THE ROLE AND CLINICAL UTILITY OF ACUTE KIDNEY INJURY BIOMARKERS IN THE SETTING OF RENAL TRANSPLANTATION

by

Melanie Field

A thesis submitted to the University of Birmingham for the degree of Doctorate of Medicine

Department of Infection and Immunity

School of Medicine

University of Birmingham Medical School

2014

Abstract

Aim:

To evaluate the role and utility of acute kidney injury biomarkers in renal transplantation.

Method:

Analysis of levels of a panel of biomarkers in deceased organ donors correlated to renal graft outcome; ABO incompatible renal transplant recipients correlated to rejection and HLA incompatible renal transplant correlated to rejection.

Results:

The panel of biomarkers showed comparable ability to creatinine in predicting the outcome of the renal graft following transplant from deceased donors. Of the panel of biomarkers tested to predict rejection, notably NGAL and IP-10 had good ability to predict those HLAi recipients who subsequently developed rejection.

Conclusion:

Biomarkers previously identified in the context of AKI may have a role in the assessment of deceased organ donor suitability but more promisingly have an excellent ability to identify those patients at risk of rejection following HLAi transplant and would now benefit from evaluation in a wider population prior to adoption in a clinical trial.

Dedication/acknowledgments

I would very much like to thank the Birmingham QE renal unit and the Queen Elizabeth Hospital Charities for supporting this work. In particular my supervisors Nick Inston and Mark Cobbold need thanks for their endless support and enthusiasm for the project.

I would also like to thank my husband (David) and children (Ben and Darcey) for their unending support and encouragement throughout this research.

List of Abbreviations

AMR	Antibody mediated rejection
ATN	Acute tubular necrosis
CV	Co-efficient of variation
DBD	Donation after brainstem death
DCD	Donation after circulatory death
DGF	Delayed graft function
DSA	Donor specific antibody
ELISA	Enzyme-linked immunosorbant assay
HLA	Human Leukocyte Antigen
IFN-γ	Interferon-γ
IGF	Intermediate graft function
IP-10	Interferon-inducible protein-10
KIM-1	Kidney injury molecule 1
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
NGAL	Neutrophil Gelatinase Associated Lipocalin
ROC	Receiver operator curve
TNF- α	Tumour Necrosis Factor α
VEGF	Vascular Endothelial Growth Factor

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Chapter 1: Introduction

1.1 Historical Background to Renal Transplantation

Early attempts at renal transplantation were first made in the early 20th century in response to the lack of any other treatment modality for end stage renal failure. Some of those attempts showed promise, involving transplanting animal kidneys into humans, but although urine was produced the kidneys only functioned for an hour before ceasing to function and the patient died within 2 weeks (Morris, 1994). The first human to human kidney transplant was performed in 1933 by Voronoy in Russia. The graft failed within 48hrs, due to mismatches in donor and recipient ABO blood groups which were not known about at the time, highlighting the lack of knowledge around immunology and blood antigens at the time, (Hamilton and Reid, 1984).

The first successful human renal transplant was undertaken in 1954 at the Brigham and Women's Hospital in Boston by Joseph Murray. The transplant was undertaken between identical twins and the graft survived without the need for immunosuppression (Murray et al., 1958).

This initial successful transplant worked because it serendipitously bypassed the immune barriers, the mechanics of which were poorly understood at that time. Despite advances in the understanding of compatibility between individuals previously no measures were available to prevent the response that had been termed rejection by Medawar and Brent a decade before (Medawar, 1944),

Despite the immune response remaining a barrier to transplantation this single operation on December 23rd 1954 by Joseph Murray, which successfully transplanted a kidney, remains one of the most important achievements in modern science, a feat recognised by the award of the Nobel prize to Murray in 1990.

This accomplishment demonstrated that not only was kidney transplantation technically feasible it reinvigorated a field that for over 50 years had met with failure.

1.1.1 **Barriers to Transplantation**

The barriers to transplanting incompatible individuals were identified from skin graft experiments. It was known that skin grafts transplanted between non-identical mice became necrotic and sloughed off rapidly whilst those from identical mice were preserved. It was also noted that a second graft was rejected more vigorously indicating the presence of an innate and adaptive immune response. This led to testing of this response *ex vivo* and the development of cross matching and subsequently mixed lymphocyte reactions.

1.1.2 **The Development of Immunosuppression**

Despite the advances in the understanding of tissue compatibility and the identification of the major histocompatibility complex, laboratory models of whole organ transplantation were still largely unsuccessful (Dausset, 1980). Whilst transplants undertaken in animal models, specifically dogs would work briefly, they stopped working and were rejected within days until the advent of mechanisms to overcome the immune system (Calne, 1960).

Following the realisation that the immune system was a key component in the failure of renal transplants, efforts were focused on suppression of the immune system.

Initially this used whole body irradiation which was known to render the patient profoundly immunosuppressed (Murray et al., 1960). The first clinical trial had only one success (transplantation between non-identical brothers) and most failures were due to bone marrow suppression, agranulocytosis and overwhelming infection.

The most significant breakthrough in immunosuppression came with the advent of 6-mercaptopurine and its later derivative azathioprine (Calne, 1960, Schwartz and Dameshek, 1959). These agents alone and more importantly, in combination with other anti-rejection agents allowed the recipients to delay rejection without rendering the recipients severely immune-deficient.

The combination of improved surgical technique and, perhaps more importantly, the development of new immunosuppressive agents or combinations of existing agents is responsible for the growth of renal transplantation to current levels.

1.2 **Success of Renal transplantation**

Renal transplantation is now highly successful and the gold standard treatment for end stage renal failure (ESRF) with graft survival continuing to improve. Currently anticipated graft survival in the UK is greater than 94% at 1 year and 88% at 5 years (UKTransplant, 2009, 2013).

The majority of early graft losses are due to surgical complications and sepsis. Chronic graft loss occurs at the rate of around 5% of grafts a year and whilst the causes of this are not fully understood acute rejection is still important. However;

despite the advances in the prevention of acute rejection, it still remains an important cause of early transplant failure and, importantly, impacts on the long term function of the allograft (Hariharan, 2001).

1.3 Classifying Allograft Rejection

The understanding of the process of rejection has increased exponentially since the early days of transplantation; however, the precise mechanisms underpinning rejection have still not been fully elucidated. This increased understanding has allowed different characteristics and processes to be used for the classification of rejection.

Broadly, rejection can be divided into either cellular or humoral with cellular rejection mediated by lymphocytes and humoral rejection antibody mediated.

The chronology and mechanism of rejection allows broad categorisation namely; hyperacute, acute and chronic or by histological characteristics (Banff criteria).

1.3.1 Hyperacute and accelerated rejection

Hyperacute or accelerated rejection occurs immediately following transplantation and is caused by the presence of preformed anti-donor antibodies against blood group antigens or tissue type and complement (thus humoral in nature). Accelerated rejection occurs in the first few days following transplantation and is due to reactivation of T cells. it is similar and relies on pre-sensitisation with an accelerated and aggressive response of sensitised T cells predominately humoral in nature with a smaller component being cellular.

1.3.2 **Acute rejection**

Acute rejection occurs over days to weeks and is due to the primary activation of T cells. This process is normally sub-divided into cellular and vascular processes. It is currently thought that cellular rejection has less humoral and more cellular mechanism, while vascular has more humoral than cellular mechanics. Cellular mechanisms are mediated by lymphocytes activated against donor antigens with the donor dendritic cells acting as antigen presenting cells. Humoral injury is mediated primarily by antibody and complement. The antibodies are either pre-existing or formed following transplant against the donor. The presence of pre-formed donor specific antibodies has been shown to correlate to poorer graft outcomes (Patel et al., 2007).

1.3.3 **Chronic rejection**

Chronic rejection occurs over months to years, whilst the multiple components of chronic allograft nephropathy (CAN) are both immunological and non-immunological factors. Chronic rejection is also composed of both humoral and cellular mechanistic factors (Chan).The classification of the mechanism of rejection is vital to the initiation of the correct treatment. The main method used in the histological diagnosis of rejection is based upon the Banff criteria. This grades the histological findings based on a renal biopsy specimen to indicate the nature of rejection.

1.3.4 **Banff criteria**

The Banff criteria represents standardised criteria for the histologic diagnosis of renal allograft rejection. It developed following a consensus meeting of surgeons, nephrologists and pathologists in Banff, Canada in 1991 and until then there had been considerable hetereogeneity in the biopsy reporting across different centres. The classification is updated regularly. This standardisation also provides a guide for therapy and allows standardised endpoints for research purposes.

Table 1-1: Different classifications of rejection based on the histological features in the Banff system (Solez and Racusen, 2013)

1.	Normal
2.	Antibody mediated rejection (AMR) a) Acute AMR i) Acute Tubular Necrosis-like Complement factor 4 fragment d ($C4d^{+)}$, minimal inflammation ii) Capillary margination and/or thromboses, $C4d^{+}$ b) Chronic Active AMR Glomerular double contours and/or peritubular capillary basement membrane multilayering and/or intersitital fibrosis/tubular atrophy and/or fibrous intimal thickening in arteries $C4d^{+}$
3.	Borderline changes: suspicious for T cell mediated rejection, no intimal arteritis, foci of tubulitis
4.	T-cell mediated rejection a) Acute T-cell mediated rejection b) Chronic active T-cell mediated rejection Chronic allograft arteriopathy
5.	Tubular atrophy/interstitial fibrosis, no evidence of specific aetiology i) Mild (<25% cortical area) ii) Moderate (26-50%) iii) Severe (>50%)
6.	Other: changes not considered to be due to rejection

1.3.5 **Effect of Rejection on Outcome**

Chronic allograft nephropathy (also called interstitial fibrosis/tubular atrophy) has become the dominant cause of kidney-transplant failure and represents the step-wise loss of nephron mass following cumulative smaller injuries. It is characterised by the fibrosis, vascular and glomerular damage that occurs in the renal allograft following injury and represents the final common pathway for damage (Chapman et al., 2005, Nankivell et al., 2003). Recurrent disease is also a cause of progressive decline in and ultimately failure of the transplanted kidneys (Hariharan et al., 1999). One of the most important determinants of chronic rejection or nephropathy is acute rejection which in turn has been shown to be linked to high panel reactive antibody (PRA) levels (established by reacting recipient blood to that of 100 donors to give a percentage of the population that the recipient has pre-formed antibodies against), long cold ischaemic times and delayed graft function. In one study, recipients of deceased transplant who did not experience acute rejection had 5-yr graft survival of 92% compared to those with one or more episodes of rejection who had an overall graft survival of 45% (Kahan, 1993) although it should be noted that newer immunosuppressive regimes may have improved this. The precise mechanisms underlying the effects of acute rejection on longer term transplant function are not fully elucidated however, it appears that late acute rejection and sub-clinical rejection are implicated in worse longer term function. This may be because of the “silent” nature of the insult as compensatory hyperfiltration by neighbouring unaffected nephrons may maintain a stable serum creatinine (Nankivell et al., 2003).

Therefore it seems intuitive that mechanisms to decrease the incidence of acute rejection would impact on graft survival although this has so far not been demonstrated (Hariharan et al., 2000). Acute rejection occurring within 0-30, 31-365

and >365 days following transplant and adjusted to account for creatinine at the time has been shown to give a RR for graft loss of 2.4, 7.1 and 21.8 respectively (Nett et al., 2005). The most critical factors for the development of chronic damage following acute rejection are the rejection occurring within the first three months, recurrent episodes, predominantly vascular rather than interstitial rejection and incompletely treated episodes (Leggat et al., 1997).The combination of factors that seem associated with the development of CAN are: acute rejection (perhaps through the mechanism of causing fibrosis), donor factors, prolonged cold ischaemic time and nephrocalcinosis (Schwarz et al., 2005).

1.4 Determinants of outcome in kidney transplantation

1.4.1 Early vs. Late loss

It can be seen that rejection, particularly severe rejection, impacts on survival and advances in immunological management have improved outcomes, however, it can be seen from survival curves that this has been predominately due to early effects, that is within the first three months.

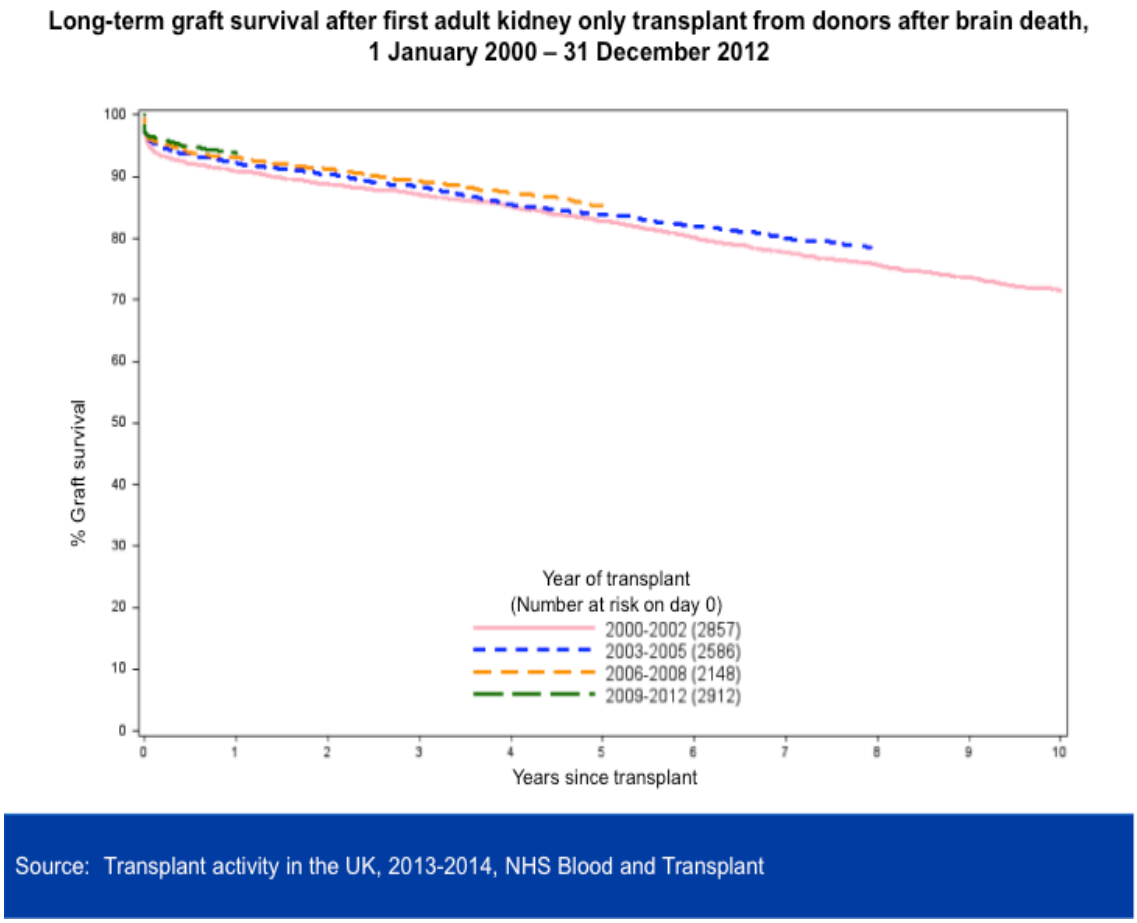


Figure 1-1 : Long-term survival after first adult kidney only transplant from donors after brain death 2000 - 2012

The attrition of kidneys following this has been around five percent per year irrespective of the era of immunosuppression. In fact prior to the introduction of cyclosporine A, long term survival of kidney transplants on azathioprine demonstrated lower attrition rates.

The reasons for long-term loss of kidneys are multi-factorial but can be broken down simplistically into pre-transplant (donor) and post-transplant (recipient) factors and these are expanded below.

1.5 Donor factors affecting outcome

1.5.1 Sources of Organs for transplantation

The landmark first successful transplant was between live donors and until the diagnosis of brain stem death criteria were proposed in 1976 it was the main source of organs for transplant. With the field of transplantation expanding to other organs such as liver (1964 Starzl), pancreas (Lillihei 1966) and heart (Barnard 1967) it was recognised that recently deceased donors were the necessary source of vital organs. The unmet demand from this source led to live donor kidney transplantation being revisited and today both sources are used in large numbers. Both deceased donors and live donors are specifically classified as follows.

1.5.1.1 Deceased donors

Within the deceased donor pool there exist two broadly different groups of donors. These are the donation after brainstem death (DBD) donors and the donation after circulatory death (DCD) donors. These donors can further be sub-divided into standard criteria and extended criteria donors.

1.5.1.1.1 Donation after Brainstem Death donors

DBD donors are those in whom a diagnosis of brainstem death has been made. The donor is then legally determined to be deceased and organ donation proceeds in a controlled manner with the patients circulation and respiratory systems supported until organ perfusion is established.

The process of brainstem death places enormous physiological strain on the body. At the point of brain death the brainstem is compressed leading to the Cushing reflex which comprises bradycardia and hypertension (Cushing, 1902). This is then followed by an autonomic storm driven by massive catecholamine release. These factors lead to increased systemic vascular resistance followed by loss of vascular tone, hypotension, endocrine organ damage, cellular metabolism derangement and blood shunting leading to end-organ hypoperfusion (Eicbaum and Bissetti, 1971). This process also causes massive inflammatory activation and cytokine release (Vergoulas et al., 2009) with significant increases in inflammatory cytokines including IL-6, IL-10, TNF-α, TGF-β and MIP-1a in the brain dead donor group when compared to the live donors. Such is the effect of this immune activation that it has been linked to more severe ischaemia reperfusion injury following transplantation (Weiss et al., 2007). The effect seems to be great enough that the outcomes from live kidney donors who are fully mismatched immunologically do better than deceased donors who are a closer immunological match (Nijboer et al., 2004). Studies in rats have shown that the duration of haemodynamic instability in the donor is also critical to the degree and speed of onset of immune activation. This process enhances ischaemia reperfusion injury (the injury that occurs to the organ following perfusion after loss of blood supply) and predisposes to acute rejection (Van der Hoeven et al., 2003).

It has been proposed that improving management of the potential donor may help mitigate these factors and improve outcomes (Schnuelle et al., 1999). The main principles of donor management are directed towards adequate fluid management, minimisation of inotropic support if possible, and minimisation of the effects of brainstem injury in inducing diabetes insipidus. High dose vasoconstrictors are

avoided because of their detrimental effect on donor organs (Nygaard et al., 1990) and their inhibitory effect on pituitary hormone function therefore vasopressin is recommended (Kustogiannis et al., 2006). Whilst the effect of specific interventions is difficult to determine targeted management has been suggested to result in greater procurement and end utilisation of organs (Rosendale et al., 2003).

DBD donors can be divided into standard criteria donors (SCD) or extended criteria donors (ECD).

ECD donors are those donors aged over 60 or donors 50 – 59 with at least two of the following: cerebrovascular accident as cause of death, terminal serum creatinine >1.5mg/dl or a history of hypertension (Port et al., 2002).

One of the principal difficulties that exist with deceased donors is that their complete medical history is often unknown and sometimes the effects of the pre-terminal event make interpretation of biochemical tests extremely difficult.

1.5.1.1.2 Donation after Circulatory Death (DCD) donors

DCD donors are classified according to the Maastricht criteria which can be seen below, however, most UK DCD donors are category 3 (Koostra et al., 1995). In this group of donors there must be circulatory asystole for a period of 5 minutes prior to the start of the organ donation operation as the criteria for brainstem death cannot be met. As circulation and respiratory support has, by definition, stopped prior to the commencement of surgery the operation must then be undertaken swiftly to establish replacement of circulatory volume with organ preservation solution to ensure that organs are cooled and preserved rapidly.

In the last few years there has been a large increase in the number of donation after circulatory death donors. This move has been championed to increase organ supply.

Table 1-2: Maastricht criteria for the classification of DCD donors

Category 1: Dead on arrival at hospital.
Category 2: Unsuccessful resuscitation.
Category 3: Awaiting circulatory death. Patients in whom death is inevitable but who do not fulfil brainstem-dead criteria.
Category 4: Circulatory arrest in brainstem-dead individual.
Category 5: Unexpected circulatory arrest in a patient on ITU

While DCD patients are not subject to the physiological storm of brainstem death they are subject to the process of asystole and the period of physiological instability leading up to asystole which may be very variable in length. One group who looked at the effect of duration of donor instability prior to asystole determined higher primary non function rates in the prolonged instability group but noted that rejection rates were higher in the group with shorter duration of instability, however, this was based on the results of a single centre and has not been consistent elsewhere (Goldsmith et al., 2010).

Rates of primary non-function and delayed graft function following DCD are higher than after DBD however, kidneys that do not experience these problems seem to have comparable outcomes to DBD kidneys (Hoogland et al., 2010). Indeed an analysis of UK data comparing DCD to DBD outcomes showed no difference in 5 year graft survival or function (Summers et al., 2010).

1.5.1.2 Living Donors

Living organ donation in the UK follows guidelines from the British Transplantation Society and the Renal Association. This covers best practice for all aspects of living kidney donation (British Transplantation Society and Renal Association 2005). Living donation may be considered between blood relatives, non-genetically related relatives, friends and altruistic donation (including both directed altruistic and non-directed). Prior to donation the donor is assessed extensively to ensure that no contraindications to donation exist either because of increased risk of future renal problems for the donor themselves and to exclude other health problems that could be worsened by the effect of surgery. Kidney function is assessed to ensure that sufficient renal reserve exists to tolerate losing a kidney and that no underlying kidney disease exists in the donor.

In the process of live donation usually both donor and recipient operations are undertaken in the same hospital and therefore cold ischaemic times are significantly lower than for deceased donors. The mechanism of nephrectomy is also more controlled as the patient is usually anaesthetically more stable (being intrinsically fit and healthy) and therefore the kidney is unlikely to be subject to the variations in blood pressure or to large doses of inotropic or other drugs that might subsequently effect the outcome of transplantation.

1.6 The impact of donor type on graft survival

Live donation has increased the number of kidney donors available and results from live donation are superior to DBD or DCD donors due to the combination of these factors. UK registry data from 2013 showed 1yr graft survival between the groups

demonstrated rates of 97% for live donors vs. 93% for DBD and 93% for DCD donors. UK registry data from 2005-2007 demonstrated5yr graft survival of 89% for live donors compared to 85% for DBD and 86% DCD donors (UKTransplant, 2009, 2013).

1.7 Delayed Graft Function (DGF)

1.7.1 Definition of DGF

DGF is mainly a manifestation of acute kidney injury (AKI) within the transplanted kidney. AKI, in a non-transplant setting, is defined as a rise in creatinine within 48hrs of the precipitating event; the timescale for the manifestation of DGF is less defined. DGF is historically defined as the need for dialysis in the first seven days following transplantation (Siedlecki et al., 2011), IGF refers to the absence of dialysis in the first 7 days but with a creatinine of >3mg/dL on the 5th day following transplant but is a less commonly used terminology. Other definitions have been proposed including functional DGF defined as a failure of serum creatinine to decrease by at least 10% on 3 successive days during the first week post-transplant, irrespective of dialysis requirement. Whilst definitions relating to dialysis requirement inherently are harder to apply to pre-emptive transplant recipients it is still the most commonly adopted definition, and therefore the definition employed here within.

1.7.2 Pathogenesis of Delayed Graft Function (DGF)

DGF reflects the impact of ischaemia and the physiological insult that the kidney has been subject to in the pathway from donation to implantation and the impact of post-

implantation factors. In part due to the mechanism of donation, rates of delayed graft function are much lower amongst the live donor kidneys, 5-10% (Shoskes and Cecka, 1997) when compared to rates of delayed graft function for either DBD or DCD kidneys. Overall figures suggest that there is a rise in rates of delayed graft function and this may be in part due to increased use of DCD kidneys and expanded criteria donors.

One of the main influences on the development of delayed graft function is the cold ischaemic time with a 23% increase in the risk of DGF for every 6hrs of cold ischaemia, which has also been shown to be a factor in the subsequent development of rejection (Ojo et al., 1997, Pratschke et al., 1999). The precise mechanisms of the damage that occurs are not fully elucidated but include the switch from aerobic metabolism and the consequent accumulation of reactive oxygen species and “free radicals”.

The development of the mechanisms that trigger DGF in the recipient can also be related to the whether the donor is DBD or DCD. The catecholamine and cytokine storm that ensues after brainstem death can produce complement activation and inflammatory immunological responses within the graft well in advance of organ recovery. As such both the length of stay in ITU and the duration of time from brainstem death to organ procurement are correlated to the development of DGF (Blasco et al., 2007, Giral et al., 2007).The administration of dopamine in this cohort has been shown to have some beneficial effect on rates of DGF (Schnuelle et al., 2004).

Within the DCD pool, which historically has had the highest rates of DGF, one of the main determinants of DGF is the duration of warm ischaemia i.e. the period between the withdrawal of therapy and the onset of asystole. During this period the kidney is

exposed to anaerobic metabolism and subsequently acute tubular necrosis can occur. Following reperfusion peritubular oedema can occur compounding the ischaemia and rarely the graft may have primary non-function. Pig models have elegantly demonstrated that longer periods of warm ischaemia as seen in DCD correlate to the development of DGF (Rojas-Pena et al., 2010).

1.7.3 **The impact of DGF**

The early effects, within the first few weeks following transplantation, of DGF are oliguria, increased risk of acute rejection and increased allograft immunogenicity. It has been suggested that this results from the effects of ischaemic injury in increasing expression of MHC class I and II (Perico et al., 2004). It also increases the risk of early graft loss and this holds true for both DGF and IGF (Raimundo et al., 2013).

DGF increases the attrition rate for kidneys and this effect is enhanced by the presence of rejection. Data from United Network for Organ Sharing (UNOS) demonstrated that, independent of rejection, delayed graft function decreased mean graft survival and when combined graft survival was reduced from 9.4 to 6.2 years (Shoskes and Cecka, 1997). Similar analysis of the link between delayed graft function and death with graft function of 44,630 living donor US registry patients demonstrated that DGF was associated with an increased risk of death with a functioning graft. 1,3 and 5yr survival being 91.9%, 86.8%, 81.6% in those with DGF and 98%, 95.2% and 91.6% in those without DGF, although the mechanism underlying this remains unclear (Narayanan et al., 2010).

1.7.4 **Delayed Graft Function and rejection**

Whilst it is clear that DGF alone impacts on both graft and patient outcomes, as demonstrated above, it is more difficult to delineate the combined effect of DGF and rejection on graft and patient outcomes. One of the potential pitfalls associated with DGF is that its presence may mask acute rejection and as such most units routinely biopsy DGF to ensure that acute rejection is not being hidden. Inherently therefore, acute rejection may have been present for a few days prior to diagnosis with subsequent damage. Individual studies have suggested that the incidence of acute rejection in the first year following transplant is higher in those patients with DGF (51.9% vs. 33.3%) (Miglinas et al., 2013). In analysis of primary deceased renal transplants from US data the combination of both DGF and acute rejection led to poor 5 year survival of only 35% (Ojo et al., 1997). A meta-analysis of studies examining DGF suggested higher rates of acute rejection in the first year following transplant in those patients with DGF (49%) as opposed to those without (35%) perhaps suggesting a mechanistic link (Yarlagadda et al., 2009).

1.7.5 **Acute Kidney Injury in the donor and Delayed Graft Function**

AKI in the organ donor has many definitions e.g. doubling of admission serum creatinine, terminal creatinine over 1.5mg/dL. Whilst the presence of donor AKI seems to correlate to higher incidence of DGF in recipients this does not seem to confer worse longer term outcome (Farney et al., 2013). Indeed one analysis demonstrated comparable outcomes in terms of graft survival and renal function at 1 year between SCD and ECD donors with or without AKI (Klein et al., 2013) however, the effect on longer term function has yet to be demonstrated. Historically the presence of AKI in a potential organ donor has led to a reticence to accept the

organs for transplantation, however, the recognition that AKI may not necessarily translate into inferior outcomes has led to an increase in utilisation of kidneys from donors with higher terminal serum creatinine. Outcomes from these donors seem to show comparable long term function although rates of DGF are higher (Jung et al., 2013, Farney et al., 2013).

1.7.6 **Predictors of DGF**

A number of clinical factors have been identified as predictors of delayed graft function. These include factors relating to procurement such as: kidneys from DCD donor, inotropic support of the donor, cold storage preservation of the graft and the duration of cold ischaemic time and factors relating to the donor such as: age over 55 and marginal kidneys from hypertensive donors. Recipient factors for example recipient hypovolaemia, number of prior transplants, inherited thrombophilia and ureteric obstruction. These factors have been combined, weighting attributed and many nomograms are available for determining the likelihood of developing DGF taking into account both recipient and donor factors however, this remains an inexact science. One such nomogram is demonstrated below demonstrating which factors and their weighting contribute to predicting the likelihood of DGF. This set of criteria was established following analysis of 19,706 deceased recipients and their outcomes in US registry data (Irish, 2003).

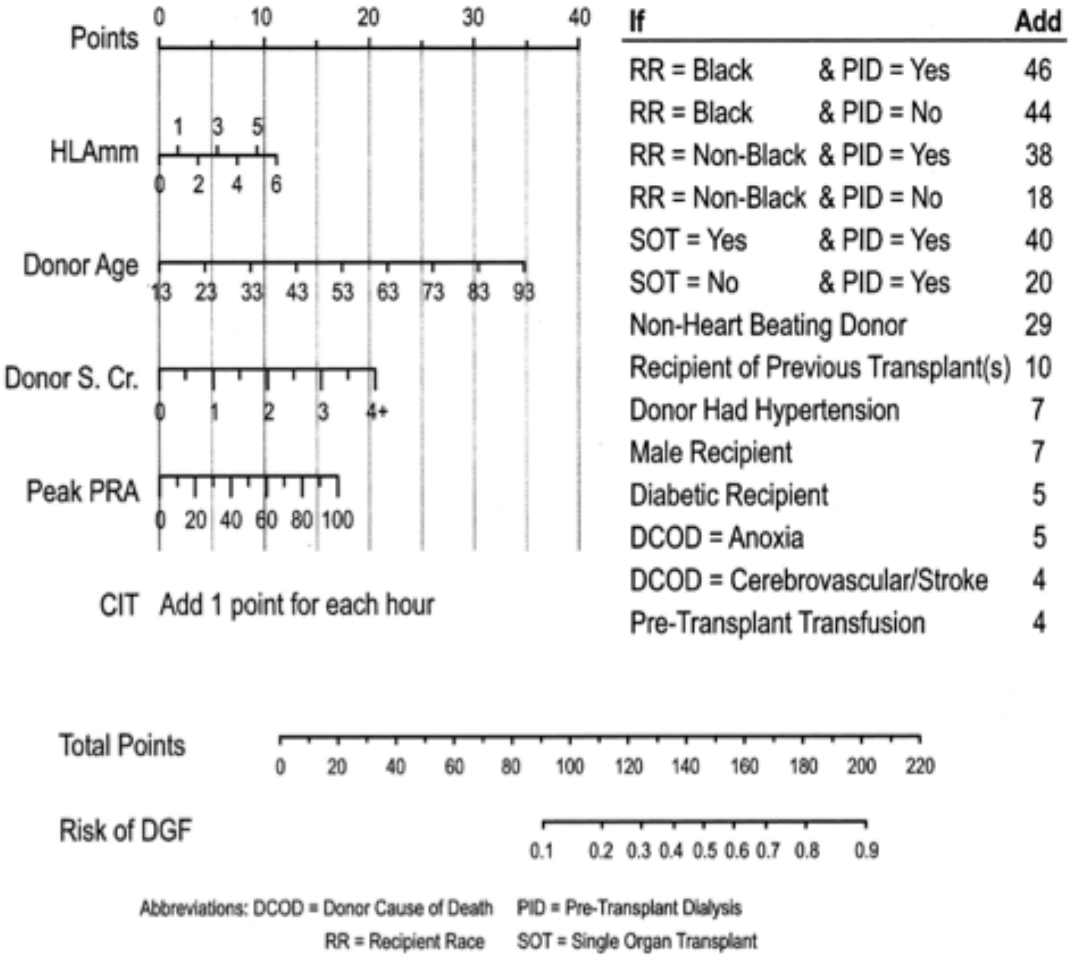


Figure 1-2: Nomogram for predicting the likelihood of delayed graft function in adult deceased transplant recipients (Irish, 2003)

1.7.7 **Interventions for DGF**

Efforts to ameliorate DGF tend to target the prevention of DGF occurring. From the nomogram illustrated above it can be seen that some of the factors implicated in the development of DGF can only be influenced by being selective of donor kidneys offered, at the expense of potential grafts. Other strategies to prevent DGF have looked at ischaemic pre-conditioning in large animal studies with some promising early results (Hanto et al., 2010). Efforts to avoid vasoconstriction and its potentially deleterious effects have employed the use of endothelin antagonists in animal

models and calcium channel blockers which have shown some benefit in the post transplant period (Kuypers et al., 2004). Attention has also been focused on inhibiting leucocyte adhesion. Similarly the use of induction immunosuppression has been shown to decrease the rate of DGF by suppressing leucocyte related vascular congestion and resultant endothelial injury (Siedlecki et al., 2011).

1.7.8 **Machine perfusion**

The aim of organ preservation and storage following organ retrieval is to prolong the potential window of opportunity for transplantation by limiting organ metabolic activity and preventing damage to cells within the organ and to ensure the organs are in optimal condition. One of the more recent areas of interest in the field of organ preservation is the use of machine perfusion. This offers the ability to ensure that the organ is properly cooled and circulated with preservation fluid. Dynamic readouts as to pressures within the kidney can give a potential indication as to the 'usability' of the organ. Whilst some studies have suggested a potential benefit, particularly to marginal donors (Wight et al., 2003, Treckmann et al., 2011), a prospective trial examining their use in DCD donors was halted early due to lack of efficacy (Watson et al., 2010). Whilst further research into the role of machine perfusion is on going the most recent meta-analysis concluded that there maybe a decrease in the rate of DGF in kidneys that undergo machine perfusion prior to implantation (O'Callaghan et al., 2012). Whilst research into machine perfusion and its role continues, normothermic perfusion has emerged as a potential method of improving donated kidneys and shown promising early results (Nicholson and Hosgood, 2013).

1.8 **Transplantation across immunological barriers**

The matching of donor kidneys to recipients is complex and varied across countries and organ sharing schemes. The balance of providing fair and equitable access to all recipients whilst ensuring best long term outcomes is based on multiple models beyond the scope of this thesis. In the UK the scheme of deceased organ allocation is a points based algorithm based predominantly on Human Leukocyte Antigen (HLA) matching but also takes into account sensitisation and other factors.

In living donor transplantation matching is much less specific but historically based on blood group compatibility and HLA compatibility.

Recently these barriers have been crossed using immunoadsorption techniques. The drive to cross immunological barriers in renal transplantation arose because of: the shortage of donors for renal transplantation; the ever increasing waiting list; and the increasing number of patients on the waiting list with high CRF. In 2005 it was estimated that in the USA there were approximately 6,000 patients waiting for a kidney transplant who had a willing but incompatible live donor (Segev et al., 2005). The number of patients on the transplant waiting list with any degree of sensitisation in the USA is 25% with 12.5% having a PRA of >80% indicative of those who are highly sensitised (Hippen, 2006). These individuals, because of previous sensitisation either due to transplantation, pregnancy or previous blood transfusion, are likely to have a positive crossmatch.

1.8.1 **ABO incompatible (ABOi) transplantation**

1.8.1.1 Early ABOi transplantation

Blood groups, as defined by the antigens on the red cells, were described in the early 1900s and have led to the classically known A, B, AB and O system (Landsteiner, 1961). The corresponding antibodies produced have hampered transplantation since its inception.

Transplantation across blood group incompatibility in the early days of transplantation resulted in universally poor outcomes and was therefore considered impossible (Rydberg, 2001). When performed, the histological examination of the specimens from these failed transplants showed occluding thrombus of the renal artery, reactive neutrophil infiltrates and fibrin deposition at the intima (Wilbrandt et al., 1969). These changes were very similar to those seen in hyperacute rejection (as it is essentially hyperacute rejection) and it was therefore suggested that that the reason ABO incompatible grafts failed was prompt binding of pre-formed circulating antibodies to A and B antigens present in the vascular wall of the graft (Porter, 1976).

Initial trials of ABO incompatible transplantation involved transplantation from A_2 to O recipients. The rational for this was that the expression of A antigens on erythrocytes from A_2 individuals was much weaker than expression in A_1 individuals (Economidou et al., 1967). This work was then strengthened by skin studies in transplantation which demonstrated that skin grafts from A and B donors to O recipients were rejected immediately whereas transplants of skin from A_2 individuals lasted a comparable time to those from O donors (Ceppellini et al., 1969). Based on this initial work a clinical trial was set up which performed 20 transplants from A_2 to O and A_2B to B recipients. Eight of the grafts were lost in the first month, the remaining

12 had long term function (Rydberg et al., 1987). Following this titres of anti-A were examined and graft survival was shown to be higher amongst those grafts where the titre was less than 1:64 (Welsh et al., 1987).

1.8.1.2 Advances in ABO incompatible transplantation

This has included consensus that splenectomy is not needed as part of the ABOi protocol. This is usually in-conjunction with the use of anti B cell depleting anti-CD20 antibody induction (Rituximab) and with careful monitoring of antibody titres in the post-transplant period and implementation of therapy to lower blood group antibodies during the initial post-transplant period (Gloor and Stegall, 2007).

1.8.1.3 Outcomes of ABOi transplantation

From these early experiments ABO incompatible transplantation has rapidly grown and is now performed with increasing frequency in transplant centres in North America, Europe and Japan. Evaluation of the outcomes from Japanese registry ABOi transplantation showed 1, 3, 5 and 10 year graft survival of 86, 82, 74 and 53% compared to 96, 89, 81 and 56 in historical controls. However, evolution of protocols and more experience has meant that those patient who underwent transplantation after 2001 had significantly better outcomes (Takahashi et al., 2006).

Despite the pre-conditioning that patients undergo as part of the ABOi protocol these transplants can still be associated with acute humoral mediated rejection due to either a DSA or a blood group specific antibody. A recent randomised control study further confirmed that ABO compatible patients with humoral rejection require

immunoadsorption and antibody removal and that therapy directed against only cellular rejection was inadequate (Bohmig et al., 2007).

1.8.2 **HLA incompatible (HLAi) transplantation**

The HLA system is responsible for coding the antigens that are present on the cell surface and hence critical for establishing tissue compatibility as they are used to determine self from non-self.

Patients who have been exposed to sensitising factors for example blood transfusion, pregnancy or prior organ transplant may develop antibodies that make it difficult to achieve a negative cross match prior to organ transplant.

The seminal paper by Patel and Terasaki in 1969 demonstrated that hyperacute rejection can result from transplantation in the presence of preformed donor specific anti-HLA antibodies (Patel and Terasaki, 1969). This made a positive cross-match an absolute contraindication to transplantation. Current programs have the ability to remove donor specific HLA antibodies to achieve a negative crossmatch and therefore prevent hyperacute rejection.

1.8.2.1 HLA desensitisation programs

Careful patient selection is critical for desensitisation programs as the process itself is rigorous. The likelihood of successful desensitisation must also be assessed prior to initiation as some antibodies prove particularly difficult to remove. Whilst early protocols employed the use of routine splenectomy most now utilise plasmapharesis or plasma exchange. The exact protocol to achieve desensitisation varies amongst

different units and local experience, however, most employ a combination of plasmapheresis, low dose IVIG, ATG and maintenance immunosuppression similar to standard transplant recipients (Sharif et al., 2012).

1.8.2.2 Outcomes of HLAi transplantation

However, despite enhanced immunosuppressive protocols rates of rejection remain high in this group with subsequent effects on long-term graft function and survival. Whilst rates of rejection, graft survival and patient survival differ between groups overall they are slightly inferior to standard transplants. Rejection rates vary between 12-53% of recipients, 2yr patient survival varies between 89-95% and 2yr graft survival between 80 and 89% (Gloor et al., 2010, Thielke et al., 2009, Higgins et al., 2011).

1.9 Monitoring transplant function and biomarkers in current use

Following renal transplantation in all categories of donor type the monitoring of the transplanted organ is essential. Simple clinical parameters such as urine output may be used but delayed graft function and native outputs render this as a guide only. Biochemical monitoring (using creatinine) is the mainstay using renal function although longer term other forms of monitoring may be helpful.

1.9.1 **Biochemical monitoring**

Laboratory blood tests of renal function routinely rely on urea, creatinine, creatinine clearance and estimated glomerular filtration rate (GFR) and these are the current "biomarkers" in use.

1.9.1.1 Urea

Urea is synthesised in the body as part of the urea cycle following oxidation of ammonia or amino acids. Urea is produced in the kidney, dissolved in the blood and then primarily excreted in the urine. Blood urea nitrogen (BUN) is used a mechanism of monitoring renal function, however, its levels are more vulnerable to being affected from factors unrelated to the kidney for example upper gastrointestinal haemorrhage or heavy protein intake. The reabsorption of urea in the tubules of is also variable but may be as high as 40% (Lamb et al., 2005, Traynor et al., 2006).

1.9.1.2 Creatinine

Creatinine is a break down product of creatine phosphate in muscle. It is produced at a fairly constant rate but is dependent on muscle mass. Creatinine is mainly filtered by the kidneys and very little is reabsorbed in the renal tubules although some is secreted in the proximal tubule. If the filtering mechanism within the kidney is defective the level of creatinine within plasma will increase. Whilst a useful measure in chronic disease and as a baseline of renal function using alterations to blood levels of creatinine as a method for assessing renal damage is flawed.

Creatinine is a breakdown product that needs time to accumulate and hence does not present a real-time indicator of renal function. For example a rise in creatinine normally indicates damage that occurred at least 24 if not 48 hrs prior to the rise. This inherently means that the opportunity to intervene and stop the process that has led to the rise has been partly missed. The delay actually indicating renal damage that has occurred is also reliant on the magnitude or renal injury. Creatinine levels remain remarkably stable in kidney injury and will only increase once a significant volume of nephron mass has been lost. Therefore in order for creatinine levels to be increased a significant mass of the transplanted kidney must be damaged (Martensson et al., 2012).

Creatinine levels are also affected by pre-renal problems such as inadequate hydration and may artificially, therefore, be increased and appear to indicate renal damage when actually the problem is over concentration and the kidneys themselves are functioning normally.

Plasma creatinine can also be affected by factors that affect degradation of creatine phosphate in muscle for example nutrition and infection and may show high inter individual variability. Similarly in some conditions because of the comparative hypersecretion of creatinine in the proximal tubule the levels of creatinine do not accurately reflect the underlying renal function, particularly in glomerulonephritis (Shemesh et al., 1985).

The imprecise and delayed response seen using creatinine would support the use of other more rapidly responding and accurate biomarkers of kidney dysfunction (Perrone et al., 1992). However, despite the recognised need for improved markers

of renal function none have yet completed the transition from research tool to clinical utility.

1.9.1.3 Creatinine clearance

Creatinine clearance is a mechanism of estimating the glomerular filtration rate by measuring the urinary creatinine in a set period multiplying it by the volume produced and dividing by the serum creatinine. The creatinine clearance tends to overestimate GFR due to tubular secretion of creatinine, however, as long as secretion remains at a fairly constant rate then changes to renal function can be monitored (Traynor et al., 2006). Whilst creatinine clearance has a role, in the setting of acute changes in transplant function it has limited use due to the necessity to collect urine output for 24hrs.

1.9.1.4 Estimated GFR (eGFR)

Many laboratories now routinely report eGFR calculated using the modification of diet in renal disease formula (MDRD). This uses the patient's age, sex, race, serum urea, serum creatinine and serum albumin. This provides a good way of estimating the renal function in relatively stable patients and has superseded the Cockcroft-Gault equation as a method of estimating GFR (Cockcroft and Gault, 1976, Levey et al., 1999).

1.9.1.5 Other methods of detecting renal function

Inulin is a polymer of fructose that can be administered to achieve a constant blood level and then renal excretion and blood levels measured to determine glomerular filtration rate. Whilst highly accurate its use lies in research rather than clinical practice.

Radioisotopes can also be administered and their clearance from the blood stream calculated to determine GFR. Again these are predominantly used for research as they provide very accurate measurements but are too time intensive and laborious to incorporate into the dynamic situation immediately post transplant.

1.9.1.6 Other modalities

Within the context of renal function in the specific transplant population a number of other modalities for inferring renal function exist. Following antibody incompatible (either HLA or ABO transplantation) titres of antibody can be measured. Whilst these do not directly correlate to renal function they are important in monitoring the immunological state of the graft. Similarly biopsy of the graft and pathology examination of tissue can help monitor the graft for the development of rejection or help determine the level of underlying chronic damage.

1.10 Biomarkers in Disease Monitoring

1.10.1 The Definition of a Biomarker

A biomarker has been defined by the National Institutes of Health Biomarkers definitions working group as: “a characteristic that is objectively measured and

evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Group, 2001).” Therefore although biomarkers are seen as “new” actually any blood result or indeed clinical measurement e.g. blood pressure that is linked to a disease process or normal response could be described as a biomarker. Whilst other definitions have been proposed there is significant overlap between them (Strimbu and Tavel, 2010).

1.10.2 **Characteristics of Biomarkers**

A number of characteristics have been described as being desirable in a biomarker (Emerging safety science: Workshop summary, 2008). These will be discussed below using Troponin as an example.

- A biomarker should be visible early, prior to histopathological changes and should be indicative of active damage. Troponin levels increase within 3-12 hours following injury, peak at 24-48hrs and return to normal over 5-14 days.
- A biomarker should be sensitive, but it should also correlate with the severity of damage. Troponin has the highest sensitivity and specificity of any marker for myocardial infarction and levels have been correlated to the risk of death.
- The biomarker should be accessible in peripheral tissue and troponin is measured in blood.
- A biomarker should be analytically stable so it can be measured after the event as in the case of troponin.

- A biomarker should ideally be translational and a bridge across species, and troponin has been shown to be useful in other species using drug development.
- The mechanism of the biomarker should be known. Troponin is a contractile protein that is only detectable in serum following myocardial necrosis. As a biomarker should also be able to localize damage troponin satisfies this requirement.

As can be seen Troponin is a very good biomarker, in part explaining its widespread usage in clinical practice.

A key element in the evaluation of a biomarker is the sensitivity, specificity, negative predictive and positive predictive value. These help interpret how useful a biomarker is and the clinical setting in which it will be useful. Whilst an ideal biomarker would be highly sensitive and specific in reality many biomarkers are either highly specific or sensitive rather than both, however, they still have a clinical role. A very sensitive biomarker, e.g. d-dimer in the context of deep vein thrombosis, has a low specificity but it very useful in clinical practice as a negative value means it is extremely unlikely that the patient has had a deep vein thrombosis. Whilst a high value does not specifically relate to any one diagnosis the absence of an elevated level makes it an excellent screening test in ruling out a diagnosis.

1.10.3 **The Evolution of Biomarkers**

The flaws in the currently available markers highlighted above make monitoring the renal graft difficult and result in damage occurring before the warning signs are apparent. These difficulties in determining true damage within kidneys are not confined to renal transplantation but to also determining both acute and chronic kidney injury in native kidneys. For these reasons, there has been a drive to determine improved techniques of monitoring for renal damage in both native kidneys and transplanted kidneys within the renal community in recent years.

The search for non-invasive biomarkers crosses specialities with examples of diagnostic advances in myocardial infarction and deep vein thrombosis. Until the late 20thcentury the diagnosis of acute myocardial infarction was made on the basis of clinical history, electrocardiogram changes and later angiography. However, work has led to the identification of cardiac troponin I in 1987 and troponin T in 1989 (Cummins et al., 1987, Katus et al., 1989). The troponins are structural proteins of the cardiac myofilament that are exclusively expressed in cardiomyocytes. Upon irreversible cardiac injury troponin T is released and peaks at 24hrs after the insult giving vital supportive information to the diagnosis. In deep vein thrombosis, a diagnosis with poor clinically documented signs, the use of d-dimers has resulted in a non-invasive screening diagnostic test that is not sensitive but is very specific i.e. it has a very high negative predictive value.

1.11 **Biomarker discovery and application**

As mentioned above a biomarker can be developed from any point from gene upregulation to end product.

A number of methods exist for the identification of biomarkers including genetic studies, genomics, proteomics, metabolomics and microarray techniques. All these processes essentially aim to determine molecules that are associated with specific disease or pathological processes. Following the identification of biomarkers they must then be validated in a variety of populations and settings to establish both the sensitivity and specificity of the marker.

1.12 **Biomarkers in Renal Disease**

Interest in renal biomarkers has increased due to the recognised problems with those currently available and the requirement of invasive methods of diagnosis and monitoring that are currently required.

The kidneys are involved in systemic responses both acute and chronic and may represent the overall patients condition for example on intensive care when AKI is common but often detected too late. Immunological disease may also affect the kidney and diagnosis of renal injury is often late. This obvious need for accurate and early screening test for early renal dysfunction, specifically monitoring renal function and ideally with prognostic ability for recovery has driven the quest to discover biomarkers that may fulfil these roles. Disease specific biomarkers would also be of great benefit. In chronic diseases with renal involvement, such as vasculitis, the current biomarkers such as ANCA, CRP etc. give disease information but not direct information on renal injury.

A number of potential candidates for biomarkers in renal medicine and renal transplantation have been proposed although as yet none have entirely made the transition from research to clinical utilisation. The reasons for this are multi-factorial.

One of the main problems is that there is not a universally agreed and well delineated pathway from discovery to clinical introduction. One of the initial hurdles that many biomarkers have faced is that easily employable laboratory methods of analysis have been lacking. Even getting through this barrier the scrutiny that is placed on newly developed biomarkers is, quite rightly, more rigorous than when the currently used methods were introduced.

Those that are applicable to this research are described below. This panel of biomarkers were chosen for a number of reasons. As diseases that affect the kidney can affect many different areas within the kidney a number of biomarkers are needed as some are very specific for certain areas within the nephron. Similarly some of the biomarkers were chosen as they reflect the underlying activity within the immune system rather than the damage caused. The biomarkers selected represent those for which there was good evidence in the literature (as described below) that they may have a role in renal transplantation either for monitoring in the recipient or for assessment in the potential donor.

1.12.1 **Kidney Injury Molecule – 1 (KIM-1)**

1.12.1.1 Identification and characteristics

KIM-1 was first identified following experiments that identified the genes that were upregulated following ischaemia in animal studies (Ichimura et al., 1998). It is produced in the proximal tubule when the epithelium is induced to dedifferentiate following an insult. It is also produced at much lower levels in lymphocytes.

In normal kidneys KIM-1 is undetectable in urine. Following renal damage, for example after an ischaemic insult, the KIM-1 ectodomain is cleaved and it becomes detectable in urine. Animal studies have suggested KIM-1 may react more quickly to damage than other biomarkers measured (including plasma creatinine, blood urea, urinary N-acetyl-B-glucosaminidase (NAG), proteinuria) (Vaidya et al., 2006).

Currently KIM-1 is measured via lab based research tools such as enzyme linked immunosorbent assay (ELISA). The R&D systems ELISA kit has been validated and used to show the reference range for the healthy population falls between 59-2146pg/ml and the assay of detects up to 17ng/ml (Chaturvedi et al., 2009).

Much of the early research was undertaken using gene expression which would be cumbersome in clinical practice. For this reason urinary and serum levels have been analysed and shown to correlate to renal KIM-1 expression, the degree of renal morphological damage, renal function and proteinuria (van Timmeren et al., 2007b) enabling these more easily measured levels to be used in place of gene expression.

One of the key qualities of any biochemical test is that the analyte to be measured has to show a relatively good stability otherwise by the time the sample of urine or

blood is analysed the levels will be inaccurate. The stability of KIM-1 has been determined under a range of pH ranges and determined that the urinary pH has no effect on the stability of KIM-1. However, the measurement of KIM-1 from one of the commercially available kits is pH dependent so adjustment of sample to neutral pH prior to testing is suggested (Pennemans et al., 2010).

1.12.1.2 KIM-1 in Acute Kidney Injury

Following the early work that identified KIM-1 as a potential biomarker of renal injury its use has been examined in a variety of different settings to establish its potential role. Levels of KIM-1 have been shown to be elevated in acute tubular necrosis (caused by ischaemia, sepsis) when compared to patients without acute tubular necrosis and the expression of KIM-1 has been demonstrated to be confined to the proximal tubule (Han, Bailly et al. 2002) suggesting that KIM-1 may have application in a wide range of settings.

A significant cause of renal injury are drugs but both in drug development and following exposure to potentially nephrotoxicant drugs detecting the injury early has proved challenging. KIM-1 has been shown to act as a marker of injury following renal exposure to a range of nephrotoxicants including: Cisplatin and TFEC (Ichimura, 2004), cadmium (Prozialeck et al., 2007), mercury, gentamicin and chromium (Zhou et al., 2007). In these studies it has been shown to be sensitive and specific and outperform traditional methods of detecting the renal injury. Importantly it has also been shown not to react to selective cardiotoxicants and hepatotoxicants (Vaidya et al., 2010). KIM-1 has been shown to outperform traditional markers of kidney injury in preclinical studies (compared to urea, serum

creatinine, NAG) particularly detecting nephrotoxicity before considerable loss of renal function occurs and correlating to the degree of histopathological damage better. It has been shown to have a role in nephrotoxicants affecting all segments of the proximal tubule (Chiusolo et al., 2010).

KIM-1 levels seem to return to normal in line with recovery of renal function and as such KIM-1 is one of a panel of biomarkers that are undergoing FDA approval as monitoring mechanisms for drug development (Ozer et al., 2010, Tonomura et al., 2010).

One of the challenges faced with AKI is predicting mortality. Urinary levels of KIM-1 have been demonstrated to be higher in patients with AKI than without but also to be a significant predictor of death or need for dialysis (Liangos et al., 2007, Vaidya et al., 2008).

Cardiac surgery has long carried the risk of AKI and this, in part, explains its high associated mortality. It has also been suggested that KIM-1 levels (either individually or in combination with other biomarkers) may allow the early detection of postoperative AKI in adults after cardiac surgery prior to a rise in serum creatinine. A mechanism of early identification could be valuable in instituting strategies to minimise the impact of renal dysfunction and to improve patient outcome. A study of 36 post-operative cardiac surgery patients who developed AKI (defined as a rise in serum creatinine >0.3mg/dl within 72hrs of surgery) examined urinary KIM-1, NAG and NGAL at five time points over the first 24hrs post operatively. The AUC for KIM-1 alone to predict AKI was 0.68 immediately post-op and 0.65 3hrs post-op. Combining all three biomarkers gave AUC of 0.75 immediately and 0.78 at 3hrs

showing promise as a mechanism for directing early treatment in those highlighted as likely to develop AKI post-operatively (Han et al., 2009).

It has been established that one of the best indicators for a poorer outcome following hospital admission is the presence of renal dysfunction at presentation and again it may be that KIM-1 is sensitive enough to have a role in this setting. A study examined admission levels of KIM-1, NGAL and β-2M with comparison to creatinine in a paediatric emergency admission centre. The urinary levels of these biomarkers detected the presence of AKI and severity more rapidly than creatinine in the acute setting, although no severe cases of AKI were seen in the study (Du et al., 2010).

1.12.1.3 KIM-1 in Chronic renal disease

As mentioned above the monitoring of renal function in chronic disease states, particularly systemic diseases, is difficult. Any mechanism that would serve to improve this would be welcome. Excretion of KIM-1 has been shown to be higher amongst patients with Adult Polycystic Kidney Disease (APKD) when compared to controls. KIM-1 has been shown to be positively associated with total renal volume independently of albuminuria and a potential role in monitoring disease progression has been suggested (Meijer et al., 2010).

The effect of therapeutic interventions to decrease proteinuria in renal disease on KIM-1 levels has been examined. By altering the levels of proteinuria in patients with Chronic Kidney Disease (CKD) by medication changes the levels of urinary KIM-1 decreased in parallel to those of proteinuria. This seems to suggest that modification of the treatment leads to amelioration of proteinuria-induced tubular damage. The suggestion from the authors is that KIM-1 levels could be used to tailor treatment in

patients with CKD and proteinuria to improve outcomes (Waanders et al., 2009, Kramer et al., 2009).

Urinary KIM-1 level has also been shown to have a role in predicting future risk for kidney disease independently from albuminuria (Peralta et al., 2012).

1.12.1.4 KIM-1 in kidney donation

When attempting to assess the potential kidney donor, to determine the underlying level of existing renal injury, it seems intuitive that a marker previously suggested to be sensitive for AKI might have a role. As such levels of KIM-1 expression have been used in small studies to compare the level of renal injury in DBD donors with live donors. This work has demonstrated that there is higher expression of KIM-1 and higher urinary levels of KIM-1 in the DBD group when compared to the live donor population. Levels have also shown to be predictive of the recipients creatinine at 14 days and at 6 months post transplant (Nijboer et al., 2009, Kamińska et al., 2011).

1.12.1.5 KIM-1 in transplant recipients

Some initial work as to the utilisation of KIM-1 in the monitoring of renal transplant function has been undertaken. A cross-sectional study measured 24hr KIM-1 excretion at a single time point in 606 transplant recipients at a mean of 6yrs post-transplant and correlated higher levels of KIM-1 positively to graft loss resulting in return to dialysis or re-transplantation (adjusted for donor age and creatinine) (van Timmeren et al., 2007a).

KIM-1 expression in transplant biopsy tissue (indicated both because of rejection and protocol driven) showed KIM-1 to be more sensitive than histology in transplant biopsies for detecting early tubular injury, correlated to the potential for the kidney to recover and sensitively and specifically identified proximal tubule injury (Zhang et al., 2007).

KIM-1 expression has also been related to differing causes of renal transplant graft dysfunction. Biopsies from patients suffering a) ATN with superimposed rejection, (n=21) b) ATN (n=11) c) acute rejection (n=12) d) calcineurin inhibitor nephrotoxicity (n=4) and e) interstitial fibrosis and tubular atrophy (n=11) were correlated with levels of KIM-1 expression. Levels of KIM-1 expression were highest amongst the calcineurin inhibitor nephrotoxicity group and the interstitial fibrosis group and lower amongst the ATN and ATN and rejection group (Nogare et al., 2010).

Urinary KIM-1 has been shown to correlate strongly with creatinine in a group of transplant recipients. Levels of KIM-1 were higher amongst those recipients who were hypertensive when compared to non-hypertensive recipients and higher amongst diabetics than non-diabetics. Levels of KIM-1 amongst all recipients were significantly higher than amongst controls (Malyszko et al., 2010).

1.12.1.6 Immunological role of KIM-1

KIM-1 is also known as TIM-1 (T cell immunoglobulin mucin domains-1) and is expressed at low levels by sub-populations of activated T cells. It is also known as hepatitis A virus cellular receptor-1 (HAVCR-1) and is expressed by hepatocytes. KIM-1 has been shown to co-localize to the site of internalisation of apoptotic cells

and mediates this by binding specifically to phosphatidylserine and oxidized lipid epitopes on the surface of the apoptotic cell (Ichimura et al., 2008).

The role of TIM-1/KIM-1 in the regulation of the immune system is not clearly defined. However, it seems that TIM-1/KIM-1 may have a role in regulating the initiation of phase of T cell response rather than differentiation (Degauque et al., 2007). It has also been shown that TIM-1/KIM-1 provides a co-stimulatory function that affects CD4 T cell function (Umetsu et al., 2005). It has been shown that TIM-1/KIM-1 blockade by administration of an agonist prevents the induction of peripheral-type transplant tolerance in vitro. It is hypothesised that this occurs by enhancing the commitment and expansion of alloreactive T cells in the Th1/Th17 T effectors, deprogramming natural T regs at the molecular and functional level and inhibiting the conversion of regulatory $Foxp3^{+}$ cells from the naive $Foxp3^{-}$ cells (Degauque et al., 2008). It has been shown in experimental models that targeting TIM-1/KIM-1 may provide an approach to overcoming resistance to tolerance in a cardiac mouse transplantation model (Yuan et al., 2009, Ueno et al., 2008). TIM-1/KIM-1 blockade has been shown to ameliorate hepatocyte damage in ischaemia reperfusion models. This is hypothesised to be through macrophage function in the local inflammatory response (Uchida et al., 2010). A study demonstrated that TIM-1/KIM-1 is a physiological ligand for LMIR-5 and that this interaction is pivotal in neutrophil accumulation related to tissue damage in kidney ischaemia reperfusion injury. Therefore, it may be that blocking this interaction might be a novel therapeutic strategy for acute renal tubular damage (Yamanishi et al., 2010). Mouse models of cresenteric glomerulonephritis have shown that blocking KIM-1 can also protect mice from proliferative and cresenteric glomerulonephritis via effects on the TH1 and

TH17 subsets with concomitant reductions in proteinuria, crescent formation and intrarenal leukocyte infiltration (Nozaki et al., 2012).

Whilst KIM-1 has been the subject of intense study in many different fields within renal medicine its specific role remains nebulous and its transition to clinical utility is still awaited. However, it seems likely that it will make the transition.

1.12.2 **Neutrophil Gelatinase Associated Lipocalin (NGAL)**

1.12.2.1 Discovery and characteristics

Neutrophil gelatinase-associated lipocalin (NGAL) is a member of the lipocalin protein family, a large group of small extracellular proteins. These proteins have roles in transport, enzyme synthesis, immunomodulation, olfaction, pheromone signalling and cell regulation (Flower, 1996).

NGAL is one of the emerging potential biomarkers for renal injury and is as such under intense scrutiny (Haase et al., 2010). Early work utilising genome-wide interrogation to identify those genes up-regulated during renal ischaemia models in animals identified NGAL as one of the most up-regulated genes (Supavekin et al., 2003, Mishra et al., 2003).

1.12.2.2 NGAL in AKI

Initial clinical work involving plasma NGAL utilised the setting of patients post cardio-pulmonary bypass (CPB) who have a comparatively high incidence of development of acute kidney injury. This initial work focused on children for their lack of

confounding factors (for example atherosclerosis). A study of 71 children undergoing CPB showed 20 children (28%) developed acute kidney injury. In this group the diagnosis of acute kidney injury utilising serum creatinine was only possible 1-3 days after the surgical insult. However, urine NGAL concentrations rose from a mean of 1.6ug/l at baseline to 147ug/l 2h after CPB and the serum levels increased from 3.2ug/l to 61ug/l at 2h after CPB. A significant correlation was shown between acute renal injury and urine and serum NGAL 2h after CPB. Using a cut-off value of 50ug/l a sensitivity of 1.0 and a specificity of 0.98 was demonstrated (Mishra et al., 2005).

A US study looked at NGAL in post CPB children to validate NGAL and a rapid point-of-care Triage NGAL device. Their initial results showed good correlation between ELISA and Triage calculated NGAL levels (r=0.94) so a further 120 children were enrolled in the study of whom 45 developed AKI. Within this group mean NGAL levels increased threefold within 2hrs of CPB surgery and remained elevated whereas diagnosis by serum creatinine took 2-3 days. The authors determined that for the 2h NGAL level using a cut-off of 150ng/ml a sensitivity of 0.84 and a specificity of 0.94 for prediction of AKI. They also determined that the 12h NGAL correlated strongly to mortality (Dent et al., 2007). Similar results were generated in a study to determine the utility of a standardized clinical platform analyser which examined 196 children undergoing CPB of who 99 developed AKI (Bennett et al., 2008). Other studies seemed to have confirmed the potential utility of NGAL in this setting (Wagener et al., 2006, Haase et al., 2009a).

It has also been suggested that combining NGAL with KIM-1 and NAG (N-acetyl-b-d-glucosaminidase) could prove useful in detecting early post-operative AKI after cardiac surgery prior to a rise in serum creatinine. An improved sensitivity and

specificity on combining the biomarkers giving areas under the curve of 0.75 for determining immediately and 0.78 at 3h post-op (Han et al., 2009).

A study in 2008 aimed to determine whether utilising a panel of biomarkers would be more sensitive and specific for AKI than an individual biomarker. The underpinning theory behind this was that renal structural heterogenicity and the varied causes of renal injury may render a single biomarker insufficient. A panel of nine biomarkers were examined in 204 patients with and without AKI. Median urinary concentrations of each biomarker were higher in patients with AKI than those without. The authors applied a logistical regression model to a combination of the markers and determined that a combination of specific biomarkers (NGAL, HGF, Protein and KIM-1) showed greater AUC than individual biomarkers. This suggested that combining different biomarkers may move the search forward and help biomarkers make the transmission from research to clinically usable measure (Vaidya et al., 2008).

A study of NGAL in the intensive care examined 451 critically ill patients of whom 14% developed AKI within 24h and 19% within 48h. Urinary NGAL levels were higher among those who developed AKI at 48h than those who didn't and urine NGAL was independently associated with the development of AKI independent of age, creatinine, sepsis and illness severity. NGAL demonstrated moderate predictive utility for the development and severity of AKI during hospitalisation but as a single point measure its utility might be limited (Siew et al., 2009).

Admission levels of urinary NGAL (and β-2-M and KIM-1) in a paediatric emergency setting have been analysed and comparison made to creatinine. Urinary levels of the biomarkers detected the presence of AKI and severity more rapidly than creatinine in the acute setting, although no severe cases of AKI were seen.

NGAL levels have also been studied in septic patients with and without AKI to determine whether the presence of sepsis would distort NGAL's ability to demonstrate AKI. A study of patients in intensive care with and without sepsis showed that interpretation of plasma NGAL was difficult in the presence of sepsis and that urinary NGAL was a better marker in this situation (Mårtensson et al., 2010) (Du et al., 2010).

1.12.2.3 NGAL in chronic kidney disease

A study looking at NGAL in the role of monitoring chronic kidney disease in children determined in their sample of 45 children with CKD stage 2 - 4 serum NGAL (r=0.62), Cystatin C (r=0.71) and eGFR (r=0.71) were all significantly correlated with measured GFR while at lower GFRs serum NGAL (r=0.62) correlated better with measured GFR than eGFR or Cystatin C (r=0.41). The authors suggested that NGAL may prove useful in the quantification of CKD particularly at lower GFRs (Mitsnefes et al., 2006).

Urinary levels of NGAL have also been correlated to the degree of proteinuria and inversely with the residual renal function in patients with primitive membranous and membrano-proliferative glomerulonephritis, although the mechanism of the correlation could not be determined (Bolignano et al., 2008).

They have also been shown to be associated with chronic renovascular hypertension thought to be due to a combination of on-going kidney and systemic inflammation (Eirin et al., 2012).

The levels of NGAL and IL-18 have also been studied in the setting of polycystic kidney disease in both animal models and human samples. These studies have shown that NGAL and IL-18 are elevated in cyst fluid in both human and animal models. The levels of NGAL in urine increased in a statistically significant manner over a period of 3 years, however, the change in levels did not correlate with total kidney function or total kidney volume which the authors surmised could be due to the lack of communication between the individual cysts and the urinary collecting system (Parikh et al., 2012). They have also been shown to be a marker of renal function in diabetic chronic kidney disease however, overall in CKD NGAL has not been shown to give more accurate information that GFR (Woo et al., 2012). Monospecific assays for NGAL have been demonstrated to correlate with the histology in biopsy specimens that typify progressive CKD (Nickolas et al., 2012).

1.12.2.4 NGAL in transplantation

Urinary NGAL levels were examined prospectively in a cohort of living donor and deceased donor renal transplant recipients to determine whether levels were able to predict delayed graft function. The group consisted of 23 living donor recipients, 20 deceased recipients with prompt graft function and 10 deceased recipients with delayed graft function. Urine NGAL levels at day 0 was significantly different between the 3 groups and highest amongst the delayed graft function group. After adjustment for other variables urine NGAL levels on day 0 were shown to predict the trend in serum creatinine in the post-transplant period and therefore could have a role as an early predictive biomarker for delayed graft function (Parikh et al., 2006). A similar study looked at NGAL levels and ability to predict DGF and demonstrated day 1 post-operative levels were predictive of dialysis within 1 week of transplant.

This predictive ability remained after adjustment for cold ischaemic time, age (donor and recipient) and urine output. NGAL also predicted graft recovery at 3 months (Hall et al., 2009). Urinary NGAL day 1 post-transplant has also been shown to correlate to prolonged DGF that led to poorer graft survival (Hollmen et al., 2010).

A study looking at serum NGAL levels rather than urine NGAL levels post deceased kidney transplant failed to show a difference between serum levels of NGAL between the group of patients who experienced delayed graft function and those who had immediate or slow graft function (Hall et al., 2011). The expression of NGAL in biopsy specimens from renal allografts taken at 0 hour by RT-PCR has also demonstrated a 3.78 fold change in NGAL level in the delayed graft function group, however odds ratios could not reach significance in the multivariate analysis involving donor age. The AUC was 0.75 and suggested that NGAL expression in 0 hour biopsies has the potential to act as an early biomarker for delayed graft function (Korbely et al., 2011).

Urinary NGAL levels have also been examined to determine whether they can differentiate between sub-clinical tubulitis in stable transplant patients and stable transplant patients with normal tubular histology to provide a non-invasive mechanism for detecting early injury. Whilst significantly higher levels of urinary NGAL were seen in a group of patients with clinical evidence of tubulitis NGAL was unable to differentiate between the stable patients whether they had sub-clinical evidence of tubulitis (Schaub et al., 2007). Similarly in a spot study of 182 patients attending clinic NGAL levels in urine were found to be significantly higher in those patients with acute allograft rejection even when compared to the other causes of AKI. This study determined at a cut-off value of 100ng/ml which demonstrated an area under the curve of 0.98 (Heyne et al., 2012).

NGAL levels have also been investigated as an early biomarker of graft injury in animal models. Porcine kidneys subject to differing degrees of warm and cold ischaemia were assessed for levels of NGAL, endothelin-1, IL-6 and TNF-α. Levels of NGAL and endothelin-1 were significantly higher in those kidneys exposed to the longest periods of warm ischaemia prior to cold storage. The authors suggest that NGAL could be used as a marker of the severity of the ischaemic injury sustained (Hosgood et al., 2012).

1.12.2.5 Immunological aspects of NGAL

NGAL has also been shown to be pivotal to the innate immune response to bacterial infection via iron sequestering. NGAL limits bacterial growth by sequestering iron-laden siderophores that are required by many bacteria to grow, this has been demonstrated in vivo in NGAL knockout mice who, when infected with *Escherichia coli H9049,* demonstrate substantial increases in bacteraemia and bacterial burden (1000 fold greater bacteraemia in NGAL deficient mice). Upon encountering bacteria innate immune cells produce and secrete NGAL which limits bacterial growth by iron sequestration (Flo et al., 2004).

The complex generated by the interaction of NGAL with iron binding siderophores has also been suggested to convert renal progenitors into epithelial tubules. It has therefore been postulated that administration of NGAL could help protect the kidney in ischaemia-reperfusion. In a mouse model the administration of NGAL during the initial phase of disease (ischaemia-reperfusion) dramatically protected the kidney and mitigated azotemia (Mori et al., 2005, Mishra et al., 2004). Whether NGAL provides a reservoir to absorb excess iron released from damaged cells preventing a

reactive molecule from the site of injury or whether NGAL may recycle iron into viable cells stimulating renal epithelial cells after injury is unclear (Mishra et al., 2004).

In order to demonstrate the role of NGAL as a surrogate marker an NGAL reporter mouse was created by inserting a double-fusion reporter gene encoding luciferase-2 and mCherry into the NGAL locus. This mouse then recapitulated and illuminated injuries in vivo in real time. A sensitive, rapid, dose-dependent, reversible and organ and cell specific relationship with tubular stress was shown. This correlated to the level of urinary NGAL (Paragas et al., 2011).

A meta-analysis undertaken in 2009 examined all studies of NGAL in AKI and concluded that NGAL level was a useful early predictor of AKI over a range of clinical settings. It was concluded that urine or plasma levels were equally useful, that standardized clinical platforms using a cut-off of 150ng/ml were superior to research assays and that NGAL level had prognostic value for clinical outcomes such as progression to renal replacement therapy and mortality (Haase et al., 2009b). For this reason it seems likely that NGAL too will make the transition to clinical utility in the foreseeable future.

1.12.3 **B-2-microglobulin (β2-M)**

β2-microglobulin (β2-M) is a low molecular weight protein that is detected on the cell membrane of all nucleated cells. It is associated with the class-I HLA proteins and other membrane proteins. β2-M is freely filtered by the glomerular basement

membrane and then reabsorbed and degraded in the proximal tubular cells. It is thought that under normal circumstances the levels of β2-M in serum are stable but that it is a potential surrogate marker of GFR. For this reason it has been proposed as a potential substitute to creatinine.

As far back as the early 1980's research was underway to determine whether β2-M had a potential role in monitoring the function of renal allografts post-transplantation. A study group of 70 deceased transplants and 23 living related transplants showed that in those grafts with stable renal function the daily serum levels of β2-M varied very little. The study group had 90 episodes of rejection in whom serum β2-M levels were significantly elevated at least 1 day prior to the elevation of serum creatinine in 49% of patients. They felt that serum β2-M was a useful adjunct to the monitoring of a renal allograft, particularly as serum β2-M appeared to be cleared by a transplanted kidney even in the face of transplant oliguria confirming delayed graft function rather than allograft failure (Edwards et al., 1983).

Following on from this a group in Singapore compared urine β2-M levels in healthy subjects with levels in post-renal transplant recipients and demonstrated that significant differences existed between the levels of urinary β2-M in the healthy and the transplant population and that urine levels of β2-M correlated positively with serum creatinine. They also suggested that serum β2-M might have a role in differentiating renal function in the transplant population irrespective of creatinine (Woo et al., 1985).

One of the significant limitations of the early work into serum and urine β2-M was that the laboratory methods of assessing β2-M were difficult and expensive whereas

now β2-M levels can be assessed more readily by relatively easily available routine tests such as ELISA.

These newer methods of measuring β2-M levels reinvigorated research and in 1995 a group looked at 53 stable renal transplant recipients and compared renal function (both serum creatinine and creatinine clearance) and serum β2-M. They also looked at 40 healthy individuals and 28 renal donors. All patients included in the study were at least 6months post-transplant. They demonstrated a poor correlation between β2-M and creatinine clearance, although they demonstrated that serum β2-M levels were higher in the transplanted patients than either the healthy controls or the donors. The authors felt that the reason that despite the excellent allograft function the recipients still had elevated levels of serum β2-M even compared to the donors who also only had one kidney was due to on-going relationship between the graft, the immune system and the immunosuppressive therapy (Pacheco-Silva et al., 1995). Having established a correlation between β2-M levels and creatinine the next step was to determine whether there was a predictive ability of β2-M levels. Serial measurement of β2-M in 46 renal transplant recipients was undertaken and 10 developed acute rejection. They demonstrated higher mean serum values of β2-M in the rejection group as opposed to the group without rejection 6.24 ±0.68mg/l as opposed to 4.2 ± 0.23 in the stable function group ($p<0.05$). The serum creatinine between the groups was not significantly different. A β2-M:creatinine ratio of above 4 at the start of follow up was associated with a relative risk of rejection of 22.9 times and a negative predictive value of 89% for rejection and a sensitivity of 70% was demonstrated. The authors felt that this measure's main applicability would probably lie in identifying those recipients who would be good candidates for weaning of immunosuppression (Saraiva Camara et al., 1998).

β2-M serum levels have also been analysed in the context of post-transplant infection and shown to be a potential indicator of CMV infection. A study looked at sequential serum β2-M levels and correlated them to CMV infection. OF the 18 cases the group had of CMV 16 patients showed increases in their β2-M levels prior to any other indicators of CMV infection. In these cases levels went form a base line of 5mg/l to a mean value of 7.7mg/l at the time of diagnosis. This seems to suggest that β2-M is a non-specific indicator of problems within the transplant allograft (Carvalho Matos et al., 2004).

Interestingly using modern proteomic methods to identify urine peaks of renal allograft rejection suggested β2-M. This group then analysed these urine protein peaks and determined that they predominately related to cleaved products from β2-M. The authors went on to demonstrate that in order for cleavage products of β2-M to be produced the urine had to be more acidic and aspartic proteases had to be present. They also demonstrated that patients with rejection had more acidic urine than those with stable function and healthy individuals (Schaub et al., 2005). In 2006 a US group used MALDI-TOF MS to analyse urine from 30 renal transplant recipients with biopsy proven acute rejection and 15 recipients without rejection. They similarly identified β2-M as the peak that most strongly correlated and showed a sensitivity of 83.5% and specificity of 80% with a positive predictive value of 89% and a negative predictive value of 70.6%. The authors suggested that the real application of urine β2-M could lie in combination with other biomarkers (Oetting et al., 2006).

Following on from the increased interest in β2-M in transplantation a Singapore group in 2008 aimed to delineate the levels of β2-M in acute renal failure, chronic renal failure, stable transplant and renal transplant rejection patients. The study

group comprised 23 patients with ARF, 22 patients with CRF, 6 cases of transplant rejection and 7 stable transplants and 28 healthy controls. They demonstrated that the highest levels of serum β2-M were the chronic renal failure patients (all patients had elevated levels, 12.97 +/- 3.83 ug/ml), compared to acute renal failure (all patients had elevated levels, 11.75 +/- 2.09ug/ml), transplant rejection (50% had moderate elevation, 50% had marked elevation), and compared to stable transplant recipients of whom 42.8% had mild elevation of serum β2-M levels. Similarly 21.4% of healthy controls were shown to have mildly elevated levels of serumβ2-M. For this reason and based on the overlap between each of the groups the authors felt that serum β2-M was not superior to serum creatinine (Sonkar et al., 2008).

In line with research undertaken on other biomarkers urine β2-M has also been examined in the context of predicting acute renal failure in those undergoing coronary artery bypass grafting in a group of markers. Urine β2-M levels were shown to rise in all patients following the stress of surgery with levels resolving after day 1 post-op but with a second peak of levels at day 3-5 consistent with cleaved products that was absent from the group who did not go on to develop acute kidney injury, suggesting that they may have a role as predictors of acute kidney injury (Ho et al., 2009).

Urinary levels of β2-M have also been demonstrated in animal models to have a better diagnostic performance than urea and creatinine for detecting glomerular injury. Application has been made based on these animal studies for the use of β2-M as part of a panel of biomarkers to assess kidney injury during drug development (Dieterle et al., 2010).

Whilst research focusing on β2-M levels has been undertaken for nearly 30 years its transition from research to bedside test seems to be progressing in combination with other biomarkers rather than a stand alone test.

1.12.4 **Fractalkine**

Fractalkine is an unusual member of the chemokine family i.e. the chemokines and their receptors which exhibit tissue and cellular specificity. As molecules involved in the trafficking of cells to tissue sites where receptors are expressed as well as their ligands in endothelium they are intrinsically involved in both homeostasis and disease and are capable of up and down regulating as required.

It has three amino acids between the first two cysteine residues and is classified as CX3CL1. It exists on the surface of cells and interacts with leukocytes and captures cells. It may also be cleaved to produce a soluble form. Expression of CX3CR1 (the receptor for Fractalkine) has been demonstrated for T cells, natural killer cells and neutrophils. The pathway Fractalkine-CX3CR1 is important in leukocyte trafficking and activation and may be important in acute rejection (Imai et al., 1997, Haskell et al., 2000).

This is further implicated when the expression of CX3CR1 in transplant patients with acute rejection was examined. In those patients with acute rejection (cellular or vascular) higher levels of expression of CX3CR1 were seen than in those patients with no biopsy evidence of rejection (Segerer et al., 2002). The role of ligand Fractalkine can also be correlated to acute rejection. This study, undertaken in China, recruited 215 allograft recipients of whom 67 patients experienced acute rejection. In those recipients with stable renal function Fractalkine levels remained

stable. Patients with rejection secreted Fractalkine at significantly higher levels than patients with no abnormal histological findings. In the same study patients with chronic allograft nephropathy and acute tubular necrosis also showed increased urinary levels of Fractalkine though significantly lower than those experiencing rejection. Higher levels of urinary Fractalkine were also shown to correlate to steroid-resistant rejection and graft loss at an early stage after acute rejection (Peng et al., 2008).supporting the role hypothesised for CXC3L1/Fractalkine in the mechanism of acute rejection.

1.12.5 **Cystatin C**

Cystatin C is a non-glycosylated low molecular weight protein that is produced by all nucleated cells at a stable rate. Blood concentration therefore should reflect glomerular filtration rate (GFR).

Studies have utilised cystatin C either in combination with other markers (e.g. creatinine) and other parameters to try to improve the ability of clinicians to estimate GFR. Some studies have shown that using serum cystatin C level as part of a formula including serum creatinine proved superior to using creatinine alone (Tidman et al., 2007, Ma et al., 2007, Roos et al., 2007). An improvement in calculation of GFR has also been demonstrated in cirrhotic liver patients by the inclusion of cystatin C (Poge, 2006). However, some authors have raised issues with the use of cystatin C in the calculation of GFR amongst transplant recipients and other clinical presentations and suggested its use as a GFR measure should be in the chronic kidney disease population (Rule et al., 2006). One study looked at the use of cystatin

C in comparison to the calculation of GFR using the Modification of Diet in Renal Disease (MDRD) equation in renal transplant patients and concluded that they were essentially equivalent but that as the availability of the MDRD was superior it should be used, although cystatin C could provide a useful adjunct particularly amongst those with well-functioning grafts (Poge et al., 2006). In a comparison between cystatin C and creatinine one study showed that cystatin C had a greater specificity but lower sensitivity than creatinine for detecting dysfunction in transplant patients (Tsai et al., 2010). However, despite the developments of the potential inclusion of cystatin C, either as a component of the equations or as a separate measure, caution is has been advised in interpretation (Hergetrosenthal et al., 2007).

Confounding factors in the variability of cystatin C levels are multiple and include age, male gender, increasing BMI, height, smoking status and higher CRP levels. These factors may impact on the interpretation of cystatin C levels (Knight et al., 2004). It has also been suggested that concurrent glucocorticoid therapy may alter cystatin C levels, necessitating the development of specific reference ranges for patients receiving immunosuppression including steroids. This may also go part way to explaining the differences seen in cystatin C levels between patients with similar GFRs but with and without renal transplants (Risch et al., 2001).

In liver transplantation cystatin C has been shown in a single study to be a sensitive marker of post orthoptic liver transplantation kidney function. The authors suggested that if the pre-operative cystatin C level exceeded a certain cut-off then a deterioration of renal function post-transplant was likely (Nemes et al., 2010). It has been shown to have a potential role in the post-operative monitoring of children after liver transplant proving significantly more sensitive than GFR calculated using creatinine (Brinkert et al., 2010).

Serum Cystatin C has also been utilised in monitoring for renal complications during drug development in pre-clinical animal models. Cystatin C has been shown to be more sensitive and specific than creatinine in monitoring generalised renal function in rat nephrotoxicity models (Ozer et al., 2010). As such the FDA has now recognised the use of cystatin C as part of a panel of biomarkers for monitoring renal safety during drug development (Dieterle et al., 2010).

Another potential role for cystatin C has been in the early detection of AKI. It has been suggested that serum cystatin C can detect AKI up to 48hrs prior to a rise in creatinine (Herget-Rosenthal et al., 2004).

A study in 2008 looked at the possible role of urinary cystatin C in detecting AKI. Median urinary concentration of Cystatin C was shown to be significantly higher in patients with AKI than those without, however, levels did not correlate to progression to renal replacement therapy or death in contrast to previous studies (Herget-Rosenthal, 2004), (Vaidya et al., 2008). This is in contrast to a study of 200 patients with AKI in which serum cystatin C was shown to perform similarly to creatinine for predicting dialysis progression or in-hospital death (Perianayagam et al., 2009).

A further study in 2009 demonstrated a role for cystatin C in AKI following cardiac surgery. Levels immediately post-operatively correlated with and independently predicted duration and severity of AKI in adult patients (Haase et al., 2009a).

Similarly, in a study of AKI due to radiographic contrast, cystatin C level at 24hrs post contrast media administration rose <10% from baseline then kidney function was unlikely to be affected (Briguori et al., 2010) in comparison to those with elevated levels who suffered a change to creatinine. Urinary cystatin C has also been used in an intensive care setting and is independently associated with AKI,

sepsis and death within 30 days (Nejat et al., 2010). This ability to diagnose and predict AKI using cystatin C has been improved by stratifying levels according to GFR, with AUC for cystatin C with an eGFR<60 of 0.69 at 6 hours after admission to ITU and an AUC of 0.88 at 12-36hr being predictive of AKI (Endre et al., 2011).

Cystatin C has been examined in the context of monitoring of renal transplant recipients. In 30 adults receiving renal transplants levels of plasma cystatin C correlated to plasma creatinine (r=0.741; p=<0.0001). In those cases of rejection of the allograft the increase in plasma cystatin was seen to be more prominent than that of creatinine (Le Bricon et al., 1999). This has also been seen in other studies (Liu, 2012).

Serum cystatin C has also been used to predict early graft function after deceased donor kidney transplant. In a cohort of 78 recipients of whom 26 had delayed graft function and 29 had slow graft function serum cystatin C outperformed serum creatinine on the first day post-operatively for predicting the clinical course (area under the curve of 0.83 (DGF) and 0.85 (non-immediate graft function) compared to 0.65 and 0.53 respectively for serum creatinine) (Hall et al., 2011).

1.12.6 **Interferon-inducible protein-10 (IP-10)**

IP-10 is a small cytokine that is secreted by several cell types in response to interferon-gamma. These include monocytes, endothelial cells and fibroblasts. It elicits its response by binding to the cell surface chemokine receptor CXCR3. Several roles have been attributed to IP-10 including chemo-attractant, promotion of T cell adhesion to endothelial cells, anti-tumour activity, and inhibition of

angiogenesis. Studies have also shown that CXCR3 also has a role in mediating host alloresponses and graft destruction (Hancock et al., 2000).

In a US study of 58 renal transplant recipients (of which 27 had acute rejection, 16 had chronic allograft nephropathy) the levels of IP-10 mRNA in urine predicted rejection. ROC showed that at a (log-transformed) mRNA levels of 9.11 copies acute rejection could be predicted with a sensitivity of 100% and a specificity of 78%. Urinary expression of IP-10 was absent in grafts with stable function (Tatpudi et al., 2004).

A study of IP-10 in urine in 99 transplant recipients was undertaken to determine the ability of urinary IP-10 to detect injury. Of the 99 transplants 28 had acute rejection, 9 had borderline rejection, 6 had BK nephropathy and 10 had acute tubular injury. Samples from 16 healthy, non-transplanted individuals were also analysed. Quantities of IP-10 in the urine samples were measured by Luminex™ assay. Patients with acute rejection, BK virus and acute tubular injury had higher levels of IP-10 while stable function and chronic rejection had lower levels. The level of IP-10 also fell as treatment was instigated for acute rejection with most patients reaching a level under 100pg/ml. The authors suggest that IP-10 might have a role as an adjunct in deciding whether to biopsy patients but was not individually diagnostic (Hu et al., 2004).

IP-10 has also been correlated to overall graft function. Urinary levels and mRNA have been correlated to episodes of rejection, in which increases in IP-10 precede rises in serum creatinine, but also levels in the first 4 weeks correlate to outcome of the graft at 6 months. Patients with urinary tract infections or CMV infection showed no increase in IP-10 levels in urine. The authors suggest that IP-10 expression

correlates to specifically intra-graft immune activation that leads to acute rejection and that measuring levels of IP-10 might allow stratification of patients on the basis of immune risk and therefore allow individual tailoring of immunosuppression (Matz et al., 2006).

IP-10 levels pre-transplant have also been correlated to early severe rejection and to chronic allograft nephropathy in serum from 316 graft recipients and in biopsy specimens from 22 patients with acute rejection. In this cohort pre-transplant serum levels greater than 150pg/ml carried an increased risk for severe acute rejection, infact they demonstrated increased pre-transplant levels carried a relative risk of 2.81 for graft loss (Lazzeri et al., 2005, Rotondi et al., 2004).

Studies undertaken in cardiac allografts in mice demonstrated that MHC-mismatched mice who were transplanted had longer survival times if they were null, i.e. could not express, for cxcr3 the receptor for IP-10. This was further extrapolated to demonstrate that the use of CXCL10 (IP-10) monoclonal antibody blocking this pathway resulted in prolonged survival (Hancock et al., 2000, Hancock et al., 2001).

1.12.7 **Vascular Endothelial Growth Factor (VEGF)**

Vascular endothelial growth factor is a heparin-binding protein with a multitude of functions. It was initially described because of its ability to increase vascular permeability and stimulate the growth of endothelial cells (Ferrara et al., 1991). It has also been found to act as a mediator in inflammation by acting in a chemo-attractant manner to monocytes and promoting adhesion (Neufeld et al., 1999). Within the kidney VEGF expression is most prominent in the glomerular and peritubular endothelial cells. It is needed for glomerular and tubular hypertrophy in response to

nephron loss and is associated with glomerulosclerosis and fibrosis within the kidney (Schrijvers et al., 2004).

A study examined urinary VEGF excretion during acute rejection to assess whether urinary VEGF levels were associated with rejection and with response to treatment. Of 215 patients enrolled, 67 had biopsy proven rejection. A statistically significant difference was demonstrated between the VEGF levels in patients with rejection (28.57 +-6.21 pg/ml) and without rejection (3.05 +-0.45 pg/ml). At a cut-off of 3.64pg/umol creatinine the sensitivity of VEGF was 85.1% and the specificity was 74.8%. Higher levels of VEGF predicted graft loss and steroid resistance. The authors concluded that VEGF levels in urine might have a role in monitoring transplant function and predicting rejection episodes (Peng et al., 2007).

Research has also been undertaken to explore whether the genetic variations in VEGF expression influences the development of transplant rejection and has suggested that 2 specific genotypes are strongly associated with higher risk of acute rejection and have the potential to be useful markers of rejection risk (Shahbazi et al., 2002).

1.12.8 **Interleukin-22**

IL-22 is a member of the IL-10 cytokine family. It is produced by activated dendritic Cells and T cells and initiates innate immune response. It can be produced by Th17 cells which are thought to play a key role in autoimmune disease and in inflammation and tissue injury. With the massive increase in the size of population of dendritic cells and T cells that occurs post transplantation it may be that IL-22 is a useful indicator of rejection within the renal allograft. The anti-CD20 antibodies that are

routinely used in transplantation also reduce the TH17 cell response so theoretically should decrease the levels of detectable IL-22, therefore, it may be that higher levels of IL-22 indicate those individuals who could require more intensive immunosuppressive therapy.

1.12.9 **Tumour Necrosis Factor-α (TNF-α)**

TNF-α is made by T-cells, NK-cells, macrophages and other cell types. It has a wide variety of functions but is centrally involved in inflammation and apoptosis. Early tissue expression of TNF-α has been shown to mediate neutrophil infiltration and injury after renal ishaemia-reperfusion (Donnahoo et al., 1999). It has also been shown that unilateral renal ischaemic injury induces bilateral TNF-α production and neutrophil infiltration through a TNF-α dependent pathway (Meldrum et al., 2002).

A study in the UK looked at whether serum or urine levels of TNF-α could be used to discriminate between the causes of renal allograft dysfunction in order to avoid renal biopsy. TNF-α levels were measured at the same time as renal biopsy was undertaken to determine the cause of the transplant dysfunction but no difference in levels of TNF was seen between the causes of graft dysfunction (Newstead et al., 1993).

Early work suggested that TNF-α levels reacted to episodes of rejection and work undertaken to determine whether serum levels of TNF-alpha could therefore be of use clinically. Work looking at TNF-α levels during episodes of acute rejection found that plasma levels might have a role in the early detection and differential diagnosis of acute rejection. This hypothesis was based on the role of TNF-α as an important mediator of renal allograft rejection by causing depression of the intra-graft

thrombomodulin pathway and contributing to intra-graft fibrin deposition (Tsuchida et al., 1992).

One study showed that TNF-α could predict episodes of rejection with a sensitivity of 70-80% and a specificity of 89% (Dorge et al., 1994) and other studies have supported the potential role of TNF- α (Sonkar et al., 2013) however it has not been incorporated into clinical use.

1.12.10 **Interferon-γ (IFN-γ)**

IFN-γ is a cytokine that is critical for the innate and adaptive immune system, although those patients who have a primary deficiency of IFN-γ are normal. When expression of IFN-γ is disordered it is associated with autoimmune diseases. It is produced by natural killer cells and by CD4 and CD8 cells. It also stimulates secretion of IP-10 by monocytes, endothelial cells and fibroblasts.

A French group have also looked at the expression of IFN-γ as a method of determining between subclinical and clinical glomerulitis. Biopsy specimens were compared between 22 patients with normal biopsy specimens at 3 months post-transplant, 17 patients with clinically evident glomerulitis and 20 patients with subclinical glomerulitis. The expression of IFN-γ was significantly higher in patients with clinical glomerulitis than in subclinical glomerulitis, however the expression was also higher in patients with subclinical findings than those with normal biopsy results (Buob et al., 2011).

It has also been suggested that following transplantation in patients with early graft function the ratio of IFN-gamma to IL-4 is significantly higher than in delayed graft

function as a resultant effect of changes to the resident T cell populations present in the graft and the characterisation of delayed graft function as a Th1 driven immune response (Loverre et al., 2011).

Similarly in another study pre-transplant urine cytokine profiles to predict acute kidney rejection after transplant and demonstrated significantly higher levels IFN-γ in those who subsequently went on to develop rejection of the graft. The authors suggest this higher level of IFN may reflect an on-going non-specific Th1 immune response that is capable of amplifying the alloimmune response rapidly in the early post-surgery phase (Karczewski et al., 2010).

The interferon pathway may also have a critical role in mediating renal ischaemia/reperfusion injury as demonstrated in animal models (Freitas et al., 2011).

1.12.11 **Cathepsin L**

Cathepsin L is a lysosomal protease involved in intracellular protein catabolism. It plays a role in antigen processing and growth regulation. It has been shown to have a role in antigen processing in turnover in intracellular and secreted proteins. Work examining transplant vasculopathy as an alloimmune form of vascular disease with enhanced neointimal formation and T-cell targeted allograft endothelial damage has also implicated Cathespin L (Soulez et al., 2010).

Cathepsin L has been shown to have increased activity in the grafts of patients who have undergone transplant nephrectomy following chronic rejection (Paczek et al., 1994). It is also implicated and under intense scrutiny for its role in the development of proteinuric kidney disease, specifically it seems Cathepsin L may have a critical

role in the mediating proteolysis in many forms of proteinuric renal disease (Sever et al., 2007).

1.12.12 **Immunoglobulin Light Chains**

Serum free light chain levels both kappa and lambda have been shown to vary amongst the renal transplant population in a way similar to that that occurs in the renal failure population i.e. that with increasing level of renal dysfunction the level of free light chain detectable increases (Sanchez-castanon et al., 2010).

Serum free lights chains are primarily removed by catabolism in the proximal epithelial cells in the kidney. In normal kidney function this equates to approximately a serum half-life of between 2-6hrs. In severe renal failure the kidney's ability to clear free light chains dramatically reduces and the main mechanism of clearance becomes the reticuloendothelial system so the half-life increases to 32h or more (Basanyake et al., 2011).

This gives a potential role in monitoring the adaptive immune system using free light chains, although many facets of this potential application remain unexplored (Hutchinson and Landgren, 2011).

1.12.13 **Summary**

The major challenges of transplantation have progressed from those of early immunological loss to the long-term attrition of transplanted kidneys and to extending the long-term success of implanted kidneys. The question – Why a

kidney transplanted from a 20 year old donor only lasts 15-20 years in a recipient rather than the expected 40 plus years had it remained in situ - cannot be answered.

The multi-factorial basis of this can be simplified into the functioning mass of the kidney and the loss of nephrons. This is based on the start point (chronic damage in the donor), peri-transplant events (acute injury in the donor and at retrieval) and recipient factors (acute and chronic injury). The ability to intervene prior to irreparable injury is a key aspect to preventing long-term damage. Currently monitoring of kidney injury in both donor and recipient is reliant on crude and imprecise methods, the mainstay being serum creatinine. This is particularly unsuited as a biomarker in kidney transplantation as it is delayed and represents established damage rather than an early indicator of acute insult that with intervention may allow reversal.

As part of this MD I wish to test two major hypotheses:

Hypothesis 1

> Acute kidney injury can be detected in deceased organ donors using biomarkers and can predict organ outcome once transplanted.

Hypothesis 2

> Acute kidney injury post-transplant can be detected using biomarkers and help monitor transplant recipients.

The application of these hypotheses to organ allocation, peri-transplant organ manipulation (e.g. machine perfusion, normothermic perfusion, drug treatment) and post-transplant follow up may have major impact on clinical outcomes but also allow an easier, quicker and cheaper method of transplant care.

2 Chapter 2: Materials and Methods

2.1 Developing an assay to measure the biomarkers

In order to be able to reliably quantify the amount of biomarkers in each sample an assay is required. Whilst a variety of different types of assay are available the selection of the optimum assay, in this case Luminex, was based on a number of factors. The most important characteristic of the assay for this research was the ability to test for a number of different analytes on a sample of comparatively small volume. Whilst commercial ELISA assays are available for the analytes in question these are run in series on samples rather than in parallel on a single sample and hence the sample volume required would have been prohibitive. Similarly the cost of a commercially developed Luminex assay would have been high. However, an in-house developed assay was deemed on balance the most logical solution.

2.2 Principles of a Luminex™ assay

Luminex™ assays allow the simultaneous analysis of up to 100 parameters in a single sample. This makes it ideal for running many different parameters on very small sample volumes.

The technology is based around microspheres (spherical polystyrol particles) that act as the solid phase for the molecular detection reagents. The microspheres or beads have defined spectral properties. Each microsphere is labelled with a two distinct fluorescent dyes (red and infrared) which emit light in different regions of the optical

spectrum. The combination of these dyes allows the microspheres to be differentiated and identified on the analysis system.

The microspheres are conjugated to protein-specific capture antibodies for the analyte in question. The microspheres are then exposed to the analyte in the sample and the target protein binds to the capture antibody that has been conjugated to the microsphere. A biotinylated detection antibody is then added which binds to the analyte. Streptavidin-conjugated fluorescent protein, R-Phycoerythrin (SAV-RPE) is added which binds to the biotinylated detector antibody. This means that a four member solid phase sandwich has been formed. The microspheres then pass through the Luminex™analyser which can then read the spectral properties of the beads and in conjunction with the amount of fluorescence from the streptavidin determine the amount of analyte present.

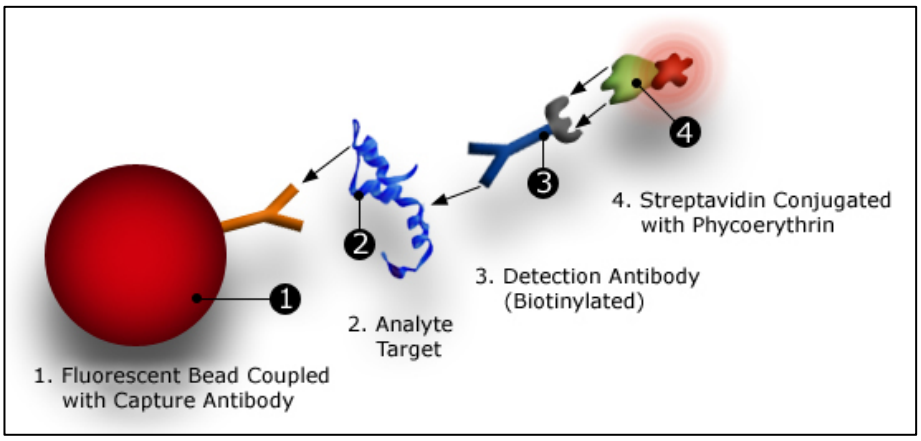


Figure 2-1: Diagram of ELISA sandwich assay principle in the context of multiplexed assay (www.evetechnologies.com)

2.2.1 **Conjugation of proteins to microsphere beads**

In order for the Luminex™ assay to work the first stage in development is to successfully couple the capture antibody to the Luminex™ microsphere beads. This involves the capture antibody being re-constituted in an environment free from

sodium azide, Bovine Serum Albumin, Glycine, Tris or amine-containing additives as these interfere with the cross-linking. This is achieved by meticulous attention to the constituents of all components of reconsitution. In order to couple the protein to the microsphere bead the carboxyl group of the polystyrene bead is first activated with the carbodiimide derivative EDC (1-Ethyl-3-[dimethylaminopropyl]carbodiimide hydrochloride) to form an intermediate that is stabilised with S-NHS (N-hydroxysulfosuccinimide). The intermediate then reacts with a proteins primary amine (NH_2 groups) to form a covalent bond (amide linkage). This is shown below.

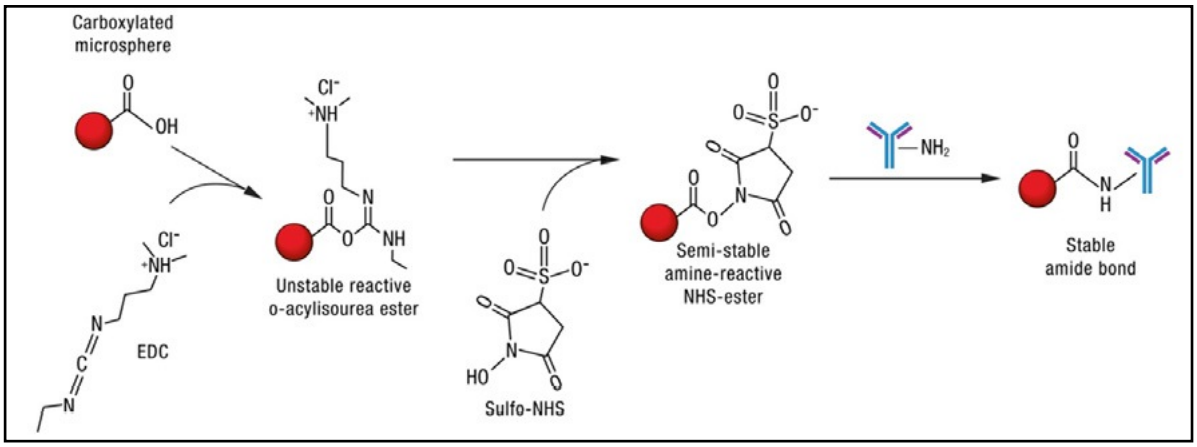


Figure 2-2: Illustration of the chemical reaction that occurs during conjugation of capture protein to the microsphere beads

In practice to achieve conjugation of the bead region a micro pellet is dispersed in a vortex using a mini shaker for 30s @2500/min. The microsphere pellet is then sonicated for 1 to 3 minutes in an ultrawave sonicator to disperse the microspheres pellet. 200ul of the microsphere pellet is then placed into a micro well plate and the fluid is aspirated and the pellet is washed twice with 200ul of activation fluid (250ml distilled water, 3g NaH2PO4 (Sigma S0751) adjust to pH 6.2), and then re-suspended in 200ul of activation fluid during the preparation of the coupling buffer. Coupling buffer is prepared by making fresh solution of sulpho-NHS (sigma 56485)

at 50mg/ml, fresh EDC (Sigma e6383) at 50mg/ml and mixing 10ul EDC + 10ul NHS + 80ul of activation buffer for each well required. The activation buffer is then aspirated from the microspheres and 100ul of coupling buffer placed in each well. This is then incubated for 30mins in the dark at room temperature and the coupling buffer is aspirated and the beads washed with 200ul of PBS .100ul of antibody (at 100ug/ml) is then added to each well and incubated for 3hrs in the dark at room temperature. Following this incubation the beads are washed with three times with storage buffer (200ml PBS, 2g BSA (Sigma A4503), 100ul Tween20 (Sigma P1379), 0.2g Sodium Azide) and re-suspended in a total of 600ul of storage buffer. Following this the conjugated beads are stored at 4°C.

2.2.2 **Antibodies used**

Antibody pairs and standards were supplied as follows:

Table 2-1: Antibody pairs and standards as used in the final assay.

Biomarker	Capture antibody	Detection antibody	Standard
NGAL	R&D systems cat no: MAB17571 Monoclonal Rat IgG	R&D systems cat no: BAF 1757 Polyclonal Goat IgG	R&D systems cat no: 1757-LC recombinant human, murine myeloma cell line
KIM-1	R&D systems cat no: AF1750 Polyclonal Goat IgG	R&D systems cat no:BAF1750 Polyclonal Goat IgG	R&D systems cat no:1750-TM-050 recombinant human, murine myeloma cell line
Cystatin C	R&D systems cat no:DY1196 MouseIgG	R&D systems cat no:DY1196 Mouse IgG	Prospec cat no#: PRO-656 recombinant human
Cathepsin-L	R&D systems cat no DY952 Goat IgG	R&D systems cat no DY952 Goat IgG	R&D systems cat no DY952 recombinant human
IFN-gamma	Peprotech cat no: 900-k27 rabbit origin	Peprotech cat no: 900-k27 rabbit origin	Peprotech cat no: 900-k27 recombinant human
TNF-alpha	Peprotech cat no: 900-k25 rabbit origin	Peprotech cat no: 900-k25 rabbit origin	Peprotech cat no: 900-k25 recombinant human
VEGF	Peprotech cat no: 900-k10 rabbit origin	Peprotech cat no: 900-k10 rabbit origin	Peprotech cat no: 900-k10 recombinant human
IL-22	Peprotech cat no: 900-k426 rabbit origin	Peprotech cat no: 900-k426 rabbit origin	Peprotech cat no: 900-k426 recombinant human
IP-10	Peprotech cat no: 900-k39 rabbit origin	Peprotech cat no: 900-k39 rabbit origin	Peprotech cat no: 900-k39 recombinant human
Fractalkine	Antigenix America cat no#: RHF 660 Polyclonal rabbit	Antigenix America cat no#: RHF 660 Polyclonal rabbit	Antigenix America cat no#: RHF 660 recombinant human

2.2.3 **Determining the concentration of secondary antibody**

To determine the concentration of secondary antibody to be added, standard curves for each analyte were run with varying concentrations of secondary antibody were generated.

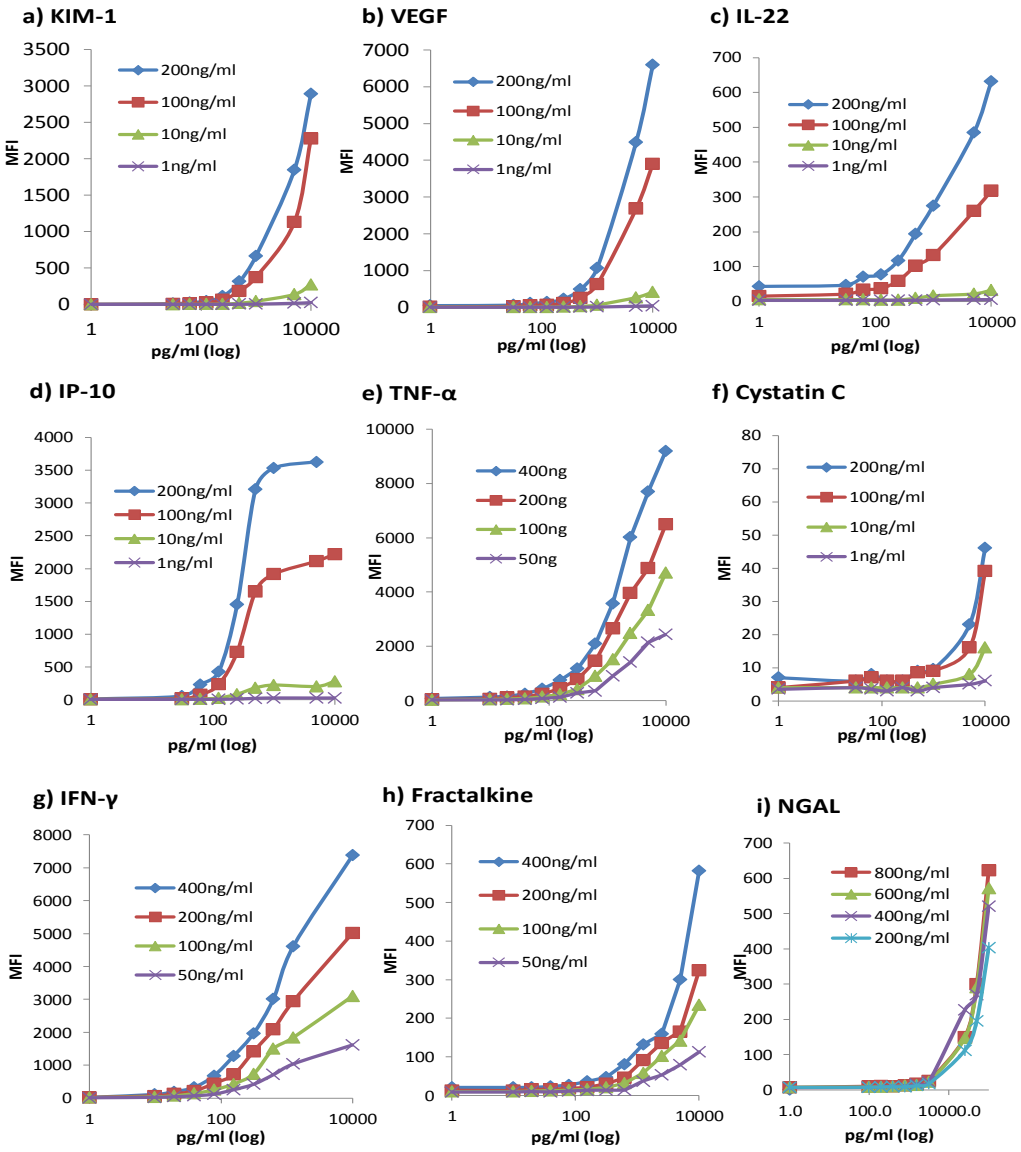


Figure 2-3: The effect of varying the concentration of secondary antibody on each biomarker to determine the optimum concentration showing the different standard curves produced.

These curves are shown in figure 2-3. From these results the optimum concentration of secondary antibody was determined to be: 200ng/ml for KIM-1, IL-22, VEGF, IP-10, Cystatin C, IFN-γ, TNF-α and was determined at 400ng/ml for NGAL and Fractalkine. Optimum concentration was decided on the basis of providing the best curve, particularly over the expected dynamic range of the assay which was determined from review of the available literature.

2.2.4 **Determining the protocol steps**

The protocol for the assay development required modification in a number of wash and incubation steps to optimise. Following preparation of a 96 well plate with Luminex buffer 50ul of microsphere (1:100) was added to each well. 50ul of standard or sample was subsequently added and a first incubation step followed by wash step (incubation/wash 1). 50ul of detection antibody was then added and a second incubation step and wash stage performed (incubation/wash 2). 50ul of streptavidin phycoerythritin was added and a 30 minute incubation period and a wash step performed. Re-suspension in Luminex buffer and 30 seconds of agitation followed by analysis on the Luminex machine.

The incubation/wash cycles (1&2) required offsetting the balance of maximising analyte detection with practicality of developing a practical and usable assay.

Experiments to determine wash cycles and incubation times were determined on VEGF, IL-22, IP-10, Cystatin C and KIM-1. Once these had been established the protocol was then run on all analytes to ensure it worked. Particular note was made of the effect on cystatin C as MFI was noted to be very low and efforts were undertaken to improve this.

2.2.5 **Altering the duration of the first incubation step**

The basic protocol steps as detailed above were run using standard of 3 washes at each wash step of 200ul Luminex™ buffer and using a secondary antibody incubation step of 1.5hrs.

The duration of incubation of the initial incubation step was run at 4, 5 and 6 hrs and the effect shown below for 3 of the biomarkers (KIM-1, VEGF and IP-10).

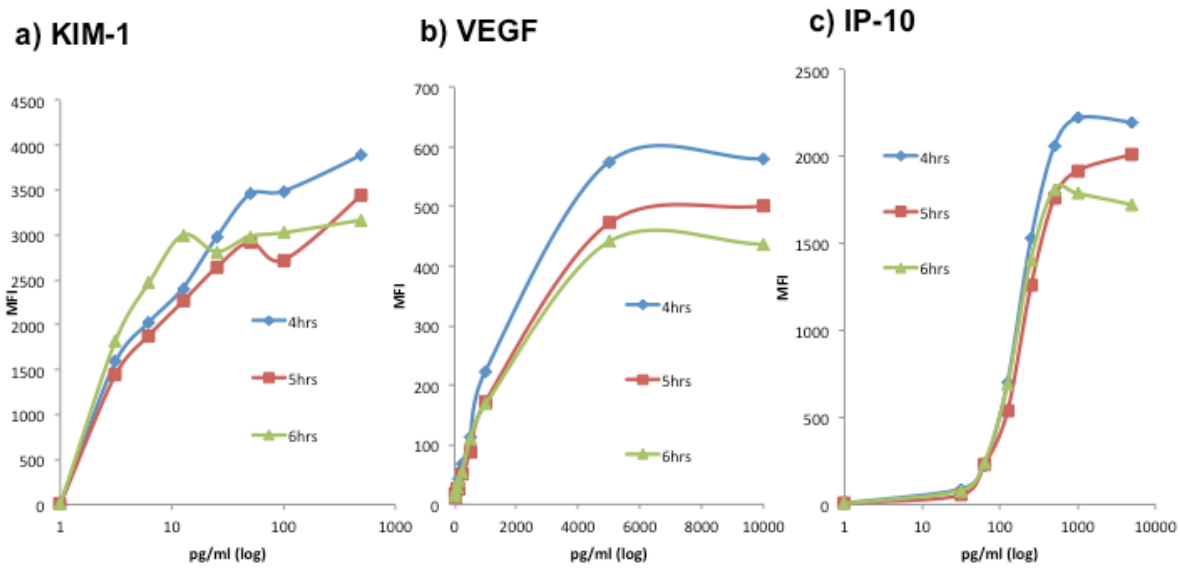


Figure 2-4: Effect of altering the duration of the first incubation step on MFI on the standard curve of KIM-1, VEGF, IP-10 to illustrate that a shorter incubation time described better standard curves.

As can be seen from the above graphs, incubation time seemed to be optimal at 4 hours or less. MFI were noted to be consistently very low for cystatin C (<70; not shown). These results seemed to indicate that running the assay with shorter first stage incubation time is beneficial not only from a practicality perspective but also from an assay optimisation.

2.2.6 **Altering the duration of the second incubation step**

The basic protocol steps as outlined above were used with 3 washes at each wash step of 200ul Luminex™ buffer. In view of the effect of shortening the first incubation step as shown above the first incubation period was set at 2.5hrs. The second incubation step was

then altered to either 3,4 or 5hrs to determine whether this improved the performance of the assay. Data below is shown for KIM-1, IP-10 and VEGF.

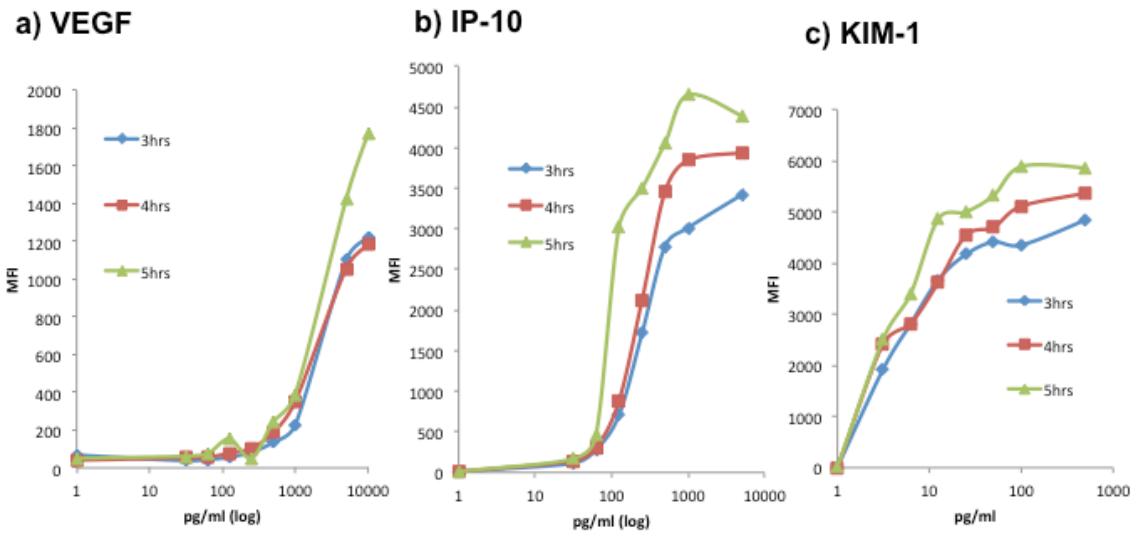


Figure 2-5: The effect of altering the duration of the second incubation step on the standard curves of 3 of the biomarkers (VEGF, IP-10 and KIM-1) demonstrating that lengthening the second incubation step did not benefit the assay.

From the graphs above it can seen that there was little added benefit to lengthening the second incubation period and from a practical perspective shorter incubation periods make the assay more manageable.

However, the results for cystatin C (not shown) were still extremely poor with maximum MFI's achieved in the range of 50-80. It was decided to look at whether a property of the cystatin C was being affected by the cycles of washing or whether the antibody pair being used was just unsuitable for application to Luminex™ technology.

2.2.7 **Determining the effect of one-step assay (removing wash steps)**

To determine whether the assay would show any benefit from being run as a one-step assay a similar protocol to the basic protocol described above was utilised. However, after adding the standard to each well the secondary antibody was added immediately and a total incubation time of 4 hrs. allowed. After this standard wash cycles the addition of streptavidin followed. Results of this are shown below for 3 biomarkers including Cystatin C.

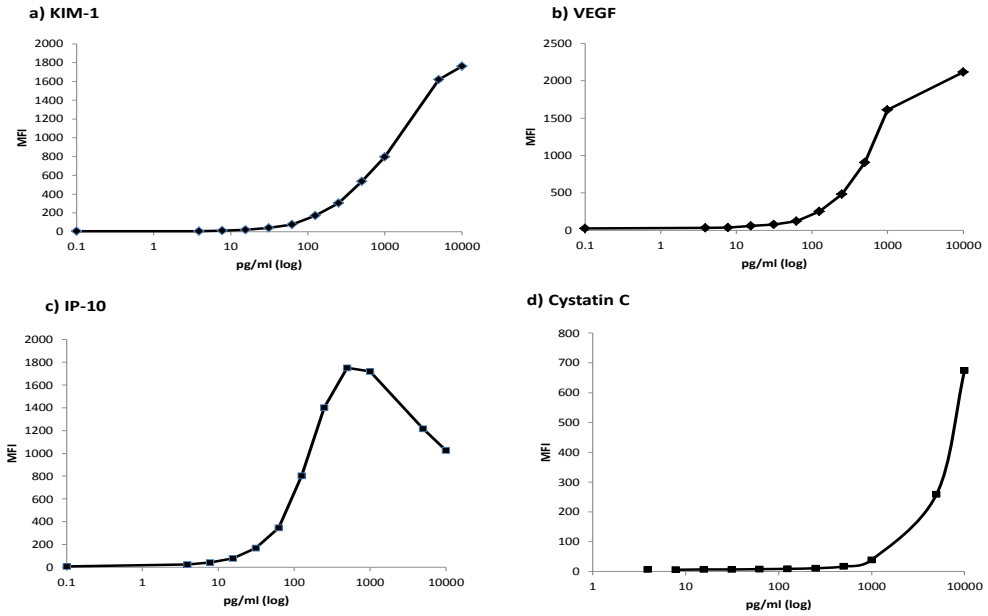


Figure 2-6: The effect of running the assay as a “one-step assay”. Results shown for KIM-1, VEGF. IP-10 and particularly Cystatin C demosntrating that undertaking the assay in this way signficiantly improved the MFI of Cystatin C.

This method of performing the experiment is less reliable and has the potential to increase errors. However, it did show that for Cystatin C to work in this assay the incubation had to occur between the analyte, microsphere and secondary antibody simultaneously rather than as 2 separate steps. It was hypothesised that this was because Cystatin C exhibited tetramisation.

As the “one step assay” was determined to be less reliable overall it was decided that the standard for the protocol should include separate incubation steps for all biomarkers between the microsphere and the analyte, then the excess analyte washed away and the secondary antibody introduced except for Cystatin which seemed to be exhibiting tetramisation and so all steps would be combined only for Cystatin C.

2.2.8 **Standardisation of optimised protocol**

The new protocol based on the experiments detailed above was then tested to ensure that there was no interference between the biomarkers and that altering the steps of the protocol solely for cystatin C still allowed the protocol to work. Two standard curves were run for each analyte to ensure that the assay worked reliably. Results for each biomarker are shown figure 9.

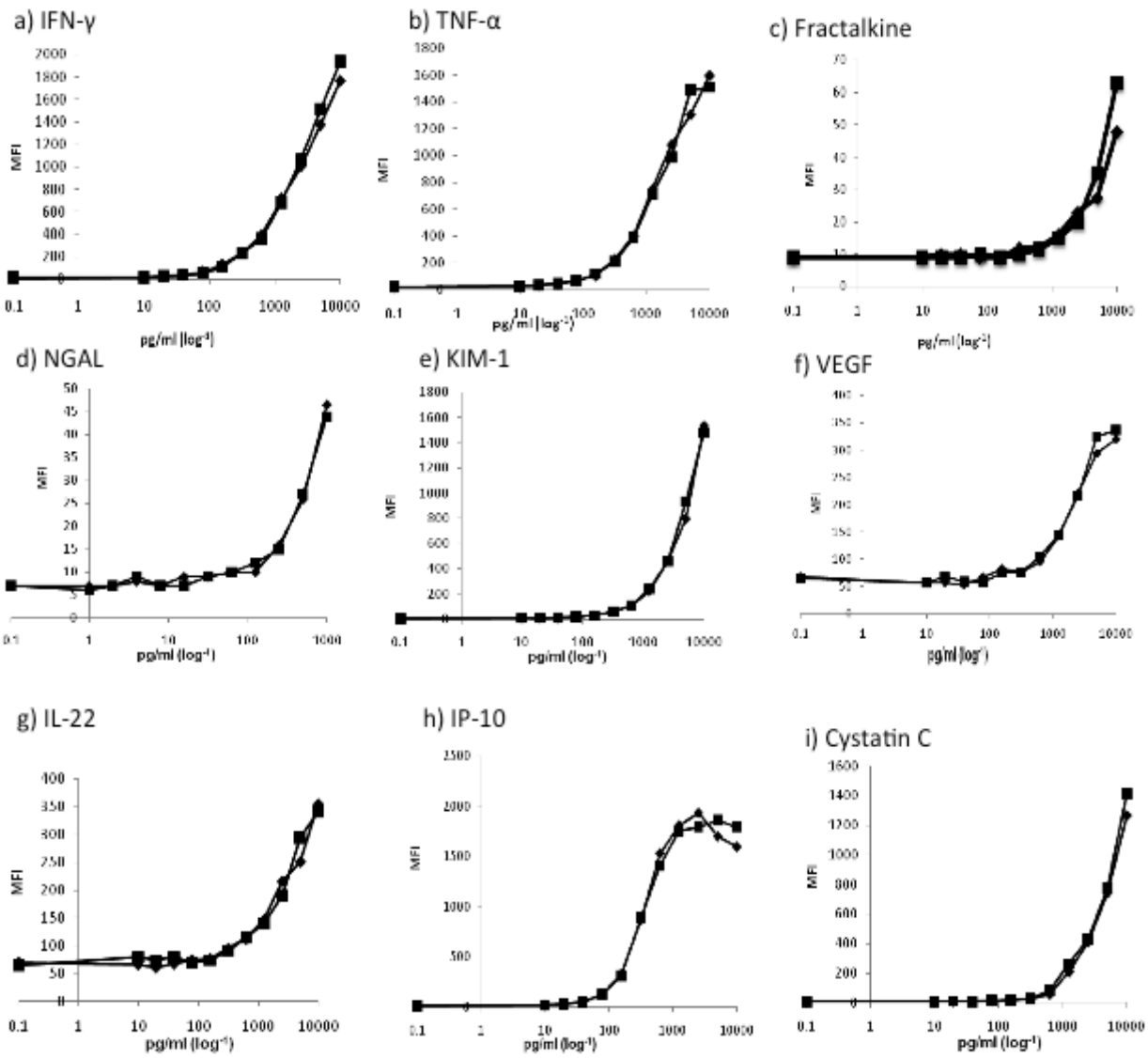


Figure 2-7: Viability of the assay following the modifications made to the protocol including altering incubation steps and running Cystatin C in the format of a “one step” assay demonstrating that the assay overall works well although NGAL and Fractalkine levels were low (later determined to be due to antibody and standard degradation over time).

The steps of the protocol are shown above to work well as can be seen above although the levels for particularly NGAL and Fractalkine were disappointingly low necessitating trial of different antibody pairs. This demonstrated improved MFI.

2.2.9 **Exclusion of confounding factors**

2.2.9.1 Determining the effect of freeze and thaw cycles

The nature of collecting samples, storing and then analysing them in batches inherently means they will be subject to freeze/thaw cycles. To determine the effect of freeze thaw cycles on the concentration of biomarkers, urine samples had to be made that contained artificial levels of the biomarkers as the majority of the markers are undetectable in urine under normal circumstances.

Three separate samples of urine were used and artificially altered to contain IP-10, VEGF, KIM-1, NGAL, Fractalkine, TNF-α, IFN-γ, IL-22 and Cystatin C. Each sample was then subjected to either 1 or 2 or 3 or 4 freeze thaw cycles. After thawing, samples remained at room temperature for 30mins prior to re-freezing. Each freezing episode was for a minimum for 24hrs. The samples were then analysed to determine the effect of repeated freeze-thaw cycles. The results of this can be seen in the table below. Changes to the amount of biomarker measured are averaged for the 3 different samples used. All percentage changes in measureable biomarker level are related to level measured from the sample that underwent only a single freeze thaw cycle.

Table 2-2: The effect of freeze thaw cycles on the levels of biomarkers present in the urine of 3 patient samples. Values given are mean across the 3 samples to show the change in measurable level from the original sample.

Biomarker	Freeze/thaw x2	Freeze/thaw x3	Freeze/thaw x4
IP-10*	0.5% ↓	3.8%↓	8.6%↓
VEGF*	39%↓	31%↓	52%↓
KIM-1	16%↑	4%↑	8.5%↓
NGAL	12%↓	17%↓	11.6%↓
TNF-alpha	7%↑	13%↓	33%↓
IFN-gamma*	20%↑	1%↓	6.3%↓
Cystatin C	30%↓	31%↓	33%↓

↓ represents a decrease in measurable level

Results for Fractalkine and IL-22 were too low to be discriminatory and are therefore not shown. Results marked with * indicate those in which one patients levels were too low to be discriminatory and therefore only 2 patients data was used.

These results show that VEGF and Cystatin C seem to be more prone to adverse effects of repeated freeze/thaw cycles and are relatively unstable, however, predominately the biomarkers variations are relatively small and while repeat freeze/thaw cycles will be avoided certainly 2 cycles seems to give results that are acceptable.

2.2.9.2 Determining the effect of urine pH

Within the normal population urine pH can vary between pH 4-8. For the purposes of this assay it needed to be determined whether the pH of the sample affected the assay or the concentration of the biomarker and whether the samples needed to be buffered.

Three separate patient samples of urine were artificially altered to contain measurable amounts of each of the biomarkers (as normally many of these are un-measurable). The pH of the samples was assessed. The samples were then aliquoted and the pH adjusted to pH 4,5,6,7,8 and 9 (using 1M NaOH or 1M HCL). One aliquot of each sample at each pH was kept and either 25ul of Luminex™ buffer added or the sample was buffered with 25ul of 0.2M $NaCCO_3$. Samples were then run according to standard protocol and the quantity of biomarker present compared to the baseline of the sample run without the addition at pH7. Samples were not corrected to urinary creatinine.

Results of this experiment demonstrated that at the extremes of pH (i.e. pH 4 and 9) for all biomarkers the most noticeable difference in the amount of biomarker detected was seen. The addition of buffer showed no improvement in the variation and therefore the addition of buffer was determined as unnecessary. The differences in the amount of measurable marker at differing levels of pH could be related to either the artificial process of altering the pH of the samples or the effect of the pH on the assay mechanics or the effect of pH on the stability of the markers. However, although variations at differing pH were seen these were relatively small generally under 30% and may also therefore fall into assay variability. This mirrors work previously done looking at the pH stability of KIM-1(Pennemans et al., 2010).

2.2.9.3 Determining the effect of delay in freezing samples

As the samples collected as part of the prospective trial are processed by a clinical lab they are subject to a delay in freezing after collection. This experiment was to determine the effect of delaying freezing on the amount of biomarker present in 3 artificially created samples. Samples were frozen either immediately after collection or were allowed to be stored in a fridge at 4°C for 24hrs, 48hrs or 72hrs. They were then analysed as per the protocol outlined above.

Table 2-3: The effect of the delay in freezing 3 samples on the measurable amount of biomarker contained within each sample. Separate aliquots of each of 3 patient samples were stored in in the fridge and frozen at: immediately, 24hrs, 48hrs and 72hrs following collection and then thawed and analysed. Results presented are mean values compared to the sample frozen immediately on collection.

Biomarker	**24hr delay**	**48hr delay**	**72hr delay**
IP-10	5%↓	13%↓	28%↓
VEGF	6%↓	21%↓	29%↓
KIM-1	18%↓	40%↓	38%↓
NGAL*	17%↓	29%↓	27%↓
TNF - α	33%↓	70%↓	76%↓
IFN - γ	36%↓	57%↓	68%↓
Cystatin C	15%↓	7%↑	23%↓

Fractalkine and IL-22 levels were too low to be accurately measured.

These results show that particularly for TNF-α and IFN-γ delay in processing and freezing the samples may have to be taken into account in analysing the samples as it may affect the results. For the other biomarkers the amount of effect that delaying freezing appears to have is smaller (<30%) and therefore is less likely to effect the interpretation of the results.

2.2.10 **Assay Variability**

To determine the variability of the assay on urine samples, 3 samples were altered to contain either: low, medium or high quantities of biomarkers. Each sample was then run 4 times according to standard protocol.

Results of the Coefficient of Variation at different biomarker concentrations can be seen in the table 2-4.

Table 2-4: The assay variability for each of the different biomarkers at low, medium and high concentrations of biomarker level showing the coefficient of variation of each biomarker at differing levels.

Biomarker	**Low**	**Medium**	**High**
NGAL	6-13%	6-14%	15-23%
KIM-1	2-5%	18-34%	
VEGF	15-32%	5-16%	4-29%
IFN- γ	7-9%	2-19%	2-12%
TNF- α	4-7%	3-8%	7-13%
Fractalkine	16-23%	8-17%	6-30%
IL-22		7-12%	10-24%
IP-10	5-12%	9-23%	
Cystatin C	4-13%	4-18%	9-33%

2.2.11 **Adapting the assay to add extra biomarkers**

The assay, whilst optimised, may be adapted to add extra markers without altering the existing setup. To test this a further biomarker of interest was added. Whilst the protocol steps remain unaltered it was felt important to exclude confounding factors to the new analyte. This can be seen below.

Table 2-5: Effect of delay in freezing on three samples of urine containing Cathepsin, levels compared to the sample analysed having been frozen immediately on collection to demonstrate degradtion to sample with increasing delay in freezing.

24hr delay	**48hr delay**	**72hr delay**
27.5%↓	9.3%↓	2.1%↑

Table 2-6: Freeze/thaw characteristics of Cathepsin L underatken on three samples of urine to demonstrate the effect of multiple freeze thaw cycles as comapred to single freeze/thaw cycle in terms of levels of measurable cathepsin L.

Freeze/thaw x2	**Freeze/thaw x3**	**Freeze/thaw x4**
32.15↑	13.3↑	1.4↑

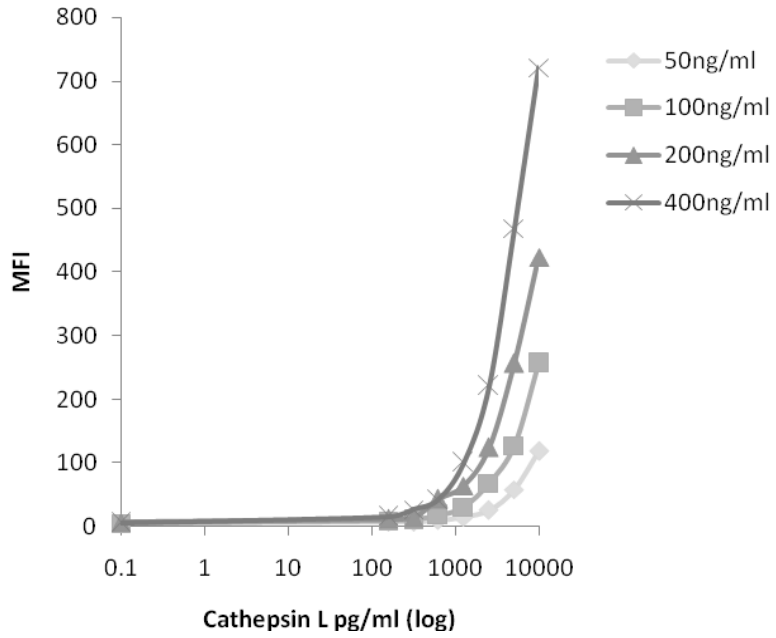


Figure 2-8: Effect of varying the concentration of secondary antibody on MFI for Cathepsin L standard curves showing that optimum standard curves were generated with secondary antibody concentration of 200ng/ml.

Following this the secondary concentration was set at 200ng/ml.

The entire panel of biomarkers was run again to check that the addition of Cathepsin L did not affect the other biomarkers. Cathepsin L was also run as a single step assay (as for cystatin C) to see if this altered its performance but the standard curve was not improved so it was kept as two–stage assay as per the other biomarkers.

2.2.12 **Trouble shooting the assay**

Amongst the issues that became apparent during assay development was an issue with storage of the secondary antibodies. Through an iterative process of trial and error the optimum method of storing the secondary antibody was identified as being

in large volumes at -20°C although storage in small aliquots and storage in BSA and -80°C were trialled with poorer results.

Similarly storage of standards required larger volumes (i.e. 0.5ml minimum quantity) and -20C°.

Another issue that became apparent was the long-term stability of the standards and secondary antibodies was variable between the different biomarkers. This was addressed by assay quality control measures as detailed below.

2.2.13 **Assay Quality Control**

In order to monitor the reliability of the assay and ensure consistent results a number of strategies were used.

Prior to any batch analysis of samples a standard curve was run to ensure that all antibody and standards were functioning as expected. MFI's for the top of the standard curve were compared to previously run standard curves.

On each plate two standard curves (8-point) were employed. This enabled calculation of the unknown samples biomarker concentrations using standard curves calculated from 2 curves.

Each plate was run with two different control samples to control for both inter-plate variability and inter-batch variability.

2.3 **Assessing KIM-1 levels using Lateral Flow device**

Renastick™ is a device that allows the quantification of the level of KIM-1 in urine in a near patient format. Renastick™ is a lateral flow device. These work by allowing the antigen to bind to travel down a porous surface and if present to bind to an indicator strip causing a colour change that becomes readable as a positive test result. The same technology is available in over the counter versions for example to screen for pregnancy.

The lateral flow device can be used with a handheld reader to give a quantitative readout of the level of KIM-1. This has been validated by the manufacturer. The lateral flow device was employed on a sample of the deceased donor urine samples as described below as per manufacturer's instructions/protocol.

Samples of urine from the deceased donors were frozen prior to analysis, whilst the aim of the lateral flow is to measure levels in fresh samples at the patients bed side. To determine whether using the Renasticks on previously frozen samples would give comparable results a group of urine samples were analysed "fresh" and following and episode of freezing. The results of this demonstrated excellent correlation in the results with a Pearson r^2 of 0.98and p value of <0.0001 as can be seen below. For analysis of the samples using the lateral flow device results are presented without normalisation to creatinine unless otherwise stated.

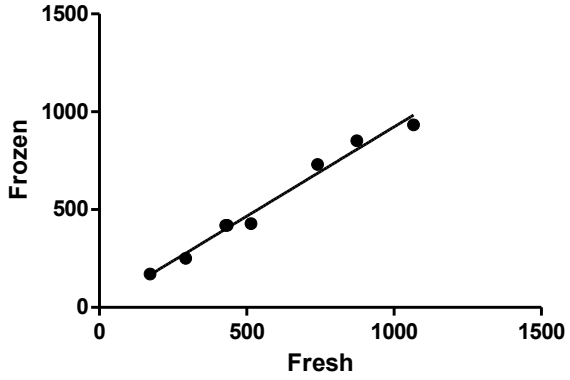


Figure 2-9: Correlation between KIM-1 levels in samples measured freshly or following freezing, by lateral flow device, demonstrating excellent correlation (Pearson r^2 of 0.98and p value of <0.0001) and confirmed that freezing or analysing freshly does not alter the lateral flow reading.

2.4 **Statistical analysis methods**

Various statistical methods are employed throughout the analysis. These will be described in general and then with regard to the analysis they are used for.

2.4.1 **T-test**

T-test is used to determine whether two sets of data are significantly different from each other. This is carried out in both paired (when the same patient but samples from 2 different time-points are analysed) and un-paired (when different cohorts are compared) within the analysis. The test is used to prove or disprove the null hypothesis when the data is parametric.

2.4.2 **Mann-Whitney**

The Mann-Whitney U test is a non-parametric test of the null hypothesis and is used in the analysis when the data is non-parametric.

2.4.3 **Contingency table analysis**

Contingency table analysis is undertaken to compared the frequency distribution of variables to determine whether a statistical difference occurs.

2.4.4 **Spearman rank correlation coefficient**

Spearman rank analysis is undertaken to determine whether there is an association between two variables where the data is non-parametric. The analysis can be undertaken on both continuous and discrete variables.

2.4.5 **Receiver operator curve (ROC) analysis**

ROC curves and the area under the curve (AUC) are used to determine whether a test has the ability to discriminate between individuals with a disease and those without. A test that has an AUC of 0.5 is not better than choosing at random and therefore worthless. A perfect test has an AUC of 1.0.

2.4.6 **Binary logistic regression analysis**

Logistic regression analysis is undertaken within the analysis to determine the effect of multiple variables where the outcome is either of two specific outcomes. It determines the relationship between a categorical dependent variable and one or more independent variables.

2.4.7 **Chi squared**

Chi square test is undertaken on categorical data to determine the likelihood that differences seen between the results from sets of data arose due to random chance.

2.5 **Analysis of Deceased Donor Data**

2.5.1 **Initial Analysis**

The samples for the deceased donors were analysed on the basis of outcome following transplantation. The samples were split into 2 groups overall:

- Those samples from which both kidneys worked immediately (immediate/undamaged function group)
- Those samples from which both kidneys had aberrant function either delayed graft function or primary non function (aberrant/damaged function group)

For each of the markers analysed the results were analysed according to whether there was significant difference in the level of the marker between the2 groups using t-test (Mann-Whitney).

2.5.2 **Further Analysis**

Spearman Rank correlation analysis was undertaken to determine whether statistically significantly different biomarkers were associated with any of the other factors that were different between the two outcome groups. For example KIM-1 levels were assessed to determine the level of association with age in the donors.

For those biomarkers that showed a significant difference between the two outcome groups ROC analysis was undertaken to determine the predictive ability of the biomarker to predict the outcome of aberrant early function.

In order to allow for other clinical factors that might act in conjunction with the degree of underlying AKI determined by the biomarker level binary logistic regression analysis was undertaken using those factors that were statistically different between the two donor groups. This was conducted with using SPSS 19 (IBM, Chicago, Il) using effect method for binary logistic regression analysis.

2.6 **Analysis of Recipient Data**

Transplant recipient data was analysed in a number of ways. In order to determine differences between groups who did and didn't get rejection Mann Whitney was performed (all data treated as non-parametric). This was undertaken comparing the whole cohorts of those who did get rejection and those who didn't where the numbers were large enough to support this (HLAi data) and using paired t-test looking at the relative change in biomarker levels where numbers were smaller (ABOi data). Analysis was also undertaken between those recipients who developed delayed graft function and those who did not in the non-immunologically complex transplants using t-test.

Similarly biomarkers that were significantly different between those recipients who did or did not develop rejection were also subject to ROC analysis. Contingency table analysis was undertaken using Chi square or Fishers exact test.

2.7 Sample collection for studies

2.7.1 Deceased organ donor samples

As part of a wider research project looking into circulatory organ donor outcomes 934 potential cardiac/multi-organ donors families were approached to take part. In those that agreed a sample of urine was taken from the donor prior to organ donation. Samples were taken after confirmation of brainstem death but prior to procurement, then stored at -80°C and transported to the central research lab. Prior to analysis the specimens were defrosted, spun for 7 min at 3000rpm at 20°C to remove the cellular component and aliquoted before re-freezing at -80°C.

2.7.2 Live organ donor samples

All samples (both blood and urine) were processed by spinning for 7 min at 3000rpm, aliquoted and stored at -80°C until batch analysis was undertaken.

2.7.3 ABOi donor samples

Blood samples were spun, aliquoted and serum stored at -40°C prior to analysis.

2.7.4 HLA incompatible transplant samples

Samples were stored in the NHSBT Histocompatibility and Immunogenetics laboratory in Birmingham following collection from patients at various time-points pre- and post-transplantation. The study had ethical approval and all patients consented to serum samples being taken and stored as part of the on-going

research study. All sera were separated from clotted whole blood and stored at -40°C prior to testing.

2.7.4.1 HLA Crossmatch methods

These were undertaken by NHSBT but are included for completeness.

2.7.4.1.1 CDC Crossmatch

A total of 2ul of patient serum and 1ul donor cells (standardised to $2x10^6$/ml) were mixed and incubated at 22°C for 60 minutes, with and without DTT. This was followed by addition of 5ul rabbit serum as a source of complement and then incubated for a further 60 minutes at 22°C. Cellular cytotoxicity was visualised using acridine orange/ethidium bromide cocktail under UV light microscopy. Anti-human globulin (AHG augmentation) is a means of enhancing the CDC match. It is a light-chain specific antibody used to amplify the complement binding capacity of HLA-bound IgG. It is sometimes used but not routinely and was not used in this cohort.

2.7.4.1.2 Flow Cytometry Crossmatch

A total of 25ul patient serum was incubated with 25ul donor cells ($10x10^6$/ml) at 22°C for 30 minutes. Cells were washed and then 100ul of goat anti-human IgG-FITC (Sigma- Aldrich, UK) was added and samples were incubated at 4°C in the dark for 15 minutes. After washing with 100ul of mouse anti-human CD3-PE (Dako) was added for the T-cell crossmatch and CD19-PE (Dako) for the B-cell crossmatch and incubated for a further 15 minutes at 4°C in the dark. Samples were then resuspended in phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) and analysed on a Becton Dickinson FacsCanto II flow cytometer. For each test sample the median channel shift for IgG-FITC was divided by that of

the negative control serum. The cut-off for positivity in HLAi transplant cases was defined as >2.5x the negative channel fluorescence.

2.7.4.1.3 Single Antigen Bead (SAB) Analysis

HLA class I and class II specific antibodies were detected using SAB manufactured by One Lambda (Canoga Park, USA). All assays were performed according to manufacturer's instructions. For each case, the antibody specificities were determined prior to antibody removal therapy. Donor specific and third party antibody specificities were defined using a positive cut-off of 1000MFI, although knowledge of HLA epitopes was considered in each case to more accurately assign anti-donor reactivity.

2.7.4.2 Desensitisation Protocol for HLA incompatible transplants

Recipients underwent double filtration Plasmapheresis with the aim being to achieve a negative crossmatch prior to transplantation as previously described. In thos group the standard maintenance immunosuppression was mycophenolate mofetil 1000mg twice a day, tacrolimus 0.15mg/kg/day in two divided doses aiming for a target trough level of 10-15ug/l in the first month, prednisolone 20mg once a day, intravenous 500mg methylprednisolone intraoperatively and basiliximab 20mg on day 0 and day 4.

2.8 **Sample analysis**

2.8.1 **Analysis of deceased samples**

Urine samples were analysed initially at 1:4 dilution (diluted with Luminex™ buffer). Those samples that fell on the standard curve range too dilute were then re-run at 1:2 dilution. For those samples that were too concentrated at 1:4 were run again at 1:8. For each plate run two standard curves were included. The CV between standard curve points was then looked at between curves on each plate and between plates. This can be seen below.

Table 2-7: CV for biomarkers assessed in the analysis of the deceased donor urine samples showing acceptable CV for a research tool

	KIM-1	NGAL	VEGF	IFN-γ	TNF-α	Fractalkine	Cystatin C
CV averages across each point standard curve % (intrassay variability)	4.4 - 11.5	8.2 - 32	5.0 - 25.0	10.0 - 29	4 - 24.0	9.0 - 19	17.0- 34
CV averages across plates % (interassay variability)	4.6 - 9.7	11.0 - 30	8.0- 27	8.2 - 19	7.4 – 23	5.0 - 33	17.0 - 35

Analysis was undertaken of the biomarkers with and without normalisation of the levels to urinary creatinine.

2.8.2 **Analysis of HLAi samples**

Serum samples were analysed for the panel of biomarkers as detailed previously using the Luminex™ based method. Serum biomarker levels were correlated with acute rejection occurring within the first 30 days after transplantation. Analysis was undertaken at 1 in 5 dilution and 1 in 100 dilution

Antibody mediated rejection (AMR) was suspected on decline in renal function, confirmed by the presence of donor specific antibody (DSA) and a biopsy showing the cellular changes of acute antibody-mediated rejection (peritubular capillaritis and/or glomerulitis) and negative for C4d (i.e. a biopsy 'suspicious' for AMR by Banff '07 criteria and confirmed in Banff 2013 as AMR). (Solez et al., 2008, Higgins et al., 2010, Solez and Racusen, 2013) (Solez et al., 2008, Higgins et al., 2010, Solez and Racusen, 2013)

2.8.3 **Analysis of Renal Transplant Recipient samples**

Blood and urine samples were collected from transplant recipients pre-operatively, during the inpatient stay and at each out-patient clinic visit. Blood and urine samples were spun, aliquoted and stored at -80°C. Samples were analysed for the panel of biomarkers as detailed previously using the Luminex™ based method. Analysis was undertaken at 1 in 5 dilution and 1 in 100 dilution.

2.8.4 **Ethical approval**

For the study undertaken on non-antibody incompatible transplant recipients and on the live donors REC approval was gained REC reference: 10/H1206/65. All samples used from the Heart study and About-K were undertaken with ethical approval.

3 Chapter 3: Results

3.1 Biomarkers in the assessment of deceased organ donors

There are many difficulties in adequately assessing the potential deceased kidney donor. Often the patient's history is incomplete and it is difficult to determine whether there is any underlying potential kidney damage. Similarly it is difficult to determine from external visual examination how successful the kidney transplant is likely to be. While biopsy specimens taken pre-transplant have been shown to have a role in identifying some underlying damage to the kidney these are often logistically difficult to arrange and result in extending the cold ischaemic time (Mazzucco et al., 2010). Whilst it is possible to identify some of the underlying chronic damage that may have accumulated, accurately determining acute kidney injury is more difficult. For this reason interest in biomarkers as a mechanism of predicting the potential outcome from the kidney donor has increased. It would be beneficial to have the knowledge about the degree of AKI in the donor before the process begins as part of the profiling of the donor. The advantage in identifying donor derived AKI is that immunosuppressive regimes could be tailored or recipient selection altered to attempt to optimize outcome and negate the impact of delayed graft function (DGF). DGF is associated with kidney injury and has been linked with acute rejection, increased biopsy requirements, longer post-transplantation hospital stays and worse 1-yr outcomes for the graft (Quiroga et al., 2006, Dominquez et al., 2009, GIral-Classe et al., 1998).

3.1.1 **Study design**

The study was approved under the authority of National Health Service Blood and Transplant service.

Of the 934 donors in the study assessed 917 (98.2%) of donated kidneys functioned. 563 (60.2%) of donors provided kidneys that both showed immediate function. 133 of these donors were selected for analysis of urine samples for biomarker levels. Selection criteria for analysis were based on random choice of those samples with sufficient sample volume and the completeness of clinical data.

The remaining 371 (39.7%) donors produced kidneys in which various presentations of aberrant initial function was observed. Both donated kidneys exhibited delayed graft function in 39 (4%) whilst 24 (2.5%) donors produced kidneys where one kidney showed DGF and one exhibited non-function. Urine samples from 32 (32/39=82%) of the former and 17 (17/24=70.8%) of the latter groups were analysed for biomarker levels and compared to the levels in the primary function group. For the purposes of this study the NHSBT definition of DGF of dialysis in the first week following transplant was used.

308 (33.0%) of donors provided kidneys from whom one kidney had delayed graft function and one had immediate function were excluded from this analysis since it is probable that the difference between the outcomes of the two grafts resulted from events occurring after procurement and as such not detectable pre-donation.

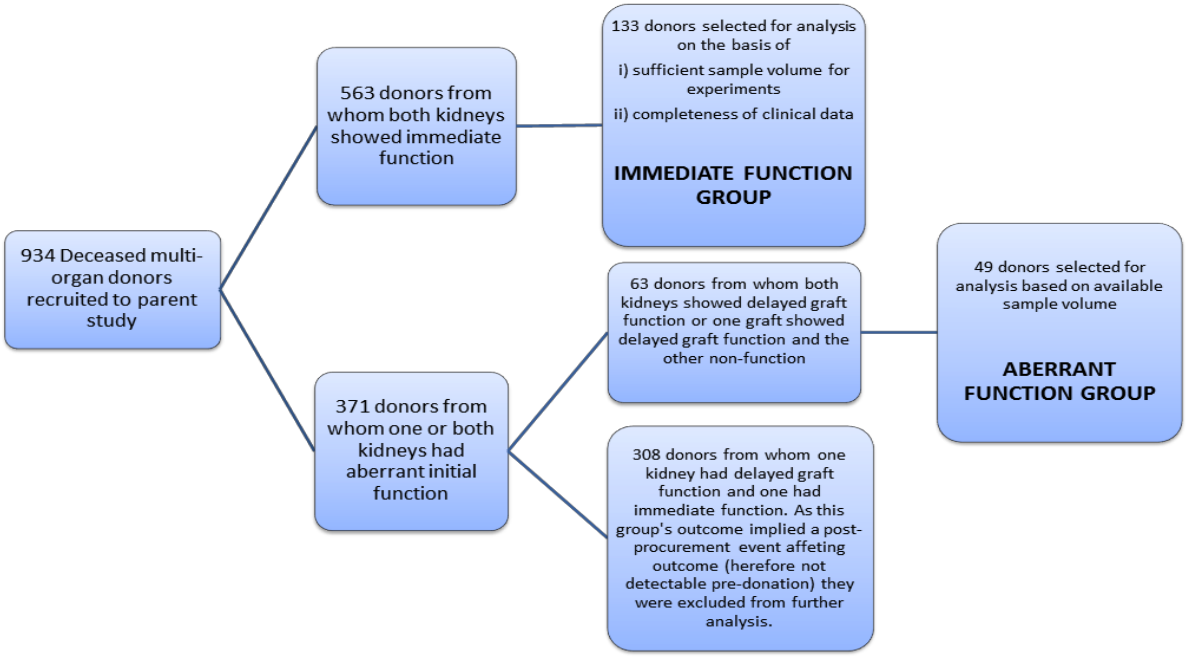


Figure 3-1: Illustration of the selection of samples for inclusion within the study

3.1.2 **Method of analysis**

Samples of urine were analysed in 2 different ways.

Urinary levels of β2M and albumin to creatinine ratio were measured by the Clinical Immunology Service of the Queen Elizabeth Hospital Birmingham. These were measured using the Roche Cobas c501 turbidimeter for urinary albumin, creatinine and B2M and an in-house Luminex™ multiplexed assay for the light chains.

Urinary levels of VEGF, TNF-α, IFN-γ, KIM-1, NGAL, Cystatin C and Fractalkine were measured using the Luminex™ assay, developed as described in methods.

3.1.3 **Donor demographics**

Table 3-1: Demographic details of the overall deceased donor pool in the study. Denominator values vary due to variations in available information from NHSBT database.

Mean age	41.7 (range 16-65; SD 12.7)
Female	41% (74/180)
Male	59% (106/180)
Cause of death	
• Hypoxic brain damage	8.8% (16/180)
• Intracranial haemorrhage	65.6% (118/180)
• Trauma	8.3% (15/180)
• Intracranial thrombosis	2.7% (5/180)
• Meningitis	1.1% (2/180)
• Brain tumour	2.2% (4/180)
Mean creatinine (mmol/l)	87.7 (range 35-303; SD 30.2)
History of hypertension	16.7% (29/174)
Need for CPR	17.7% (29/164)
Use of inotropes	91.8% (156/170)

The overall characteristics of the donors analysed can be seen in the table detailing the main causes of death. Donors had a mean age of 41.7.

3.1.4 **Kidney Donation data**

With the aim of identifying only AKI in the donors, rather than procurement damage, data relating the procurement procedure was examined. Organ procurement data demonstrated perfusion as good or fair in 99.7% (as subjectively assessed and documented by the retrieval surgeon at the time of retrieval). Surgical damage that was noted to have occurred during the procurement procedure included: stripping of the kidney capsule 2/346 (0.6%), haematoma evident on kidney 2/346 (0.6%) and

damage to renal vein 6/346 (1.7%). This demonstrated very low levels of surgical damage noted to have occurred at the time of procurement.

3.1.5 **Recipient Demographics**

Recipients had a mean age of 41.7 years. The differences between the recipients of the two outcome groups can be seen below (table 3-2). The only statistically significant difference between the recipients in the two groups was age; although different the actual difference was relatively small (40.4 vs. 45.5).

Table 3-2: The differences between the recipients or either the kidneys from donors termed ”aberrant early function donors” or “immediate function donors” in terms of demographics

	Recipients of kidneys from donors which both kidneys demonstrated Immediate function donors	**Recipients of kidneys from donors which both kidneys showed Aberrant early function donors**	**p value**
Gender (%female)	41% (108/264)	32% (31/96)	0.144*
Age (mean)	**40.4 (n=263; CI 38.6-42.1))**	**45.5 (n=96;CI 42.5-48.4)**	**0.004****
Cause ESRF			0.057*** overall
• **Diabetes**	25% (66/266)	11% (11/98)	
• **Glomerulonephritis + IgA nephropathy**	9.8% (26/266)	14% (14/98)	
• **Polycystic disease**	8% (22/266)	7% (7/98)	
• **Pyelonephritis**	8.6% (23/266)	4% (4/98)	
• **Other**	19.5% (53/266)	22.4% (22/98)	
• **Not reported**	28.6% (76/266)	40.8% (40/98)	

* Fischers exact test, ** Mann Whitney, *** Chi square

3.1.6 **Group analysis**

The differences between the characteristics of the two donor groups can be seen in the table below divided by the immediate function and the aberrant function group classification described above. P values were considered significant if <0.05.

Table 3-3: Differences in characteristics between those donors whose graft displayed immediate function or aberrant function.

	Immediate function Donors (n=117)	**Aberrant function donors (n=49)**	**p value**
Gender (%female)	37% (43/117)	53% (20/43)	0.0566***
Mean Age	**38.5 (n=117)**	**50.4 (n=44)**	**< 0.0001****
Mean Creatinine	**85.9 (n=124)**	**92.7 (n=44)**	**0.043****
Cold Ischaemic time	15.8hrs (n=254)	16.4hrs (n=93)	0.399**
Donor Hypertension	**8.4% (11/131)**	**41% (18/44)**	**<0.0001*****
Cardiac arrest	23% (10/120)	16% (19/44)	0.3564*
Treatment with inotropes	93% (116/125)	89% (40/44)	0.5267*

* Fischers exact test, ** Mann Whitney, *** Chi square

As can be seen there were statistically significant differences between the two groups in mean age, mean creatinine and the presence of hypertension. This tallies with published data in terms of the outcomes from donors with higher terminal creatinine, history of hypertension and older age have poorer outcomes, so it is reassuring that this cohort is in concordance with data published previously.

3.1.7 **Biomarker Panel Analysis**

To assess whether the previously described AKI biomarkers were different between the two groups of donors, suggesting that the presence of AKI might be different between the two groups, Luminex™ analysis was undertaken. The results of the biomarker testing via the Luminex™ method can be seen below and represented graphically in Figure 3-2.

Table 3-4: Differences in urine biomarker levels between the groups (mean, 95% confidence intervals in brackets, statistically analysed using Mann Whitney)

Biomarker	Donors giving immediate functioning kidneys (n=133)	Donors giving aberrant functioning kidneys (n=49)	P value
KIM-1 (ng/ml)	**2.53 (2.0 – 3.0)**	**3.98 (2.9 – 5.1)**	**0.011**
NGAL (ng/ml)	92.17 (69.5-114.8)	92.63 (66.3-119)	0.309
Fractalkine (ng/ml)	1.95 (1.7-2.2)	2.75 (2-3.5)	0.053
VEGF (pg/ml)	357 (260-454)	726 (247-1206)	0.096
IFN-γ (pg/ml)	63 (54-72)	64 (48-80)	0.710
Cystatin C (ng/ml)	1083 (898-1268)	1218 (886-1549)	0.447
TNF-α (pg/ml)	78 (57-99)	91 (51-131)	0.677
β2-microglobulin (mg/l)	5.38 (9.1-15.4)	4.18 (5-11.4)	0.655
Albumin: Creatinine mg/mmol	38.7 (7.6-85)	14.7 (8.7-20.6)	0.210

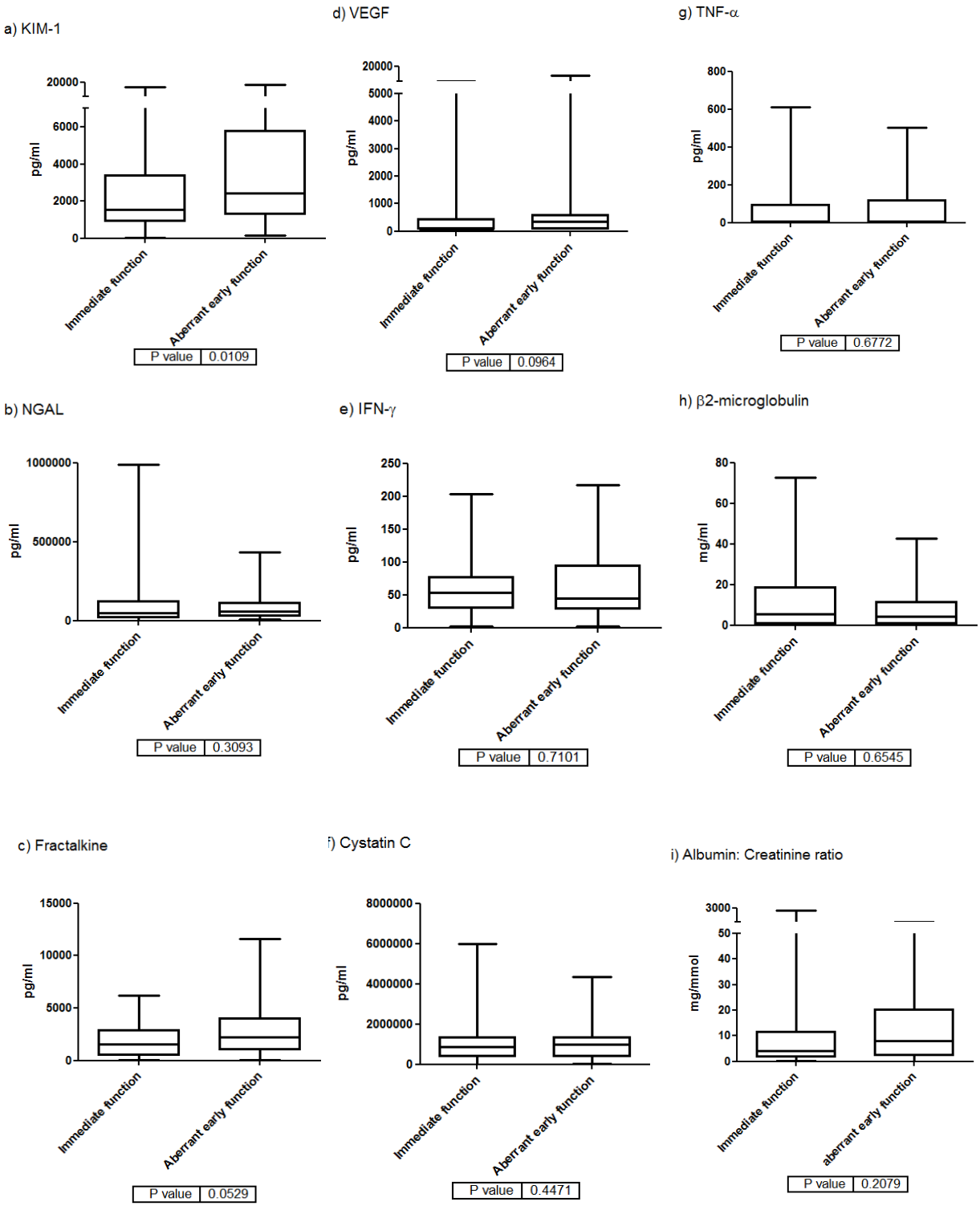


Figure 3-2: Differences between urine biomarker levels in deceased donors between those whose grafts displayed immediate function and those with aberrant early function (median, 25% to 75% percentiles).

Most candidate biomarkers showed no difference between the two groups. However, KIM-1 levels (Figure 3-2a) were significantly higher in the groups of donors whose grafts subsequently displayed aberrant early function (p=0.011). Fractalkine levels tended to be higher in the aberrant group but failed to reach significance (p=0.053) (Figure 3-2c).

To exclude the possibility that the concentration capacity of the donor kidney, either as a result of iatrogenic or intracranial events, had influenced urinary biomarker concentrations, levels were normalized to urine creatinine levels and re-analysed and expressed as biomarker: creatinine ratio as can be seen below. Only those biomarkers from whom the initial analysis showed a difference that was more significant than p<0.4 were normalised.

Table 3-5: Biomarker levels following normalisation to urinary creatinine compared between the donors whose grafts displayed immediate function and those who displayed aberrant early function

Biomarker	**Donors giving immediate functioning kidneys (n=133)**	**Donors giving aberrant functioning kidneys (n=49)**	**P value**
KIM-1: creatinine ratio	**428.5 (357 – 500)**	**728.7 (533-925)**	**0.0068**
NGAL: creatinine ratio	20050 (15290-24810)	21660 (13840-29490)	0.5643
Fractalkine: creatinine ratio	**402.7 (343-463)**	**701.3 (325-1078)**	**0.0038**
VEGF: creatinine ratio	211.9 (144-280)	225 (62-388)	0.7205

The KIM-1: Creatinine ratio and Fractalkine: Creatinine ratios were both significantly higher in the group with aberrant function. No significant difference was seen with the other potential markers. To further delineate the ability of these markers to predict outcome ROC analysis was undertaken.

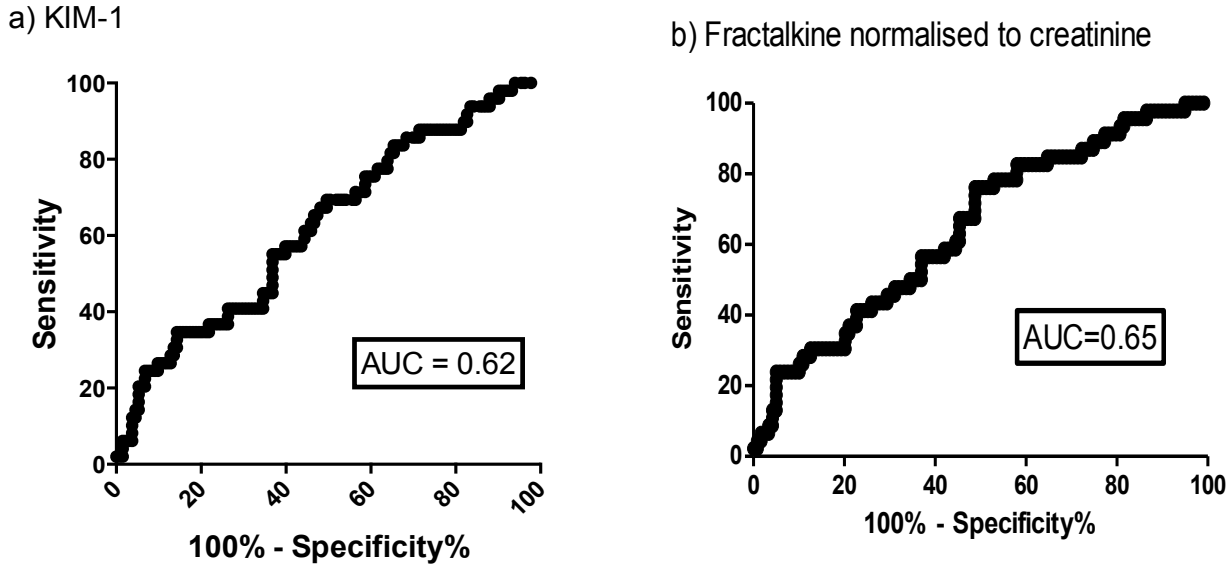


Figure 3-3: ROC analysis of deceased donor biomarker levels a) KIM-1 and b) Fractalkine: creatinine in predicting the outcome of aberrant early function demosntrating AUC of 0.62 and 0.65 respectively.

To determine whether KIM-1 and Fractalkine were associated with other variables Spearman rank correlation statistics was performed between donor age and donor creatinine and KIM-1 and Fractalkine. The results, shown below, revealed no association.

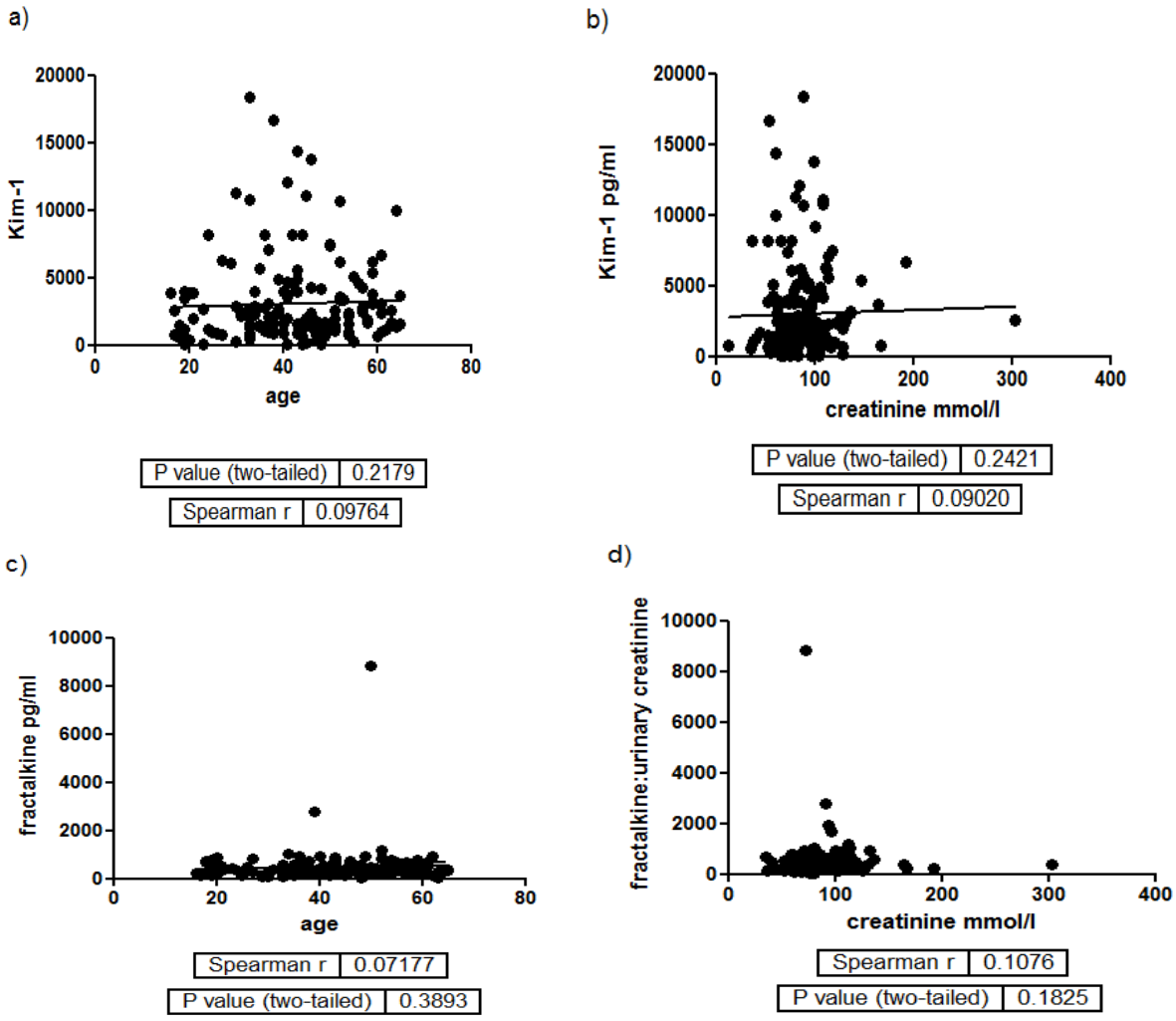


Figure 3-4: Correlation graphs of deceased donor markers (KIM-1 and Fractalkine) with donor age or donor creatinine

3.1.8 **Lateral Flow Assay**

KIM-1 was also measured using a point-of-care, lateral flow device (Renastick™, BioassayWorks, USA) as per manufacturers protocol along with a portable reader (Vaidya et al., 2009). This device is designed to be used at the bedside and for this reason the results generated were not compared as a ratio to urine creatinine as in a clinical setting this would be not be possible.

To assess whether this assay could be used to measure KIM-1 it was employed on a

sample of the population comprised of aberrant early outcome donors (n=49) and immediate function outcome donors (n=40). Results from this approach showed significant difference between the groups (p=0.03) with mean urine level in the former group of 8.0ng/ml and in the latter group 6.1ng/ml as can be seen below. ROC curve analysis showed an AUC of 0.63, similar to the multiplex assay.

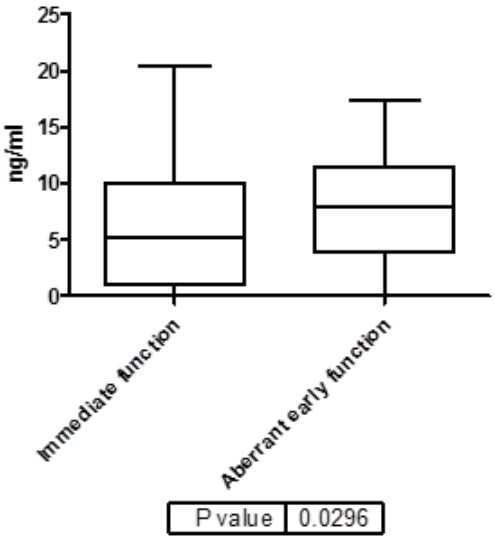


Figure 3-5: Differences in urine KIM-1 levels as assessed by a Lateral flow device (Renastick™) in deceased donors (medians, 25-75% percentile shown). Results are not normalised to urinary creatinine.

3.1.9 **Binary logistic regression analysis**

The number of factors that interplay in the potential outcome of the graft suggest that a simple univariate analysis may be criticised for oversimplying the actual clinical real-world situation. For this reason a mechanism to try to integrate a number of potentially influential factors and determine the interplay between them is important. This is provided by Binary Logistic Regression analysis. Binary logistic regression analysis was undertaken using those variables that were identified as being statistically different between the donor groups of early dysfunction and good

immediate function (age, donor creatinine and history of hypertension). This was then combined in different permutations with either:

a) KIM-1 level as determined by the Luminex™ assay

Table 3-6: Results of binary logistic regression analysis using KIM-1 from Luminex™ and those variables previously identified as staistically signficantly different between the two outcome groups.

Variables in analysis	**PPV**	**NPV**	**Sensitivity**	**Specificity**
Donor age, donor creatinine, history hypertension, Kim-1 level	0.68	0.83	0.45	0.93
Donor age, history hypertension, Kim-1 level	0.70	0.81	0.40	0.94
Donor age, history hypertension, donor creatinine	0.61	0.80	0.37	0.92
Donor age, history hypertension	0.64	0.80	0.35	0.93

b) KIM-1 and Fractalkine normalised to urinary creatinine

Table 3-7: Results of binary logistic regression analysis using KIM-1 normalised to urinary creatinine and those variables previously identified as staistically signficantly different between the two outcome groups.

Variables in analysis	**PPV**	**NPV**	**Sensitivity**	**Specificity**
Donor age, donor creatinine, history hypertension, Kim-1 level, Fractalkine level	0.69	0.83	0.47	0.93
Donor age, donor creatinine, history hypertension, kim-1 level	0.67	0.83	0.47	0.92
Donor age, history hypertension, kim-1 level, Fractalkine level	0.68	0.82	0.43	0.93

c) KIM-1 as determined by lateral flow assay

Table 3-8: Results of binary logistic regression analysis using KIM-1 from Lateral flow device (Renastick™) and those variables previously identified as staistically signficantly different between the two outcome groups.

Variables in analysis	**PPV**	**NPV**	**Sensitivity**	**Specificity**
Donor age, donor creatinine, history hypertension, Kim-1 level on lateral flow	0.80	0.77	0.77	0.79
Donor age, donor creatinine, history hypertension	0.76	0.78	0.81	0.74
Donor age, hypertension, Kim-1 on lateral flow	0.84	0.81	0.82	0.83

This analysis suggested that the most important donor characteristics predicting renal damage sustained pre-donation (essentially analogous to AKI) were age and history of hypertension as evidenced by their positive predictive ability. Surprisingly donor serum creatinine, although different between the two outcome groups, did not appear on logistic regression to differentiate between the outcomes. This concurs with the hypothesis suggesting that higher creatinine is not a reliable marker of eventual graft outcome. The addition of KIM-1 (measured by either method) increased the differentiation of aberrant early function from good immediate function donors as evidenced by the rise in positive predictive value.

Binary logistic regression analysis of KIM-1 level as determined on lateral flow assay maintained the viability, in that addition of KIM-1 as measured by lateral flow method maintains its positive predictive value whilst using a more clinically applicable means

of investigation.

The combination of factors that seems to be best identify those donors at risk of producing early dysfunction in the recipient are: the donor's age, history of hypertension and urinary KIM-1 level on LFD.

3.2 Kidney biomarker changes in living organ donors

A group of live donors was recruited in order to establish a control group for the effects of surgical intervention on biomarker levels. This was to ensure that any changes in biomarker levels seen in subsequent transplantation studies were truly reflective of changes resultant from the transplanted kidney rather than as a result of the surgery.

3.2.1 Study design

3.2.1.1 Recruitment and Sample collection

This study was undertaken following national ethical approval. Patients undergoing live donor nephrectomy were approached to take part in the study to act as a control group. 15 patients were recruited. Blood and urine samples were taken at the following time points: pre-donation, 24 and 48hrs following donation.

3.2.1.2 Demographics of cohort

Table 3-9: Demographics of the live donor nephrectomy patients

Characteristic	**Live donors (n=15)**
Age, years (mean)	51 (range 22-73)
Male sex no. (%)	40%
Operative time (mean)	104 mins
Baseline	
- Creatinine mmol (mean)	72
- GFR ml/min (mean)	80

The demographics of the cohort can be seen in the table above demonstrating mean age of 51 and average baseline GFR of 80ml/min which is in concordance with guidelines from the British Transplant Society.

3.2.2 **Biomarker Panel Analysis from living donors**

Urine and serum samples were analysed using the Luminex™ method described previously.

Levels of IP-10, Fractalkine, IFN-γ and TNF-α were below the threshold for detection, as would be expected.

Comparison at the time points of 24hr and 48hr to the pre-donation levels was performed for both urine and serum (figure 18).

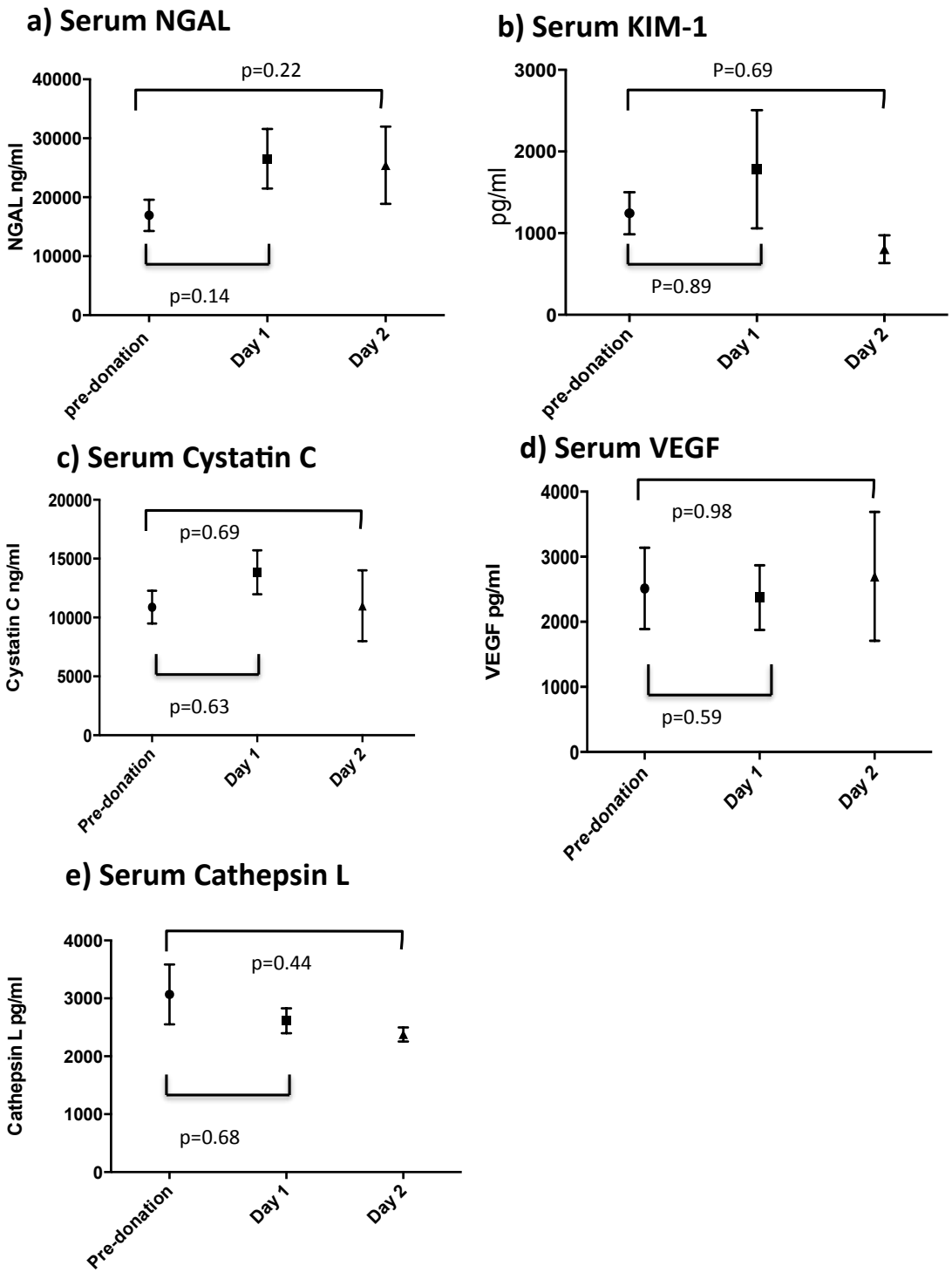


Figure 3-6: Biomarker changes in live donor serum levels from pre-donation to 24hrs and 48hrs following donation.

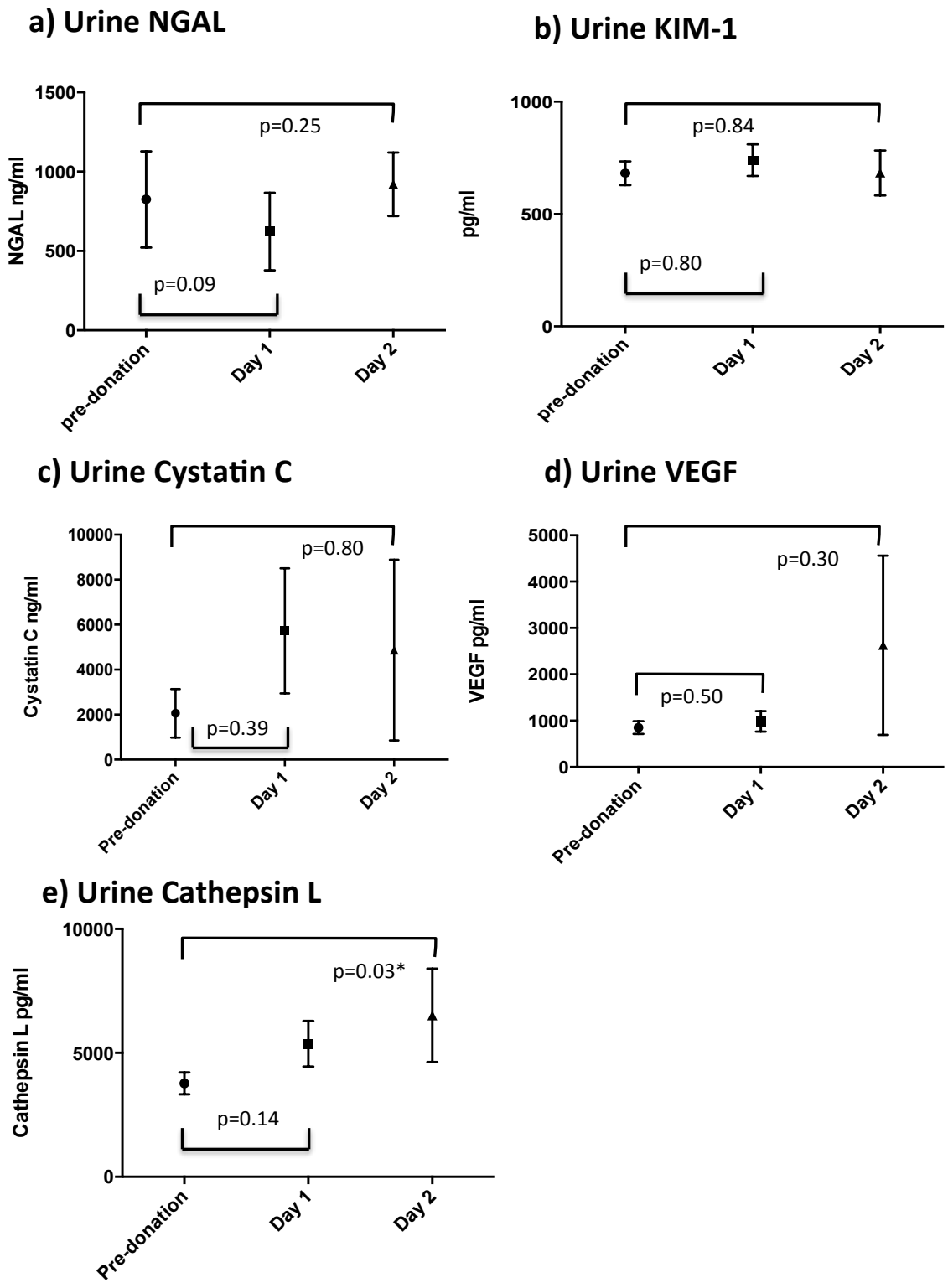


Figure 3-7: Urinary biomarker levels at the time points of pre-donation, 24hrs following and 48hrs after live donor nephrectomy. Urine levels are not normalised to urinary creatinine.

As shown, levels of all biomarkers except Cathepsin L were not significantly different at 24hrs or 48hrs following organ donation.

The lack of changes of biomarkers following surgery in this group is useful as they provide a point of comparison for the analysis undertaken on patients undergoing transplantation. By inference any changes seen in the transplant recipient population should be arising as a result of the transplanted kidney rather than as a result of the effects of surgical stress.

3.3 ABO incompatible transplants

Transplantation across ABO incompatible blood groups has increased significantly since its inception using a desensitisation program in 1987 (Thielke et al., 2007). Whilst results, as previously described, are good and continue to improve they represent a different cohort of transplant recipients to the immunologically uncomplicated by virtue of the desensitisation program and often modified immunosuppressive regime they follow. Biomarkers that potentially detect or monitor the rejection process may, therefore, behave differently within this population. We aimed to determine whether biomarkers that had been suggested as markers of rejection in non-immunologically complex transplantation and as markers of acute kidney damage would have a clinical role in this sub-group of transplant recipients.

3.3.1 Study design

3.3.1.1 Recruitment and sample collection

Patients undergoing ABOi transplantation were recruited to the ABOUT-K study (an observational, UK wide study evaluating antigen-specific antibody removal protocol for ABO-incompatible renal transplantation with national ethical approval). Blood samples were collected at time points including: pre-initiation of antibody treatment, post-treatment but prior to transplantation and at varying time points post transplantation as part of the ABOUT-K protocol.

Overall 99 patient serum samples were available for analysis. All patients underwent ABOi transplantation between January 2009 and April 2012. Samples were collected from nine centres undertaking ABOi transplantation within the UK (Birmingham

Queen Elizabeth Hospital, Guys Hospital London, Northern General Hospital Sheffield, Royal Free Hospital London, University Hospital Wales Cardiff, St James Hospital Leeds, St Georges Hospital London, City Hospital Nottingham, Freeman Hospital Newcastle).

Of the 99 patients recruited 17 (17%) displayed rejection in the first month following transplant. This was predominately cellular rejection (71%) with a small proportion of antibody mediated (18%) and mixed (12%).

Table 3-10: Table demonstrating serum biomarkers levels in ABOi transplants between those patients who developed rejection and those who did not at various time points. Values are median.

Biomarker	Time point	Rejection	No rejection	P value
NGAL (ng/ml)	Pre-transplant	7020	8058	0.49
	Day 3	5426	4672	0.99
	Day 5	6340	5669	0.44
	Day 30	4160	3717	0.88
KIM-1 (pg/ml)	Pre-transplant	1233	1097	0.43
	Day 3	1130	1036	0.46
	Day 5	1203	1112	0.49
	Day 30	1223	1034	0.05*
VEGF (pg/ml)	Pre-transplant	874	1028	0.27
	Day 3	580	683	0.21
	Day 5	603	628	0.89
	Day 30	495	624	0.38
IP-10 (pg/ml)	Pre-transplant	168	174	0.61
	Day 3	145	128	0.51
	Day 5	143	147.5	0.78
	Day 30	141	137	0.51
Cystatin C (ng/ml)	Pre-transplant	4022	4391	0.23
	Day 3	4325	5285	0.36
	Day 5	4761	3467	0.04*
	Day 30	3452	3141	0.26
Cathepsin L (pg/ml)	Pre-transplant	6134	6503	0.19
	Day 3	4773	4948	0.56
	Day 5	5020	5007	0.95
	Day 30	5357	4741	0.21

In view of the small numbers with rejection and the mixed nature of the rejection (combination of both cellular and antibody mediated) analysis was undertaken to determine whether examining the change in levels might differ between the two groups.

In the group who developed rejection in the first month NGAL levels were not statistically different between the pre-transplant and the day 3 level, however in the group who did not develop rejection there was a drop in NGAL levels from pre-transplant to day 3 (although this just failed to achieve significance). The range of values for the non-rejectors was much greater. Comparison was also made between NGAL levels those who developed rejection and those who did not at the time points of pre-transplant and at day 3 and this showed no difference between the two groups. This can be seen below.

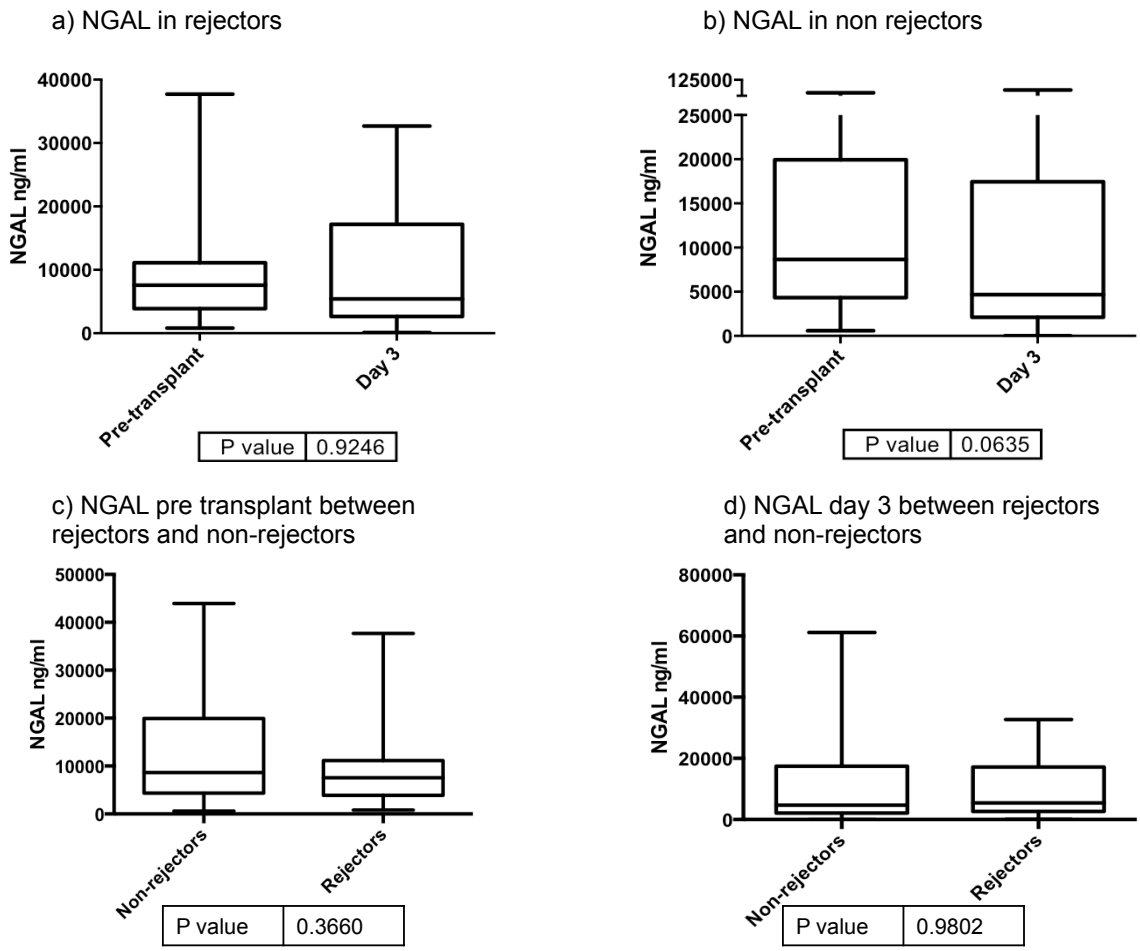


Figure 3-8: Serum NGAL levels in a) ABOi rejectors and b) ABOi non-rejectors pre-transplant and day 3 and c) comparson netween NGAL levels pre transplant between rejectors and non-rejectors and d) NGAL levels day 3 between rejectors and non-rejectors

Although this failed to reach statistical significance it does seem to suggest that those patients who subsequently suffer from early rejection fail to clear their NGAL levels by day 3 post-transplant.

A similar pattern is seen in KIM-1 with patients who subsequently develop rejection failing to demonstrate a drop in KIM-1 levels and those who do not develop rejection showing a fall in KIM-1 between the pre-transplant and the day 3 levels although it is worth noting the wide range of levels in the non-rejectors.

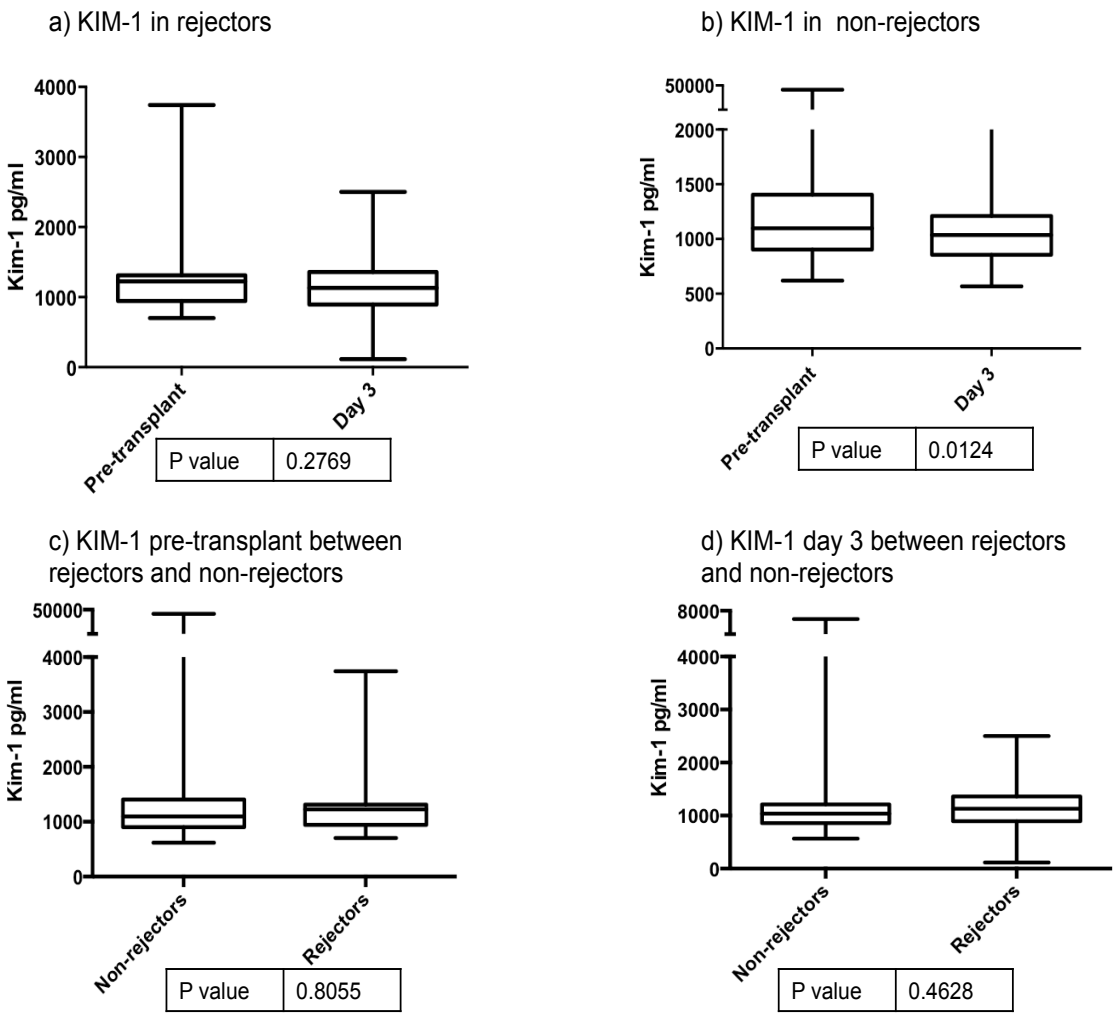


Figure 3-9: Serum KIM-1 levels in a) ABOi rejectors and b) ABOi non-rejectors pre-transplant and day 3 and c) comparison between KIM-1 levels pre transplant between rejectors and non-rejectors and d) KIM-1 levels day 3 between rejectors and non-rejectors

Interestingly in both groups VEGF levels fell at day 3, however, the fall was more noticeable in the non-rejectors although the range of levels was much greater than for rejectors. No statistical difference was seen between the levels either pre-transplant or at day 3 between those who did or did not subsequently develop rejection.

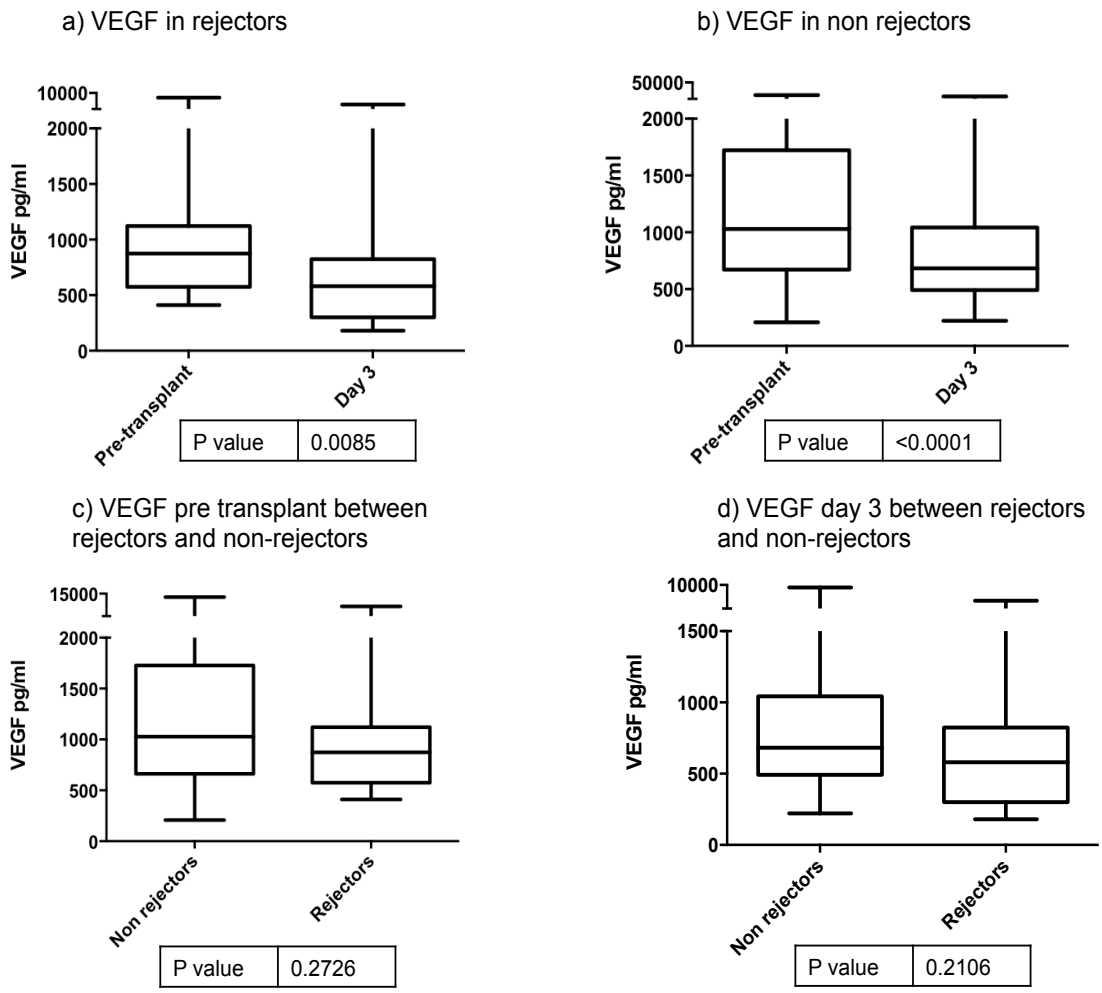


Figure 3-10: Serum VEGF levels in a) ABOi rejectors and b) ABOi non-rejectors pre-transplant and day 3 and c) comparison between VEGF levels pre transplant between rejectors and non-rejectors and d) VEGF levels day 3 between rejectors and non-rejectors

Although the decrease in IP-10 levels failed to reach significance as can be seen below (figure 3-11) The change in levels in the non-rejector group tended towards significance (p=0.0869) and this may have failed to reach significance in part due to the small number of patients with rejection. Although statistical significance was not reached the degree of change was still different to that in the rejectors group where no change was seen. Levels directly compared between the rejectors and non-rejectors at pre-transplantation and day 3 were not different suggesting that it is not

just that there are higher levels for example pre-transplant between the two groups that could explain the trend.

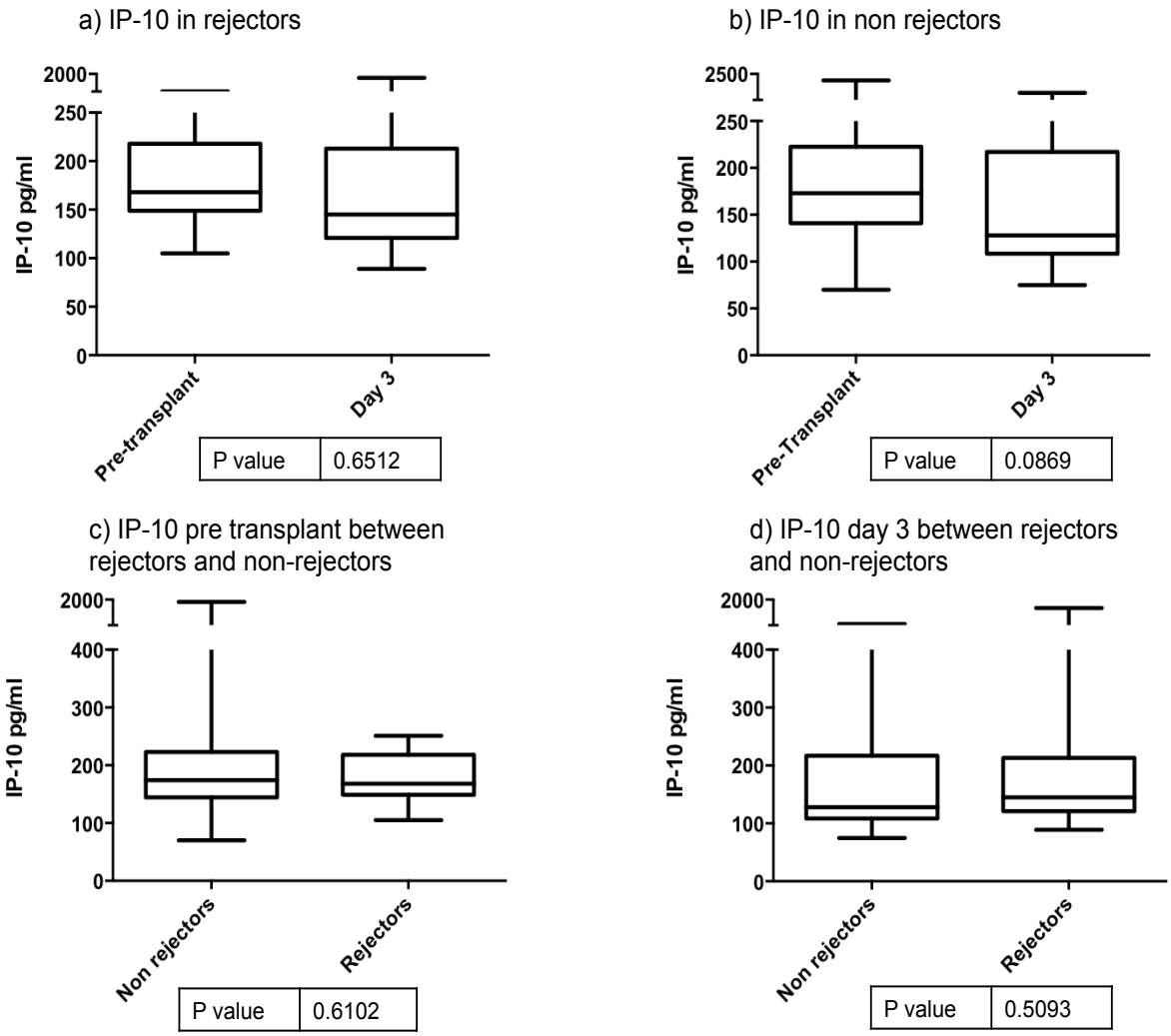


Figure 3-11: Serum IP-10 levels in a) ABOi rejectors and b) ABOi non-rejectors pre-transplant and day 3 and c) comparison between IP-10 levels pre transplant between rejectors and non-rejectors and d) IP-10 levels day 3 between rejectors and non-rejectors

A similar pattern is also shown for Cystatin C with a significant drop in levels on day 3 from the pre-transplant levels in those who did not develop rejection whereas the rejection cohort failed to show a change in Cystatin C level. Again, comparison of the levels pre-transplant demonstrated that there was no significant difference suggesting that the change is not purely a product of different starting levels. The

observed drop between pre-transplant levels and day 3 levels may be reflective of the ability of the transplant not developing rejection to clear cystatin C effectively.

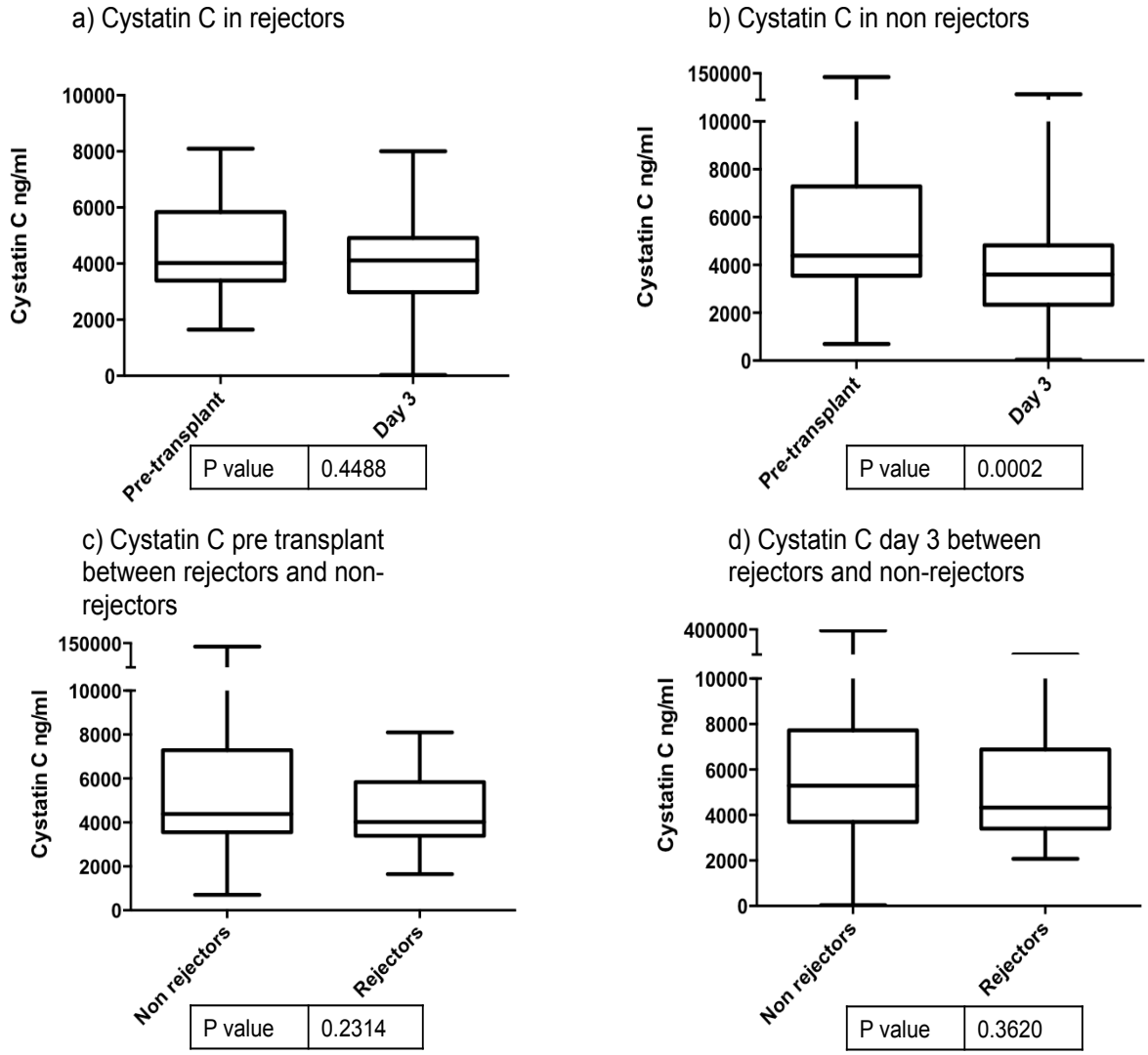


Figure 3-12: Serum Cystatin C levels in a) ABOi rejectors and b) ABOi non-rejectors pre-transplant and day 3 and c) comparison between Cystatin C levels pre transplant between rejectors and non-rejectors and d) Cystatin C levels day 3 between rejectors and non-rejectors

For Cathepsin L both groups showed significant decreases in levels from pre-transplant to day 3 although the difference was more marked in the non-rejector cohort than in those patients who subsequently developed rejection.

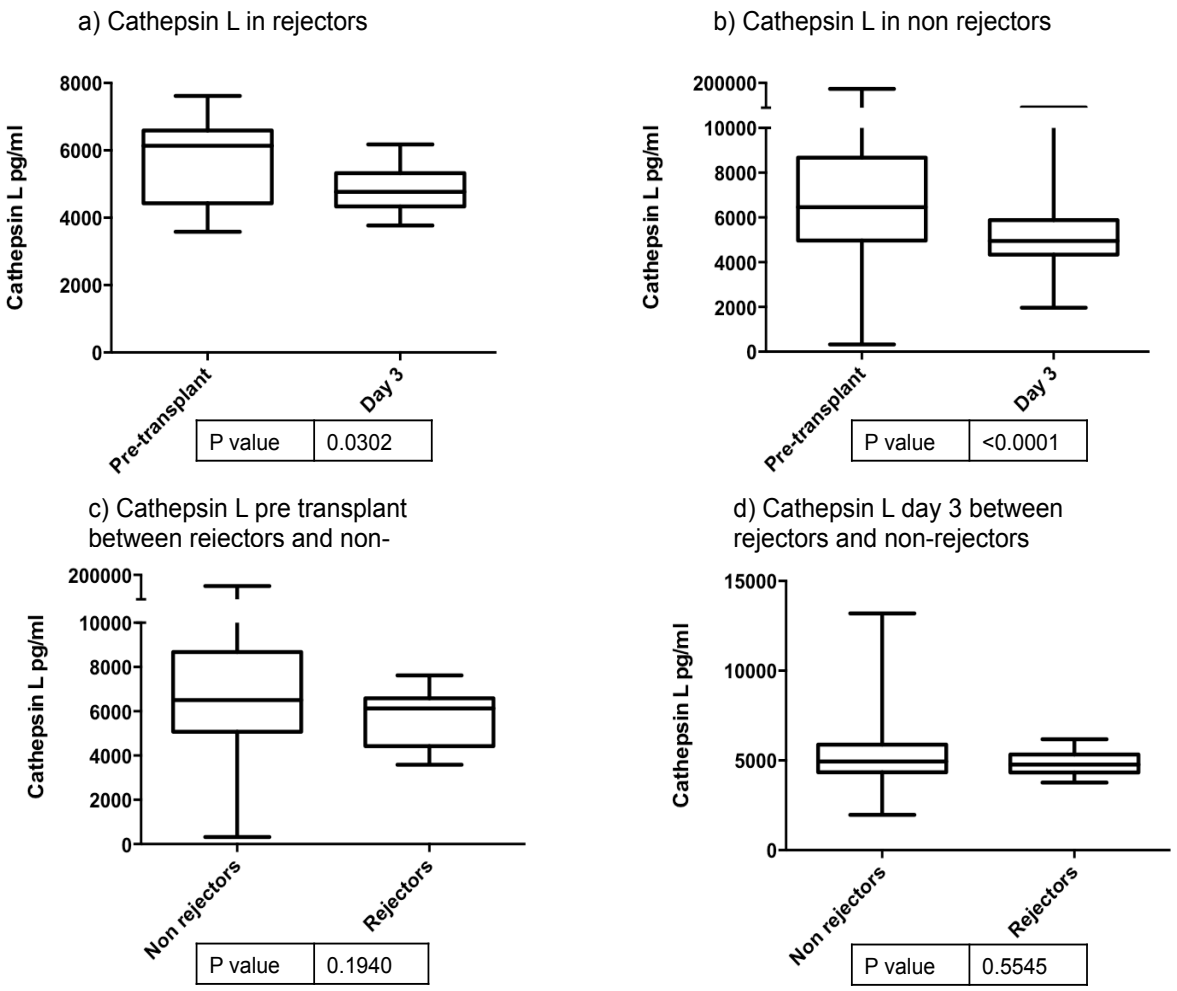


Figure 3-13: Serum Cathepsin L levels in a) ABOi rejectors and b) ABOi non-rejectors pre-transplant and day 3 and c) comparison between Cathepsin L levels pre transplant between rejectors and non-rejectors and d) Cathepsin L levels day 3 between rejectors and non-rejectors

For all the biomarkers there was a greater decrease in the levels between the pre-transplant levels and the day 3 levels that was more marked in those who did not develop rejection. Speculatively this is likely to be due a combination of factors; it may be that the rejection process is detectable at this very early time period by these biomarkers prior to an effect on creatinine or it may represent better function within those kidneys that do not develop rejection enabling them to clear the biomarkers more effectively.

3.4 HLA incompatible transplants

With the ever growing waiting list for transplantation and re-transplantation, inevitably the population of potential kidney recipients who are highly sensitized will increase. Transplantation across previously impossible HLA barriers (defined as transplantation against a positive crossmatch) is now considered routine in many centres, however, it is still limited by poorer long term outcomes, perhaps in part due to the effects of rejection with rates of rejection between 12-53% (Gloor et al., 2010, Thielke et al., 2009, Vo et al., 2010, Haririan et al., 2009, Higgins et al., 2009). In this sub-group of transplantation recipients, with a very high rate of rejection, any mechanism of reducing and potentially improving long term outcomes would be extremely useful. The potential of biomarkers to help elucidate those at higher risk could also be invaluable in increasing the use of transplantation across this immunological barrier.

3.4.1 Study design

3.4.1.1 Patient Selection

Retrospective analysis was performed on a library of sera from ninety-four HLA sensitised patients who underwent HLAi renal transplantation between 2003 and 2012. Patients were selected for inclusion in the University Hospital Coventry and Warwickshire program if they had current reactivity with donor-specific HLA mismatches as measured by complement dependent cytotoxic crossmatch (CDC), flow cytometric crossmatch (FC) or single antigen bead (SAB) assay as undertaken by the NHSBT Histocompatibility and Immunogenetics laboratory (for further detail see methods).

Patients undergoing HLA incompatible transplantation between 2003 and 2012 at a single centre were recruited. Serum samples were taken at time points of prior to transplantation (including prior to therapy to achieve desensitisation), day 1 following transplantation and at day 30 after transplantation.

Donor Specific Antibody levels prior to pre-transplant antibody removal, day 1 post-transplant and at 30 days following transplant were also recorded. Donor specific antibody levels were also correlated with the rejection occurring during the first 30 days following transplantation and to serum biomarker findings.

3.4.2 **Demographics of the cohort**

Between 2003 and 2012, 94 patients underwent HLAi transplant of whom 44 (46%) developed acute rejection in the first month. The mean time to onset of rejection was 4.2 days. The majority of organs were from live donors with only 8 from deceased organ donors. There were no significant differences between patient demographics although differences between pre-transplantation antibodies were seen between the group of rejectors and non-rejectors. Baseline demographics between those patients who did or did not develop rejection can be seen in the table below (Table 3-11).

Table 3-11: Comparison of demographic data between those with rejection and no rejection of the HLA transplants

		No rejection (n=50)	**Rejection (n=44)**	**P value**
Age mean (median, range)		41.8 (42, 18-67)	44.2 (43.5, 22-68)	0.38
Sex (M:F)		18:26	21:29	1.0
Time on dialysis (mean, months)		70	79	0.37
Time on waiting list (mean, months)		53	59	0.41
Donor specific antibody		Class I – 25 Class II – 14 Both – 11	Class I – 17 Class II – 11 Both - 16	0.30
Number of mismatches (Median)		3 (0-5)	3 (1-5)	0.24
Number of previous transplants (median)		1 (0-3)	1 (0-3)	0.80
Crossmatch	CDC+/FC+/SAB+	11	13	0.18
	CDC - /FC+/SAB+	23	24	
	CDC-/FC-/SAB+	16	7	
DSA MFI Total pre-transplant (mean, range) Day 1 (mean, range)		6574 (221-36360) 2963 (97-17760)	8594 (124-33730) 3278 (75-13950)	0.123 0.630
Creatinine Day 1 (mean and 95%CI)		331 (284-378)	369 (318 -420)	0.262

3.4.2.1 Initial Biomarker Analysis

Analysis was undertaken between the cohort who went on to reject in the first 30days and those who did not for each of the time points. The results of this for all biomarkers can be seen below.

a) Comparison between biomarker levels in rejectors and non-rejectors pre-transplantation

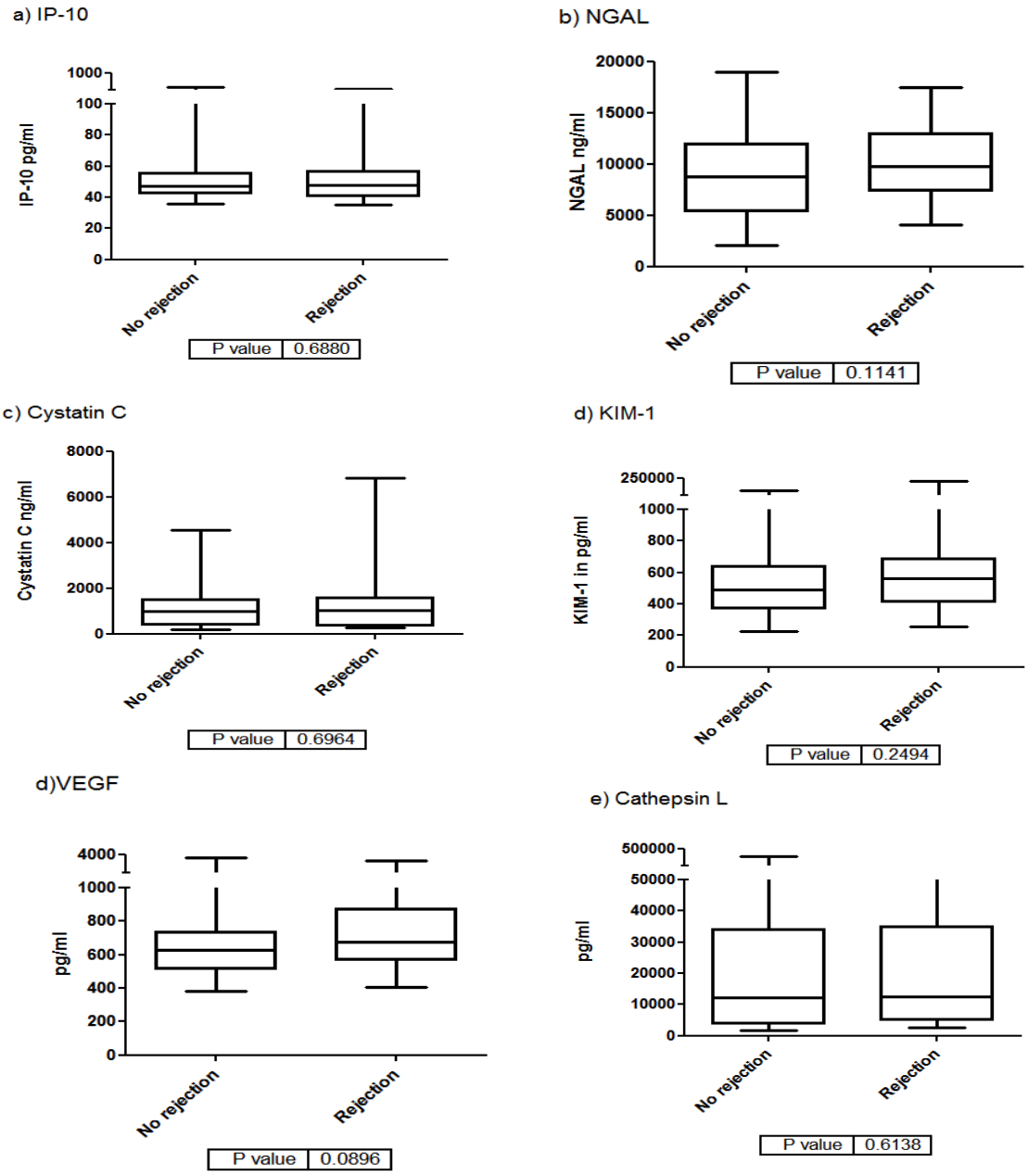


Figure 3-14: Comparison of serum biomarker levels in HLAi transplant recipients pre-transplantation between those who subsequently developed rejection and those who did not.

b) Comparison between biomarker levels in rejectors and non-rejectors at day 1 following transplantation

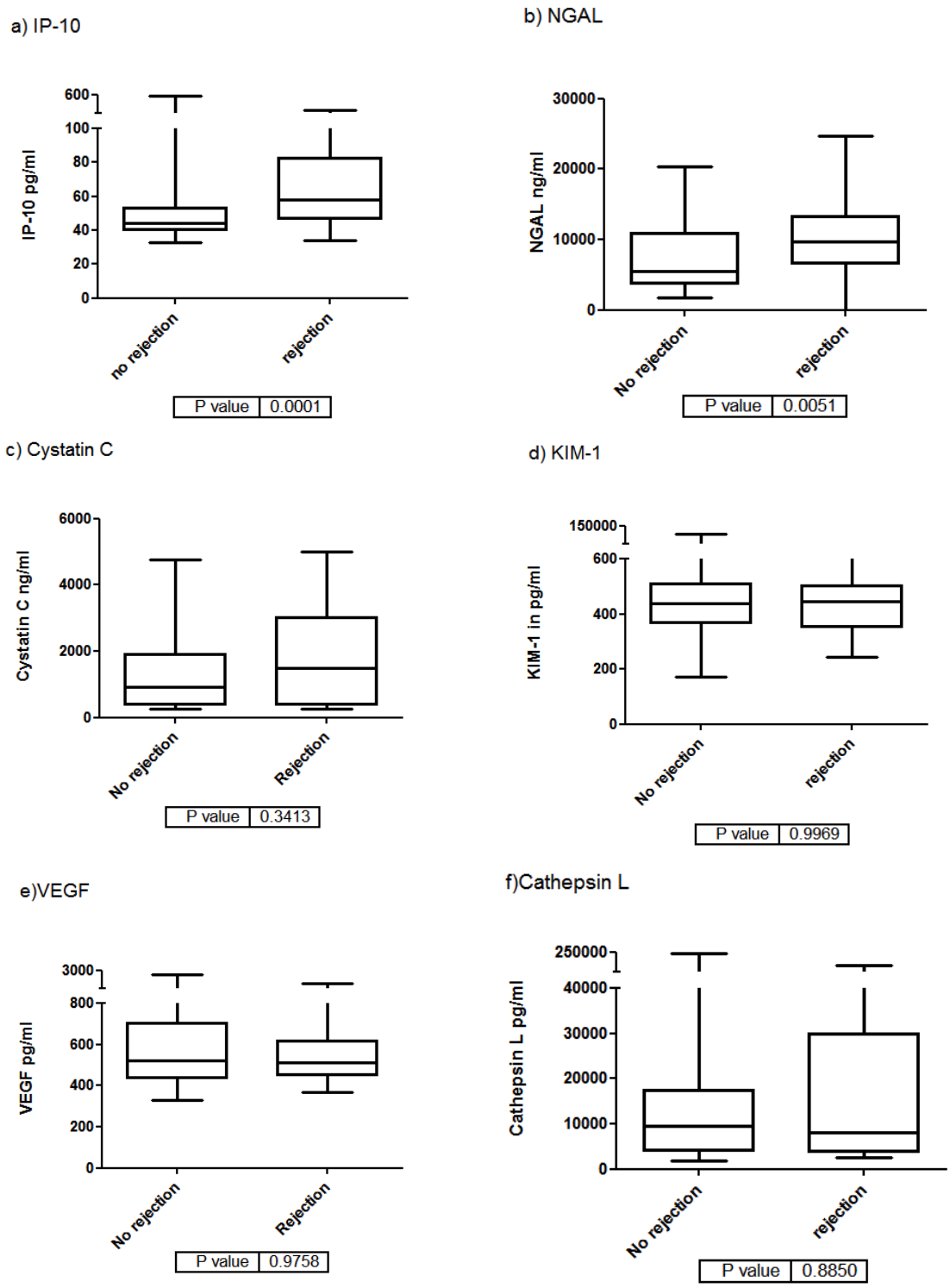


Figure 3-15: Comparison of serum biomarker levels in HLAi transplant recipients day 1 between those who subsequently developed rejection and those who did not

c) Comparison between biomarker levels in rejectors and non-rejectors at day 30 following transplantation

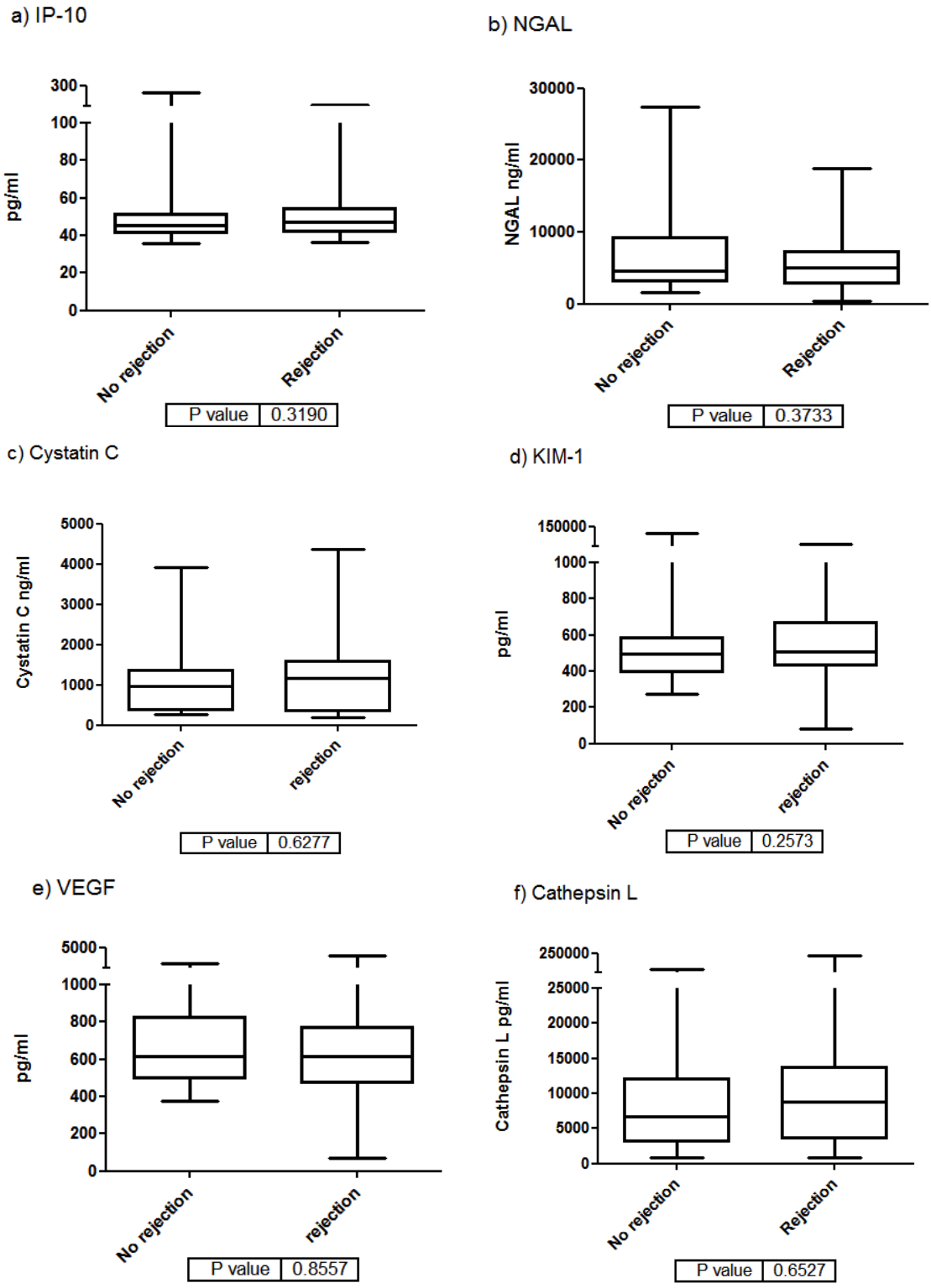


Figure 3-16: Comparison of serum biomarker levels in HLAitransplant recipientsat day 30 between those who subsequently developed rejection and those who did not

Levels for IFN-γ, TNF-α, IL-22 and Fractalkine were below the level of detection to be accurately assessed in the cohort.

Overall analysis revealed no significant differences in biomarker levels either pre-transplant or at 30 days following transplant.

However, significantly elevated levels of NGAL (p=0.005, means of 7657 ng/ml vs. 10080 ng/ml) and IP-10 (p=0.0001, means of 54.5pg/ml vs. 74.2pg/ml) were seen on day 1 in recipients who subsequently rejected in the first 30 days (Figure 3-15). There was no significant difference between the two groups with regards to other evaluated biomarkers. Analysis was also undertaken to determine the relationship between levels of NGAL and IP-10 and demonstrated a weak correlation indicated by a Spearman r of 0.25 (CI 0.45-0.44, p=0.015). This suggested a weak association between the two levels.

ROC analysis was undertaken to determine the ability of Day 1 NGAL and IP-10 to predict rejection within the first 30 days. This demonstrated an AUC of 0.67 and 0.73 respectively (Figure 3-16). At a cut off of 50pg/ml IP-10 demonstrated a specificity of 72% and sensitivity of 70% for predicting rejection. At a cut off of 6300ng/ml NGAL demonstrated a specificity of only 56% and but a sensitivity of 86% for predicting rejection. At this level NGAL also had a negative predictive value of 82% (PPV 63%) suggesting that it might have a role as a screening test i.e. in a clinical setting if the NGAL level was under 6300ng/ml it would be reassuring that rejection was unlikely.

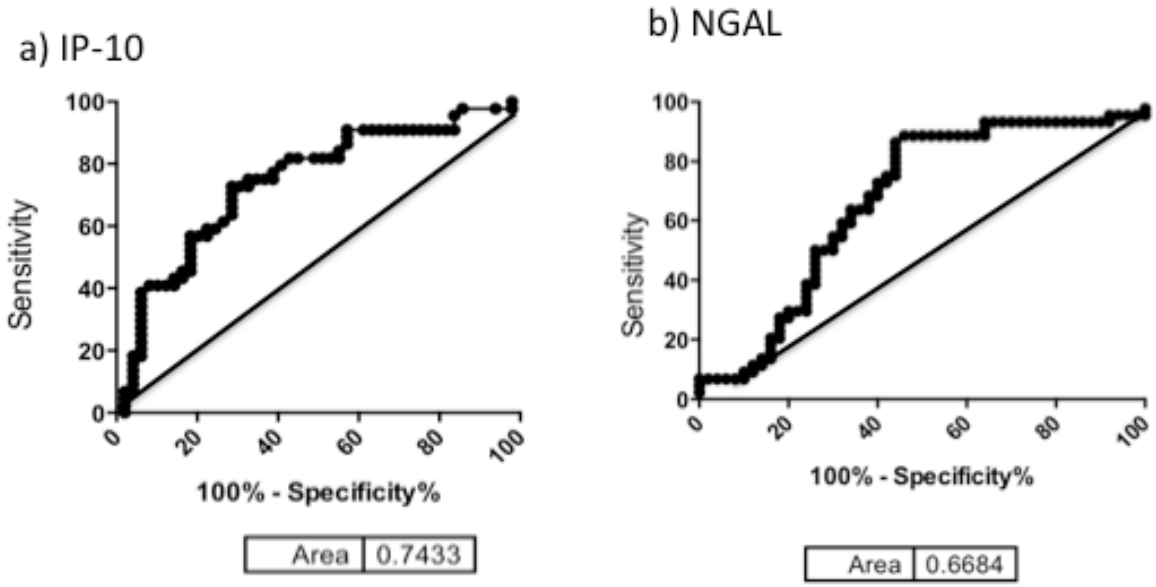


Figure 3-17: ROC analysis of HLAi biomarkers for IP-10 and NGAL

DSA levels were compared to biomarker levels at the time points described previously. DSA levels at these time points did not correlate with levels of any of the biomarkers at the same time points. Analysis was also undertaken using a pre-transplant DSA cut off MFI of 2000 which demonstrated significantly higher chance of rejection in those patients with a pre-transplant DSA level above 2000 (28% vs. 54% p=0.048). There was no significant association between IP-10 and NGAL with DSA levels on day 1 (p=0.77, p=0.133 respectively).

Levels of IP-10 and NGAL were also correlated to the time of onset of rejection to determine whether the magnitude of biomarker level on day 1 was predictive of the timescale of rejection. Spearman r for NGAL was -0.21 with a p value of 0.17 indicating no significant association between the level of NGAL on day 1 post-transplant and the timeframe for developing rejection. For IP-10 a Spearman r value of -0.354 was generated with a p value of 0.02 demonstrating that the level of IP-10

on day 1 post -transplant is weakly associated with the timeframe for developing rejection.

Patients who subsequently developed acute rejection demonstrated a trend towards higher rates of delayed graft function (DGF) than those without rejection, but this was not significant (38% vs. 25% p=0.47). NGAL and IP-10 levels have been suggested to be elevated in DGF in other cohorts and the differences between levels in this cohort were examined to ensure this was not causative in the association with acute rejection. Analysis revealed that NGAL and IP-10 levels were not significantly different on day 1 between those who had DGF or primary function overall (p=0.32 and 0.12 respectively). Similarly, no significant differences were seen between NGAL or IP-10 levels in those patients with rejection whether they developed DGF or not (p=0.99 and 0.51 respectively). This implies that the elevated levels of IP-10 and NGAL on day 1 in those who later develop rejection are not a function of their higher rates of DGF. Whilst there is evidence in the literature suggesting that NGAL is associated with DGF the lack of association between IP-10/NGAL and DGF in this study could be explained because of the high proportion of live donors and the lower percentage of DGF in this population.

3.5 Biomarkers in the monitoring of renal transplant recipients

To provide a counterpoint for the results of the biomarker assessments in the immunologically complex transplant recipient a comparable group of non-immunologically complex recipients were studied. A prospective trial was established with ethical approval for recruitment of both living donor transplant recipients and deceased donated kidneys. Although recruitment was prospective all analysis was undertaken as a batch retrospectively.

3.5.1 Study Design

3.5.1.1 Patient recruitment

Patients undergoing both live and deceased renal transplantation were approached to take part in a prospective study to determine the use of Biomarkers for monitoring renal transplant function. Blood and urine (where possible) samples were taken both pre-operatively and post-operatively as an in-patient and then at regular scheduled out-patient follow up appointments.

Results were analysed comparing the levels of the biomarkers at pre-transplant and day 1 post transplant, pre-transplant and at day 30 post-transplant to act as control group for the changes seen in the immunologically difficult transplants. Biomarker levels were also compared between the groups who developed delayed graft function to determine whether levels on day 1 post-transplant were significantly different and able to predict delayed graft function.

3.5.2 **Recruitment and outcomes**

36 patients were recruited who proceeded to successful kidney transplantation. Of the 36 patients 30 were transplanted from deceased donors (24 DBD and 6 DCD) and 6 were live donors. The mean recipient age within this group was 42.5 and the mean donor age was 49. Of the 36 patients 13 were female (36%) and 23 were male (64%).

Within this cohort 2 patients developed rejection, giving an acute rejection rate of 5.5%. The lower than perhaps anticipated level of rejection means that planned analysis of the samples to determine their ability to predict rejection is unfortunately not possible. However, this does mean they are able to act as a control for those changes seen in the immunologically complex transplants.

Below can be seen the biomarker changes seen in serum between pre-transplant samples and day 1 post-operative samples demonstrating no significant difference between the two time points (figure 3-18). In this analysis the 2 patients with rejection were excluded.

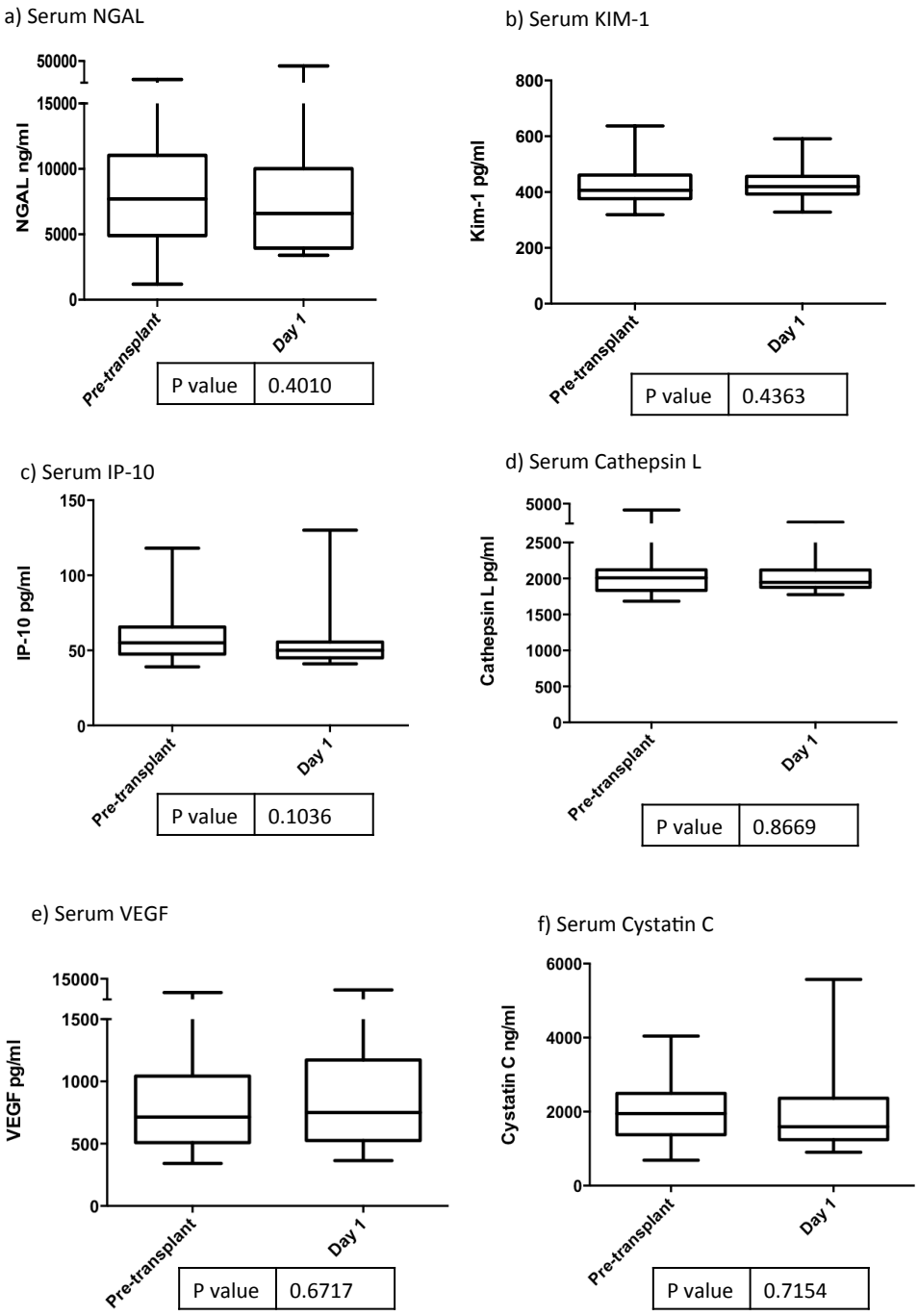


Figure 3-18: Serum biomarker levels in immunological straightforward transplants at pre-transplant and day 1 showing no statistically significant differences.

With the cohort 14 patients had delayed graft function (defined as the need for dialysis in the 7 days). This gives a DGF rate of 39% permitting analysis of

biomarker predictors of delayed graft function. Day 1 Serum and urine biomarkers were compared between those who developed delayed graft function and those who did not. These results can be seen below (figure 3-19).

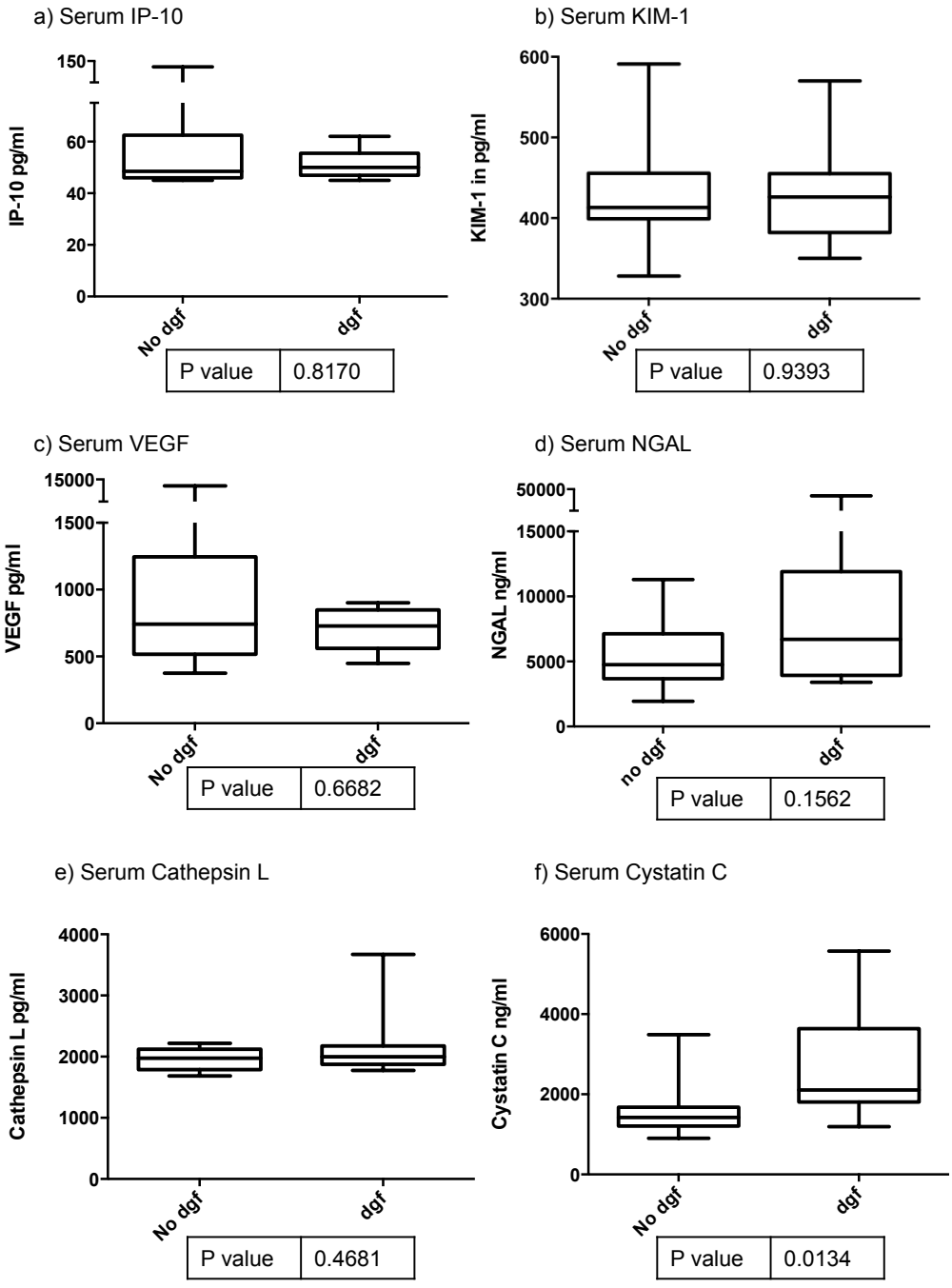


Figure 3-19: Differences in serum day 1 levels of biomarkers in immunologically uncomplicated transplants between those recipients with DGF and those with immediate function

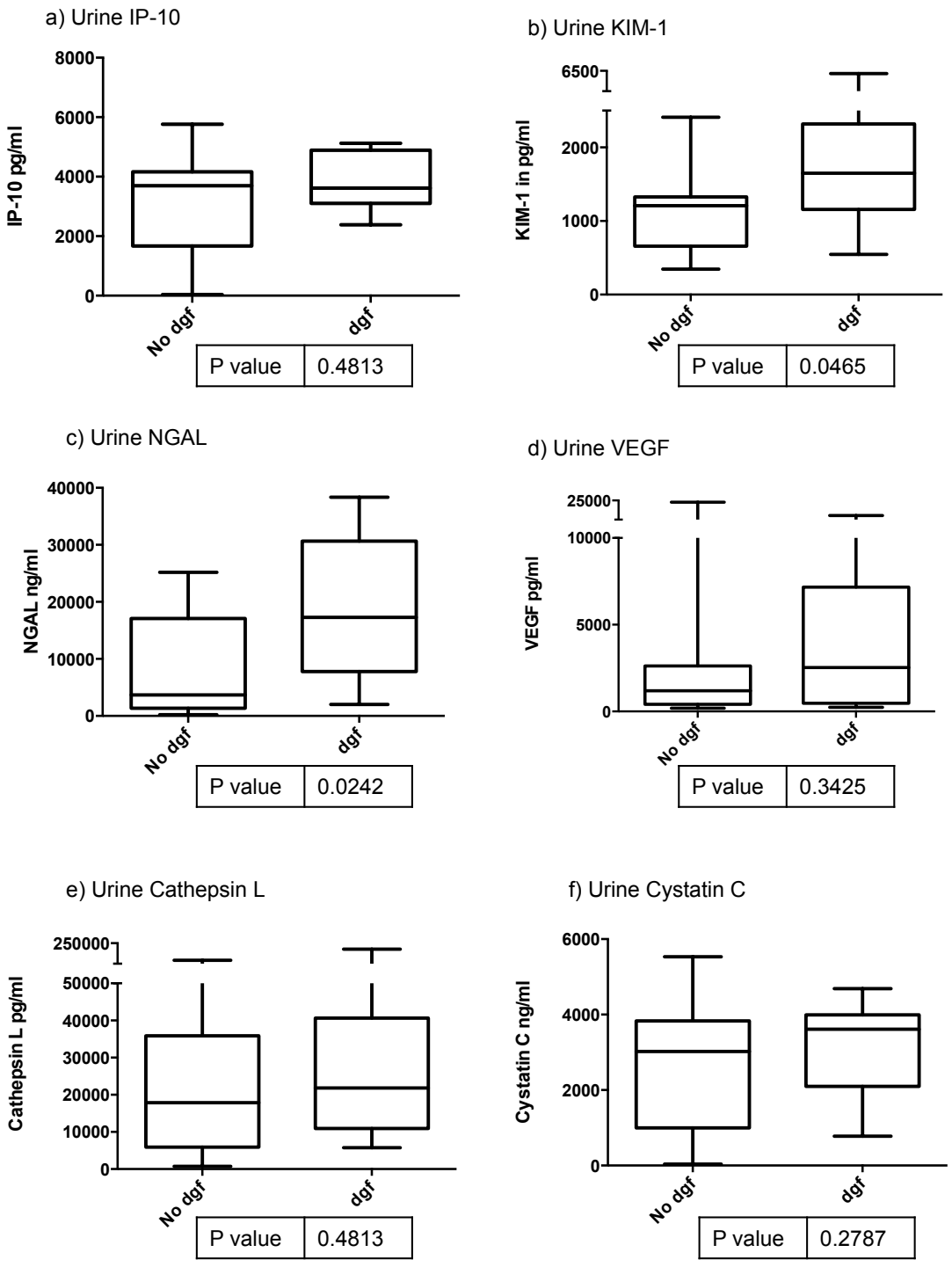


Figure 3-20: Differences in urine day 1 levels of biomarkers in immunologically uncomplicated transplants between those recipients with DGF and those with immediate function.

This demonstrated that serum levels of cystatin C and urine levels of KIM-1 and NGAL were statistically different on day 1 between those recipients with delayed graft function and those with immediate function. Whether these markers are detecting underlying AKI that leads to DGF or are a surrogate marker of decreased renal clearance is difficult to say.

To determine the predictive ability of those markers that were identified as being statistically different between the two groups of those who did and didn't develop delayed graft function ROC analysis was undertaken. The results of this can be seen below (Figure 3-21).

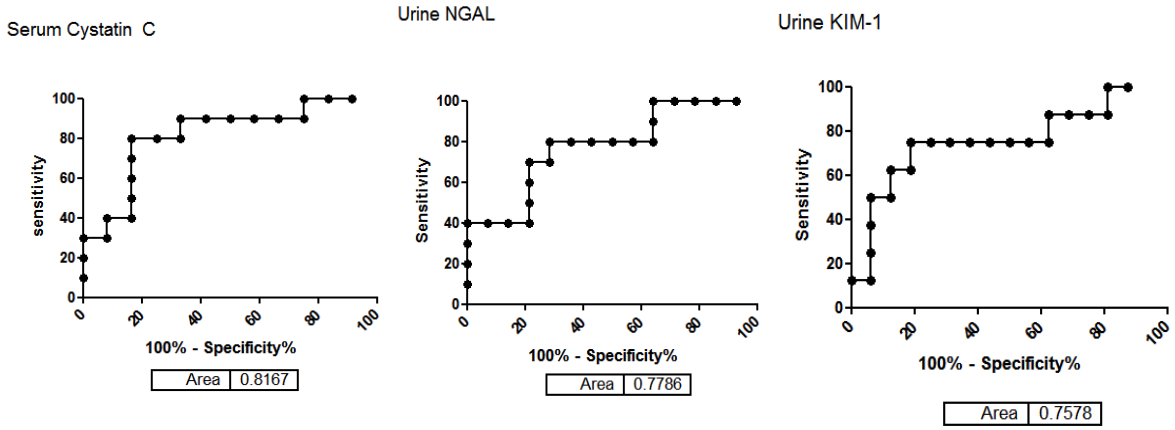


Figure 3-21: Graphs demonstrating ROC analysis of DGF predictors for serum Cystatin C and urinary NGAL and urinaryKIM-1

As can be seen day 1 serum Cystatin C has excellent predictive ability for delayed graft function and urinary levels of KIM-1 and NGAL also have good predictive power although with the caveat that many of those patients with delayed graft function will not have passed any urine on day 1.

At a cut off of 1807ng/ml serum Cystatin C demonstrated a sensitivity of 80% and a specificity of 83%. At a cut off of 1421 pg/ml urinary KIM-1 displayed a sensitivity of

75% and a specificity of 81%. At a level of 8517ng/ml urinary NGAL showed sensitivity of 80% and specificity of 71%. It may be that the value of biomarkers in this setting is as a screening tool; at a cut of level of 1500ng/ml Cystatin C has a negative predictive value of 89% suggesting that day 1 levels may prove an excellent screen for DGF.

Predictors of delayed graft function were also assessed in the original 60 HLAi transplants (ABOi transplants were excluded as the earliest post-transplant sample available for analysis was day 3).

From the cohort of HLAi transplants 12 patients demonstrated delayed graft function from the total with available outcomes of 54. This gives a rate of delayed graft function of 22%. This is lower than the rate in the immunologically uncomplicated transplants probably reflecting the higher proportion of living donors in the HLAi cohort.

Analysis of biomarker levels on day 1 between those recipient who did or did not have DGF can be seen below (Figure 3-22).

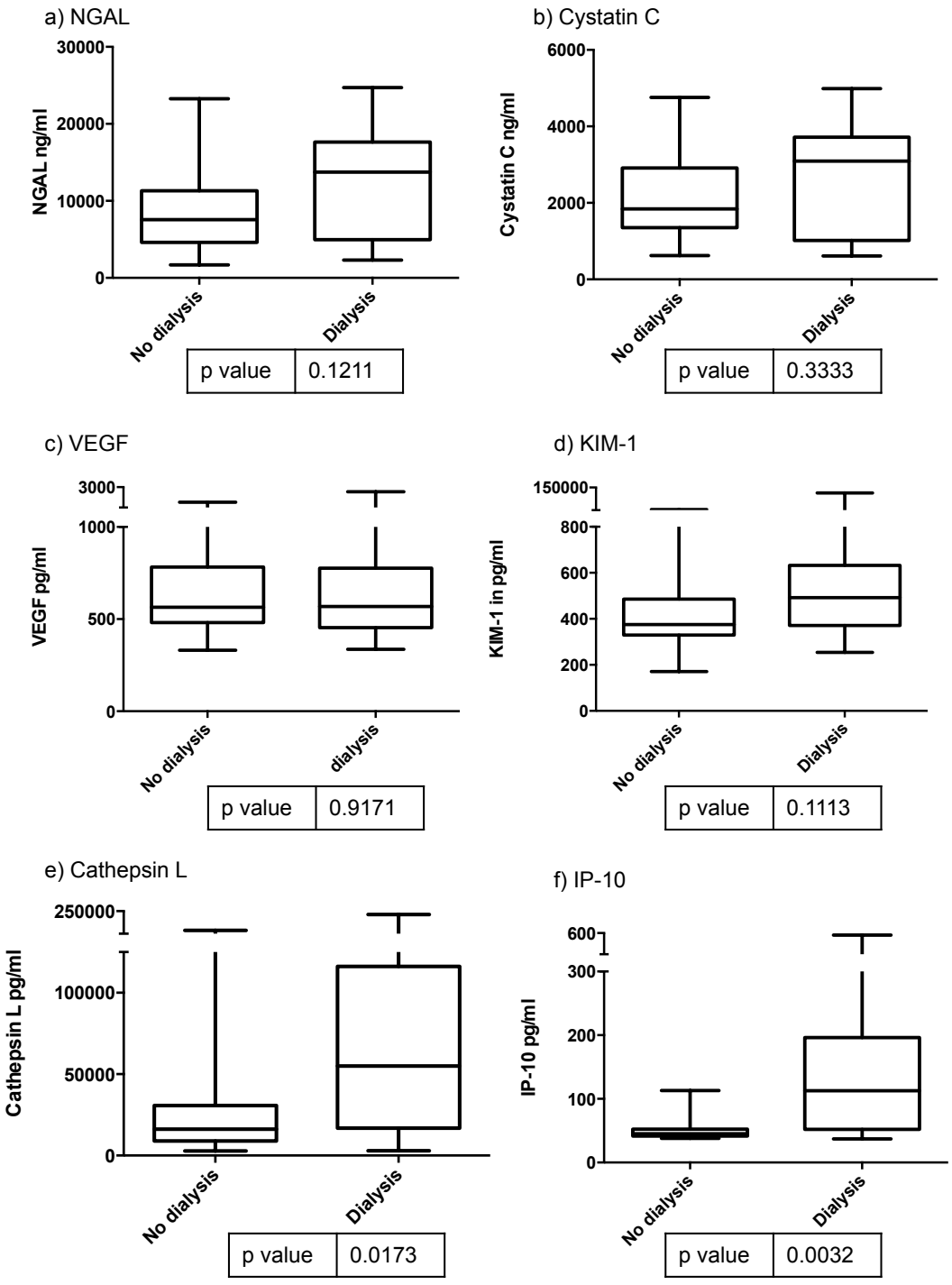


Figure 3-22: Differences between biomarker levels in the HLAi transplant group on day 1 between those with or without DGF

As can be seen in this HLAi cohort of patients serum Cystatin C levels were not statistically different between those with DGF and those with immediate function

which is in contrast to the immunological straightforward transplants. Interestingly in this cohort Cathepsin L and IP-10 were statistically different. ROC analysis demonstrated AUC of 0.75 for Cathepsin L and 0.78 for IP-10. It is difficult to interpret the IP-10 results in isolation as of those patients with DGF in the HLAi cohort 50% also had rejection and as previously demonstrated IP-10 levels are elevated on day 1 in those patients who subsequently develop rejection. Cathepsin L, however, was not predictive of rejection so may be a more reliable predictor of delayed graft function in this cohort.

Levels of Cathepsin L were compared within those patients with delayed graft function between those who also had rejection and those who only had delayed graft function. This can be seen below (Figure 3-23).

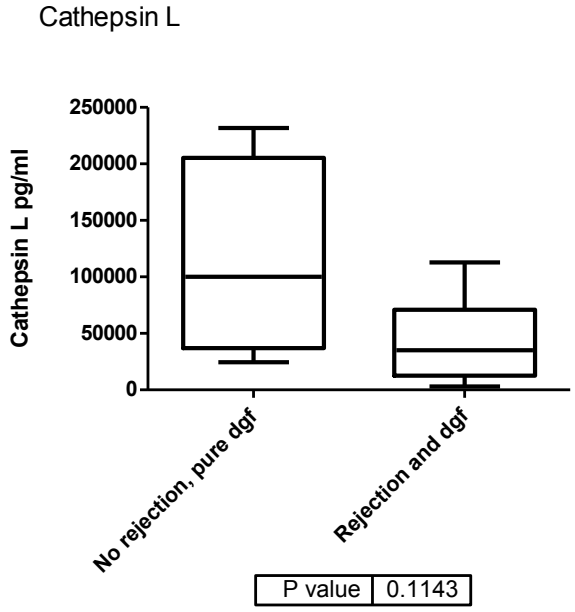


Figure 3-23: Graph to demonstrate differences in Cathepsin L level between those patient with DGF and either rejection or no rejection

The graph demonstrates that levels of Cathepsin L are much higher in the group with pure delayed graft function rather than the mixed picture of rejection and delayed graft function, although this failed to reach significance perhaps due to the small

numbers in each group (n=4 in the pure DGF group, n=6 in the combined group). This is in contrast to IP-10 levels which are very similar between the two groups (p=0.81).

Levels of biomarkers were also compared between the HLA rejectors, HLA non-rejectors and the non-immunologically complex transplants at the time-points pre-transplantation, day 1 and day 30.

Results of this can be seen below for NGAL, IP-10 and Cystatin C (figures 3-24, 3-25 & 3-26).

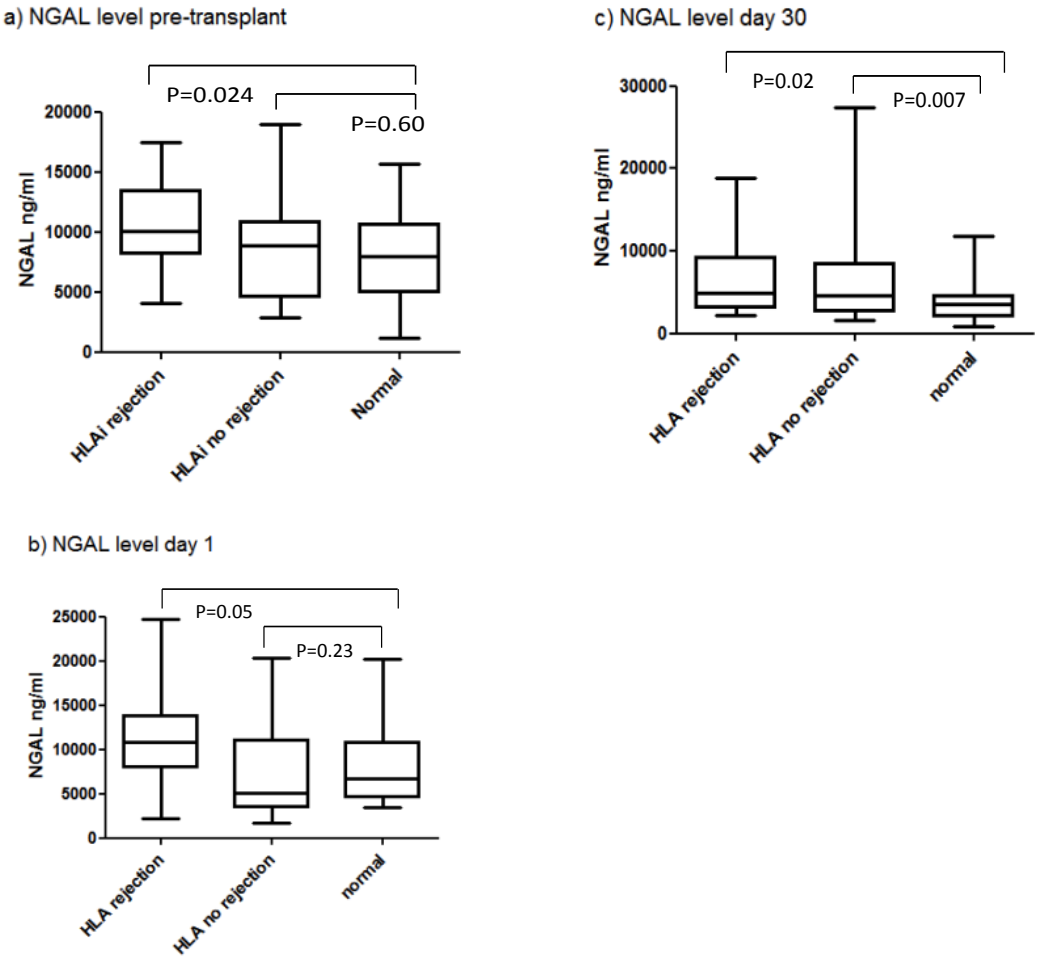


Figure 3-24: Differences between serum NGAL levels in the HLAi transplant recipients with rejection, HLAI transplant recipients without rejection and “normal” transplant recipient groups

NGAL levels were significantly higher pre-transplant in the HLAi group who subsequently developed rejection when compared to the normal transplant group whereas the HLAi group who did not develop rejection were no different from the normal group.

At day 1 post-transplant levels of NGAL amongst the HLA rejection cohort were higher than the normal transplants and HLAi with no rejection were no different from the normal transplants. At 30 days following transplant NGAL levels in the “normal transplants” were significantly lower than either the HLA rejectors or the HLA non-rejectors whose levels had equalised (as shown in Figure 3-24).

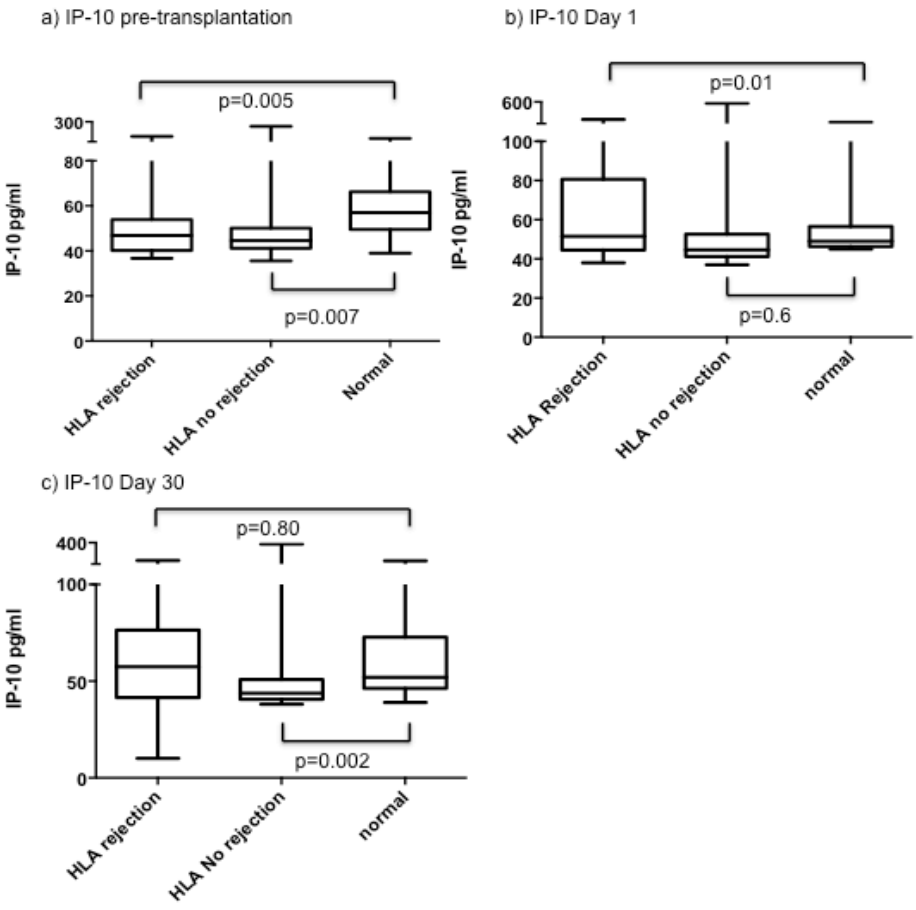


Figure 3-25: Differences between IP-10 levels in the HLAi transplant recipients without rejection, HLAi transplant recipients with rejection and “normal” transplant recipient groups

The reasons for this marked difference could be speculated to relate to the presence of donor specific antibodies in the HLAi group, however, more study would be needed to confirm this.

IP-10 levels were significantly higher in normal transplants than in the HLA group whether they subsequently developed rejection or not. At Day 1 post-transplant IP-10 levels were significantly lower in the HLA non-rejection group whilst the levels amongst the normal and HLA rejector levels were not different. At 30 days following transplant HLA group without rejection were still significantly lower than either the HLA rejection group or the normal group (as shown in Figure 3-25).

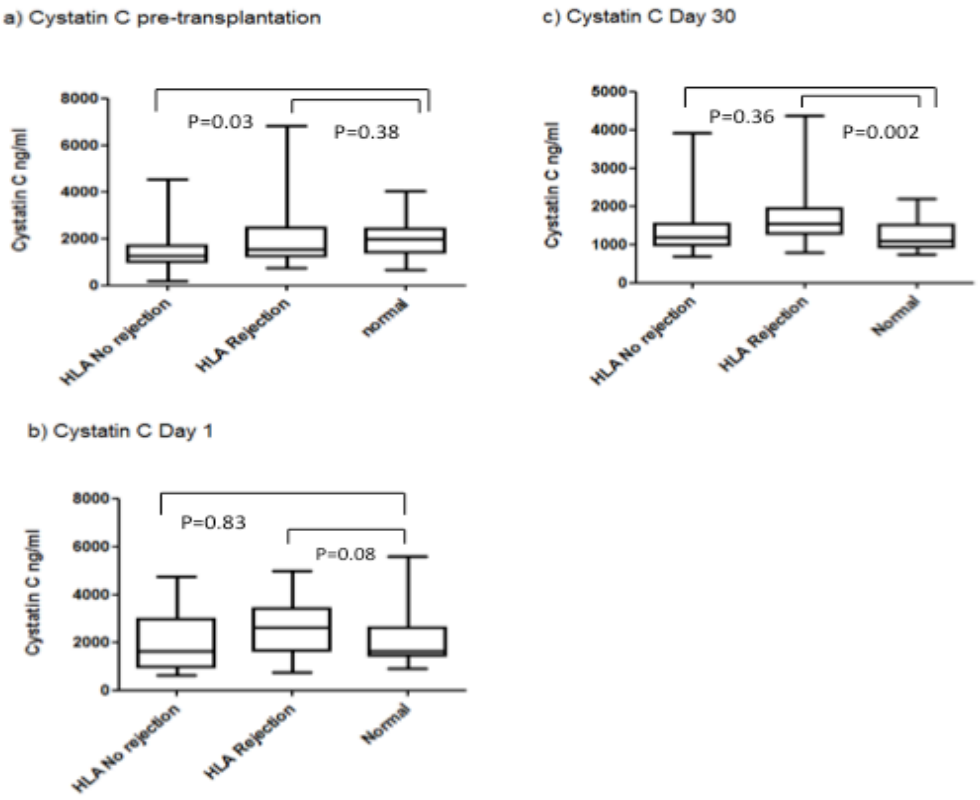


Figure 3-26: Differences between Cystatin C levels in the HLAi transplant recipients and non-antibody incompatible recipient groups

Cystatin C levels were not significantly different between the HLAi rejectors and the normal transplants pre-transplant but the levels in the HLA group without rejection were significantly lower than the normal or HLA rejection group. At Day 1, although not statistically different, levels in the HLA rejectors were higher than the normal transplants levels. This rise was sustained at 30 days following transplant and did reach significance (as shown in Figure 3-26).

4 Chapter 4: Discussion

4.1 Deceased donor biomarker analysis

Deceased organ donation is complex and many events may impact upon outcome including any chronic and acute kidney injury pre-existing in the donor at the time of donation. This includes factors relating to the donor, the organ retrieval process, the cold ischaemic time and the recipient factors as discussed earlier. Most donors have a history of complex, and often traumatic, events preceding donation that carry the risk of AKI.

Chronic damage is a non-modifiable factor that leads to long-term, suboptimal renal function after transplantation but may not impact on the graft's initial functional status. Hence a graft with chronic damage may achieve primary function but longevity and the baseline function may be suboptimal. By contrast AKI is more likely to be associated with delayed, or rarely, primary non-function. The combination of AKI in a chronically damaged kidney is poor for the outcome but difficult to distinguish in practice.

Nevertheless, assessing the level of AKI at the time of donation can be difficult. In the situation of the critically ill patient urine output and creatinine can be difficult to interpret. Creatinine is recognised as a poor marker of AKI as plasma levels are influenced by non-renal events affecting its generation, distribution and excretion(Port et al., 2002, Star, 1998).

When offered a kidney for transplant the surgeon has few tools to discriminate a "good" from a "bad" kidney.

This study cohort assesses the ability of the previously identified AKI biomarkers to predict which donors have sustained AKI as reflected in post-transplant graft dysfunction. This is not always a clear relationship as many events between donation and the early post-transplant period can induce AKI. However, these variables were controlled for (as much as possible) allowing donor-derived AKI to be assessed in isolation. Hence, all donors were deemed suitable for cardiac donation, were young with low probability of chronic renal damage. There were no obvious adverse events reported at procurement or implantation and donors were excluded when events appeared most likely to have occurred i.e. where only one donated kidney showed aberrant function - suggesting a post-donation event.

Even in a cohort of optimized, near ideal donors, in whom the process of procurement appears uncomplicated, 6.5% of donors still produce two kidneys in which post-transplant dysfunction was observed.

The availability of a point-of-care test for KIM-1, that could be routinely performed on candidate donors and predict recipient kidney transplant outcome, could facilitate greater matching of donor-recipient pairs and increase the availability of the organ pool by identifying those organs with AKI rather than poor function due to irreversible chronic damage.

Machine perfusion may have a role in determining the quality of organs and failure of parameters to improve has been shown to correlate to higher discard of organs previously (Patel et al., 2012). Similarly histological examination of biopsy tissue can help determine chronic damage, but it has been suggested that significant problems

can occur with the examination of biopsy tissue and lead to higher than necessary discard rates (Kasiske et al., 2014). With pressure to keep cold ischaemic times as low as practicable both these options are not necessarily ideal in every case and a immediate indicator to perhaps indicate which kidneys would benefit from further evaluation would be useful.

The point of care lateral flow device (Renastick™) allows the user to easily assess at the patient's bedside the level of KIM-1 in the urine. This raises the possibility of incorporating the KIM-1 level into the assessment of the potential donor on the intensive care unit, particularly in combination with other known potential risk factors for poorer outcomes following organ donation. This could help donor management, perhaps aiming to modify those risk factors that are modifiable such as cold ischaemic time, machine perfusion or recipient selection. Whilst clearly further studies need to confirm these findings it may be that the lateral flow assay may be the catalyst that drives the transition for biomarkers from the research laboratory to widespread clinical adoption.

Crucially whilst KIM-1 in deceased donors did seem to have predictive ability for DGF it's role in directly predicting long term outcome is currently not known.

4.2 **Biomarker changes following Live donor nephrectomy**

Patients undergoing laparoscopic donor nephrectomy show no changes to biomarker levels either in urine or serum as a result of the surgical intervention. This allows the inference to be made that any changes that are then seen amongst the transplant

population reflect changes as a result of the transplanted kidney rather than as a result of the physiological stress of surgery.

Previous authors have suggested that donor nephrectomy leads to cytokine and biomarker changes as a result of hypertrophy and up regulation within the remaining kidney (Yap et al., 2012). This wasn't seen in our cohort of patients. Possible reasons for this may be due to the shorter operative time in our cohort or different operative technique (hand assisted laparoscopic donor nephrectomy vs. fully laparoscopic) used in the published literature.

4.3 **Biomarker changes in the HLAi transplant population**

This study demonstrates that the biomarker IP-10 has a good ability to predict rejection in the first 30 days in this group of transplants, as evidenced by the AUC of 0.73. Whilst levels of NGAL were significantly different between the two groups its ability to differentiate was inferior, with an AUC of 0.67. However, both merit further evaluation as they may have differing roles as screening tools.

The analysis of biomarkers of rejection usually involves the comparison of biomarker levels at the time of diagnosis of rejection (e.g. upon biopsy for rising creatinine) with biomarker levels in a cohort with stable renal function. This strategy does not identify the rejection process at an earlier time point than standard current practice. The ability of a single biomarker or a panel of biomarkers to predict rejection earlier than current methods would expedite diagnosis and treatment of the episode. It may also identify those patients at higher risk of rejection and guide surveillance. Since increased glomerular margination can be detected as early as 30 minutes following perfusion in biopsy specimens of those who later go on the develop rejection, it is not

unreasonable to suggest that a more peripheral investigation such as a blood test could detect changes that similarly predict rejection soon after transplantation (Higgins et al., 2010)

The correlation statistics between IP-10, NGAL and the timeframe for developing rejection suggests that the higher levels of IP-10 on day 1 may be associated with more rapidly developing rejection.

Interestingly, in contrast to our results a 2005 study showed high pre-transplant levels of serum IP-10 in immunologically uncomplicated transplants was correlated to worse graft outcomes and rejection in the first 30 days whilst our pre-transplant levels were not different (Lazzeri et al., 2005). However, in our study cohort day 1 post-transplant levels of IP-10 did show good predictive ability for rejection within the first month. IP-10 levels at day 30, following treatment of the rejection episode, normalised to the same as the levels of those without rejection suggesting that IP-10 may also be able to demonstrate sufficient immunosuppression has been achieved.

The difference in IP-10 levels demonstrated in this and other cohorts also the raises the possibility of IP-10 as a potential target or pathway, as well as a marker of immunological activity and this certainly merits further study.

DSA levels have been previously shown; pre-transplant, to be suggestive of development of rejection. Work undertaken demonstrated higher rates of rejection amongst patients with pre-transplantation DSA's of above 2000u (Higgins et al., 2009). Within our cohort rates of rejection were significantly higher in the group whose pre-transplant antibody levels were above 2000 (28% vs. 54% p=0.048). However, levels at other time points provided no additional information in predicting rejection. This is probably because of the combined effects of antibody removal prior

to transplantation and absorption of antibody by the kidney immediately following transplantation. It is important to consider that these biomarkers can be predictive of rejection when detected as early as 1 day post-transplant. In the majority of our cohort at this time point there is very little, if any, detectable DSA, due to reasons outlined earlier such as antibody absorption via epitopes expressed on the kidney and it is unknown whether this may also have an indirect effect on biomarker levels. As such the level of DSA in the very early post-transplant period is of little predictive value, thus augmenting the importance of the discovery of new informative biomarkers.

4.4 Biomarker levels between antibody incompatible vs. antibody compatible

The differences between the HLAi group who reject, the HLAi group who do not and the non-antibody incompatible transplants are interesting. At the pre-transplant timepoint the HLA rejection group NGAL levels are higher than non-antibody incompatible or HLA non-rejectors and this is sustained at day 1 suggesting that NGAL is reflecting the underlying reactivity or response to the immune mediated process. Perhaps more interestingly is that at day 30 there is no difference between the HLA rejection and no rejection group whilst the normal transplant levels are much lower. It is difficult to explain exactly why these differences exist. It would be interesting to explore them further to determine whether they reflect the on going difference between the HLA transplant recipient population and the non-immunologically complex transplants. It could be hypothesised that the NGAL levels reflects a higher basal level of neutrophil trafficking within the HLA grafts (as a result

of the mismatch) that might be causing sub-clinical damage, however, without further study this remains purely speculative.

The cystatin C changes show higher levels in HLA rejectors at day 1 and this is sustained at day 30 although HLA non-rejectors levels are similar to those of the normal transplants. This seems to suggest that the damage caused by the rejection episode may cause sustained or long term damage that isn't detected by conventional methods.

The differences in the IP-10 levels are the most interesting as their trends are different from the other markers. IP-10 being a tissue expressed chemokine ligand supports its role in activated T cell recruitment. IP-10 secretion is upregulated by fibroblasts, monocytes and endothelial cells in response to IFN-γ. It has been shown to have functions including promoting recruitment and adhesion of T cells. At all time-points the levels of IP-10 are lower amongst the HLA non-rejectors than the normal transplants perhaps reflective of the heavier burden of immunosuppression. Whilst the levels in HLA rejectors are lower pre-transplant than the normal patients levels they are much higher at day 1 although not statistically significant. At day 30 the HLA no rejection group's levels are much lower than either of the other 2 groups.

What this seems to suggest overall is that there are notable differences in the biomarker profile between the HLA and the "normal" transplants. This means that when considering the use of biomarkers in the HLA group it maybe that they will need to be utilised in a way that is fundamentally different from their application in other potential transplant groups. For example it may be that comparison with baseline "normal" levels of biomarkers in the HLA group will have to be used as the benchmark rather than levels from the "normal" transplant population. It will also be

important to bear these differences in mind on other biomarker research that may not segregate the immunologically complex from the immunologically uncomplicated transplants. This may in part be due to the effects of previous transplants in the sensitisation process.

Historical attempts to predict antibody mediated rejection in HLAi transplants using standard cross-match and IgG levels were unsuccessful. They did however demonstrate that detection of preformed C4d antibody did link to outcome (Bartel et al., 2013), although levels of soluble CD30 and CD27 in the HLAi renal transplants failed to find an association with episodes of acute rejection (Hamer et al., 2010).

4.5 Biomarker changes in ABOi transplant recipients

Despite a large cohort of patients undergoing ABOi transplantation the levels of rejection in the cohort are low when compared to the frequency of rejection in the HLAi population. This may suggest that blood group incompatibility is easier to overcome than HLA incompatibility.

From a clinical perspective, in terms of the utilisation of this technique to increase the numbers of living donors, this is excellent. It does mean that levels of rejection in this cohort were low from the perspective of analysis of biomarker levels, particularly as the levels of antibody mediated rejection were so low.

Analysis initially undertaken compared the entire cohort changes in a manner similar to that used for the HLA transplants but this failed to demonstrate any differences. However, analysis examining the change in levels in each separate group revealed some very interesting findings.

Amongst those patients who did not reject in the first month levels of NGAL, KIM-1, VEGF, IP-10, Cystatin C and Cathepsin L all decreased between the pre-transplant levels and levels at day 3 (IP-10 levels did not reach significance although they approached it). However, in the patients who did experience rejection, levels either did not show a reduction of significance (NGAL, KIM-1, IP-10 and cystatin C) or showed a less significant reduction (VEGF and Cathepsin L).

This raises the possibility the markers NGAL, KIM-1, IP-10 and Cystatin C could have a role in predicting rejection in the ABOi transplants. Interestingly these are the same markers (other than the inclusion of KIM-1) that showed promise in the HLA analysis.

It could be hypothesised that in those patients who do not go on to develop rejection adequate immunosuppression has been achieved as evidenced by the fall in the levels of the biomarkers, raising the possibility that monitoring of levels and their changes could allow tailoring of immunosuppression.

Absence of a fall in levels may be important and raises the question of whether at this early time-point post transplantation that rejection is affecting the kidney or the biomarkers are acting as surrogate markers of immune activation within the graft or systemically. In order to determine this non-immunologically mediated injury would have to be compared to determine whether that caused similar changes to marker levels.

4.6 Non-antibody incompatible transplants and predicting Delayed Graft Function

As mentioned previously the occurrence of acute rejection in the recruited cohort was so low that any analysis looking for indicators of rejection amongst the biomarkers is impossible. This is common to many transplant studies using acute rejection and large cohort studies are undoubtedly necessary. However, the changes in marker levels do provide interesting counter point for those changes seen in the more immunologically complex transplants.

The nature of the recipient recruitment also ensured that a greater proportion of the patients recruited were deceased donor transplants and therefore rates of delayed graft function in the “normal” group are comparatively large. This enabled the assessment of these biomarkers in the setting of predicting delayed graft function from the recipient perspective.

As can be demonstrated the only serum biomarker for delayed graft function was cystatin C which had an AUC of 0.82 at day1 post-transplant. This has potentially useful clinical implications as a serum test which may offer advantages over a urine test as it is not subject to the same potential confounders as a urine marker e.g. oliguria post operatively, underlying native urine produced by damaged kidneys and dilution resultant from intravenous fluid shifts. The potential negative to a serum test, compared to urine test, is that levels may be influenced by dialysis and some patients undergoing transplantation do require dialysis immediately post-operatively as a result of hyperkalaemia produced by the surgery.

Analysis of the urine demonstrated that KIM-1 and NGAL levels were also predictive of DGF (AUC 0.76,0.78). With the availability of a point of care urine test for KIM-1

levels it is conceivable that it might be possible to determine at a very early time-point post-transplant how likely the patient is to develop DGF. Whilst direct mechanisms to intervene and change the clinical course of delayed graft function are currently unavailable this is an area of growing interest.

The analysis of the HLAi cohort predictors of delayed graft function is difficult to interpret due to the high incidence of rejection in those with delayed graft function. This is especially true of IP-10 whose levels have been shown to correlate to rejection on day 1. Although difficult to analyse, as the number are small, Cathepsin L, does appear to differentiate DGF on day 1 in the HLA group. While drawing hard and fast conclusions seems premature it could be hypothesised that this difference in Cathepsin L reflec a combination of the differing neointimal changes already set in motion within the kidneys and the differing degrees of apoptosis within those kidneys with DGF and those with immediate function.

It is interesting that Cystatin C, which showed good predictive power in the “normal” transplants failed to differentiate in the HLAi transplants. This suggests that findings between the different groups of transplants (immunologically uncomplicated, ABOi and HLAi and possibly even live donor vs. deceased donor) may well display different biomarker changes, suggesting differing underlying processes or affects. This means that any finding in one group needs to be verified in the other rather than purely accepted as being generalizable to the entire transplant population.

5 Limitations of study

Perhaps one of the biggest limitations with the study is that all samples were stored and analysed in batches retrospectively. This raises a number of issues, not least that some of the biomarkers may have degraded over time so levels may not entirely reflect levels in fresh samples. Whilst the experiments detailed before aimed to demonstrate the effects of freeze thaw and delay in freezing the effect of long term storage which some of the samples have been subject to is more difficult to assess. The only way round this is prospectively analysed fresh samples. The numbers of participants in the ABOi and the HLAi study groups are large, however, this has meant that collection over a protracted period was necessary (particularly for the HLAi). This not only raises the storage issue but seemingly small changes to treatment protocol or immunosuppressive regimes over the period of recruitment may be present.

The deceased donor biomarker work was undertaken only on DBD cardiac donors who represent a very specific group of organ donors, so prior to any firm conclusions being drawn extrapolation to a full clinical trial would be essential.

The non-antibody incompatible transplant analysis is limited by recruitment numbers. It would be extremely interesting to examine biomarker changes in this cohort with regard to rejection, however, the low rates of rejection mean that for meaningful statistical analysis to be undertaken significantly larger numbers of patients undergoing transplantation would need to be recruited. Despite this they still act as a

good counterpoint to the changes in the other groups although they are a hetergenous group.

A further limitation of the study is that for the non-antibody incompatible transplant group and the live donor group the urine biomarker levels were analysed without normalising to the urinary creatinine. There remains debate on when or how urinary biomarkers should be normalised to urinary creatinine as in some situations, for example established AKI, it may be that non-normalised biomarker levels may more accurately represent the clinical situation (Ralib et al., 2012). However, it may be that normalisation of the biomarker levels to creatinine might have provided a different insight in these groups and, therefore, it remains a limitation.

Renal transplant recipients represent a very heterogenous group with a widespread aetiology of renal failure and other co-morbidities. Inherently this makes it difficult to generalise and biomarker work needs to be carried out across much larger study populations than has been undertaken to date so that any differences as a result of underlying disease process rather than the transplant outcome can be controlled for.

6 Development of the work from this thesis

1. Role of KIM-1 in assessing deceased organ donors

Following the identification of urinary KIM-1 as a mechanism of identifying AKI in cardiac organ donors (DBD) and its correlation to early graft outcome this should be assessed in a wider range of organ donors. Most notably it would be good to extrapolate this work in the form a prospective clinical trial encompassing all organ donors. If the hypothesis that KIM-1 can help identify AKI holds true for a wider donor cohort then the use of a point-of-care test to routinely identify this would potentially be extremely useful and could find a role in routine donor assessment.

2. Role of IP-10 in predicting rejection

The changes in IP-10 levels between rejectors and non-rejectors both in the HLAi group and the ABOi are very interesting. More work looking into whether IP-10 is a biomarker or actually a potential target in the process would be worthwhile, however, it would be useful to set up a prospective clinical trial stratifying HLAi patients into low and high risk of rejection on day 1 post-transplant and to potentially modify their surveillance.

3. Role of biomarkers in identifying delayed graft function

Given the demonstration of both urinary and serum biomarkers that can predict delayed graft function a trial using some novel interventions to try to ameliorate the

delayed graft function would be useful to demonstrate a real function and it may be that changes to biomarker levels actually provide standardised endpoints for such research.

In this research I have demonstrated that Biomarkers can help inform on renal transplants in a number of settings. A possible role in the identifying DBD donors whole have incurred AKI which may translate to DGF has been identified. Similarly potential markers in immunologically complex transplants that may help predict early rejection have been suggested.

7 References:

Brtish Transplantation Society (2004): Guidelines relating to solid organ transplant from non-heart beating donors. London.

Emerging safety science: Workshop Summary (2008). *Institute of Medicine (US) Forum on Drug DIscovery, Development and Translation.* Washington DC: National Academies Press (US).

2005. British Transplantation Society Renal Association (2005). Guidelines for living kidney donation. 2nd ed.: British Transplantation Society and Renal Assoication.

March 2010. Chest pain of recent onset. NICE Clinical Guideline.

BARTEL, G., WAHRMANN, M., SCHWAIGER, E., KIKIC, Z., WINZER, C., HORL, W., MUHLBACHER, F., HOKE, M., ZLABINGER, G., REGELE, H. & BOHMIG, G. 2013. Solid phase detection of C4d-fixing HLA antibodies to predict rejection in high immunological risk kidney transplant recipients. *Transplant International,* 26**,** 121-30.

BASANYAKE, K., STRINGER, S., HUTCHINSON, C. & COCKWELL 2011. The biology of immunoglobulin free light chains and kidney injury. *Kidney Int,* 79**,** 1289-1301.

BENNETT, M., DENT, C. L., MA, Q., DASTRALA, S., GRENIER, F., WORKMAN, R., SYED, H., ALI, S., BARASCH, J. & DEVARAJAN, P. 2008. Urine NGAL Predicts Severity of Acute Kidney Injury After Cardiac Surgery: A Prospective Study. *Clinical Journal of the American Society of Nephrology,* 3**,** 665-673.

BLASCO, V., LEONE, M., BOUVENOT, J., GEISSIER, A., ALBANESE, J. & MARTIN, C. 2007. Impact of intensive care on renal function before graft harvest: results of a monocentric study. *Crit Care* 11**,** r103.

BOHMIG, G., WAHRMANN, M., REGELE, H. & 2007. Immunoadsorption in severe C4d-positive acute kidney allograft rejection: a randomised controlled trial. *Am J Transplant,* 7**,** 117-121.

BOLIGNANO, D., COPPOLINO, G., CAMPO, S., ALOISI, C., NICOCIA, G., FRISINA, N. & BEUEMI, M. 2008. Urinary neutrophil gelatinase-associated lipocalin (NGAL) is associated with the severity of renal disease in proteinuric patients. *Nephrol Dial Transplant,* 23**,** 414-416.

BRIGUORI, C., VISCONTI, G., RIVERA, N. V., FOCACCIO, A., GOLIA, B., GIANNONE, R., CASTALDO, D., DE MICCO, F., RICCIARDELLI, B. & COLOMBO, A. 2010. Cystatin C and Contrast-Induced Acute Kidney Injury. *Circulation,* 121**,** 2117-2122.

BRINKERT, F., KEMPER, M. J., BRIEM-RICHTER, A., VAN HUSEN, M., TRESZL, A. & GANSCHOW, R. 2010. High prevalence of renal dysfunction in children after liver transplantation: non-invasive diagnosis using a cystatin C-based equation. *Nephrology Dialysis Transplantation*.

BUOB, D., HAZAN, M., HOMS, S., MATIGNON, M., MANSOUR, H., AUDARD, V., DESVAUX, D., REMY, P., NOEL, C., BASTUJI-GARIN, S. C., J, LANG, P. & GRIMBERT, P. 2011. Intrarenal IFN-gamma mRNA expression differentiates clinical and subclinical glomerulitis in renal transplant recipients. *Transplantation,* 92**,** 170-5.

CALNE, R. 1960. The rejection of renal homografts. Inhibition in dogs by 6-mercaptopurine. *Lancet,* 1**,** 417-418.

CARVALHO MATOS, A. C., DURÃO JR, M. S. & PACHECO-SILVA, A. 2004. Serial beta-2 microglobulin measurement as an auxilliary method in the early diagnosis of cytomegalovirus infection in renal transplant patients. *Transplantation Proceedings,* 36**,** 894-895.

CEPPELLINI, R., BAGLIANI, S., CURTONI, E. & LEIGHEB, G. 1969. Experimental allotransplantation in man: II. The role of A_1, A_2 and B antigens; III Enchancement by circulating antibody. *Transplantation Proceedings,* 1**,** 390-394.

CHAN, L. Atlas of diseases of the kidney. *In:* SCHRIER, R. (ed.) *Transplant rejection and its treatment.* ISN Informatics Commission

CHAPMAN, J., O'CONNELL, P. & NANKIVELL, B. J. 2005. Chronic Renal Allograft Dysfunctio. *J Am Soc Nephrol,* 16**,** 3015-3026.

CHATURVEDI, S., FARMER, T. & KAPKE, G. 2009. Assay Validation for KIM-1: human urinary renal dysfunction biomarker. *Int J Biol Sci,* 5**,** 128-134.

CHIUSOLO, A., DEFAZIO, R., ZANETTI, E., MONGILLO, M., MORI, N., CRISTOFORI, P. & TREVISAN, A. 2010. Kidney Injury Molecule-1 Expression in Rat Proximal Tubule after Treatment with Segment-Specific Nephrotoxicants: A Tool for Early Screening of Potential Kidney Toxicity. *Toxicologic Pathology,* 38**,** 338-345.

COCKCROFT, D. & GAULT, M. 1976. Prediction of creatinine clearance from serum creatinine. *Nephron,* 16**,** 31-41.

CUMMINS, B., AUCKLAND, M. & P, C. 1987. Cardiac-specific troponin-I radioimmunoassay in the diagnosis of acute myocardial infarction . *Am Heart J,* 113**,** 1333-44.

CUSHING, H. 1902. Some experimental and clinical observation concerning states of increased intracranial tension. . *AM J Med Sci,* 124**,** 375-400.

DAUSSET, J. 1980. Nobel lecture: The major histocompatibility complex in man - past, present and future concepts. *Physiology or medicine*.

DEGAUQUE, N., MARIAT, C., KENNY, J., SANCHEZ-FUEYO, A., ALEXOPOULOS, S. P., KUCHROO, V., ZHENG, X.-X. & STROM, T. B. 2007. Regulation of T-Cell Immunity by T-Cell Immunoglobulin and Mucin Domain Proteins. *Transplantation,* 84**,** S12-S16.

DEGAUQUE, N., MARIAT, C., KENNY, J., ZHANG, D., GAO, W., VU, M. D., ALEXOPOULOS, S., OUKKA, M., UMETSU, D. T., DEKRUYFF, R. H., KUCHROO, V., ZHENG, X. X. & STROM, T. B. 2008. Immunostimulatory Tim-1–specific antibody deprograms Tregs and prevents transplant tolerance in mice. *Journal of Clinical Investigation,* 118**,** 735-741.

DENT, C. L., MA, Q., DASTRALA, S., BENNETT, M., MITSNEFES, M. M., BARASCH, J. & DEVARAJAN, P. 2007. Plasma neutrophil gelatinase-associated lipocalin predicts acute kidney injury, morbidity and mortality after pediatric cardiac surgery: a prospective uncontrolled cohort study. *Critical Care,* 11**,** R127.

DIETERLE, F., PERENTES, E., CORDIER, A., ROTH, D. R., VERDES, P., GRENET, O., PANTANO, S., MOULIN, P., WAHL, D., MAHL, A., END, P., STAEDTLER, F., LEGAY, F., CARL, K., LAURIE, D., CHIBOUT, S.-D., VONDERSCHER, J. & MAURER, G. 2010. Urinary clusterin, cystatin C, β2-microglobulin and total protein as markers to detect drug-induced kidney injury. *Nature Biotechnology,* 28**,** 463-469.

DOMINQUEZ, J., LIRA, F., REBOLLEDO, R., TRONCOSO, P., ARAVEN, C., ORTIZ, M. & GONZALEZ, V. R. 2009. Duration of delayed graft function is an important predictors of 1-year serum creatinine. *Transplant Proc,* 41**,** 131-2.

DONNAHOO, K., MENG, X., AYALA, A., CAIN, M., HARKEN, A. & MELDRUM, D. 1999. Early kidney TNF-alpha expression mediates neutrophil infiltration and injury after renal ischaemia-reperfusion. *Am J Physiol* 277**,** R922-9.

DORGE, S., ROUX-LOMBARD, P., DAYER, J., KOCH, K., FREI, U. & LONNERMANN, G. 1994. PLasma levels of tumour necrosis factor (TNF) and soluble TNF receptors in kidney transplant recipients. *transplantation,* 58**,** 1000-8.

DU, Y., ZAPPITELLI, M., MIAN, A., BENNETT, M., MA, Q., DEVARAJAN, P., MEHTA, R. & GOLDSTEIN, S. L. 2010. Urinary biomarkers to detect acute kidney injury in the pediatric emergency center. *Pediatric Nephrology,* 26**,** 267-274.

ECONOMIDOU, J., HUGH-JONES, N. & GARDNER, B. 1967. Quantitative measurements concerning A and B antigen sites. *Vox Sang,* 12**,** 321-328.

EDWARDS, L., HELDERMAN, J., HAMM, L., LUDWIN, D., GAILIUNAS, P. & HULL, A. 1983. Noninvasive monitoring of renal transplant function by analysis of beta-2-microglobulin. *Kidney Int,* 23**,** 767-770.

EICBAUM, F. & BISSETTI, P. 1971. Cardiovascular disturbances following increases in intracranial pressure. *Cardiovas Res,* 5.

EIRIN, A., GLOVICZKI, M. L., TANG, H., RULE, A. D., WOOLLARD, J. R., LERMAN, A., TEXTOR, S. C. & LERMAN, L. O. 2012. Chronic renovascular hypertension is associated with elevated levels of neutrophil gelatinase-associated lipocalin. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*.

ENDRE, Z. H., PICKERING, J. W., WALKER, R. J., DEVARAJAN, P., EDELSTEIN, C. L., BONVENTRE, J. V., FRAMPTON, C. M., BENNETT, M. R., MA, Q., SABBISETTI, V. S., VAIDYA, V. S., WALCHER, A. M., SHAW, G. M., HENDERSON, S. J., NEJAT, M., SCHOLLUM, J. B. W. & GEORGE, P. M. 2011. Improved performance of urinary biomarkers of acute kidney injury in the critically ill by stratification for injury duration and baseline renal function. *Kidney International,* 79**,** 1119-30.

FARNEY, A., ROGERS, J., ORLANDO, G., AL-GEIZAWI, S., BUCKLEY, M., FAROOQ, U., AL-SHRAIDEH, Y. & STRATTA, R. 2013. Evolving experience using kidneys from deceased donors with terminal acute kidney injury. *J Am Coll Surg,* 216**,** 645-55.

FERRARA, N., HOUCK, K., JAKEMAN, L., WINER, J. & LEUNG, D. 1991. THe vascular endothelial growth factor family of polypeptides. *J Cell Biochem,* 47**,** 211-218.

FLO, T., SMITH , K., SATO, S., RODRIQUEZ, D., HOLMES, M., STRONG, R., AKIRA, S. & ADEREM, A. 2004. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestering iron. *Nature,* 432**,** 917-921.

FLOWER, D. 1996. The lipocalin protein family: structure and function. *Biochem J,* 318**,** 1-14.

FREITAS, M., UCHIDA, Y., LASSMAN, C., DANOVITCH, G., BUSUTTIL, R. & KUPIEC-WEGLINSKI, J. W. 2011. Type 1 interferon pathway mediates renal ischaemi/reperfusion injury. *Transplantation,* 92**,** 131-8.

GIRAL, M., BERTOLA, J., FOUCHER, Y., VILLERS, D., BIRONNEAU, E., BLANLOEIL, Y., KARAM, G., DAGUIN, P., LERAT, L. & SOULILLOU, J. 2007. Effect of brain-dead donor resuscitation on delayed graft function: results of a monocentric analysis. *Transplantation,* 83**,** 1174-81.

GIRAL-CLASSE, M., HOURMANT, M., CANTAROVICH, D., DANTAL, J., BLANCHO, G., DAQUIN, P., ANCELET, D. & SOULILLOU, J.-P. 1998. Delayed graft function of more than six days strongly decreases long-term survival of transplanted kidneys. *Kidney Int,* 54**,** 972-8.

GLOOR, J. & STEGALL, M. 2007. ABO incompatible kidney transplantation. *Current Opinion in Nephrology and Hypertension,* 16**,** 529-534.

GLOOR, J., WINTERS, J., CORNELL, L., FIX, L., DEGOEY, S., KNAUER, R., COSIO, F., GANDHI, M., KREMERS, W. & STEGALL, M. D. 2010. Baseline donor-specfici antibody levels and outcomes in positive crossmatch kidney transplantation. *Am J Transplant,* 10**,** 582-9.

GOLDSMITH, P., PINE, J., RIDGWAY, D., ECUYER, C., POLLARD, S., ATTIA, M., MENON, K. & AHMAD, N. 2010. Renal transplantation following donation after cardiac death: impact of duration from withdrawl to asystole. *Transplantation Proceedings,* 42**,** 3966-3967.

GROUP, B. D. W. 2001. Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clinical Pharmacology & Therapeutics,* 69**,** 89-95.

HAASE, M., BELLOMO, R., DEVARAJAN, P., MA, Q., BENNETT, M. R., MÖCKEL, M., MATALANIS, G., DRAGUN, D. & HAASE-FIELITZ, A. 2009a. Novel Biomarkers Early Predict the Severity of Acute Kidney Injury After Cardiac Surgery in Adults. *The Annals of Thoracic Surgery,* 88**,** 124-130.

HAASE, M., BELLOMO, R., DEVARAJAN, P., SCHLATTMANN, P. & HAASE-FIELITZ, A. 2009b. Accuracy of Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Diagnosis and Prognosis in Acute Kidney Injury: A Systematic Review and Meta-analysis. *American Journal of Kidney Diseases,* 54**,** 1012-1024.

HAASE, M., BELLOMO, R. & HAASE-FIELITZ, A. 2010. Neutrophil gelatinase-associated lipocalin. *Curr Opin Crit Care,* 16**,** 526-532.

HALL, I. E., DOSHI, M. D., POGGIO, E. D. & PARIKH, C. R. 2011. A Comparison of Alternative Serum Biomarkers With Creatinine for Predicting Allograft Function After Kidney Transplantation. *Transplantation,* 91**,** 48-56.

HALL, I. E., YARLAGADDA, S. G., COCA, S. G., WANG, Z., DOSHI, M., DEVARAJAN, P., HAN, W. K., MARCUS, R. J. & PARIKH, C. R. 2009. IL-18 and Urinary NGAL Predict Dialysis and Graft Recovery after Kidney Transplantation. *Journal of the American Society of Nephrology,* 21**,** 189-197.

HAMER, R., ROCHE, L., SMILLIE, D., HARMER, A., MITCHELL , D., MOLOSTVOV, G., LAM, F., KASHI, H., TAN, L., IMRAY, C., FLETCHER, S., BRIGGS, D., LOWE, D., ZEHNDER, D. & HIGGINS, R. 2010. Soluble CD30 and Cd27 levels in patients undergoing HLA antibody incompatible renal transplantation. *Transplant Immunology,* 23**,** 161-165.

HAMILTON, D. & REID, W. 1984. Yu Yu Voronoy and the first human kidney allograft. *Surg Gynecol Obstet,* 159**,** 289-97.

HAN, W. K., WAGENER, G., ZHU, Y., WANG, S. & LEE, H. T. 2009. Urinary Biomarkers in the Early Detection of Acute Kidney Injury after Cardiac Surgery. *Clinical Journal of the American Society of Nephrology,* 4**,** 873-882.

HANCOCK, W., GAO, W., CSIZMADIA, V., FAIA, K., SHEMMERI, N. & LUSTER, A. 2001. Donor-derived IP-10 initiates development of acute allograft rejection. *J Exp Med,* 193**,** 975-80.

HANCOCK, W., LU, B., GAO, W., CSIZMADIA, V., FAIA, K., KING, J., SMILEY, S., LING, M., GERARD, N. & GERARD, C. 2000. Requirement of the Chemokine Receptor CXCR3 for Acute Allograft Rejection. *Journal of Experimental Medicine* , 192**,** 1515-1519.

HANTO, D., MAKI, T., YOON, M., CSIZMADIA, E., CHIN, B., GALLO , D., KONDURU, B., KURAMITSU, K., SMITH, N., BERSEENBRUGGE, A., ATTANASIO, C., THOMAS, M., WEGIEL, B. & OTTERBEIN, L. 2010. Intraoperative administration of inhaled carbon monoxide reduces delayed graft function in kidny allografts in swine. *Am J Transplant,* 10**,** 2421-30.

HARIHARAN, S. 2001. Long-term kidney transplant survival. *American Journal of Kidney Diseases,* 38**,** S44-S50.

HARIHARAN, S., ADAMS, M., BRENNAN, D., DAVIS, C., FIRST, M., JOHNSON, C., OUSEPH, R., PEDDI, V., PELX, C., ROZA, A., VINCENTI, F. & GEORGE, V. 1999. Recurrent and de novo glomerular disease after renal transplantation:a report from Renal Allograft Disease Registry (RADR). *Transplantation,* 68**,** 635-41.

HARIHARAN, S., JOHNSON, C., BRESNAHAN, B., TARANTON, B., MCINTOSH, M. & STABLEIN, D. 2000. Improved graft survival after renal transplantation in the United States, 1988 to 1996. *N Engl J Med,* 342**,** 605-12.

HARIRIAN, A., NOGUEIRA, J., KUKURUGA, D., SCHWEITZER, E., HESS, J., GURK-TURNER, C., JACOBS, S., DRACHENBERG, C., BARTLETT, S. & COOPER, M. 2009. Positive cross-match living donor kidney transplantation: longer-term outcomes. *Am J Transplant,* 9**,** 536-42.

HASKELL, C., CLEARY, M. & CHARO, I. 2000. Unique role of the chemokine domain of fractalkine in cell capture. Kinetics of receptor dissociation correlate with cell adhesion. *j Biol Chem,* 275**,** 34183-9.

HERGET-ROSENTHAL, S. 2004. Prognostic Value of Tubular Proteinuria and Enzymuria in Nonoliguric Acute Tubular Necrosis. *Clinical Chemistry,* 50**,** 552-558.

HERGET-ROSENTHAL, S., MARGGRAF, G., HUSING, J., GORING, F., PIETRUCK, F., JANSSEN, O., PHILLIP, T. & KRIBBEN, A. 2004. Early detection of acute renal failure by serum cystatin C. *Kidney Int,* 66**,** 1115-1122.

HERGETROSENTHAL, S., BOKENKAMP, A. & HOFMANN, W. 2007. How to estimate GFR-serum creatinine, serum cystatin C or equations? *Clinical Biochemistry,* 40**,** 153-161.

HEYNE, N., KEMMNER, S., SCHNEIDER, C., NADALIN, S., KONIGSRAINER, A. & HARING, H. 2012. Urinary Neutrophil Gelatinase-Associated Lipocalin accurately detects acute allograft rejection among other causes of acute kidney injury in renal allograft recipients. *Transplantation,* 93**,** 1252-1257.

HIGGINS, R., LOWE, D., HATHAWAY, M., LAM, F., KASHI, H., TAN, L., IMRAY, C., FLETCHER, S., CHEN, G., KHRISHNAN, N., HAMER, R., ZEHNDER, D. & BRIGGS, D. 2009. Rises and falls in donor-specific and third-party HLA antibody levels after antibody incompatible transplantation. *Transplantation,* 87**,** 882-888.

HIGGINS, R., LOWE, D., HATHAWAY, M., WILLIAMS, C., LAM, F., KASHI, H., TAN, L., IMRAY, C., FLETCHER, S., CHEN, K., KRISHNAN, N., HAMER, R., DAGA, S., EDEY, M., ZEHNDER, D. & BRIGGS, D. 2011. Human leukocyte antigen antibody-incompatbile incompatible renal transplantation: excellent medium-term outcomes with negative cytotoxic crossmatch. *Transplantation,* 92**,** 900-6.

HIGGINS, R., ZEHNDER, D., CHEN, K., LOWE, D., MCKINNELL, J., LAM, F., KASHI, H., TAN, L., IMRAY, C., FLETCHER, S., KRISHNAN, N., HAMER, R. & BRIGGS, D. 2010. The histological development of acute antibody-mediated rejection in HLA antibody-incompatible renal transplantation. *Nephrol Dial Transplant,* 25**,** 1306-1312.

HIPPEN, B. 2006. The sensitized recpient: What is to be done? *Am J Transplant,* 6**,** 2230-31.

HO, J., LUCY, M., KROKHIN, O., HAYGLASS, K., PASCOE, E., DARROCH, G., RUSH, D., NICKERSON, P., RIGATTO, C. & RESLEROVA, M. 2009. Mass Spectrometry–Based Proteomic Analysis of Urine in Acute Kidney Injury Following Cardiopulmonary Bypass: A Nested Case-Control Study. *American Journal of Kidney Diseases,* 53**,** 584-595.

HOLLMEN, M. E., KYLLÖNEN, L. E., INKINEN, K. A., LALLA, M. L. T. & SALMELA, K. T. 2010. Urine neutrophil gelatinase-associated lipocalin is a marker of graft recovery after kidney transplantation. *Kidney International,* 79**,** 89-98.

HOOGLAND, E., SNOEIJIS, M. & VAN HEURN, L. 2010. DCD kidney transplantation: results and measures to improve outcome. *Curr opin organ transplant,* 15**,** 177-82.

HOSGOOD, S., HUNTER, J. & NICHOLSON, M. 2012. Early Urinary Biomarkers of Warm and Cold Ischaemic Injury in an Experimental Kidney Model. *J Surg Research,* 174**,** e85-e90.

HU, H., AIZENSTEIN, B. D., PUCHALSKI, A., BURMANIA, J. A., HAMAWY, M. M. & KNECHTLE, S. J. 2004. Elevation of CXCR3-Binding Chemokines in Urine Indicates Acute Renal-Allograft Dysfunction. *American Journal of Transplantation,* 4**,** 432-437.

HUTCHINSON, C. & LANDGREN, O. 2011. Polyclonal immunoglobulin free light chains as a potential biomarker of immune stimulation and inflammation. *Clinical Chemistry,* 57**,** 10.

ICHIMURA, T. 2004. Kidney injury molecule-1: a tissue and urinary biomarker for nephrotoxicant-induced renal injury. *AJP: Renal Physiology,* 286**,** 552F-563.

ICHIMURA, T., ASSELDONK, E. J., HUMPHREYS, B. D., GUNARATNAM, L., DUFFIELD, J. D. & BONVENTRE, J. 2008. Kidney injury molecule-1 is a phophatidylserine receptor that confers a phagocytic phenotye on epithelial cells. *Journal of Clinical Investigation,* 118**,** 1657-1668.

ICHIMURA, T., BONVENTRE, J., BAILLY, V., WEI, H., HESSION, C., CATE, R. & SANICOLA, M. 1998. Kidney Injury Molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain is up-regulated in renal cells after injury. *Journal of Biological Chemistry,* 273**,** 4135-4142.

IMAI, T., HIESHIMA, K., HASKELL, C., BABA, M., NAGIRAN, M., NISHIMURA, M., KAKIZAKI, M., TAKAGI, S., NOMIYAMA, H., SCHALL, T. & YOSHIE, O. 1997. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell,* 91**,** 521-530.

IRISH, W. D. 2003. Nomogram for Predicting the Likelihood of Delayed Graft Function in Adult Cadaveric Renal Transplant Recipients. *Journal of the American Society of Nephrology,* 14**,** 2967-2974.

JUNG, C., PARK, K., KIM, S., KIM, S., KIM, M., JO, S., CHO, W. & KIM, H. 2013. Clinical outcomes in kidney transplantation patients from deceased donors with acute kidney injury. *Transplant Proc,* 45**,** 2941-5.

KAHAN, B. 1993. Toward a rational design of clinical trials of immosuppressive agents in transplantation. *Immunological Reviews,* 136**,** 29-49.

KAMIŃSKA, D., KOŚCIELSKA-KASPRZAK, K., DRULIS-FAJDASZ, D., HAŁOŃ, A., POLAK, W., CHUDOBA, P., JAŃCZAK, D., MAZANOWSKA, O., PATRZAŁEK, D. & KLINGER, M. 2011. Kidney ischemic injury genes expressed after donor brain death are predictive for the outcome of kidney transplantation. *Transplant Proc,* 43**,** 2891-4.

KARCZEWSKI, J., KARCZEWSKI, M. & WIKTOROWICZ, K. 2010. Pretransplant urine cytokine pattern predicts acute kidney rejection. *Cytokine,* 51**,** 10-11.

KASISKE, B., STEWART, D., BISTA, B., SALKOWSKI, N., SNYDER, J., ISRANI, A., CRARY, G., ROSENDALE, J., MATAS, A. & DELMONICO, F. 2014. The role of procurement biopsies in acceptance decicions for kidneys retrieved for transplant. *Clin J Am Soc Nephrol,* 9**,** 562-71.

KATUS, H., REMPPIS, A., LOOSER, S., HALLERMEIER, K., SCHEFFOLD, T. & KUBLER, W. 1989. Enzyme linked immunoassay of cardiac troponin T for the detection of acute myocardial infarction in patients. *J Moll Cell Cardiol,* 21**,** 1349-53.

KLEIN, R., GALANTE, N., DE SANDES-FREITAS, T., DE FRANCO, M., TEDESCO-SILVA, H. & MEDINA-PESTANA, J. 2013. Transplantation with kidneys retrieved from deeased donors with acute renal failure. *Transplantation,* 95**,** 611-6.

KNIGHT, E., VERHAVE, J., SPIEGELMAN, D., HILLEGE, H., ZEEUW, D., CURHAN, G. & DE JONG, P. 2004. Factors influencing serum cystatin C levels other than renal function and the impact on renal function measurement. *Kidney Int,* 65**,** 1416-1421.

KOOSTRA, G., DAEMOIN, J. & OOMEN, A. 1995. Categories on non-heart beating donors. *Transplant Proc,* 27**,** 2893.

KORBELY, R., WILFLINGSEDER, J., PERCO, P., KAINZ, A., LANGER, R., MAYER, B. & OBERBAUER, R. 2011. Molecular biomarker candidates of acute kidney injury in zero-hour renal transplant needle biopsies. *Transplant International,* 24**,** 143-149.

KRAMER, A., VAN TIMMEREN, M. M., SCHUURS, T., VAIDYA, V., BONVENTRE, J., VAN GOOR, H. & NAVIS, G. 2009. Reduction of proteinuria in adriamycin-induced nephropathy is associated with reduction of renal kidney injury molecule (Kim-1) over time. *Am J Physiol Renal Physiol,* 296.

KUSTOGIANNIS, D., PAGLIARELLO, G., DOIG, C., ROSS, H. & SHEMIE, S. 2006. Medical management to optimize donor organ potential : a review of the literature. *Can J Anaesth,* 53**,** 820-30.

KUYPERS, D., NEUMAYER, H., FRITSCHE, L., BUDDE, K., RODICIO, J., VANRENTERGHEN, Y. & GROUP, L. S. 2004. Calcium channel blockade and preservation of renal graft function in cyclosporin-treated recipients: a prospective randomized placebo-controlled 2-year study. *Transplantation,* 78**,** 1204-11.

LAMB, E., TOMSON, C. & RODERICK, P. 2005. Clinical sciences reviews committee of the association for clinical biochemistry: Estimating kidney function in adults using formulae. *Ann Clin Biochem,* 42**,** 321-45.

LANDSTEINER, K. 1961. On agglutination of normal human blood. *Transfusion* 1**,** 5-8.

LAZZERI, E., ROTONDI, M., MAZZINGHI, B., LASAGNI, L., BUONAMANO, A., RODATI, A., PRADELLA, F., FOSSOMBRONI, V., LA VILLA, G., GACCI, M., BERTONI, E., SERIO, M., SALVADORI, M. & ROMAGNANI, P. 2005. High CXCL10 expression in rejected kidneys and predictive role of pretransplant serum CXCL10 for acute rejection and chronic allograft rejection. *Transplantation,* 79**,** 1215-1220.

LE BRICON, T., THERVET, E., BENLAKEHAL, M., BOUSQUET, B., LEGENDRE, C., ERLICH, D. & 1999. Changes in plasma Cystatin C after renal transplantation and acute rejection in adults. *Clin Chem ,* 45**,** 2243-2249.

LEGGAT, J., OJO, A., LEIGHTMAN, A., PORT, F. K., WOLFE, R., TURENNE, M. & HELD, P. 1997. Long-term renal allograft survival: prognostic implication of the timing of acute rejection episode. *Transplantation,* 63**,** 1268-72.

LEVEY, A. S., BOSCH, J., LEWIS, J., GREENE, T., ROGERS, N. & ROTH, D. 1999. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease study group. *Ann Intern Med,* 130**,** 461-70.

LIANGOS, O., PERIANAYAGAM, M. C., VAIDYA, V. S., HAN, W. K., WALD, R., TIGHIOUART, H., MACKINNON, R. W., LI, L., BALAKRISHNAN, V. S., PEREIRA, B. J. G., BONVENTRE, J. V. & JABER, B. L. 2007. Urinary N-Acetyl-beta-(D)-Glucosaminidase Activity and Kidney Injury Molecule-1 Level Are Associated with Adverse Outcomes in Acute Renal Failure. *Journal of the American Society of Nephrology,* 18**,** 904-912.

LIU, J. 2012. Evaluation of Serum Cystatin C for Diagnosis of acute rejection after renal transplantation. *Transplant Proc,* 44**,** 1250-1253.

LOVERRE, A., DIVELLA, C., CASTELLANO, G., TATARANNI, T., ZAZA, G., ROSSINI, M., DITONNO, P., BATTAGLIA, M., PALAZZO, S., GIGANTE, M., RANIERI, E., SCHENA, F. & GRANDALIANO, G. 2011. T helper 1,2 abd 17 cell subsets in renal transplant patients with delayed graft function. *Transplant International,* 24**,** 233-242.

MA, Y. C., ZUO, L., CHEN, J. H., LUO, Q., YU, X. Q., LI, Y., XU, J. S., HUANG, S. M., WANG, L. N., HUANG, W., WANG, M., XU, G. B. & WANG, H. Y. 2007. Improved GFR estimation by combined creatinine and cystatin C measurements. *Kidney International,* 72**,** 1535-1542.

MALYSZKO, J., KOC-ZORAWSKA, E., MALYSZKO, J. S. & MYSLIWIEC, M. 2010. Kidney Injury Molecule-1 Correlates With Kidney Function in Renal Allograft Recipients. *Transplantation Proceedings,* 42**,** 3957-3959.

MÅRTENSSON, J., BELL, M., OLDNER, A., XU, S., VENGE, P. & MARTLING, C.-R. 2010. Neutrophil gelatinase-associated lipocalin in adult septic patients with and without acute kidney injury. *Intensive Care Medicine,* 36**,** 1333-1340.

MARTENSSON, J., MARTLING, C. & BELL, M. 2012. Novel biomarkers of acute kidney injury and failure: clinical applicability. *Br J Anaesth,* 109**,** 843-850.

MATZ, M., BEYER, J., WUNSCH, D., MASHREGHI, M. F., SEILER, M., PRATSCHKE, J., BABEL, N., VOLK, H. D., REINKE, P. & KOTSCH, K. 2006. Early post-transplant urinary IP-10 expression after kidney transplantation is predictive of short- and long-term graft function. *Kidney International,* 69**,** 1683-1690.

MAZZUCCO, G., MAGNANI, C., FORTUNATO, M., TODESCO, A. & MONGA, G. 2010. The reliability of pre-transplant donor renal biopsies (PTDB) in predicting the kidney state. A comparative single-centre study on 154 untransplanted kidneys . *NDT,* 25**,** 3401-3408.

MEDAWAR, P. 1944. Behaviour and fate of skin autografts and skin homografts in rabbits. *J Anat,* 78**,** 176-199.

MEIJER, E., BOERTIEN, W. E., NAUTA, F. L., BAKKER, S. J. L., VAN OEVEREN, W., ROOK, M., VAN DER JAGT, E. J., VAN GOOR, H., PETERS, D. J. M. & NAVIS, G. 2010. Association of Urinary Biomarkers With Disease Severity in Patients With Autosomal Dominant Polycystic Kidney Disease: A Cross-sectional Analysis. *American Journal of Kidney Diseases,* 56**,** 883-895.

MELDRUM, K., MELDRUM, D., MENG, X., AO, L. & HARKEN, A. 2002. TNF-alpha-dependent bilateral renal injury is induced by unilateral renal ischemia-reperfusion. *AM J physiol Heart Circ Physiol,* 282**,** H540-6.

MIGLINAS, M., SURANAVICIENE, L., MATEIKAITE, K., SKEBAS, K. & KUBILIENE, A. 2013. Delayed graft function: risk factors and the effects of early function and graft survival. *Transplant Proc,* 45**,** 1363-7.

MISHRA, J., DENT, C., TARABISHI, R., MITSNEFES, M., MA, Q., KELLY, C., RUFF, S., ZAHEDI, K., SHAO, M. & BEAN, J. 2005. Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. *The Lancet,* 365**,** 1231-1238.

MISHRA, J., MA, Q., PRADA, A., MITSNEFES, M., ZAHEDI, K., YANG, J., BARASCH, J. & DEVARAJAN, P. 2003. Identification of Neutrophil Gelatinase-Associated Lipocalin as a Novel Early Urinary Biomarker for Ischemic Renal Injury. *Journal of the American Society of Nephrology,* 14**,** 2534-2543.

MISHRA, J., MORI, K., MA, Q., KELLY, C., YANG, J., MITSNEFES, M., BARASCH, J. & DEVARAJAN, P. 2004. Amelioration of Ischemic Acute Renal Injury by Neutrophil Gelatinase-Associated Lipocalin. *Journal of the American Society of Nephrology,* 15**,** 3073-3082.

MITSNEFES, M. M., KATHMAN, T. S., MISHRA, J., KARTAL, J., KHOURY, P. R., NICKOLAS, T. L., BARASCH, J. & DEVARAJAN, P. 2006. Serum neutrophil gelatinase-associated lipocalin as a marker of renal function in children with chronic kidney disease. *Pediatric Nephrology,* 22**,** 101-108.

MORI, K., LEE, H., RAPOPORT, D., DREXLER, I., K, F., YANG, J., SCHMIDT-OTT, K. M., CHEN, X., LI, J., WEISS, S., MISHRA, J., CHEEMA, F., MARKOWITZ, G., SUGANAMI, T., SAWAI, K., MUKOYAMA, M., KUNIS, C., D'AGATI, V., DEVARAJAN, P. & BARASCH, J. 2005. Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. *Journal of Clinical Investigation,* 115**,** 610-621.

MORRIS, P. 1994. *Kidney Transplantation: principles and practice.*, WB Saunders.

MURRAY, J., MERRILL, J. & DAMMIN, G. 1960. Study of transplantation immunity after whole body irradiation: clinical and experimental investigation. *Surgery,* 48**,** 272.

MURRAY, J., MERRILL, J. & HARRISON, J. 1958. Kidney transplantation between seven pairs of identical twins. *Ann Surg,* 148.

NANKIVELL, B. J., BORROWS, R. J., FUNG, C. L. S., O'CONNELL, P., ALLEN, R. & CHAPMAN, J. 2003. The natural history of chronic allograft nephropathy. *N Engl J Med,* 349**,** 2326-33.

NARAYANAN, R., CARDELLA, C., CATTRAN, D., COLE, E., TINCKAM, K., SCHIFF, J. & KIM, S. 2010. Delayed graft function and the risk of death with graft function in living donor kidney transplant recipients. *Am J Kidney Dis,* 56**,** 961-70.

NEJAT, M., PICKERING, J. W., WALKER, R. J., WESTHUYZEN, J., SHAW, G. M., FRAMPTON, C. M. & ENDRE, Z. H. 2010. Urinary cystatin C is diagnostic of acute kidney injury and sepsis, and predicts mortality in the intensive care unit. *Critical Care,* 14**,** R85.

NEMES, B., ZÁDORI, G., GELLEY, F., GÁMÁN, G., GÖRÖG, D., DOROS, A. & SÁRVÁRY, E. 2010. Can a Cutoff Value for Cystatin C in the Operative Setting Be Determined to Predict Kidney Function After Liver Transplantation? *Transplantation Proceedings,* 42**,** 2323-2326.

NETT, P., HEISEY, D., SHAMES, B., FERNANDEZ, L., PIRSCH, J. & SOLLINGER, H. 2005. Influence of kidney funtion to the impact of acute rejection on long term kidney transplant survivial. *Transplant International,* 18**,** 385-9.

NEUFELD, G., COHEN, T., GENRINOVITCH, S. & POLTARAK, Z. 1999. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J,* 13**,** 9-22.

NEWSTEAD, C., LAMB, W., BRENCHLEY, P. & SHORT, C. 1993. Serum and urine IL-6 and TNF-alpha in renal transplant recipients with graft dysfunction. *Transplantation,* 56**,** 831-5.

NICHOLSON, M. & HOSGOOD, S. 2013. Renal transplantation after ex vivo nortmothermic perfusion: the first clinical study. *Am J Transplant,* 13**,** 1246-52.

NICKOLAS, T. L., FORSTER, C. S., SISE, M., BARASCH, N., SOLA-DEL VALLE, D., VILTARD, M., BUCHEN, C., KUPFERMAN, S., CARNEVALI, M., BENNETT, M., MATTEI, S., BOVINO, A., ARGENTIERO, L., MAGNANO, A., DEVARAJAN, P., MORI, K., ERDJUMENT-BROMAGE, H., TEMPST, P., ALLEGRI, K. & BARASCH, J. 2012. NGAL monomer is associated with tubulointerstitial damage in chronic kidney disease. *Kidney Int,* 82**,** 718-722.

NIJBOER, W., SCHUURS, T., VAN DER HOEVEN, J. A. B. & PLOEG, R. 2004. Effect of brain death and donor treatment on organ inflammatory response and donor organ viability. *Curr Opin Organ Transplant,* 9**,** 110-115.

NIJBOER, W. N., SCHUURS, T. A., DAMMAN, J., VAN GOOR, H., VAIDYA, V. S., VAN DER HEIDE, J. J. H., LEUVENINK, H. G. D., BONVENTRE, J. V. & PLOEG, R. J. 2009. Kidney Injury Molecule-1 is an Early Noninvasive Indicator for Donor Brain Death-Induced Injury Prior to Kidney Transplantation. *American Journal of Transplantation,* 9**,** 1752-1759.

NOGARE, A. L., JOELSONS, G., PEDROSO, J. A. R., VERONESE, F. J. V., GONÇALVES, L. F. & MANFRO, R. C. 2010. Quantitative Analyses of Kidney Injury Molecule-1 Messenger RNA in Kidney Transplant Recipients With Graft Dysfunction. *Transplantation Proceedings,* 42**,** 473-474.

NOZAKI, Y., NIKOLIC-PATERSON, D. J., SNELGROVE, S. L., AKIBA, H., YAGITA, H., HOLDSWORTH, S. R. & KITCHING, A. R. 2012. Endogenous Tim-1 (Kim-1) promotes T-cell responses and cell-

mediated injury in experimental crescentic glomerulonephritis. *Kidney International,* 81**,** 844-55.

NYGAARD, C., TOWNSEND, R. & DIAMOND, D. 1990. Organ donor management and organ outcome: a 6 year review from a level 1 trauma center. *J Trauma,* 30**,** 728-32.

O'CALLAGHAN, J., MORGAN, R., KNIGHT, S. & MORRIS, P. 2012. Systematic review and meta-analysis of hypothermic machine perfusion versus static cold storage of kidney allografts on transplant outcome. *BJS,* 94(suppl)**,** 277.

OETTING, W., ROGERS, T., KRICK, T., MATAS, A. & IBRAHIM, H. 2006. Urinary β2-Microglobulin Is Associated With Acute Renal Allograft Rejection. *American Journal of Kidney Diseases,* 47**,** 898-904.

OJO, A., R, W., HELD, P., PORT, F. K. & SCHMOUDER, R. 1997. Delayed graft fucntion: Risk factors and implications for renal allograft survival 1. *Transplantation,* 63**,** 968-974.

OZER, J. S., DIETERLE, F., TROTH, S., PERENTES, E., CORDIER, A., VERDES, P., STAEDTLER, F., MAHL, A., GRENET, O., ROTH, D. R., WAHL, D., LEGAY, F., HOLDER, D., ERDOS, Z., VLASAKOVA, K., JIN, H., YU, Y., MUNIAPPA, N., FOREST, T., CLOUSE, H. K., REYNOLDS, S., BAILEY, W. J., THUDIUM, D. T., TOPPER, M. J., SKOPEK, T. R., SINA, J. F., GLAAB, W. E., VONDERSCHER, J., MAURER, G., CHIBOUT, S.-D., SISTARE, F. D. & GERHOLD, D. L. 2010. A panel of urinary biomarkers to monitor reversibility of renal injury and a serum marker with improved potential to assess renal function. *Nature Biotechnology,* 28**,** 486-494.

PACHECO-SILVA, A., NISHIDA, S., SILVA, M., RAMOS, O., AJZEN, H., PESTANA, J. & PEREIRA, A. 1995. Increased production of beta-2 microglobulin in stable renal transplant patients. *Transplantation,* 59**,** 914-917.

PACZEK, L., PAZIK, J., TESCHNER, M., SCHAEFER, R., ROWINSKI, W., SZMIDT, J., LAO, M., ABRAGAROWICZ, K., GRADOWSKA, L., MORZYCKA-MICHALIK, M. & HEIDLAND, A. 1994. Human chronic kidney allograft rejection is accompanied by increased intraglomerular cathepsin B and L activity. *Transpl Int,* 7**,** S311-3.

PARAGAS, N., QIU, A., ZHANG, Q., SAMSTEIN, B., DENG, S.-X., SCHMIDT-OTT, K. M., VILTARD, M., YU, W., FORSTER, C. S., GONG, G., LIU, Y., KULKARNI, R., MORI, K., KALANDADZE, A., RATNER, A. J., DEVARAJAN, P., LANDRY, D. W., D'AGATI, V., LIN, C.-S. & BARASCH, J. 2011. The Ngal reporter mouse detects the response of the kidney to injury in real time. *Nature Medicine,* 17**,** 216-222.

PARIKH, C. R., DAHL, N., CHAPMAN, A., BOST, J., EDELSTEIN, C. L., COMER, D., ZELTNER, R., TIAN , X., GRANTHAM, J. & SOMLO, S. 2012. Evaluation of urine biomarkers of kidney injury in polycystic kidney disease. *Kidney Int,* 81**,** 784-790.

PARIKH, C. R., JANI, A., MISHRA, J., MA, Q., KELLY, C., BARASCH, J., EDELSTEIN, C. L. & DEVARAJAN, P. 2006. Urine NGAL and IL-18 are Predictive Biomarkers for Delayed Graft Function Following Kidney Transplantation. *American Journal of Transplantation,* 6**,** 1639-1645.

PATEL, A., PANCOSKA, C., MULGAONKAR, S. & WENG, F. 2007. Renal transplantation in patients with pre-transplant donor-specific antibodies and negative flow cytometry crossmatches. *7,* 10**,** 2371-7.

PATEL, R. & TERASAKI, P. 1969. Significance of the postivie cross-match test in kidney transplantation. *N Engl J Med,* 280**,** 735-9.

PATEL, S., PANKEWYCZ, O., NADER, N., ZACHARIAH, M., KOHIL, R. & LAFTAVI, M. 2012. Prognostic utility of hyporthermic machine perfusion in deceased donor renal transplantation *Transplant Proc,* 44**,** 2207-12.

PENG, W., CHEN, J., JIANG, Y., SHOU, Z., CHEN, Y. & WANG, H. 2007. Acute renal allograft rejection is associated with increased levels of vascular endothelial growth factor in the urine. *Nephrology,* 0**,** 070918212946005-???

PENG, W., CHEN, J., JIANG, Y., WU, J., SHOU, Z., HE, Q., WANG, Y., CHEN, Y. & WANG, H. 2008. Urinary fractalkine is a marker of acute rejection. *Kidney International,* 74**,** 1454-1460.

PENNEMANS, V., DE WINTER, L. M., FAES, C., VAN KERKHOVE, E., REYNDERS, C., RIGO, J.-M., SWENNEN, Q. & PENDERS, J. 2010. Effect of pH on the stability of kidney injury molecule 1 (KIM-1) and on the accuracy of its measurement in human urine. *Clinica Chimica Acta,* 411**,** 2083-2086.

PERALTA, C., KATZ, B., BONVENTRE, J., SABBISETTI, V. S., SISCOVICK, D., SARNAK, M. & SHILPAK, M. 2012. Associations of urinary levels of Kidney Injury Molecule (KIM-1) and Neutrophil Gelatinase-Associated Lipocalin (NGAL) with kidney function decline in the multi-ethnic study of atherosclerosis. *Am J Kidney Dis*.

PERIANAYAGAM, M. C., SEABRA, V. F., TIGHIOUART, H., LIANGOS, O. & JABER, B. L. 2009. Serum Cystatin C for Prediction of Dialysis Requirement or Death in Acute Kidney Injury: A Comparative Study. *American Journal of Kidney Diseases,* 54**,** 1025-1033.

PERICO, N., CATTANEO, D., SAYEGH, M. & REMUZZI, G. 2004. Delayed graft function in kidney transplantation. *Lancet,* 364**,** 1814-1827.

PERRONE, R., MADIAS, N. & LEVEY, A. S. 1992. Serum creatinine as an in index of renal function: new insights into old concepts. *Clin Chem,* 38**,** 1933-1953.

POGE, U. 2006. Calculation of glomerular filtration rate based on Cystatin C in cirrhotic patients. *Nephrology Dialysis Transplantation,* 21**,** 660-664.

POGE, U., GERHARDT, T., STOFFEL-WAGNER, B., PALMEDO, H., KLEHR, H.-U., SAUERBRUCH, T. & WOITAS, R. P. 2006. Prediction of glomerular filtration rate in renal transplant recipients: cystatin C or Modification of Diet in Renal Disease equation? *Clinical Transplantation,* 20**,** 200-205.

PORT, F., BRAGG-GRESHAM, J., METZGER, R., DYKSTRA, D., GILLESPIE, B., YOUNG, E., DELMONICO, F. L., WYNN, J. J., MERION, R., WOLFE, R. & HELD, P. 2002. Donor characteristics associated with reduced graft survival: an approach to expanding the pool of kidney donors. *Transplantation,* 74**,** 1281-1286.

PORTER, K. 1976. The effects of antibodies on human renal allografts. *Transplantation Proceedings,* 8**,** 189-197.

PRATSCHKE, J., WILHELM, M., KUSAKA, M., BASKER, M., COOPER, D., HANCOCK, W. & TILNEY, N. 1999. Brain death and its influence on donor organ quality and outcome after transplantation. *Transplantation,* 67**,** 343-348.

PROZIALECK, W. C., VAIDYA, V. S., LIU, J., WAALKES, M. P., EDWARDS, J. R., LAMAR, P. C., BERNARD, A. M., DUMONT, X. & BONVENTRE, J. V. 2007. Kidney injury molecule-1 is an early biomarker of cadmium nephrotoxicity. *Kidney International,* 72**,** 985-993.

QUIROGA, I., MCSHANE, P., KOO, D., GRAY, D., FRIEND, P., FUGGLE, S. & DARBY, C. 2006. Major effects of delayed graft function and cold ischaemia time on renal allograft survival. *Nephrol Dial Transplant,* 21**,** 1689-96.

RAIMUNDO, M., GUERRA, J., TEIZEIRA, C., SANTANA, A., SILVA, S., HOMENS, C. & DA COSTA, A. 2013. Intermediate early graft function is associated with increased incidence of graft loss and worse long-term graft function in kidney transplantation. *Transplant Proc,* 45**,** 1070-2.

RALIB, A., PICKERING, J., SHAW, G., DEVARAJAN, P., EDELSTEIN, C. L., BONVENTRE, J. & ENDRE, Z. 2012. Test characteristics of urinary biomarkers depend on quantification method in acute kidney injury. *J Am Soc Nephrol,* 23**,** 322-333.

RISCH, L., HERKLOTZ, R., BLUMBERG, A. & HUBER, A. 2001. Effects of Glucocorticoid Immunosuppression on Serum Cystatin C Concentrations in Renal Transplant Patients. *Clinical Chemistry,* 47**,** 2055-2059.

ROJAS-PENA, A., REOMA, J., KRAUSE, E., BOOTHMAN, E., PADIYAR, N., COOK, K., BARTLETT, R. & PUNCH, J. 2010. Extracorporeal support: improves donor renal graft function after cardiac death. *Am J Transplant,* 10**,** 1365-74.

ROOS, J., DOUST, J., TETT, S. & KIRKPATRICK, C. 2007. Diagnostic accuracy of cystatin C compared to serum creatinine for the estimation of renal dysfunction in adults and children—A meta-analysis. *Clinical Biochemistry,* 40**,** 383-391.

ROSENDALE, J., KAUFFMAN, H., MCBRIDE, M., CHABALEWSKI, F., ZAROFF, J., GARRITY, E., DELMONICO, F. & ROSENGARD, B. 2003. Aggressive pharmacologic donor management results in more transplanted organs. *Transplantation,* 75**,** 482-7.

ROTONDI, M., ROSATI, A., BUONAMANO, A., LASAGNI, L., LAZZERI, E., PRADELLA, F., FOSSOMBRONI, V., CIRAMI, C., LIOTTA, F., LA VILLA, G., SERIO, M., BERTONI, E., SALVADORI, M. & ROMAGNANI, P. 2004. High pretransplant serum levels of CXCL10/IP-10 are related to increased risk of renal allograft failure. *Am J Transplant,* 4**,** 1466-74.

RULE, A. D., BERGSTRALH, E. J., SLEZAK, J. M., BERGERT, J. & LARSON, T. S. 2006. Glomerular filtration rate estimated by cystatin C among different clinical presentations. *Kidney International,* 69**,** 399-405.

RYDBERG, L. 2001. ABO incompatibility in solid organ transplantation. *Transfusion Medicine,* 11**,** 325-342.

RYDBERG, L., BREIMER, M., SAMUELSSON, B. & BRYNGER, H. 1987. Blood group ABO-incompatible (A2 to O) kidney transplantation in human subjects: a clinical, serologic and biochemical approach. . *Transplantation Proceedings ,* 19**,** 4528-4537.

SANCHEZ-CASTANON, M., GAGO, M., FERNANDEZ-FRESNEDO, G., GOMEZ-ALMILLO, C., RUIZ-CRIADO, J., LOPEZ-HOYOS, M. & ARIAS, M. 2010. Quantitative assessment of serum free light chains in renal transplantation. *transplantation Proceedings,* 42**,** 2861-2863.

SARAIVA CAMARA, N., NISHIDA, S., PESTANA, J., PEREIRA, A., SESSO, R. & PACHECO-SILVA, A. 1998. Monitoring serum Beta-2 Microglobulin is useful for detecting patients wirth increased risk of acute rejection during reduction in immunosuppresion. *Transplantation Proceedings,* 30**,** 4158-4159.

SCHAUB, S., MAYR, M., H??NGER, G., BESTLAND, J., STEIGER, J. R., REGENITER, A., MIHATSCH, M. J., WILKINS, J. A., RUSH, D. & NICKERSON, P. 2007. Detection of Subclinical Tubular Injury After Renal Transplantation: Comparison of Urine Protein Analysis With Allograft Histopathology. *Transplantation,* 84**,** 104-112.

SCHAUB, S., WILKINS, J. A., ANTONOVICI, M., KROKHIN, O., WEILER, T., RUSH, D. & NICKERSON, P. 2005. Proteomic-Based Identification of Cleaved Urinary beta2-microglobulin as a Potential Marker for Acute Tubular Injury in Renal Allografts. *American Journal of Transplantation,* 5**,** 729-738.

SCHNUELLE, P., LORENZ, D. & A, M. 1999. Donor catecholamine use reduces acute allograft rejection and improves graft survival after cadaveric renal transplantation. *Kidney Int,* 56**,** 738-746.

SCHNUELLE, P., YARD, B., BRAUN, C., DOMINGUEZ-FERNANDEZ, E., SCHAUB, M., BIRCK, R., STURM, J., POST, S. & VAN DER WOUDE, F. 2004. Impact of donor dopamine on immediate graft function after kidney transplantation. *Am J Transplant,* 4**,** 419-26.

SCHRIJVERS, B., FLYVBERG, A. & VRIESE, A. 2004. The role of vascular endothelial growth factor (VEGF) in renal pathophysiology. *Kidney Int,* 65**,** 2203-17.

SCHWARTZ, D. & DAMESHEK, W. 1959. Drug induced immunoloigcal tolerance. *Nature,* 183**,** 1682-1683.

SCHWARZ, A., MENGEL, M., GWINNER, W., RADEMACHER, J., HISS, M., KREIPE, H. & HALLER, H. 2005. Risk factors for chronic allograft nephropathy after renal transplantation: a protocol biopsy study. *Kidney Int,* 67**,** 341-8.

SEGERER, S., HUGHES, E., HUDKINS, K., MACK, M., GOODPASTER, T. & ALPERS, C. 2002. Expression of the fractalkine receptor (CX3CR1) in human kidney diseases. *Kidney Int,* 62**,** 488-495.

SEGEV, D., GENTRY, S., WARREN, D., REEB, B. & MONTGOMERY, R. 2005. Kidney paired donation and optimising the use of live donor organs. *JAMA,* 293**,** 1883-1890.

SEVER, S., ALTINTAS, M., NANKOE, S., MOLLER, C., KO, D., WEI, C., HENDERSON, J., DEL RE, E., HSING, K., ERICKSON, A., COHEN, C., KRETZLER, M., KERJASCHKI, D., RUDENSKY, A., NIKOLIC, B. & REISER, J. 2007. Proteolytic processing of dynamin by cytoplasmic cathepsin L is a mechanism for proteinuric kidney disease. *J Clin Invest,* 117**,** 2095-104.

SHAHBAZI, M., FRYER, A., PRAVICA, V., BROGAN, I., RAMSAY, H., HUTCHINSON, I. & HARDEN, P. 2002. Vascular endothelial growth factor gene polymorphisms are addociated with acute renal allograft rejection. *JASN,* 13**,** 260-4.

SHARIF, A., ALACHKAR, N. & KRAUS, E. 2012. Incompatible kidney transplantation: a brief overview of the past, present and future. *QJM,* epub.

SHEMESH, O., GOLBETZ, H., KRISS, J. & MYERS, B. 1985. Limitations of reatinine as a filtration marker in glomerulopathic patients. *Kidney Int,* 28**,** 830-8.

SHOSKES, D. & CECKA, J. 1997. Effect of delayed graft function on short and long term kidney graft survival. *Clin Transplant***,** 297-303.

SIEDLECKI, A., IRISH, W. & BRENNAN, C. 2011. Delayed graft function in the kidney transplant. *American Journal of Transplantation,* 11**,** 2279-2296.

SIEW, E. D., WARE, L. B., GEBRETSADIK, T., SHINTANI, A., MOONS, K. G. M., WICKERSHAM, N., BOSSERT, F. & IKIZLER, T. A. 2009. Urine Neutrophil Gelatinase-Associated Lipocalin Moderately Predicts Acute Kidney Injury in Critically Ill Adults. *Journal of the American Society of Nephrology,* 20**,** 1823-1832.

SOLEZ, K., COLVIN, R. B., RACUSEN, L. C., HAAS, M., SIS, B., MENGEL, M., HALLORAN, P. F., BALDWIN, W., BANFI, G., COLLINS, A. B., COSIO, F., DAVID, D. S., DRACHENBERG, C., EINECKE, G., FOGO, A. B., GIBSON, I. W., GLOTZ, D., ISKANDAR, S. S., KRAUS, E., LERUT, E., MANNON, R. B., MIHATSCH, M., NANKIVELL, B. J., NICKELEIT, V., PAPADIMITRIOU, J. C., RANDHAWA, P., REGELE, H., RENAUDIN, K., ROBERTS, I., SERON, D., SMITH, R. N. & VALENTE, M. 2008. Banff 07 classification of renal allograft pathology: updates and future directions. *Am J Transplant,* 8**,** 753-60.

SOLEZ, K. & RACUSEN, L. C. 2013. The Banff classification revisited. *Kidney Int,* 83**,** 201-6.

SONKAR, G., SINGH, S., SONKAR, S., SINGH, U. & SINGH, R. 2013. Evaluation of serum interleukin 6 and tumour necrosis factor alpha levels, and their association with various non-immunological parameters in renal transplant recipients. *Singapore Med J,* 54**,** 511-5.

SONKAR, G., USHA & SINGH, R. 2008. A preliminary study on the significant value of beta-2-microglobluin over serum creatinine in renal transplant rejection and renal failure. *Singapore Med J,* 49**,** 786.

SOULEZ, M., SIROIS, I., BRASSARD, N., RAYMOND, M., NICODEME, F., NOISEUX, N., DUROCHER, Y., PSHEZHETSKY, A. & HEBERT, M. 2010. Epidermal growth factor and perlecan fragments produced by apoptotic endothelial cells co-ordinately activate ERK 1/2-dependent antiapoptotic pathways in mesenchymal stem cells. *Stem Cells,* 28**,** 810-20.

STAR, R. 1998. Treatment of acute renal failure. *Kidney Int,* 54**,** 1817-31.

STRIMBU, K. & TAVEL, J. 2010. What are biomarkers? *Curr Opin HIV AIDS,* 5**,** 463-466.

SUMMERS, D., JOHNSON, R., ALLEN, J., FUGGLE, S., COLLETT, D., WATSON, C. & BRADLEY, J. 2010. Analysis of factors that affect outcome after transplantation of kidneys donated after cardiac death in the UK: a cohort study. *Lancet,* 376**,** 1303-11.

SUPAVEKIN, S., ZHANG, W., KUCHERLAPATI, R., KASKEL, F., MOORE, L. & DEVARAJAN, P. 2003. Differential gene expression following early renal ischemia/reperfusion. *Kidney Int,* 63**,** 1714-24.

TAKAHASHI, K., SAITO, K. & SHIRO, T. 2006. Present status of ABO-incompatible kidney transplantation in Japan. . *Xenotransplantation,* 13**,** 118-122.

TATPUDI, R., MUTHUKUMAR, T., DADHANIA, D., DING, R., LI, B., SHARMA, V., LOZADA-PASTOIO, E., SEETHARAMU, N., HARTONO, C., D, S., SESHAN, S., KAPUR, S., HANCOCK, W. & SUTHANTHIRAN, M. 2004. Noninvasive detection of renal allograft inflammation by meaurements of mRNA and IP-10 and CXCR3 in urine. *Kidney Int,* 65**,** 2390-2397.

THIELKE, J., KAPLAN, B. & BENEDETTI, E. 2007. The role of ABO-incompatible living donors in kidney transplantation: state of the art. *Sem Nephrol,* 27**,** 408-413.

THIELKE, J., WEST-THIELKE, P., HERREN, H., BAREATO, U., OMMERT, T., VIDANOVIC, V., CAMPBELL-LEE, S., TZVETANOV, I., SANKRAY, H., KAPLAN, B., BENEDETTI, E. & OBERHOLZER, J. 2009.

Living donor kidney transplantation across positive crossmatch: the Unviersity of Illinois at Chicago experience. *Transplantation,* 87**,** 268-73.

TIDMAN, M., SJOSTROM, P. & JONES, I. 2007. A Comparison of GFR estimating formulae based upon s-cystatin C and s-creatinine and a combination of the two. *Nephrology Dialysis Transplantation,* 23**,** 154-160.

TONOMURA, Y., TSUCHIYA, N., TORII, M. & UEHARA, T. 2010. Evaluation of the usefulness of urinary biomarkers for nephrotoxicity in rats. *Toxicology,* 273**,** 53-59.

TRAYNOR, J., MACTIER, R., GEDDES, C. & FOX, J. 2006. How to measure renal function in clinical practice. *BMJ,* 333**,** 733-7.

TRECKMANN, J., MOERS, C., SMITS, J., GALLINAT, A., MAATHUIS, M., VAN KASTEROP-KUTZ, M., JOCHMANS, I., HOMAN VAN DER HEIDE, J. J., SQUIFFLET, J., VAN HEURN, E., KIRSTE, G., RAHMEL, A., LEUVENINK, H., PIRENNE, J., PLOEG, R. & PAUL, A. 2011. Machine perfusion versus cold storage for preservation of kidneys from expanded criteria donors after brain death. *Transpl Int,* 24**,** 548-54.

TSAI, J. P., WU, S. W., HUNG, T. W., KAO, W. T., HONG, C. L., LIAN, J. D. & CHANG, H. R. 2010. Diagnostic Performance of Serum Cystatin C and Serum Creatinine in the Prediction of Chronic Kidney Disease in Renal Transplant Recipients. *Transplantation Proceedings,* 42**,** 4530-4533.

TSUCHIDA, A., SALEM, H., THOMSON, N. & HANCOCK, W. 1992. Tumour necorsis factor production during human renal allograft rejection is associated with depression of plasma protien C and free protein S levels and decreased intragraft thrombomodulin expression. *J Exp Med,* 175**,** 81-90.

UCHIDA, Y., KE, B., FREITAS, M. C. S., JI, H., ZHAO, D., BENJAMIN, E. R., NAJAFIAN, N., YAGITA, H., AKIBA, H., BUSUTTIL, R. W. & KUPIEC-WEGLINSKI, J. W. 2010. The emerging role of T cell immunoglobulin mucin-1 in the mechanism of liver ischemia and reperfusion injury in the mouse. *Hepatology***,** NA-NA.

UENO, T., HABICHT, A., CLARKSON, M. R., ALBIN, M. J., YAMAURA, K., BOENISCH, O., POPOOLA, J., WANG, Y., YAGITA, H., AKIBA, H., ANSARI, M. J., YANG, J., TURKA, L. A., ROTHSTEIN, D. M., PADERA, R. F., NAJAFIAN, N. & SAYEGH, M. H. 2008. The emerging role of T cell Ig mucin 1 in alloimmune responses in an experimental mouse transplant model. *Journal of Clinical Investigation,* 118**,** 742-751.

UKTRANSPLANT. 2009, 2013. *UK Transplant 2009, 2013* [Online]. Available: http://www.organdonation.nhs.uk/ukt/statistics/statistics.jsp.

UMETSU, S. E., LEE, W.-L., MCINTIRE, J. J., DOWNEY, L., SANJANWALA, B., AKBARI, O., BERRY, G. J., NAGUMO, H., FREEMAN, G. J., UMETSU, D. T. & DEKRUYFF, R. H. 2005. TIM-1 induces T cell activation and inhibits the development of peripheral tolerance. *Nature Immunology,* 6**,** 447-454.

VAIDYA, V. S., FORD, G. M., WAIKAR, S. S., WANG, Y., CLEMENT, M. B., RAMIREZ, V., GLAAB, W. E., TROTH, S. P., SISTARE, F. D., PROZIALECK, W. C., EDWARDS, J. R., BOBADILLA, N. A., MEFFERD, S. C. & BONVENTRE, J. V. 2009. A rapid urine test for early detection of kidney injury. *Kidney International,* 76**,** 108-114.

VAIDYA, V. S., OZER, J. S., DIETERLE, F., COLLINGS, F. B., RAMIREZ, V., TROTH, S., MUNIAPPA, N., THUDIUM, D., GERHOLD, D., HOLDER, D. J., BOBADILLA, N. A., MARRER, E., PERENTES, E., CORDIER, A., VONDERSCHER, J., MAURER, G., GOERING, P. L., SISTARE, F. D. & BONVENTRE, J. V. 2010. Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. *Nature Biotechnology,* 28**,** 478-485.

VAIDYA, V. S., RAMIREZ, V., ICHIMURA, T., BOBADILLA, N. A. & BONVENTRE, J. V. 2006. Urinary kidney injury molecule-1: a sensitive quantitative biomarker for early detection of kidney tubular injury. *Am J Physiol Renal Physiol,* 290**,** F517-29.

VAIDYA, V. S., WAIKAR, S. S., FERGUSON, M. A., COLLINGS, F. B., SUNDERLAND, K., GIOULES, C., BRADWIN, G., MATSOUAKA, R., BETENSKY, R. A., CURHAN, G. C. & BONVENTRE, J. V. 2008.

Urinary biomarkers for sensitive and specific detection of acute kidney injury in humans. *Clin Transl Sci,* 1**,** 200-8.

VAN DER HOEVEN, J. A. B., MOLEMA, G., TER HORST, G., FREUND, R., WIERSEMA-BUIST, J. & REINOU 2003. Relationship between duration of brain death and heomdynamic instability on progressive dysfucntion and increased immunologic activation of donor kidneys.

VAN TIMMEREN, M. M., VAIDYA, V. S., VAN REE, R. M., OTERDOOM, L. H., DE VRIES, A. P. J., GANS, R. O. B., VAN GOOR, H., STEGEMAN, C. A., BONVENTRE, J. V. & BAKKER, S. J. L. 2007a. High Urinary Excretion of Kidney Injury Molecule-1 Is an Independent Predictor of Graft Loss in Renal Transplant Recipients. *Transplantation,* 84**,** 1625-1630.

VAN TIMMEREN, M. M., VAN DEN HEUVEL, M. C., BAILLY, V., BAKKER, S. J. L., VAN GOOR, H. & STEGEMAN, C. A. 2007b. Tubular kidney injury molecule-1 (KIM-1) in human renal disease. *The Journal of Pathology,* 212**,** 209-217.

VERGOULAS, G., BOURA, P. & EFSTRATIADIS, G. 2009. Brain dead donor kidneys are immunologically active: is intervention justified? *Hippokratia,* 13**,** 205-210.

VO, A., PENG, A., TOYODA, M., KAHWAJI, J., CAO, K., LAI, C., REINSMOEN, N., VILLICANA, R. & JORDAN, S. 2010. Use of intravenous immun globulin and rituximab for desnsitization of highly HLA-sensitized patients awaiting kidney transplantation. *Transplantation,* 89**,** 1095-102.

WAANDERS, F., VAIDYA, V., VANGOOR, H., LEUVENINK, H., DAMMAN, K., HAMMING, I., BONVENTRE, J., VOGT, L. & NAVIS, G. 2009. Effect of Renin-Angiotensin-Aldosterone System Inhibition, Dietary Sodium Restriction, and/or Diuretics on Urinary Kidney Injury Molecule 1 Excretion in Nondiabetic Proteinuric Kidney Disease: A Post Hoc Analysis of a Randomized Controlled Trial. *American Journal of Kidney Diseases,* 53**,** 16-25.

WAGENER, G., JAN, M., KIM, M., MORI, K., BARASCH, J., SLADEN, R. & THOMAS LEE, H. 2006. Association between increases in urinary neutrophil gelatinase-associated lipocalin and acute renal dysfunction after adult cardiac surgery. *Anaesthesiology,* 105**,** 485-91.

WATSON, C., WELLS, A., ROBERTS, R., AKOH, J., FRIEND, P., AKYOL, M., CALDER, F., ALLEN, J., JONES, M., COLLETT, D. & BRADLEY, J. 2010. Cold macine perfusion versus static cold storage of kidneys donated after cardiac death:a UK multientre randomized controlled trial. *Am J Transplant,* 10**,** 1991-9.

WEISS, S., KOTSCH, K., FRANCUSKI, M., REUTZEL-SELKE, A., MANTOUVALOU, L., KLEMZ, R., KUECUEK, O., JONAS, S., WESSLAU, C., ULRICH, F., PASCHER, A., VOLK, H. D., TULLIUS, S. G., NEUHAUS, P. & PRATSCHKE, J. 2007. Brain Death Activates Donor Organs and Is Associated with a Worse I/R Injury After Liver Transplantation. *American Journal of Transplantation,* 7**,** 1584-1593.

WELSH, K., VAN DAM, M., KOFFMAN, C., BEWICK, M., RUDGE, C., TAUBE, D. & CLARK, A. 1987. Transplantation of blood group A_2 kidneys into O or B recipients: The effect of pre-transplant anti-A titres on graft survival. *Transplantation Proceedings,* 19**,** 4565-4567.

WIGHT, J., CHILCOTT, J., HOLMES, M. & BREWER, N. 2003. Pulsatile machine perfusion vs. cold storage of kidneys for transplantation: a rapid and systematic review. *Clin Transplant,* 17**,** 293-307.

WILBRANDT, R., TUNG, K., DEODHAR, S., NAKAMOTO, S. & KLOFF, W. 1969. ABO blood group incompatibility in human renal homotransplantation. *American Journal of Clinical Pathology,* 51**,** 15-23.

WOO, K., CHOI, J., KIM, B., AN, W. & HAN, J. 2012. Urinary neutrophil gelatinase-associated lipocalin levels in comparison with glomerular filtration rate for evaluation of renal function in patients with chronic kidney disease. *Diabetes Metab J,* 36**,** 307-313.

WOO, K., LEE, E., LAU, Y. & LINM, C. 1985. Beta-2-microglobulin in the assessment of renal function in the transplanted kidney. *Nephron* 39**,** 223-227.

YAMANISHI, Y., KITAURA, J., IZAWA, K., KAITANI, A., KOMENO, Y., NAKAMURA, M., YAMAZAKI, S., ENOMOTO, Y., OKI, T., AKIBA, H., ABE, T., KOMORI, T., MORIKAWA, Y., KIYONARI, H., TAKAI, T., OKUMURA, K. & KITAMURA, T. 2010. TIM1 is an endogenous ligand for LMIR5/CD300b: LMIR5 deficiency ameliorates mouse kidney ischemia/reperfusion injury. *Journal of Experimental Medicine,* 207**,** 1501-1511.

YAP, S., PARK, S., EGAN, B. & LEE, H. 2012. Cytokine elevation and transaminitis after laparoscopic donor nephrectomy. *Am J Physiol Renal Physiol,* 302**,** 1104-11.

YARLAGADDA, S. G., COCA, S. G., FORMICA, R., POGGIO, E. D. & PARIKH, C. R. 2009. Association between delayed graft function and allograft and patient survival: a systematic revew and meta-analysis. *Nephrol Dial Transplant,* 24**,** 1039-47.

YUAN, X., ANSARI, M. J., D'ADDIO, F., PAEZ-CORTEZ, J., SCHMITT, I., DONNARUMMA, M., BOENISCH, O., ZHAO, X., POPOOLA, J., CLARKSON, M. R., YAGITA, H., AKIBA, H., FREEMAN, G. J., IACOMINI, J., TURKA, L. A., GLIMCHER, L. H. & SAYEGH, M. H. 2009. Targeting Tim-1 to overcome resistance to transplantation tolerance mediated by CD8 T17 cells. *Proceedings of the National Academy of Sciences,* 106**,** 10734-10739.

ZHANG, P. L., ROTHBLUM, L. I., HAN, W. K., BLASICK, T. M., POTDAR, S. & BONVENTRE, J. V. 2007. Kidney injury molecule-1 expression in transplant biopsies is a sensitive measure of cell injury. *Kidney International,* 73**,** 608-614.

ZHOU, Y., VAIDYA, V. S., BROWN, R. P., ZHANG, J., ROSENZWEIG, B. A., THOMPSON, K. L., MILLER, T. J., BONVENTRE, J. V. & GOERING, P. L. 2007. Comparison of Kidney Injury Molecule-1 and Other Nephrotoxicity Biomarkers in Urine and Kidney Following Acute Exposure to Gentamicin, Mercury, and Chromium. *Toxicological Sciences,* 101**,** 159-170.

8 Appendix A

Characteristics of an Ideal Biomarker

It should be visible early, prior to histopathological changes, and should be indicative after active damage
It should be sensitive, but it should also correlate with the severity of damage
It should be accessible in the peripheral tissue; in the case of the kidney, for example, it should be measurable in either the blood or the urine
It should be analytically stable in tissue so it can be measured after
It should be translational; that is, it should bridge across species.
It should be associated with a known mechanism.
A biomarker should be able to localize damage

9 Appendix B

Published work arising from this thesis:

Urinary biomarkers of acute kidney injury in deceased organ donors--kidney injury molecule-1 as an adjunct to predicting outcome.

Clin Transplant. 2014 Jul;28(7):808-15.

Field M, Dronavalli V, Mistry P, Drayson M, Ready A, Cobbold M, Inston N.

The use of NGAL and IP-10 in the prediction of early acute rejection in highly sensitized patients following HLA-incompatible renal transplantation.

Transpl Int. 2014 Apr;27(4):362-70

Field M, Lowe D, Cobbold M, Higgins R, Briggs D, Inston N, Ready AR.

TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

The use of NGAL and IP-10 in the prediction of early acute rejection in highly sensitized patients following HLA-incompatible renal transplantation

Melanie Field,[1] David Lowe,[2] Mark Cobbold,[3] Robert Higgins,[4] David Briggs,[2] Nicholas Inston[1] and Andrew R. Ready[1]

1 Department of Renal Transplantation, University Hospital Birmingham Foundation Trust, Edgbaston, UK
2 Histocompatibility and Immunogenetics, NHSBT Birmingham, Edgbaston Birmingham, UK
3 MRC Centre for Immune Regulation, School of Immunity and Infection, Medical School, University of Birmingham, Birmingham, UK
4 Department of Nephrology, University Hospitals of Coventry and Warwickshire, Coventry, UK

Keywords
HLA, IP-10, kidney transplant, NGAL, rejection.

Correspondence
Miss Melanie Field, Queen Elizabeth Hospital, Middleton Way, Edgbaston, Birmingham. B15 2WB, UK.
Tel.: 0121 3712000;
e-mail: melfield@doctors.org.uk

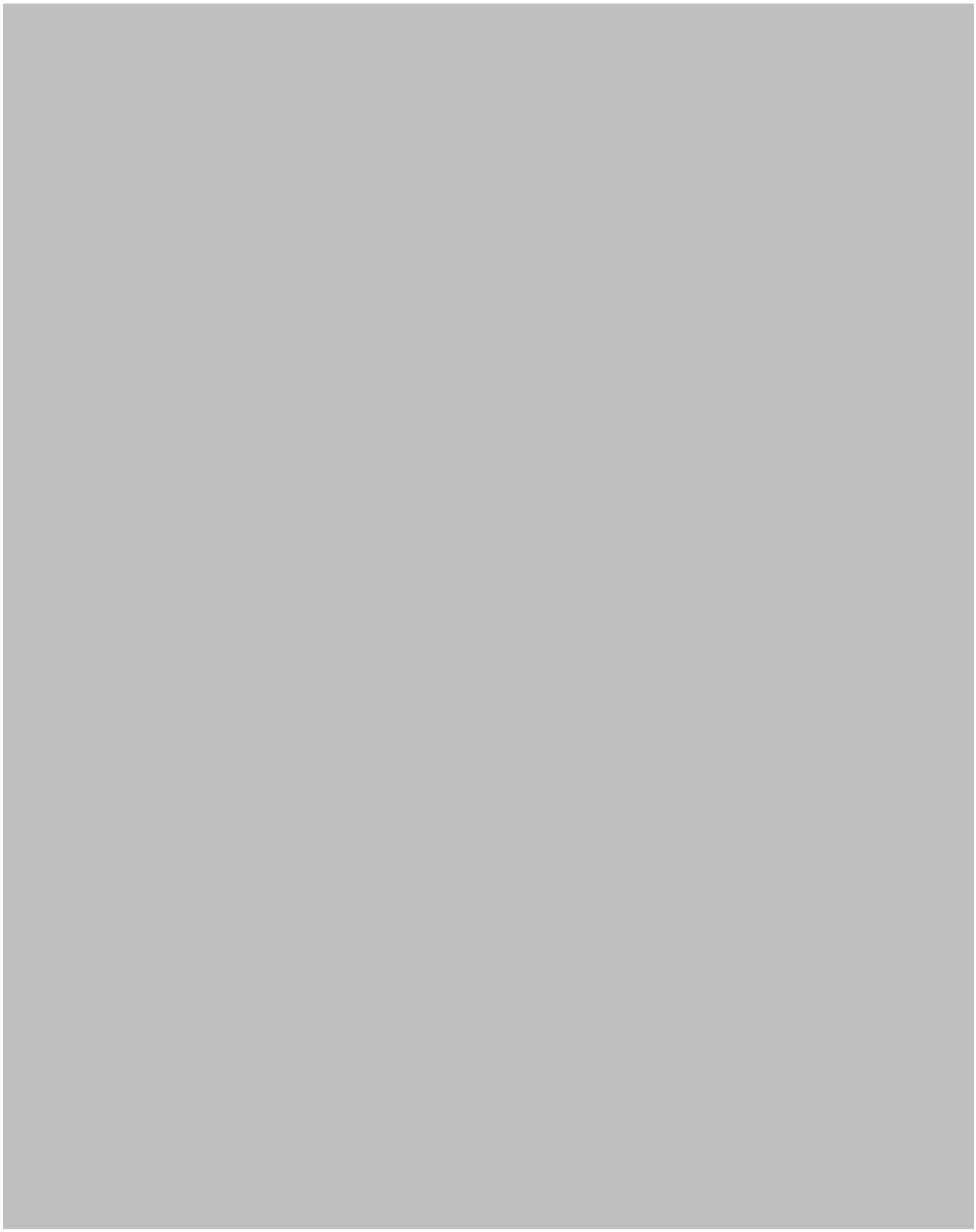
Conflicts of interest
The results of this study have not been published elsewhere in whole or in part.

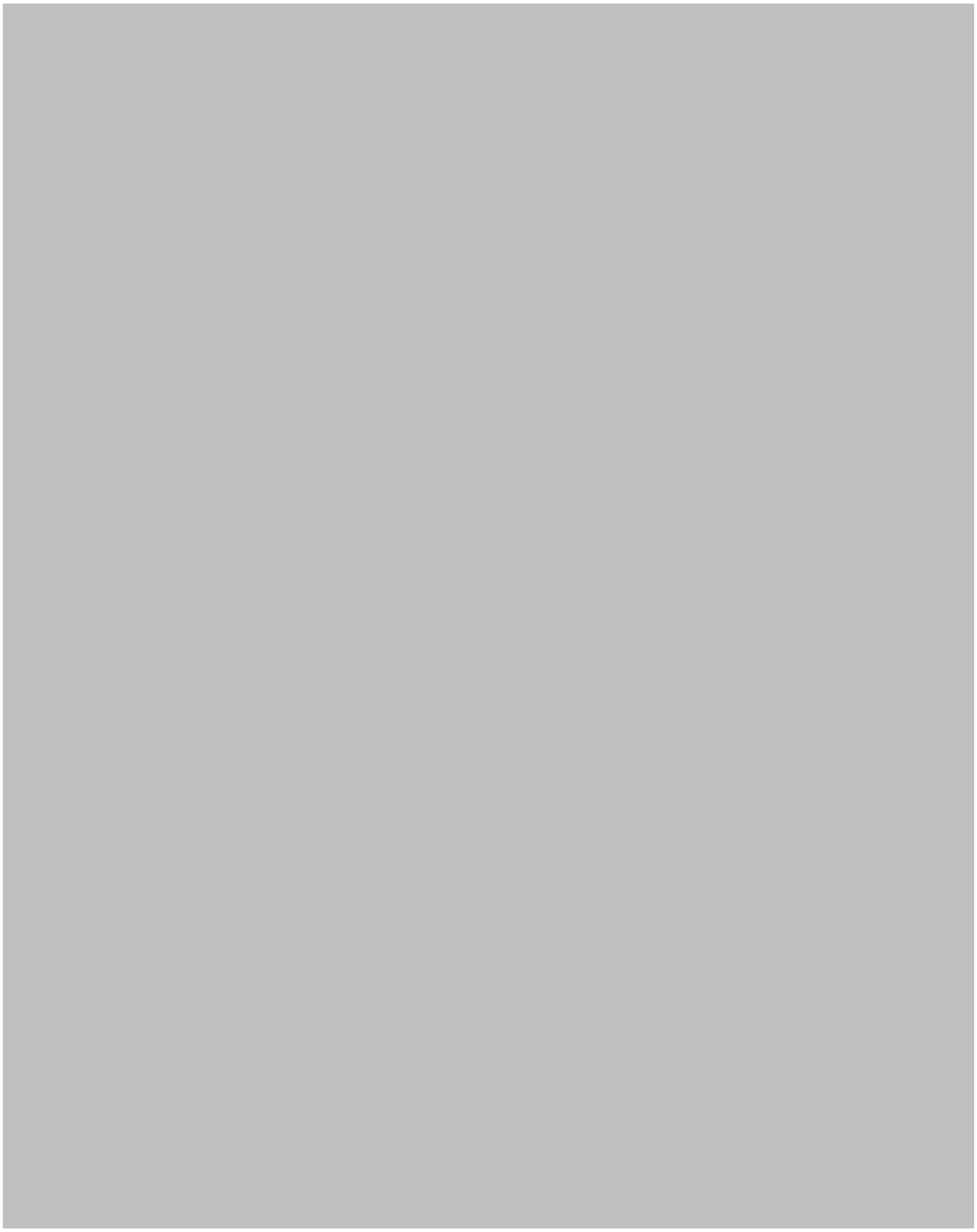
Received: 14 July 2013
Revision requested: 1 September 2013
Accepted: 13 January 2014
Published online: 11 February 2014

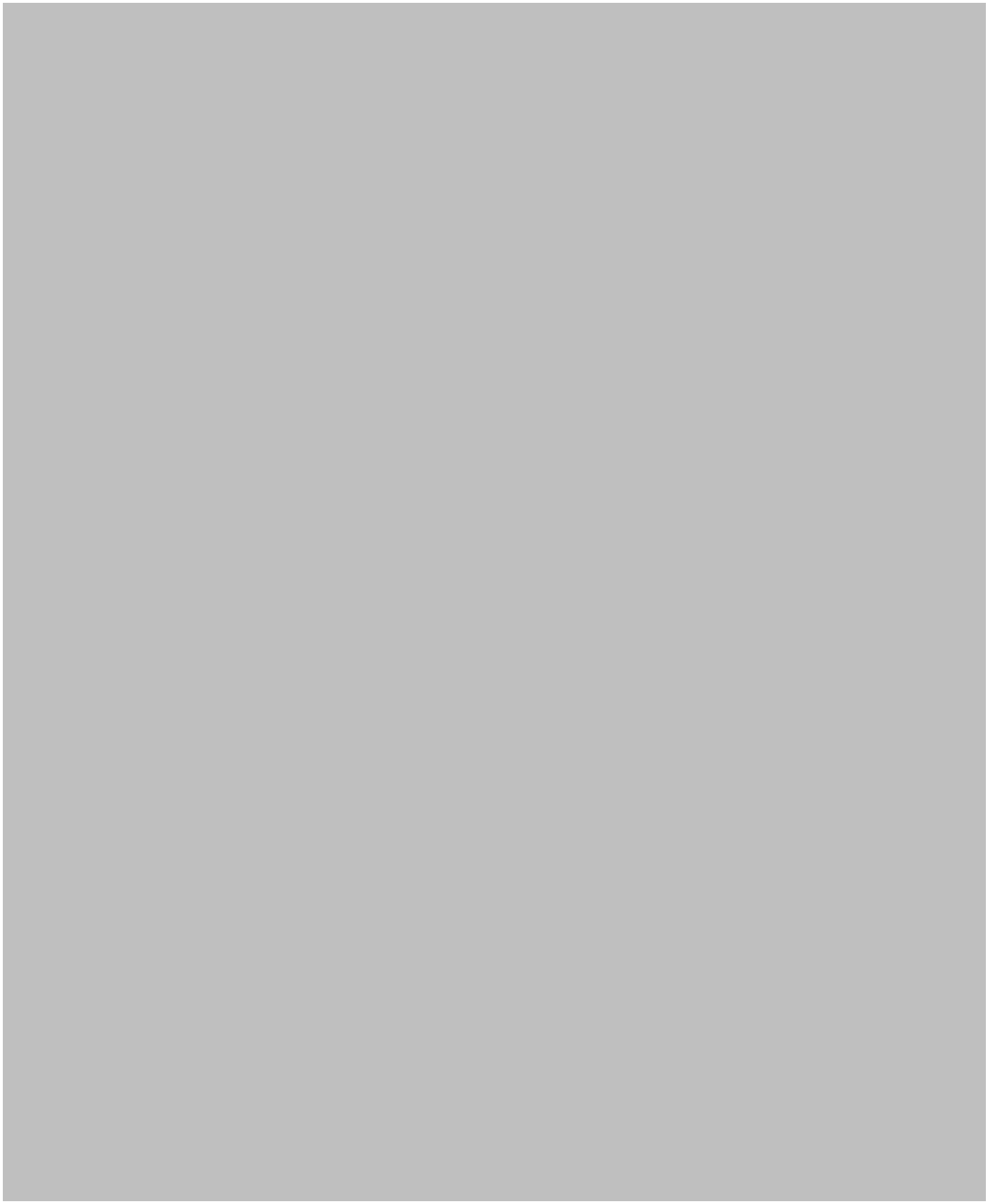
doi:10.1111/tri.12266

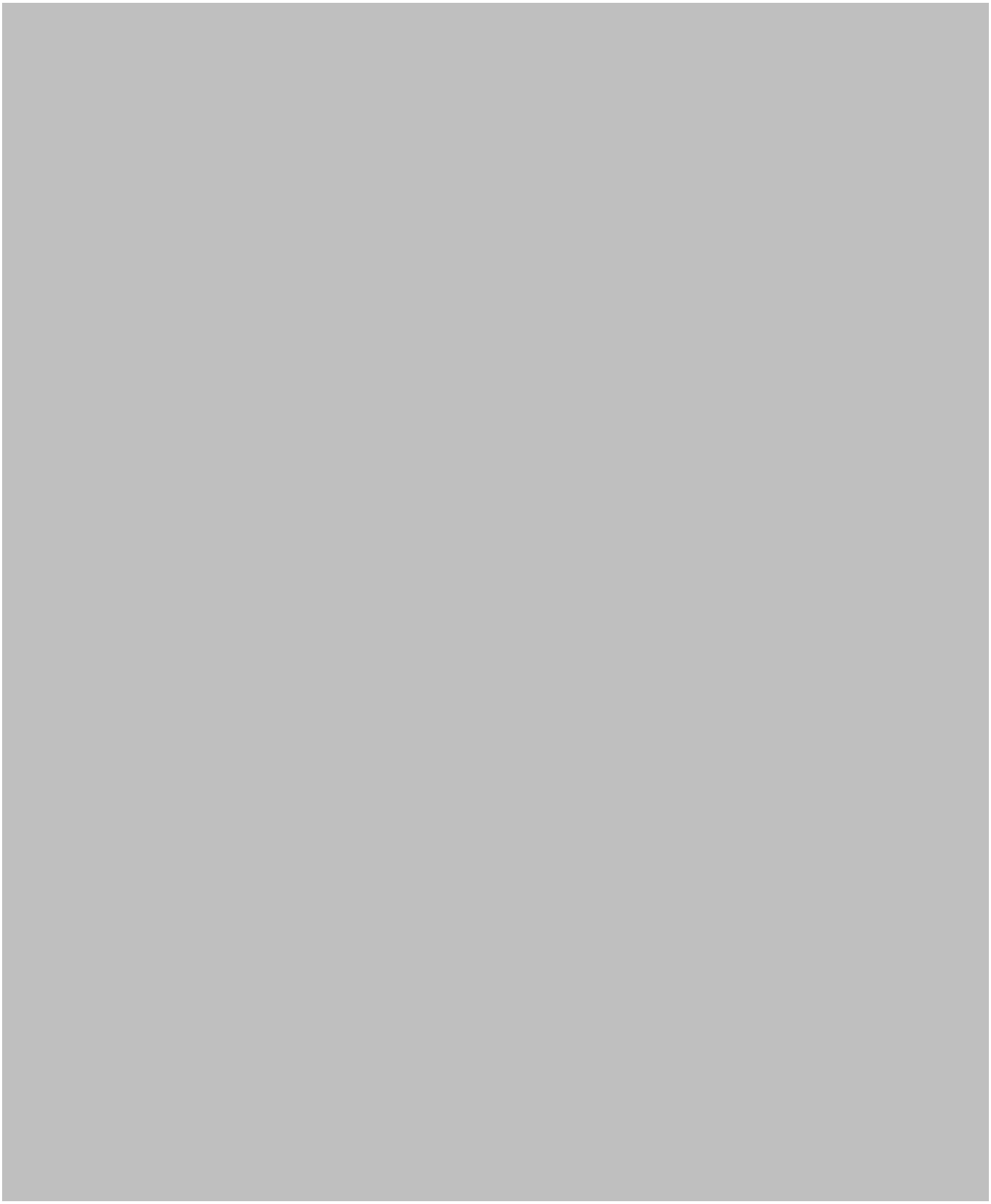
Summary

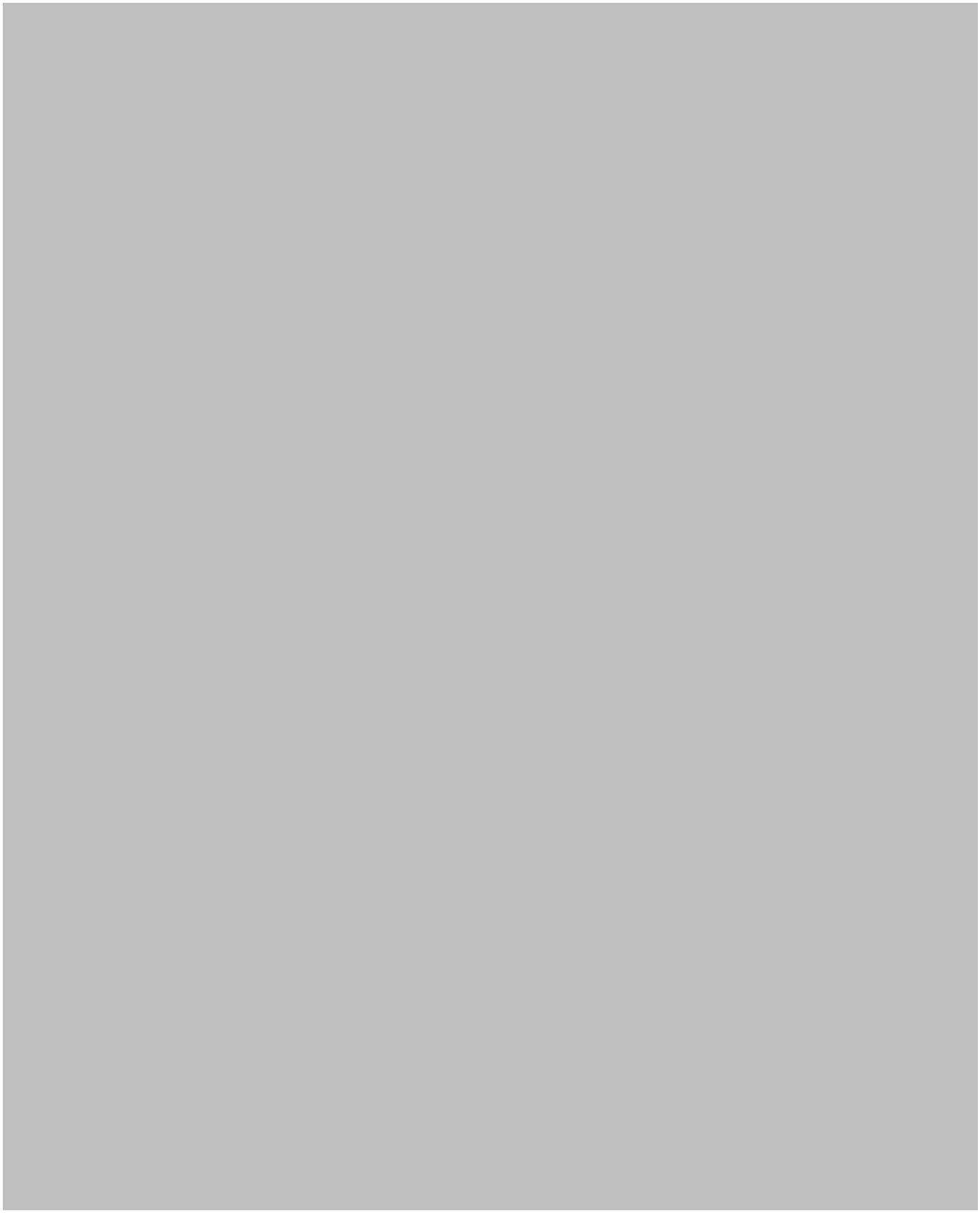
Acute rejection is a significant problem for patients undergoing HLA-incompatible renal transplantation, affecting between 12 and 53% of patients. Any mechanism of detecting rejection in advance of current methods would offer significant benefit. This study aimed to evaluate whether serum biomarkers could predict rejection in HLAi transplants recipients. Sera from 94 HLAi transplant recipients from a single centre were analysed for a panel of biomarkers including: NGAL, KIM-1, IP-10, cystatin C, cathepsin L and VEGF. Biomarker levels pre-operatively, day 1 and at day 30 post-transplant were correlated with the development of early rejection. Significantly higher levels of IP-10 and NGAL were seen on day 1 following transplant in those patients who developed acute rejection ($P < 0.001$ and 0.005) and generated AUC of 0.73 and 0.67, respectively. No differences were seen for the other biomarkers or at the other time points. In this study cohort, IP-10 and NGAL have demonstrated good predictive ability for the development of acute rejection at a very early time point. They may have a role in identifying patients at higher risk for rejection and stratifying immunosuppression or surveillance.

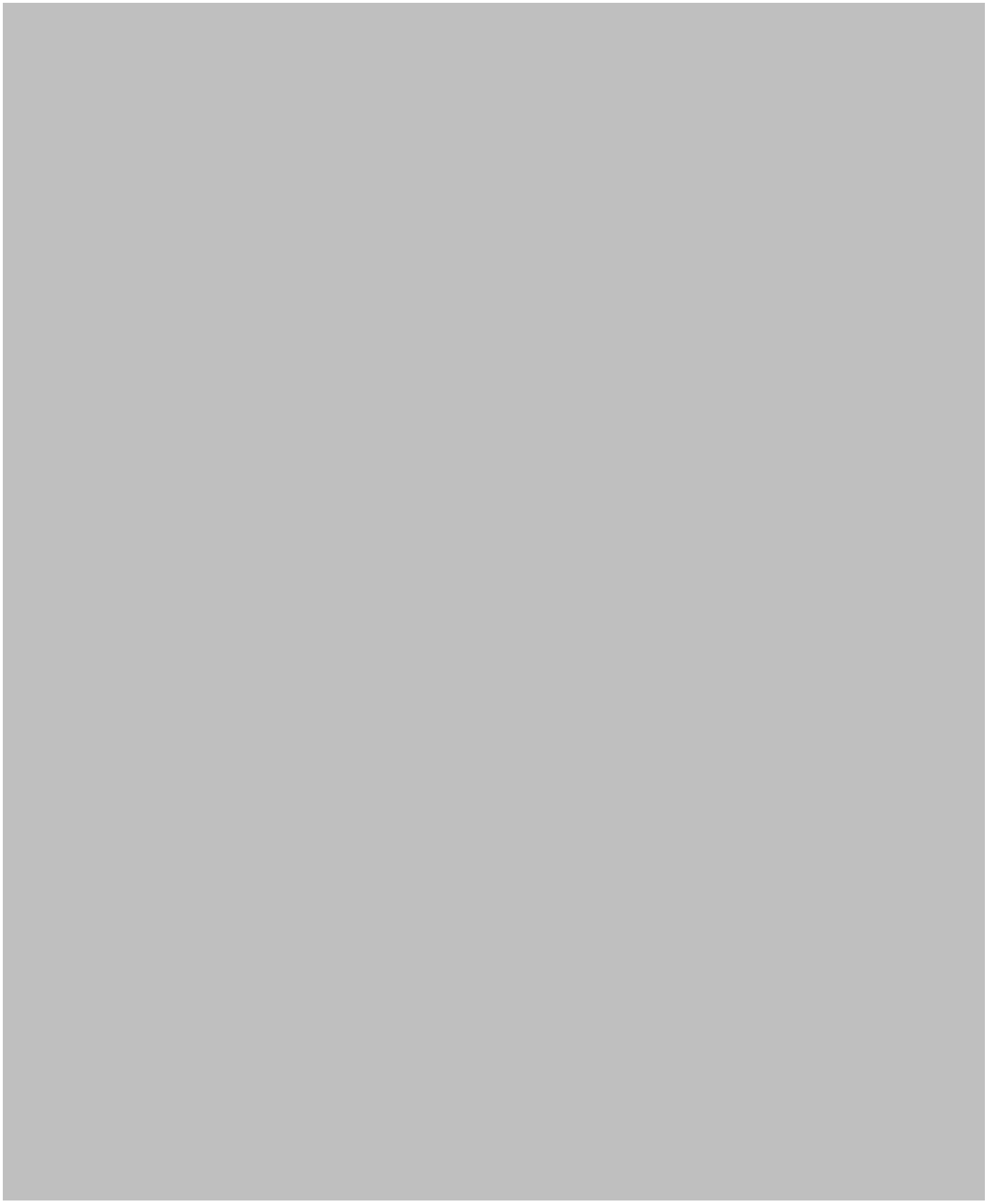


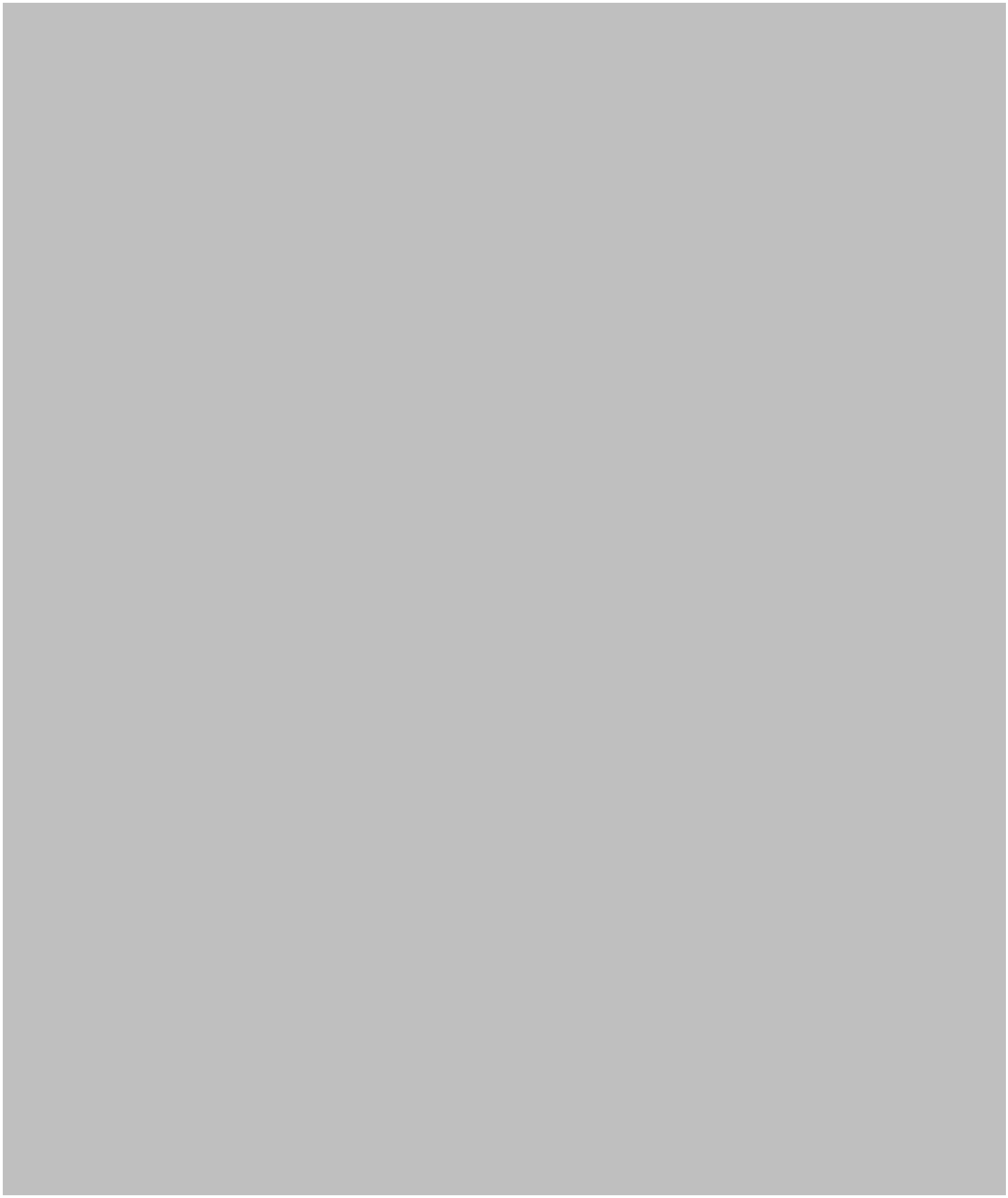












Clin Transplant 2014: 28: 808–815 DOI: 10.1111/ctr.12383

Clinical Transplantation

Urinary biomarkers of acute kidney injury in deceased organ donors – kidney injury molecule-1 as an adjunct to predicting outcome

Field M, Dronavalli V, Mistry P, Drayson M, Ready A, Cobbold M, Inston N. Urinary biomarkers of acute kidney injury in deceased organ donors – kidney injury molecule-1 as an adjunct to predicting outcome.

Abstract: Background: Deceased kidney donors are increasingly "marginal," and many have risk factors for acute kidney injury (AKI) that may impact on subsequent renal transplant outcome. Despite this, determining the presence of AKI at the time of deceased organ donation remains difficult.
Methods: Urine samples from 182 brainstem dead multi-organ donors (all of whom donated hearts that were transplanted) were analyzed for a Luminex™ panel of biomarkers linked with AKI. This included KIM-1, NGAL, IFN-γ, TNF-α, cystatin C, Fractalkine and vascular endothelial growth factor. Levels were correlated to early renal transplant outcomes, most specifically delayed graft function.
Results: Donor urinary KIM-1 levels were significantly higher in donors whose kidneys displayed aberrant early function ($p = 0.011$). Fractalkine levels showed a trend toward elevation in such donors but uncorrected this did not attain significance. No correlation occurred with the remaining biomarkers.
Conclusions: KIM-1 appears to show promise as a marker for AKI in deceased cardiac organ donors. The availability of a lateral flow device (Renastick™) for KIM-1 that also demonstrates higher urinary KIM-1 levels in donors whose kidneys show aberrant initial function ($p = 0.03$), makes KIM-1 a potential indicator of AKI that may merit further evaluation for its application at the donor bedside.

Melanie Field[a,b], Vamsi Dronavalli[c], Punam Mistry[b], Mark Drayson[b], Andrew Ready[a], Mark Cobbold[b] and Nicholas Inston[a]

[a]Department of Renal Transplantation, University Hospital Birmingham Foundation Trust, [b]MRC Centre for Immune Regulation, School of Immunity and Infection, Medical School, University of Birmingham and [c]Department of Cardiothoracic Surgery, University Hospital Birmingham Foundation Trust, Birmingham, UK

Key words: biomarkers – deceased organ donor – Kidney Injury Molecule-1 – Transplantation

Corresponding author: Melanie Field, MBChB, MRCS, MRC Centre for Immune Regulation, School of Immunity and Infection, Medical School, University of Birmingham, Birmingham B15 2TT, UK.
Tel.: 01216272000;
e-mail: melfield@doctors.org.uk

Conflict of interest: This work was undertaken using financial support from University Hospital Birmingham Charities. The authors of this manuscript have no conflicts of interest to disclose.

Accepted for publication 30 April 2014