Shinkai T, Suzuki S, et al. Oxidative damage of rat cerebral cortex and hippocampus, and changes in antioxidative defense systems caused by hyperoxia. Free Radic Res. 2003 Apr;37(4):367–72.
132. Munshi L, Kiss A, Cypel M, Keshavjee S, Ferguson ND, Fan E. Oxygen Thresholds and Mortality During Extracorporeal Life Support in Adult Patients. Crit Care Med. 2017 Dec;45(12):1997–2005.
133. Mather A, Pollock C. Glucose handling by the kidney. Kidney Int Suppl. 2011

Mar;(120):S1–6.
134. Stumvoll M, Meyer C, Mitrakou A, Nadkarni V, Gerich JE. Renal glucose production and utilization: new aspects in humans. Diabetologia. 1997 Jul;40(7):749–57.
135. Meyer C, Stumvoll M, Dostou J, Welle S, Haymond M, Gerich J. Renal substrate exchange and gluconeogenesis in normal postabsorptive humans. Am J Physiol Endocrinol Metab. 2002 Feb;282(2):E428–34.
136. Ross BD, Espinal J, Silva P. Glucose metabolism in renal tubular function. Kidney Int. 1986 Jan;29(1):54–67.
137. Zhang W, Edwards A. A model of glucose transport and conversion to lactate in the renal medullary microcirculation. Am J Physiol Renal Physiol. 2006 Jan;290(1):F87–102.
138. Bartlett S, Espinal J, Janssens P, Ross BD. The influence of renal function on lactate and glucose metabolism. Biochem J. 1984 Apr 1;219(1):73–8.
139. Hui S, Ghergurovich JM, Morscher RJ, Jang C, Teng X, Lu W, et al. Glucose feeds the TCA cycle via circulating lactate. Nature. 2017 Nov;551(7678):115–8.
140. biomarker | Definition of biomarker in English by Oxford Dictionaries [Internet]. Oxford Dictionaries | English. [cited 2018 Sep 5]. Available from: https://en.oxforddictionaries.com/definition/biomarker
141. Carlomagno N, Incollingo P, Tammaro V, Peluso G, Rupealta N, Chiacchio G, et al. Diagnostic, Predictive, Prognostic, and Therapeutic Molecular Biomarkers in Third Millennium: A Breakthrough in Gastric Cancer. BioMed Res Int [Internet]. 2017;2017. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5637861/
142. Metabolomics. In: Wikipedia [Internet]. 2018. Available from: https://en.wikipedia.org/w/index.php?title=Metabolomics&oldid=858012609
143. Peng B, Li H, Peng X-X. Functional metabolomics: from biomarker discovery to metabolome reprogramming. Protein Cell. 2015 Sep;6(9):628–37.
144. Proteomics. In: Wikipedia [Internet]. 2018. Available from: https://en.wikipedia.org/w/index.php?title=Proteomics&oldid=858012069
145. Cowland JB, Borregaard N. Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. Genomics. 1997 Oct 1;45(1):17–23.
146. Devarajan P. Review: neutrophil gelatinase-associated lipocalin: a troponin-like biomarker for human acute kidney injury. Nephrol Carlton Vic. 2010 Jun;15(4):419–28.
147. Bonventre JV. Kidney injury molecule-1 (KIM-1): a urinary biomarker and much more. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc. 2009 Nov;24(11):3265–8.
148. Han WK, Alinani A, Wu C-L, Michaelson D, Loda M, McGovern FJ, et al. Human kidney injury molecule-1 is a tissue and urinary tumor marker of renal cell carcinoma. J Am Soc Nephrol JASN. 2005 Apr;16(4):1126–34.
149. Smathers RL, Petersen DR. The human fatty acid-binding protein family: evolutionary divergences and functions. Hum Genomics. 2011 Mar;5(3):170–91.
150. Obata Y, Kamijo-Ikemori A, Ichikawa D, Sugaya T, Kimura K, Shibagaki Y, et al. Clinical usefulness of urinary liver-type fatty-acid-binding protein as a perioperative marker of acute kidney injury in patients undergoing endovascular or open-abdominal aortic aneurysm repair. J Anesth. 2016 Feb;30(1):89–99.
151. Kamijo A, Sugaya T, Hikawa A, Kimura K. [Urinary fatty acid binding protein as a new clinical marker for the progression of chronic renal disease]. Rinsho Byori. 2003 Mar;51(3):219–24.

152. Bonventre JV, Vaidya VS, Schmouder R, Feig P, Dieterle F. Next-generation biomarkers for detecting kidney toxicity. Nat Biotechnol. 2010 May;28(5):436–40.
153. Bacci MR, Chehter EZ, Azzalis LA, Costa de Aguiar Alves B, Fonseca FLA. Serum NGAL and Cystatin C Comparison With Urinary Albumin-to-Creatinine Ratio and Inflammatory Biomarkers as Early Predictors of Renal Dysfunction in Patients With Type 2 Diabetes. Kidney Int Rep. 2017 Mar;2(2):152–8.
154. Kawai A, Kusaka M, Kitagawa F, Ishii J, Fukami N, Maruyama T, et al. Serum liver-type fatty acid-binding protein predicts recovery of graft function after kidney transplantation from donors after cardiac death. Clin Transplant. 2014 Jun;28(6):749–54.
155. Sharma A, Demissei BG, Tromp J, Hillege HL, Cleland JG, O'Connor CM, et al. A network analysis to compare biomarker profiles in patients with and without diabetes mellitus in acute heart failure. Eur J Heart Fail. 2017;19(10):1310–20.
156. 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.
157. Wijermars LGM, Schaapherder AF, de Vries DK, Verschuren L, Wüst RCI, Kostidis S, et al. Defective postreperfusion metabolic recovery directly associates with incident delayed graft function. Kidney Int. 2016;90(1):181–91.
158. 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 Apr;99(4):754–9.
159. Human Metabolome Database: Browsing metabolites [Internet]. [cited 2018 Sep 17]. Available from: http://www.hmdb.ca/metabolites
160. Akram M. Citric Acid Cycle and Role of its Intermediates in Metabolism. Cell Biochem Biophys. 2014 Apr 1;68(3):475–8.
161. Møller N, Meek S, Bigelow M, Andrews J, Nair KS. The kidney is an important site for in vivo phenylalanine-to-tyrosine conversion in adult humans: A metabolic role of the kidney. Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1242–6.
162. Kopple JD. Phenylalanine and tyrosine metabolism in chronic kidney failure. J Nutr. 2007;137(6 Suppl 1):1586S – 1590S; discussion 1597S – 1598S.
163. Poll VD, Cg M, Soeters PB, Deutz NE, Fearon KC, Dejong CH. Renal metabolism of amino acids: its role in interorgan amino acid exchange. Am J Clin Nutr. 2004 Feb 1;79(2):185–97.
164. D'Alessandro A, Nemkov T, Kelher M, West BF, Schwindt RK, Banerjee A, et al. Routine Storage of Red Blood Cell Units in Additive Solution-3: a comprehensive investigation of the RBC metabolome. Transfusion (Paris). 2015 Jun;55(6):1155–68.
165. Pubchem. Pidolic acid [Internet]. [cited 2018 Sep 12]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/7405
166. Pubchem. Indoleacetic acid [Internet]. [cited 2018 Sep 12]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/802
167. Wakil SJ. The Mechanism of Fatty Acid Synthesis. Am J Clin Nutr. 1960 Sep 1;8(5):630–44.
168. UniProt [Internet]. [cited 2018 Sep 5]. Available from: https://www.uniprot.org/
169. Sigdel TK, Lee S, Sarwal MM. Profiling the proteome in renal transplantation. Proteomics Clin Appl. 2011 Jun;5(5-6):269–80.
170. Sigdel TK, Salomonis N, Nicora CD, Ryu S, He J, Dinh V, et al. The identification of novel potential injury mechanisms and candidate biomarkers in renal allograft rejection by quantitative proteomics. Mol Cell Proteomics MCP. 2014 Feb;13(2):621–31.

171. Perseus [Internet]. [cited 2018 Sep 5]. Available from: http://www.biochem.mpg.de/5111810/perseus
172. STRING: functional protein association networks [Internet]. [cited 2018 Sep 5]. Available from: https://string-db.org/cgi/input.pl
173. Seong S-Y, Matzinger P. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. Nat Rev Immunol. 2004 Jun;4(6):469–78.
174. Damage-associated molecular pattern. In: Wikipedia [Internet]. 2018. Available from: https://en.wikipedia.org/w/index.php?title=Damage-associated_molecular_pattern&oldid=851371620
175. PDHA1 pyruvate dehydrogenase E1 alpha 1 subunit [Homo sapiens (human)] - Gene - NCBI [Internet]. [cited 2018 Sep 13]. Available from: https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=ShowDetailView&TermToSearch=5160
176. HIBADH 3-hydroxyisobutyrate dehydrogenase [Homo sapiens (human)] - Gene - NCBI [Internet]. [cited 2018 Sep 13]. Available from: https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=ShowDetailView&TermToSearch=11112
177. Key M. A tutorial in displaying mass spectrometry-based proteomic data using heat maps. BMC Bioinformatics. 2012;13 Suppl 16:S10.
178. Gatto L, Breckels LM, Naake T, Gibb S. Visualisation of proteomics data using R and bioconductor. Proteomics. 2015 Apr;15(8):1375–89.
179. Reference GH. AGT gene [Internet]. Genetics Home Reference. [cited 2018 Aug 14]. Available from: https://ghr.nlm.nih.gov/gene/AGT
180. Satou R, Shao W, Navar LG. Role of stimulated intrarenal angiotensinogen in hypertension. Ther Adv Cardiovasc Dis. 2015 Aug;9(4):181–90.
181. Carbonic anhydrase. In: Wikipedia [Internet]. 2018. Available from: https://en.wikipedia.org/w/index.php?title=Carbonic_anhydrase&oldid=854816633
182. Lawson C, Wolf S. ICAM-1 signaling in endothelial cells. Pharmacol Rep PR. 2009 Feb;61(1):22–32.
183. PCK1. In: Wikipedia [Internet]. 2017. Available from: https://en.wikipedia.org/w/index.php?title=PCK1&oldid=799429119
184. Turner J-E, Becker M, Mittrücker H-W, Panzer U. Tissue-Resident Lymphocytes in the Kidney. J Am Soc Nephrol JASN. 2018 Feb;29(2):389–99.
185. Munro DAD, Hughes J. The Origins and Functions of Tissue-Resident Macrophages in Kidney Development. Front Physiol. 2017;8:837.
186. Takada M, Nadeau KC, Hancock WW, Mackenzie HS, Shaw GD, Waaga AM, et al. Effects of explosive brain death on cytokine activation of peripheral organs in the rat. Transplantation. 1998 Jun 27;65(12):1533–42.
187. van der Hoeven JAB, Molema G, Ter Horst GJ, Freund RL, Wiersema J, van Schilfgaarde R, et al. Relationship between duration of brain death and hemodynamic (in)stability on progressive dysfunction and increased immunologic activation of donor kidneys. Kidney Int. 2003 Nov;64(5):1874–82.
188. Nijboer WN, Schuurs TA, van der Hoeven JAB, Fekken S, Wiersema-Buist J, Leuvenink HGD, et al. Effect of brain death on gene expression and tissue activation in human donor kidneys. Transplantation. 2004 Oct 15;78(7):978–86.
189. Lechler RI, Batchelor JR. Immunogenicity of retransplanted rat kidney allografts. Effect of inducing chimerism in the first recipient and quantitative studies on immunosuppression of the second recipient. J Exp Med. 1982 Dec 1;156(6):1835–41.

190. Boardman DA, Jacob J, Smyth LA, Lombardi G, Lechler RI. What Is Direct Allorecognition? Curr Transplant Rep. 2016;3(4):275–83.
191. Anti-inflammatory cytokines list [Internet]. [cited 2018 Sep 4]. Available from: http://www.sinobiological.com/Anti-inflammatory-cytokines-list.html
192. Opal SM, DePalo VA. Anti-inflammatory cytokines. Chest. 2000 Apr;117(4):1162–72.
193. Greig SL, Scott LJ, Plosker GL. Epoprostenol (Veletri®, Caripul®): a review of its use in patients with pulmonary arterial hypertension. Am J Cardiovasc Drugs Drugs Devices Interv. 2014 Dec;14(6):463–70.
194. Mubarak KK. A review of prostaglandin analogs in the management of patients with pulmonary arterial hypertension. Respir Med. 2010 Jan;104(1):9–21.
195. Epoprostenol 1.5 mg Powder and Solvent for Solution for Infusion - Summary of Product Characteristics (SmPC) - (eMC) [Internet]. [cited 2018 Sep 6]. Available from: https://www.medicines.org.uk/emc/product/5292/smpc
196. Pubchem. Epoprostenol [Internet]. [cited 2018 Sep 6]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/5280427
197. 6-keto Prostaglandin F1α (CAS 58962-34-8) | Cayman Chemical [Internet]. [cited 2018 Sep 6]. Available from: https://www.caymanchem.com/product/15210
198. Johnson RA, Lincoln FH, Thompson JL, Nidy EG, Mizak SA, Axen U. Synthesis and sterochemistry of prostacyclin and synthesis of 6-ketoprostagliandin F1alpha. J Am Chem Soc. 1977 Jun 8;99(12):4182–4.
199. Förstermann U, Neufang B, Hertting G. Rapid and effective conversion of 6-keto-prostaglandin F1 alpha to 6,15-diketo-13, 14-dihydro-prostaglandin F1 alpha-immunoreactive material in vivo. Prostaglandins Leukot Med. 1982 Sep;9(3):277–84.
200. Chaumais M-C, Jobard M, Huertas A, Vignand-Courtin C, Humbert M, Sitbon O, et al. Pharmacokinetic evaluation of continuous intravenous epoprostenol. Expert Opin Drug Metab Toxicol. 2010 Dec;6(12):1587–98.
201. Brash AR, Jackson EK, Saggese CA, Lawson JA, Oates JA, FitzGerald GA. Metabolic disposition of prostacyclin in humans. J Pharmacol Exp Ther. 1983 Jul;226(1):78–87.
202. Nicolas LB, Krause A, Gutierrez MM, Dingemanse J. Integrated pharmacokinetics and pharmacodynamics of epoprostenol in healthy subjects. Br J Clin Pharmacol. 2012 Dec;74(6):978–89.
203. Herner SJ, Mauro LS. Epoprostenol in primary pulmonary hypertension. Ann Pharmacother. 1999 Mar;33(3):340–7.
204. Securon I.V. - Summary of Product Characteristics (SmPC) - (eMC) [Internet]. [cited 2018 Sep 6]. Available from: https://www.medicines.org.uk/emc/product/979/smpc
205. Mooy J, Schols M, v Baak M, v Hooff M, Muytjens A, Rahn KH. Pharmacokinetics of verapamil in patients with renal failure. Eur J Clin Pharmacol. 1985;28(4):405–10.
206. Verapamil Pharmacology [Internet]. [cited 2018 Sep 6]. Available from: https://www.infomed.ch/100drugs/veraphar.html
207. Hashimoto S, Kobayashi A. Clinical pharmacokinetics and pharmacodynamics of glyceryl trinitrate and its metabolites. Clin Pharmacokinet. 2003;42(3):205–21.
208. Seth P, Fung HL. Biochemical characterization of a membrane-bound enzyme responsible for generating nitric oxide from nitroglycerin in vascular smooth muscle cells. Biochem Pharmacol. 1993 Oct 19;46(8):1481–6.
209. Feelisch M, Noack EA. Correlation between nitric oxide formation during

degradation of organic nitrates and activation of guanylate cyclase. Eur J Pharmacol. 1987 Jul 2;139(1):19–30.
210. Feelisch M, Brands F, Kelm M. Human endothelial cells bioactivate organic nitrates to nitric oxide: implications for the reinforcement of endothelial defence mechanisms. Eur J Clin Invest. 1995 Oct;25(10):737–45.
211. Glyceryl Trinitrate 1mg/ml solution for infusion - Summary of Product Characteristics (SmPC) - (eMC) [Internet]. [cited 2018 Sep 7]. Available from: https://www.medicines.org.uk/emc/product/6296/smpc
212. Simpson DP. Citrate excretion: a window on renal metabolism. Am J Physiol. 1983 Mar;244(3):F223–34.
213. Li K, Xu Y. Citrate metabolism in blood transfusions and its relationship due to metabolic alkalosis and respiratory acidosis. Int J Clin Exp Med. 2015;8(4):6578–84.
214. Monchi M. Citrate pathophysiology and metabolism. Transfus Apher Sci Off J World Apher Assoc Off J Eur Soc Haemapheresis. 2017 Feb;56(1):28–30.
215. Hamm LL. Renal handling of citrate. Kidney Int. 1990 Oct;38(4):728–35.
216. Bellomo R. Bench-to-bedside review: Lactate and the kidney. Crit Care. 2002;6(4):322–6.
217. [cited 2018 Sep 8]. Available from: https://ntp.niehs.nih.gov/nnl/urinary/kidney/rtvac/kidney-tubule-vacuolation-cytoplasmic-pdf_508.pdf
218. Soltran - Summary of Product Characteristics (SmPC) - (eMC) [Internet]. [cited 2018 Sep 8]. Available from: https://www.medicines.org.uk/emc/product/1873/smpc
219. [cited 2018 Sep 8]. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2005/013684s091lbl.pdf
220. Nomani AZ, Nabi Z, Rashid H, Janjua J, Nomani H, Majeed A, et al. Osmotic nephrosis with mannitol: review article. Ren Fail. 2014 Aug;36(7):1169–76.
221. *Primum non nocere*. In: Wikipedia [Internet]. 2018. Available from: https://en.wikipedia.org/w/index.php?title=Primum_non_nocere&oldid=843992827
222. Charles Lindbergh's Perfusion Pump | At the Smithsonian | Smithsonian [Internet]. [cited 2018 Aug 22]. Available from: https://www.smithsonianmag.com/smithsonian-institution/save-his-dying-sister-law-charles-lindbergh-Invented-medical-device-180956526/
223. The Lifeport Kidney Transporter [Internet]. INDEX: Design to Improve Life®. 2011 [cited 2018 Aug 22]. Available from: https://designtoimprovelife.dk/the-lifeport-kidney-transporter/
224. Hypothermic perfusion preservation: The future of organ preservation revisited? Cryobiology. 2007 Apr 1;54(2):129–45.
225. Kox J, Moers C, Monbaliu D, Strelniece A, Treckmann J, Jochmans I, et al. The Benefits of Hypothermic Machine Preservation and Short Cold Ischemia Times in Deceased Donor Kidneys. Transplantation. 2018 Aug;102(8):1344–50.
226. DiRito JR, Hosgood SA, Tietjen GT, Nicholson ML. The future of marginal kidney repair in the context of normothermic machine perfusion. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2018 Jun 7;
227. Hosgood SA, Nicholson ML. The evolution of donation after circulatory death donor kidney repair in the United Kingdom. Curr Opin Organ Transplant. 2018 Feb;23(1):130–5.
228. Hosgood SA, Saeb-Parsy K, Wilson C, Callaghan C, Collett D, Nicholson ML. Protocol of a randomised controlled, open-label trial of ex vivo normothermic perfusion versus static cold storage in donation after circulatory death renal

transplantation. BMJ Open. 2017 23;7(1):e012237.
229. Yates PJ, Hosgood SA, Nicholson ML. Leukocyte and platelet depletion improves blood flow and function in a renal transplant model. J Surg Res. 2012 Jan;172(1):159–64.
230. Hamar M, Urbanellis P, Kaths MJ, Kollmann D, Linares I, Ganesh S, et al. Normothermic Ex Vivo Kidney Perfusion Reduces Warm Ischemic Injury of Porcine Kidney Grafts Retrieved After Circulatory Death. Transplantation. 2018 Aug;102(8):1262–70.
231. Weissenbacher A, Lo Faro L, Boubriak O, Soares MF, Roberts IS, Hunter JP, et al. Twenty-four-hour normothermic perfusion of discarded human kidneys with urine recirculation. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2018 May 14;
232. Eos ECMO [Internet]. [cited 2018 Aug 23]. Available from: http://www.sorin.com/products/cardiac-surgery/perfusion/long-term-use/eos-ecmo
233. Lequier L, Horton SB, McMullan DM, Bartlett RH. Extracorporeal Membrane Oxygenation Circuitry. Pediatr Crit Care Med J Soc Crit Care Med World Fed Pediatr Intensive Crit Care Soc. 2013 Jun;14(5 0 1):S7–12.
234. [cited 2018 Aug 23]. Available from: https://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm073670.pdf
235. Vincent J-L. Intensive Care Medicine: Annual Update 2009. Springer Science & Business Media; 2010. 989 p.
236. Parikh CR, Hall IE, Bhangoo RS, Ficek J, Abt PL, Thiessen-Philbrook H, et al. Associations of Perfusate Biomarkers and Pump Parameters With Delayed Graft Function and Deceased Donor Kidney Allograft Function. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2016;16(5):1526–39.
237. Hoogland ERP, de Vries EE, Christiaans MHL, Winkens B, Snoeijs MGJ, van Heurn LWE. The value of machine perfusion biomarker concentration in DCD kidney transplantations. Transplantation. 2013 Feb 27;95(4):603–10.
238. Hosgood SA, Nicholson ML. An Assessment of Urinary Biomarkers in a Series of Declined Human Kidneys Measured During Ex Vivo Normothermic Kidney Perfusion. Transplantation. 2017;101(9):2120–5.
239. Devarajan P. Neutrophil gelatinase-associated lipocalin: a promising biomarker for human acute kidney injury. Biomark Med. 2010 Apr;4(2):265–80.
240. Devarajan P. Review: neutrophil gelatinase-associated lipocalin: a troponin-like biomarker for human acute kidney injury. Nephrol Carlton Vic. 2010 Jun;15(4):419–28.
241. Cravedi P, Ruggenenti P, Remuzzi G. Proteinuria should be used as a surrogate in CKD. Nat Rev Nephrol. 2012 Mar 6;8(5):301–6.
242. Seeman T. Management of proteinuria in the transplanted patient. Pediatr Nephrol Berl Ger. 2015 Jun;30(6):889–903.
243. D'Amico G, Bazzi C. Pathophysiology of proteinuria. Kidney Int. 2003 Mar;63(3):809–25.
244. Andrews PM, Cooper M, Verbesey J, Ghasemian S, Rogalsky D, Moody P, et al. Mannitol infusion within 15 min of cross-clamp improves living donor kidney preservation. Transplantation. 2014 Oct 27;98(8):893–7.
245. Hamburger J, Halpern B, Funck-Brentano JL. [Variety of anuria induced by excessive hydration of the renal cells]. Presse Med. 1954 Jun 23;62(46):972–6.
246. [cited 2018 Aug 23]. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2005/013684s091lbl.pdf

247. Shi J, Qian J, Li H, Luo H, Luo W, Lin Z. Renal tubular epithelial cells injury induced by mannitol and its potential mechanism. Ren Fail. 2018 Nov;40(1):85–91.
248. Naesens M, Kuypers DRJ, Sarwal M. Calcineurin Inhibitor Nephrotoxicity. Clin J Am Soc Nephrol. 2009 Feb 1;4(2):481–508.
249. Satou R, Shao W, Navar LG. Role of stimulated intrarenal angiotensinogen in hypertension. Ther Adv Cardiovasc Dis. 2015 Aug;9(4):181–90.
250. Acetazolamide. In: Wikipedia [Internet]. 2018. Available from: https://en.wikipedia.org/w/index.php?title=Acetazolamide&oldid=846970242
251. Wood KJ, Bushell A, Hester J. Regulatory immune cells in transplantation. Nat Rev Immunol. 2012 May 25;12(6):417–30.
252. Stone JP, Ball AL, Critchley WR, Major T, Edge RJ, Amin K, et al. Ex Vivo Normothermic Perfusion Induces Donor-Derived Leukocyte Mobilization and Removal Prior to Renal Transplantation. Kidney Int Rep. 2016 Nov;1(4):230–9.
253. Shahzad A, Knapp M, Lang I, Köhler G. Interleukin 8 (IL-8) - a universal biomarker? Int Arch Med. 2010 Jun 15;3:11.
254. Matz M, Lorkowski C, Fabritius K, Wu K, Rudolph B, Frischbutter S, et al. The selective biomarker IL-8 identifies IFTA after kidney transplantation in blood cells. Transpl Immunol. 2016;39:18–24.
255. Ulbing M, Kirsch AH, Leber B, Lemesch S, Münzker J, Schweighofer N, et al. MicroRNAs 223-3p and 93-5p in patients with chronic kidney disease before and after renal transplantation. Bone. 2017;95:115–23.
256. Friedman BH, Wolf JH, Wang L, Putt ME, Shaked A, Christie JD, et al. SERUM CYTOKINE PROFILES ASSOCIATED WITH EARLY ALLOGRAFT DYSFUNCTION IN PATIENTS UNDERGOING LIVER TRANSPLANTATION. Liver Transpl. 2012 Feb;18(2):166–76.
257. De Perrot M, Sekine Y, Fischer S, Waddell TK, McRae K, Liu M, et al. Interleukin-8 release during early reperfusion predicts graft function in human lung transplantation. Am J Respir Crit Care Med. 2002 Jan 15;165(2):211–5.