AN INVESTIGATION OF EPO AS A TISSUE PROTECTIVE AGENT IN HUMAN KIDNEY TRANSPLANTATION

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Dr Declan de Freitas

School of Medicine

Faculty of Medical and Human Sciences

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Abstract

Ischaemia-reperfusion injury (IRI) has been identified as a major contributor to both short and long term kidney transplant failure. Experimental evidence from the literature suggests that Erythropoietin (EPO) is tissue protective, reducing both inflammation and apoptosis following IRI. We performed a randomised, double blind, placebo controlled trial examining the tissue protective effect of high dose EPO (100,000iu over 3 days) in 39 recipients of an extended criteria donor kidney or a non-heart-beating donor kidney. The primary endpoints of the study were difference in plasma and urinary biomarker levels (NGAL, IL-18 and KIM-1) in addition to changes in gene expression. Secondary endpoints included safety, clinical data and differences in metabolomics profiles. There was no difference detected between the treatment groups in terms of biomarkers, gene expression, metabolomics profiling or clinical parameters. No adverse events related to EPO therapy were recorded. In addition, we developed a cell model of kidney transplantation using primary tubulo-epithelial cells and HMEC-1 cells, with which to confirm the protective effects of EPO. Treatment with 50U/ml one hour prior to undergoing cold hypoxia resulted in the maximum degree of tissue protection, as measured using an MTT and an LDH assay. No evidence of EPO toxicity was demonstrated. Tubulo-epithelial cells expressed EPOR mRNA and protein. No CD131 receptor could be demonstrated. In summary, EPO confers tissue protection in a cell model of kidney transplantation but this has not been shown to occur in a clinical trial using high dose EPO in recipients of marginal donor kidneys.

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Finally, I want to thank all my patients for their selfless bravery in entering into this clinical trial so that others may benefit. None of this would have been possible without your help.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

CHAPTER 1 INTRODUCTION

1.1 General introduction

In the financial year ending on the 31st March 2010, there were 2501 kidney transplants performed in the UK, equating to just over 1/3rd of the waiting list transplanted that year (1). Despite the marked increase in the number of deceased organ donations (7%), the active waiting list remained stable with 7183 people awaiting a kidney transplant. In fact, since the year 2000, the number of patients registered on the active kidney transplant list has risen by nearly 50%. In contrast, the number of deceased donor kidney transplants, which make up almost 2/3rd of transplants performed, has increased just 27%. Figure 1.1 illustrates the recent national trends in the waiting list and numbers of deceased donor transplants performed.

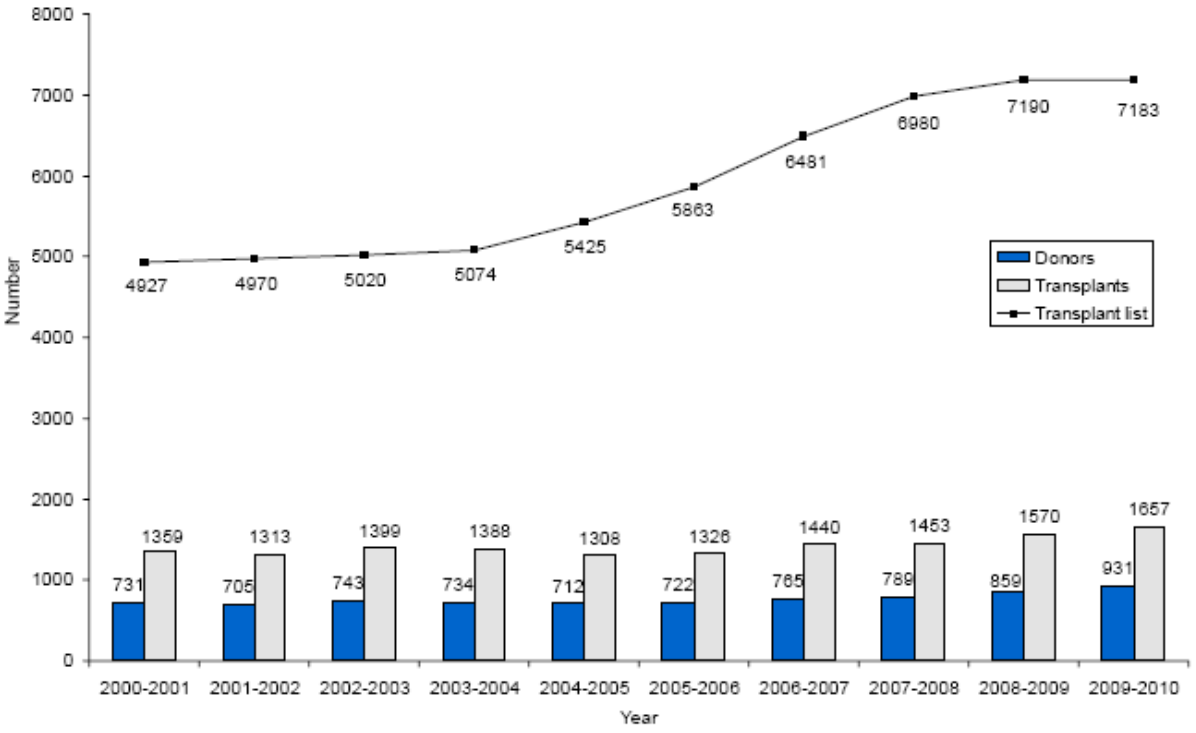


Figure 1-1: Changes in the UK waiting list and transplant rate over the last 10yrs.
This shows the number of deceased donors, transplants and growth in the waiting list from 1st April 2000 – 31st March 2010(1).

In parallel with this, two other issues have affected organ donation and the waiting list. Firstly, donor quality is changing with the increased use of more marginal donors. Secondly and linked with the first point, a significant proportion of those active on the UK transplant

list (~25%), despite advances in immunosuppression, are waiting for a re-transplant (Source: personal communication, UK Transplant 2007).

1.2 Defining donor type

1.2.1 Standard criteria donor (SCD) kidneys

The ideal organ donor is a young patient, who until suffering irreparable brain injury, was otherwise well, with excellent multi-organ function and no medical history. With the expansion of the donor pool to include marginal heart beating donors, these have subsequently been classified as standard criteria donors (SCDs), to reflect their better outcomes in comparison to extended criteria donors (2). By definition, they do not fulfil extended criteria or non-heart beating donor criteria.

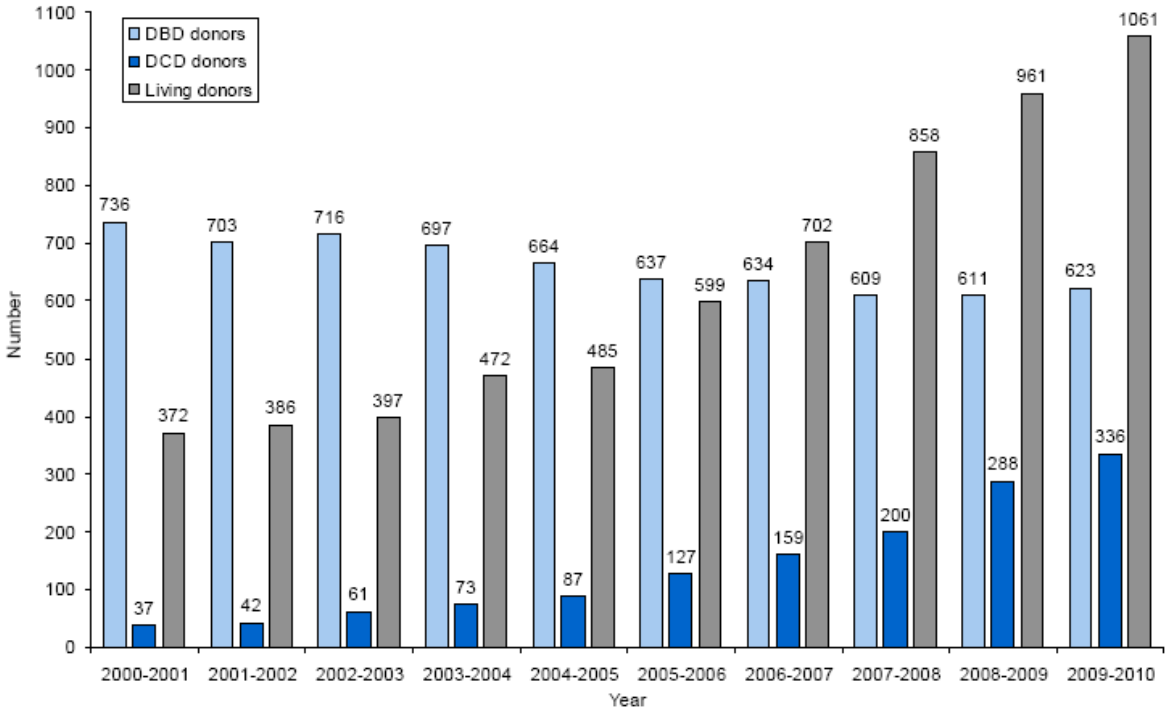


Figure 1-2: Rate of deceased and live donor transplantation over the last 10yrs.
Number of deceased and living donors in the UK, 1 April 2000 – 31 March 2010. DBD: donation after brain death (can be standard or extended criteria donors); DCD: donation after cardiac death or non-heart-beating donors (1).

Numbers of these SCD have fallen over time due to improved neurosurgical care and motor vehicle safety (Figure 1.2) (1). In addition, the rising donor age, increasing comorbidity and the need to declare brain death prior to becoming a SCD, increase the likelihood that donors will be either classified as an extended criteria donor or a non-heart beating donor respectively, thus increasing the frequency of marginal donor kidney transplants (Figure 1.2-1.5) (1;3).

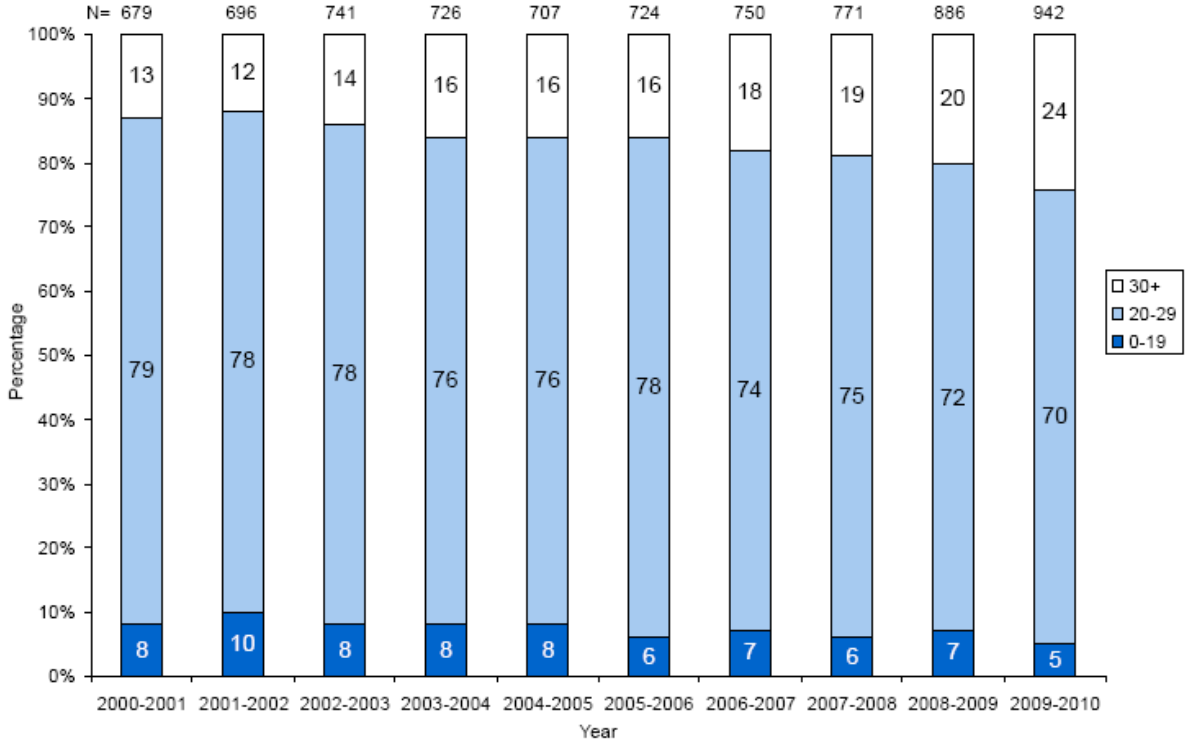


Figure 1-3: BMI as a marker of increased comorbidity in donors.
Body Mass Index of deceased donors in the UK, 1st April 2000 – 31st March 2010, as a marker of co-morbidities (1).

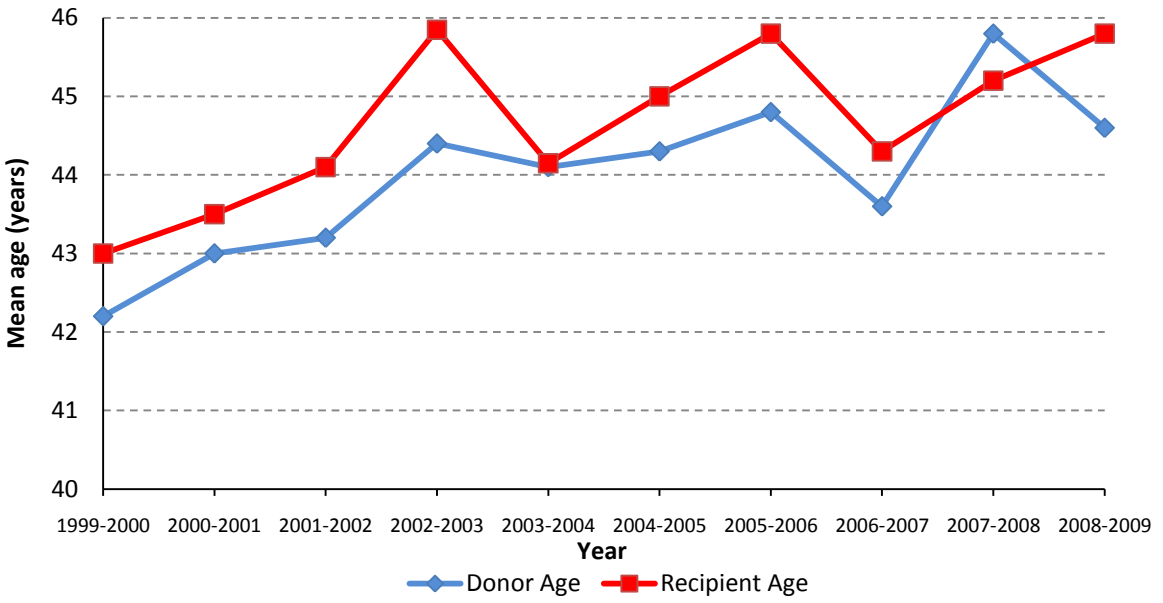


Figure 1-4: Changes in donor and recipient age with time.
Mean age of deceased donor solid organs and transplant recipients in the UK, 1 April 1999 – 31 March 2009(3)

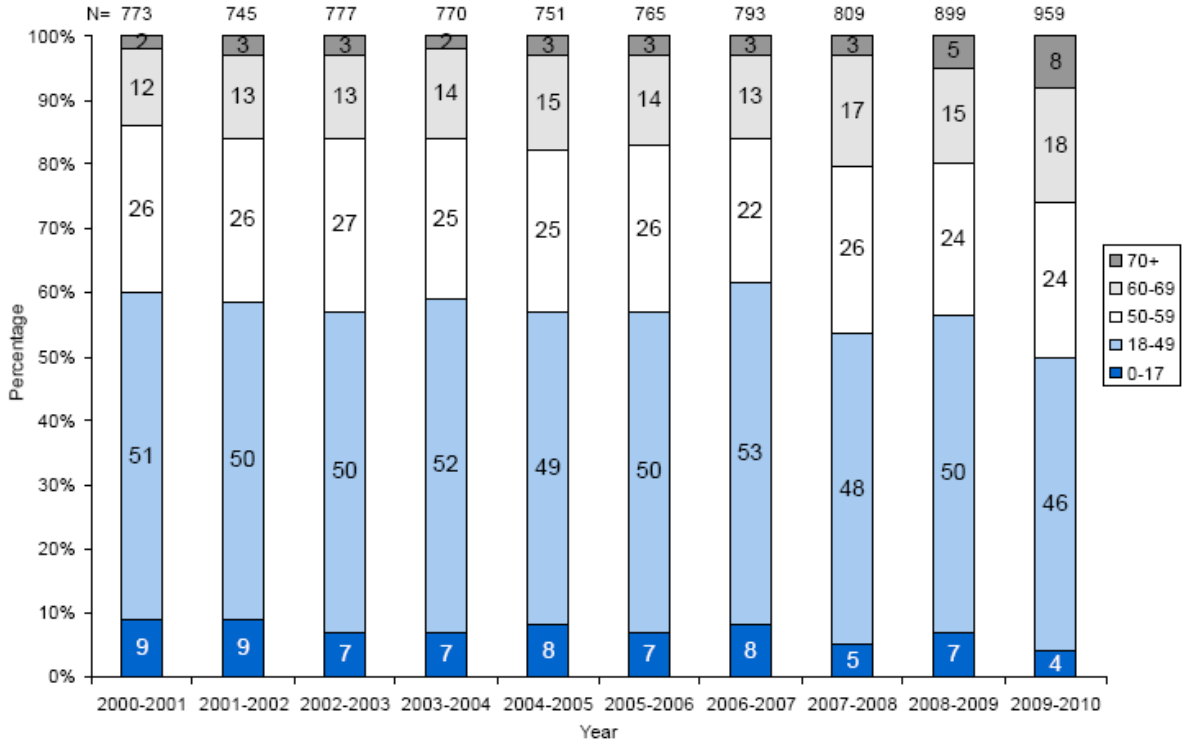


Figure 1-5: Changes in donor age profile over the last 10yrs in the UK.
Age distribution of deceased donors from 1st April 2000 – 30th March 2010 (1)

1.2.2 Extended criteria donors (ECD) kidneys

Port et al. identified four donor factors associated with a relative risk of graft failure of greater than 1.7 when compared to ideal kidneys, defined by: donor age 10-39yrs; absence of hypertension, death not due to a cerebrovascular accident; and a pre-donation serum creatinine under 133μmol/L (2). These 4 donor factors have been accepted as the definition of an extended criteria donor (ECD):

- Donor age ≥60yrs
- Donors aged 50-59yrs with at least two of three additional risk factors
    - Cerebrovascular accident as a cause of death
    - A history of hypertension
    - Serum creatinine >133μmol/L

By definition, both heart-beating donors (HBD) and non-heart-beating donors (NHBD) can meet these criteria, but they are more commonly found in the former. Numbers are increasing, in part due to the increasing donor age as shown above (Figure 1.3 and 1.4). The ECD definition has led to reduced waiting times and organ discard rates, increasing the pool of donor organs (4). The caveat to this has been the recognition that these donors may lead to shortened graft life and an earlier return to dialysis, with the added complication of sensitising the recipient and thus decreasing the chance of receiving a future transplant (5).

1.2.3 Non-heart-beating donor (NHBD) kidneys

Non-heart-beating donor (NHBD), also known as donation after cardiac death (DCD), kidneys are increasingly being used now for kidney transplantation, comprising approximately 20% of the donor pool (Figure 1.2) (1). These kidneys are procured following cardiorespiratory arrest, without the need for brain stem death testing. Typically donors have had a catastrophic brain injury which makes survival impossible but are unable to be certified as brain dead using standard brain stem death criteria. In contrast to heart beating donors in whom organ perfusion is interrupted under controlled conditions that minimise ischaemia, NHBD organs may experience significant periods of warm ischaemia, while the surgeon awaits cessation of the donor heart. Furthermore, it is recommended that asystole of 10

minutes duration should elapse to ensure the patient is deceased, prior to initiating in situ cold perfusion, thus exposing the organs to a significant period of warm ischaemia (the warm ischaemic time (WIT)) (6).

1.3 Outcomes of marginal donor kidney transplantation

1.3.1 Graft survival

North American registry data demonstrates a 1yr and 3yr graft survival in ECD kidney recipients of 83.6% and 55.1% respectively compared to a 1yr and 3yr graft survival of 90% and 70% in SCD recipients (5;7). The adjusted graft survival is on average 8% lower at 1yr and 15-20% lower at 3-5yrs after transplantation compared with standard criteria donors (4). No recent UK data exists but multicentre data from the cyclosporine era suggests kidneys from donors >60yrs have a relative risk for graft loss of 2.15 in comparison to donors aged 18-29yrs, with a 5yr graft survival of 44% compared to 68% in young donors (8). Thus, using ECD kidneys predisposes patients to sub-optimal graft function and earlier return to dialysis due to graft failure. Furthermore, inherent in this risk is that the chance of receiving a subsequent transplant decreases due to increased sensitization and increased waiting times as a result of the first transplant. Thus ECD kidneys should be carefully considered in young patients, with evidence that a donor-recipient age ratio >1.10 (donor age 55yrs to recipient age ≤50yrs) is associated with a 3-fold increase in graft loss (9).

A recent meta-analysis of outcomes of NHBD and HBD renal transplants demonstrated the 5 year graft survival rates were similar between the two groups, as was the serum creatinine (sCr) level at 12 months (10). UK data from the Leicester group also showed similar graft survival up to 10yrs (44.2% versus 58.5% for HBD; p = 0.052), but with a p value approaching significance (11). Interestingly, these NHBD kidneys had higher serum creatinine levels at 10yrs suggesting longer term graft survival may not be as good.

There is conflicting data on graft survival in NHBD-ECD kidneys. Japanese reports show inferior 10yr survival in organs from NHBD >60yrs of age compared to SCD grafts (30% vs 47%; p=0.001) (12;13). The Johns Hopkins group studied over 2500 controlled NHBD and

showed that kidneys from donors older than 50yrs of age had lower 5yr graft survival (14). The Dutch experience however demonstrates no difference in graft survival from donors older or younger than 65yrs (15). Finally, US registry data did not show a difference in 5yr graft survival between NHBD+ECD and NHBD kidneys (16).

1.3.2 Patient survival

Patient survival is inferior in recipients of ECD kidneys in comparison to SCD kidneys. Metzger et al. reported an adjusted patient survival of 90.6% and 69% at 1 and 5 years for ECD kidneys compared to 94.5% and 81.2% for non-ECD kidneys (17). However, if receiving an ECD transplant offers improved overall patient survival, then inferior graft outcome may represent an acceptable compromise. Ojo et al demonstrated an average increase in life expectancy of 5yrs compared to remaining on dialysis (18). ECD kidneys conferred a survival advantage over remaining on dialysis waiting for a SCD in patients older than 40yrs perceived to have a long waiting time and in patients with diabetes (19). It is important to note however that these studies are based on US registry studies, using intermittent hospital haemodialysis three times per week, which is sub-optimal compared to home haemodialysis (20). Therefore this increased life expectancy may not hold true when measured against the dialysis gold standard.

In comparison, NHBD appear to have similar 1 and 5 year patient survival when compared to standard heart beating donors (6;10). This may be related to the relatively short period of follow up, particularly if graft half-life is attenuated leading to an earlier return to dialysis (21).

1.4 Delayed graft function

Historically, delayed graft function (DGF) has been defined as the need for dialysis within the first week post renal transplantation but many definitions exist (22). However, using the need for dialysis alone will result in under-diagnosis, depending on the underlying renal function and physician preference. Decisions to dialyse patients can depend on the immediate postoperative fluid status and potassium levels which can be iatrogenic in origin, and

individual physician's criteria for dialysis, which may differ. There are up to 18 alternative definitions of DGF, defining it as a functional factor distinct from the need for dialysis (22). For example:

a) The number of days to achieve an eGFR >10mls/min calculated by the Cockroft Gault formula (23)
b) A serum creatinine level of >264µmol/L on day 5 post operatively (24)
c) The need for dialysis within 72 hours post transplantation (25)
d) If the serum creatinine level increases or remains unchanged or fails to decrease by >10% per day on three consecutive days within the first week (26)
e) A serum creatinine level more than the pre-op value, or a urine output of <300mls within 6 hours of transplantation, despite adequate hydration and diuretics (27)
f) A urine output of <1L in the first 24 hrs, or a decrease in the serum creatinine of <20-30% (28)
g) A serum creatinine reduction ratio of ≤30% (29)

Thus, depending on the definition used it may occur in up to 83% of cases (11;30;31).

The aetiology of DGF is multi-factorial, depending on donor factors, recipient factors and transplant procedure factors (Figure 1.6).

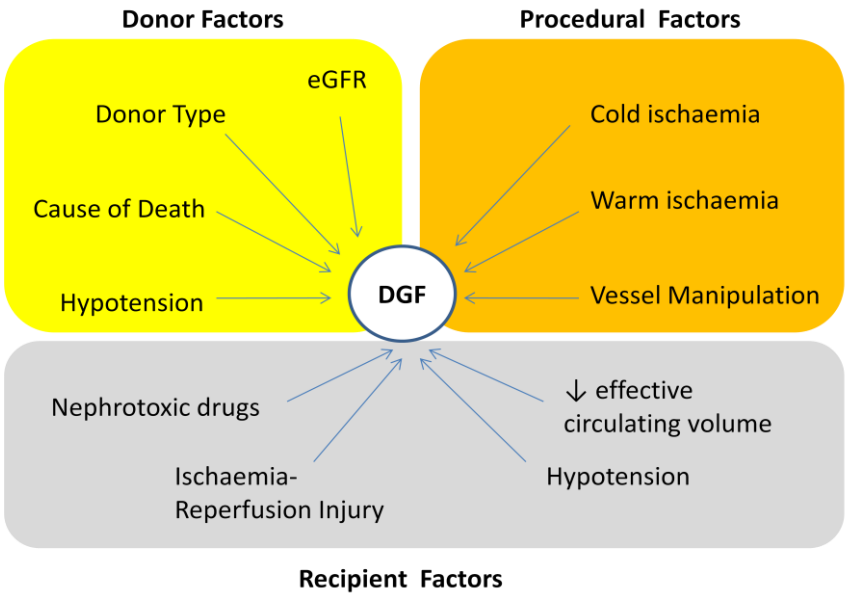


Figure 1-6: Aetiology of delayed graft function.

There is much debate on the impact of DGF on graft survival (Figure 1.7), with some authors reporting a deleterious effect on graft survival (23;32) and others demonstrating inferior graft survival only in the presence of acute rejection (33-35). Potential explanations for this include registry data versus prospective trials, duration of follow up, variation in the definition of DGF, donor quality and immunosuppressive regimes (36).

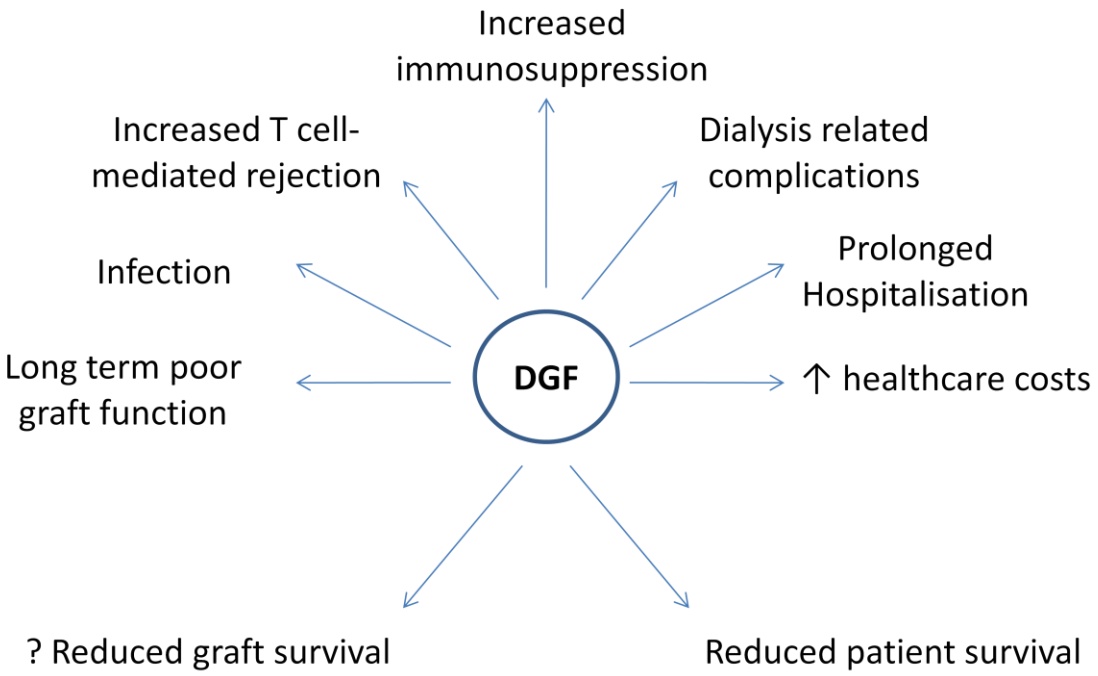


Figure 1-7: Consequences of delayed graft function.

1.5 Mechanisms of injury

Transplantation constitutes a severe physical stress on the donor kidney. Ischaemia followed by reperfusion of the kidney involves an interrelated sequence of events which culminate in cell injury, dedifferentiation, apoptosis and necrosis. The pathogenesis of IRI involves a complex interrelationship between renal microvasculature dysfunction, tubular dysfunction and inflammation. Three phases of injury can be discerned during the process of organ transplantation and each of these phases is potentially eligible for therapeutic intervention: (1) donor derived injury; (2) preservation injury; and (3) reperfusion injury and post-operative injury (Figure 1.8).

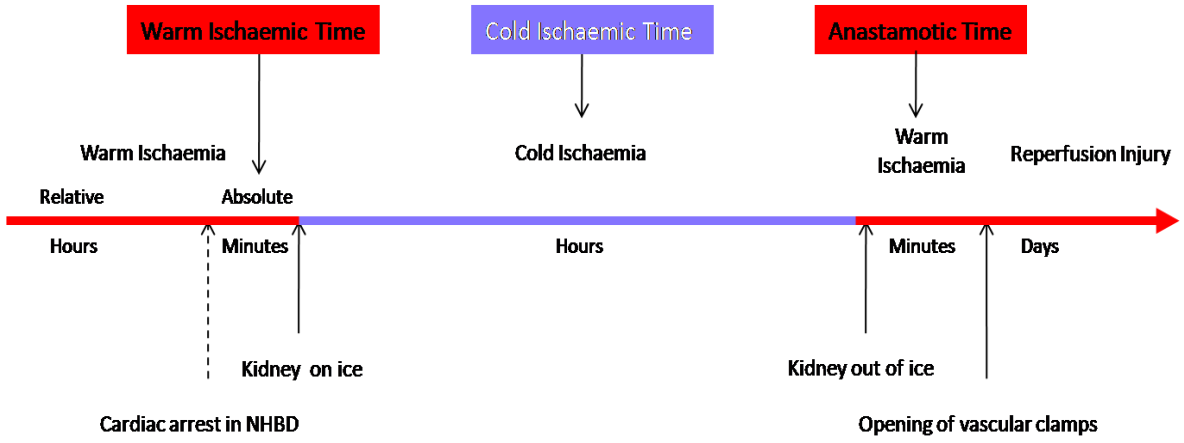


Figure 1-8: Ischaemia-reperfusion injury timeline

1.5.1 Donor derived injury

The initial insult to the donor kidney can occur long before organ retrieval. Donor age is a well recognised risk factor for DGF and reduced graft survival (2). Longitudinal studies of the ageing kidneys reveal a progressive reduction in the number and size of glomeruli, in addition to an increase in interstitial fibrosis (37-39). Furthermore, renal biopsies in donors over 40yrs of age show intimal fibrosis in the smaller arteries and arteriolar hyalinosis, a factor potentially exacerbating reperfusion injury (40). Thus, a reduction in the renal reserve, along with functional constraints diminish the kidneys ability to respond to challenges (41).

Events peri-mortem also result in donor organ injury, such as underlying disease processes (e.g. sepsis), manoeuvres instituted to treat the disease (e.g. nephrotoxic dugs) or to maintain blood pressure after brain death (e.g. inotropes) (42). Brain death itself is associated with complex haemodynamic, neurohormonal and immunological alterations, which can all result in organ dysfunction and injury. An initial increase in parasympathetic tone is rapidly followed by excessive sympathetic activation, which leads to increased catecholamines and severe arterial hypertension (43). The resulting vasoconstriction leads to local tissue ischaemia, disruption of ATP production, free radical generation, increased intra-cellular calcium, and activation of a number of enzymes including nitric oxide synthase and various endonucleases (44). A severe reduction in sympathetic outflow follows, leading to hypotension, further hypoperfusion and warm ischaemia in the vasoconstricted organ (45).

Microscopically, kidneys retrieved from brain dead donors have demonstrated morphological changes, including glomerular hyperaemia, glomerulitis, endothelial proliferation and acute tubular necrosis (46). At a molecular level, marked increased transcript expression of the pro-inflammatory mediators TNFa, IFNg, IL-1, IL-6, IL-10, IL-15, MCP-1 and Rantes has been demonstrated within 30 minutes of brain death (47;48). Interestingly, the intensity of IL-1, IL-6, IL-10, IL-15, IFNg and TNFa gene up-regulation matched or was greater than that seen during episodes of acute rejection. Cell adhesion markers E-selectin and P-selectin have also been shown to be markedly up-regulated, facilitating leukocyte infiltration (48). Recognition of non-self antigens is facilitated by the early up-regulation in major histocompatability complex, class I and II, which can be observed after just one hour. Finally, granulocyte infiltration begins immediately after brain death (48). Thus, the kidneys are not only injured prior to donation, but primed for further injury on reperfusion.

All heart beating deceased donors must have brain stem testing to confirm brain death prior to commencing the retrieval process. In contrast, no brain stem testing is needed prior to donation after cardiac death (DCD). Brain stem death, with its ensuing consequences, is likely to occur in these donors also, particularly following withdrawal of supportive treatment. However, the short time interval between withdrawal of support and asystole (varying from 2-4 hrs) means its duration is likely to be short but still significant. Interestingly, evidence exists that a longer duration of brain death may be associated with better graft outcomes (49). In ischaemic pre-conditioning, initial sub-lethal insults may induce priming mechanisms that subsequently protect the subject when exposed to a second insult. The cytoprotective gene haemoxygenase-1 (HO-1) is progressively up-regulated over a 4hr time period, most likely as a result of ischaemia induction (48;50). Therefore, the optimal time to retrieve a donor organ may not actually be the shortest possible time in all situations (49).

1.5.2 Preservation injury

All transplanted organs must by definition suffer a variable duration of ischaemia. This is typically described as being either warm ischaemia or cold ischaemia, depending on the temperature of the organ.

Relative warm ischaemia may occur in a hypotensive or vasoconstricted donor prior to retrieval. However, only NHBD, by definition, experience a significant period of warm ischaemia. This occurs between cardiac asystole and in situ cold perfusion of the organs with a preservation solution, the so-called warm ischaemic time (WIT). Typically, the WIT is between 10 and 30 minutes, which includes a mandatory 10 minutes 'hands off' period to confirm donor death. Errors in line placement can result in inadequate flushing/cooling of the organ contributing to warm ischaemia (42).

All donor organs experience a variable period of cold ischaemia, the so-called cold ischaemic time, from the time of organ retrieval to just before implantation. The organ is initially flushed in situ with cold preservation solution before undergoing hypothermic storage at 0 to 4°C on ice. Traditionally, refrigeration in combination with a specialised preservation solution has been relied upon to preserve the organ and limit ischaemic damage. Cooling slows cellular metabolism by 10-12 fold while the preservation solution contains an impermeant which limits cellular oedema. The type of preservation fluid may also be an important factor, with University of Wisconsin (UW) solution being superior to Euro Collins solutions or HTK in limiting injury (51;52). Cold storage does not halt metabolism completely, as anaerobic metabolism continues to occur. This leads to accumulation of metabolic breakdown products which are later metabolised on reperfusion, leading to the generation of toxic molecules, particularly oxygen free radicals. The degree of damage occurring during organ storage is therefore proportional to the length of the cold ischaemic time and the warm ischaemic time.

As the kidney is prepared and the blood vessels anastamosed (the time period known as the anastamotic time), a short but potentially more hazardous period of warm ischaemia occurs. Manipulation of the renal arteries may also result in vasospasm on reperfusion, further potentiating the ischaemic injury (42).

1.5.3 Reperfusion injury

The final physical insult follows the return of recipient blood to the organ, so-called reperfusion injury, which occurs over minutes to hours, following release of the vascular clamps. The reintroduction of oxygen leads to the changes in intracellular ion concentrations, redox state and cell viability resulting in marked changes in the microvasculature and tubules.

Further episodes of hypotension both intra-operatively and during the early post-operative period compound this injury.

1.5.3.1 Alterations in intracellular ion concentrations

Oxygen deprivation leads to almost complete ATP depletion within 4 hours(53). The resulting loss of the active ion transporters, Na^+/K^+ and Ca^{2+}/Mg^{2+} ATPase, leads to an accumulation of Na^+, Cl^- and Ca^{2+} in the cells (54-56). Hypothermia and acidosis during cold preservation further depress ATPase dependent active transport mechanisms, compounding the problem (54;57). High intracellular Na^+ levels result in an influx of water leading to cellular oedema, injury and dysfunction. Endothelial cell swelling restricts blood flow resulting in renal ischaemia, despite reperfusion of the organ.

Increased cytosolic Ca^{2+} levels activate Ca^{2+} dependent enzymes such as cysteine proteases and phospholipases which destroy the cell cytoskeleton leading to cell death (58). However, high intra-cellular calcium levels may also promote recovery from acute kidney injury through up-regulation of proteins such as annexin A2 and S100A6, which promote cell proliferation (59).

1.5.3.2 Alterations in the cell redox state

The reintroduction of oxygen leads to the formation of reactive oxygen species promoting protein oxidation, lipid peroxidation and DNA damage leading to apoptosis and necrosis (60). Metabolism of hypoxanthine by xanthine oxidase generates superoxide and hydrogen peroxide, which reacts with free iron to form a hydroxyl radical. This forms the premise for adding allopurinol, a xanthine oxidase inhibitor, to preservation solutions to prevent free radical formation. Ischaemia also induces nitric oxide synthase and NO formation, which interact with superoxide to form peroxynitrate. This results in cell damage via protein nitrosylation and oxidant injury (61). Finally, the inflammatory infiltrate containing neutrophils and CD4+ T cells, also contributes to the abundance of reactive oxygen species, overcoming the intrinsic anti-oxidant defence mechanisms leading to cell injury.

1.5.3.3 Alterations in cell viability – Apoptosis and Necrosis

The majority of cells remain viable after reperfusion injury, reflected simply by graft survival. These cells either escape injury altogether or are sub-lethally injured allowing recovery. For the small population that are lethally injured, death occurs in two forms and is dependent on the nephron segment involved and the severity of injury.

Apoptosis or programmed cell death is the mechanism through which cell death occurs in an organised manner. It is a tightly regulated process requiring energy and can be triggered by a variety of stimuli, both extrinsically or intrinsically, such as by binding of ligands (e.g. TNF, FasL) to their cell surface receptors or by DNA damage from oxidative stress. Apoptosis predominates in the distal tubule and areas of less severe injury. The Fas-FADD pathway (62), the p53 pathway (63) and an imbalance between the pro- and anti-apoptotic Bcl2 family members (64;65) appear to be the most important signalling pathways for mediating apoptosis in tubular cells (Figure 1.9).

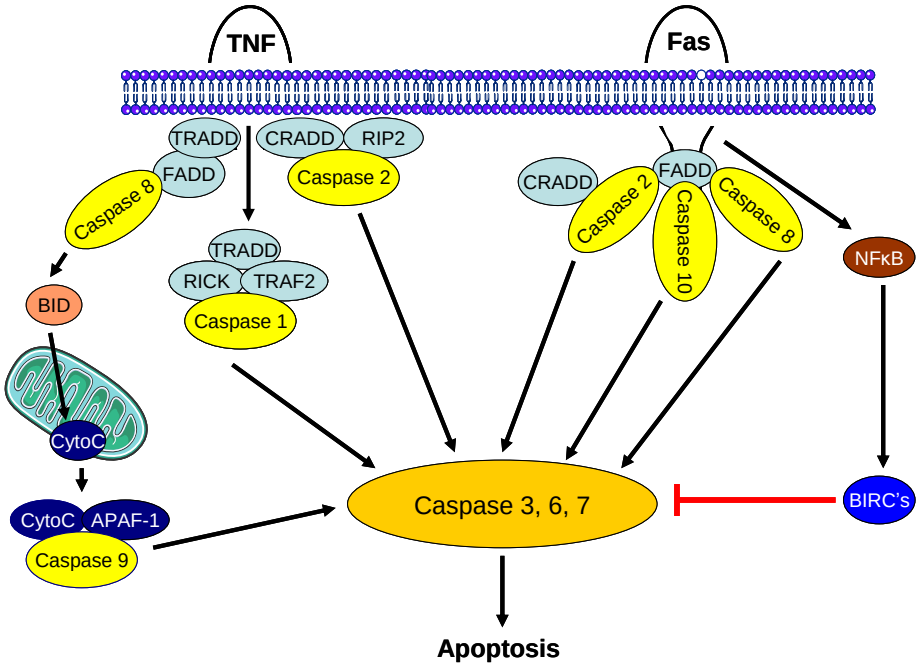


Figure 1-9: The extrinsic pathway of apoptosis.

Necrosis occurs when cells suffer a severe insult, resulting in a catastrophic loss of membrane integrity and cell swelling ending in rupture of the cell. Its contents, including LDH, are released into the surrounding environment as opposed to being packaged awaiting phagocytosis. This induces an inflammatory response injuring adjacent cells. Necrosis is usually found in the most susceptible nephron segments such as the outer medullary segment of the proximal tubule, where cells already exist under low oxygen conditions (66;67). It also reflects more severe injury. Figure 1.10 represents a day 7 biopsy from a kidney allograft with severe cortical necrosis demonstrating necrosis in the tubules with intra-luminal necrotic debris, glomerular congestion and an inflammatory cell infiltrate in response to the injury.

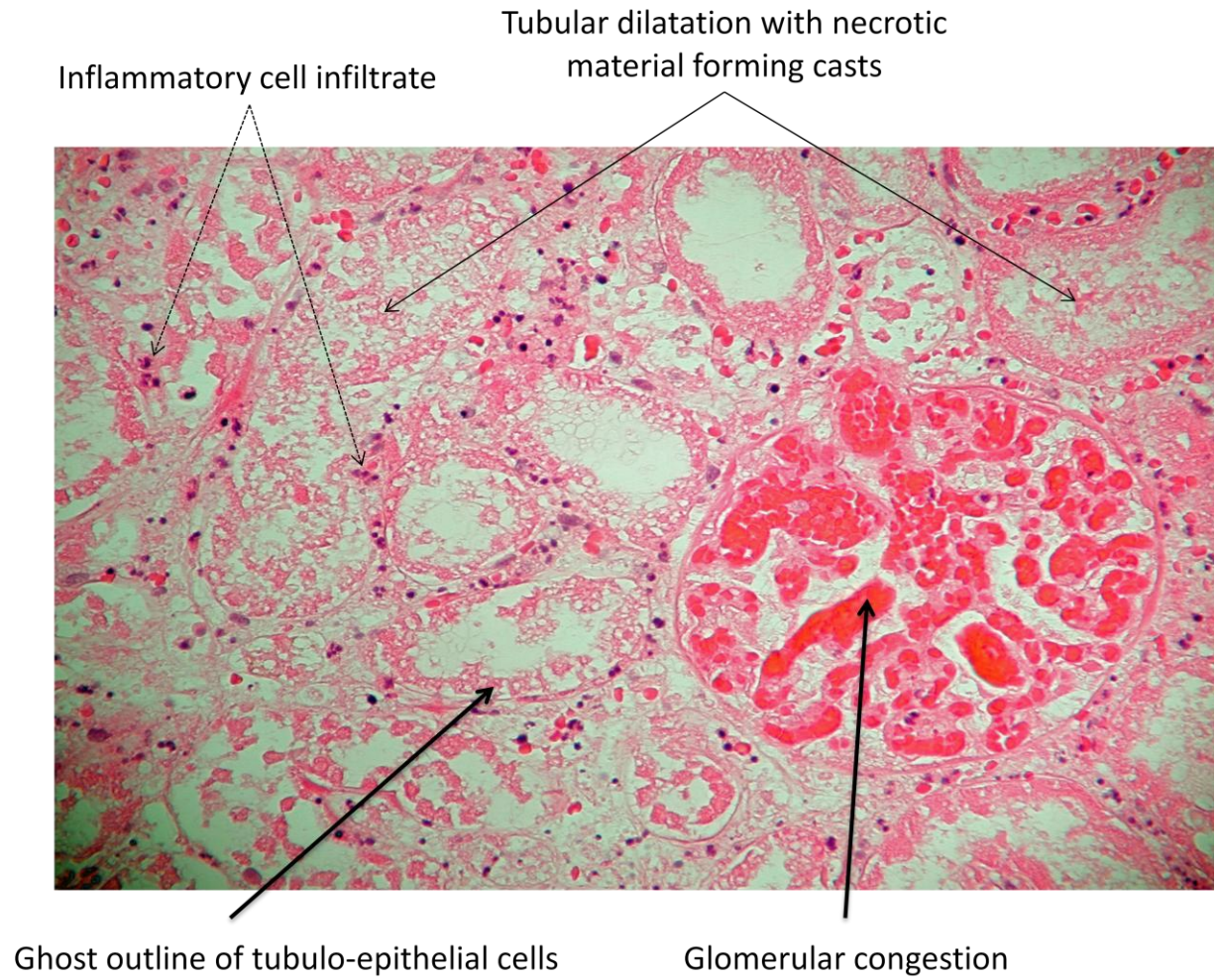


Figure 1-10: Histology of severe ischaemia-reperfusion injury.
Severe cortical necrosis in day 7 transplant kidney biopsy for DGF

However, despite the frequent histological diagnosis of acute tubular necrosis (ATN) as the cause of DGF, little evidence of necrosis is seen in biopsies. In fact, dysfunction is due to widespread tubule de-differentiation which is not readily detectable using a microscope, but has a clear molecular phenotype.

1.5.3.4 Activation of inflammation

The inflammatory response plays a major role in ischaemia reperfusion injury and results in injury to, and the eventual death of renal cells. It has also been implicated in the aetiology of later allograft rejection. It can begin at the time of donor brain death but is exaggerated following reperfusion. Pro-inflammatory cytokines (e.g. TNFa, IL-6, IL-1B) (48;68;69), chemotactic cytokines (e.g. MCP-1, IL-8, C5a) (48;69;70) and adhesion molecules (e.g. ICAM-1, VCAM-1, P-selectin) (71) are produced by tubular cells, endothelial cells, infiltrating leukocytes and donor derived dendritic cells in response to injury. Studies in the peri-operative transplant period have demonstrated that levels of the pro-inflammatory cytokines TNFa, IL-1B, IL-6 and IL-8 are higher in kidneys undergoing greater ischaemia-reperfusion injury (69;72). In native acute kidney injury (AKI), levels of IL-6 and IL-8 predict mortality (73), while CXCR3-binding chemokine levels have also been shown to be elevated in the urine post transplantation and to predict AKI (74). Some of these factors may just be markers of injury, but the net effect of these molecules is likely to enhance injury.

Toll-like receptors play a significant role in the inflammatory cascade after reperfusion injury, through recognition of damage-associated molecular patterns (75). These are endogenous ligands, released from necrotic cells, which engage TLR's which initiate cytokine and chemokine release, up-regulate co-stimulatory molecules, which together amplify local inflammation (76;77). Examples include heat shock proteins, Tamm-Horsfall protein, fibrinogen and heparan sulphate (77;78). TLR4 is expressed in both proximal and distal tubular cells, with higher expression in pre-anastamosis biopsy sections obtained from deceased donor kidneys compared with living donors (79). Ischaemia increases TLR4 expression in tubular cells, further increasing IL-6, TNFa and MCP-1 transcript levels, augmenting inflammation and apoptosis (79;80).

1.5.3.5 Alteration in the microvasculature

It its constitutive state, the endothelium regulates vascular tone, vasopermeability, coagulation and leukocyte migration. Following injury, the endothelium loses its controlling ability with the occurrence of hypoperfusion, oedema, clotting abnormalities and the infiltration of inflammatory cells.

The post-ischaemic kidney displays marked regional variations in blood flow patterns (Figure 1.11). This is most prominent in the outer medullary region of the kidney which houses the most metabolically active nephron segments and exists in relative hypoxia as a result (81). During reperfusion, hypoperfusion occurs, particularly in this region, worsening the relative hypoxia and cellular injury, resulting in a decrease in the glomerular filtration rate (82). Hypoperfusion occurs due to a number of mechanisms. The rapid increase in the intra-vascular pressure on unclamping the vessels results in a reactive vasospasm. Congestion occurs in the renal microcirculation, especially in the vasa recta, due to the accumulation of erythrocytes and leucocytes, shunting blood away from the outer medulla (83;84). Ischaemic injury results in endothelial cell oedema, denudation and death. Sites with exposed basement membranes are prone to prolonged vasoconstriction, hampering blood flow (85). Local imbalances in vasoactive molecules such endothelin and endothelium derived nitric oxide (NO) occur, further narrowing the lumen (86).

IRI is associated with the release of chemotactic compounds and expression of adhesion molecules, promoting endothelial-leukocyte interactions which compound congestion. These include increased endothelial expression of intra-cellular adhesion molecule-1 (ICAM-1), E selection and platelet derived P-selectin (87) (71;88).

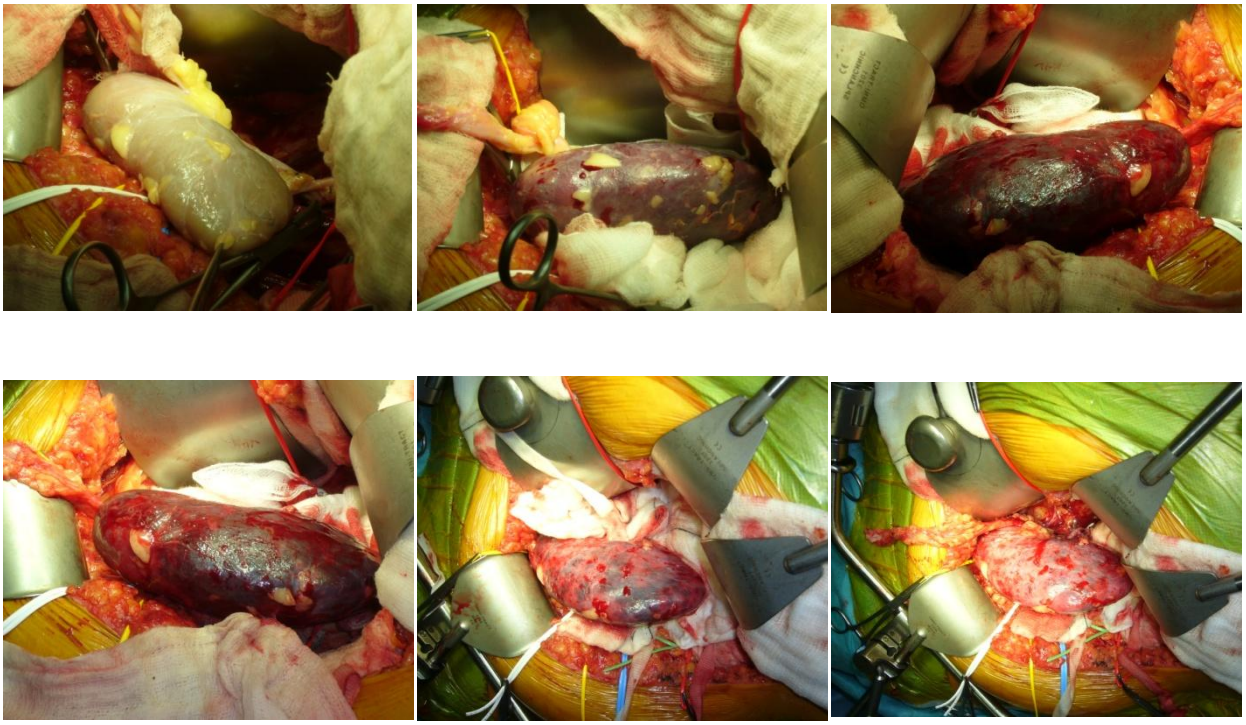


Figure 1-11: Photographic sequence of reperfusion injury.
Reperfusion injury in a 35yr NHBD kidney with a CIT of 29hrs 05mins and a 0:0:0 mismatch over 12mins from reperfusion. Note the patchy perfusion.

1.5.3.6 Alterations in tubular function

A confusing feature of early transplant biopsies is the lack of correlation between delayed graft function and histological appearances down the microscope. Patients are often said to have acute tubular necrosis (ATN) due to the severity of the kidney dysfunction, but little evidence of this is seen histologically. The most common appearances seen include loss of the epithelial brush border, patchy loss of tubule cells, tubular dilatation and intra-luminal casts (Figure 1.12). This discrepancy between function and findings is best explained by the finding of poorly functioning sub-lethally injured tubular cells, altered blood flow, obstruction of the tubular lumens and cells undergoing varying stages of apoptosis (65).

Loss of the tubular basolateral Na/K ATPase and brush border leads to impairment of Na reabsorption in the proximal tubule with a consequent rise in the fractional excretion of sodium, which is a diagnostic feature of acute kidney injury (89).

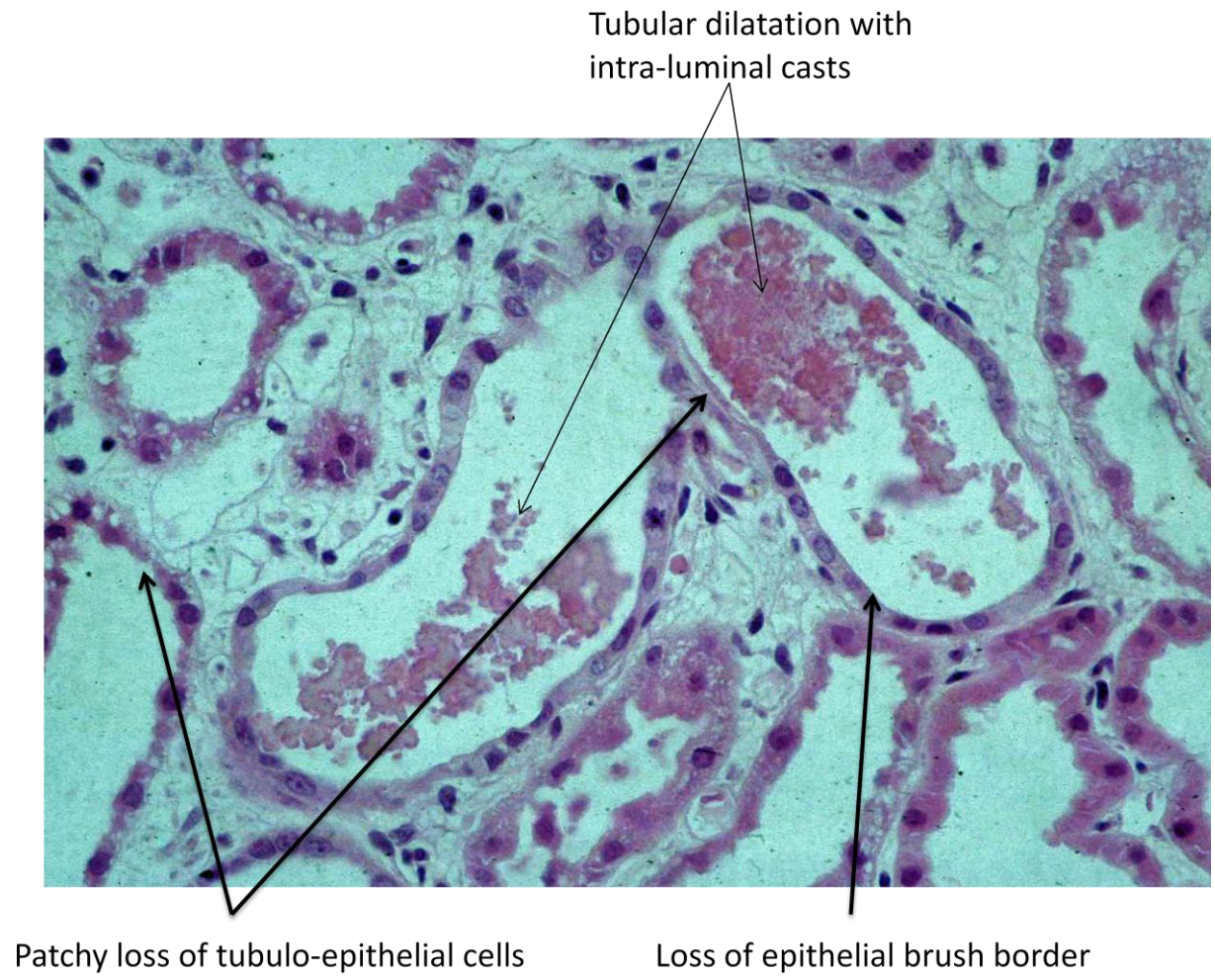


Figure 1-12: Histology of ATN
ATN in a day 7 kidney transplant biopsy for delayed graft function.

1.6 Non-invasive monitoring of kidney injury

1.6.1 Overview

There is much interest in the ability to predict or detection of DGF in the very early post-operative period, with a view to minimising further renal injury and the development of therapeutic strategies to ameliorate acute kidney injury. Most definitions of DGF are registry derived, relying on the evolution of serum creatinine or urine output over many days e.g. a serum creatinine level of >264µmol/L on day 5 post operatively (24). In addition, interpretation of these clinical features requires knowledge of prior measurements and interceding events such as a dialysis episode or diuretic usage, both of which can make values

un-interpretable. Furthermore, while they predict DGF, they do not predict recovery. Therefore, there is a need for a non-invasive biomarker, which can rapidly and accurately predict the onset of delayed graft function as well as functional recovery.

There are a number of novel biomarkers, identified as early markers of acute kidney injury in the general population, which have recently been translated into kidney transplantation, to predict DGF, including:

- Neutrophil gelatinase lipocalin (NGAL)
- Kidney injury molecule 1 (KIM_1)
- Interleukin-18 (IL-18)
- Fatty acid binding proteins (FABPs)
- Hepatocyte growth factor

1.6.2 Neutrophil gelatinase-associated lipocalin (NGAL)

Neutrophil gelatinase-associated lipocalin (NGAL) or lipocalin 2 (LCN2) (90) is one of the earliest and most induced genes and proteins following acute kidney injury (AKI). This has led to much interest in its use not only as a biomarker of kidney injury that offers potential prognostic significance, but also as a potential therapeutic molecule. First described in neutrophils, NGAL is expressed in most tissues exposed to microorganisms such as epithelial cells in the gastrointestinal tract, respiratory tract and genitourinary tract including the kidneys.

NGAL has been shown to have a variety of functions including fatty acid and iron transport, apoptosis induction, the suppression of bacterial proliferation and modulation of inflammation (91-95). More recently, NGAL has been shown to be involved in recovery following renal ischaemia with up-regulation in tubule-epithelial cells undergoing proliferation (96;97). Oxidative stress has also been shown to induce NGAL expression. One mechanism for this is through a marked induction of haemoxygenase-1(HO-1) expression, which is linked to the promotion of intracellular iron release and formation of anti-oxidants such as carbon monoxide and biliverdin (98). Exogenously administered NGAL has been suggested to confer tissue protection in this setting (99-101). However, this latter finding is

controversial as NGAL -/- mice exposed to IRI and given exogenous NGAL did not show a treatment benefit (102).

In the normal kidney, filtered NGAL is almost completely reabsorbed in the proximal tubule by megalin, with only small quantities (~5ng/ml) found in normal urine. By comparison, a paediatric study of acute kidney injury following cardiopulmonary bypass, demonstrated >10 fold (>50ng/ml) increases in urine NGAL from baseline within 2-6 hours of injury, contrasting with a 50% increase in serum creatinine occurring 24-72 hours later (103). Multivariate analysis confirmed urine NGAL as the most powerful independent predictor of severe kidney damage with an area under the curve (AUC) of 0.998 for the 2hr urine NGAL measurement. In a study of AKI in children with diarrhoea associated haemolytic uraemic syndrome, urine NGAL excretion >200ng/ml successfully predicted the need for dialysis (104). A further study in children who developed AKI following cardiac surgery demonstrated that peak urinary NGAL levels were independently associated with the duration of AKI (105). Thus, in the paediatric population, NGAL increases early following acute kidney injury and is highly predictive of both acute kidney injury and its duration. Although these findings in children have been confirmed in adults with AKI following cardiac surgery (106), the reported AUCs were less favourable, with a 3hr NGAL AUC of 0.74 and 18hr NGAL AUC of 0.80. One suggested reason for this difference is the increased frequency of comorbidity in adults acting as a confounding variable, including obesity or bacterial infection (107).

Kidney transplantation is analogous to acute ischaemic kidney injury and thus NGAL findings would be expected to extrapolate into this area. In biopsies of allografts within 1hr of reperfusion, NGAL staining intensity strongly correlated with cold ischaemic time and the development of delayed graft function (108). In addition, day 0 urine NGAL levels predicted the need for dialysis within the first week with an AUC of 0.9 and preceded peaks in serum creatinine levels by 2-4 days (109). However, with a high incidence of DGF and anuria, recipients of organs from donors with cardiac death are more likely to be anuric and thus urine biomarkers are unlikely to be useful in this population. However, serum NGAL has been shown in this group to predict organ recovery (110). A biphasic peak was noted in this population and it has been suggested that the negative slope of NGAL following this second peak predicts organ recovery(110). However, one caveat to the use of NGAL in

transplantation is that levels are also higher in patients with pre-existing renal disease potentially confounding post-operative measurements (111).

1.6.3 Kidney injury molecule-1

Kidney injury molecule-1 (KIM-1) is a type 1 membrane glycoprotein found on the apical membrane of de-differentiated or proliferating proximal tubular epithelium but not expressed (mRNA or protein) in the normal kidney, by other renal cell types or by other tissues (112;113).

KIM-1 is a phosphotidylserine receptor which binds phophatidyserine on the apoptotic cells surface, transforming the tubular epithelium into semi-professional phagocytic cells, enhancing the clearance of adjacent apoptotic cells and necrotic debris (114). Functionally, the ability to prevent and clear tubule obstruction may be key to restoring glomerular filtration in the nephron. Furthermore, it may serve to attenuate the pro-inflammatory response to cell necrosis, through prevention of the necrotic debris activating the inflammatory cascade.

In response to tubular injury (primary or secondary), KIM-1 mRNA and protein levels are dramatically increased, with the ectodomain rapidly cleaved into the tubular lumen, allowing detection of KIM-1 in the urine (115;116). Ischaemia, drug nephrotoxicity and immune mediated damage (as in the transplant setting) have all been shown to increase urine KIM-1 levels (117-119). Tissue KIM-1 levels are closely related to urinary levels, correlating with the severity of damage and persisting until recovery of the tubule-epithelium (120). KIM-1 levels were predictive of the need for dialysis and mortality in hospitalised patients with acute kidney injury (117). One advantage of using KIM-1 to detect tubular injury is that it appears to be more specific for ischaemic injury and relatively unaffected by chronic kidney disease or urinary tract infections (121).

In kidney transplantation, tissue KIM-1 expression and urinary KIM-1 was > 2 fold higher in brain dead donors when compared to living donors prior to transplantation (122). This may be explained by its correlation with the donor eGFR at the time of procurement acting as a marker for acute kidney injury in the donor (123). In this study, urine KIM-1 was a positive predictor of recipient serum creatinine at 14 days and 1yr, possibly relating to the severity of

peri-operative tubular injury leading to a greater fixed renal deficit. Surprisingly, it was not predictive of DGF, nor was it associated with duration of brain death, the need for vasopressors or the duration of cold ischaemic time. As energy is required for the generation and shedding of KIM1 into the urine, cold storage and the resulting energy depletion may limit KIM-1 detection at this time point, limiting its usefulness in the peri-transplant period (112). A further study also showed that urine levels were not predictive of DGF in the early post-operative period, casting some doubt on the performance of KIM-1 as a biomarker at this time (124).

1.6.4 Interleukin-18 (IL-18)

Interleukin-18 is a pro-inflammatory cytokine involved in both the innate and acquired immune system, playing a role in the host defence against viruses or malignant cells. A wide variety of cells have been shown to express IL-18 including dendritic cells, mononuclear cells and renal epithelial cells.

Emerging evidence in experimental models suggest a role for IL-18 in mediating ischaemic kidney injury. Neutralisation of IL-18 in lethal endotoxaemia and the use of IL-18 anti-serum in ischaemic acute kidney injury both result in protection from injury (125;126). IL-18 has been shown to up-regulate cell adhesion molecules, leading to leukocyte infiltration, which plays a pathogenic role in ischaemic injury (127).

It is induced and cleaved in the proximal tubule following acute kidney injury and is readily detectable in the urine. Cross sectional studies reveal that it is relatively specific for ischaemic acute kidney injury with little change in urinary levels in other conditions: urinary tract infections, chronic kidney disease, nephritic syndrome or pre-renal failure (128). However, levels can be affected by concomitant conditions such as endotoxaemia and immunologic injury (129). Plasma levels are known to be influenced by other systemic inflammatory diseases including systemic lupus erythematosus and inflammatory bowel disease.

Urine IL-18 has been shown to predict acute kidney injury up to 24hrs before the rise in serum creatinine in critically ill patients and in children undergoing cardiopulmonary bypass

surgery, where urine IL-18 levels increased around 6 hours and peaked at 12 hrs (105;130). There is no information regarding plasma IL-18 in detecting acute kidney injury.

In the transplant setting, urinary IL-18 levels have been shown to predict DGF, with an AUC of 0.95 and to reflect the rate of kidney function recovery out to 3 months (109;124).

1.6.5 Fatty acid binding proteins

Fatty acid binding proteins are a family of 15kDa proteins known to facilitate long chain fatty acid transport, regulate gene expression via PPARs and have anti-oxidant effects in ischaemic injury through their capacity to bind long-chain fatty acid oxidation products (131-133). Among the family of fatty acid binding proteins, two have been shown to be important in kidney injury: liver-type fatty acid-binding protein (L-FABP or FABP1) and heart type fatty acid binding protein (H-FABP or FABP3) (134). These proteins were named after the tissue in which they were first described but are not exclusive to these tissues: FABP1 is expressed in the liver, gut and kidney; FABP3 is expressed in the heart, brain, skeletal muscle and kidney. In the kidney, FABP1 appears to be specifically expressed in the cytoplasm of proximal tubules (134). In contrast, FABP3 is mainly expressed in the distal tubular cells of the kidney, but also occurs in proximal tubular cells to a lesser degree (134). Glomeruli and collecting tubules do not appear to express either protein on immunostaining (134).

Under physiologic conditions, free fatty acids bound to albumin are filtered by the glomerulus and absorbed in the proximal tubule. Here free fatty acids are released and bound by FABPs which can deliver them to the cell organelles or nucleus (135). When ischaemia occurs, lipid peroxidation products accumulate in the tubular cell inducing cellular injury. FABPs are capable of binding these lipid peroxidation products and transporting them into the urine, indicating a potential role as a cellular anti-oxidant during oxidative stress. Using imunohistochemical staining on 1 hr post reperfusion kidney transplant biopsies compared to pre-reperfusion biopsies, Yamamoto et al demonstrated a shift of FABP1 from the proximal tubule cytoplasm to the tubular lumen, illustrating its possible mechanism of action. Increased levels of FABP1 have also been shown to be protective in animal models of unilateral ureteral obstruction and diabetic nephropathy (136;137).

Plasma FABP3 levels are significantly influenced by age, sex and circadian rhythm with levels increasing with age, muscle mass and during the night. A plasma upper reference limit of 6μg/L has been proposed (138;139). FABP1 plasma levels are not influenced by age or sex but are higher at night also. No reference limit has been proposed. Both FABP1 and FABP3 are freely filtered by the glomerulus before being reabsorbed in the proximal tubule, which could partly explain the increase in urinary FABP levels following proximal tubular injury. No urine upper reference limit has been proposed. A urine FABP1 upper reference limit has been proposed at 17 μg/g creatinine (140). FABPs appear to be very stable proteins, which can undergo multiple freeze thaw cycles and still maintain immunoreactivity (131).

Elevated levels in urine have been detected in progressive kidney disease, as well as following ischaemic acute kidney injury in cardiac bypass surgery and kidney transplantation (141;142). Post cardiac bypass surgery, urine FABP1 levels rise early (~4hrs) and exponentially followed later by a more modest rise in plasma FABP1 levels. In addition to diagnosis, urinary FABP1 levels appear to be predictive of risk of developing AKI and the severity of injury. In patients undergoing a non-emergency coronary angiogram, those with elevated urine FABP1 all developed acute kidney injury following the procedure (143). A further study of patients with septic shock demonstrated that FABP1 levels predict severity of injury with non-survivors having higher levels and a smaller reduction in levels after treatment compared to survivors (144).

Compared to NGAL, KIM-1 and IL-18, there has been little research performed examining the performance of the FABPs as biomarkers in kidney transplantation. One study examined the use of plasma FABP3 levels in the donor to predict DGF but did not find any relationship (145). Urinary FABP1 levels in live donor renal allograft recipients negatively correlated with the peri-tubular capillary blood flow and directly correlated with ischaemic time (146). FABP3 has been shown to differentiate between controlled and uncontrolled NHBD kidneys on machine perfusion, with higher perfusate levels (at 4hrs) of H-FABP seen in uncontrolled donors with a longer WIT reflecting greater injury (147). High perfusate FABP3 levels, while predictive of DGF, were not associated with outcome (145).

1.6.6 Hepatocyte growth factor (HGF)

HGF is a 103kDa polypeptide primarily produced by non-epithelial cells, including fibroblasts, endothelial and mesangial cells (148). However, its c-met receptor is constitutively expressed on all renal cells, including epithelial cells (149).

It is a multifunctional peptide, with regenerative, anti-fibrotic and anti-apoptotic effects. Although first described in the liver, where it promotes growth and differentiation in hepatocytes, HGF has pleiotropic effects on multiple tissues including the kidney, particularly following acute kidney injury (148). Animal models demonstrate a role for HGF in preventing apoptosis and promoting tubular regeneration, which is lost with anti-HGF therapy (150). HGF reduces cell adhesion molecule expression on endothelial cells thereby reducing neutrophil infiltration, which is known to compound ischaemic injury (151). Its angiogenic properties prevent endothelial cell apoptosis, reduce capillary permeability and promote angiogenesis (152;153). HGF reduces macrophage production of IL-18, reducing the interferon gamma response and favours the formation of Tregs (154;155). Finally, through its antagonism of TGF-β1, it has both anti-fibrotic properties and anti-apoptotic properties on tubulo-epithelial cells in chronic injury states (148). Administration of HGF has been shown to be renoprotective in experimental models of ischaemic renal injury (156) and toxic renal injury (157).

In healthy individuals, trace amounts of HGF are found in the urine. Although acute kidney injury results in a marked rise in levels >0.5ng/mg creatinine, no reference limit for urine or plasma HGF levels in acute kidney injury have been proposed.

There have only been two studies examining HGF in native acute kidney injury which demonstrated the potential for HGF to be used as a biomarker (158;159). In the first study, acute kidney injury resulted in a marked rise in HGF which mirrored injury severity and persisted until urine output increased. Chronic kidney disease including glomerular disease and polycystic kidney disease had similar levels to healthy controls. The second study assessed the utility of HGF as a diagnostic tool and found that alone it had a low AUC 0.23 for acute kidney injury, although it was able to significantly differentiate a composite endpoint of mortality or need for dialysis.

Only one study has examined HGF as a biomarker for acute kidney injury in the early post transplant period. Kwiatkowska et al. found the highest HGF levels occur on post operative day 1 with the greatest decline occurring between day 1 and day 7(160). They did not assess its diagnostic utility or its association with DGF.

1.6.7 Combinations of biomarkers

A single study assessed 9 biomarkers in a cross sectional study on native acute kidney injury: KIM-1, NGAL, IL-18, HGF, cystatin C, N-acetyl-beta-D-glucosaminidase, vascular endothelial growth factor, chemokine-inducible interferon protein 10 and total protein (159). Each biomarker was assessed individually and then using a logistic regression model, a composite equation was formed: risk score equation (2.93*(NGAL > 5.72 and HGF > 0.17) + 2.93*(PROTEIN > 0.22) − 2*(KIM < 0.58) with an AUC of 0.94. Interestingly, IL-18 was not used in the risk score and its AUC outperformed any individual biomarker.

1.7 Interventions for acute transplant injury

Any intervention to reduce inflammation or confer cytoprotection may be more productive when carried out as early as possible in the transplant timescale. In clinical practice, this is not possible as patients become donors after catastrophic injuries have occurred, such as brain death. To intervene earlier in potential donors, before they are officially classed as such, is unethical. However, as soon as a patient is categorized as a donor, be it by brain death or by NHBD attributes, protection of the organs could be considered. Efforts are ongoing to develop novel therapeutic interventions aimed at ameliorating cell death or accelerating the recovery process.

Erythropoietin (EPO) is a drug which has been in widespread use to treat renal anaemia and more recently, anaemia associated with malignancy. There is accumulating evidence from animal and human studies suggesting that EPO confers tissue protection, attenuating both inflammation and apoptosis following ischaemia-reperfusion injury, resulting in improved organ function. If EPO can reduce transplant dysfunction peri-operatively, potential benefits

include improved early graft function and improved long term graft and patient survival, with associated cost savings in terms of financial and organ resources.

1.8 Erythropoietin (EPO)

1.8.1 EPO overview

Erythropoietin (EPO) is a primary mediator of the body's physiological response to hypoxia. By stimulating growth, inducing differentiation and preventing apoptosis of erythroid progenitor cells, there is an increase in the oxygen carrying capacity of the blood, thus correction of hypoxia. Since its introduction in 1989, recombinant human erythropoietin (EPO) has been a major advance in the management of anaemia associated with chronic kidney disease, enhancing patient cognitive function, physical activity and quality of life (161-163). In addition, there is now increasing evidence that EPO has pleiotropic effects on the body beyond the erythroid compartment, with the discovery of EPO and its receptor in non-haematopoietic cells, such as in the kidney, heart or brain (164-167). EPO promotes survival in a variety of cells by mediating anti-apoptotic and anti-inflammatory effects, as well as having a trophic effect.

1.8.2 EPO structure

Erythropoietin is a 30,400 Da glycoprotein, made up of 165 amino acids and four carbohydrate side chains, secreted by the EPO gene located on chromosome 7 (q11-q22) (168-171). The gene, existing as a single copy, consists of five exons and four introns which encode a protein precursor of 193 amino acids (172). Cleavage of the 27-amino acid leader sequence and loss of the C-terminal arginine produces a final circulating form of 165 amino acids(173). The EPO molecule undergoes N-glycosylation at three asparagine residues at amino acid positions 24, 38 and 83 and O-linked glycosylation at a serine residue, amino acid position 126 (170;174)**.** While the O-linked sugar appears to have no important function, the N-linked sugars improve the stability of the molecule in the circulation and prolong in vivo survival (173). In addition, deglycosylated EPO remains biologically active, but has a markedly reduced half life due to removal via galactose receptors on hepatocytes (175).

There are two structure stabilizing disulfide bonds between amino acids 7 and 161, amino acids 29 and 33, which are critical for bioactivity (174). The tertiary structure of EPO is made up of four anti-parallel α helices.

1.8.3 Regulation of EPO production

Following birth, the production of EPO is regulated primarily by tissue oxygen supply. Upon hypoxic exposure, activation of the HIF-1 pathway leads to increased EPO production, through binding of HIF-1 to the transcription enhancer at the 3′-flanking region of the EPO gene (176). Other factors known to increase local erythropoietin production include oestrogen in the female reproductive tract and raised intra-cellular calcium or hypoglycaemia in the CNS (177;178). Cytokines which regulate EPO production include TNFα, IL-1β and IL-6 (179).

1.8.4 Erythropoietic agents

Epoetin alfa (Eprex, Janssen-Cilag) was the first agent approved for the treatment of anaemia associated with renal failure, followed by epoetin beta (NeoRecormon®, Roche Pharmaceuticals) and darbopoetin alfa (Aranesp, Amgen Pharmaceuticals). Both epoetin alfa and epoetin beta are recombinant forms of endogenous erythropoietin with identical amino acid sequences to the endogenous form, but with an altered glycosylation pattern. They demonstrate similar pharmacokinetic profiles in healthy subjects. However, epoetin beta has a greater volume of distribution and a prolonged elimination following IV use, when compared to epoetin alfa. The sialic acid content of an EPO molecule is directly associated with its half life. Darbopoetin alfa is a novel erythropoiesis stimulating protein that contains a higher sialic acid carbohydrate content than endogenously produced erythropoietin. There are two additional N-linked carbohydrate chains, each containing four additional sialic acid residues. Thus it has five carbohydrate side chains compared with three in recombinant human erythropoietin. The result of this modification is a two to three fold longer half-life, but approximately four fold lower binding affinity for the erythropoietin receptor, when compared to epoetin alfa and epoetin beta. Despite its lower receptor binding affinity, it is significantly more potent in vivo than epoetin alpha or beta. When its administration is once weekly, it is approximately fourteen fold more potent than epoetin beta at the same dosing schedule.

Carbamylated EPO or CEPO is new form of EPO which lacks haematopoietic activity but retains its tissue protective effects. This is thought to arise via signalling through a heterodimer consisting of an EPO-R and a common β receptor (βcR) subunit (also known as CD131) (180). This remains controversial as EPO has been shown to be tissue protective in the absence of CD131 (181). CEPO is currently under investigation in phase 3 clinical trials.

1.8.5 Pharmacokinetics of EPO

EPO is present in the circulation at concentrations of approximately 15IU/L or 1-7pmol/L (182;183). It has a low volume of distribution indicating that the majority of EPO remains in the circulation and has a terminal half life of 8.1-9.4 hrs, before being removed by receptor mediated elimination (183). Using mathematical modelling in healthy volunteers, the fraction of occupied EPORs was 3.1% at baseline, increasing up to 98.7% with a dose of 1000U/kg. At this latter dose, receptors remained maximally saturated for 55.6hrs at this dose.

To convert IU/L to pM/L one must assume that 7.7mcg of EPO is equivalent to 1000IU and the molecular weight of EPO is 30.4kDa (184). One unit of EPO approximately corresponds to 10ng of EPO protein (185).

1.9 EPO receptor

1.9.1 The EPO-R gene

The human EPO-R gene is located on chromosome 19pter-q12 and is approximately 6kb in length (186).

1.9.2 Overall structure

The erythropoietin receptor (EPO-R) is a transmembrane (type 1) receptor is a member of the cytokine receptor superfamily, which also includes receptors for granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), growth hormone, prolactin, leukaemia inhibitory factor (LIF), Il-2, IL-3, Il-4, IL-5, IL-6, IL-7, all of whom are involved in haematopoiesis (187). The special characteristic of this family

of receptors is that they are activated and transduce signal following formation of a homo- or hetero-oligomer (dimers or trimers).

The mature receptor is a 484 amino acid glycoprotein, that ranges from 66 to 78kD and consists of 8 exons (extracellular domains 1-5; membrane spanning domain 6; and intracellular domains 7-8) (Figure 1.13) (188;189).

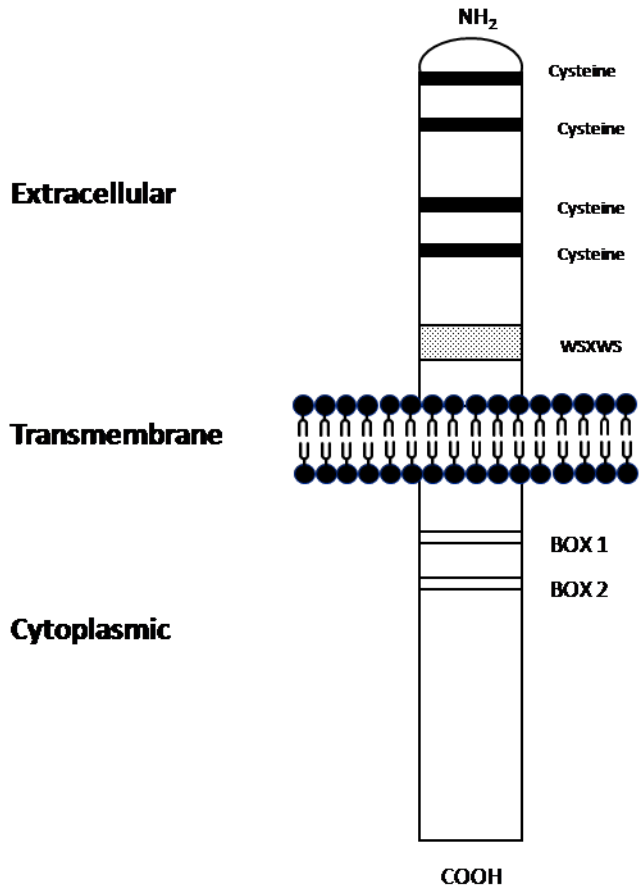


Figure 1-13: The basic structure of EPO-R and CD131 receptor.

1.9.3 The extracellular domain of EPO-R

The extracellular ligand-binding domain contains two pairs of cysteines and a conserved motif, Trp-Ser-X-Trp-Ser (WSXWS), which is situated close to the transmembrane domain. Two binding sites of differing affinities for EPO have been demonstrated with EPO dissociation constants (K_d) of 1nM and 1μM (190). Whether these different binding sites on

the extracellular domain result in the activation of different intracellular signalling pathways is unknown. However, human erythroblasts exhibit a single high affinity binding site for EPO (191). Haematopoietic activity of EPO requires binding of EPO to both sites, as site directed mutations which prevent binding to either site, inhibit EPO dependent haematopoiesis (192). Previously it had been reported that EPO-R and EPO form 1:1 complexes. However, as a result of the weak binding interaction of the second EPO-R (approximately 1000 fold weaker than the first binding), complexes are easily dissociated during chromatography, forming the 1:1 complex which was reported previously (190). Thus, one molecule of EPO activates two EPO-R by homodimerisation, changing the conformation of the receptor, bringing the JAK2 molecules in to close proximity which is necessary for autophosphorylation and activation (188;193). The relative orientation of the extracellular domain is important as dimerizing antagonists of EPO (EMP33) show that inhibitory dimeric conformations exist (194;195). Also, the relative dimeric orientation of the transmembrane and cytoplasmic domains is also important for activation (195). Finally, the degree of receptor binding depends on the carbohydrate content of the EPO with an inverse relationship existing (196;197). The carbohydrate portion of the ligand prevents receptor binding through electrostatic forces (198). Thus, the prolonged half-life of hyperglycosylated EPO analogues such as darbopoietin may be explained by reduced receptor binding and internalisation (196).

1.9.4 The cytoplasmic domain of EPO-R

Following ligand binding, some or all of the 8 conserved phosphotyrosine sites within EPOR's distal cytoplasmic domain are phosphorylated, despite the EPO-R not possessing endogenous tyrosine kinase activity (Figure 1.14) (199). This is performed by Janus Kinase (JAK)2, a cytoplasmic tyrosine kinase, which is associated with the membrane-proximal domain (box1/box2) of the receptor (173;200). Activated JAK2 phosphorylates both itself and distal phosphotyrosine sites, which then act as docking stations for signalling molecules containing SRC homology 2 (SH2) domains (201;202). Of these sites, tyrosine at position 343 binds STAT5 leading to anti-apoptotic Bcl-X gene expression (203;204), tyrosine at position 479 binds p85/PI-3-kinase, leading to the promotion of Akt survival pathways (205)and tyrosine at position 460 leads to calcium channel activation (206).

The activation of these signalling pathways is transient, despite continued presence of EPO, with a return to basal levels after 30-60 minutes of stimulation (207). The effect of EPO is

terminated by dephosphorylation of JAK2 by haematopoietic cell phosphatase (HCP), also known as SHP-1(208;209) and by Cis1, which promotes EPO receptor degradation (210). Failure to switch off the receptor, either due to mutations in the EPO-R cytoplasmic domain or functional deficiency of HCP, results in erythrocytosis (211;212). A further mechanism for receptor down-regulation is the internalisation and degradation of the internalised receptor (207). The proteasome controls the down-regulation of EPO-Rs in EPO stimulated cells, by inhibiting the cell surface replenishment of internalised EPO-Rs. Thus, proteasome inhibitors prolong the EPO-R signalling duration by allowing continuous replenishment of the cell surface pool of EPO-R (207). Gross and Lodish, using Ba/F3 and UT-7/Epo cells demonstrated that 60% of internalised EPO is re-secreted, while 40% is degraded (213). It remains to be shown whether this re-secreted EPO is biologically active. Few if any EPO-Rs are recycled back to the membrane (214).

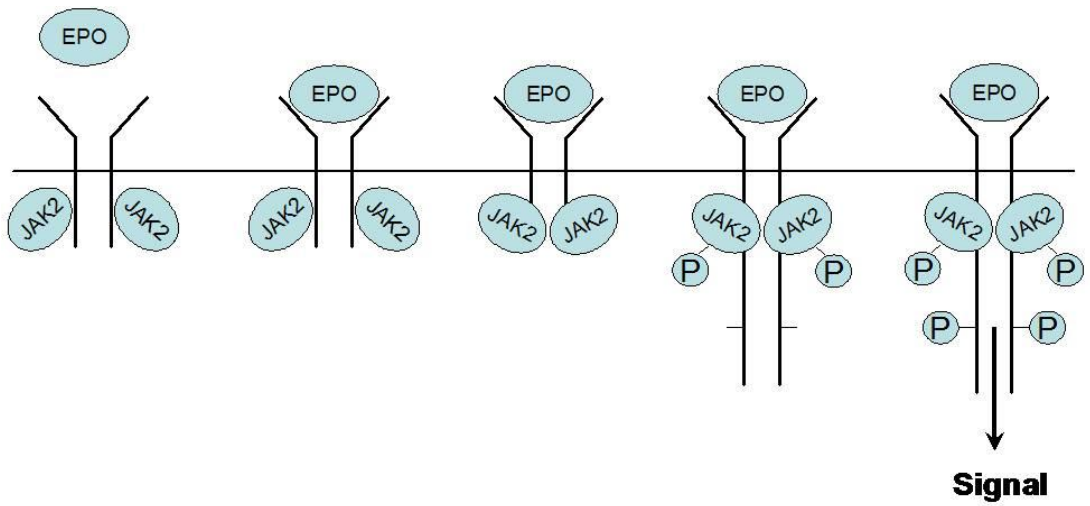


Figure 1-14: EPO induced conformational change of the EPO-R.

1.9.5 Sites of EPO-R expression

EPO-R is expressed mainly on erythroid progenitor cells with 100-300 receptors per erythroblast (191). In addition, EPO-R protein and/or EPO-R mRNA have been identified in a variety of tissues outside the haematopoietic system. In the kidney, all areas including the mesangium, proximal and distal tubular cells, express EPO-R and this is common to human, mouse and rat kidneys (164). Thus EPO receptors have also been detected in cyst epithelia

from polycystic kidneys and in malignant kidney cells. EPO-R expression has been demonstrated in a variety of cell lines originated from the cardiovascular system. In the mouse, complete knockout of the EPO-R results in severe cardiac malformations and foetal death by day 13.5, most likely as a result of anaemia and its role in vasculogenesis (215). Tissue specific EPO-R knockout mice excluding haemopoietic tissue, exhibit no abnormalities (216). Neonatal and adult rat cardiac myocytes express the receptor, as do endothelial cells and ventricular myocytes in human hearts (165;166). In the human brain, EPO-R becomes detectable 8 weeks following conception (167;217). It is expressed in neuronal cells, astrocytes and brain capillary endothelial cells (218;219). In EPO or EPO-R knockout mice, severe embryonic neurogenesis defects occur (220). Furthermore, mice with brain specific deletion of EPO-R have impaired migration of regenerating neurons post brain injury (220). Either EPO-R mRNA or EPO-R have also been demonstrated in breast carcinoma, lung carcinoma and female reproductive tract malignancies (221-224). However, reports of EPO-R expression, using anti-EPO-R antibodies for immunoblotting and immunostaining, should be viewed with caution due to the poor specificity of some of these antibodies (225).

1.9.6 Regulation of EPO-R expression

The EPO-R is constitutively expressed but increased expression has been demonstrated in haematopoietic tissue and brain in the presence of hypoxia and anaemia (226;227). In breast cancer cell lines, exposure to hypoxia has only a small effect on EPO-R mRNA expression in an 8hr period but causes a marked up-regulation of cell surface receptor in that time, suggesting that a reservoir of EPO-R exists intra-cellularly (221). Activation of the hypoxia-inducible factor 1 (HIF-1) pathway appears central to this (228). Other identified regulators of EPO-R gene expression include stem cell factor(229), IL-1α (230) and TNFα (179) which are positive regulators and interferon-γ (231), which is a negative regulator.

1.10 Beta-common receptor (CD131)

1.10.1 CD131 may be involved in EPO mediated tissue protection

One group have proposed that the tissue protective effects of EPO are mediated via a heterodimer consisting of an EPO-R and a common β receptor (βcR) subunit, also known as

CD131 (180;232). Carbamylated EPO (CEPO) does not bind to the EPO-R and thus has no haematopoietic activity. It has however been shown to be cytoprotective both in vitro and in vivo in a number of models including ischaemia, inflammation and trauma (232). Using CD131 knockout mice and cardiomyocytes, Brines et al. failed to demonstrate a cytoprotective effect of both EPO and CEPO in response to injury (180). They postulated that a hetero-complex of EPO-R and CD131 is responsible for the cytoprotective effects. In contrast, Um et al demonstrated tissue protection in neuroblastoma SH-SY5Y cells which lacked CD131 (181). Thus, it is unclear which receptor is responsible for EPO's tissue protective effects.

1.10.2 The CD131 gene

The gene for CD131 is located on chromosome 22 in humans (233).

1.10.3 CD131 overall structure

Like EPO-R, CD131 is a transmembrane receptor and a member of the cytokine receptor superfamily. Interleukin-3 (IL-3), IL-5 and GM-CSF receptors are heterodimers containing unique α chains and shared use of a common β receptor subunit (CD131). This common subunit has the dual function of modifying the initial ligand binding from a low affinity to a high affinity state, as well as being the major signal transducer for the receptor system (234). In addition to heterodimers, signal transduction from CD131 homodimers has been demonstrated. Using chimeric molecules expressing extracellular GM-CSF receptor α chain or EPOR and cytoplasmic β receptor domains, signalling in the presence or absence of ligand has been demonstrated (235;236). Furthermore, spontaneous homodimers have also been observed in primary cells, which are not phosphorylated in the absence of ligand (234).

Molecular cloning of the cDNA encoding human βcR predicts the protein to be an 880-amino acid molecule with a molecular weight of 120-140kDa. The CD131 gene consists of 12 exons, which encode 4 extracellular domains (organised in two cytokine receptor modules), a single transmembrane domain and a cytoplasmic domain (234;237).

1.10.4 The CD131 extracellular domain

The extracellular ligand-binding domain contains two pairs of cysteines and a conserved motif, Trp-Ser-X-Trp-Ser (WSXWS), which is situated close to the transmembrane domain (234;237).

1.10.5 The CD131 cytoplasmic domain

After activation of the receptor, there is tyrosine phosphorylation of several proteins, performed by JAK2 associated with the membrane-proximal domain (box1/box2) (233). The cytoplasmic region of the EPO receptor has significant amino acid homology with the intracellular portion of CD131. Thus, as with EPO-R, the principal signalling pathways are JAK/STAT pathway, the MAPK pathways and the PI3-K pathway. Like the activated EPO-R, HCP is involved in down-regulating CD131 activation and internalised receptors may be recycled (233).

1.10.6 Sites of CD131 expression

CD131 is expressed predominantly in haematopoietic cells and has yet to be convincingly demonstrated in kidney cells (233).

1.11 Signal Transduction Pathways used by the EPO-R

1.11.1 Phosphorylation of the EPO-R

Following binding of EPO to its receptor homodimer, the resulting conformational change leads to activation of a number of kinases and other signalling molecules. These include JAK2 kinase, protein kinase C (PKC), phophatidylinositol 3-kinase, Lyn kinase and Tec kinase (200;238-241). Jak2 and Lyn kinase phosphorylate tyrosine residues on the EPO-R cytoplasmic domain, resulting in docking of SH2 domain containing proteins, such as Grb2, STAT5, Shc and CrkL (200;240;242). This sequence of events represents the beginning of EPO-R signal transduction.

1.11.2 The JAK2-STAT5 pathway

The signal transducers and activators of transcription (STAT) family are substrates of JAKs, which regulate gene expression. Phosphorylation of the carboxy-terminal end of STATS leads to dimerization of STATS through the SH domains, translocation to the nucleus and binding to target genes, with eventual regulation of gene expression. EPO is known to activate STAT1, STAT3, STAT5A and STAT5B (243)**.** The activated JAK2 – STAT5 signalling pathway appears to be important in preventing apoptotic injury to a number of cells. EPO induces Bcl-XL through STAT5, and the STAT5 – PI3K interaction, which is thought to be responsible for differentiation and proliferation (cell cycle progression) in the haematopoietic system (204;244).

1.11.3 The PI-3K/Akt pathway

The second signalling pathway induced by EPO is through induction of phosphatidylinositol 3-kinase and the resulting phosphorylation of serine/threonine kinase (Akt) or protein kinase B (PKB), which is thought to be pivotal in maintaining cell survival (Figure 1.15) (243;245-248). Activation of Akt has been shown to be dependent on PI3K. Loss of Akt1 activity with gene silencing of Akt1 expression or pharmacological blockade of PI3K pathway, results in loss of its protective effect (248;249).

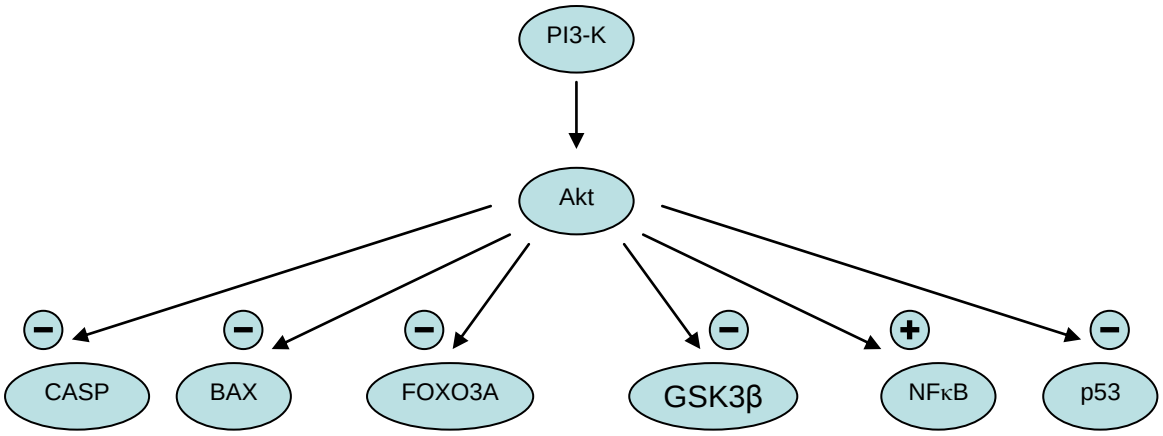


Figure 1-15: The PI3-K/Akt anti-apoptotic pathway

1.11.3.1 Akt and Bcl2 family gene expression

Activated Akt phosphorylates the pro-apoptotic factor Bcl-2-associated death-promoting protein (BAD), causing it to dissociate from the cell survival factor Bcl-XL and bind with the cytosolic protein 14-3-3 (250). This confers cytoprotection via its interaction with the pro-apoptotic protein BAX, preventing BAX translocation to the mitochondria and release of cytochrome c, in addition to activation of caspase-3 and caspase-9 (251). Akt activation also maintains Bcl-XL expression following injury, maintaining the pro-survival ratio of anti-apoptotic:pro-apoptotic Bcl2 family members (252) and preventing caspase 1, 3 and 9 induction following injury (253).

1.11.3.2 Akt and FOXO3a

The Forkhead transcription factors are involved in a number of cellular processes including apoptosis (254). Upon activation, FOXO3a translocates to the nucleus where it binds to specific DNA sequences and mediates the activation of transcription of genes involved in apoptosis such as the death receptor Fas ligand and BIM (254;255). BIM functions upstream of BAX – mediated cytochrome c release from the mitochondria. Phosphorylation of FOXO3a by Akt results in the export of FOXO3a into the cytosol, in association with the protein 14-3-3 (254). This inhibits any disruption of the mitochondrial membrane and subsequent cytochrome c release, thereby conferring cytoprotection.

1.11.3.3 Akt and GSK-3beta / β-catenin / c-Jun

Glycogen synthase kinase-3beta (GSK-3beta) is a serine/threonine kinase which mediates cell survival through the regulation of its multiple targets, including β-catenin and c-Jun. β-catenin signalling has been shown to be important in cell survival in endothelial cells and neuronal cells (256;256;257). c-Jun is a transcription factor produced in response to cell stress that activates a number of pro-apoptotic genes, including BIM, in neurons and endothelial cells (258;259). GSK-3beta negatively regulates β-catenin and up-regulates c-Jun. Following oxidative stress, GSK-3beta is associated with increased levels of caspase 3, BIM and cytosolic cytochrome c, leading to neuronal cell death (260). In erythroid progenitor cells, it has been shown to induce a conformational change in BAX associated with apoptosis induction (261). When over-expressed, GSK-3beta is also known to contribute to apoptosis in

vascular smooth muscle cells (262), endothelial cells (256) and cardiomyocytes (263). GSK-3beta activity is suppressed in response to EPO induced Akt activity (261).

1.11.3.4 Akt and the NFκB family

Akt is also cytoprotective through its effect on the NFκB complex (264). NFκB induces the expression of a number of anti-apoptotic genes in response to cellular injury or cytokine stimulation, including the inhibitor of apoptosis family (cIAP1, cIAP2 and XIAP) (265). These proteins inhibit the executioner caspases, caspases 3 and 6, as well as the initiator caspases, caspase 9. NFκB has also been shown to directly activate Bcl2-XL (266) and to suppress TNFα induced apoptosis through cIAP1 and cIAP2 inhibition of caspase 8 (267), as well as possibly through Bcl-2 homolog Bfl-1/A1 (268). In resting conditions, NFκB is bound in the cytosol to the IκB family. In response to TNF or other apoptotic stimuli, IKK (IκB kinase), which contains an IKKα and a IKKκ catalytic subunit, phosphorylates IκB causing it to release NFκB, which then translocates to the nucleus and activates transcription of target genes. Akt activates the IKKα subunit resulting in NFκB activation.

1.11.3.5 Akt and the caspases

Caspases are a family of cysteine proteases which have key roles in initiating and transducing apoptosis. Akt can indirectly suppress caspase activity, through the mechanisms described above. Akt has also been demonstrated to directly modulate caspase 9 activity through phosphorylation, thus protecting against TNF-α induced apoptosis (269).

1.11.3.6 Akt and p53

The tumour suppressor gene p53 plays an important role in apoptosis induced by DNA damage. Akt phosphorylates murine double minute (Mdm2), a ubiquitin ligase, to inactivate p53 (270). Activated Akt has also been shown to suppress p53-dependent transcriptional activation of BAX(271).

1.11.4 The Ras-MAPK pathway

EPO also signals through the ras-mitogen-activated kinase (MAPK) signalling pathway which promotes cell survival by increased transcription of pro-survival genes such as CREB (cAMP response element binding protein) and by suppressing BAD-mediated apoptosis.

1.11.5 EPO and aquaporins

Aquaporins (AQP) are a family of membrane proteins that function as water channels. 4 aquaporins have been identified in the kidney: AQP1, AQP2, AQP3 and AQP4. In the proximal tubule and thin descending loop of Henle, AQP1 has been found to be abundant, where it plays a role in constitutive water reabsorption (272). AQP2 is found on the apical membrane of collecting duct principal cells and participates in vasopressin-regulated water absorption (273). AQP3 and AQP4 mediate water transport across the basolateral membrane of the principal cells (274;275). In rat IRI models, EPO treatment at the time of injury prevented down-regulation of AQP2 and AQP3 protein expression in the kidney and attenuated down-regulation of AQP1, although not completely (276). This finding is consistent with the functional improvement in the urinary concentration ability of the rat kidney exposed to IRI. Interestingly, when EPO treatment was given 4hours post injury, it failed to prevent AQP2 down-regulation (276). One proposed mechanism for this is prevention of inducible nitric oxide production, which has been shown to inhibit the vasopressin induced increase in collecting duct water permeability. Furthermore, inhibition of nitric oxide protects against hypoxic injury of rat proximal tubules.

1.11.6 EPO and sodium transporters

Renal tubular water absorption is driven by active sodium transport. Thus disturbances in sodium transporter function, which have been demonstrated following IRI, can have profound effects on how the kidney regulates water balance. Protein expression of the sodium transporters Na/K ATPase, apical type 3 Na/H exchanger and thiazide sensitive sodium chloride channel has been shown to be decreased following IRI in the rat kidney (276). EPO treatment at the time of IRI has been shown to completely prevent this down-regulation. Delayed treatment with EPO until 4 hrs after IRI has also been shown to protect Na/K ATPase protein expression (276). These findings are consistent with the functional

improvement in renal sodium handling in EPO treated IRI kidneys compared to IRI kidneys alone.

1.11.7 EPO and intra-cellular calcium

Regulation of intracellular calcium by EPO is one of the signalling mechanisms controlling proliferation and differentiation of erythroid cells (277-280). Phosphorylation of PLC-γ1 leads to hydrolysis of PIP_2 to IP_3, which induces release of calcium from intracellular stores(281). Calcium channel proteins also become phosphorylated following EPO-R activation, further increasing calcium levels (278;279). More recently, this effect on intracellular calcium has been demonstrated in non-erythroid cells. Binding of EPO to its receptor has been shown to increase extracellular calcium influx in myoblasts and neuronal cell lines (282-284). In nerve growth factor deprived neuronal cells, EPO has been shown to increase cell viability and intra-cellular calcium. These effects were blunted by nicardipine and anti-EPO antibody, suggesting that EPO may stimulate neuronal function and viability through activation of calcium channels (285).

1.12 EPO as a tissue protective agent

EPO has been used both in the laboratory and clinically to ameliorate injury in cardiac, neurological and renal disease.

1.12.1 Cardioprotection

There have been a number of animal studies demonstrating EPO induced cardioprotection from ischaemia-reperfusion injury (180;286). Following acute coronary syndromes, EPO has been shown to protect cardiomyocytes and facilitate myocardial regeneration, with functional preservation in terms of left ventricular size and ejection fraction (287-289).

The mechanism of this protection has been elucidated by specific inhibitors of MAPK (p38 and p42/44) (290), protein kinase C (PKC) (291), potassium channels (290)and the PI3K/Akt pathway (292), all of which appear to be important in EPO mediated cardioprotection. The role of different pathways also appears to be time dependent, with activation of PKC by EPO

important prior to and during ischaemia but the PI3K and PAK pathways important post ischaemia (291;293). In addition to reducing apoptosis, EPO cardioprotection may have other explanations. The myocardial inflammatory response following ischaemia-reperfusion injury may be reduced with EPO pre-treatment, by up-regulation of nitric oxide production and by preventing activated myocytes switching to a pro-inflammatory phenotype (294). EPO also increases haemoglobin concentrations, thus increasing oxygen delivery to the tissues and reducing the degree of ischaemia. However, using carbamylated EPO, Fiordaliso et al in the rat model showed that the cardioprotective effect is independent of the erythropoietin effect (295). EPO also stimulates endothelial progenitor cell mobilization and activation, leading to neorevascularization, with a significant increase seen at 72 hours post single dose therapy (296-299).

EPO mobilises endothelial progenitor cells. The mechanism of protection in doxorubicin-induced cardiomyopathy may be related to preservation of EPC proliferation, migration and adhesion to areas of injury (300;301). A randomised, placebo controlled, pilot study of single dose darbopoietin 300ug was performed in 40 patients who underwent successful percutaneous coronary revascularisation for their first MI. FACS analysis demonstrated almost 3-fold increase in CD34+/CD45- cells in the blood at 72hrs post EPO treatment (298).

1.12.2 EPO promotes vascular integrity and angiogenesis

EPO confers vascular protection that extends beyond the preservation of individual endothelial cellular integrity, such as protection from oxidative stress, prevention of apoptosis and maintenance of cell to cell junctions (178;302-304). EPO has been shown to play a key role in angiogenesis through mitogenic and chemotactic effects on endothelial cells (305), promotion of vessel formation (299) and through mobilisation of endothelial progenitor cells (297). Thus, while EPO may provide direct protection to endothelial cells, its angiogenic effects may indirectly contribute to parenchymal cell protection and to better functional recovery through improved vascular perfusion.

1.12.3 Neuroprotection

In addition to EPO-R's, EPO is produced locally in the CNS and has been found in the CSF of humans (306). Astrocytes from rat foetuses produce EPO in an oxygen dose dependent manner (307). Interestingly, this EPO had a lower molecular weight, when compared to the circulating form, due to its lower sialic acid content. In vivo, much work has been done examining the effects of EPO on experimental brain injury in animal models. Using rodent models, epoetin alfa has been shown to ameliorate blunt force brain injury, experimental autoimmune encephalomyelitis and toxin-induced seizures. Studies by Brine et al have also demonstrated epoietin alfa to be efficacious in reducing brain injury by 50-75% when given up to 6 hours after induction of focal ischaemia (308).

There have been a number of studies in humans using EPO to prevent or treat CNS damage related to ischaemia. In the acute stroke trial, 20 patients were given 33,000iu IV daily for 3 days within 5 hours of onset of symptoms, compared with a control group (309). CSF EPO was 60-100 times higher in the EPO group. Treated patients had better and earlier improvements in follow up and outcome scores, as well as smaller infarct sizes at 1 month, when compared with controls. However, a follow-up study to this trial failed to show a benefit from high dose (40,000U) EPO administration, primarily due to a significantly increased mortality in the treatment group (310). Thrombolysis may have been a contributory factor to this outcome, through its associated increased risk of intra-cerebral bleeding complications.

EPO has been shown to reduce the incidence of ischaemic cerebrovascular accidents in patients undergoing surgical revascularisation of their heart with the use of a heart-lung machine (311). 10 patients received 24,000iu of EPO on three consecutive days, starting peri-operatively. None of the EPO group had an ischaemic event as assessed by MRI brain imaging compared to 40% of the control group.

Other studies have also shown better cognitive function and quality of life, both in renal and cancer patients, who are treated with epoetin alfa for anaemia (312).

There are many potential mechanisms of action of EPO in terms of neuroprotection, including direct anti-apoptotic effects on neurons, neoangiogensis and reducing the effects of glutamate (313) (314).

1.12.4 Renoprotection

1.12.4.1 Animal models

Much in vivo work has been done in rodents looking at the response of experimentally-induced ischaemia reperfusion injury leading to acute renal failure to EPO therapy (Table 1.1). In addition to establishing whether EPO confers renoprotection, these studies have examined its effects when administered before or at the initiation phase and/or during the recovery phase of the injury.

Yang et al. were the first group to suggest that EPO may protect the kidney from ischaemia-reperfusion injury (315). By administering 3,000iu/kg intra-peritoneally (IP) 24 hours before clamping bilateral renal arteries for 45 minutes, they were able to show a significant functional difference from the control group. There was attenuation in the rise of serum creatinine, reduced proximal tubular epithelial cell death by TUNEL staining, reduced caspase-3 production and increased HSP-70 and Bcl-$_2$ expression. Of note, HSP-70 increased in a dose dependent manner.

Vesey at al. later showed that a single high dose of EPO (5,000iu/kg), administered 30min prior to ischaemia in Sprague-Dawley rats, was associated with increased tubular regeneration (316). Increased tubular mitosis at 24hr was observed and confirmed by increased immunostaining for proliferating nuclear cell antigen (PCNA) within proximal tubular cells.

Patel et al. used a murine ischaemia reperfusion model to compare a regimen of 1,000iu/kg/day of EPO for 3 days prior to surgery to a single dose of 1,000iu/kg, at the time of reperfusion, with the higher dose based on a regimen known to stimulate endothelial progenitor cell production (although the authors didn't measure endothelial progenitor levels) (317). Uraemia is known to decrease circulating endothelial progenitor cells (318) thus potentially limiting vascular repair. While pre-conditioning conferred a greater reduction in

serum creatinine in this model, both arms of the study gave significant protection from ischaemia-reperfusion injury, with less injury, less neutrophil infiltration and less lipid peroxidation.

Authors, ref	Model	Dose	Timing	Outcome
	Acute Kidney Injury			
Yang et al.(315)	Rat: 45 min I/R injury	3,000iu/kg IP	24 hrs pre-injury	Reduced apoptosis Functional protection
Vesey et al.(316)	Rat: 45 min I/R injury	500-3,000iu/kg IP	30 mins pre-injury	Reduced apoptosis Reduced necrosis Functional protection
Patel et al.(317)	Mouse: 30 min I/R injury	1,000iu/kg SC	72 hrs pre-injury or On reperfusion	Anti-inflammatory
Abdelrahman et al.(319)	90mins I/R model	300iu/kg IV	At reperfusion	Functional protection Decreased CASP3
Sharples et al.(248)	Rat: 45 min I/R injury	300iu/kg IV	30mins pre-injury or At reperfusion or 30 mins post reperfusion	Reduced apoptosis Functional protection
Ishii et al.(320)	Monkey 90 min I/R model	12,000iu x 2 doses	At injury and post-injury	Reduced apoptosis Functional protection
Johnson et al.(321)	Rat 45 min I/R injury	5,000iu/kg IV	At injury or 6hrs after reperfusion	Reduced Apoptosis Functional protection
Forman et al.(322)	Pig 60mins I/R injury	5,000iu/kg IV stat + 1,000iu/kg SC daily	At injury then for 5 days post injury	Functional protection Decreased cell death
Kitamura et al.(323)	Rat Ureteral obstruction	CEPO	Pre-injury	Functional protection
Bahlmann et al.(324)	Rat 5/6th nephrectomy	0.1µ/kg/week	Weekly x 6	Decreased apoptosis Vascular protection
	Chronic CyA Toxicity			
Lee et al.(325)	Rat CyA nephrotoxicity	100iu/kg	3 doses/week for 4 weeks	Less fibrosis Anti-apoptotic Anti-inflammatory
	Transplant Model			
Bagul et al.(326)	Pig DCD transplant model	5000iu	Start of cold storage or At reperfusion	No benefit
Maio et al.(327)	Pig DCD transplant model	1000iu/kg I|V	Pre-cardiac arrest	Anti-inflammatory Functional protection

Table 1-1: Animal models of EPO tissue protection in acute kidney injury.

Using a similar model, Sharples et al. used a single low dose of EPO (300iu/kg) IP in 3 groups at 30 minutes prior to, at reperfusion and 30 minutes post reperfusion (248). They showed better kidney function, with preservation of the tubular architecture, when compared with the control group. At a molecular level, they found increased XIAP and Bcl_2 expression and reduced caspase -3, -6 and -9 expression, favouring cell survival. Importantly, EPO administration 30 minutes post reperfusion was also associated with a significant protection at 6 hours, albeit to a lesser extent than the other two groups.

In a primate model of ischaemia reperfusion injury, EPO 12000u was given to monkeys with a single kidney 5 minutes before clamping the left renal pedicle for 90 minutes and 5 minutes before releasing the clamp (320). The EPO group had significantly better serum creatinine and cystatin C levels, with less apoptotic cells compared to the placebo group. Interestingly, IL-6 levels were also lower in the EPO group suggesting an anti-inflammatory effect of the EPO.

As early as 1994, Vaziri et al. looked at the effects of EPO on tubular recovery, in a model of cisplatin induced nephrotoxicity in the rat (328). A dose of 100iu/kg administered daily for 9 days was associated with a higher creatinine clearance by day 9 when compared to a control group. Using ^{3}H-thymidine incorporation in cortical tissue as a marker of tubular regeneration, they showed that the enhanced functional recovery was associated with increased tubular regeneration.

Forman et al examined EPO dosing both at the initiation and recovery phase of ischaemia reperfusion injury in a pig model, where the kidney was clamped for 1 hour and then followed for 5 days (322). EPO conferred significant function protection with differences in the creatinine clearances between EPO and placebo groups occurring in as little as 12 hours. Interestingly, these differences were lost by 36 hours. Apoptosis and necrosis were considered together as ‘cell death’, which was significantly lower in the EPO group, although no difference was seen in the mitotic rate.

To address if late administration of EPO could still confer tissue protection, Johnson et al administered EPO (5000U/kg) to rats at the beginning of 45mins of ischaemia or 6 hrs after reperfusion (321). Both regimens were significantly better than controls in maintaining renal function and reducing both apoptosis and apoptotic gene expression (BAX), with no

functional or expression differences seen between the two time points. This study also showed the darbopoietin conferred similar degrees of tissue protection to erythropoietin.

In a cyclosporine rat model, Lee et al. administered 100iu/kg thrice weekly and found a renoprotective effect (325). Histologically, EPO treated rats showed less tubulointerstitial fibrosis and less macrophage infiltration. At a molecular level, there was increased Bcl_2 and caspase-3 expression, favouring cell survival; decreased pro-inflammatory mediators osteopontin and C-reactive protein; and decreased pro-fibrotic mediators transforming growth factor β1 (TGFβ1) and TGFβ1-inducible gene-h3.

In a DCD transplant model of ischaemia reperfusion injury, pigs were given 1000u/kg of EPO IV 30 minutes prior to cardiac arrest, before undergoing 30 minutes of warm ischaemia followed by 24hrs cold static storage. The organs were then transplanted and followed for 4 hours, at which point glomerular function (as measured by creatinine clearance) and tubular function were assessed. Creatinine clearance, urine flow and fractional excretion of sodium were significantly better in the EPO group. EPO had anti-inflammatory effects resulting in a reduction in serum inflammatory markers, IL-1 and IL-6, as well as reduced neutrophil infiltration, as measured by myeloperoxidase levels.

In contrast to this study, a porcine model of controlled DCD kidneys given 5000u IV of EPO at the beginning of reperfusion and/or the beginning of cold storage did not show a tissue protective benefit or functional difference (326). This study examined EPO given at the start of 18hrs of cold storage or EPO given before 16hrs static cold storage and 2hrs normothermic perfusion. All kidneys underwent an initial 10 minutes of warm ischaemia prior to initiating the study. The only difference seen was improved oxygen consumption compared to control in EPO group undergoing cold storage and normothermic perfusion.

In addition to epithelial and endothelial cells, EPO also appears to confer cytoprotection on podocytes. Darbopoetin in a rat model, when given prior to injury and in increasing doses, reduced proteinuria and podocyte injury, independent of haematocrit (329). This protective effect was correlated with immunohistochemical amelioration of markers of podocyte injury, desmin and the immune co-stimulator molecule B7-1, with the preserved nephrin expression in the slit diaphragms. Podocyte foot process retraction and effacement along with actin filament rearrangement, were all reduced by EPO treatment. Interestingly, terminal

deoxynucleotidyl (TdT)-mediated deoxyuridin triphosphate (dUTP) nick end labeling (TUNEL) staining of rat glomeruli and cultured podocytes showed no significant difference between treated and control groups, suggesting the EPO effect is unlikely to relate to a reduction in apoptosis. This contrasts with previous experiments in tubular epithelial cells and glomerular endothelial cells, where EPO is anti-apoptotic (248;324). Binding of EPO to its receptor activates protein kinase B (Akt) which is anti-apoptotic (330). However, Akt co-immunoprecipitation with actin has also been demonstrated and this direct interaction of Akt with actin may be the beneficial mechanism through which EPO protects podocytes (329;331;332).

Thus almost all animal studies examining acute kidney injury in the native and transplant setting have shown functional improvements, in addition to anti-apoptotic or anti-inflammatory effects after EPO administration, either before, during or after the injury has taken place. Most importantly, the demonstration that EPO can be given after a period or warm ischaemia or following reperfusion is important for interpreting its potential benefits in human transplantation, since little can be done to ameliorate injury until after the donation process has taken place.

1.12.4.2 Human studies

There have been several studies examining the potential renoprotective benefits of erythropoietin both in native and transplant acute kidney injury (Table 1.2).

Song YR et al administered 300mg/kg of erythropoietin beta IV to 36 adults undergoing coronary artery bypass grafting at induction of anaesthesia and noted a reduction in the incidence of acute kidney injury (EPO 8% vs Placebo 29%, p=0.03), defined as a 50% increase in serum creatinine over baseline in the first 5 post-operative days (333). In the EPO group, there was attenuation of the rise in serum creatinine post-operatively and a reduction in the delta GFR. No adverse events were attributed to the study drug. Poulsen et al examined the effect of high dose EPO (500iu/kg IV 12-18hrs pre-op and at induction) in patients undergoing CABG with no apparent difference between treatment and placebo groups with regard to serum creatinine (334). It must be noted that this was a secondary endpoint, although they also did not find any anti-inflammatory effect of EPO. In fact, they found EPO augmented the TNFα response to cardiopulmonary bypass.

A French multicentre placebo controlled trial examined the effect of 40,000U EPO IV administered before kidney transplantation, at 12 hours, 7 days and 14 days post-operatively on the incidence of DGF(335). There was no difference in DGF rates between the groups EPO (32% vs placebo 29%, p=ns), with similar eGFRs at 1 month post-implantation. Importantly, there was no difference in adverse events reported in the two groups.

Table 1-2: Human studies examining EPO tissue protection in acute kidney injury.

Authors, ref	Study	Disease	Patient No.	Dose	Timing	Outcome
Song et al.(333)	RCT	AKI 2° to CABG	71	300iu/kg IV	pre-operatively	Less AKI
Martinez et al.(335)	RCT	DGF 2° to transplantation	104	30,000iu IV	pre-op, 12hrs, day 7, 14	No effect
Poulsen et al.(334)	RCT	SIRS in CABG	43	500iu/kg IV	12-18 hrs pre and at induction	No effect
Mohiuddin et al.(336)	Case control	DGF 2° to transplantation	207	Variable SC	Anaemia maintenance	No effect
Kamar N et al.(337)	Case control	DGF 2° to transplantation	181	250iu/kg SC	Anaemia maintenance	No effect

Mohiuddin et al performed a retrospective review of patients receiving anaemia maintenance doses of EPO at the time of transplantation and found no difference in the DGF rate or haemoglobin out to three months compared to those not on EPO (338). A second retrospective study in France compared recipients on 250U/kg/week of EPO at the time of transplantation to recipients not receiving EPO and also found no difference in graft function or haemoglobin level at 1 month (337).

Thus, despite the overwhelming experimental evidence for the efficacy of EPO to prevent ischaemic injury, there is little clinical evidence to support these tissue protective effects in human acute kidney injury.

1.13 EPO toxicity

Rat brain vascular endothelial cells exposed to anoxia and increasing EPO demonstrated a ceiling effect, where doses higher than 10ng/ml (~1U/ml EPO) were not associated with increased survival (302). Other studies have demonstrated similar effects. In a model of cerebral ischaemia, EPO at a dose of 50U/day or 500U/day was ineffective at ameliorating neuronal damage, when compared to doses of 25U/day or less (185). One possibility is that cells respond to EPO within a limited concentration range and that high concentrations may induce down-regulation or internalisation of the EPOR, preventing further signalling (185).

In doxorubicin-induced cardiomyopathy in rats, EPO at a dose of 20U/dose was associated with increased survival and better myocardial contractility compared to 200U/dose (300).

1.13.1 Safety of EPO

Numerous clinical trials have shown the benefit and safety of using EPO to treat anaemia associated with renal failure and malignancy. In a review of controlled studies using EPO, it was noted that the adverse events varied from indication to indication and perhaps were influenced by the underlying illness (339). Side effects of erythropoietin treatment include hypertension, thromboembolism, headache, seizure, myalgia and flu-like symptoms.

1.13.1.1 Hypertension

Hypertension is a well established complication of long term EPO therapy, with approximately 25% of renal patients developing new onset hypertension or worsening of their blood pressure control (340). The cause is likely to be multi-factorial as a number of potential mechanisms have been identified as contributing to this phenomenon. Initially, it was proposed that changes in blood pressure where as a result of increases in haematocrit (341). A higher haematocrit is thought to raise vascular resistance through an increase in blood viscosity and adversely affect hypoxia-induced vasodilation (342-344). However, Besarab et al. demonstrated that blood pressure did not differ between two groups of haemodialysis patients with haematocrits of 42% and 30% respectively (345). Furthermore, repeated blood transfusions did not increase arterial pressure, despite normalising haemoglobin levels in uraemic rats (341). Thus, it is unlikely that increases in the red cell mass are a major

contributory factor. The main pathophysiological mechanisms are likely to be related to changes in endothelial and vascular smooth muscle cell function.

Firstly, EPO enhances endothelin-1 (ET-1) expression by up-regulating its transcription via increasing cytosolic free calcium (346;347). Miyashita et al. gave haemodialysis patients a single injection of 9,000iu of EPO and monitored blood pressure and ET-1 levels (348). Approximately 70% of patients had a 0-4mm Hg rise in their mean blood pressure (MBP), while approximately 25% had a 5-9mm Hg rise in their MBP. In patients who had a blood pressure rise, ET-1 expression increased also. In another study, intravenous administration, rather the sub-cutaneous administration of EPO was associated with elevated mean arterial blood pressure and ET-1 levels in haemodialysis patients (349). This may be as a consequence of IV doses being higher to achieve the same duration of effect as a SC dose.

Nitric oxide appears to have a role in EPO-induced hypertension. The proposed mechanisms for this are multi-factorial, including reduced responsiveness to its vasodilatory effect, increase in nitric oxide synthase inhibitors as well as reduced expression of nitric oxide and nitric oxide synthase (350;351). The vasodilator effect of nitric oxide is mediated by a cyclicGMP-induced fall in intracellular calcium, which EPO antagonises. Interestingly, calcium channel blockade in uraemic animals treated with EPO restores normal intra-cellular calcium levels and increases vascular and renal nitric oxide synthase expression.

Increased noradrenaline levels and responsiveness to noradrenaline may be contributory (352), as may changes in the ratio of vasodilatory to vasoconstrictory prostaglandin levels (353;354). The role of the renin-angiotensin system is controversial, but the presence of the T235 variant of the angiotensinogen gene polymorphism may be associated with EPO-induced hypertension (355).

EPO has a direct effect on vascular smooth muscle cells, causing an increase in smooth muscle tone via increased intra-cellular calcium. This vasoconstrictor effect increases peripheral vascular resistance and reduces the response to vasodilators (356).

Interestingly, none of the randomised controlled trials examining the effects of high dose EPO for tissue protection or to prevent blood transfusion dependency have listed hypertension as an adverse event (see below).

1.13.1.2 Thromboembolism

EPO has beneficial effects on platelet function and haemostasis through increases in platelet serotonin content and release, the number of glycoprotein IIb-IIIa molecules on the platelet membrane and also increased thrombin-induced phosphorylated platelet proteins (357-359). Reductions in protein C, protein S and TPA levels have also been noted (360-363), as have increased levels of plasminogen activator inhibitor 1 (PAI-1). Indirectly, it improves platelet function by correcting anaemia, which is associated with a prolonged bleeding time in patients with renal failure (364).

There have been numerous studies examining the role of EPO in venous thromboembolism, with conflicting results. Henry et al compared 80,000iu every 2 weeks with 40,000iu weekly in patients with chemotherapy induced anaemia (target haemoglobin of 12g/dL) and reported an 8% incidence of venous thrombosis in each group (365). A placebo controlled trial in children with non-myeloid malignancies given 600-900iu/kg/week over 16 weeks showed no difference in the adverse event rate (366). A clinical study conducted in surgery patients, not participating in an autologous blood donation program, with pre-treatment haemoglobins of >10g/dl to </=13g/dl showed the rate of deep venous thrombosis was similar between epoetin alfa and placebo (367). Sowade et al reported no significant increased thromboembolic risk in a placebo controlled trial in which patients undergoing cardiac surgery who received 5 doses of 500iu/kg epoietin beta IV over 14 days pre-surgery (368). All patients received S/C heparin pre-operatively. In a study examining the pharmacokinetics of EPO in serum and CSF, four patients with malignancies were given 1500iu/kg of epoietin alfa (369). The drug was well tolerated except in one patient who developed a deep femoral thrombosis 5 days later.

The Breast Cancer Erythropoietin Survival Trial (BEST) (target haemoglobin of 12-14g/dL) showed an increase in venous thromboembolism in the EPO (40,000iu epoietin alfa weekly) group of 16% versus 14% in the placebo group (370). The mean haemoglobin in the study was 12.5g/dl. This trial has been criticized for its failure to include stratification factors such as performance status in its randomization process such that the EPO arm tended to have sicker patients (371). A second trial by Henke et al, was also stopped early due to a decrease in survival in the EPO treatment group (300iu/Kg 3/week), with an increase in thromboembolic phenomenon (11% versus 5%) (372). This may well be related to the high

target haemoglobin set in the trial, with 15.4g/dl being achieved at the end of the study in men. The target haemoglobin in this group of patients is 12-13g/dl according to the European Organisation for Research and Treatment of Cancer 2006 guidelines (373). A recent meta-analysis by Bohlius et al, concluded that there was no significant increase in the relative risk of venous thromboembolism with EPO treatment versus placebo (RR=1.58, 95% CI=0.94 to 2.66) (374).

An increased risk of arterial thrombosis has not been identified with the use of EPO, when compared with placebo (375;376).

Thrombotic rates in EPO studies performed with high target haemoglobin levels or no ceilings must be interpreted with caution due to convincing evidence that artificially raised haemoglobins are associated with increased mortality which may be a product of increased red cell mass, rather than a direct effect of the drug itself (377). This is particularly true in studies of cancer patients, who already have elevated thrombotic risk.

1.13.1.3 Malignancy

Prominent EPO-R expression has been demonstrated in several human cancer cell lines (221;223). In regions of tumours adjacent to viable and necrotic cells (hypoxic centres), large numbers of apoptotic nuclei have been demonstrated. However, apoptotic nuclei were rarely seen in cells expressing EPO and EPOR, suggesting that their presence conferred protection from hypoxia induced injury (221). Activation of the EPO-R by EPO has been shown to inhibit apoptosis in haematopoietic cells. If present in cancer cells, in addition to hypoxia mediated up-regulation of the EPO-R, this could lead to a population of cells relatively resistant to both apoptosis and potential treatments.

1.13.1.4 Dose safety:

Healthy adults have received doses of epoietin alfa up to 1,500iu/kg three times per week for three to four weeks, without any direct toxic effects (367). Adults have also received doses of 3,000iu/kg in a single day with no adverse events (367). Following accidental administration of a total dose of 384,000iu of erythropoietin over three days to two patients, the only observed adverse events included elevated AST levels and a mild rise in haemoglobin (up to

1.7g/dl increase) (378). Both of these adverse events had resolved spontaneously by 3 months.

Table 1.3 summarises the randomised controlled trials of high dose EPO in tissue protection. In addition to these studies, high dose EPO has been used to reduce blood transfusion requirement and its potential side effects. Weltert et al. gave 14,000iu SC two days pre-operatively, 14,000iu on the next day, 8000iu on the morning of surgery, 8,000iu one day post-op and 8,000iu the following day(379). Interestingly, while patients in the EPO group were more likely to have raised liver enzymes, fewer patients suffered neurological or thrombotic events.

Table 1-3: Randomised controlled trials of high dose EPO for tissue protection.
***Number of patients treated with erythropoietin; ** darbopoietin dose equivalent to 100,000iu EPO**

Authors, ref	Patient No. Treated	Dose	Timing	Outcome
Lipsic et al. (298)	11	300µg**	Pre-angioplasty	No adverse events
Mocini et al. (380)	20	40,000iu	Pre-op	No adverse events
Silver et al. (381)	42	40,000iu	Weekly	No adverse events
Ehrenreich et al.(309)	21	33,000iu	Daily x 3	No adverse events
Ehrenreich et al. (310)	256	40,000iu	Daily x 3	? ↑mortality
Song et al.(333)	36	300iu/kg	pre-operatively	No adverse events
Poulsen et al.(334)	22	500iu/kg	<18 hrs pre-op and at induction	No adverse events
Martinez et al.(335)	51	30,000iu	pre-op, 12hrs, day 7, 14	No adverse events
Lakic et al. (311)	10	24,000iu x 3		No adverse events
Weltert et al. (379)	158	14,000iu x 2, then 8,000 x 3	Daily	?neurological sequelae ?thrombotic sequelae Raised liver enzymes

1.14 Conclusion

There is a pressing need to improve both short and long term outcomes in kidney transplantation, particularly with the increasing frequency of non-heart beating donors and extended criteria donors making up the donor pool. There is abundant evidence that erythropoietin confers tissue protection in a number of organs in animal models examining different injury settings. However, there is disappointingly little evidence that this can be translated into human disease. The following EPO trial was designed and instigated after the publication of the first stroke study that first outlined the potential benefits of EPO in human ischaemic medicine.

CHAPTER 2 RESEARCH QUESTIONS

2.1 Primary hypothesis:

High dose EPO administered intra-venously peri-operatively protects extended criteria and non-heart beating donor kidneys from ischaemia-reperfusion injury, as evidenced by a reduction in the incidence and severity of delayed graft function, reduced acute kidney injury biomarker levels and a differential gene expression between EPO and placebo treated groups, in keeping with a reduced degree of injury.

2.2 Secondary hypothesis:

The mechanism of action of high dose EPO can be unravelled through a combination of in vitro cell culture models and analysis of the in vivo responses of biomarkers and gene expression to EPO treatment.

2.3 Expected value

Both the clinical and laboratory aspects of this project will contribute to the design of a multi-centre study to examine the role of EPO in human kidney transplantation. Knowledge acquired from this may have important implications at a local, national and international level, both to patients and institutions. Reducing early graft injury is likely to lead to improved outcomes both in the short and long term. This improvement would likely translate into shorter hospital stays post transplantation, reduced need for dialysis post transplantation and in the longer term, longer graft survival and reduced requirement for maintenance dialysis. As a result, there are potential substantial economic benefits and improvements in patient quality of life and life expectancy.

More generally, this project will lead to a better understanding of ischaemia-reperfusion injury. The possible wider application of this work includes all areas of ischaemic medicine, including non-renal organ transplantation, myocardial infarction, ischaemic stroke and ischaemic kidney injury in various settings.

CHAPTER 3 GENERAL METHODS

3.1 Introduction

General methods are described in this chapter with specific details or modifications described in the "Specific Methods" section in each result chapter.

Details of the clinical trial methodology, including assay methodology used, are described in Appendix A.

3.2 Cell lines

Both primary cells and immortalised cell lines were used in the development of a cell model mimicking transplantation.

3.2.1 Human Primary Renal Proximal Tubular Epithelial Cells (RPTECs)

Primary renal proximal tubular cells (Lonza Group Ltd, Switzerland) were grown using a Clonetics™ REGM™ Bulletkit™ (CC-3190): Renal Epithelial Cell Basal Medium, 500mls; hydrocortisone, 0.5ml; hEGF, 0.5ml; foetal bovine serum, 2.5ml; epinephrine, 0.5ml; insulin, 0.5ml; triiodothyroine, 0.5ml; transferrin, 0.5ml; GA-1000, 0.5ml. All cells are performance assessed and tested negative for HIV-1, mycoplasma, Hepatitis B, Hepatitis C, bacteria, yeast and fungi.

3.2.2 Immortalised human proximal tubular cells (HK-2)

HK-2 cells (American Type Culture Collection, Manassas, VA), an immortalised human proximal tubular epithelial cell line, were grown and passaged in 75-cm^2 cell culture flasks that contained KSF media supplemented with 5% FCS, antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin), 25mg bovine pituitary extract and 2.5 μg recombinant epidermal growth factor. Cells were tested negative for mycoplasma.

3.2.3 Immortalised human microvascular endothelial cells (HMEC-1)

HMEC-1 cells, an immortalised dermal microvascular endothelial cell line, were grown and passaged in 75-cm^2 cell culture flasks that contained MCDB-131 media supplemented with 10% FCS, antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin) and 200mM L-Glutamine. Cells were tested negative for mycoplasma.

3.3 Cell culture

3.3.1 General conditions

Cell culture was performed under sterile conditions in a humidified 5% CO_2 atmosphere at 37°C. All solutions and equipment were sterile when obtained from the manufacturer. 0.2µm filters were used to filter sterilise any solution that was not guaranteed to be sterile. Class II biohazard extraction hoods were wiped with 2% Virkon solution prior to and following cell work, following which surfaces were subject to UV light treatment. The following sized cell culture flasks and plates were used: 75cm^2 and 25cm^2 flasks; 96 and 6 well plates. 75cm^2 flasks had 20µm filtered lids to allow atmosphere equilibration and maintenance of sterility. The volume of culture medium / University of Wisconsin solution used for each size of vessel was as follows: 10mls for a 75cm^2 flask; 5mls for a 25cm^2 flask, 2mls for each well of a 6 well plate and 100µl for each well of a 96 well plate. Cell culture medium was changed 3 times per week.

3.3.2 Trypsinisation and passaging of cells

Medium was aspirated from confluent flasks and cells were washed, depending on the cell type and manufacturer guidelines:

- RPTECs were only passaged using solutions supplied by Lonza (CC-5034): Cells were first washed with HEPES-BSS (5mls/25cm^2), followed by trypsinisation (2mls/25cm^2) for up to 5mins at 37°C, which was then neutralised with trypsin

neutralising solution (6mls/25cm^2). Cells were then centrifuged at 1200rpm for 5mins before the medium was aspirated off.

- HK-2 cells were washed 3 times with HANKs solution, while HMEC-1 cells were washed 5 times in HANKs solution, at volumes equivalent to the recommendations for medium use in that vessel. Trypsin and EDTA was added at a volume of 5mls/75cm^2 for 5 mins at 37°C before neutralisation with medium. Cells were centrifuged at 1200rpm for 5 minutes before the medium was aspirated off.

3.3.3 Cryopreservation

Cell pellets for cryopreservation were re-suspended in 1ml of freezing medium, consisting of 10% DMSO, 30% FCS and 60% culture medium. Aliquots were transferred into cryotubes, labelled with the date, cell type, number of cells and passage number where appropriate, before storage in a -80°C freezer for 24 hrs. Cells were then transferred to liquid nitrogen.

3.3.4 Thawing cells

Cryotubes were removed and immediately thawed in warm water. The cell suspension was added to the correct volume of medium for the plating surface. Cells were incubated at 37°C to allow adherence and proliferation until cells reached 70-80% confluency before undergoing passage or beginning experiments.

3.4 Characterisation of cells

3.4.1 Flow cytometry

Cells in a T75cm^3 flask were trypsinised when 90% confluent and washed 3 times in HANKs solution. After the final wash, cells were suspended in 1ml of HANKs. 25 µl of cells (~1-2 x 10^5 cells) were each added to 20 µl of fluorescein-conjugated anti EPO-R reagent (FAB307IF, R&D Systems), 20 µl of fluorescein-conjugated mouse IgG isotype as a control, EPOR mouse monoclonal antibody (38409.11, R&D Systems) and CD131 mouse monoclonal antibody (140516, R&D Systems). Cells were incubated for 30mins at 4°C prior

to being washed in PBS. For indirect staining, a FITC donkey anti-mouse antibody was added for a further 30mins at 4°C before flow cytometry analysis.

3.4.2 Western blotting

Five T75 flasks each of HMEC-1 and HK-2 cells were grown to confluency, washed thoroughly in serum free medium x 3 and extracted with ProteoExtract, native membrane protein extraction kit (Calbiochem) according to the method. Extracts were assayed for protein content by BioRad Folin assay and stored at -80°C until use. 10µg of protein was loaded per track onto a 4-20% gradient SDS gel together with molecular weight standards and electrophoresed for 30 minutes. The gels were electroblotted onto PVDF membranes in CAPs buffer in a semi-dry blotter for 60 minutes and blocked in protein blocker (Pierce labs) overnight. Blots were exposed to biotinylated antibodies specific for either EPO receptor or CD131 for 4 hours and following washing x3 in PBS Tween, the blots were incubated in streptavidin-HRP for 2 hours. Following further washing in PBS Tween x 3, blots were incubated in chemiluminescent substrate for 5 minutes, dried, wrapped in clingfilm and exposed to Polaroid film in a film cassette for between 30 seconds and 2 minutes. The Polaroid film was developed to reveal the banding pattern.

3.4.3 Immunofluorescence

Cells in a T75cm^3 flask were trypsinised when 90% confluent and washed 3 times in HANKs solution. After the final wash, cells were suspended in 1ml of cold PBS. 25 µl of cells (~1 x 10^5 cells) were each added to 20 µl of fluorescein-conjugated specific anti-receptor reagent and 20 µl of fluorescein-conjugated mouse IgG isotype as a control. Cells were incubated for 30mins at 4°C prior to being washed in PBS and undergoing microscopy under UV light.

3.4.4 Receptor gene expression

EPO and CD131 gene expression was identified using the method described below.

3.5 Gene expression

3.5.1 RNA extraction and cDNA synthesis

3.5.1.1 RNA stabilisation

Adherent cells underwent lysis and RNA stabilisation using 1 ml of RNAprotect® reagent (Qiagen, West Sussex, UK) per 25cm^2 area, before being transferred to RNase free centrifuge tubes. Aliquots were stored at -20°C for at least 24 hours prior to RNA extraction.

3.5.1.2 Use of RNase free plastics and solutions

RNase free plastics and solutions were used for all steps.

3.5.1.3 RNA extraction and purification

RNA was extracted using the RNeasy Plus Mini RNA Extraction Kit (Qiagen) as per the kit protocol. Briefly, cells were mechanically disrupted in buffer then mixed with equal volumes of 70% ethanol. RNA from suspensions was separated on the RNeasy mini columns, washed with a series of buffers and eluted using RNase free water. RNA solutions were stored at -80°C prior to reverse transcription to cDNA.

3.5.1.4 DNA elimination

DNA elimination was performed during RNA extraction and purification by placing the samples in a gDNA eliminator column and centrifuging the sample, prior to the addition of 70% Ethanol.

3.5.1.5 Quantification and purity estimation of RNA

Quantification and purity estimation of RNA was performed using the Nanodrop ND-1000 UV-Vis Spectrophotometer. 1µl of purified RNA per sample was used in this step, with the option of retrieving this from the nanodrop spectrophotometer. Quantity was expressed as µg/µl and purity expressed as 260/280 ratios. A ratio of >1.8 was required to proceed.

3.5.1.6 Synthesis of cDNA

All reactions were set up on ice to inhibit premature cDNA synthesis and minimize RNA degradation. The RNA concentration of each sample was standardized by correcting each sample volume against the lowest sample RNA concentration. One microgram of total RNA was reverse transcribed to one microgram of cDNA.

A template RNA mix was formed by the addition of 1 μL of random hexamers, 1 μL of 10mM dNTP mix and the standardised sample volume of RNA, aiming for a volume of 8 μL of RNA for the lowest concentration sample. This was made up to 13 μL with RNase free water and subsequently heated to 65°C for 5 minutes after which it was incubated on ice for 1 minute.

A reverse transcription master mix solution for each sample was formed by the combination of 4 μL of 5xFS buffer, 1 μL of 0.1% DTT, 1 μL of RNase inhibitor and 1 μL of DNA Polymerase (SuperScript III, Invitrogen). 13 μL aliquots of template RNA mix were combined with 7 μL aliquots of reverse transcription master mix and incubated at 25°C for 5 minutes then at 50°C for 45 minutes.

Reactions were inactivated following heating to 70°C for 15mins and then stored on ice.

3.5.2 Low density array Real Time Polymerase Chain Reaction (RT-PCR)

Quantification of mRNA expression was performed using TaqMan Low Density Arrays (TLDA) (Applied Biosystems, Warrington, UK) with the Applied Biosystems 7900HT Fast Real-Time PCR System. The TaqMan Gene Signature Arrays are pre-designed array cards containing TaqMan Gene Expression Assays matching genes specific to target pathways e.g. apoptosis. Each array contains a 384-well micro fluidic card. Genes are chosen from published data, pathway analysis tools and collaborator input.

3.5.2.1 TLDA PCR protocol

A 100 μL reaction mix containing 50 μL of TaqMan Master Mix (2X), 3 μL of cDNA and 47 μL of RNase free water was added to each fill port of the card. The cards were centrifuged and then sealed.

The thermal profile parameters were 50°C for 2 minutes, 94.5°C for 10 minutes and then 40 cycles of 97°C for 30 seconds followed by 1 minute at the primers annealing temperature of 59.7°C.

3.5.3 qRT-PCR

Candidate gene expression was quantified using quantification reverse transcription polymerase chain reaction (qRT-PCR) on a TaqMan 7500HT real time PCR system (Applied Biosystems, Warrington, UK) .

3.5.3.1 qRT-PCR protocol (96 well plates)

All housekeeper primer probe pairs were validated for constant efficiency across experimental conditions. Samples were run in triplicate in a 96 well optical plate in a 25 μL final volume. Samples underwent a standard 90 minute cycle in a TaqMan 7500 thermocycler (Applied Biosystems). The thermal profile parameters were 50°C for 2 minutes, 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds followed by 1 minute at the primers annealing temperature of 60°C.

3.5.3.2 Relative gene expression quantification methods for RT-PCR

Measurements were performed in triplicate (or duplicate where stated) for each sample. The threshold cycle time (C_T) was set within the exponential phase of the PCR and checked manually. The C_T values of the target gene were normalized by subtracting the C_T value of the housekeeping gene, 18s or GAPDH. The relative expression/quantification (RQ) between experimental conditions was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta C_T = (C_{T\ Target} - C_{T\ Housekeeper})_{Intervention} - (C_{T\ Target} - C_{T\ Housekeeper})_{Control}$. As three replicate PCR's were performed on the same sample, C_T data was averaged for each sample prior to performing the $2^{-\Delta\Delta Ct}$ calculation. C_T is an exponential value derived from a log-linear plot of the PCR signal versus the cycle number. Therefore, to graphically represent up- and down-regulated genes, all RQ values are converted to a linear form using the formula: $\text{Log}_{10}(RQ)$.

To determine inter-sample variation among replicates, the individual C_T values are converted to a linear form using 2^{-Ct} and a mean ± SD are calculated allowing calculation of the coefficient of variation.

3.5.4 Housekeeper gene validation

The Applied Biosystems TaqMan® Gene Expression Assays, 18s, β-Glu and GAPDH, were purchased and validated as the potential housekeeper genes to normalise the target gene expression (Table 4.1).

3.5.5 Candidate gene validation

The Applied Biosystems TaqMan® Gene Expression Assays, CASP3, NGAL, IL-18 and EPOR, were purchased and validated as the potential candidate genes to examine the effects of EPO on gene expression (Table 3.1). These genes were chosen based on a literature review, with CASP3 as a representative of apoptosis, NGAL and IL-18 as biomarkers of acute kidney injury, and EPOR as a measure of the effect of EPO on its own function (248;388).

Table 3-1: Applied Biosystems TaqMan® Gene Expression Assays.

Molecule	Gene Name	Assay Number
18s RNA	18s	Hs99999901_s1
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Hs99999905_m1
Actin beta	ACTB	Hs99999903_m1
Neutrophil gelatinase lipocalin	NGAL	Hs00194355_m1
Interleukin 18	IL-18	Hs01038788m1
Caspase 3	CASP3	Hs00234387_m1
Erythropoietin receptor	EPOR	Hs00959427_m1

3.6 Assessment of assays measuring apoptosis and cell death

3.6.1 Caspase activity (EnzChek Caspase-3 Assay Kit #1 (Invitrogen))

Protease activity in HK-2 cells was assessed using the EnzChek Caspase-3 Assay Kit #1 with Z-DEVD-AMC substrate. Optimal cell numbers (1 x 10^6 cells) were determined in preliminary experiments. Cells were exposed to known apoptosis stimulators camptothecin and staurosporine. Both induced and control cells were then harvested, lysed and assayed as described in the kit protocol. Reactions were carried out at room temperature and fluorescence was measured in a fluorescence microplate reader using excitation at 360±20nm and emission detection at 460±20nm after the indicated amount of time.

Cells were washed once in ice-cold PBS and then lysed for 10min on ice in a buffer containing 200mM TRIS, pH 7.5, 2M NaCl, 20mM EDTA, 0.2% Triton^{TM} X-100. After centrifugation (10,000g for 5min at 4C), caspase activity was determined in the supernatant in a 100µL reaction volume using the caspases-3/7 specific coloured substrate, acetyl-Asp-Glu-Val-Asp-7-amino-4methylcoumarin (Ac-DEVD-AMC from Molecular Probes). Protein extracts were incubated in 10mM substrate in 50mM PIPES, pH 7.4, 10mM EDTA, 0.5% CHAPS. When used, the caspases-3/7 inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO from Molecular Probes) was added for 10min prior to addition of the substrate peptide. Production of cleaved AMC from the tetrapeptide substrate Ac-DEVD-AMC was followed at a wavelength of 460±20nm using a microplate reader (PerSeptive Biosystems CytoFluor Series 4000), allowing quantification of the total DEVD-specific protease activity. Assays were performed in duplicate and results are presented as average emission ± SEM.

3.6.2 DNA fragmentation (Cell Death Detection ELISAPLUS Kit (Roche))

Briefly, cells were harvested from a T25cm^2 flask and diluted with medium to a concentration of 1 x 10^4 cells per 100 µL and allowed to adhere overnight in a 96 well plate. Cells were then incubated under experimental conditions. The microplate was centrifuged for 10 min at 200 x g and the supernatant was removed. Following re-suspension of the cell pellet in 200 µL of lysis buffer, cells were incubated at room temperature for 30mins. Finally, the lysate was centrifuged at 200 x g for 10 minutes and 20 µL of the supernatant was transferred into the streptavidin coated microplate for immediate analysis. 20 µL of positive,

negative and background controls were also transferred. 80 µL of the immunoreagent was added to each well. The plate was covered and incubated on a microplate shaker (300rpm) for 2 hours at room temperature. The solution was removed and the wells were washed 3 times with 250 µL of incubation buffer. 100 µL of ABTS solution was pipetted into each well and incubated on a plate shaker at 250 rpm for approximately 20 minutes (until the colour development was sufficient). 100 µL of ABTS Stop Solution was added and the absorbance was measured at 405nm.

3.6.3 LDH assay (LDH-Cytotoxicity Assay Kit II, Biovision)

Briefly, cells were seeded onto a 96 well plate and exposed to experimental conditions. The plate was gently shaken at the end of incubation to ensure an even distribution of LDH in the culture medium. The plate was centrifuged at 600g for 10min to precipitate the cells. 10 µL/well of the clear medium solution was transferred into an optically clear 96-well plate. 100 µL of LDH Reaction Mix was added to each well, mixed and incubated for 30mins at room temperature. The absorbance was measured at 450nm at multiple time points. The reference wavelength was 650nm. The percentage cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity \%} = \frac{\text{(Test sample – low control (100 µL of cells))}}{\text{(High control (100 µL of cells + 10 µL Cell lysis soln) – low control}} \times 100\%$$

3.6.4 Cell viability assay (Live/Dead® Viability/Cytotoxicity Kit Molecular Probes, (Invitrogen))

To determine optimum concentrations of calcein and ethidium for each cell type, the cells were grown until 90% confluent, trypsinised and washed in PBS twice. Cells were re-suspended in 2mls of PBS and 30 µL of cells were placed in each well of a fluorimeter plate (Corning). 70 µL of PBS was added to the live cells, while 70 µL of 70% methanol was added for 30 minutes to kill the ‘dead’ cells. Varying concentrations of calcein (0.3-5 µM) and ethidium (0.25-8 µM) were added to the cells, based on recommendations from

Molecular Probes. Excitation and emission wavelengths for calcein (495nm and 530nm respectively) and ethidium (530nm and 645nm respectively) were measured every ten minutes. The percentage of live cells was calculated from the fluorescence readings:

$$\% \text{ Live cells} = \frac{\text{((unknown C + E at 530nm) – (All cells alive Ethidium at 530nm))}}{\text{((All cells alive Calcein at 530nm)-(All cells alive ethidium at 530nm))}}$$

$$\% \text{ Dead cells} = \frac{\text{((unknown C + E at 645nm) – (All cells dead calcein at 645nm))}}{\text{((All cells dead ethidium at 645nm)-(All cells dead calcein at 645nm))}}$$

3.6.5 MTT assay

The MTT assay is based on the principle that the vital dye 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) (Sigma Chemicals) is actively transported by respiring mitochondria which leads to intra-mitochondrial formazan crystal precipitation which can be quantified using a fluorimeter(389;390). The amount of precipitation is reflective of the mitochondrial mass, and is thus used to provide an index of cell number.

Briefly, cells were seeded onto a 96 well plate and underwent experimental conditions. At the end of experiment, the supernatant was removed and 90µL of fresh medium was added in addition to 10µL of MTT. Cells were then incubated at 37°C for 4 hours. Following incubation, the wells were washed in 1 x PBS before the precipitated formazan crystals were dissolved in 100 µL of DMSO for 60 seconds through pipetting. The absorbance was measured on an ELISA reader (x) at 565nm using a 630nm reference wavelength. The percentage viability was calculated by dividing the OD of the experimental condition by the OD of the control group, before multiplying by 100.

Serial dilutions of RPTECs (20,000, 10,000, 7,500, 5,000, 2,500 cells per well) indicated a direct relationship between cell number and absorbance ($r = 0.979$, $p=0.0037$). Therefore, an increase or decrease in absorbance indicates a change in cell number.

Technical notes:

- Initial experiments used isopropyl alcohol to dissolve the formazan crystals. However, a cloudy precipitate formed when it mixed with residual solution. DMSO was used without any interaction.
- OD readings were higher when cells were washed with 1 x PBS first before adding DMSO.
- This assay could not be used to assess cell viability immediately after cold storage as no MTT uptake occurred at 4°C due to a reduction in cell metabolism at that temperature.

3.7 Statistical analysis

3.7.1 In vitro study

Data analysis included the Student's t test, Mann-Whitney, ANOVA and chi-squared test as appropriate. Data is presented as mean ± SEM for parametric data, median ± IQR (range from the 25^{th} to the 75^{th} percentile) for non-parametric data or as frequencies for categorical variables. Error bars are shown when 3 or more replicates are used in an experimental condition. Results from TLDA experiments was summarised into gene sets (e.g. TNF family, anti-apoptotic genes etc) before performing a paired t test to compare experimental conditions.A p value less than 0.05 will be considered statistically significant.

3.7.2 Clinical trial

The statistical methods were discussed with the CMMC Trust statistician, Dr Steven Roberts, Senior Lecturer in Medical Statistics, University of Manchester, when designing the study. Dr David Broadhurst, Associate Professor, University of Alberta and with Dr Jeff Reeve,

Principal Statistician, ATAGC, University of Alberta were involved in the analysis of the metabolomics and microarray data, respectively. Data analysis was perfmored using GraphPad Prism, SoftmaxProv4 for ELISA work, Bioconductor version 2.4 and R version 2.9.1.

Data is presented as mean ± SEM for parametric data, median ± IQR (range from the 25^{th} to the 75^{th} percentile) for non-parametric data or as frequencies for categorical variables. Data analysis was performed using a Student's t test, Mann-Whitney, Wilcoxon signed rank test, chi squared test or fishers exact test as appropriate. Significance was set at a p value ≤ 0.05. The survival times of the grafts and patients are summarised using Kaplan-Meier estimates of the survivor function for EPO and placebo. PBT scores were calculated as the average fold change across transcripts for a particular PBT set from nephrectomies.

CHAPTER 4 CREATING A CELL MODEL OF KIDNEY TRANSPLANTATION

4.1 General introduction

Classically, tubulo-epithelial cells have been thought of as the predominant cell type that is injured in acute kidney injury, resulting in the clinical condition known as acute renal failure. Histologically, this is seen as acute tubular necrosis, tubular dilatation and atrophy (391). More recently, increasing recognition is being given to vascular injury as a component of acute kidney injury, contributing to the clinical picture, with morphological features such as endothelial swelling and vascular sludging and occlusion being recognised(391).

To elucidate the mechanism of how EPO may confer tissue protection in renal transplantation, an in vitro model, utilising both tubulo-epithelial cells and endothelial cells, was developed to mimic injuries occurring peri-transplantation: cold storage; re-warming; hypoxia; and free radical injury. To assess this model, assays measuring early apoptosis, late apoptosis, necrosis and changes in gene expression were tested to develop the model and to assess treatment strategies.

Initial experiments employed immortalised cell lines, chosen for their ease of handling and general robustness. HK-2 cells (392), an immortalised kidney tubule-epithelial cell line, and HMEC-1 cells (393), an immortalised endothelial cell line were used. Later, primary proximal tubulo-epithelial cells (RPTECs) were used to more accurately reflect the transplanted kidney, particularly given the immortalised cell lines relative resistance to injury.

4.2 Assessing caspase activity

4.2.1 General introduction

Caspase activation is an early marker of apoptosis with caspase -3, -6 and -7, known as executioner caspases, comprising the final common pathway to proceed to apoptotic cell

death (394). The EnzChek Caspase-3 Assay Kit #1(Molecular Probes) is based on caspase 3 and -7 having substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD), which is linked to a reagent that fluoresces when cleaved.

4.2.2 Specific methods

Experiment 1: HK-2 cells were exposed to 4°C in University of Wisconsin (UW) perfusion solution and medium for 24 hours, before caspase activity was assessed as described in section 3.6.1. **Experiment 2**: HK-2 cells were exposed to the pro-apoptotic agents, camptothecin (0.6µg/ml) and staurosporine (1µM), in fresh medium for 4 hours duration.

4.2.3 Results

When stored in medium at 4°C, the cells did not exhibit a significant change in caspase activity compared to cells incubated at 37°C (Figure 4.1). In comparison, cold storage in UW was associated with a significant decrease in activity when compared to both the control group ($p<0.05$) and cold storage in medium ($p<0.001$).

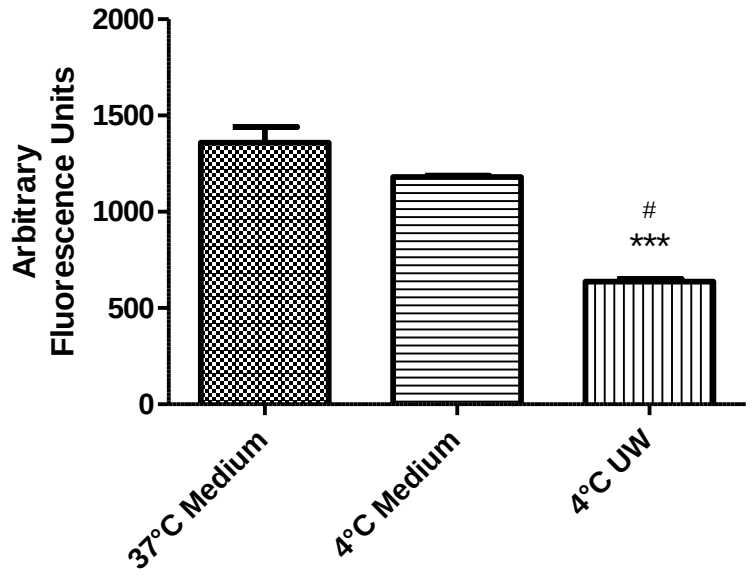


Figure 4-1: Caspase 3 activity in HK-2 cells following cold storage.
HK-2 cells were washed once in UW to remove any trace of medium before UW at 4°C was added. Cells were stored at 4°C for 24hrs before harvesting for assessment of caspase activity. The control group was the same passage no. cells in fresh medium stored at 37°C and 5% CO2. (n=2; #p<0.05 vs 37°C medium; ***,p<0.001 vs 4°C Medium)

Due to the high level of caspase activity seen in the previous experiment's control group, a second experiment was performed using cell free medium as a negative control and the pro-apoptotic agents, camptothecin (CT) and staurosporine (STS), as positive controls (Figure 4.2). A high level of caspase activity was seen in the control group, when compared to medium alone, which was attenuated by the introduction of a caspase inhibitor. Introduction of staurosporine resulted in a marked increase in caspase activity, which was again abolished in the presence of a caspase inhibitor. The camptothecin treated group had significantly less caspase activity when compared to the control group, which was further reduced by the addition of the inhibitor.

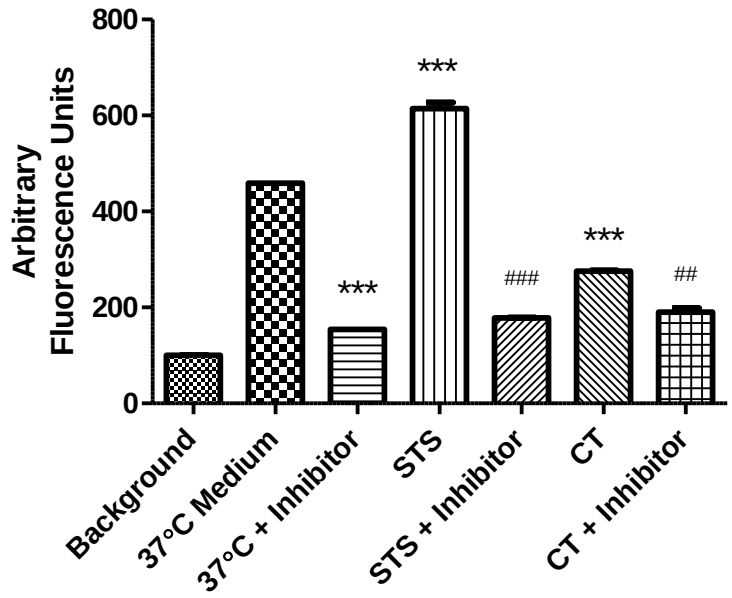


Figure 4-2: Caspase activity after staurosporine and camptothecin treatment.
HK-2 cells were incubated in fresh medium containing either camptothecin (CT) or staurosporine (STS) at 37°C for 4 hours before harvesting for caspase activity assessment. Background caspase activity was measured in fresh cell free medium. (n=2; ##p<0.01 vs CT without inhibitor; ###p<0.001 vs STS without inhibitor; ***p<0.001 vs 37°C Medium)

4.2.4 Discussion

Initial experiments revealed a high level of caspase activity in the control group, which was reduced through the addition of a caspase inhibitor. This is a surprising finding in cells under

normal growing conditions. It is known that the cells undergo contact inhibition when fully confluent, which may lead to an increase in apoptosis. In these experiments, cells were used when approximately 80% confluent to limit this possibility, although this is subjective observation and cells may be in contact with other cells. High constitutive caspase expression in a relatively apoptosis resistant immortalised cell line may also explain this finding.

When exposed to conditions mimicking transplantation, UW appeared to confer protection from apoptosis when compared to medium at low temperatures, in keeping with its known role in maintaining organ viability. Alternatively, cells stored in UW lack sufficient energy to undergo apoptosis, when compared to those stored in complete medium. Thus, it may be more appropriate to use this assay during the re-warming period when energy is readily available for apoptosis to occur.

Finally, cells appear to be more sensitive to Staurosporine-induced apoptosis than camptothecin-induced apoptosis, potentially reflecting their immortalised nature with immortalised cells possibly having a greater resistance to DNA topoisomerase I inhibitors as opposed to the ATP binding inhibitors.

Limitations of this assay included cost and the requirement for a large number of cells per experimental condition (1×10^6). It was not therefore considered suitable as a medium to high throughput assay. The high background level of caspase 3 activity in the control group reduced its sensitivity in detecting apoptosis under the experimental conditions. However, since caspase activation occurs early, this assay is suitable to detect early apoptosis in comparison to DNA fragmentation which occurs later. It is also a relatively quick and straightforward assay to perform and can be used in conjunction with a supernatant assay, such as LDH activity.

4.3 Assessment of DNA fragmentation

4.3.1 General introduction

Subsequent to caspase activation, DNAases degrade DNA into 180bp fragments or multiples thereof (e.g. 360, 540 etc), producing the classic laddering effect, before the formation of apoptotic bodies and their phagocytosis. The Cell Death Detection ELISAPLUS Kit (Roche) is a sandwich enzyme immunoassay which uses mouse monoclonal antibodies directed against DNA and histones. This allows the specific determination of mono- and oligo-nucleosomes in the cytoplasmic fraction of cell lysates. The anti-histone-biotin-antibody binds to histones H1, H2A, H2B, H3 and H4. The anti-DNA-POD-antibody reacts with single and double stranded DNA.

4.3.2 Specific methods

HK-2 cells were exposed to 4°C in UW and medium for 24 hours before quantification of DNA fragmentation was assessed as described in section 3.6.2.

4.3.3 Results

DNA fragmentation was significantly reduced by cold storage in both UW and medium for 24hrs when compared to the control group (Figure 4.3). Cells maintained in UW at 37°C for 24 hours displayed a significant degree of DNA fragmentation.

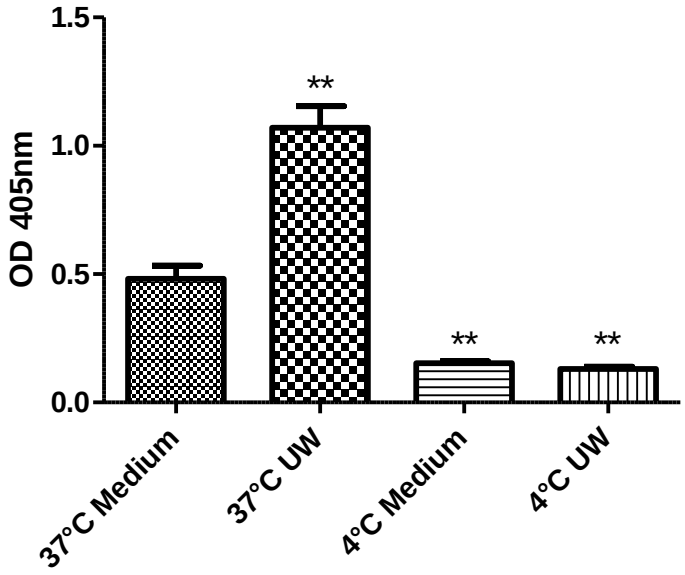


Figure 4-3: DNA fragmentation in HK-2 cells following cold storage.
HK-2 cells were incubated in fresh medium or UW for 24 hours at 37°C or 4°C before harvesting for quantitation of DNA fragmentation. The control group was cells stored in fresh medium at 37°C. (n=2; **p<0.01 vs 37°C Medium)

4.3.4 Discussion

Similar to the caspase assay results, a significant degree of apoptosis, as measured by DNA fragmentation, occurs in HK-2 cells under standard growing conditions at 37°C in complete medium. This again may be related to their degree of confluence, with cells for these experiments were approximately 80% confluent before entering the experiment. Cells were stored at 37°C in UW as a positive control and a significant increase in DNA fragmentation occurred. UW is a specialised preservation solution that does not contain any growth factors or nutrient supply. Thus, serum starvation may be responsible for the increase in DNA fragmentation. Alternatively, drug toxicity from the allopurinol or adenosine in UW may have been a factor. In contrast to the caspase assay, no difference was seen in DNA fragmentation rates between cells stored in medium or UW at 4°C for 24 hours. Caspase activity occurs early in the process of apoptosis, with DNA fragmentation occurring later in the pathway. It is probable that cold storage for 24 hours is too early following injury to detect significant DNA fragmentation, which occurs in late apoptosis. Again this process is energy and temperature dependent and is likely to be more marked following re-warming.

However, one group have demonstrated that DNA laddering is unusual in the early re-warming period following hypoxia (first 24hrs) (395), suggesting that this assay is unlikely to be useful for the time points we are evaluating (24hrs cold followed by up to 24 hrs re-warming).

The time course over which this assay is performed, in addition to its cost make this a less than ideal measure of apoptosis in our cell model. It can however be used with an assay measuring compounds in the supernatant, such as the LDH assay.

4.4 Assessment of LDH

4.4.1 General introduction

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that is released upon damage of the cell plasma membrane. This occurs in cell necrosis but not apoptosis allowing quantification of necrosis in response to an injurious stimulus. The degree of enzyme activity in cell culture supernatants correlates directly with the proportion of cells lysed. The Cytotoxicity Detection Kit (Roche) utilises the enzyme coupling reaction of LDH oxidising lactate to generate NADH, which subsequently reacts with a tetrazolium salt (pale yellow) to generate formazan (red). The intensity of the colour generated directly correlates with the amount of formazan formed, during a limited time period and is thus proportional to the number of lysed cells.

4.4.2 Specific methods

Experiment 1: HK-2 cells were exposed to 1mM hydrogen peroxide for 4 hours, before measurement of LDH activity as described in section 3.6.3. **Experiment 2**: RPTECs were exposed to increasing concentrations of H_2O_2 (0.02 - 2mM) in serum free medium and University of Wisconsin (UW) solution at 37ºC for 4 hours. **Experiment 3**: Purified LDH was exposed to increasing concentrations of hydrogen peroxide in complete medium before measurement of LDH activity.

4.4.3 Results

It was not possible to measure LDH activity in HK-2 cells exposed to hydrogen peroxide, despite multiple attempts. This experiment was performed exactly as published in (248). I repeated the experiment in a different cell line (RPTECs) and thus, different culture media, to no avail.

In order to explain this finding, purified LDH was measured in the presence of increasing concentrations of hydrogen peroxide. Hydrogen peroxide produced a loss of LDH activity over 4 hours that was inversely proportional to its concentration (Figure 4.4).

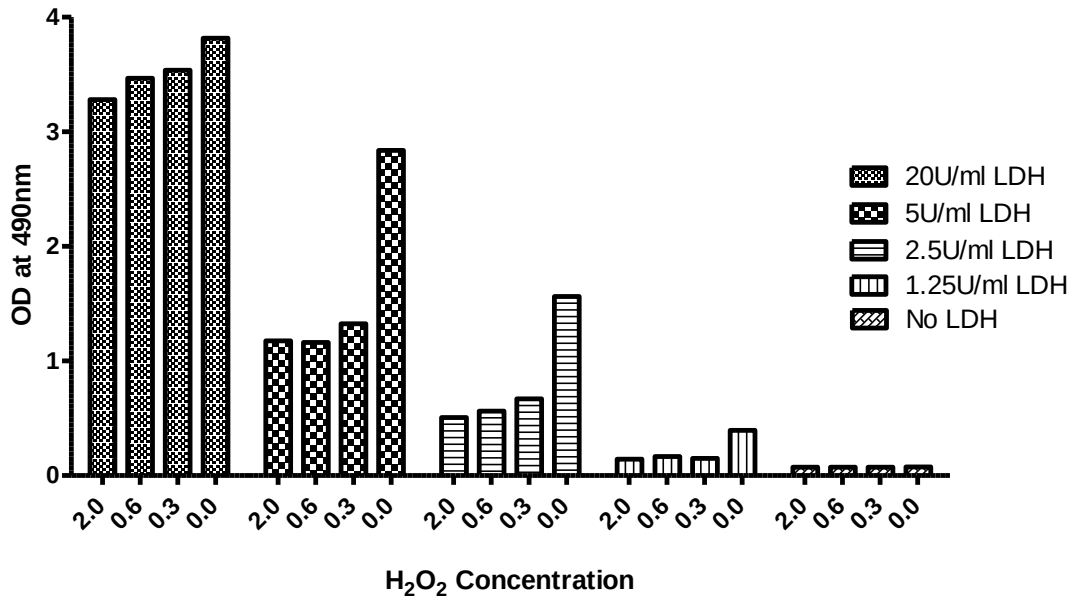


Figure 4-4: Purified LDH is degraded by hydrogen peroxide in a dose dependent manner.
Increasing concentrations of hydrogen peroxide were added to complete medium containing increased concentrations of purified LDH and incubated at 37°C for 4 hours in a 96 well plate, before aspirating the medium for LDH quantification using spectrophotometric quantification. Data is expressed as mean OD reading ± SEM.

4.4.4 Discussion

Sharples et al. and Abdelrahman et al, in their cell model of hydrogen peroxide-induced free radical injury of kidney tubulo-epithelial cells, demonstrated that erythropoietin reduced cell necrosis by measuring LDH release using the same assay as used in these experiments (LDH Cytotoxicity Assay, Roche Applied Sciences)(248;319). In their model, hydrogen peroxide resulted in a >40% total cytotoxicity rate in comparison to the control rate of <10%. Both EPO 10U/ml and EPO 50U/ml, but not EPO 1U/ml, significantly abrogated this necrosis rate to <25%. However, I was not able to replicate their results despite testing different cell types and different media. Using purified LDH as a positive control, I have shown that the technique is unsound as LDH released into the incubation medium is susceptible to degradation over time by hydrogen peroxide. Thus, in vitro models mimicking reperfusion injury-induced free radical damage using hydrogen peroxide should not use an LDH assay to quantify cytotoxicity. Furthermore, measured LDH levels were lower in UW solution when compared to medium, despite the addition of the same concentration of LDH, suggesting that UW also has an impact on LDH levels (data not shown).

The Roche LDH assay was cheap and very easy to use, allowing high throughput. It lends itself particularly to our model, in the re-warming period where cells are in medium. However, it is not applicable to situations where hydrogen peroxide is used to mimic free radical injury or when cells are cold stored in UW. An added advantage of this assay is that it is compatible with other assays not requiring the culture supernatant.

4.5 Assessment of cell viability using calcein and ethidium

4.5.1 General introduction

The Live/Dead® Viability/Cytotoxicity Kit is based upon the uptake or exclusion of vital dyes by live or dead cells. Calcein and ethidium can be used to differentiate between cells with intact cell membranes (alive) and damaged cell membranes (dead/dying). Live cells are distinguished from dead cells by the presence of ubiquitous intracellular esterase activity. The polyanionic dye calcein predominantly enters live cells, producing an intense uniform green

fluorescence (Ex/Em ~495nm/~515nm). In contrast, Ethidium only enters cells with damaged membranes and undergoes a 40 fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (Ex/Em ~495nm/ ~635nm).

4.5.2 Specific methods

HK-2 cells that were 80% confluent were refrigerated at 4°C in UW and medium for 24 hours or stored at 37°C in UW (positive control) or medium for 24 hours, before the percentage of live/dead cells was assessed as per section 3.6.4.

4.5.3 Results

Storage of the cells in UW at 37°C resulted in a 50% decrease in cell survival at 24hrs (Figure 4.5). Interestingly, storage in fresh complete medium at 37°C was associated with a 10% death rate. When stored in UW at 4°C, the cells appeared to have a survival advantage over those stored in complete medium, as might be expected in a solution specifically designed to attenuate cell injury in response to cold storage. However, the sum of alive and dead percentages is >100% suggesting that cell numbers were not equal between the groups.

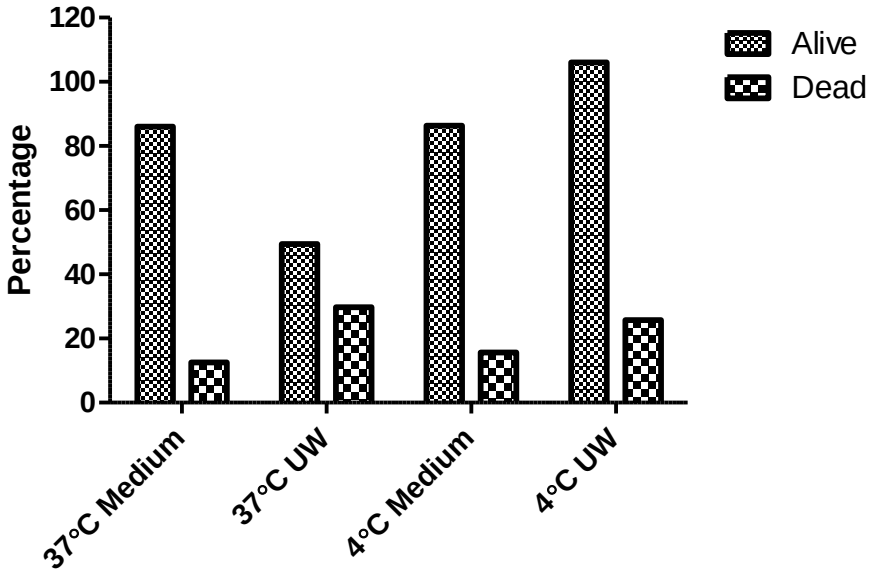


Figure 4-5: Cell viability in following cold storage assessed using calcein and ethidium.
HK-2 cells were incubated in fresh medium or UW for 24 hours at 37°C or 4°C before exposure to calcein or ethidium. The control group was cells stored in fresh medium at 37°C. (n=1)

4.5.4 Discussion

This experiment supports the previous caspase and DNA fragmentation experiments, with decreased cell viability in cells stored at 37°C in fresh medium. Furthermore, storage of cells in UW at 4°C appears to be associated with improved viability compared to storage at the same temperature in medium. As expected, cell viability was markedly reduced in UW when stored at 37°C, supporting the increased caspase activity and DNA fragmentation seen under these experimental conditions previously.

This assay was difficult to use due to its complexity in terms of experimental design and thus not suitable for modelling transplantation environments. It was not deemed suitable as a high throughput assay. It can however be used in conjunction with an assay measuring compounds in the supernatant, such as the LDH assay.

4.6 Assessment of cell viability using MTT

4.6.1 General introduction

Metabolism of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in the mitochondria of living cells results in the formation of the water insoluble product, formazan. The amount of precipitation is reflective of the mitochondrial mass, and is thus used to provide an index of cell number. Formazan must be dissolved in DMSO, producing a colour change from yellow to red/purple.

4.6.2 Specific method

Equal numbers of RPTECs (passage number 3) were seeded to 96 well plates and allowed to adhere overnight. Cell viability was assessed in the control group at this time to ensure that cell proliferation did not account for discrepancies between results. One plate was washed with UW to remove all traces of medium before adding UW to each well and storing the plate at 4°C for 24 hours. A third plate was incubated at 4°C for 24 hours before having the UW replaced with complete medium and incubating the cells at 37°C for 24 hours. Cell viability was assessed as described in 3.6.5.

4.6.3 Results

After 24 hours of cold storage in UW, there was no decrease in cell viability compared to the control group (Figure 4.6). In contrast, cells undergoing cold storage and re-warming had a significant reduction in cell viability, as measured by the optical density of the resulting formazan precipitate ($p<0.001$).

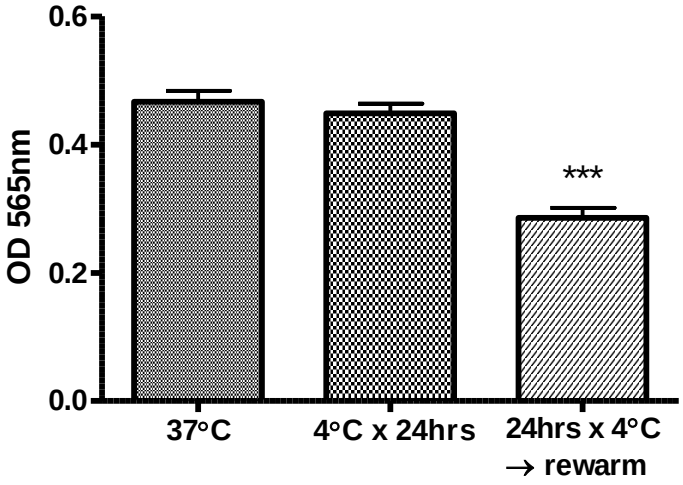


Figure 4-6: Cell viability in RPTECs undergoing cold storage and re-warming using MTT.
Equal numbers of passage 3 RPTECs were seeded on 96 well plates and left to adhere overnight. MTT was assayed in the 8 control wells at the beginning of the experiment. Cells were incubated in UW for 24 hours at 4°C before MTT was added at 37C for 3 hours or undergoing re-warming for 24 hours in complete medium before MTT was added. Data is expressed as mean OD reading ± SEM. (n=2; ***p<0.001 vs 37°C Medium and 4°C UW).

4.6.4 Discussion

Cold storage was not associated with a decrease in cell viability, in keeping with previous experiments and the specific features of UW solution. However, re-warming was associated with a significant decrease in cell viability as would be expected with the increase in metabolism and the provision of energy.

This assay was inexpensive and very simple to use, making it ideal for high throughput. It could not however be used in isolation to measure the effects of cold storage on cells, due to the requirement for a 3-4hr period at 37°C to allow the cells to take up the MTT. This effectively represents a re-warming period following cold storage. A further advantage of this assay is that it can be used with the LDH assay.

4.7 Discussion of assays used to monitor tissue protection

The above experiments have examined and attempted to optimise assays assessing early apoptosis, late apoptosis, necrosis and cell death (a combined endpoint of apoptosis and necrosis). Findings were similar in that little cell death occurred following cold storage, particularly when performed in UW solution. To test EPO will require a period of re-warming to ensure the control group has a decrease in cell viability.

A marked difference in both sensitivity and practicality was demonstrated between the assays in terms of usefulness in the model of transplantation. The easiest, least expensive and most applicable assays in terms of simplicity, throughput and timing were the LDH assay and the MTT assay, despite problems with LDH degradation in UW and H_2O_2. Furthermore, both assays are reproducible under controlled conditions in the cell model.

4.8 Validation of the housekeeping gene for quantitative gene expression under experimental conditions

4.8.1 General introduction

Housekeeper genes are required as a common denominator to which target gene expression is normalised. An ideal housekeeper gene expression is not modified under the experimental conditions used. In qRT PCR it serves three important functions: it allows standardisation of the amount of RNA between samples and allows the combination of data from multiple experiments; it ensures that transcriptional changes seen are selective rather than a general

transcriptional increase; and finally, the housekeeper acts as a positive control because it is constitutively expressed in all cells.

4.8.2 Specific methods

Cells were grown until 70-80% confluent under normal conditions before being exposed to 4°C for 24 hours in complete medium. RNA extraction and cDNA synthesis were performed as described in section 3.5.1. ACTB, GAPDH and 18s gene expression were quantified in both HK-2 and HMEC-1 cell lines using TLDA (TaqMan Low Density Array, on the Applied Biosystems 7900HT Fast Real-Time PCR System) comparing 4°C normoxia to 37°C control as described in section 3.5. Experimental repeats were n=2 and within each experiment, samples were run in triplicate. In RPTECs the effect of temperature variation, hypoxia and EPO at different time points on 18s gene expression was also validated. Data was analysed using TaqMan 7900HT system software to generate amplification plots and Ct values. Ct values were then analysed in GraphPad Prism 5 to generate mean Ct values and SEM. Results and graphs shown are representative.

4.8.3 Results

Of the three housekeeper genes, 18s proved to exhibit the least variation under experimental conditions in both HK-2 and HMEC-1 cells. A representative amplification plot in HMEC-1 cells is shown in Figure 4.7, demonstrating the least variation in Ct values occurs with 18s and the most variation occurs with ACTB. Furthermore, 18s appears earliest, signalling the highest gene expression, while ACTB appears the latest demonstrating the lowest gene expression.

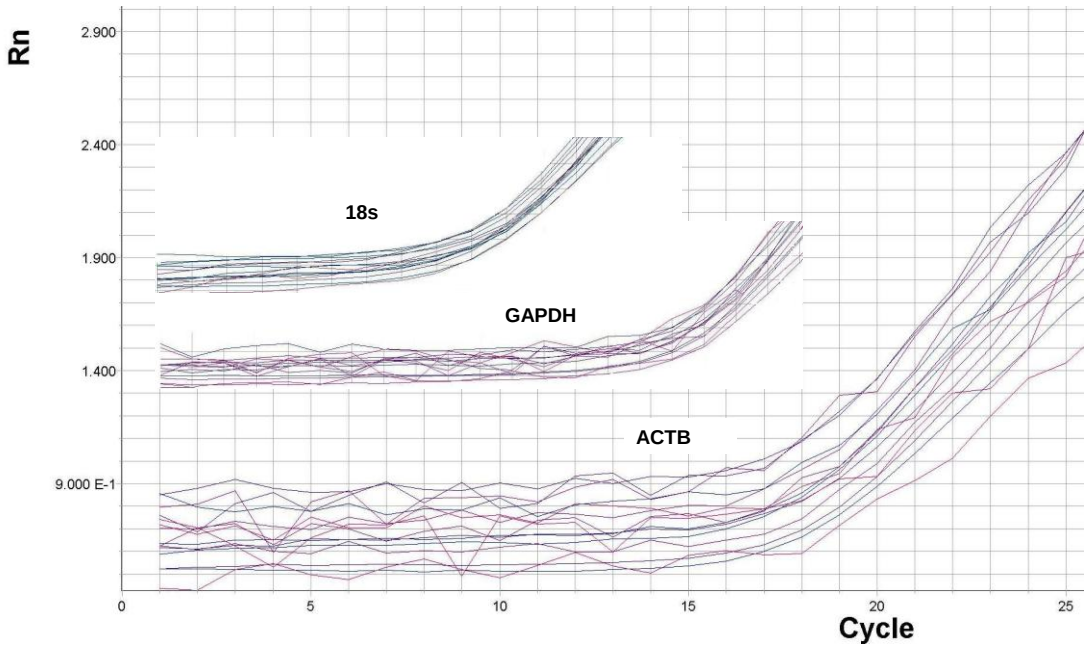


Figure 4-7: TLDA amplification plots for 18s, GAPDH and ACTB in HMEC-1 cells
HMEC-1 cells were grown to 80% confluence before undergoing storage at 4°C in complete medium for 24 hours. Cells were lysed in RNAlater before RNA was extracted. Each cycle represents a doubling of message, with the earlier appearance of message reflecting increased abundance.

Under experimental conditions of time, hypothermia, normothermia, hypoxia and EPO, little variation in 18s gene expression occurred in RPTECs with a Ct value of 14.23 ± 0.19 in this study and a normal distribution (p=0.088, D'Agostino and Pearson omnibus normality test) (Figure 4.8). In one experiment spanning 9 x 96 well plates, 11 experimental conditions and 135 replicates, the variation in 18s gene expression was small with a Ct range of 13.84-15.04, a mean of 14.37 (95% CI 14.32-14.41), a SD of 0.2747 and a SEM of 0.024.

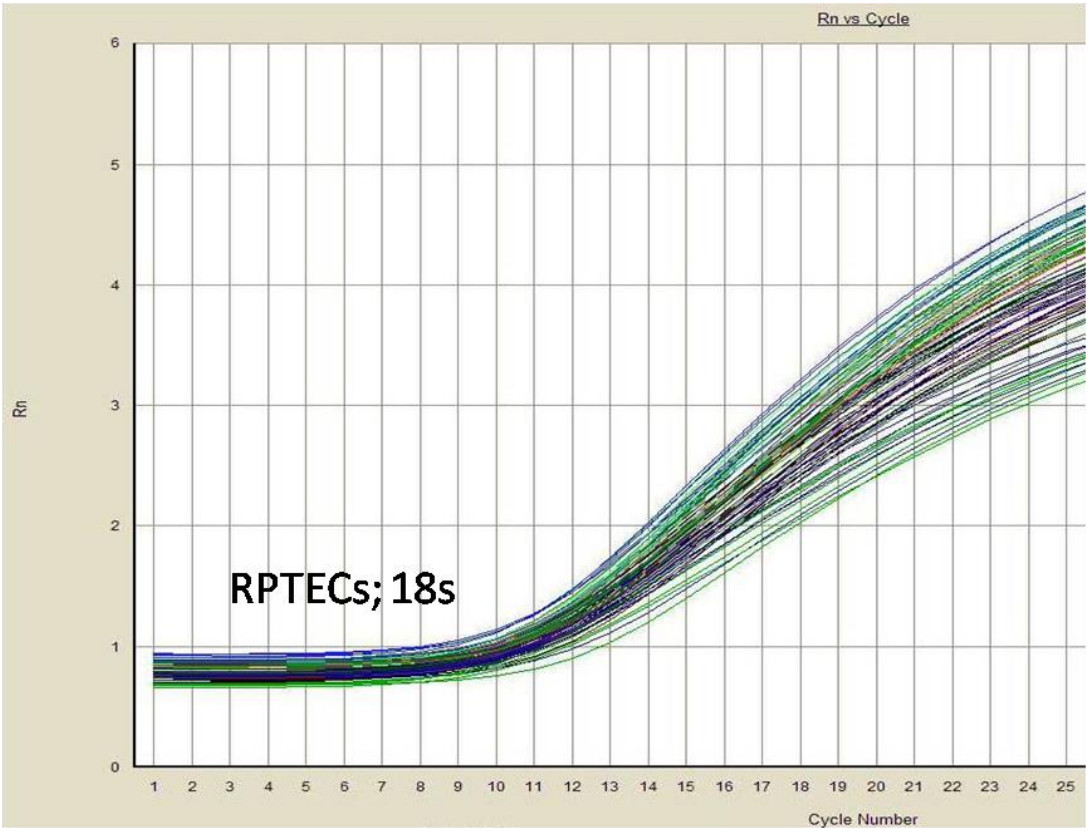


Figure 4-8: qRT-PCR amplification plot for 18s in RPTECs in the cell model
RPTECs were grown to 80% confluence before undergoing storage in UW ± EPO at 4°C and 1%O_2 for varying time points followed by a period of re-warming. Cells were lysed in RNAlater before RNA was extracted. Each cycle represents a doubling of message, with the earlier appearance of message reflecting increased abundance. 18s appears most frequently between cycle 14 and 14.5 with a Gaussian distribution (p=0.09, D'Agostino & Pearson omnibus normality test)

4.8.4 Discussion

From this work, 18s has been identified as the best housekeeping gene, with which to normalise other gene Ct values, under the experimental conditions mimicking transplantation.

4.9 Screening pre-made apoptotic gene expression cards for TLDA

4.9.1 General introduction

In order to determine the target genes to be examined in the clinical trial samples, the in vitro model was used to screen for potential target genes (novel and known). The TLDA platform

is a medium throughput assay allowing us to screen for up to 93 target genes per sample in triplicate. Applied Biosystems has developed pre-made TLDA cards including an apoptosis panel of human genes, which can be used as a screening tool, prior to potentially designing TLDA cards for the clinical trial samples. This apoptosis panel contains all key members of the apoptotic pathway.

Caspases, cysteine derived aspartate-specific proteases, are key initiators and executioners of apoptosis. The pro-apoptotic caspases can be separated into initiator caspases (Caspase -2, -8, -9, and -10) and the executioner caspases (Caspase -3, -6 and -7).

The primary function of the Bcl-2 family is to regulate mitochondrial integrity and the release of mitochondrial proteins into the cytosol. The family can be divided into pro- and anti-apoptotic factors. Pro-apoptotic Bcl-2 family members include BAD, BAK1, BAX, BBC3, BCAP31, BIK, Bcl10, Bcl2L11 and Bcl2L13. The anti-apoptotic members of the Bcl-2 family include Bcl2A1, Bcl2L1, Bcl2L2, Bcl3 and MCL1. During apoptosis, Bax and Bak may provoke or contribute to increased outer mitochondrial membrane permeability, thus allowing release of cytochrome c. The anti-apoptotic factors sequester pro-apoptotic members by binding to their BH-3 domains, preventing activation.

If the extrinsic signal via the TNFR does not generate a caspase signalling cascade sufficient to cause apoptosis, the signal can be amplified by mitochondria dependent apoptotic pathways. The link between the caspase signalling cascade and the mitochondria is provided by Bid, which is cleaved by caspase-8 to form a truncated form, tBid, which translocates to the mitochondria and acts synergistically with the pro-apoptotic family members Bax and Bak to induce the release of cytochrome c into the cytosol. Thus the caspases and Bcl2 family are interlinked in the process of apoptosis (Figure 4.9).

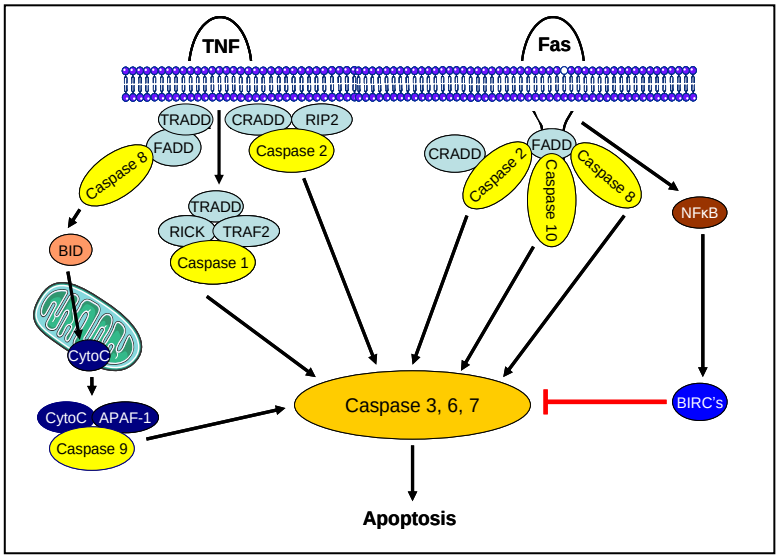


Figure 4-9: Schematic representation of apoptosis.

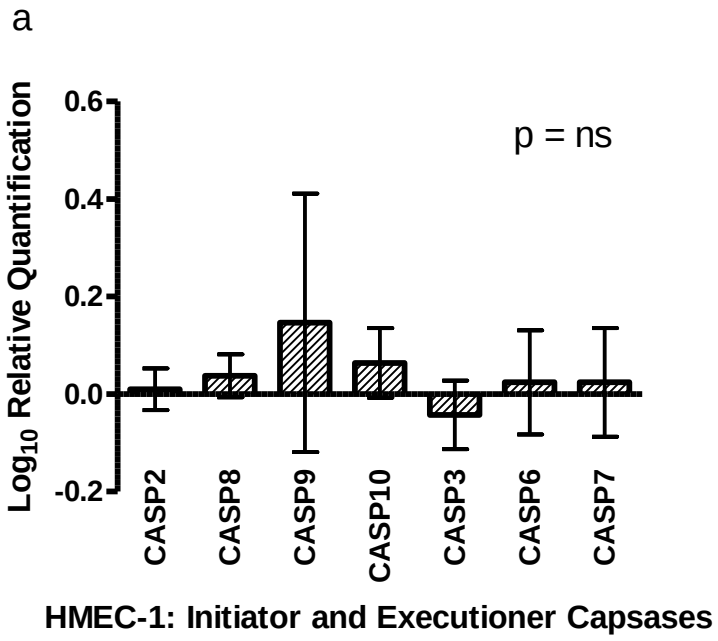
4.9.2 Specific methods

HK-2 cells and HMEC-1s were grown until 70-80% confluent under normal conditions before being exposed to 4°C for 24 hours in complete medium. RNA extraction and cDNA synthesis were performed as described in section 3.5. ACTB, GAPDH and 18s gene expression were quantified in both HK-2 and HMEC-1 cell lines using TLDA (TaqMan Low Density Array, on the Applied Biosystems 7900HT Fast Real-Time PCR System) comparing 4°C normoxia to 37°C control. Experimental repeats were n=2 and within each experiment, samples were run in triplicate. Data was analysed using TaqMan 7900HT system software to generate amplification plots and Ct values. Ct values were then analysed in GraphPad Prism 5 to generate mean Ct values and SEM. Results and graphs shown are representative.

4.9.3 Results

No significant difference in caspase gene expression was observed in the endothelial cell line compared to the control group (Figure 4.10a). In contrast, significant increases in caspase gene expression occurred at 24hrs in HK-2 cells ($p<0.0001$) (Figure 4.10b). Both initiator and executioner caspase expression increased with caspases -2, -8, -9 and -7 up-regulated by

more than two fold when compared with control. Caspases -3, -6 and -10 were up-regulated by a factor of 1.7. This suggests that tubulo-epithelial cells (HK-2) may be more susceptible to injury/apoptosis following cold storage than microvascular endothelial cells (HMEC-1).



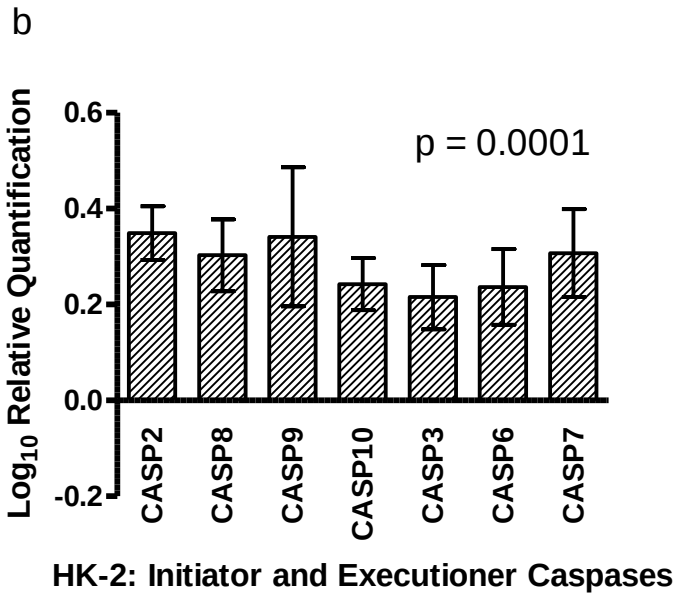


Figure 4-10: Caspase expression in (a) HMEC-1 and (b) HK-2 cells lines in response to cold storage. Cell lines were grown to 80% confluency before undergoing cold storage in fresh medium for 24 hours. Cells were lysed and the RNA stabilized using RNAlater. Data shown is mean ± SEM. Data shown is one experiment in triplicate. The x axis represents baseline gene expression at 37°C. One way ANOVA performed across all genes between conditions.

No significant difference in pro-apoptotic Bcl-2 family gene expression was noted in HMEC-1 cells undergoing cold storage (Figure 4.11a). However, the individual genes BAD, BCAP31, BID, BIK, Bcl10 and Bcl2L13 had increased expression suggesting a pro-apoptotic profile. BAD and BID were up-regulated 2.6 and 3.2 fold respectively. Bcl10 was up-regulated 94-fold. Surprisingly, cold storage had no significant effect on BAX.

Cold injury resulted in a significant increase in gene expression of the pro-apoptotic Bcl-2 family in HK-2 cells ($p=0.0218$, one way ANOVA) (Figure 4.11b). Individual genes up-regulated included BAK1, BCAP31, BID, BIK, Bcl2L11 and Bcl2L13, favouring apoptosis. The mean fold change in gene expression was 1.8 with BID up-regulated 2.6 fold. Interestingly, BAX and BAD were not up-regulated compared to baseline.

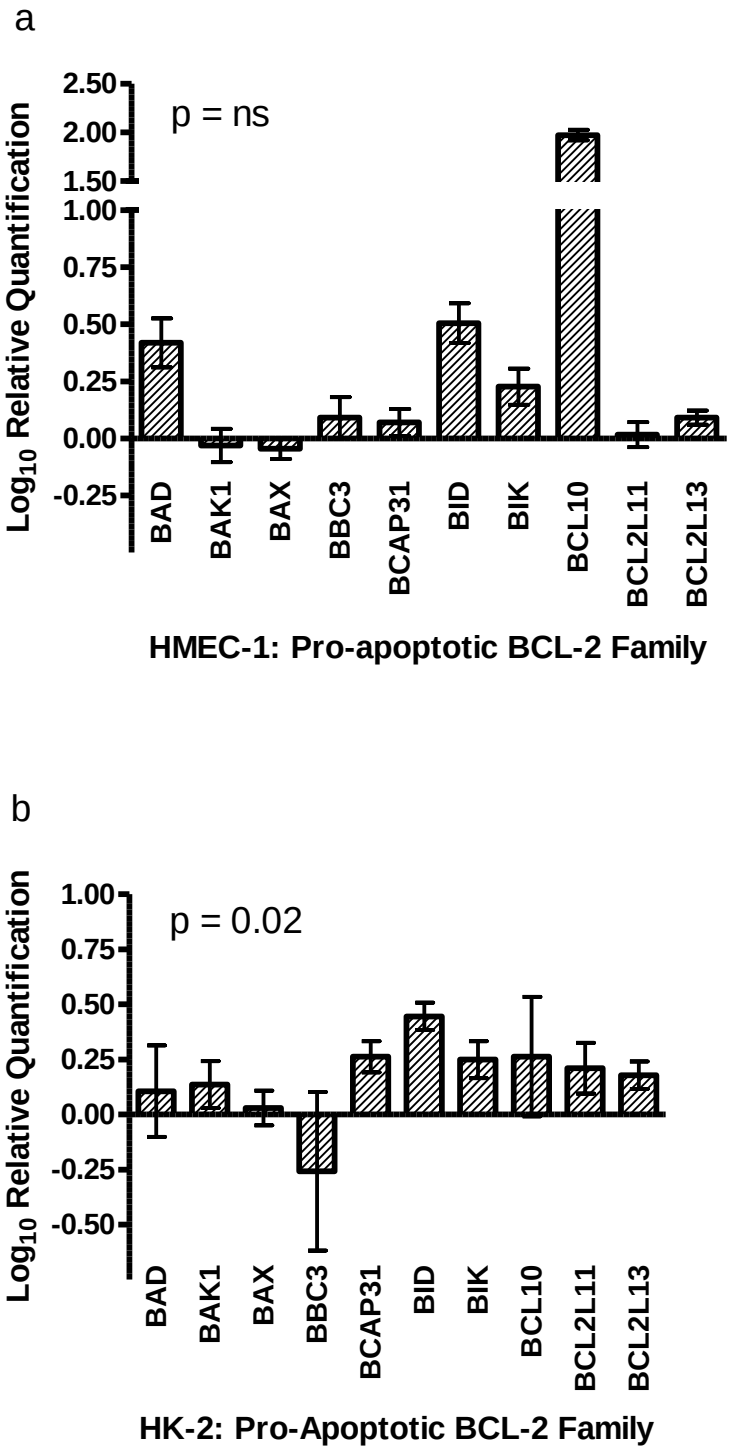


Figure 4-11: Pro-apoptotic BCL-2 family gene expression in (a) HMEC-1 and (b) HK-2 cells lines in response to cold storage.
Cell lines were grown to 80% confluency before undergoing cold storage in fresh medium for 24 hours. Cells were lysed and the RNA stabilized using RNAlater. Data shown is mean ± SEM. Data shown is one experiment in triplicate. The x axis represents baseline gene expression at 37°C. One way ANOVA

No significant difference in anti-apoptotic Bcl-2 family gene expression was noted in HMEC-1 cells exposed to cold injury compared to control ($p>0.05$) (Figure 4.12a). Both Bcl2A1 and Bcl3 expression was decreased in response to cold storage, but Bcl2L2 was up-regulated 1.6 fold. In contrast, HK-2 cells demonstrated significant up-regulation of the anti-apoptotic Bcl2 family ($p=0.0039$, one way ANOVA) gene expression in response to cold storage (Figure 4.12b). Bcl2L1 (Bcl-XL), Bcl3 and MCL1 were all up-regulated compared to control by 1.2 to 1.4 fold.

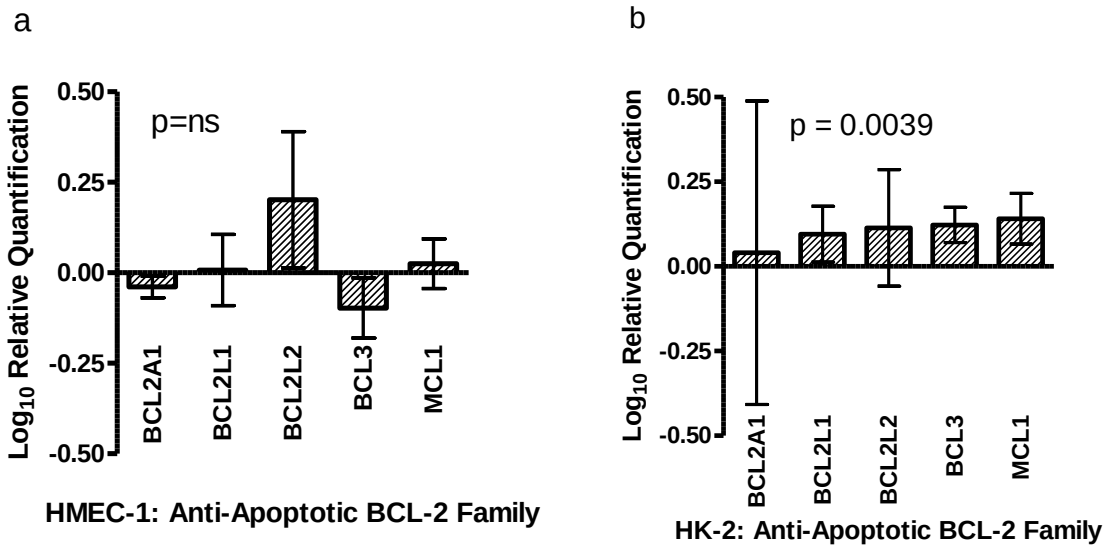


Figure 4-12: Anti-apoptotic BCL-2 family gene expression following cold storage.
(a) HMEC-1 and (b) HK-2 cell lines were grown to 80% confluency before undergoing cold storage in fresh medium for 24 hours. Cells were lysed and the RNA stabilized using RNAlater. Data shown is mean ± SEM. Data shown is one experiment in triplicate. The x axis represents baseline gene expression at 37°C. One way ANOVA.

No significant difference in TNF pathway gene expression was demonstrated in HMEC-1 cells undergoing cold storage compared to cells maintained at 37°C (Figure 4.13a). Individual genes including TNFR1 (1.5X), FADD (1.7X), RIPK2 (1.5X), BID (3.1X) and DIABLO (1.3X) appeared to be up-regulated in response to cold injury.

Highly significant gene expression up-regulation (mean fold change of 2) occurred in response to cold injury in HK-2 cells, favouring apoptosis ($p=0.0001$) (Figure 4.13b). FADD and TRADD were the only two members of the pathway not up-regulated.

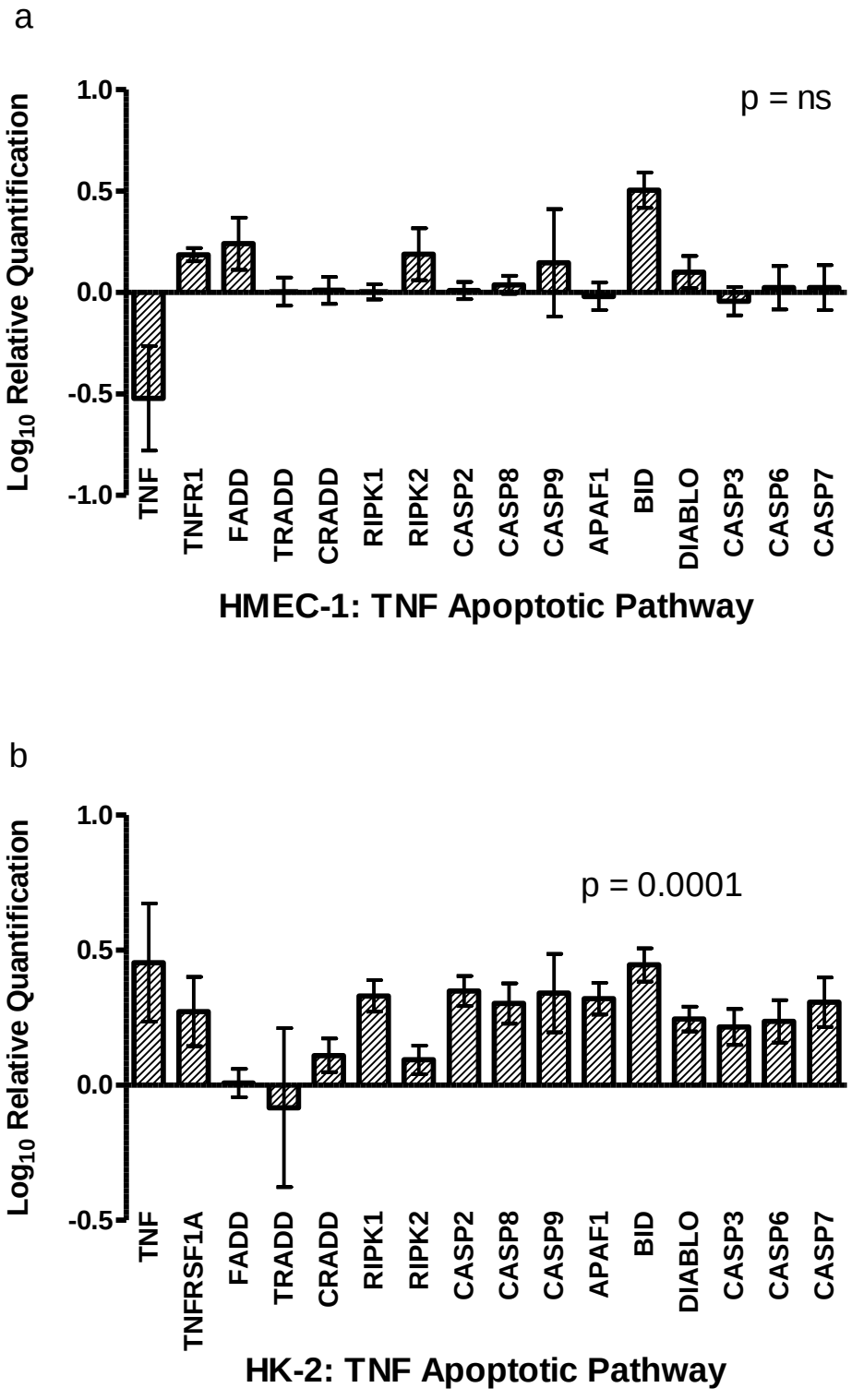


Figure 4-13: TNF apoptotic pathway gene expression in response to cold storage.
(a) HMEC-1 and (b) HK-2 cell lines were grown to 80% confluency before undergoing cold storage in fresh medium for 24 hours. Cells were lysed and the RNA stabilized using RNAlater. Data shown is mean ± SEM. Data shown is one experiment in triplicate. The x axis represents baseline gene expression at 37°C. One way ANOVA.

4.9.4 Discussion: apoptotic gene expression

Differences in apoptotic gene expression between the two cell lines, suggest that HK-2 cells, the tubulo-epithelial cell line, are much more sensitive to cold injury, as evidenced by significant up-regulation of the caspase pathway, the pro-apoptotic Bcl2 family pathway and the TNF pathway. HMEC-1 cells appear to be much more resistant to cold injury with no significant change in gene expression when compared to the control group. Apoptotic gene expression may reflect that both cell lines are transformed cell lines and thus more resistant to injurious stimuli, when compared with primary cells.

This screening tool has highlighted key members of the apoptosis pathway for further investigation in the clinical trial samples. These include caspase -3, -6 and -7, BID, Bcl2, Bcl2XL, BAX, BAD and TNF. However, gene expression was measured when cells were stored in the absence of hypoxia, in complete medium. Thus, insufficient injury may have occurred due to medium growth factor cytoprotection, in addition to probable decreased sensitivity of immortalised cell lines to apoptosis. Storage in UW, a solution specifically designed to be protective during cold storage, may also yield alternative results. Finally, gene expression was measured at 24hrs which may be too late to detect cold storage-induced up-regulation of some genes. To determine when to measure gene expression, a candidate gene approach is needed, to identify the optimal time point in the cell model to measure this.

4.10 Determining the optimal time point to measure gene expression under experimental conditions in the cell model

4.10.1 General introduction

In order to determine the optimal time at which to measure gene expression in the cell model, target genes were identified in the literature and from previous work in the laboratory and candidate gene analysis was performed using the TaqMan 7500 system. Target genes included:

- EPO and its potential receptors (EPO, EPOR(396) and CD131(180))

- Biomarkers of acute kidney injury (NGAL(96;99;388), IL-18(388;397;398), HGF(399;400), FABP1, FABP3(131;136;401))

- Potential targets of EPO (TNF, STAT5a(248), Caspase 3(248))

4.10.2 Specific methods

Passage 2 RPTECs were grown to 80% confluence in 6 well flasks prior to exposure to cold hypoxia for 2, 6, 16 and 24hrs in the presence or absence of EPO. Hypoxic treatment of cells was performed in an enclosed chamber (Hypoxia Incubator Chamber cat. no. 27310, Stemcell Technologies, France) flushed with pre-mixed gas mixture (1% O_2, 5% CO_2, 94% N_2) for the time indicated. Following 24hrs of cold hypoxia, cells were also exposed to a re-warming time point. Exactly 1 hour prior to cold hypoxia, 50U/ml of EPO was administered to the EPO wells. Medium in the non-EPO wells was also changed. At the time of initiating cold hypoxia, wells were first washed in UW before cold UW was added to the wells for the required time.

4.10.3 Results

CD131 gene expression was at the limit of sensitivity of the test under normal conditions and there was no evidence of increased expression in response to cold hypoxia. EPOR expression was decreased by cold hypoxia (Figure 5.14). EPO gene expression was also at the limit of the tests sensitivity under normal conditions suggesting small quantities of mRNA were present.

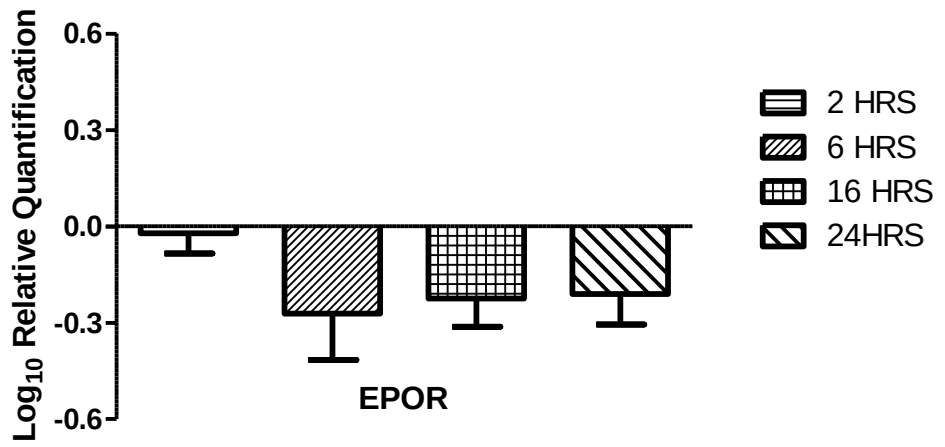


Figure 4-14: EPO-R expression in response to time and cold hypoxia.
Passage 2 RPTECs were grown to 80% confluency before undergoing cold hypoxia for the prescribed durations ± 50U/ml of EPO. Cells were lysed and the RNA stabilized using RNAlater. Data shown is mean ± SEM. (n=1, done in triplicate). The x axis represents baseline gene expression at 37°C.

Figure 4.15a-c examines the effect of cold hypoxia with time on gene expression of 3 representative apoptotic genes. The executioner caspase, caspase 3, had decreased expression compared to the control group following cold hypoxia (Figure 4.15a). STAT5a expression was increased in response to cold hypoxia favouring an anti-apoptotic response (Figure 4.15b). TNF expression also decreased in response to cold injury, which again favours cell survival (Figure 4.15c). Thus, changes in expression were maximal after 16 hours of cold hypoxia.

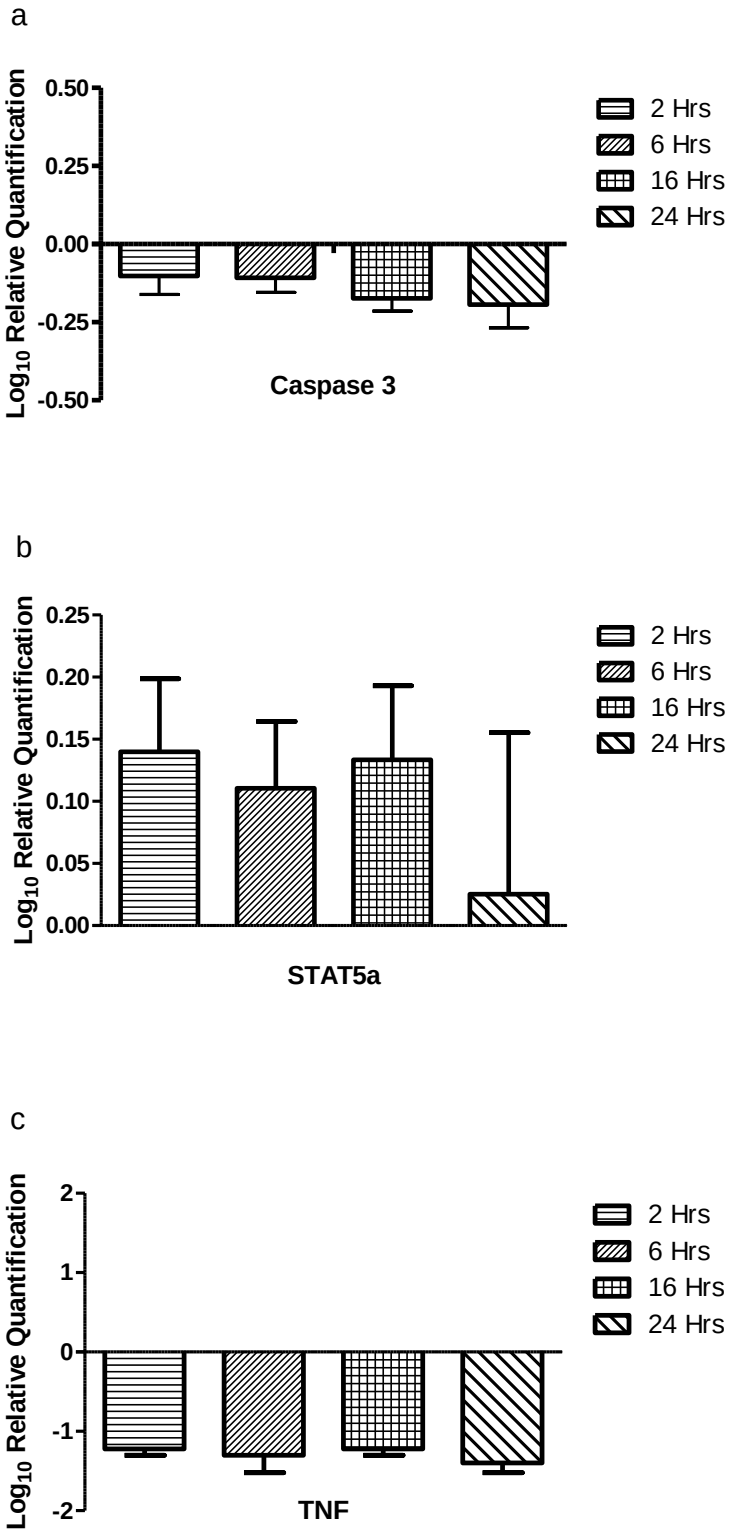


Figure 4-15: Caspase, STAT5a and TNF expression in response to time and cold hypoxia.
Passage 2 RPTECs were grown to 80% confluency before undergoing cold hypoxia for the prescribed duration. The control group was harvested under normal growing conditions at the start of the experiment. Cells were lysed and the RNA stabilized using RNAlater. (a) Caspase 3; (b) STAT5a; (c) TNF expression was measured. Data shown is mean ± SEM. (n=1, done in triplicate). The x axis represents baseline gene expression at 37°C.

Figure 4.16a+b examine the effect of cold hypoxia of potential biomarkers of acute kidney injury: IL-18; and NGAL. Interestingly, expression of all the biomarkers was increased at all time points. As for the apoptotic genes, this appeared to be maximal at 16 hours.

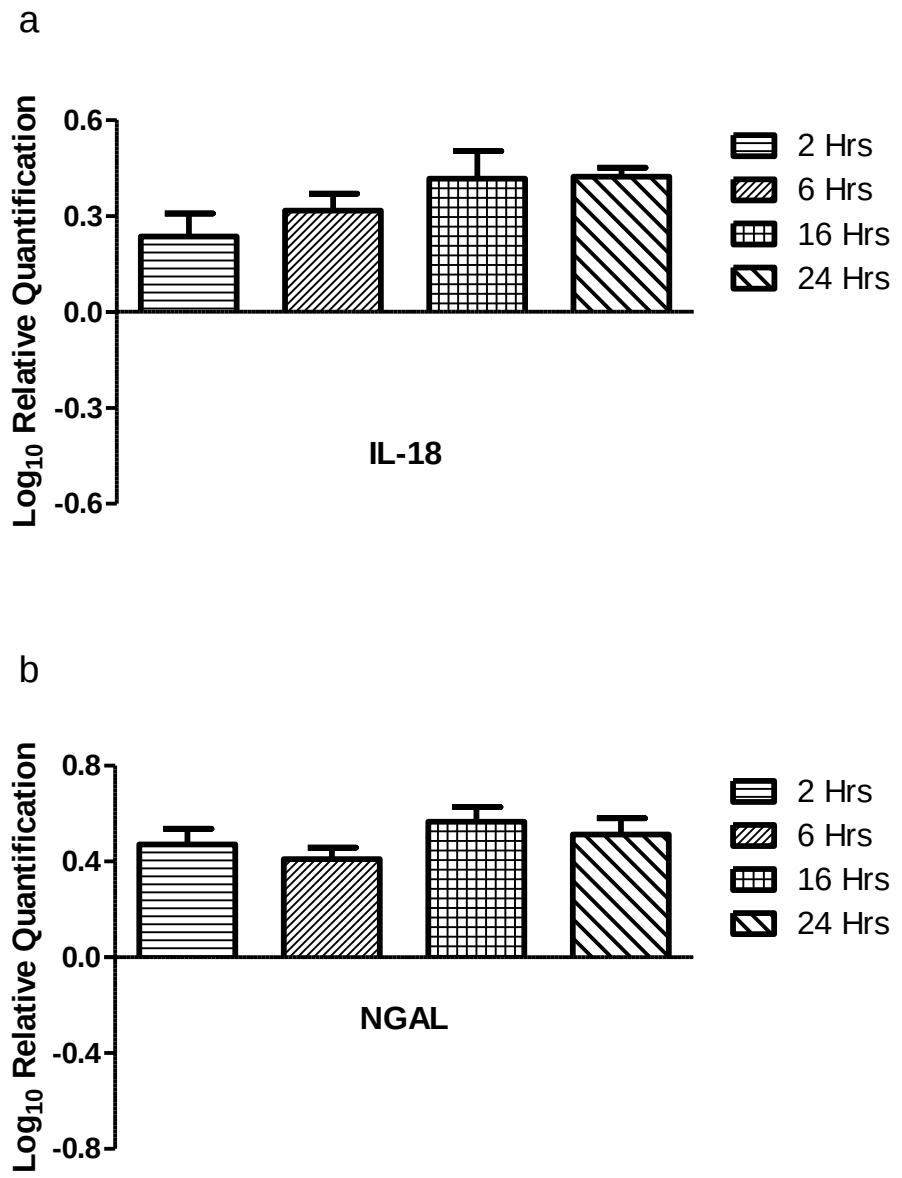


Figure 4-16: IL-18 and NGAL expression in response to time and cold hypoxia.
Passage 2 RPTECs were grown to 80% confluency before undergoing cold hypoxia for the prescribed duration. The control group was harvested under normal growing conditions at the start of the experiment. Cells were lysed and the RNA stabilized using RNAlater. (a) IL-18 and (b) NGAL expression was measured. Data shown is mean ± SEM. (n=1, done in triplicate). The x axis represents baseline gene expression at 37°C.

Cells exposed to a re-warming period following 24hrs of cold hypoxia demonstrated increased gene expression compared to the control group maintained at 37°C (Figures 4.17 and 4.18). mRNA quality was poor in samples re-warmed for 16 and 24 hrs, as indicated by a 260/280 absorbance ratio of <1.8. As a result, gene expression was not assessed in these samples.

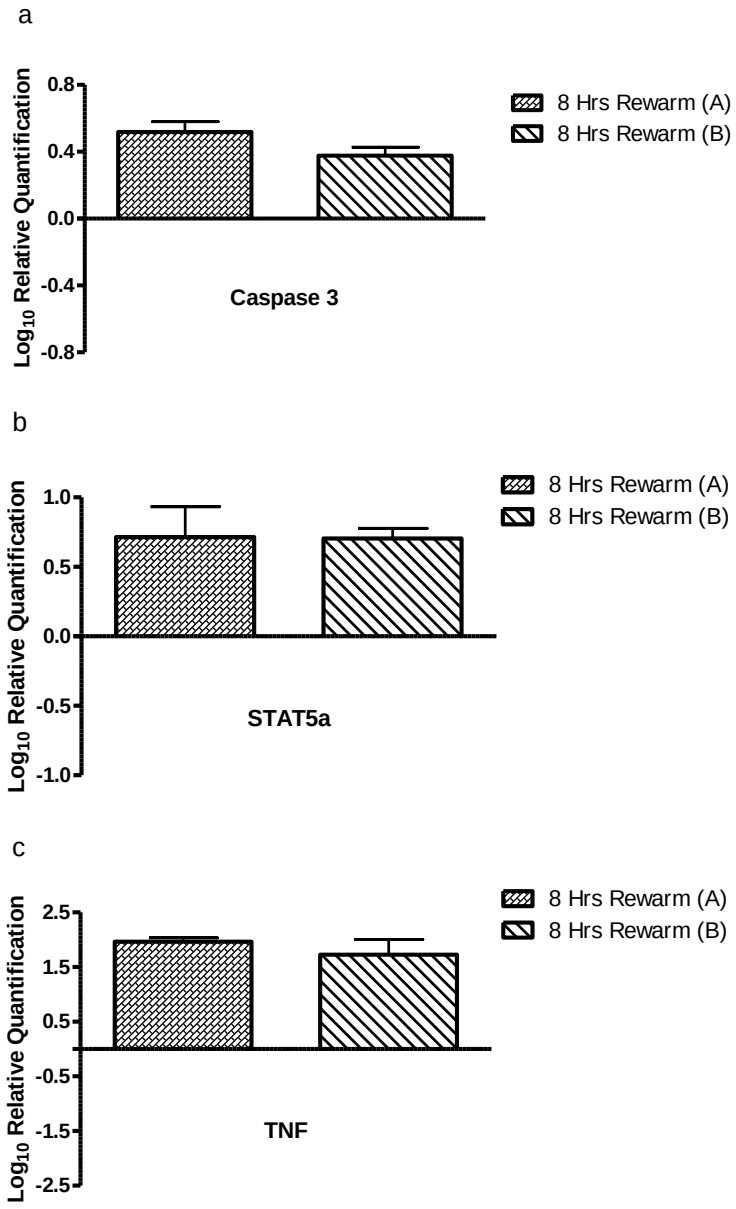


Figure 4-17: Caspase 3, STAT5a and TNF expression in response to cold hypoxia and re-warming. Passage 2 RPTECs were grown to 80% confluency before undergoing cold hypoxia for 24 hours and then re-warming for 8 hours. Cells were lysed and the RNA stabilized using RNAlater. (a) Caspase 3; (b) STAT5a; (c) TNF expression was measured. Data shown is mean ± SEM. (n=2, A and B, done in triplicate). The x axis represents baseline gene expression at 37°C.

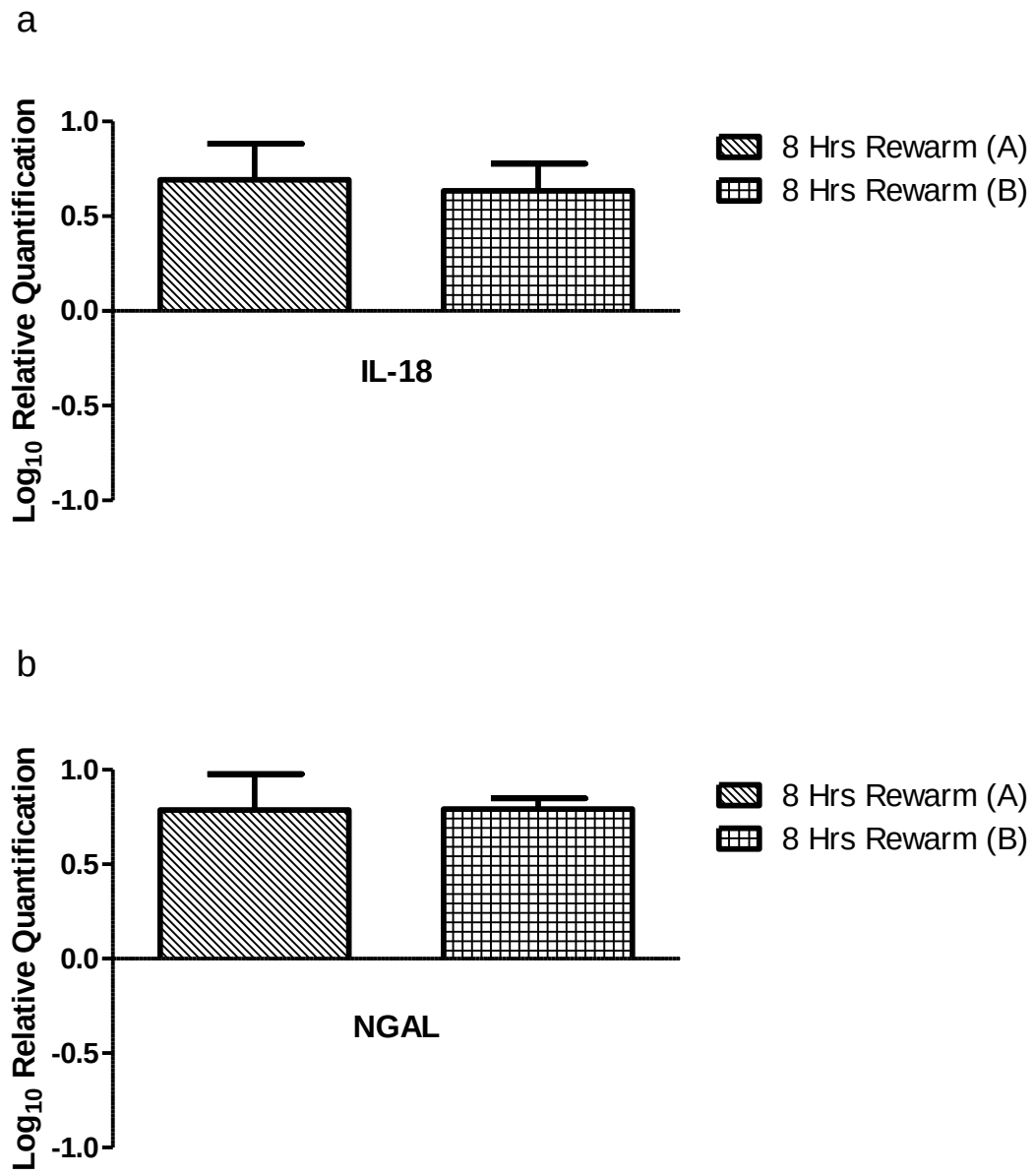


Figure 4-18: IL-18 and NGAL expression in response to cold hypoxia and re-warming.
Passage 2 RPTECs were grown to 80% confluency before undergoing cold hypoxia for 24 hours and then re-warming for 8 hours. Cells were lysed and the RNA stabilized using RNAlater. (a) IL-18 and (b) NGAL expression was measured. Data shown is mean ± SEM. (n=2, A and B, done in triplicate). The x axis represents baseline gene expression at 37°C.

4.10.4 Discussion

Based on the collective gene expression profiles, 16hrs cold hypoxia appears to be the best time point, to assess the potential effect of EPO on candidate gene expression. This is in keeping with local data (presented at BTS 2005 – Lee et al: A single centre prospective audit

of CIT and outcomes) which highlighted 16 hours as a time point after which graft outcomes are worse. Substantial mRNA degradation had occurred at the 16hrs and 24hrs re-warming time points preventing analysis of gene expression. Furthermore, the further away from the injurious stimulus, the more likely that the cell population becomes selected with a favourable gene expression profile. In summary, the time points selected for the cell model are:

- 37C control (baseline for cold hypoxia experiments)
- 16hr cold hypoxia (both apoptosis and inflammation gene sets)
- 24hr cold hypoxia and re-warming for 8 hours)

Acute kidney injury biomarkers, NGAL, IL-18 and HGF, are all increased in response to cold storage alone and when followed by a period of re-warming. This suggests that injury is occurring during cold storage, despite the use of UW solution, which is specifically designed to reduce cold storage injury (402). However, while IL-18 promotes the inflammatory response (126), induction of HGF and NGAL may be part of a protective response. Exogenous NGAL has previously been shown to ameliorate ischaemic injury in the mouse model (99) while HGF has been shown to be not only anti-apoptotic but also promoting tubular regeneration(150).

In keeping with the induction of a protective response to cold hypoxia, STAT5a expression increased in response to cold hypoxia, while potentially signalling a pro-survival response, particularly when combined with a reduction in TNF and caspase 3 expression, both pro-apoptotic proteins. Reduced caspase 3 gene expression, in response to cold hypoxia, supports earlier results showing reduced caspase 3 activity during cold storage without hypoxia.

4.11 Discussion of cell model design

Classically, tubulo-epithelial cells have been thought of as the predominant cell type that are injured in ischaemic / toxic injury, resulting in acute renal failure, as evidenced by the term

acute tubular necrosis. The literature confirms that EPO confers tissue protection on tubular cells, in situations of oxidant stress (248;319), serum starvation (248), nephrotoxic drugs (325;328) and warm ischaemia followed by reperfusion injury (248;315-317;321). However, the models used did not invoke environments akin to clinical transplantation.

Deceased donor kidneys initially undergo a period of relative warm ischaemia due to periods of relative hypotension in the donor prior to retrieval. Kidneys from non-heart beating donors undergo a further period of up to 30 minutes of absolute warm ischaemia after cardiac death, prior to perfusion of the organs with cold perfusate. Following removal of the organs from the donors, they undergo a period of cold ischaemia for a variable period of time At the time the vascular anastamosis is being performed, a further period of relative warm ischaemia occurs with the kidney inside the body cavity. Finally, reperfusion occurs with the ensuing reperfusion injury.

In the proposed model, cells undergo storage in UW solution at 4°C and a 1% O_2 atmosphere to mimic cold storage of the retrieved kidney, followed by a period of re-warming to 37°C in complete medium. Initially, immortalised cell lines were used. However, it quickly became apparent that these were relatively resistant to injury, most likely reflecting their transformed state. Subsequently, the model required primary tubular cells.

Previous published work on tubular cells maintained at 4°C and UW have demonstrated significant apoptosis (up to 8%) and necrosis (up to 16%) after 24 hours cold storage (without hypoxia), with necrosis as the predominant form of injury (395;403). 24hrs is also comparable to cadaveric cold ischaemic times in the clinical trial. Hence for the LDH and MTT assays, this time point has been used.

Re-warming injury following cold storage in the clinical situation occurs immediately and its duration is difficult to quantify. Work in HK-2 cells has demonstrated that the degree of re-warming injury is dependent on the duration of cold storage, which appears to prime the cells for greater injury. Thus re-warming of the cells for 8 hours after 16 hours of cold storage is associated with significantly more apoptosis and necrosis in comparison to 8 hours cold storage followed by 16 hours re-warming (403). In the proposed cell model, 24 hours of re-warming has been chosen as the definitive time point in an attempt to capture maximum

necrosis and reduction in viability, without surviving cell proliferation confounding the results, particularly for the MTT assay.

Optimum time points at which to measure the impact of cold hypoxia and re-warming on gene expression differ from those for monitoring cell viability, as gene expression often precedes cellular events. The time series in cold hypoxia has demonstrated that the optimum time point appears to be at 16 hours of cold hypoxia, when changes in gene expression are maximal. This can then be compared with gene expression in cells maintained at 37°C for 24 hours. To measure gene expression following re-warming, gene expression after 24 hours of cold hypoxia is measured as a baseline to quantify changes in gene expression on re-warming for 6 hours.

CHAPTER 5 ERYTHOPOIETIN AND TISSUE PROTECTION

5.1 General introduction

Erythropoietin has been shown to confer cytoprotection in cell models using tubule-epithelial cells, podocytes and dermal microvascular endothelial cells (248;319;329;404;405). Proposed mechanisms include reducing oxidative damage, stimulating growth, ameliorating apoptosis and necrosis, reducing pro-inflammatory and fibrotic gene expression, up-regulating anti-inflammatory and anti-apoptotic gene expression.

5.2 EPO confers tissue protection in a renal epithelial cell model of kidney transplantation

5.2.1 General introduction

A number of assays were examined for their suitability in assessing the potential therapeutic effect of EPO in the cell model of transplantation. LDH, measuring necrosis, and MTT, measuring cell viability were complimentary to each other. Both were easy to perform, capable of high throughout, reproducible and capable of being performed on the same experimental material.

For the final analysis of the cell model, a primary cell line was used, RPTECs, as they were the closest approximation to the un-injured proximal tubular epithelium. The immortalised cell lines had also proven to be very robust, despite hypoxic and cold environments, with excessive caspase activity, possibly related to their immortalised nature.

5.2.2 Specific methods

Passage 2 RPTECs were seeded into 96 well plates at a cell count of 10,000 cells per well (approximately 80% confluence) and allowed to adhere overnight prior to beginning the experiment. Cells were exposed to 4°C in 1% O_2 environment in UW solution for 24 hours ± EPO treatment (50U/ml), before undergoing re-warming with EPO-containing medium to 37°C in a 5% CO_2 atmosphere for 24 hours. Initial EPO treatment was given at the beginning

of injury without prior treatment. LDH was measured in the supernatants and an MTT viability assay was performed on the remaining cells as per sections 3.6.3 and 3.6.5. Supernatant was aspirated from 6 wells prior to beginning the experiment, frozen at -20°C. When running the final LDH assay, this was thawed and assayed as the 37°C control group. For the MTT assay, 8 wells were assessed at time 0 for cell viability for the 37°C control, with the development of the formazan crystals standardised to 3 hours before dissolving in DMSO and the OD being measured. Graphs shown are from representative experiments. Experiment repeats = 4. Statistical tests were done with a one way ANOVA with a Dunnett's Multiple Comparison Test.

5.2.3 Results

Cold storage and re-warming was associated with a significant increase in cell necrosis, as measured by LDH release into the supernatant ($p<0.0001$) (Figure 5.1a). EPO therapy started at the time of cold storage was associated with a significant reduction in cell necrosis at the end of the experiment compared to the un-treated group ($p<0.001$). However, EPO treatment did not prevent necrosis occurring with a significant difference in LDH levels compared to the 37°C control group ($p<0.001$).

In keeping with the LDH experiment, cold storage and re-warming was associated with a significant decrease in cell viability in the absence of EPO treatment ($p<0.001$) (Figure 5.1b). There was no significant difference in cell viability between the control group and the EPO-treated group.

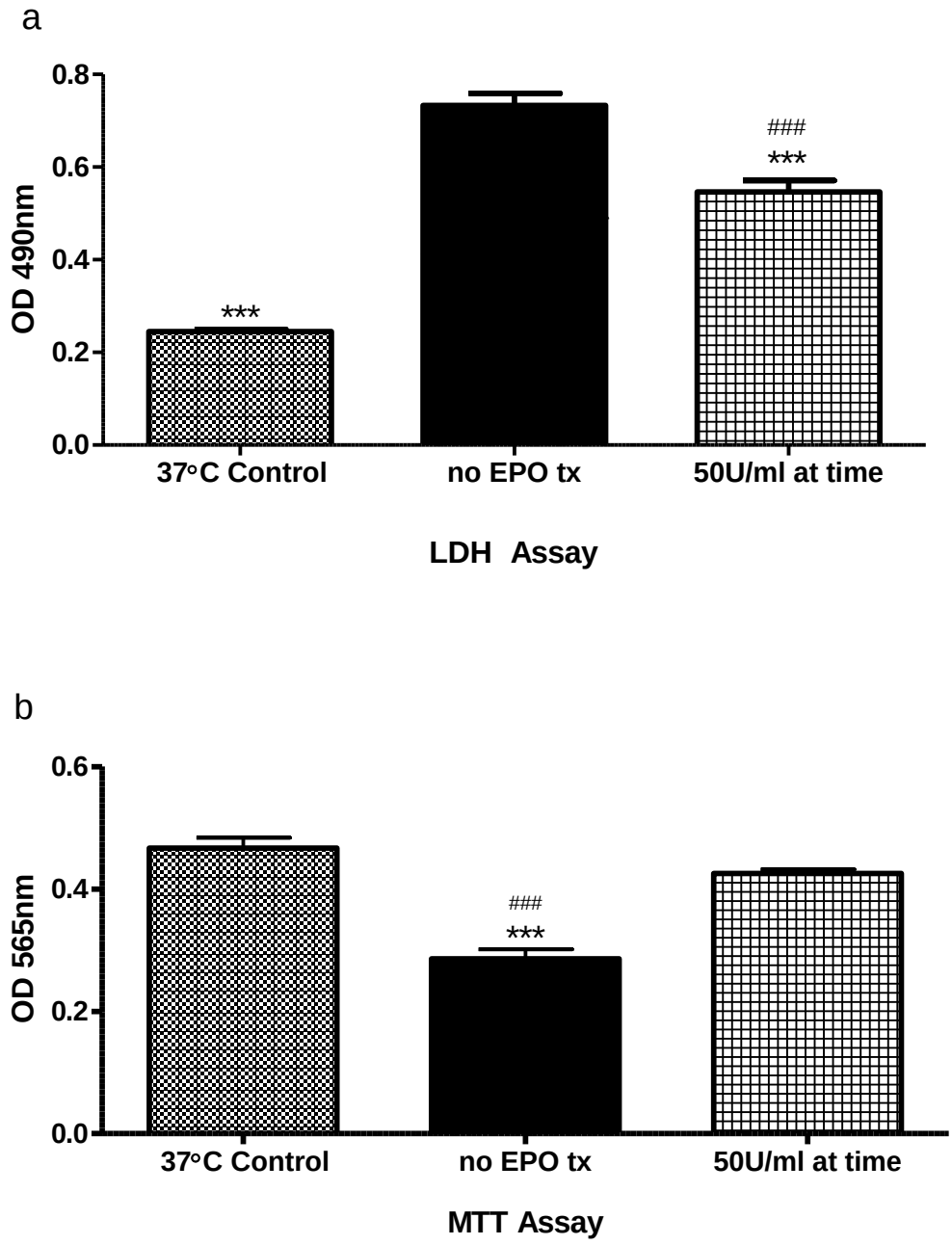


Figure 5-1: EPO confers tissue protection in a cell model of kidney transplantation.
Passage 2 RPTECs were seeded in equal numbers on to a 96 well plate and allowed to adhere overnight. UW ± 50U/ml EPO was added and the cells were exposed to 4°C in 1% O_2 environment before undergoing rapid re-warming to 37°C in a 5% CO_2 atmosphere and complete medium ± 50U/ml EPO for 24 hrs. The supernatant was then aspirated to perform the LDH assay. Data is expressed as mean OD reading ± SEM. One way ANOVA with Dunnett's Multiple Comparison Test, ***p<0.001 vs no EPO treatment, $^{###}$p<0.001 vs 37°C control. The data is representative of n=4 experiments with 6 replicates each.

5.2.4 Discussion

EPO conferred cytoprotection in a primary cell model mimicking conditions akin to human kidney transplantation. The dose and timing of EPO were chosen from experiments published by Sharples et al, examining oxidative stress in HK-2 cells (248) and were similar to those used in other studies (319).

Despite similar cell viabilities between the control group and the EPO-treated group, LDH levels were markedly higher in the EPO group, suggesting increased necrosis. This discrepancy may be partly explained by the experimental design, specifically the re-warming period. During this time, cell death via necrosis is occurring resulting in large amounts of LDH release. However, at the same time, surviving cells may begin to proliferate again, thereby augmenting the number of viable cells for the MTT assay. EPO at this dose has been shown to stimulate proliferation of HK-2 cells, even in the presence of serum starvation (248).

5.3 Determining the optimum timing of EPO treatment

5.3.1 General introduction

Interventions with EPO can occur before, during or after injury has taken place. In the context of human kidney transplantation and the EPO study, both donor-derived injury and cold storage-related injury occur prior to the recipient's first dose of EPO. I wished to interrogate the cell model as to the optimum time to give EPO therapy.

5.3.2 Specific methods

Passage 2 RPTECs were seeded into 96 well plates at a cell count of 10,000 cells per well (approximately 80% confluence) and allowed to adhere overnight prior to beginning the experiment. Cells were exposed to 4°C in 1% O_2 environment in UW solution for 24 hours, before undergoing re-warming to 37°C in a 5% CO_2 atmosphere for 24 hours. Initial EPO treatment (50U/ml) was given 24 hours prior to injury, 1 hour prior to injury or at the beginning of injury and continued throughout the experiment. LDH was measured in the

supernatants and an MTT viability assay was performed on the remaining cells as per sections 3.6.3 and 3.6.5. Supernatant was aspirated from 6 wells prior to beginning the experiment, frozen at -20°C. When running the final LDH assay, this was thawed and assayed as the 37°C control group. For the MTT assay, 8 wells were assessed at time 0 for cell viability for the 37°C control, with the development of the formazan crystals standardised to 3 hours before dissolving in DMSO and the OD being measured. Graphs shown are from representative experiments. Experiment repeats = 4.

5.3.3 Results

As shown previously, hypoxic cold storage of RPTECs resulted in a marked increase in cell necrosis and LDH release compared to the control group ($p<0.0001$) (Figure 5.2a). The presence of EPO resulted in a significant reduction in cell necrosis ($p<0.001$ at all time points) and LDH release which was minimal when EPO was given 1 hour before hypoxic cold storage ($p<0.05$ vs EPO at the time of injury and $p<0.001$ vs EPO given 24 hours prior to injury).

Similar to the previous experiment, no EPO treatment resulted in the greatest reduction in cell viability, as assessed by the MTT assay ($p<0.0001$) (Figure 5.2b). There was no difference in cell viability between any EPO group and the control group. However, treatment either 1 hour or 24 hours prior to injury resulted in better cell viability compared to treatment at the time of injury ($p<0.001$).

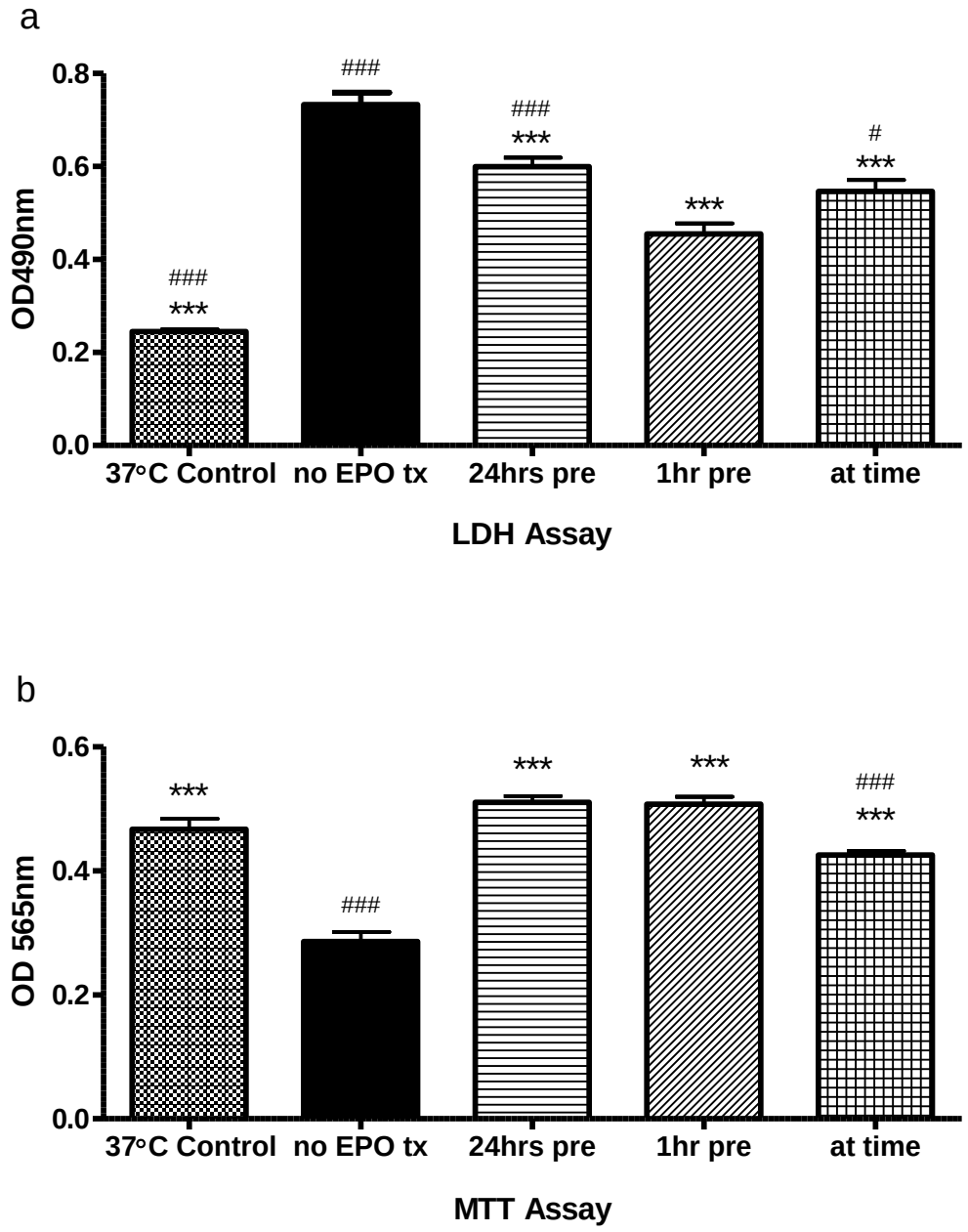


Figure 5-2: EPO tissue protection varies with duration of exposure to EPO.
Passage 2 RPTECs were seeded in equal numbers on to a 96 well plate and allowed to adhere overnight. EPO treatment began at 24 hours prior to injury, 1 hour prior to injury or at the time of injury. UW ± 50U/ml EPO was added and the cells were exposed to 4°C in 1% O_2 environment before undergoing rapid re-warming to 37°C in a 5% CO_2 atmosphere and complete medium ± 50U/ml EPO for 24 hrs. The supernatant was then aspirated to perform the LDH assay. Data is expressed as mean OD reading ± SEM. One way ANOVA with Dunnett's Multiple Comparison Test, ***p<0.001 vs no EPO treatment, ###p<0.001 vs 37°C control. The data is representative of n=4 experiments with 6 replicates each.

5.3.4 Discussion

The optimum time to administer EPO was determined to be 1 hour prior to undergoing injury, based on the LDH assay which showed a significantly higher LDH level in the supernatant at 24 hours and with no pre-treatment compared to the 1 hour pre-treatment group. There was no difference in LDH levels between the group treated for 24 hours before and the group given treatment only at the beginning of injury. There is no published evidence to suggest that high doses or prolonged exposure to EPO result in cytotoxicity. In fact, as mentioned above, EPO appears to act as a growth factor (248).

The MTT assay confirmed that 1 hour pre-treatment was associated with the highest cell viability, although in this case, there was no difference between 1 hour and 24 hour treatment. As mentioned above, prolonged exposure to EPO may result in cell proliferation and artificially enhance cell numbers and therefore cell viability resulting in the discrepancy again between the LDH and the MTT assay (248)

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5.4 Determining the optimum dose of EPO

5.4.1 General introduction

We wished to determine the optimum dose of EPO to confer tissue protection, in addition to determining its potential for cytotoxicity at high doses.

5.4.2 Specific methods

Methods were similar to the previous experiment, with the exception of a 500U/ml dose of EPO used in addition to a 50U/ml dose.

5.4.3 Results

500U/ml given one hour prior to cold hypoxia conferred similar protection from cell necrosis following injury as did 50U/ml (Figure 5.3a). Interestingly, administration of 500U/ml of EPO at any of the three time points conferred similar levels of protection, unlike EPO 50U/ml

which was most effective at one hour pre-injury (Figure 5.3b). Cell viability was greatest with 50U/ml EPO compared to 500U/ml of EPO at any time point.

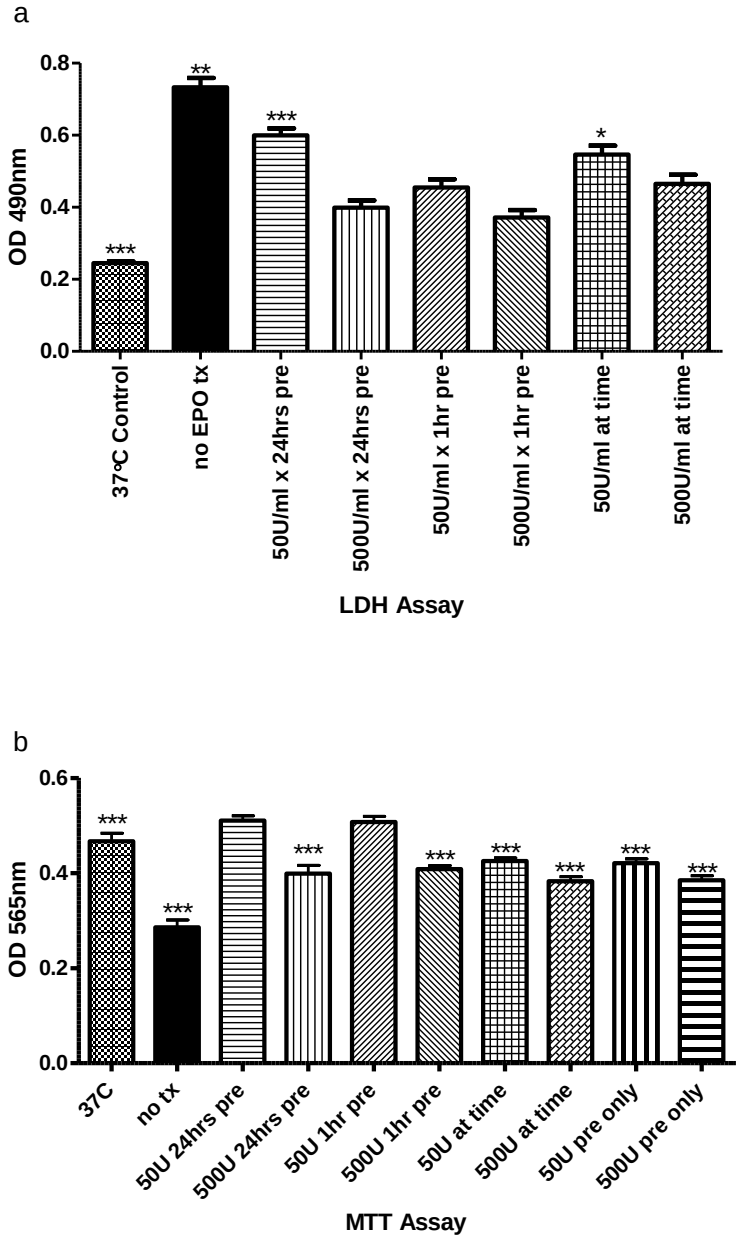


Figure 5-3: EPO tissue protection varies with dose of EPO.
Passage 2 RPTECs were seeded in equal numbers on to a 96 well plate and allowed to adhere overnight. EPO treatment began at 24 hours prior to injury, 1 hour prior to injury or at the time of injury. UW ± 50U/ml or 500U/ml EPO was added and the cells were exposed to 4°C in 1% O_2 environment before undergoing rapid re-warming to 37°C in a 5% CO_2 atmosphere and complete medium ± 50U/ml or 500U/ml EPO for 24 hrs. The supernatant was then aspirated to perform the LDH assay. Data is expressed as mean OD reading ± SEM. One way ANOVA with Dunnett's Multiple Comparison Test, ***p<0.001 vs EPO 50U/ml at 1 hour, *p<0.05 vs EPO 50U/ml at 1 hour. The data is representative of n=4 experiments with 6 replicates each.

5.4.4 Discussion

EPO administered one hour prior to injury at a dose of 50U/ml is the optimal for conferring tissue protection. Multiple time points were used for each dose to examine if different time points were more efficacious for different doses. High dose EPO appeared was associated with worse cell viability at the 1 hour time point, suggesting both a ceiling effect with EPO dosing and the possibility of EPO-induced toxicity.

Rat brain vascular endothelial cells exposed to anoxia and increasing EPO demonstrated a ceiling effect, where doses higher than 10ng/ml (~1U/ml EPO) were not associated with increased survival (302). Other studies have demonstrated similar effects. In a model of cerebral ischaemia, EPO at a dose of 50U/day or 500U/day was ineffective at ameliorating neuronal damage, when compared to doses of 25U/day or less (185). One possibility is that cells respond to EPO within a limited concentration range and that high concentrations may induce down-regulation or internalisation of the EPOR, preventing further signalling (185).

5.5 Examining the effect of EPO on cell proliferation and thus its potential for cytotoxicity

5.5.1 General introduction

Published work and prior experiments have suggested that EPO may act as a growth factor for tubular cells (248). In addition, the previous dose finding experiment raised the possibility of EPO toxicity with EPO 50U/ml at 1 hour pre-injury having significantly higher cell viability compared to 500U/ml at the same time point. I wished to explore this further.

5.5.2 Specific methods

Methods were similar to the previous experiment, with the exception that cells were incubated with 5U/ml or 50U/ml or 500U/ml EPO at 37°C for 24 hours to determine the effect of EPO on cell proliferation. The control group were harvested at the beginning of the experiment unless otherwise stated.

5.5.3 Results

Increasing doses of EPO did not result in increasing levels of proliferation, as measured by the number of cells converting MTT to formazan (Figure 5.4a). Furthermore, using 50U/ml of EPO did not alter the rate of cell proliferation over a 24 hour period compared to un-treated cells growing in fresh medium during that time (Figure 5.4b). Finally, 500U/ml of EPO did not result in a reduction in cell viability or increased cell necrosis over a 24 hour period of incubation (Figure 5.4c+d).

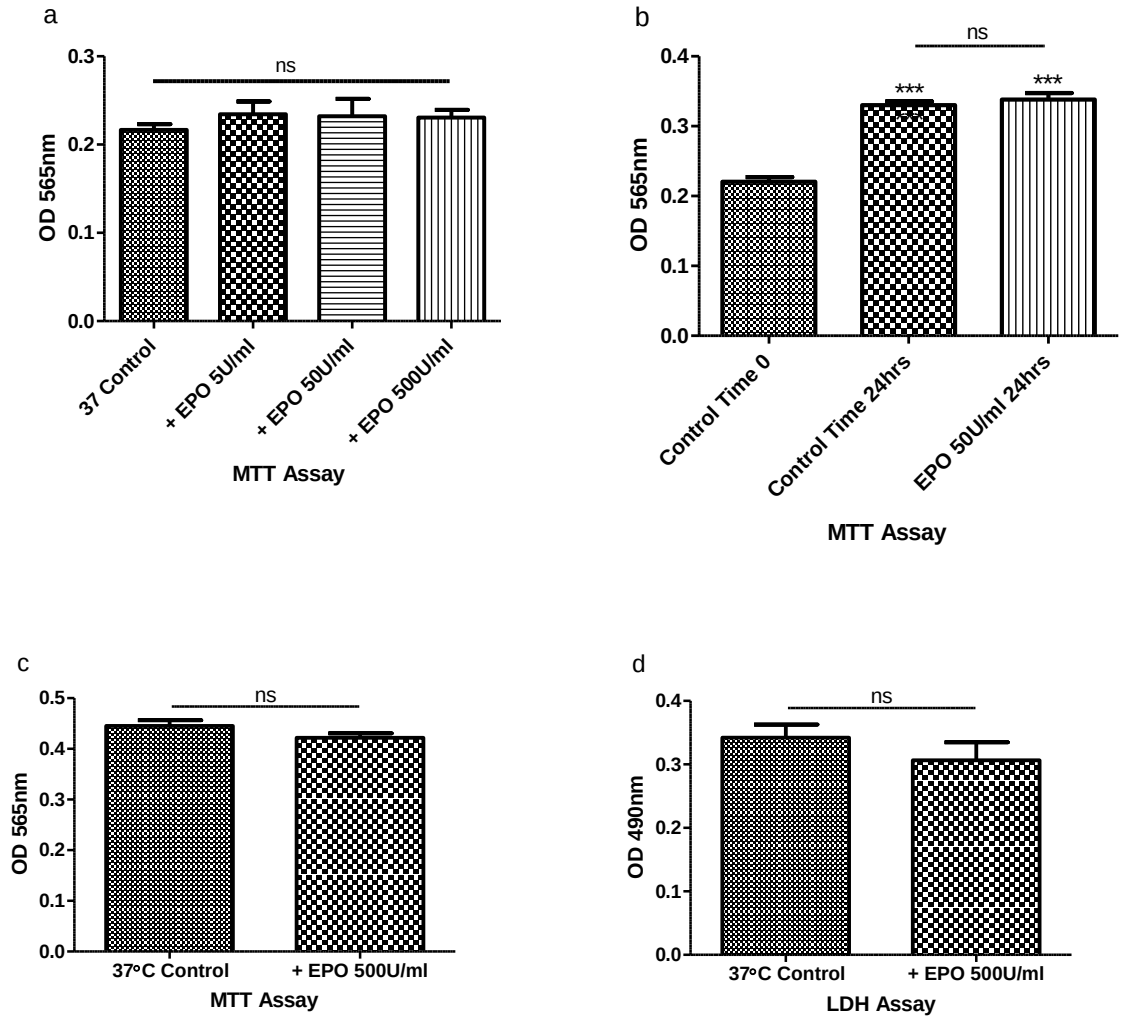


Figure 5-4: Effect of EPO on cell proliferation
Passage 2 RPTECs were seeded in equal numbers on to a 96 well plate and allowed to adhere overnight. Fresh medium ± EPO at the stated doses was added to cells incubated at 37°C in a 5% CO_2 for 24 hrs. The supernatant was then aspirated to perform the LDH assay. Data is expressed as mean OD reading ± SEM. Mann-Whitney test, ***p<0.001 vs control group. The data is representative of n=4 experiments with 6 replicates each.

5.5.4 Discussion

Increasing doses of EPO did not result in increasing RPTEC proliferation in the absence of injury. Sharples et al found that EPO at doses >10U/ml stimulated call proliferation, even in the presence of serum starvation. The main difference between studies was cell type with Sharples et al using the immortalised cell line, HK-2 cells to represent tubule-epithelium, compared to primary cells in the above experiment (248). In prior work using HK-2 cells, I have found them to proliferate freely and be very robust in the presence of injury, in keeping with their immortalised status. This is likely to explain the difference between the results.

Finally, no evidence of high dose EPO cytotoxicity was demonstrated using two different assays. This is in keeping with the lack of published in vitro evidence that EPO induces cytotoxicity. However, high doses in the clinical setting have been shown to raise aspartate aminotransferase levels suggesting hepatocyte cytotoxicity (378). Thus, this safety experiment may not translate into clinical practice.

5.6 EPO does not prevent oxidative stress

5.6.1 General introduction

One of the proposed mechanisms of EPO-induced tissue protection is through a reduction in the susceptibility to oxidative effects. I have previously shown, while developing the cell model, that LDH is an unreliable indicator of oxidative stress induced cell necrosis, due to the degrading effect of hydrogen peroxide on LDH.

Reactive oxygen species (ROS) have been implicated in the pathogenesis of renal ischaemia-reperfusion injury, with the proximal tubule appearing to be particularly susceptible to reperfusion injury (406;407). In the presence of iron, the hydroxyl radical derived from H2O2 is a powerful oxidizing agent that can cause membrane lipid peroxidation, protein denaturation and DNA damage (408).

5.6.2 Specific methods

HK-2 cells were exposed to 1mM hydrogen peroxide and to 0.1mM hydrogen peroxide in the presence of increasing doses of EPO, similar to the experiments described in (248;319). EPO pre-treatment was given for 1 hour prior to the administration of hydrogen peroxide and continues for the duration of the experiment. After 4 hours, hydrogen peroxide containing medium was aspirated and the cells washed with fresh medium before the addition of fresh medium containing MTT.

RPTECs (passage 2) were exposed to 1mM hydrogen peroxide and 50U/ml EPO. EPO treatment was begun one hour prior to the addition of hydrogen peroxide and continued for the duration of the experiment. Supernatant was aspirated for LDH assay and MTT was added to the cells as described above.

5.6.3 Results

Hydrogen peroxide (1mM) treatment of HK-2 cells was associated with a significant decrease in cell viability, which was not reversed by increasing doses of EPO treatment (Figure 5.5a). In fact, after 2 hours of hydrogen peroxide treatment, all cells in the experiment had rounded up. 0.1mM of hydrogen peroxide did not result in a decrease in cell viability.

Similarly, RPTECs treated with 1mM hydrogen peroxide had a marked increase in LDH levels were seen in response to oxidative stress which was not reversed by EPO treatment (Figure 5.5b. Furthermore, a significant reduction in cell viability occurred, which again was not ameliorated by EPO treatment (Figure 5.5c).

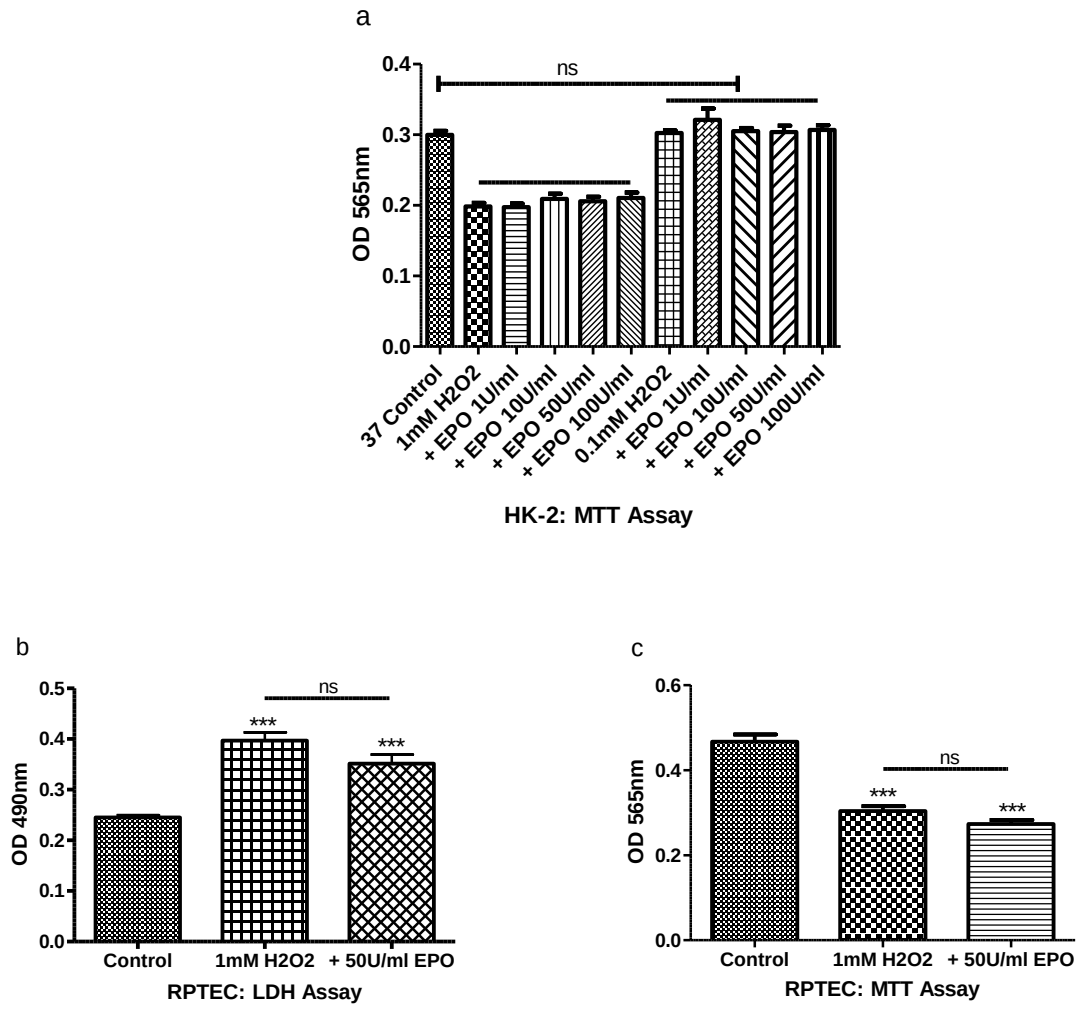


Figure 5-5: Effect of EPO on oxidative stress
HK-2 cells and passage 2 RPTECs were seeded in equal numbers on to a 96 well plate and allowed to adhere overnight. Fresh medium ± EPO at the stated doses was added to cells for 1 hour prior to the addition of hydrogen peroxide for 4 hours treatment. The supernatant was then aspirated to perform the LDH assay. Data is expressed as mean OD reading ± SEM. Mann-Whitney test, ***p<0.001 vs control group. The data is representative of n=3 experiments with 6 replicates each.

5.6.4 Discussion

Sharples and colleagues demonstrated a dose dependent decrease in necrosis in response to EPO treatment, measured by lactate dehydrogenase release, in HK-2 cells exposed to 1mM H2O2 for 4 hours (248;319). Furthermore, pre-incubation for 1hr or 24hrs with EPO 10U/ml conferred the same cytoprotection, suggesting long periods of pre-treatment do not affect the degree of tissue protection. Despite multiple attempts in different cell lines, using the same

dose and type of EPO, using the same LDH assay or the MTT assay, I have not been able to demonstrate EPO tissue protection in response to hydrogen peroxide treatment. I have already shown that hydrogen peroxide degrades purified LDH protein making the interpretation of LDH levels in these experiments tenuous. Furthermore, hydrogen peroxide treatment may also degrade EPO, eliminating its potential effect.

5.7 EPO prevents serum starvation-induced injury

5.7.1 General introduction

In HK-2 cells deprived of serum, DNA fragmentation was significantly increased at 24 hours (248). Co-incubation with EPO significantly reduced apoptotic cell death. I examined serum deprivation using the MTT assay as a means of assessing EPO cytoprotection.

5.7.2 Specific methods

RPTECs (passage 2) were cultured in serum-free medium for 24 hours before an MTT assay was performed. Cells were pre-treated with 50U/ml of EPO for 1 hour prior to the addition of serum free medium containing EPO. Control cells were harvested at the beginning of the experiment and at the end of the experiment.

5.7.3 Result

As expected, RPTECs continue to proliferate over a 24 hour period with an approximate 50% increase in cell number within the 24 hour period (control OD at 24 hours / OD at 0 hours) (Figure 5.6). Serum free medium reduced cell proliferation with less cells present after 24 hours compared to the control group ($p<0.001$), but did not stop some cell proliferation ($p<0.001$). EPO treatment had no effect on serum deprived cells.

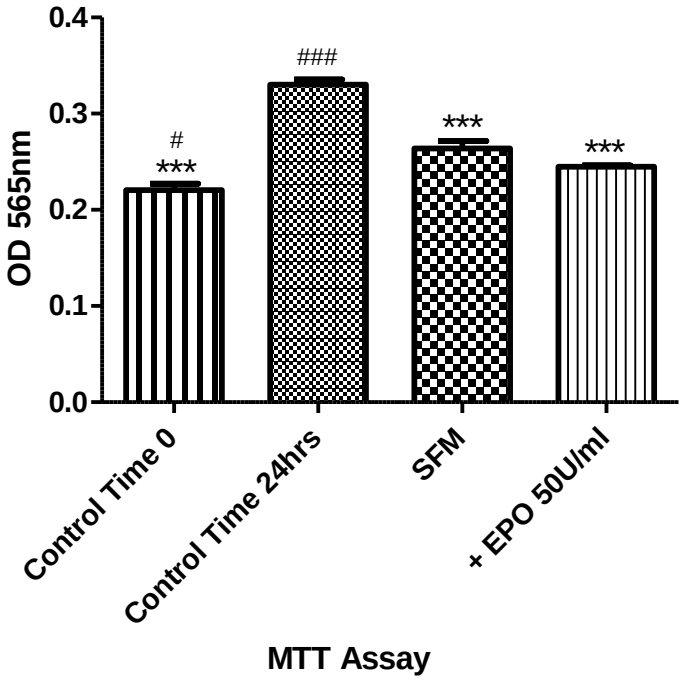


Figure 5-6: Effect of EPO on serum deprivation
Passage 2 RPTECs were seeded in equal numbers on to a 96 well plate and allowed to adhere overnight. Fresh medium ± EPO at the stated doses was added to cells for 1 hour prior to the addition of serum free medium (SFM) ± EPO for 24 hours. Data is expressed as mean OD reading ± SEM. One way ANOVA with Dunnett's Multiple Comparison Test, ***$p<0.001$ vs 24 hours control group, $^{\#}p<0.05$ compared to SFM + EPO. The data is representative of n=3 experiments with 6 replicates each.

5.7.4 Discussion

Serum deprivation resulted in a reduction in cell proliferation but not cell viability. Similar to previous experiments, EPO 50U/ml did not act as a growth factor in the absence of serum. The only difference in experimental methodology from the published literature is the cell type with immortalised cells potentially proliferating in response to EPO treatment (248). This may be important in clinical practice, given concerns that EPO may act as a growth factor for neoplastic cells(221), which have a similar phenotype to the immortalised cells proliferating in response to EPO treatment.

5.8 Identification of receptors thought to mediate EPO-associated tissue protection

5.8.1 General introduction

EPO has been shown to be tissue protective in tubulo-epithelial cells in the kidney (248). However, the receptor through which this is mediated remains unconfirmed. It is known that most cells express EPOR and conventional thought has been that it is through this receptor that EPO mediates its pleiotropic effects. This view has been challenged more recently with the development of carbamylated EPO which lacks a haematopoietic effect but maintains its tissue protective properties, suggesting an alternative mechanism of protection (180). One group have demonstrated using a CD131 knockout mouse model that EPO's tissue protective effects are mediated through a receptor heterodimer containing one EPOR and one common β chain receptor (CD131) (180). However, this has been challenged in neuroendocrine cells where EPO remains tissue protective in the absence of demonstrable CD131 (181). Furthermore, an EPOR / CD131 knockout mouse is needed to definitively prove that CD131 is responsible for the tissue protective effects. Whether tubular cells express CD131 has yet to be determined, but carbamylated EPO is renoprotective implying that CD131 is present in the kidney, if this indeed is how tissue protection is mediated (323;409;410). EPOR has been demonstrated on endothelial cells and carbamylated EPO also confers protection in the cardiovascular system (295;411). Thus, endothelial cells in the kidney should express both receptor types also.

5.8.2 EPOR and CD131 gene expression under normal conditions

5.8.2.1 General introduction

Cells were characterised to determine if EPOR and CD131 receptor mRNA was present in tubulo-epithelial and endothelial cells.

5.8.2.2 Specific methods

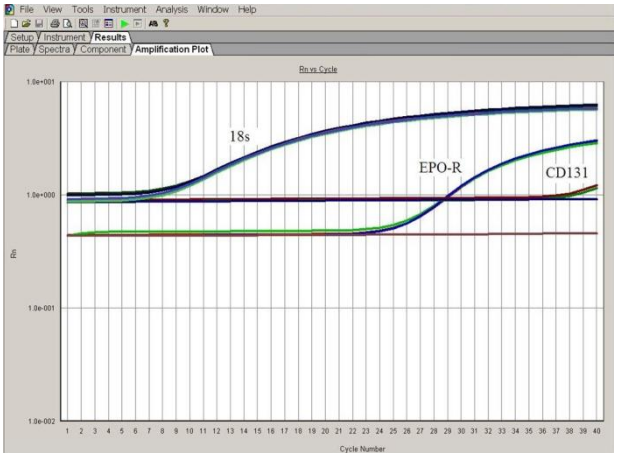
Cells were grown until 70-80% confluent under normal conditions before undergoing RNA extraction and cDNA synthesis as described in section 3.6.1. EPOR and CD131 mRNA expression were quantified in both cell types using quantitative RT-PCR as described in

section 3.6.3. Passage 2 RPTECs were used. Experimental repeats were n=3 and within each experiment, samples were run in triplicate. Data was analysed using the TaqMan 7500 system software to generate RQ values ± 95% confidence intervals.

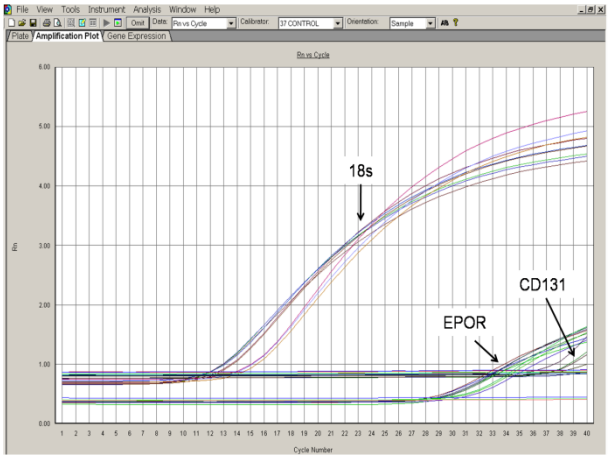
5.8.2.3 Results

The tubulo-epithelial cells HK-2 and RPTECs demonstrated good quantities of EPOR mRNA appearing between cycles 25-29 (Figure 5.7a-c).

A



B



C

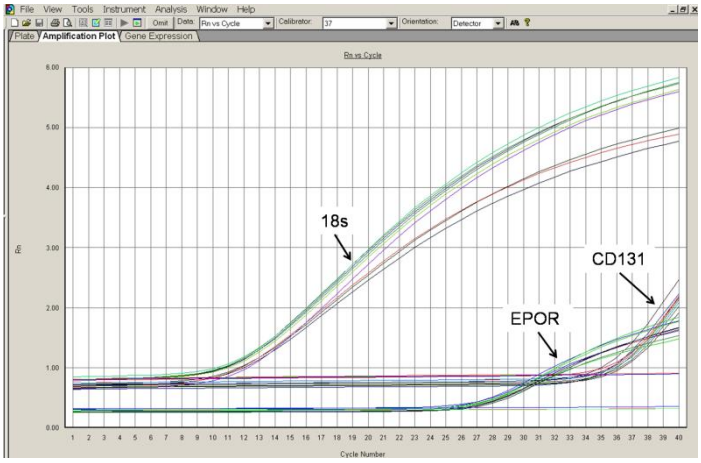


Figure 5-7: EPOR and CD131 mRNA expression under normal conditions.
Cells were grown under normal conditions before lysis and stabilization of the mRNA in RNAlater: (a) RPTEC; (b) HK-2 cells; and (c) HMEC-1. TaqMan 7500 system amplification plots are shown for 18s, EPOR and CD131 cDNA. Each cycle represents a doubling of message, with the earlier appearance of message reflecting increased abundance.

CD131 mRNA was also detected but appeared after cycle 35 suggesting very low quantities in these cells. The endothelial cell line, HMEC-1 cells, also express EPOR and CD131 mRNA with the suggestion that more CD131 message was present than was seen in the tubulo-epithelial cells. Each cycle represents a doubling of message, with the earlier appearance of message reflecting increased abundance. As such, more 18s mRNA is present compared to EPOR and CD131, with CD131 having the least abundant message.

5.8.3 EPOR and CD131 protein expression under normal conditions

5.8.3.1 General introduction

Following demonstration of receptor mRNA in the cells, the next step was to determine protein expression on the cell surface.

5.8.3.2 Specific Methods

Receptor protein expression was determined using immunofluorescence, flow cytometry and western blotting, as described in section 3.5. Cells were 70-80% confluent at 37°C before harvesting using trypsinisation. The human acute monocytic leukaemia cell line, THP-1, which is known to express CD131, was used as a positive control.

5.8.3.3 Results

Western blotting demonstrated a robust protein band at ~60-70kDa in both HK-2 and HMEC-1 cells, using EPOR polyclonal antibody (Figure 5.8). CD131 was not found at its expected band of 130kDa. Western blotting has not been performed in RPTECs.

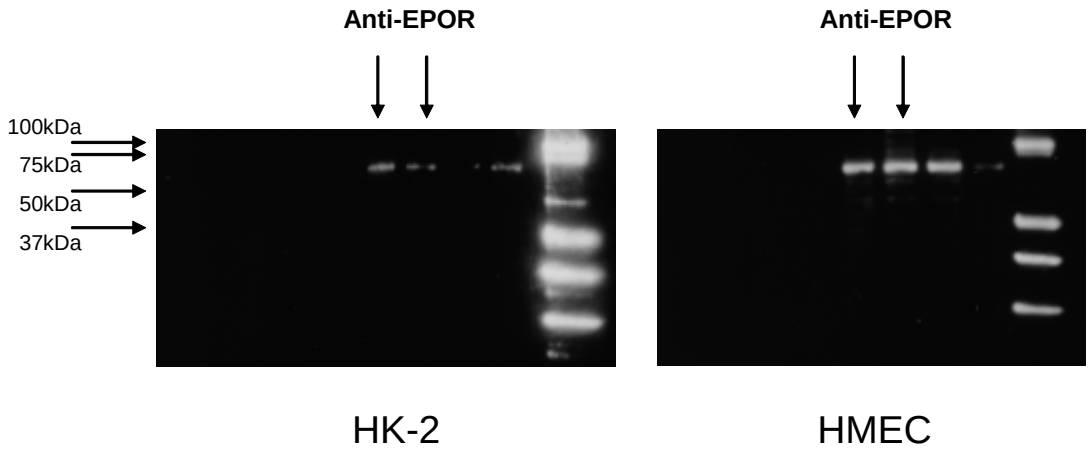


Figure 5-8: Western blotting of EPOR protein in HK-2 and HMEC-1 cells

It was not possible to confirm the presence of EPOR or CD131 on the cell surface of un-stimulated HMEC-1s, HK-2s, RPTECs or the positive control (THP-1 cells) using immunofluorescence.

EPO-R expression was demonstrated using flow cytometry and anti-EPOR antibody, as seen in HMEC-1 (Figure 5.9) and HK-2 cells (Figure 5.10).

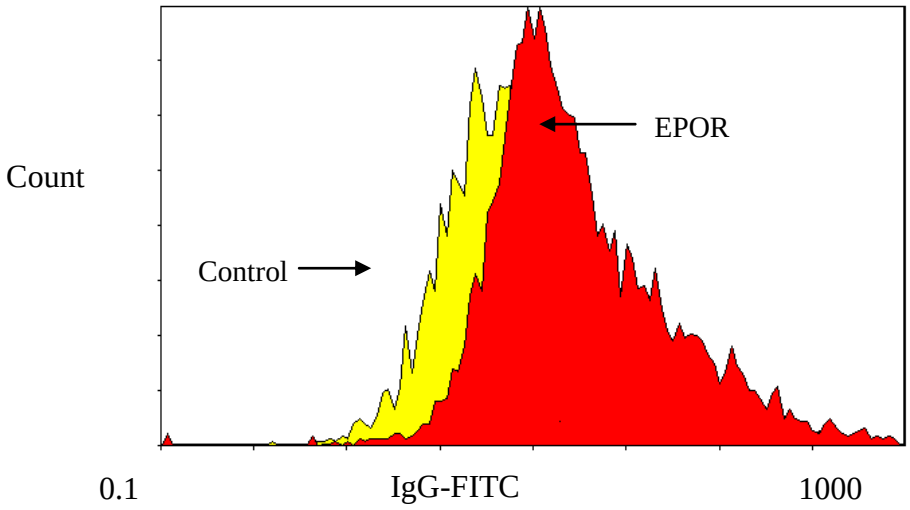


Figure 5-9: Demonstration of EPOR protein on un-stimulated HMEC-1 cells by flow cytometry. FL1 channel (FITC) versus count histogram for EPOR.

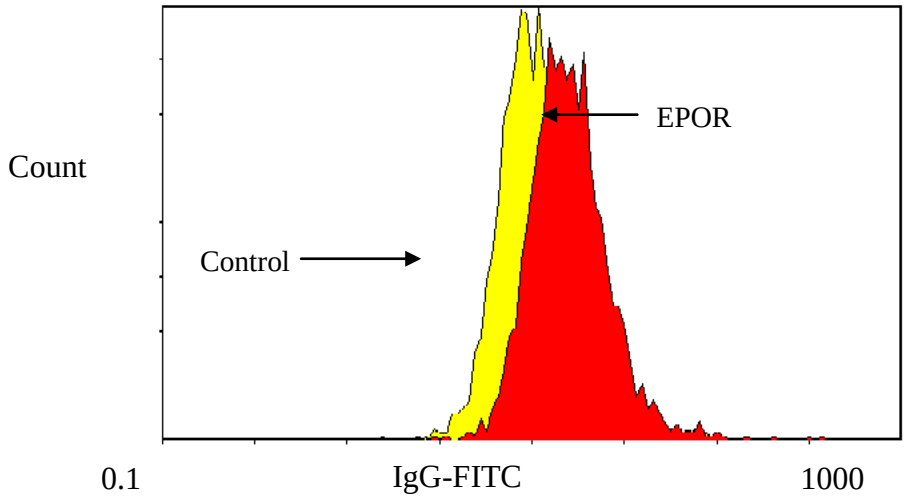


Figure 5-10: Demonstration of EPOR protein on un-stimulated HK-2 cells by flow cytometry. FL1 channel (FITC) versus count histogram for EPOR.

5.8.4 EPOR and CD131 gene expression in response to cold storage ± hypoxia ± EPO

5.8.4.1 General introduction

EPOR expression has been shown to be induced following exposure to hypoxia (223). Intriguingly, this up-regulation is enhanced by EPO itself (396). Little is known about CD131 in this regard or if EPOR is induced by conditions such as cold storage.

5.8.4.2 Specific Methods

Passage 2 RPTECs were grown until 70-80% confluent under normal conditions before undergoing cold hypoxia in UW ± EPO 50U/ml added 1 hour prior to cold hypoxia. A time course was performed where cells were exposed to cold hypoxia for 2, 6, 16 and 24 hours.

HMEC-1 and HK-2 cells in complete medium were exposed to 4°C for 24hrs in the presence or absence of EPO 10iu/ml. The control group (37°C Control) received fresh medium and were harvested after 24 hours. mRNA extraction and cDNA synthesis were performed as described in section 3.6.1. EPO-R and CD131 mRNA expression were quantified using quantitative RT-PCR as described in section 3.6.3. Experimental repeats were n=1 and within each experiment, samples were run in triplicate. Data was analysed using the TaqMan 7500 system software to generate RQ values ± 95% confidence intervals.

5.8.4.2.1 Results

EPOR gene expression is significantly reduced in response to exposure to cold and hypoxia (p=0.0002, one sample t test where RQ values are compared with theoretical value of 1), which was not altered by EPO (Figure 5.11). This reduction occurs throughout the 24hr period.

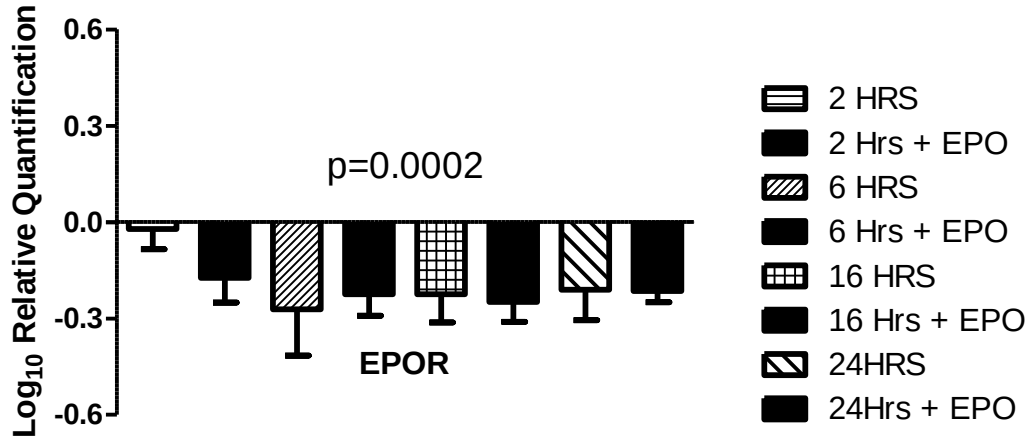


Figure 5-11: EPO-R expression in response to time, cold hypoxia.
Passage 2 RPTECs were grown to 80% confluency before undergoing cold hypoxia for the prescribed durations ± 50U/ml of EPO. Cells were lysed and the RNA stabilized using RNAlater. Data shown is mean ± SEM. (n=1, done in triplicate). The x axis represents baseline gene expression at 37°C. Relative quantification is fold change from control.

There was no significant difference in EPOR gene expression when HK-2 and HMEC-1 cells underwent cold storage in medium, without hypoxia, in the presence or absence of EPO (Figure 5.12a+b).

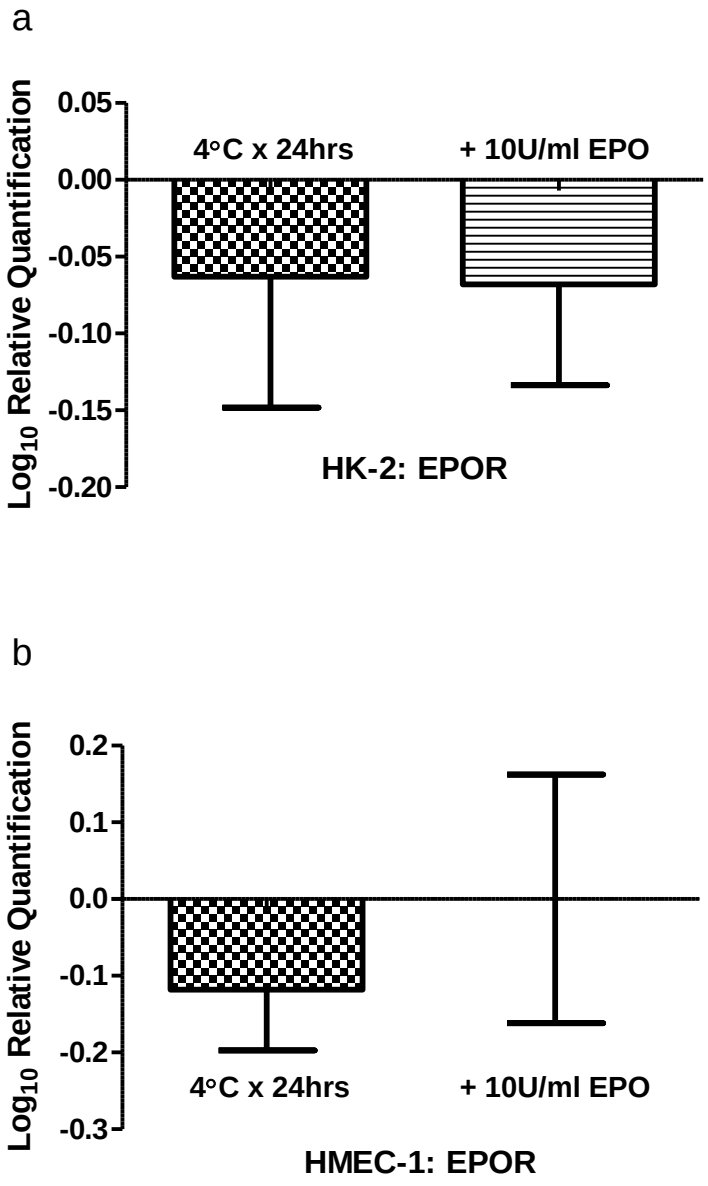


Figure 5-12: EPOR expression in response to time, cold storage and EPO in (a) HK-2 and (b) HMEC-1. Cells were grown to 80% confluency before undergoing cold hypoxia for the prescribed durations ± 10U/ml of EPO. Cells were lysed and the RNA stabilized using RNAlater. Data shown is mean ± SEM. (n=1, done in triplicate). The x axis represents baseline gene expression at 37°C. Relative quantification is fold change from control.

CD131 expression in RPTECs was at the limit of detection in the control group and was not increased by EPO or cold hypoxia. In HK-2 and HMEC-1 cells, CD131 mRNA was present but at the limit of detection, with no discernable increase in expression with EPO or cold hypoxia, resulting in wide confidence intervals.

5.8.5 Discussion: Characterisation of cell EPOR and CD131 receptor expression

HK-2 and HMEC-1 cells are capable of producing EPO-R mRNA and EPO-R protein, a finding that may explain the signalling mechanism for EPO induced tissue protection in both endothelial and kidney tubular cells. RPTECs express EPOR mRNA but EPOR protein has not been demonstrated. EPO-R may be predominantly expressed within the cell cytoplasm (412), which may explain the lack of cell surface immunofluorescence in the cells. Thus the cell could potentially be primed to respond to an injury, by converting cytoplasmic EPO-R to cell surface EPO-R.

CD131-R mRNA is expressed at the limit of RT-PCR sensitivity in HK-2 cells, HMEC-1 and RPTECs suggesting very low quantities of mRNA are present when compared to EPO-R mRNA. This may explain the difficulty in demonstrating CD131-R protein both on immunofluorescence and immunoblotting, with small numbers of receptors expressed on the cell surface. Poor sensitivity of the commercial antibody may also be responsible, given the failure to confirm CD131 on the positive control cell line, THP-1.

Thus, the possibility remains that in both of these cell lines, EPO may exert its tissue protective effects through either or both EPO-R and CD131.

5.9 Gene expression

5.9.1 General introduction

Candidate genes were chosen from previous work and the literature. Caspase 3 is an executioner caspase which plays a key role in apoptosis and is inhibited by EPO (248). IL-18 is a novel acute kidney injury biomarker that also has pro-inflammatory effects, recruiting leucocytes to areas of injury (105;127). Inhibition has been shown to be associated with tissue protection (124;126). NGAL is a sensitive marker of acute kidney injury that may or may not have tissue protective effects (99;102;109). Finally, HGF is a biomarker of acute kidney injury, with anti-apoptotic and anti-inflammatory effects (148;150).

5.9.2 Specific methods

Passage 2 RPTECs were grown to 80% confluence in 6 well flasks prior to exposure to cold hypoxia for 2, 6, 16 and 24hrs in the presence or absence of 50U/ml of EPO. Hypoxic treatment of cells was performed in an enclosed chamber (Hypoxia Incubator Chamber cat. no. 27310, Stemcell Technologies, France) flushed with pre-mixed gas mixture (1% O_2, 5% CO_2, 94% N_2) for the time indicated. Following 16hrs of cold hypoxia, cells were harvested. Exactly 1 hour prior to cold hypoxia, 50U/ml of EPO was administered to the EPO wells. Medium in the non-EPO wells was also changed. At the time of initiating cold hypoxia, wells were first washed in UW before cold UW was added to the wells for the required time. Data shown are representative of 3 experiments done in triplicate.

5.9.3 Results

As previously shown, cold hypoxia resulted in decreased expression of caspase 3, which was not further accentuated by EPO (Figure 5.13a). In contrast, cold hypoxia increased the expression of the three acute kidney injury biomarkers: IL-18, NGAL and HGF (Figure 5.13b-d). Increased expression of IL-18 was completely reversed with administration of EPO, while NGAL expression was attenuated. EPO had no effect on HGF expression.

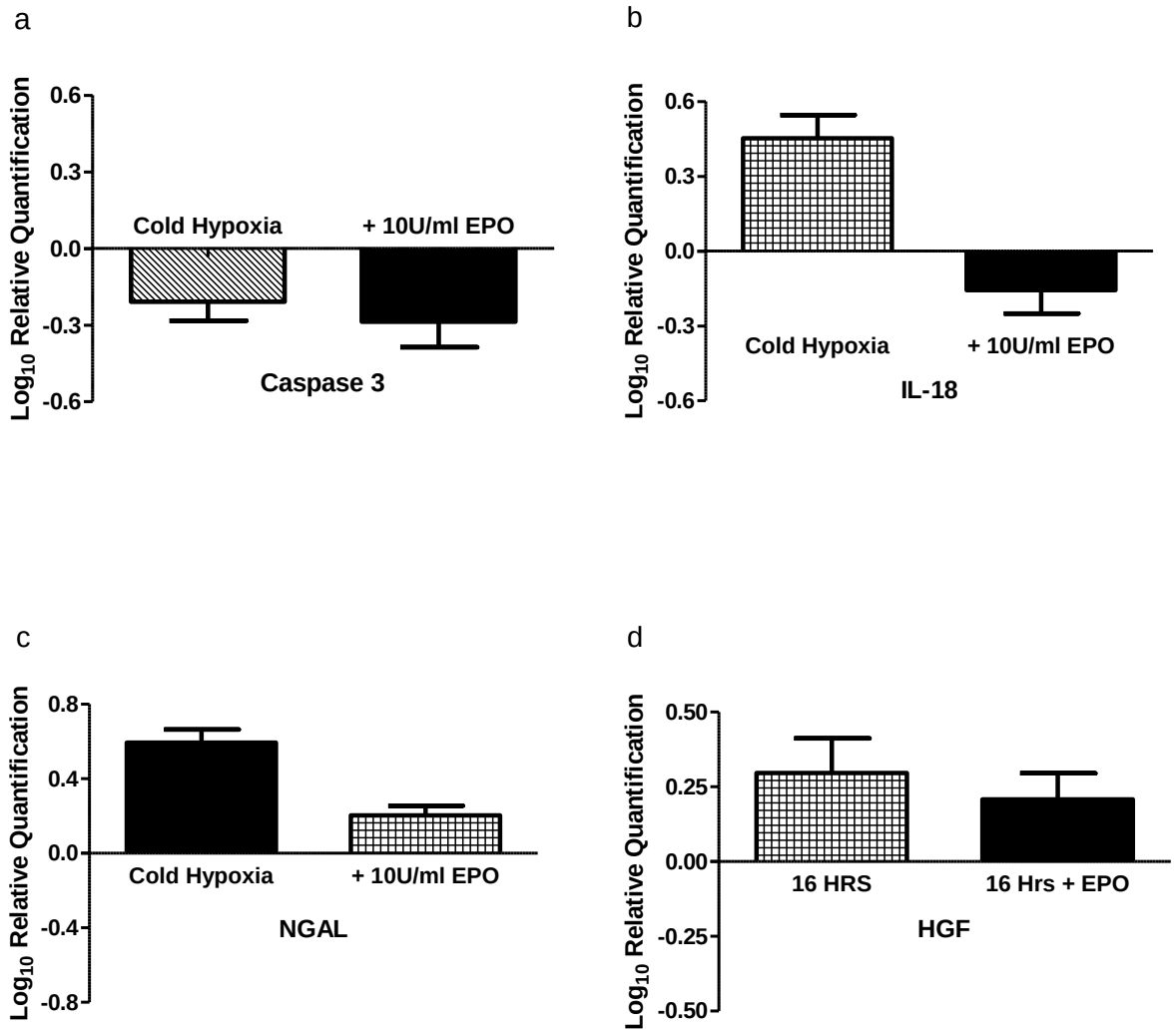


Figure 5-13: Caspase 3, IL-18, NGAL and HGF expression in response to cold hypoxia ± 50U/ml EPO Passage 2 RPTECs were grown to 80% confluency before undergoing cold hypoxia for the stated time. Cells were lysed and the RNA stabilized using RNAlater. (a) Caspase 3; (b) IL-18; (c) NGAL; and (d) HGF expression was measured. Data shown is mean ± SEM. (n=2, done in triplicate). The x axis represents baseline gene expression at 37°C.

5.9.4 Discussion of candidate gene analysis

Candidate gene analysis was chosen to focus on genes of specific interest in apoptosis and acute kidney injury. EPO has previously been shown to reduce caspase 3 protein activity in cell models of transplantation injury (413). In keeping with this, the model here demonstrated reduced caspase expression. However, previous models of transplantation injury undergo cold storage for 48 hours, prior to a re-warming period (413), which is not analogous to clinical events. Thus the injury sustained in the current experiment may not be severe enough

to significantly alter gene expression and may also be a reason why no significant effect was seen with EPO treatment. It may be that a period of re-warming is needed before any protective effect is seen.

Both IL-18 and NGAL are markers of ischaemic acute kidney injury which may also have mechanistic roles in the evolution of that injury. Thus, it is not surprising that the gene expression of both biomarkers is increased in response to cold hypoxia. IL-18 is a pro-inflammatory cytokine that is produced in the proximal tubule following acute kidney injury (129). In addition to its biomarker status in delayed graft function (124), it also functions as a stimulus to increase cell adhesion molecule expression on the endothelium, which facilitates leukocyte trafficking into areas of injury (127). Further evidence for its direct role in acute kidney injury come from animal models where IL-18 neutralisation prevents acute kidney injury (125;126). Thus it is very interesting that EPO decreases expression of IL-18, pointing to a possible novel mechanism of EPO cytoprotection. NGAL expression has previously been shown to be induced in tubular cells during oxidative stress and during the recovery phase of injury, possibly through haemoxygenase-1 induction (96;97). Its role in binding free iron may protect the kidney from further injury and indeed, animal models have suggested this (99). EPO appears to reduce NGAL expression either through a direct effect or possibly through an indirect mechanism of reducing cell injury.

5.9.5 Discussion of cell model design

Classically, tubulo-epithelial cells have been thought of as the predominant cell type that are injured in ischaemic / toxic injury, resulting in acute renal failure, as evidenced by the term acute tubular necrosis. The literature confirms that EPO confers tissue protection on tubular cells, in situations of oxidant stress(248;319), serum starvation(248), nephrotoxic drugs(325;328) and warm ischaemia followed by reperfusion injury(248;315-317;321). However, the models used did not invoke environments akin to clinical transplantation.

Sharples et al. pre-treated the HK-2 cells for 1 hour with EPO, citing that no difference was found between a 12 hour pre-treatment and 1 hour pre-treatment with EPO(248). There was a difference between pre-treatment and treatment at the time of injury, with pre-treatment conferring the most benefit. Work in rats has shown that treatment up to 6 hours after ischaemia reperfusion injury with darbopoietin was still associated with significant

renoprotection (321). No difference was seen in the proposed model between 24 hours and 1 hour pre-treatment, but both appeared to confer more protection when compared to treatment at the start of cold hypoxia. Furthermore, if cells are pre-treated for 24 hours, renoprotection is diminished if EPO treatment is not continued during cold hypoxia. Treatment on re-warming does appear to confer tissue protection, but not to the degree of 1 hour pre-treatment. Thus, the pre-treatment time for the cell model will be 1 hour pre-cold hypoxia.

The degree of tissue protection appears to be directly proportional to the EPO dose. 24 hour pre-treatment with up to 500U/ml of EPO did not appear toxic with no increase in necrosis or no change in cell viability. Using the LDH assay, 500U/ml appeared to confer the best cytoprotection. In comparison, 50U/ml demonstrated the best cytoprotection at each time point in the MTT assay. The reason for this is not clear, but may relate to differences in the time points at which these assays are assessing the model. The MTT assay compares the cell viability over the entire experiment. The LDH assay only measures necrosis occurring during the re-warming period, due to UW's effect on LDH levels. As such, the EPO dose for future experiments in the proposed model will be 50U/ml, which has been shown previously to cause effective tissue protection (248;319).

CHAPTER 6 CLINICAL TRIAL RESULTS

Erythropoietin and delayed graft function in renal allograft from marginal donors: A pilot study

6.1 Introduction

In the UK, allocation of ECD kidneys is performed as part of a national scheme run by NHS Blood and Transplant. In contrast, NHBD kidneys are allocated locally based on local rules with one kidney allocated to the best HLA match and the other to the person waiting longest on the local transplant waiting list.

The EPO study is a single centre, randomised placebo controlled trial designed to investigate the potential therapeutic effect of giving high dose erythropoietin peri-operatively to kidney transplant recipients at high risk for delayed graft function, on the incidence and duration of delayed graft function. In addition, we measured novel biomarkers of acute kidney injury in the recipient post-operatively to assess the degree of molecular injury present and any potential amelioration through EPO usage. Finally and most importantly, the safety of giving high dose EPO in the context of renal transplantation was unknown, particularly with recent trials highlighting concerns regarding thrombotic episodes and cardiovascular events(414;415).

The primary endpoints of this study were to compare the effect of EPO with placebo on acute kidney injury biomarkers (NGAL, IL-18 and KIM-1) and gene expression from biopsy material. The secondary endpoints, which are dealt with in this chapter, examined clinical outcomes between the two groups, in addition to addressing safety and feasibility issues.

6.2 Screening

Screening commenced August 1st, 2007, through to June 8th 2009. A total of 82 renal transplant recipients received either a NHBD or an ECD kidney or had a CIT > 24hrs. Potential recipients of ECD or NHBD kidneys were approached following a negative crossmatch result and informed consent was obtained.

16 recipients were excluded on the basis of a high pre-operative haemoglobin level. 3 patients were enrolled in other interventional trials at the time of recruitment. 63 fulfilled the inclusion and exclusion criteria.

- 4 refused consent
- 1 was recruited but deemed to be un-transplantable due to vascular calcification after randomisation and laparotomy
- 19 patients were not recruited due to staff unavailability

Thus 39 out of a possible 40 were recruited and underwent successful transplantation. Figure 6.1 shows a flow diagram of the 39 kidney recipients included in our analysis.

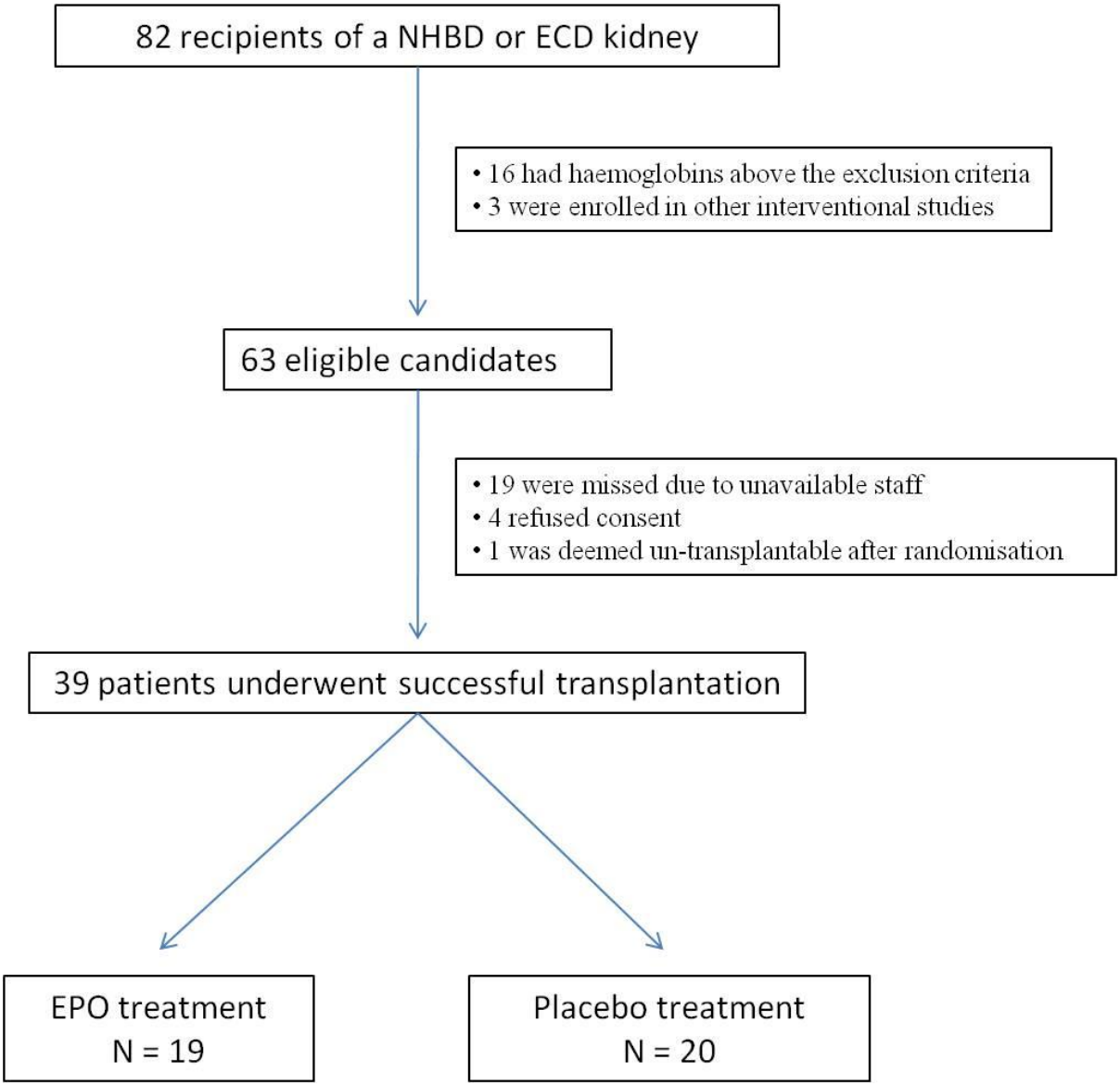


Figure 6-1: Screening flow chart.

6.3 Protocol substantial amendment

In a relatively short time period after enrolment began in the study, it became apparent that the haemoglobin level of 13g/dl, set as an exclusion criteria, was resulting in a significant exclusion rate. This threshold was set on the basis that many national guidelines at the time recommended target haemoglobin levels of between 11-12g/dl for patients with kidney disease, recognising concern regarding the risk of thrombosis associated with higher levels. 15 potential study participants had haemoglobin levels ≥ 13g/dl at the time of surgery and thus were excluded from the study, significantly impacting on expected recruitment

completion date. In view of this, a local review was performed examining both pre- and post-operative haemoglobin levels in relation to the incidence of graft thrombosis in all adult kidney or kidney pancreas transplants performed the preceding year.

At the MRI between January 1st and December 31st 2007, a total of 147 kidney transplant operations were performed in adults. The mean haemoglobin pre-operatively was 12.2 ± 1.6g/dl, with a range of 7.7-17.7g/dl in these recipients (Figure 6.2). On post-operative day 1, the mean haemoglobin was 9.9 ± 1.4g/dl, representing a mean fall in haemoglobin in the first 24hr and 48hr post-operative period following engraftment of 2.4 ± 1.8g/dl ($p<0.0001$) and 3.4 ± 2.8g/dl ($p<0.0001$) respectively. In those patients with a haemoglobin ≥ 13g/dl (n=43), the mean decrease in haemoglobin was greater at 3.3 ± 1.7g/dl. Thus at 24hrs post engraftment, only 1/43 patients had a haemoglobin persisting ≥ 13g/dl and 146/147 now had a haemoglobin level below the exclusion threshold.

On the basis of 2007 data, 29% of potential transplant recipients would have been ineligible to enrol in the EPO Study due to a haemoglobin level ≥ 13g/dl. If the exclusion haemoglobin was increased to ≥ 14g/dl, only 10% would be ineligible based on the above data. Similarly, if the exclusion haemoglobin was increased to ≥ 15g/dl, only 5% would be considered ineligible.

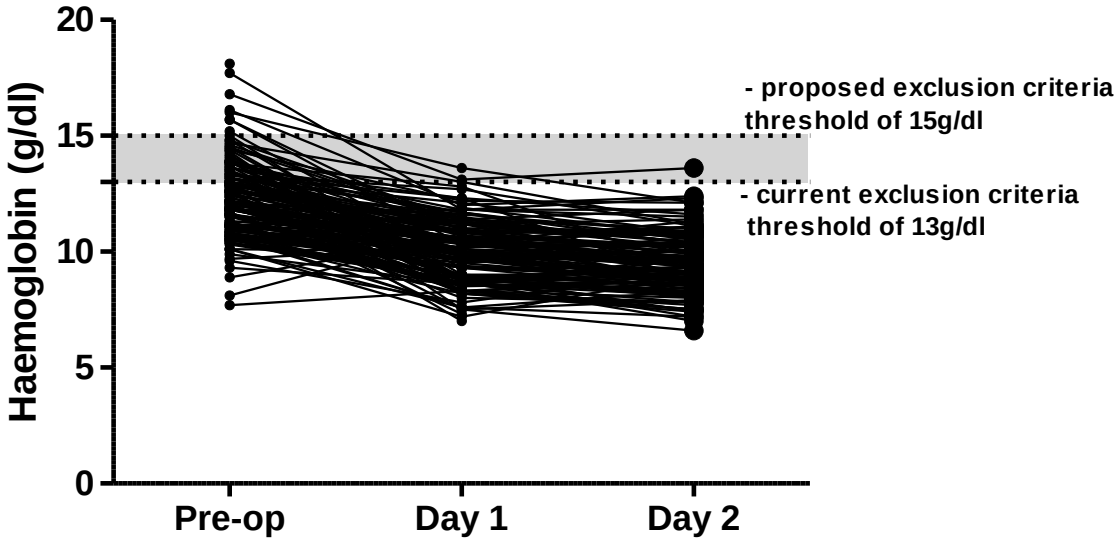


Figure 6-2: Peri-operative changes in haemoglobin levels at MRI in 2007.
Change in haemoglobin from prior to surgery to day 2 post-operatively in 147 adult kidney transplant recipients at the Manchester Royal Infirmary in 2007.

During this 2007 calendar year, there were no deaths in the first month post transplantation and three kidneys were lost due to thrombosis with haemoglobins of 11g/dl, 13g/dl and 15.7g/dl, which were felt to be unrelated to the haemoglobin level (technical failures or undiagnosed pro-thrombotic conditions).

From this evidence, it was concluded that standard medical practice at all three centres submitting patients for transplantation, allowed kidney engraftment to occur safely, in the absence of a haemoglobin level ceiling. At post-operative day 1, all but one patient had a haemoglobin level above the exclusion criteria threshold. By increasing the haemoglobin exclusion cut off to 15g/dl, only 5% of routinely transplanted patients would be ineligible for the study. Thus, a substantial amendment to the protocol was submitted to and subsequently granted by the local ethics committee, increasing the haemoglobin exclusion threshold to ≥ 15g/dl.

6.4 Study withdrawals

The first patient recruited to the study withdrew on post-operative day one, having had one infusion of 33,000iu of NeoRecormon®, but consented to allow continued collection of samples and follow-up data. This kidney of this patient had multiple vessels and the upper pole of the kidney did not perfuse following release of the vascular clamps. The kidney was removed and re-anastamosed. This brought about slow reperfusion of the upper pole. The surgical team attributed this event to an arterial intimal flap unrelated to the study drug. This patient is included in the final analyses on the basis of intention to treat.

6.5 Donor baseline characteristics

Table 6.1 lists the donor demographics. 34 donors contributed the 39 kidneys enrolled in the study with 5 NHBD's contributing a single kidney into both the EPO- and placebo- treated groups. No difference was seen in age, sex, ethnicity and BMI of the donor between the groups. Donor cause of death was predominantly an intra-cerebral event in >75% of cases.

The EPO group received more NHBD kidneys (63% vs 50%, p=0.41), particularly NHBD kidneys meeting extended criteria (33% vs 20%, p=0.65). While the incidence of diabetes was similar between groups, donors in the EPO group were more likely to be hypertensive (53% vs 25%, p=0.07).

Donors in the placebo group had a higher mean serum creatinine at the time of donation (68 vs 84µmol/L, p=0.11) with no difference in the acute kidney injury rate, defined as a change in serum creatinine of ≥ 25% from the time of admission to donation. Episodes and duration of hypotension was similar between groups, although donor kidneys from the placebo group were more likely to have been exposed to vasopressors (80% vs 63%, p=0.30). In the EPO group, donors were more likely to have received steroids preceding donation (21% vs 10%, p=0.41).

Table 6-1: Donor baseline characteristics.

Variable	Donor (n=34)		
	EPO Group	Placebo Group	*P* value
Age (yrs)*	49.3 ± 12.3	52.7 ± 14.8	Ns
Male	10 (53%)	13 (65%)	Ns
Ethnicity			
White	19 (100%)	20 (100%)	Ns
Asian	0	0	Ns
Afro-Caribbean	0	0	Ns
Body mass index (BMI)*	27.5 ± 4.3	27.6 ± 5.4	Ns
Donor cause of death			
intra-cranial haemorrhage	14 (74%)	14 (70%)	Ns
other intra-cranial event	1 (5%)	4 (20%)	Ns
extra-cranial event	4 (21%)	2 (10%)	Ns
ECD kidney	7 (37%)	10 (50%)	Ns
NHBD kidney	12 (63%)	10 (50%)	Ns
Donor hypertension	10 (53%)	5(25%)	Ns
Donor diabetes	1 (5%)	1 (5%)	Ns
Hypotensive episodes	8 (42%)	8 (40%)	Ns
duration of hypotension (mins)*	30 ± 19.8	32.75 ± 37.5	Ns
Donor treatment			
inotropic support	12 (63%)	16 (80%)	Ns
Steroids	4 (21%)	2 (10%)	Ns
ddAVP	9 (47%)	11 (55%)	Ns
Donor creatinine at retrieval*	67.5 ± 23.7	84.1 ± 37.5	Ns
Donor urine output (mls/hr)*	195 ± 175.3	180.7 ± 108.3	Ns
Donor acute kidney injury**	4 (21%)	5 (20%)	Ns

*values are means ± SD. NHBD, non-heart-beating donor; ECD, extended-criteria donor kidney; ddAVP, vasopressin, comparisons were made using a Student's t test or fishers exact test were appropriate, with the exception of non-parametric time data which used a Mann Whitney test.

**acute kidney injury was defined as a change in serum creatinine of ≥ 25% from the time of admission to donation

6.6 Recipient baseline characteristics

Recipient baseline characteristics are shown in Table 6.2. No difference was seen in age, sex or BMI of the recipients between the groups. More Asian and Afro-Caribbean recipients were

recruited into the EPO group, with significantly more white participants in the placebo group (95% vs 68%, p=0.04). Cause of end stage kidney disease, dialysis modality and duration of dialysis treatment were similar between groups. Both groups contained patients not yet requiring renal replacement therapy, with 2 in the EPO group and 1 in the placebo group.

Table 6-2: Recipient baseline characteristics.

Variable	Recipient		
	EPO Group	Placebo Group	*P* value
Age (yrs)*	51.5 ± 10.4	53.3 ± 13.6	ns
Male	10 (53%)	14 (70%)	ns
Ethnicity			
White	13 (68%)	19 (95%)	*0.04**
Asian	5 (27%)	1 (5%)	ns
Afro-Caribbean	1 (5%)	0	ns
BMI*	24.9 ± 3.5	25.8 ± 4.3	ns
Cause of ESRD			
Glomerular disease	8 (42%)	6 (30%)	ns
Hypertension	1 (5%)	3 (15%)	ns
Reflux nephropathy	4 (21%)	3 (15%)	ns
Others	6 (32%)	8 (40%)	ns
Mode of dialysis			
Haemodialysis	9 (47%)	12 (60%)	ns
Peritoneal dialysis	8 (42%)	7 (35%)	ns
Pre-dialysis	2 (11%)	1 (5%)	ns
Months on dialysis*	31.5 ± 23	45.4 ± 29.8	ns
Previous transplant	3 (16%)	5 (25%)	ns
EPO usage prior to study	17 (90%)	17 (85)	ns
time from last dose (days)*	7.1 ± 5.4	16.3 ± 32.1	ns
RAS blockade prior to transplant	9 (46%)	10 (50%)	ns
CCB usage prior to transplant	9 (46%)	7 (35%)	ns
Statin usage prior to transplant	7 (37%)	8 (40%)	ns
Steroids prior to transplant	0	6 (30%)	*0.02**
Pre-transplant EPO continued post transplant	9 (46%)	11 (55%)	ns
Anuric patients pre-transplant	6 (32%)	7 (35%)	ns
Urine rate/day >1000mls pre-transplant	6 (32%)	4 (20%)	ns
Recipient diabetes	1 (5%)	0	ns
Recipient hypertension	17 (90%)	17 (85%)	ns

*values are means ± SD. EPO, erythropoietin, RAS, renin-angiotensin system, CCB, calcium channel blocker, comparisons were made using a Student's t test or fishers exact test were appropriate, with the exception of non-parametric time data which used a Mann Whitney test.

No differences were found in diabetes or hypertension rates, with similar use of calcium channel blockers, renin-angiotensin system (RAS) blockade and statin therapy. In the placebo group, 6 patients were receiving steroid therapy prior to transplantation (p=0.02); three were on treatment for a glomerular disease; one for sarcoidosis; one was also a heart transplant

recipient; and one recipient was taking tacrolimus and steroids for a recently failed kidney transplant.

Erythropoietin usage was similar in both groups with no difference in the time from last dose to transplantation. Slightly more patients in the placebo group continued to receive maintenance EPO therapy following surgery (55% vs 46%, p=0.75), which was decided at the discretion of the attending physician.

6.7 Transplant specifics

Details specific to the organ procurement, preservation and implantation procedure are shown in Table 6.3. There were no differences between groups in terms of organ preservation techniques. No kidney underwent machine perfusion. Warm ischaemic time, defined as the time from asystole to in-situ cold perfusion in non-heart-beating donors, was similar at 17 minutes duration. In addition, the two groups were comparable with respect to the cold ischaemic time and the anastamotic times. Transplant kidney anatomy was similarly complex between groups in relation to multiple vessels. Atheromatous renal arteries were more common in the placebo group (40% vs 27%, p=0.50). The degree of HLA mismatch and prior sensitisation was not significantly different between groups.

All patients received induction immunosuppression with Basiliximab therapy given on day 0 and day 5, as well as a single dose of methylprednisolone given intra-operatively. In addition, one patient in the EPO group received ATG induction, due to a weakly positive flow crossmatch. Apart from this, the two groups were comparable with respect to the degree of HLA mismatch and the peak percent of panel reactive antibodies. Maintenance immunosuppression was recommended as triple immunosuppression in the protocol with similar numbers of protocol violations between groups during the early post-operative period. One patient in the EPO group was treated with Tacrolimus monotherapy on the basis of immediate graft function and previous unit practice.

Table 6-3: Transplant specifics.

Variable	Donor (n=34)		
	EPO Group	Placebo Group	*p* value
Preservation solution			
University of Wisconsin solution	15 (79%)	14 (70%)	ns
Marshalls solution	4 (21%)	6 (30%)	ns
Machine perfusion	0	0	ns
Warm ischaemic time (min)*	16.9 ± 3.3	16.6 ± 2.3	ns
Cold ischaemic time (min)*	1086 ± 343.5	1065 ± 310.1	ns
Anastamotic time (min)*	44.3 ± 12.1	41.6 ± 11.2	ns
Intra-operative mannitol	19 (100%)	18 (90%)	ns
Intra-operative inotropes	14 (74%)	16 (80%)	ns
Multiple arteries	8 (42%)	9 (45%)	ns
Multiple veins	3 (16%)	5 (25%)	ns
Atheromatous vessels	5 (27%)	8 (40%)	ns
CMV status			
Pos/Pos	2 (11%)	3 (15%)	ns
Pos/Neg	3 (16%)	3 (15%)	ns
Neg/Pos	9 (46%)	8 (40%)	ns
Neg/Neg	5 (27%)	6 (30%)	ns
MAP pre-EPO infusion			
Induction regimen			ns
Methylprednisolone	19 (100%)	20 (100%)	ns
Basilixmab	19 (100%)	20 (100%)	ns
Thymoglobulin	1 (5%)	0	ns
Maintenance regimen			
Tacrolimus/prednisolone/MMF	14 (74%)	17 (85%)	ns
Tacrolimus/MMF	2 (11%)	2 (10%)	ns
Tacrolimus/Prednisolone/Azathioprine	2 (11%)	0	ns
Tacrolimus/Prednisolone	0	1 (5%)	ns
Tacrolimus monotherapy	1 (5%)	0	ns
HLA mismatches			
0-1	5 (27%)	3 (15%)	ns
2-4	13 (65%)	13 (65%)	ns
>4	1 (5%)	0	ns
Any DR mismatch	9 (46%)	7 (35%)	ns
Peak panel-reactive antibody level			
0-5%	11 (57%)	13 (65%)	ns
6-84%	5 (27%)	5 (25%)	ns
≥85%	3 (16%)	2 (10%)	ns
Panel-reactive level at transplantation			
0-5%	14 (74%)	14 (70%)	ns
6-84%	4 (21%)	5 (25%)	ns
≥85%	1 (5%)	1 (5%)	ns

6.8 Incidence of delayed graft function and slow graft function

The incidence of delayed graft function, defined as the need for haemodialysis or peritoneal dialysis within the first seven days post transplantation, was not different between the two groups (Table 6.4). In the EPO group, 10 (53%) patients required dialysis in the post-operative period, including one patient who received a pre-emptive transplant. Similarly, 11 (55%) patients in the placebo group required dialysis in the first week, including one patient pre-emptively transplanted.

Slow graft function was defined as a creatinine reduction ratio at day 7 of <70%(416), with 6 patients in the EPO group and 5 in the placebo group having slow graft function.

Table 6-4: Early graft outcomes in EPO and placebo treated groups.

	EPO Group (n = 19)	Placebo group (n =20)	$p^{\#}$
Primary non-function	0	0	
Delayed graft function*	10 (53%)	11 (55%)	ns
Slow graft function**	6 (32%)	5 (25%)	ns

Values in parentheses are percentages. EPO, erythropoietin beta
Fishers exact test
*delayed graft function was defined as the need for dialysis within the first seven days post transplant.
**slow graft function was defined as a creatinine reduction ratio of <70% on day 7

The most common indication for first dialysis was hyperkalaemia, typically occurring on the first post-operative day. The median time to first dialysis was identical in both groups (post-operative day 1, range immediately post-operatively to day 3) (p=0.43). The median number of dialysis episodes was 2.5 (range 1-17) in the EPO group and 4 (range 1-19) in the placebo group (p=0.67). Finally the median time to last dialysis was 6.5 days (range 1-41) in the EPO group and 8 days (range 1-35) in the placebo group (p=0.86). Primary non-function did not occur in this study.

Five patients in the EPO group received 2 hours of haemodialysis and one patient received rapid cycling peritoneal dialysis with varying degrees of ultrafiltration immediately prior to surgery, of whom 4 subsequently developed delayed graft function (all had haemodialysis prior to surgery). 4 patients in the placebo group received 2 hours of haemodialysis prior to surgery and all developed DGF subsequently. Of interest, 17/21 (81%) patients on

maintenance haemodialysis prior to surgery developed DGF, as opposed to 3/15(20%) of patients on maintenance peritoneal dialysis (RR 4.05 (CI 1.44-11.38, p=0.0005). This is perhaps explained by patients with immediate graft function (IGF) reporting better residual renal function as measured by greater urine volume pre-surgery compared to patients with DGF (median 625 vs 50 mls/24hrs, p=0.014) (Figure 6.3). The commonest indication for dialysis was hyperkalaemia in both groups.

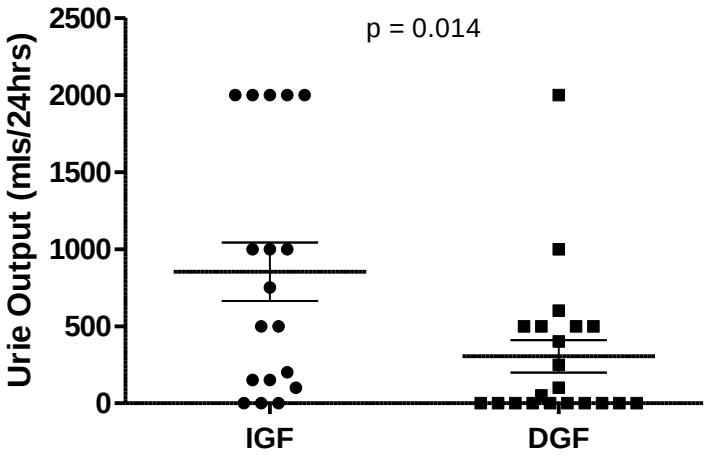


Figure 6-3: Pre-operative urine output in patients with DGF vs IGF.
Pre-operative urine output as reported by patients prior to surgery as a factor in determining the rate of delayed graft function (DGF) or immediate graft function (IGF).

Two patients in the EPO group and four in the placebo group, who received RAS blockade pre-transplant, developed DGF (p=ns). However, all patients with RAS blockade pre-transplant were less likely to develop DGF (RR 0.42 (CI 0.21-0.86), p=0.01). In contrast, calcium channel blocker and statin use had no impact on the rate of DGF (RR 0.84 (CI 0.43-1.66), p=0.75) and (RR 0.64 (CI 0.32-1.28), p=0.21) respectively. Steroid usage pre-transplant did not confer tissue protection from DGF with 4/6 placebo recipients developing DGF. EPO usage and time from last dose of EPO was similar between groups.

NHBD kidney recipients were more likely to develop DGF (15/22) than ECD kidney recipients (5/17) (RR 2.47 (CI 1.13-5.39), p=0.01). 7/12 NHBD recipients in the EPO group and 8/10 in the placebo group developed DGF (p = 0.38). Similarly 2/7 ECD recipients in the EPO group and 3/10 in the placebo group developed DGF (p = 1).

6.9 Paired kidney analysis

There were 5 pairs of non-heart-beating donor kidneys recruited to the study (Table 6.5). Both kidneys donated by 3 donors developed DGF. In one donor pair, the EPO recipient did not develop DGF, despite a longer cold ischaemic time (1117 vs 697mins). However, this may be explained by better residual renal function with the patient undergoing maintenance PD prior to surgery. In the other donor pair, again the EPO recipient did not require dialysis. However, this is likely to be unrelated to EPO usage, as it was a pre-emptive transplant and thus did not meet the criteria for delayed graft function but had slow graft function. Function was calculated by Cockcroft-Gault formula in an attempt to compensate for different recipient mass affecting creatinine clearance estimations. EPO recipients either had excellent creatinine clearance (defined as >50mls/min) or better creatinine clearance than their placebo counterparts although numbers were too small to do any meaningful statistical tests.

Table 6-5: Transplant and functional specifics amongst pairs in the EPO Study.

	Pair 1		Pair 2		Pair 3		Pair 4		Pair 5	
Trial drug	Placebo	EPO	EPO	Placebo	Placebo	EPO	Placebo	EPO	EPO	Placebo
CIT	951	1166	739	1047	697	1117	759	1291	797	1078
DGF	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No	Yes
No. of dialysis episodes	2	1	2	1	19	0	3	2	0	2
sCr at D7	796	569	407	541	378	507	645	994	205	859
sCr at D30	147	129	133	119	323	154	100	113	188	316
CC at D7	10	12	25	19	23	14	10	7	26	8
CC at D30	55	53	75	88	27	46	66	60	28	22

CIT: cold ischaemic time; DGF: delayed graft function; sCr: serum creatinine (µmol/L); CC: Cockcroft-Gault estimated creatinine clearance (mls/min) used to compensate for different body masses between recipients receiving the same donor kidney;

6.10 Renal function

Sequential serum creatinine levels (µmol/L) and 4 variable MDRD eGFR values for the first three months are shown in Figure 6.4. Overall, ANOVA demonstrated that serum creatinine

was not different between groups (p=0.54). Analysis of individual time points demonstrated that serum creatinine was not significantly higher in the placebo group at day 7 (p=0.72), day 14 (p=0.86), day 30 (p=0.21), day 60 (p=0.39) and day 90 (p=0.33). Baseline serum creatinine was not different between groups (764 ± 306 µmol/L for EPO group *vs* 729 ± 322 µmol/L, p=0.75). Kidney function was not analysed prior to day 7 due to the rate of DGF and the impact of dialysis on measured kidney function preventing meaningful analysis. Sub-group analysis by donor type did not reveal any differences with respect to serum creatinine or eGFR post-transplantation (Figure 6.5).

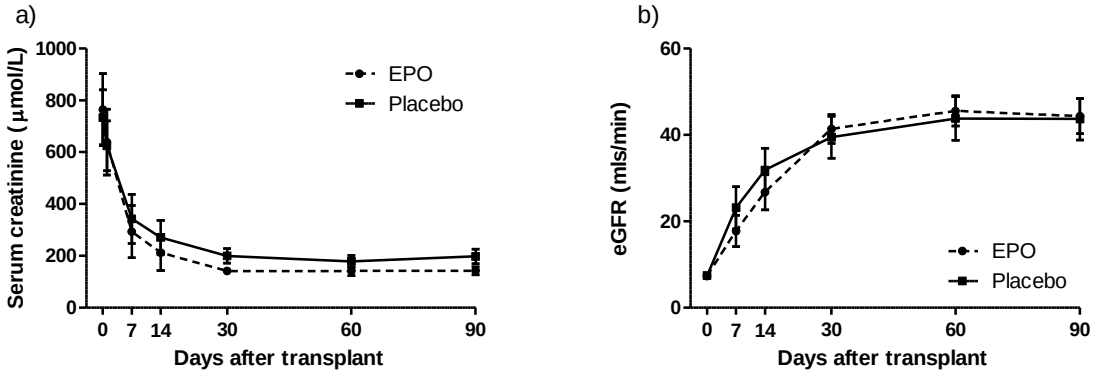


Figure 6-4: Comparative renal function measured by (a) serum creatinine and (b) eGFR (4v MDRD) between EPO and placebo treated groups.
Serum creatinine was measured pre-operatively, day 7, day 14, day 30, day 60 and day 90 post-transplantation. There was no difference at all time points. Data are expressed as mean ± SEM.

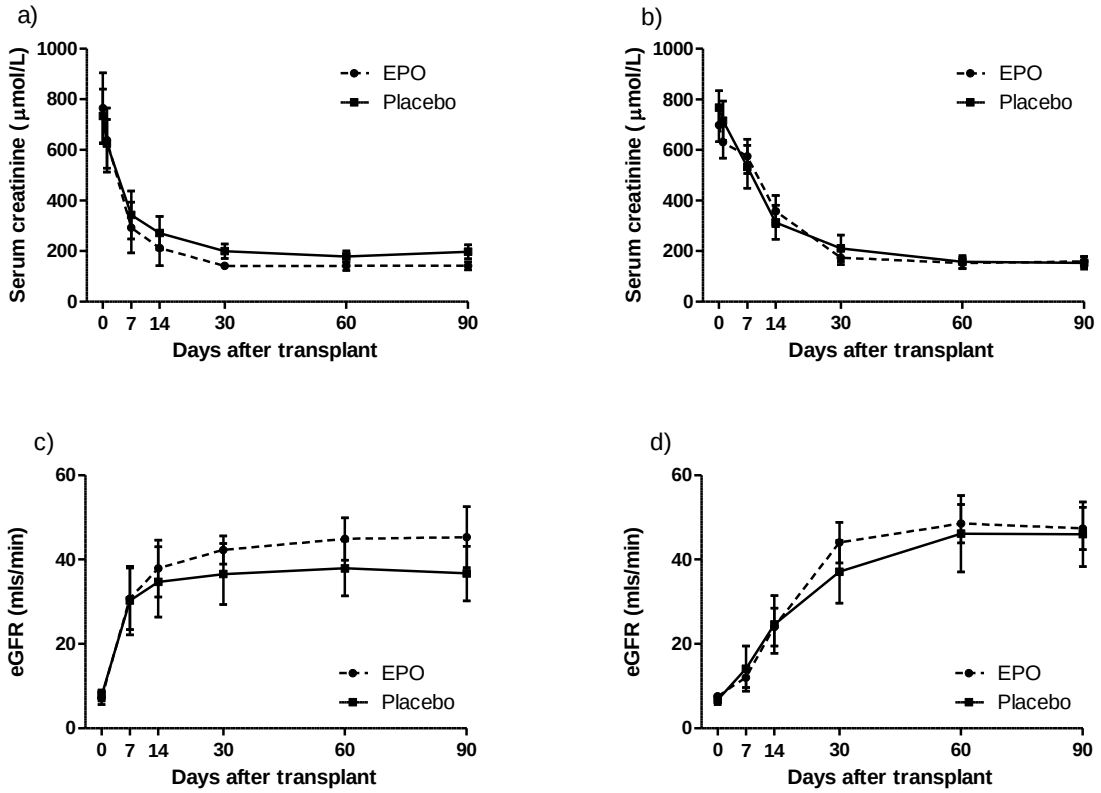


Figure 6-5: Subgroup analysis of EPO and Placebo patients examining function.
Comparative renal function measured by serum creatinine and eGFR (4 variable MDRD) in a) and c) ECD kidney recipients; b) and d) NHBD kidney recipients respectively, who received either EPO or placebo. Serum creatinine was measured pre-operatively, day 7, day 14, day 30, day 60 and day 90 post-transplantation. There was no difference at all time points. Data are expressed as mean ± SEM.

6.11 Acute rejection

Biopsy-proven acute rejection occurred within 3 months of transplantation in 5 patients in the EPO group and 3 patients in the placebo group (p=0.45). In the first two weeks post-transplantation, 4 episodes of vascular rejection occurred of which 3 were in high dose EPO recipients. Only one of these episodes was treated with anti-thymocyte globulin (Genzyme, UK) and all recovered function.

6.12 Graft and patient survival

Log rank analysis of Kaplan-Meier curves of crude survival, with graft failure and patient death as events, did not show any difference between the two groups (p=0.58) (Figure 6.6). Crude survival rates at 1, 3, 6 and 9 months were 100% at each time point for the EPO group and 100%, 100%, 95% and 95% respectively for the placebo group. At 12 months, crude survival was 95% in the EPO group and 90% in the placebo group. The findings were similar when the event of death with a functioning graft was censored to produce a death censored graft survival curve, accounting for recipient mortality (Figure 6.6). Survival rates at 1, 3, 6, 9 and 12 months were 100% until 9 months and 95% at 12 months for the EPO group and 100% at all time points for the placebo group.

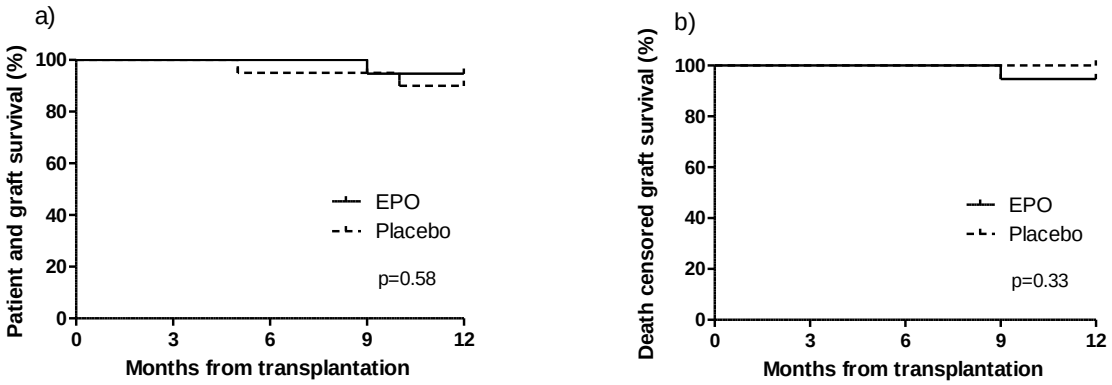


Figure 6-6: Kaplan-Meier curves for graft and patient survival in the study.
a) Overall Kaplan-Meier curves for graft and patient survival of EPO and placebo treated groups. Graft failures and patient deaths are events; b) Kaplan-Meier curves for death censored graft survival of EPO and placebo treated groups. Graft failures are events.

There were no deaths in the EPO group and two deaths in the placebo group. The first death occurred in a heart transplant recipient who subsequently received an ECD kidney which had slow graft function. Best graft function was 31mls/min on day 60 (eGFR 4v MDRD) and the patient died following an episode of sepsis, with a functioning graft. The second patient received a NHBD kidney which had a protracted period of DGF (19 haemodialysis sessions in total). The kidney never achieved good function with an eGFR of 27mls/min at 9 months. The patient subsequently died due to an episode of sepsis, with a functioning graft.

There was one failure in the EPO group, which occurred 9 months post-transplantation. This followed an attempted angioplasty for transplant renal artery stenosis, which was complicated by formation of an intimal flap and subsequent renal artery thrombosis requiring a transplant nephrectomy. Of note, transplant renal artery stenosis was reported in one other patient during the follow-up period.

6.13 Safety of high dose NeoRecormon®

High dose NeoRecormon® was not associated with an increased incidence of adverse events and serious adverse events compared to placebo (Table 6.6). No deaths occurred in either group in the first three months. The single graft lost at 10 months in the EPO group was unrelated to EPO treatment and due to a technical complication following a transplant renal artery angioplasty. No thrombotic complications occurred within the first three months in either group. Hypertension occurred in 6 high dose EPO recipients during their in-patient stay period, in comparison to 5 patients in the placebo group (p=ns). One patient in the EPO group developed generalised tonic-clonic seizures due to severe hypertension. This, however, was attributed by the clinical team and safety adviser to stoppage of anti-hypertensives peri-operatively and hypervolaemia. Interestingly, two patients in the EPO group developed transplant renal artery stenosis at 3 months and 9 months with one graft failing following a technical complication during angioplasty. No transplant renal artery stenosis occurred in the placebo group.

Table 6-6: Adverse events.

	EPO	Placebo	p value
N=	19	20	
Death	0	2	ns
Graft failure	1	0	ns
Arterial or Venous Thrombosis	0	0	ns
Hypertension	6	5	ns
MAP on day 3 Pre-infusion Post-infusion	 99.6 ± 11.5 99.6 ± 11.5	 99.6 ± 11.7 99.6 ± 11.6	 ns ns
Renal Artery Stenosis	2	0	ns
Time to discharge with a functioning graft	15.4 ± 11.5	13.8 ± 10.8	ns

6.14 Haematopoietic effects of high dose erythropoietin

There was no difference in haemoglobin levels between the EPO and placebo group on entry into the study (11.5 ± 0.3g/dl *vs* 11.3 ± 0.4 g/dl, respectively; p=0.78). Similarly, there was no difference at any time point between either group from surgery out to 90 days post transplantation (90 days (EPO group; 11.8 ± 0.3g/dl *vs* 11.2 ± 0.3 g/dl, respectively; p=0.21) (Figure 6.7a). Haematocrit levels behaved similarly to haemoglobin levels between groups at all time points, again showing a trend towards higher haematocrits in the EPO group (figure 6.7b).

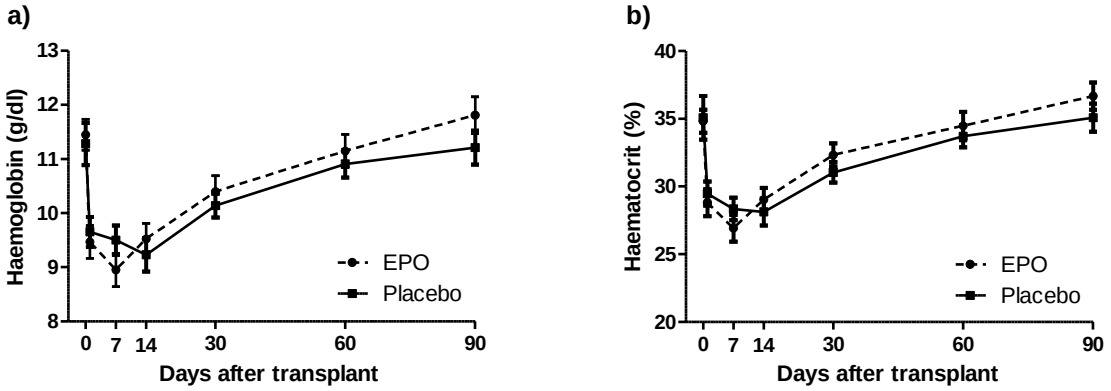


Figure 6-7: Mean serum haemoglobin and haematocrit levels over time.
Levels were measured on day 0, day 1, day 7, day 14, day 30, day 60 and day 90. High dose EPO was given in the peri-operative period to the EPO group which did not influence haemoglobin or haematocrit levels at any time point. Data shown are mean ± SEM.

The number of blood transfusion required and the number of packed red cell units used during the in-patients stay did not differ between groups. 32% of the EPO group and 55% of the placebo group required a blood transfusion (p=0.20). The median number of packed red cell units used was 1.2 ± 0.5 units in the EPO group versus 1.9 ± 0.6 units (p=0.19). As shown in table 1, both groups has similar levels of maintenance EPO usage post transplant (46% in the EPO group vs 55% of the placebo group) (p = 0.55).

Administration of high dose EPO had no effect of platelet levels at all time points (Figure 6.8)

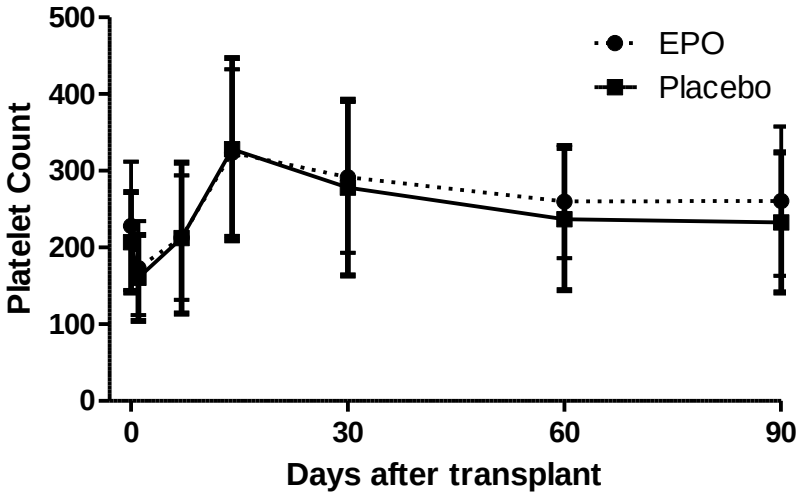


Figure 6-8: Mean platelet levels over time.
Platelet levels measured on day 0, day 1, day 7, day 14, day 30, day 60 and day 90. High dose EPO was given in the peri-operative period to the EPO group which did not influence platelet levels at any time point. Data shown are mean ± SEM.

6.15 Safety of post-reperfusion biopsies

Post-reperfusion biopsies were performed between 20 and 45 minutes post-reperfusion of the transplanted kidney, depending on when the surgeon was happy that the vascular anastamosis was intact and the ureter had been anastamosed. The decision to take a wedge or an 18G needle core biopsy was left to the attending surgeon. Wedge biopsies were performed in all but two cases. All biopsies yielded tissue sufficient to undergo microarray analysis. Only one patient returned to theatre in the early post-operative period for active bleeding around the graft. A haematoma was evacuated from the upper pole of the kidney, which is where both the pre- and post-perfusion biopsies were taken. However, there was no evidence of active bleeding from the biopsy site.

6.16 Feasibility of the study protocol

This protocol was very staff intensive, much of it occurring out of normal working hours. As a result, a number of potentially suitable patients were not recruited due to staff unavailability. All biopsy and blood samples were acquired in theatre.

6.17 Power calculation

Based on the standard deviations for estimated glomerular filtration rate (eGFR) at day 7 of the placebo population, assuming a power of 80% is required, at an alpha of 0.05, the sample sizes needed for each group were calculated. In most clinical studies, a difference of 25% between groups would be deemed a biologically significant result. However, this would translate into less than a 5ml/min difference in eGFR, which is unlikely to have any significant impact on the incidence or outcome of delayed graft function. In addition, as shown in Table 6.7, the detection of a small effect would require 300 patients in each group, changes of 15 or 20 mls/min require correspondingly smaller sample numbers of only 34 and 19, respectively in each arm. Alternatively, given the sample size in this study, only a delta eGFR of 20mls/min would have adequately powered this study (83%) (Table 6.8). Thus, I submit that even the small number of patients in this study should have been sufficient to demonstrate a meaningful biological effect of EPO in this study.

Table 6-7: Power analysis based on the EPO study control group.
The necessary sample sizes for increments of the estimated glomerular filtration rate (effect size), measured by a 4v MDRD equation calculated for a two-sample t-test with 80% power and an alpha of 0.05, assuming the standard deviation for the eGFR of the placebo group.

Effect size (ΔeGFR at day 7)	Placebo	EPO
5mls/min	300	300
10mls/min	75	75
15mls/min	34	34
20mls/min	19	19

Table 6-8: Power analysis of the EPO Study.
The power of the EPO study to detect incremental eGFR differences was calculated for the 19 EPO recipients and 20 placebo recipients.

Effect size (ΔeGFR at day 7)	Power
5mls/min	11%
10mls/min	30%
15mls/min	58%
20mls/min	83%

6.18 Discussion

The EPO study examined the benefit of early intervention with high dose EPO prior to unclamping of the transplant kidney artery, on ischaemia-reperfusion injury and its clinical sequelae. In keeping with a recently published study by Martinez et al (335) examining the impact of EPO in extended criteria donors, EPO failed to reduce the incidence and the severity of delayed graft function. Following recovery from delayed graft function, EPO did not result in better graft function out to 3 months. The number of acute rejection episodes, graft and patient survival was not different between the two groups. The study was adequately powered to detect a difference of eGFR of 20mls/min at day 7. Finally, no serious adverse events related to EPO treatment were observed, with no impact on haemoglobin levels or the number of thrombotic events.

The objective of this study was to discover whether the experimental findings of EPO-induced tissue protection in acute kidney injury (reviewed in Chapter 1) would be translated into the clinical setting of acute kidney injury and kidney transplantation in humans. At the time of this study's design, only one clinical study had been performed in humans, in the setting of cerebral ischaemia which encouragingly showed marked benefits with a reduction in infarct size and functional limitations (309). In the early post-transplant period, these protective effects were expected to translate into less delayed graft function and better functional outcomes. However, delayed graft function has many different definitions (reviewed in Chapter 1) and the definition used in this study, although in widespread clinical use, is to a certain extent subjective, based on a recipient's perceived need for dialysis in the first week post-transplantation. This clinical judgement has marked inter-individual and inter-centre variability and can also be influenced by iatrogenic events such as inattention to fluid prescriptions and the effects of anaesthesia on potassium levels. Function at day 7, as

measured by eGFR, can also be influenced by the timing of a dialysis session or the occurrence of acute rejection. Thus, rather than making these functional outcomes with their limitations the primary endpoints, we chose a biomarker approach as the primary endpoint (discussed in next chapters), with these clinical indices as secondary endpoints.

The failure of EPO to confer nephroprotection is unlikely to be due to inadequate dosing. The dose was chosen directly from the clinically efficacious stoke study, where high levels of EPO were required to cross the blood-brain barrier and provide a high concentration in the cerebrospinal fluid (309). While EPO is routinely given to renal patients, the maximum recommended dose in renal failure is 720iu/kg/week (417), which in the average 70kg patient, is half the administered dose in this study. Furthermore, mathematical modelling in healthy volunteers has also shown that a similar dose (1000U/kg) results in >98% occupation of EPO receptors, which persists for over 2 days after that dose. It has also been shown that low dose EPO (300iu/kg) is efficacious for tissue protection in the setting of non-transplantation related acute kidney injury (333). This latter finding raises the possibility that with over 85% of recipients in this study receiving maintenance EPO for anaemia associated with end-stage renal failure, tissue protection may have been afforded by low levels to both groups and thus confounded the outcome, with a dose ceiling effect for EPO. This dose ceiling effect has previously been shown in EPO-induced neuro- and cardio-protection and has been postulated to result from down-regulation or internalisation of the EPO receptor in the presence of high concentrations of EPO, preventing further tissue-protective signalling (185;300). It has also been postulated that with injury-induced dedifferentiation and cell death, EPO receptor expression and subsequent clinical effect is reduced (the group are unclear whether this is gene expression or cell surface expression and do not provide evidence for the postulate) (335). In keeping with this, interferon-gamma has been shown to be a negative regulator of EPO receptor gene expression (231). However, experimental evidence suggests that EPO receptor gene expression is increased in both haematopoietic and brain tissue in response to hypoxia (226;227). Furthermore, in breast cancer cell lines exposed to hypoxia, limited increases in EPO receptor gene expression occurred, but marked up-regulation of cell surface EPO receptors occurred, suggesting that a reservoir of EPO receptors exists within a cell (221). Thus, it is unlikely that the lack of clinical effect of EPO is due to an ischaemia-mediated decrease in gene expression or cell surface protein expression, particularly with the role of hypoxia-inducible factor 1 in ischaemia and in

regulating the EPO receptor gene and protein (228). Finally, there is no published evidence that high dose EPO is directly cytotoxic to tubular cells.

It is conceivable that a lack of efficacy is primarily a consequence of the timing of EPO administration. Injury prior to reperfusion is multi-factorial and due to recurrent insults to the kidney rather than a single ischaemic event (reviewed in Chapter 1). Typically it begins in the donor from peri-mortem events including sepsis, severe hypertension or hypotension and nephrotoxic agents. Retrieval of the kidneys is associated with the ensuing warm ischaemia phase, cold ischaemia phase and the anastamotic ischaemic phase, which all lead to further injury. Thus the lack of efficacy in this study may be explained by therapy in the experimental setting being initiated either before injury or earlier in the course of injury, whereas the intervention in this study was delayed until the final insult of reperfusion. At the time of designing the study, it was felt that the earliest time for therapeutic intervention was immediately prior to reperfusion. The ideal scenario for tissue protection would be a therapeutic intervention in the donor, prior to organ retrieval and storage. However, this would impact on all organs donated, not just the kidneys, and prevent any potential recipients from declining to partake in the study as they would potentially be declining life-saving treatment in the form of organ transplantation. Furthermore, this was a pilot study which was specifically examining the safety of high dose EPO therapy. As a result, it was not felt to be ethically appropriate to intervene at this stage. It was also not possible to intervene during cold storage as at the time, we did not have access to machine perfusion technology and again the potential recipient would not be able to decline partaking in the study. Thus, it was decided to intervene immediately prior to reperfusion, with the knowledge that at least some injury had already occurred.

The lack of a clinical effect of EPO is further evidence of the discrepancy between experimental models and human clinical trials with regard to EPO and tissue protection. No clinical benefit has been shown in three studies in kidney transplant recipients (335;337;338). The only prospective study which examined the effect of high dose EPO (30,000iu), given on 4 occasions over two weeks, on DGF and function at 1 month, failed to demonstrate any benefit over placebo (335). Two prospective studies in patients undergoing coronary artery bypass grafting have demonstrated conflicting results. Poulson et al. administered EPO at two time points prior to the onset of ischaemia with no effect on the incidence of acute kidney injury, although it must be admitted that this was a secondary endpoint (334). Song et al.

administered a single dose of EPO prior to ischaemia and demonstrated less acute kidney injury and better function in the EPO group. In another organ system, the follow-up stroke study also failed to show a benefit of high dose EPO (310). Current evidence supports the clinical outcome of this study, that experimental EPO tissue protection is not readily translated into the clinical setting. The lack of efficacy of EPO therapy that we and others have observed mirrors earlier experience with IGF-1 in the transplant setting. IGF-1 was shown in experimental models to accelerate recovery of acute renal failure (418). However, in the clinical transplant setting this was not confirmed (419).

This trial is the first to test the safety of 100,000iu of EPO, administered over 3 days, in the early transplant period. The only other study used 4 doses of 30,000iu of EPO, given pre-operatively, a 12 hours, on day 7 and day 14 (335). Neither study has demonstrated an increased risk of adverse events. Two studies examining EPO in the setting of cardiac bypass surgery, also did not demonstrate any adverse events (333;334).An increased incidence of vascular thrombosis is a recognised side effect of EPO therapy, particularly in the presence of a raised haematocrit (341). Neither vascular thrombosis nor polycythaemia occurred in this study with a mean decrease in haematocrit in the EPO group of over 5% on the first post-operative day. Furthermore, the impact of surgery-induced systemic inflammation on haematopoiesis and EPO resistance is likely to have been responsible for the lack of effect of this EPO regimen on haemoglobin concentrations at all time points. Martinez et al did demonstrate an erythropoietic effect in their study, but the prolonged administration until day 14 is the likely explanation for this. Overall, there was no increase in hypertension in the EPO group.

In conclusion, the administration of 100,000iu of erythropoietin beta, pre-reperfusion, at 24 hours and at 48 hours, does not reduce the incidence and severity of delayed graft function in high risk marginal donor kidney recipients, or improve function at any time point up to 3 months. Furthermore, it was safe and did not increase the incidence of acute rejection or vascular events. Thus, there is no evidence at this time for a role of EPO to confer tissue protection in the early post-transplant period.

CHAPTER 7 BIOMARKERS IN THE EPO STUDY

7.1 Introduction

There is much interest in the ability to predict or detect delayed graft function in the very early post-operative period, with a view to minimising further renal injury and predicting recovery of function. Typically, it is defined as the need for dialysis within the first post-operative week and can last from several days to weeks or even months. Traditionally, serum creatinine, serum urea, serum potassium, volume status and urine output are all used within the first 24 hours to indicate the need for dialysis. The importance of each of these factors is to a certain extent subjective and this influences the proportion of patients receiving dialysis and consequently the proportion designated as having delayed graft function. These are functional markers of injury that are neither sensitive nor specific for kidney injury. Serum creatinine is a poor index of renal function at this early renal injury, with a non-linear relationship between serum creatinine and GFR (420). Serum urea is raised in hypercatabolic states (e.g. following any surgery) and also by steroid therapy, the mainstay of immunosuppression. Anaesthesia and muscle trauma intra-operatively both result in the release of intra-cellular potassium which increases serum levels. Hypervolaemia is usually iatrogenic in origin with blood pressure control and blood loss in theatre frequently managed by large volume fluid replacement, resulting in positive fluid balances frequently in the order of 3-4 litres. Urine output itself is unreliable as it is affected by bladder irrigation, catheter obstruction due to blood clots and inaccurate monitoring. Furthermore, while they predict DGF, they do not predict recovery. Thus, there is a need for sensitive early markers of transplant injury which are easily quantifiable in a clinical setting offering diagnostic, prognostic and theranostic potential. At least 20 biomarkers of acute kidney injury have been identified in both plasma and urine (421). Three of the most intensively studied and potentially clinically useful biomarkers are neutrophil gelatinase-associated lipocalin (NGAL), interleukin-18 (IL-18) and kidney injury molecule-1 (KIM-1) (reviewed in Chapter 1).

In addition to plasma and urine sampling, recent evidence suggests that microarray analysis of post-implantation kidney biopsies may also be useful in predicting delayed graft function (422). Molecular changes in implant biopsies and biopsies for cause are highly reproducible between biopsy cores and reflect large scale biologic processes, including entry of T cells and macrophages into the tissue, interferon-gamma effects, and changes in the epithelium and endothelium (423). Using experimental models, cell lines and the published literature, transcripts corresponding with these events were labelled as pathogenesis based transcript sets (PBTs) (http://translant.med.ualberta.ca/), which could be used to interrogate a tissue to identify key biological processes taking place. For the purpose of this study, I focused on two specific injury PBTs: Kidney transcript set 2 (KT2); injury- and repair-associated transcripts day 3 (IRITD3). KT2s are a collection of solute carriers, specifically found in epithelial cells, whose expression decreases in response to acute kidney injury(423). Loss of KT2s may be the molecular equivalent of loss of function. IRITD3s were defined to reflect the injury-repair response in parenchymal and stromal cells in mouse isografts and ischaemic native acute kidney injury (424). In addition, a class comparison approach is useful across all 54,000 transcripts to identify novel differences between groups, not previously annotated as PBTs.

Transplantation is an excellent model to assess potential treatments aimed at ameliorating acute kidney injury using potential biomarkers and microarrays on biopsies as a sensitive means of identifying therapeutic effects. We therefore performed a single centre, randomised placebo controlled trial examining the impact of erythropoietin treatment pre-reperfusion on biomarker levels and gene expression in the first week post-transplantation.

7.2 Methods

7.2.1 Collection of samples

Ethical approval for collection of biopsy, serum and urine samples was obtained as part of the EPO Study (LREC 07/Q1407/94; EudraCT No. 2006-005373-22).

10mls of whole blood was collected in EDTA-containing plasma collection tubes (BD Vacutainers®, Oxford, UK) prior to infusion of the trial drug (~20 minutes before reperfusion), 24 hours post-reperfusion and daily for 6 days. This was stored for a maximum period of 24 hours at 4°C prior to centrifugation and plasma collection. Aliquots were then transferred into 1ml centrifuge tubes, frozen and stored at -80°C.

Urine collection was performed prior to reperfusion (if the patient was not anuric), at 2 hours, 8 hours, 24 hours and then daily thereafter until day 6. This was stored for a maximum period of 24 hours at 4°C prior to centrifugation for the removal of cells and aliquoting. Sample aliquots were then transferred into 1ml centrifuge tubes, frozen and stored at -80°C.

Implant biopsies were collected within 30 minutes of reperfusion of the transplanted kidney. Wedge or needle cortex cores were taken at the discretion of the consultant surgeon and were immediately stabilised in RNAlater at -20°C for later processing. All 39 patients had sufficient RNA material extracted to undergo microarray analysis. Sample signal was normalised to nephrectomy controls to generate a fold change difference.

7.2.2 Biomarker assay methodology

This is detailed in appendix 1.12.1.

7.3 Results

7.3.1 Quantification of the biomarkers

The assay for IL-18 was developed and evaluated in this study whereas all other biomarker assays were commercially available. The assay ranges were extensively evaluated and within acceptable ranges (Table 7.1), (Figure 7.1).

Table 7-1: Evaluation of biomarker assays for kidney injury used in the EPO Study.
***R&D Systems Europe Ltd, Abingdon, OX143NB, UK, **MBL, Naka-ku, Nagoya, Japan**

Parameters	NGAL	IL-18	KIM-1
Capture antibody	Duoset R&D Systems* DY1757	Clone 125-2H, MBL**	Duoset R&D Systems* DY1750
Detection antibody		Clone 159-12B, MBL	
Standard protein		rHuIL-18, MBL	
Assay range	78-5000 pg/ml	78-5000 pg/ml	62-4000 pg/ml

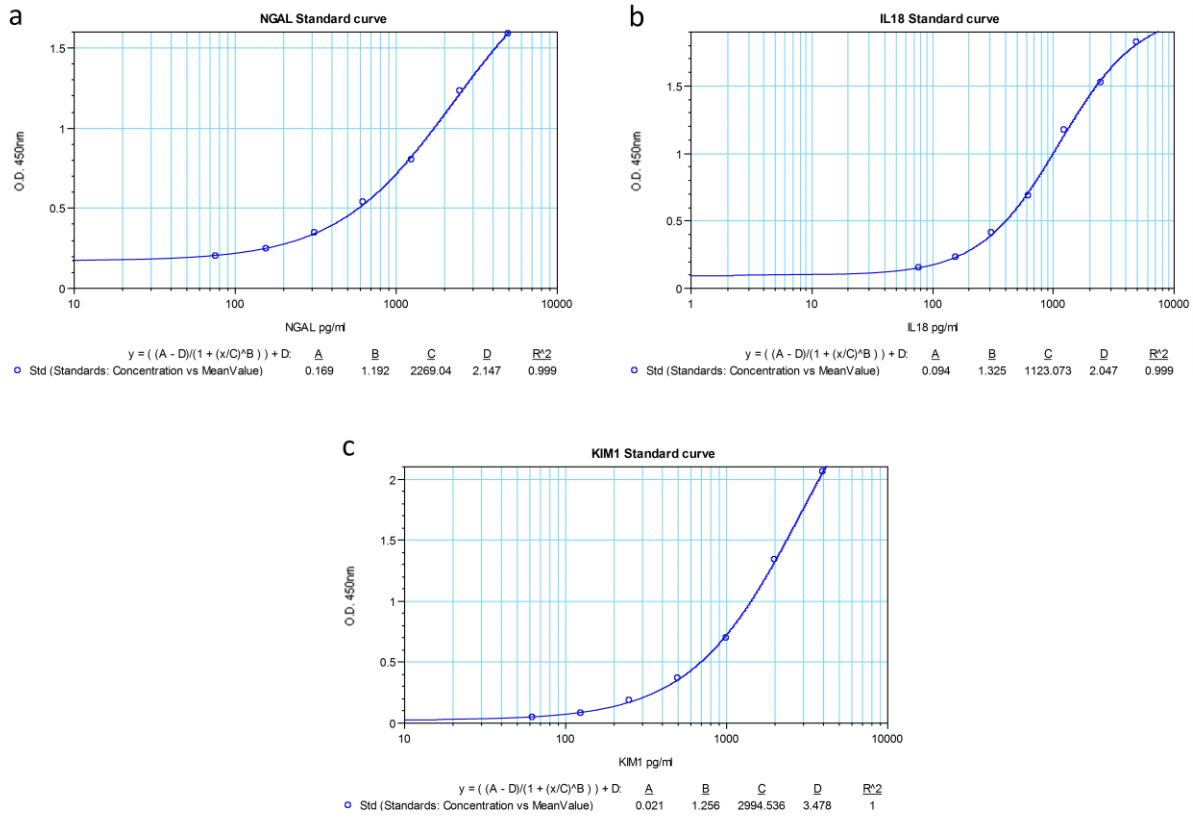


Figure 7-1: Evaluation of ELISA assays for quantification of plasma and urinary NGAL, IL-18 and KIM-1.
The standard curves were dilutions of purified recombinant proteins plotted as four parameter logarithmic curves, using SOFTmaxPROv4 software (Molecular Devices Inc.). The working ranges are shown in Table 7.1.

7.3.2 Study population

The full demographics of the study population are shown in Chapter 6, Tables 6.1-3. In brief, 39 patients were recruited to the study with 19 in the EPO arm and 20 in the placebo arm. Delayed graft function occurred in 21 patients in total with 10 patients in the EPO group and 11 patients in the placebo group. Immediate graft function occurred in 18 patients. All patients had baseline blood samples taken 15 minutes prior to reperfusion (and infusion of the study drug). Urine was collected pre-reperfusion in those with native urine output (n=21). Completeness of sample collection is shown in Table 7.2. Urine sample collection was incomplete due to delayed graft function with anuria post-operatively.

Table 7-2: Completeness of sample collection in the EPO Study.
Number of samples (blood and urine) collected at each time point for biomarker measurement. # baseline urine samples prior to reperfusion could only be collected in patients with native urine output (n=21). *Incomplete sample collection due to a patient who was discharged from hospital with a functioning graft on day 5.

Time point	Serum			Urine		
	Total samples collected	EPO (n=19)	Placebo (N=20)	Total samples collected	EPO (n=19)	Placebo (n-20)
Baseline#	39	19	20	21	10	11
15 mins	39	19	20	-	-	-
2 hrs	37	17	20	18	8	10
8 hrs	37	18	19	26	12	14
D1	39	19	20	36	17	19
D2	36	17	19	38	18	20
D3	39	19	20	38	18	20
D4	38	19	19	34	17	17
D5	39	19	20	32	14	18
D6*	38	19	19	35	16	19

7.3.3 Correlations between baseline plasma and urine levels of the three biomarkers of interest – NGAL, IL-18 and KIM-1

The relationships of plasma biomarkers to each other and their urinary counterparts were examined in pre-reperfusion (pre-trial drug) samples using Spearman correlations. With

regard to plasma and urine comparisons, these were performed on 21 pairs, with numbers limited by the number of pre-reperfusion urine samples collected. Plasma and urine NGAL baseline levels were highly correlated ($r = 0.57$, $p=0.007$), as were IL-18 baseline levels ($r = 0.82$, $p<0.0001$). In contrast, plasma KIM-1 did not correlate with urine KIM-1 ($r = -0.05$, $p=0.85$).

Plasma biomarkers, NGAL, IL-18 and KIM-1, at baseline did not correlate with other plasma biomarkers at baseline, and likewise for baseline urine biomarkers, NGAL, IL-18 and KIM-1.

7.3.4 Effect of native urine output on baseline biomarker levels

Baseline urine NGAL was the only urine biomarker that correlated with native urine output ($r = -0.66$, $p=0.001$). Baseline urine IL-18 and KIM-1, as well as all 3 plasma biomarkers did not correlate with native urine output. To study this relationship further, I examined the effects of dialysis modality and urinary volume on urine NGAL levels (Figure 7.2). Baseline urine NGAL levels were compared in haemodialysis (n=8), peritoneal dialysis (n=10) and patients not yet requiring dialysis (n=3). Of the three categories, pre-dialysis patients had the lowest urine NGAL levels (median, 102 ng/mgCr, range 0 – 560ng/mgCr). In contrast, haemodialysis patients had significantly higher urine NGAL levels (median 5316ng/mgCr, range 1818 -10367ng/mgCr) (Dunns multiple comparison testing, $p<0.01$). Peritoneal dialysis patient urine NGAL levels (median 2230ng/mgCr, range 246 – 6574ng/mgCr) did not differ significantly from pre-dialysis levels, although the range of values was much higher. Finally, native urine output prior to surgery was higher in the group who subsequently had IGF (median 625mls/24hrs) compared to those with DGF (50mls/24hrs) ($p=0.015$). In fact 18/21 DGF patients had self-reported 24 hour urine volumes of ≤500mls, compared to 9/18 in the IGF group ($p=0.035$). Thus, patients with good residual renal function and native urine output are less likely to be diagnosed with DGF, due to their ability to better control their volume status and electrolyte balance. This is also reflected in their urine NGAL levels.

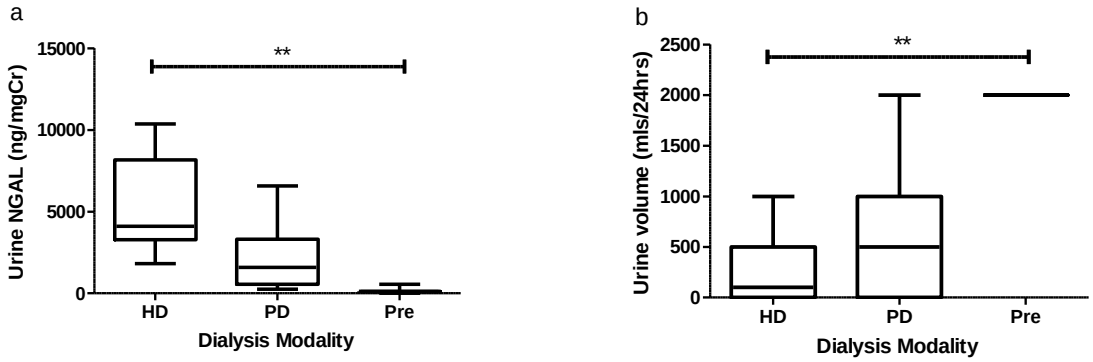


Figure 7-2: Effect of dialysis modality on urine NGAL levels and urine output.
(a) urine NGAL levels differences by dialysis modality (p=0.004, ANOVA). Levels are higher in recipients on haemodialysis (HD) compared to pre-dialysis (pre) recipients (Dunn's Multiple Comparison test p<0.01). (b) self-reported 24hr urine volume in different dialysis modalities (p=0.003, ANOVA). Pre-dialysis patients had the highest urine volumes compared to recipients on haemodialysis (p<0.01, Dunn's Multiple Comparison testing). PD: peritoneal dialysis.

7.3.5 Plasma neutrophil gelatinase-associated lipocalin

7.3.5.1 Neutrophil gelatinase-associated lipocalin plasma levels in the study population

Plasma neutrophil gelatinase-associated lipocalin levels in the study population are shown in Table 7.3 and Figure 7.3. The median plasma level was highest prior to reperfusion, and gradually declined post-operatively, becoming significantly different from baseline at 24 hours after reperfusion (p=0.0001, Wilcoxon signed rank test). The highest plasma NGAL level occurred at 2 hours post-reperfusion, representing a 6.8 fold change from baseline, and occurred in a placebo recipient (DDF14) who was later labelled as having delayed graft function.

Table 7-3: Plasma NGAL levels (ng/ml) in all 39 study participants at each time point

Plasma NGAL (ng/ml)				
Time	**Mean ± SD**	**Median**	**Range**	**Sample No.**
0	1272 ± 908	1034	156 – 4052	39
15mins	1222 ± 929	1013	177 – 4842	39
2hrs	1349 ± 1344	984	149 – 7098	37
8hrs	1000 ± 687	904	0 – 3643	37
D1	743 ± 448	646	0 – 2010	38
D2	619 ± 387	524.5	156 – 1719	36
D3	550 ± 356	502	0 – 1423	39
D4	495 ± 405	378.5	0 – 2047	39
D5	378 ± 283	351	0 – 1203	39
D6	438 ± 412	320.5	0 – 2166	37

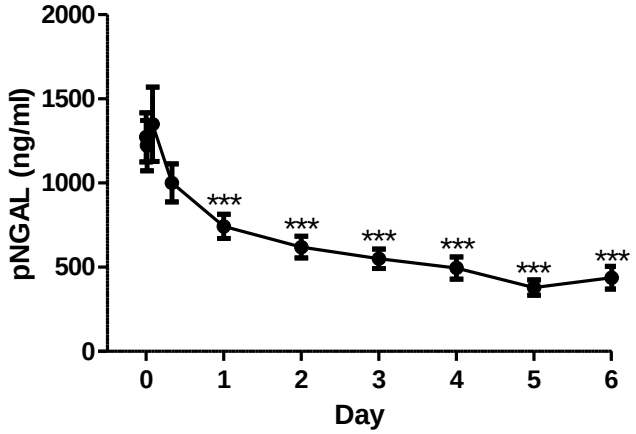


Figure 7-3: Natural history of plasma NGAL levels in the study population.
Plasma NGAL (pNGAL) levels (ng/ml) in all 39 study participants plotted over time. Mean ± SEM shown. No significant increase from baseline occurred in the early post-operative period, with a steady decline beginning after 8 hours. **p<0.01, ***p<0.001; Wilcoxon signed rank test compared to baseline.

7.3.6 Plasma NGAL (ng/ml) levels do not reliably detect DGF before 24 hours post-reperfusion

Delayed graft function (DGF) was defined as the need for dialysis within the first 7 post-operative days (n=21). Immediate graft function (IGF) was defined as any patient not meeting the criteria for DGF (n=18). Plasma NGAL levels (ng/ml) were significantly different between the DGF and IGF group from as early as 8 hours post-reperfusion (p=0.04, Mann-Whitney test) and this difference persisted until day 6 (Figure 7.4). However, in contrast to the 24 hour samples, the 8 hour NGAL measurement in both groups was not different from their respective baseline samples (p>0.10, Wilcoxon signed rank test) questioning the relevance of this observation at 8 hours.

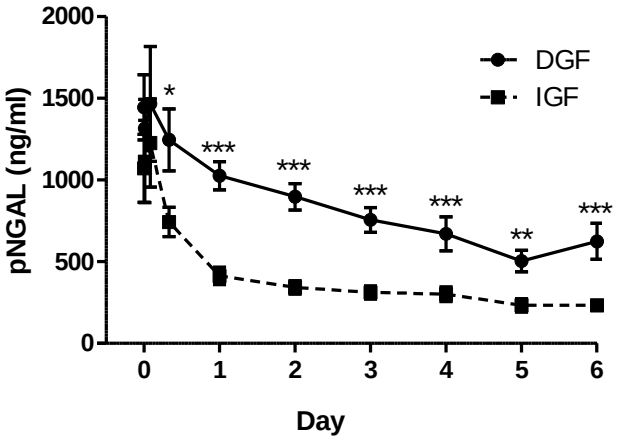


Figure 7-4: Plasma NGAL stratified by graft function.
Plasma NGAL (pNGAL) levels across time in patients stratified by the presence or absence of delayed graft function (DGF); Mean ± SEM shown. IGF: immediate graft function. Day 0 is pre-reperfusion

Examining this further, DGF (1/0) was highly correlated with plasma NGAL levels (ng/ml) at 24 hours (spearman r = 0.75, p<0.0001) but markedly less so at 8 hours (r = 0.35, p=0.03) (Table 7.4).

Using ROC curve analysis, plasma NGAL levels at 24 hours had an AUC of 0.93, (p<0.0001) with levels > 640ng/ml having a sensitivity of 85% and specificity of 83% (Figure 7.5a+b). In comparison, plasma NGAL at 8hrs had an AUC of 0.70 (0.04) with levels of >641.5ng/ml

having a sensitivity of 84% but a specificity of only 39%. In contrast, ROC curve analysis of corrected NGAL levels (fold change compared to baseline) was not significant at 8 hrs. At 24 hours, ROC curve analysis of corrected NGAL levels had a lower AUC compared to raw levels (0.75, p=0.009). However, a fold change of ≥0.80 at 24 hours gave a sensitivity of 62% and a specificity of 83% for predicting DGF.

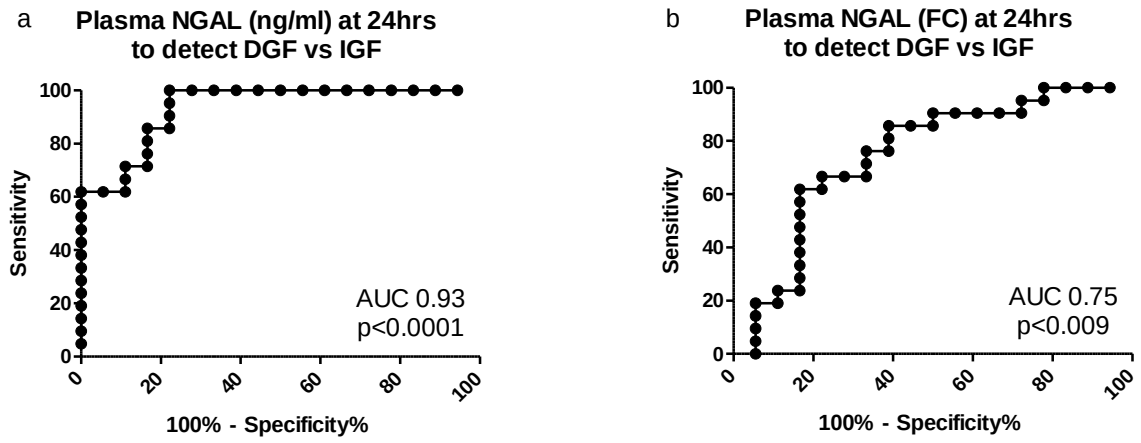


Figure 7-5: ROC curve analysis of plasma NGAL.
ROC curve analysis examining the ability of plasma NGAL (a) un-corrected (ng/ml) and corrected (FC: fold change) to differentiate delayed graft function (DGF) from immediate graft function (IGF). AUC: area under the curve.

Thus, although plasma NGAL levels (ng/ml) can distinguish between IGF and DGF groups at 8 hours, the earliest time point at which levels can reliably distinguish them with an excellent AUC is at 24 hours. Surprisingly, correcting post-reperfusion NGAL levels to baseline levels does not confer any advantage in making the diagnosis.

7.3.7 Plasma NGAL is associated with the severity of acute transplant injury

Severity of transplant injury post-reperfusion can be measured by the number of dialysis episodes required and also the number of days required to become dialysis independent (Table 7.4). The highest correlation with the number of dialysis episodes needed before function was regained was at day 2 ($r = 0.74$, $p<0.0001$), decreasing thereafter. There was no significant correlation at time points before 24 hours ($r = 0.67$, $p<0.0001$). Time to last dialysis episode (days) significantly correlated with pNGAL at 24 hours and day 2 ($r = 0.69$,

p<0.0001 and r = 0.75, p<0.0001 respectively). Finally, estimated glomerular filtration rate (4v MDRD eGFR mls/min) at day 7 was inversely correlated with pNGAL levels at all time points after 8 hours. Plasma NGAL levels at all time points did not correlate with eGFR at 1 month or 3 months. Thus, while plasma levels did not correlate with DGF any earlier than traditional time points (24 hours), higher levels suggest greater severity of acute kidney injury as measured by number of dialysis sessions required, time to last dialysis episode and function at day 7.

Table 7-4: Correlations between plasma NGAL and surrogates of injury.
Spearman correlation coefficients examining the correlation of plasma NGAL levels (ng/ml) to the need for dialysis and the severity of acute transplant injury. DGF: delayed graft function. EGFR: estimated glomerular filtration rate (4 variable MDRD equation in mls/min)*p<0.05, **p<0.01, ***, p<0.001

	Baseline	15 mins	2Hrs	8Hrs	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
No. of samples	39	39	37	37	39	36	39	38	39	38
DGF (1/0)	0.27	0.23	0.15	0.35*	0.75***	0.78***	0.69***	0.52***	0.51***	0.58***
No. dialysis sessions	0.2	0.15	0.09	0.3	0.67***	0.74***	0.65***	0.51**	0.45**	0.58***
Time to last dialysis	0.25	0.19	0.11	0.31	0.69***	0.75***	0.67***	0.53***	0.44**	0.58***
eGFR at day 7	-0.11	-0.12	-0.05	-0.39*	-0.55***	-0.74***	-0.60***	-0.46**	-0.54***	-0.69***

7.3.8 Effect of EPO on the severity of acute kidney injury as measured by pNGAL levels

Plasma NGAL levels in the EPO and placebo group were compared at each time point until day 6 post-reperfusion (Figure 7.6). There was no significant difference in plasma NGAL levels between EPO- and placebo-treated patients.

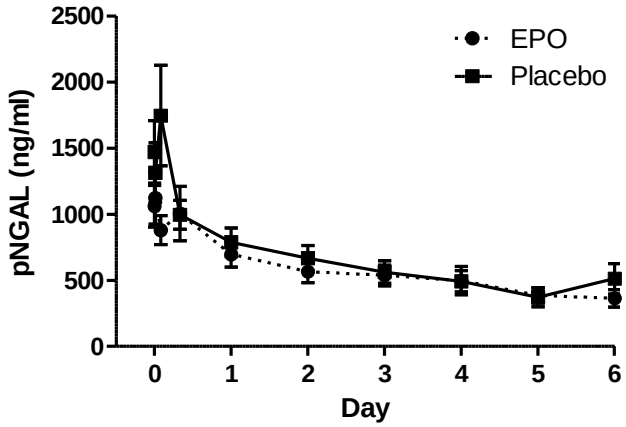


Figure 7-6: Plasma NGAL over time stratified by trial drug group.
Plasma NGAL (pNGAL) levels in EPO- and placebo-treated patients from pre-reperfusion until day 6. Mean ± SEM shown. There was no difference between the EPO and placebo groups at all time points.

7.3.9 Urine neutrophil gelatinase-associated lipocalin

7.3.9.1 Neutrophil gelatinase-associated lipocalin urine levels in the study population

Neutrophil gelatinase-associated lipocalin levels (uNGAL) levels in the study population are shown in Table 7.5 and Figure 7.7. The median urine level was highest prior to reperfusion, and gradually declined post-operatively, becoming significantly different from baseline on day 3 after reperfusion ($p=0.02$, Mann-Whitney test). A Mann-Whitney test rather than a Wilcoxon signed rank test was performed due to the paucity of pre-reperfusion samples. The highest urine NGAL level occurred on day 2, in a NHBD recipient (DDF38) with delayed graft function. Unfortunately, the patient was anuric and thus did not have a pre-reperfusion control sample.

Table 7-5: Urine NGAL levels (ng/mgCr) in all 39 study participants at each time point.

Urine NGAL (ng/mgCr)				
Time	**Mean ± SD**	**Median**	**Range**	**Sample No.**
0	3119 ± 2897	2867	0 – 10367	21
2hrs	2843 ± 2677	2473	0 – 10208	18
8hrs	2835 ± 2883	1858	0 – 9773	26
D1	2542 ± 5668	894.5	0 – 33616	36
D2	4375 ± 14447	532.5	0 – 89066	38
D3	2378 ± 4326	445	0 – 20638	38
D4	2150 ± 4453	362	0 – 24481	34
D5	2277 ± 6250	190	0 – 32761	32
D6	1920 ± 4065	174	0 – 15388	35

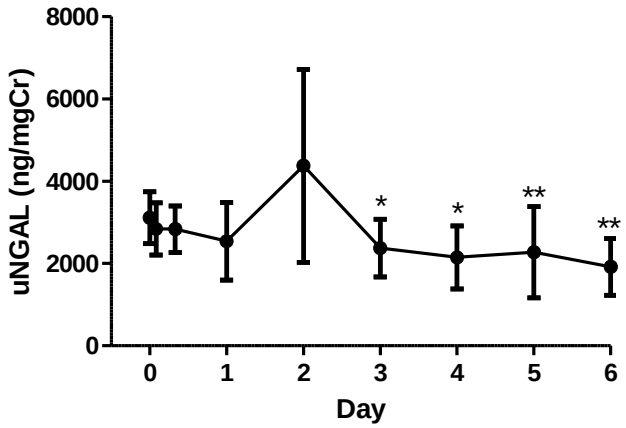


Figure 7-7: Natural history of urine NGAL in the study population.
Urine NGAL (uNGAL) levels (ng/mgCr) in all 39 study participants plotted over time. Mean ± SEM shown. Levels declined with time and reached significance at day 3 compared to baseline. Wilcoxon signed rank test. *p<0.05, **p<0.01.

7.3.9.2 Urine NGAL levels may diagnose DGF earlier than routinely used clinical markers

Urine NGAL levels (ng/mgCr) are significantly higher in patients with DGF compared to patients with IGF, beginning at 8 hours after reperfusion and continuing throughout the study

period (p=0.03, Mann-Whitney test) (Figure 7.8). In keeping with this finding, DGF (1/0) correlated with urine NGAL levels at 8 hours (spearman r = 0.45, p=0.03), with the highest correlation occurring on day 3 (r = 0.72, p<0.0001) (Table 7.6). Thus, failure for uNGAL levels to fall within 8 hours suggests that DGF may occur. Of note, baseline uNGAL levels were significantly lower in patients with IGF.

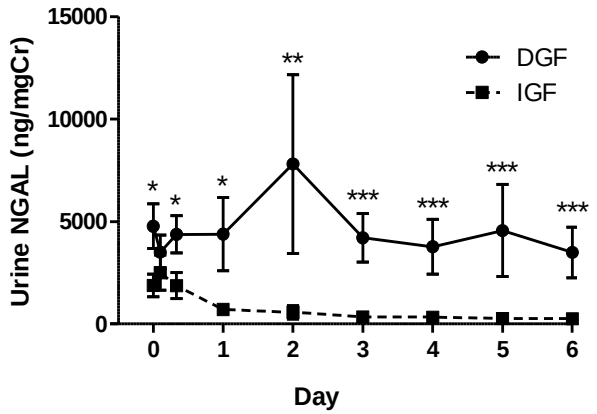


Figure 7-8: Urine NGAL (uNGAL) relationship to graft function
uNGAL across all time points in patients stratified by the presence or absence of delayed graft function (DGF); IGF: immediate graft function. Day 0 is pre-reperfusion. Mean ± SEM shown. *p<0.05, **p<0.01, ***p<0.001 Mann Whitney test

Using ROC curve analysis, urine NGAL at 8 hours does not significantly differentiate DGF from IGF (AUC=0.52, p=0.88). At 24 hours, the AUC has increased to 0.79 (p=0.003), with a urine NGAL level >674.5 having a sensitivity of 83% and a specificity of only 61% (Figure 7.9). By 48 hours, the AUC is >0.90 but at this point, the diagnosis of DGF will already have been apparent.

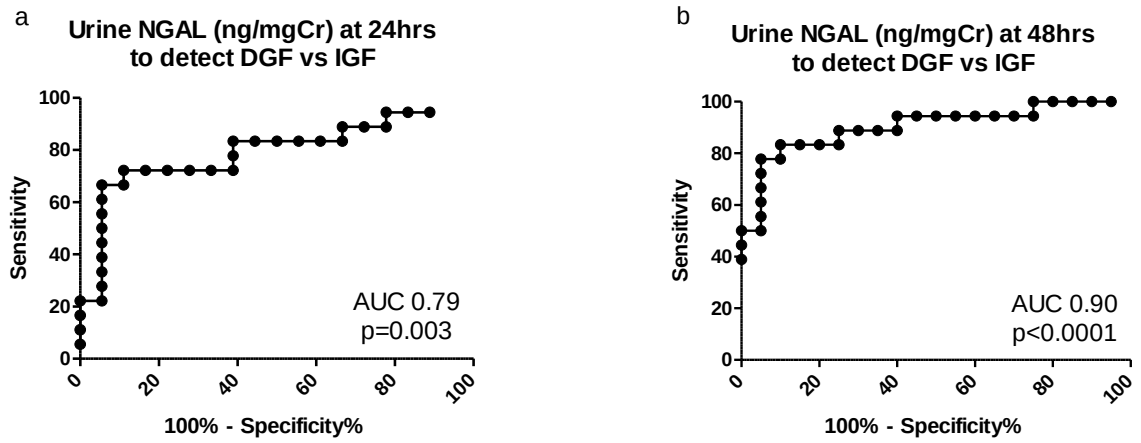


Figure 7-9: uNGAL identifies kidneys at risk of DGF.
ROC curve analysis examining the ability of urine NGAL to differentiate delayed graft function (DGF) from immediate graft function (IGF). AUC: area under the curve.

7.3.9.3 Urine NGAL is associated with the severity of acute transplant injury

Urine NGAL levels were highly correlated with the number of dialysis episodes required and the time to last dialysis (Table 7.6). These correlations were present by 8 hours and persisted throughout the sample collection period. Finally, estimated glomerular filtration rate (4v MDRD eGFR mls/min) at day 7 was inversely correlated with uNGAL levels at all time points after 8 hours. Urine NGAL levels at any time point did not correlate with eGFR at 1 month or 3 months. Similarly to pNGAL, high uNGAL levels suggest greater severity of acute kidney injury as measured by number of dialysis sessions required, time to last dialysis episode and function at day 7.

Table 7-6: uNGAL predicts the need for dialysis and the severity of acute kidney injury.
DGF: delayed graft function. eGFR: estimated glomerular filtration rate (4 variable MDRD equation in mls/min); Spearman correlations; *p<0.05, **p<0.01, ***, p<0.001

	Baseline	2Hrs	8Hrs	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
No. of samples	21	18	26	36	38	38	34	32	35
DGF (1/0)	0.52*	0.25	0.45*	0.51**	0.70***	0.72***	0.68***	0.66***	0.64***
No. dialysis sessions	0.50*	0.31	0.48*	0.51**	0.71***	0.73***	0.71***	0.69***	0.71***
Time to last dialysis	0.47*	0.31	0.47*	0.50**	0.69***	0.73***	0.71***	0.69***	0.70***
eGFR at day 7	-0.33	0.03	-0.52**	-0.72***	-0.79***	-0.81***	-0.66***	-0.79***	-0.77***

7.3.9.4 Effect of EPO on the severity of acute kidney injury as measured by uNGAL levels

Urine NGAL levels in the EPO and placebo group were compared at each time point until day 6 post-reperfusion (Figure 7.10). There was no significant difference in urine NGAL levels between EPO- and placebo-treated patients.

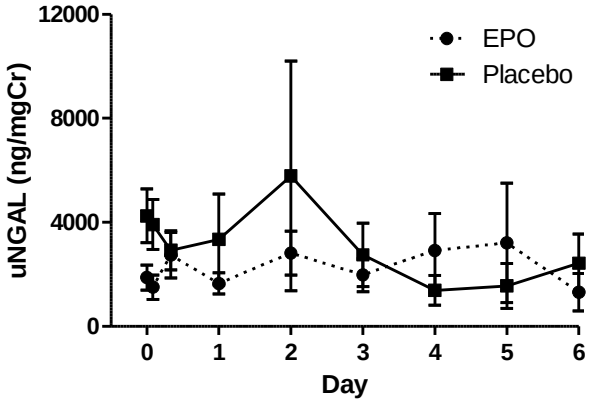


Figure 7-10: Urine NGAL over time stratified by trial drug group.
Urine NGAL (pNGAL) levels in EPO- and placebo-treated patients from pre-reperfusion until day 6. Mean ± SEM shown. There was no difference between the EPO and placebo groups at all time points.

7.3.10 Plasma interleukin-18

7.3.10.1 Interleukin-18 plasma levels in the study population

Interleukin-18 (IL-18) plasma levels in the entire study population are shown in Table 7.7 and Figure 7.11. The highest median plasma level occurred at day 6, but plasma levels were significantly different from baseline at day 5 (p=0.0002, Wilcoxon signed rank test). The highest plasma IL-18 level occurred on day 6, at 6.9 fold greater than the baseline level pre-reperfusion. This occurred in an ECD recipient (DDF18) with delayed graft function who two days later had an episode of biopsy-proven rejection.

Table 7-7: Plasma IL-18 levels (ng/ml) in all 39 study participants at each time point.

Plasma IL-18 (ng/ml)				
Time	**Mean ± SD**	**Median**	**Range**	**Sample No.**
0	525 ± 353	448	0 – 2028	39
15mins	529 ± 429	428	0 – 2526	39
2hrs	546 ± 622	422	0 – 3188	37
8hrs	589 ± 547	418	0 – 2414	37
D1	584 ± 529	368	0 – 1956	38
D2	528 ± 479	363.5	0 – 1900	36
D3	594 ± 544	445	0 – 2127	39
D4	600 ± 529	442	0 – 2410	39
D5	765 ± 546	566	0 – 2488	39
D6	910 ± 714	734.5	0 – 3486	37

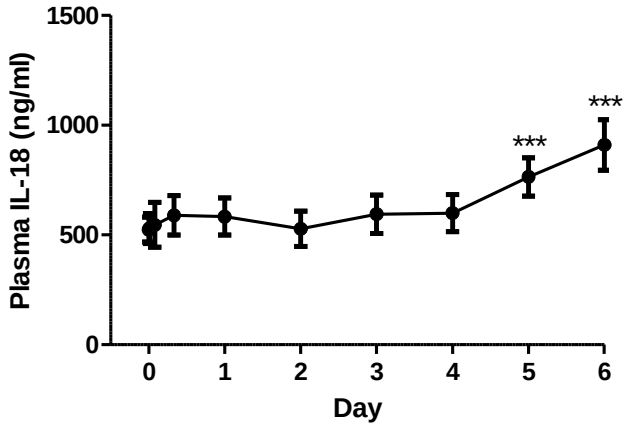


Figure 7-11: Natural history of plasma IL-18 in the study population.
Plasma IL-18 (pIL-18) levels in all 39 study participants plotted over time. Mean ± SEM shown. No significant increase from baseline in the early post-operative period until day 5. ***p<0.001; Wilcoxon signed rank test compared to pre-reperfusion samples.

7.3.10.2 Plasma IL-18 does not differentiate delayed graft function from immediate graft function.

At no point in the post-transplant period did IL-18 plasma levels separate DGF from IGF (Figure 7.12). Baseline levels were not different between the two groups. In keeping with this finding, plasma IL-18 levels did not correlate with DGF.

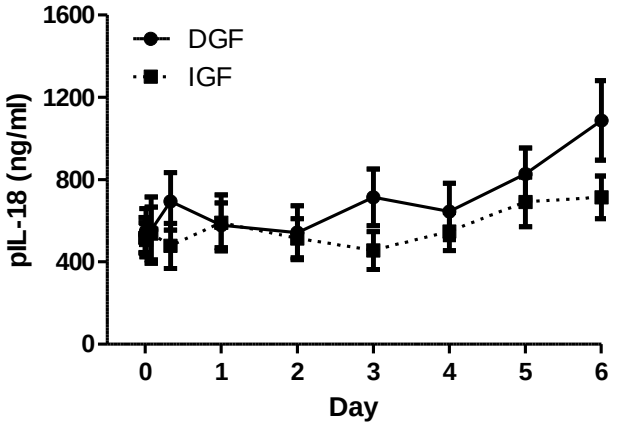


Figure 7-12: Plasma IL-18 over time stratified by graft function.
Plasma interleukin-18 (pIL-18) levels do not separate delayed graft function (DGF) from immediate graft function (IGF) at any time point. Mean ± SEM are shown.

7.3.10.3 Plasma IL-18 does not predict the severity of acute transplant injury

Plasma IL-18 levels at each time point did not correlate with the incidence of delayed graft function, the number of dialysis episodes, the duration of dialysis post-operatively and the eGFR at day 7.

7.3.10.4 Effect of EPO on the severity of acute kidney injury as measured by plasma IL-18 levels

Plasma IL-18 did not differentiate the EPO group from the placebo group at any time point (Figure 7.13).

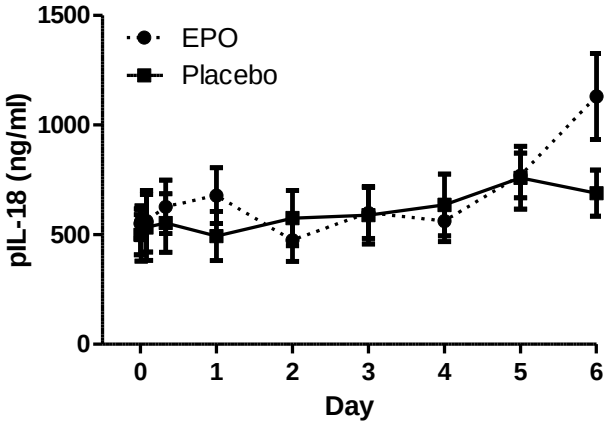


Figure 7-13: Plasma IL-18 levels over time stratified by trial drug.
The effect of EPO compared to placebo on the severity of acute kidney injury as measured by plasma IL-18.

7.3.11 Urine interleukin-18

7.3.11.1 Interleukin-18 urine levels in the study population

Urine interleukin-18 (uIL-18) levels in the study population are shown in Table 7.8 and Figure 7.14. As previously described, sample collection pre-reperfusion was limited due to anuric patients. The data was not normally distributed meaning that the highest median urine IL-18 occurred at 2 hours while the highest mean was at 2 days post-reperfusion (1222 *vs* 3036, respectively). High uIL-18 levels were seen at 2 hours compared to baseline (p=0.006) with subsequent levels declining with time. Using the 2 hour point as an alternative reference point, levels declined with time, beginning by day 3 and continuing to the end of sample collection. A Mann-Whitney test rather than a Wilcoxon signed rank test was performed due to the paucity of pre-reperfusion samples. The highest urine IL-18 level occurred on day 2, in an ECD kidney recipient (DDF32) who developed delayed graft function. Unfortunately, the patient was anuric and thus did not have a pre-reperfusion control sample.

Table 7-8: Urine interleukin-18 (uIL-18) levels (ng/mgCr) in all 39 study participants at each time point. Sample collection pre-reperfusion was incomplete due to anuria.

Urine IL-18 (ng/mgCr)				
Time	**Mean ± SD**	**Median**	**Range**	**Sample No.**
0	360 ± 625	81	0 – 2630	21
2hrs	2190 ± 3385	1222	0 – 14410	18
8hrs	1936 ± 3720	314.5	0 – 16733	26
D1	1927 ± 7012	301.5	0 – 41983	36
D2	3036 ± 14689	299.5	0 – 90833	38
D3	1109 ± 4135	181	0 – 25506	38
D4	699 ± 2015	112.5	0 – 11126	34
D5	1246 ± 4960	102	0 – 28225	32
D6	980 ± 2397	145	0 – 11367	35

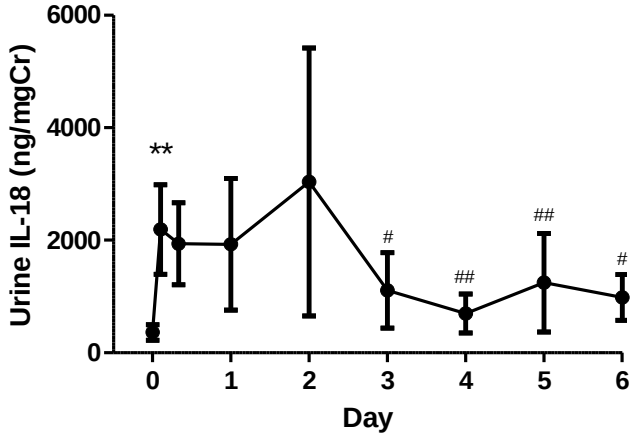


Figure 7-14: The natural history of urine IL-18 in the study population.
Urine interleukin-18 (uIL-18) levels (ng/mgCr) in all 39 study participants plotted over time. Mean ± SEM shown. Sample collection was limited pre-reperfusion and thus the apparent rise from pre- to post-reperfusion samples must be interpreted with caution (p=0.006, Mann-Whitney test). Comparing levels to the 2 hour sample revealed increasingly significant differences from day 3 onwards (p=0.01). **p<0.01, $^{\#}$p<0.05, $^{\#\#}$p<0.01

7.3.11.2 Urine IL-18 levels do not diagnose DGF earlier than routinely used clinical markers

In the early post-transplant period, there is no significant difference in urine IL-18 levels (ng/mgCr) until day 3-5, when IGF patients have lower levels (p=0.02, p=0.002, p=0.005 respectively) (Figure 7.15). Both groups have an initial increase in levels at 2 hours (p=0.67),

which slowly declines with time, in contrast to plasma IL-18 whose levels increase over time (see above). 14/39 recipients had no detectable urine IL-18 by day 4.

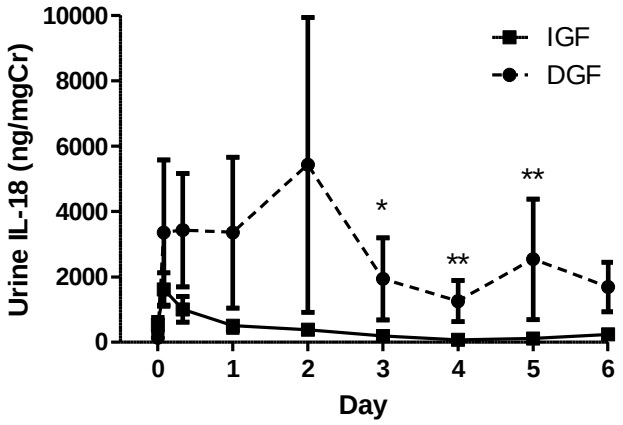


Figure 7-15: Urine IL-18 levels over time stratified by graft function.
Urine IL-18 (uIL-18) levels (ng/mgCr) across time in patients stratified by the presence or absence of delayed graft function (DGF); IGF: immediate graft function. Day 0 is pre-reperfusion. Mean ± SEM. *p<0.05, **p<0.01, Mann-Whitney test.

7.3.11.3 Urine IL-18 predicts the severity of acute transplant injury

Urine IL-18 levels were highly correlated with the number of dialysis episodes required and the time to last dialysis from day 2 onwards (Table 7.9). Estimated glomerular filtration rate (4v MDRD eGFR mls/min) at day 7 was inversely correlated with urine IL-18 levels, particularly at day 5 and 6 ($r = -0.6$, $p=0.0003$) and $r= -0.46$, $p=0.005$ respectively). Urine IL-18 levels did not correlate with eGFR at 1 month. Thus high urine IL-18 levels on day 3-5 suggest greater severity of acute kidney injury as measured by number of dialysis sessions required, time to last dialysis episode and function at day 7. Interestingly, urine IL-18 is a marker of inflammation and levels on day 6 correlated with the occurrence of a biopsy proven rejection episode (8/39) in the first 3 months ($r = 0.43$, $p=0.009$). However, 7 out of the 8 biopsy proven rejection episodes occurred with 12 days of reperfusion – the significance of which is uncertain.

Table 7-9: Correlations of urine IL-18 with surrogate markers of graft injury.
Spearman correlations examining the ability of urine IL-18 levels (ng/mgCr) to predict the need for dialysis and the severity of acute kidney injury. DGF: delayed graft function. eGFR: estimated glomerular filtration rate (4 variable MDRD equation in mls/min)*p<0.05, **p<0.01, ***, p<0.001

	Baseline	2Hrs	8Hrs	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
No. of samples	21	18	26	36	38	38	34	32	35
DGF (1/0)	-0.25	0.11	0.21	0.29	0.28	0.38*	0.53**	0.51**	0.28
No. dialysis sessions	-0.23	0.16	0.31	0.33	0.35*	0.49**	0.55***	0.57***	0.44**
Time to last dialysis	-0.20	0.17	0.30	0.32	0.39*	0.51***	0.55***	0.57***	0.39*
eGFR at day 7	0.15	-0.19	-0.10	-0.24	-0.26	-0.42**	-0.27	-0.60***	-0.46**

7.3.11.4 Effect of EPO on the severity of acute kidney injury as measured by uIL-18 levels

Urine IL-18 did not discriminate between EPO and placebo treatment at any time point (Figure 7.16).

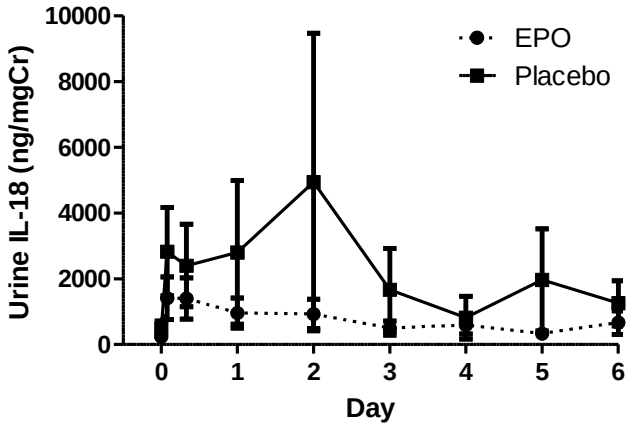


Figure 7-16: uIL-18 over time in treatment groups.
Line plot showing mean ± SEM of urine interleukin-18 (IL-18) over time in EPO and placebo groups. No significant difference at any time point.

7.3.12 Plasma kidney injury molecule-1

7.3.12.1 Kidney injury molecule-1 plasma levels in the study population

Kidney injury molecule-1 levels (KIM-1) levels in the study population are shown in Table 7.10 and Figure 7.17. Plasma KIM-1 levels increase with time, becoming significantly different from baseline at 2 hours ($p<0.001$ at all time points, Wilcoxon signed rank test). The median plasma level was highest at day 6. The highest plasma KIM-1 level occurred on day 4, representing a 10.7 fold change from baseline, and occurred in a NHBD recipient (DDF06) who was diagnosed as having delayed graft function. 13/39 patients did not have a detectable plasma KIM-1 level prior to reperfusion.

Table 7-10: Plasma KIM-1 levels (pg/ml) in all 39 study participants at each time point.

Plasma KIM-1 (pg/ml)				
Time	**Mean ± SD**	**Median**	**Range**	**Sample No.**
0	132 ± 181	88	0 – 912	39
15mins	140 ± 164	94	0 – 824	39
2hrs	180 ± 160	134	0 – 894	37
8hrs	200 ± 174	138	0 – 842	37
D1	275 ± 216	210.5	0 – 945	38
D2	380 ± 303	303	120 – 1556	36
D3	599 ± 596	427	117 – 3352	39
D4	823 ± 814	557	164 – 4164	39
D5	1067 ± 1107	551	144 – 4124	39
D6	1060 ± 786	854	160 -2996	37

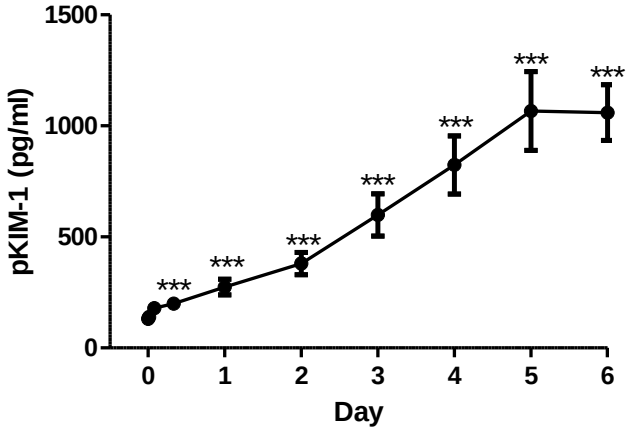


Figure 7-17: The natural history of plasma KIM-1 in the study population.
Plasma KIM-1 (pNGAL) levels (pg/ml) in all 39 study participants plotted over time. Mean ± SEM shown. Plasma levels increased from 2 hours onwards. ***p<0.001. Wilcoxon signed rank test compared to pre-reperfusion sample.

7.3.13 Plasma KIM-1 does not predict DGF earlier than routinely used clinical markers

Plasma levels increased in both DGF and IGF groups, although the slope of the IGF curve was smaller, with the curves significantly diverging at day 6 (p=0.005) (Figure 7.18). Furthermore, DGF (1/0) was only correlated with day 6 pKIM-1 levels (r = 0.47, p=0.003).

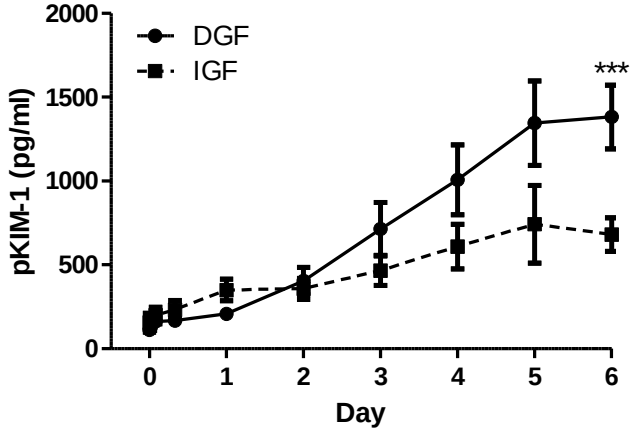


Figure 7-18: Plasma KIM-1 levels over time stratified by graft function.
Plasma KIM-1 (pKIM-1) levels across time in patients stratified by the presence or absence of delayed graft function (DGF); No differences were seen at time points up until day 6, when DGF patients had significantly higher levels ***p=0.005; Mean ± SEM; IGF: immediate graft function; Day 0 is pre-reperfusion.

ROC curve analysis revealed an AUC of 0.77 (p=0.004) at day 6 and only 0.62 (p=0.20) at 24 hours (Figure 7.19a+b).

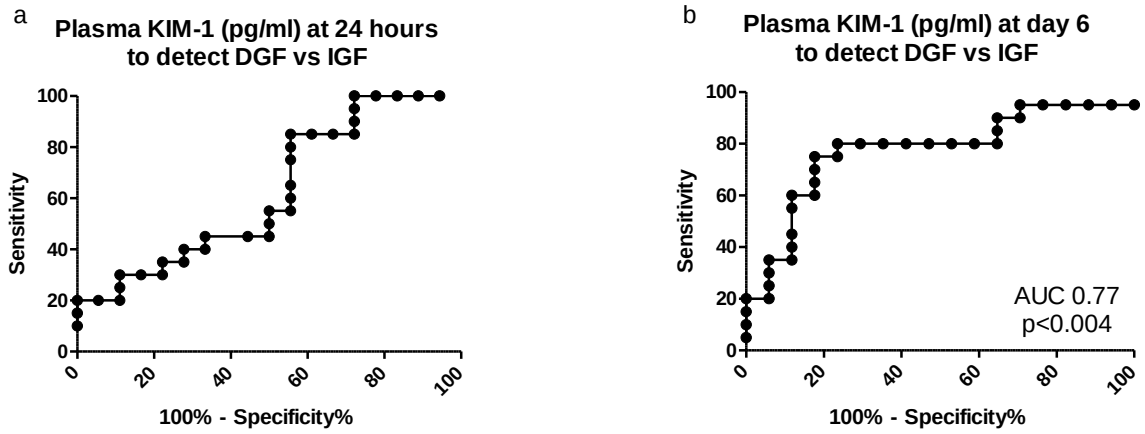


Figure 7-19: ROC curve analysis of plasma KIM-1.
ROC curve analysis examining the ability of plasma KIM-1to differentiate delayed graft function (DGF) from immediate graft function (IGF). AUC: area under the curve.

7.3.14 Plasma KIM-1 predicts the severity of acute transplant injury

The highest correlation with the number of dialysis episodes needed before function was regained was at day 6 (r = 0.54, p=0.003), with levels from day 3-6 having weak but significant correlations (Table 7.11). Time to last dialysis episode (days) was also best correlated with pKIM-1 levels on day 6 (r = 0.52, p=0.0005). Finally, estimated glomerular filtration rate (4v MDRD eGFR mls/min) at day 7 was inversely correlated with pKIM-1 levels beginning at day 3. Levels did not correlate with eGFR at 1 month or 3 months. Thus, higher levels suggest greater severity of acute kidney injury as measured by number of dialysis sessions required, time to last dialysis episode and function at day 7.

Table 7-11: Correlations of plasma KIM-1 with surrogate markers of injury.
Spearman correlation coefficients examining the correlation of plasma KIM-1 levels (pg/ml) to the need for dialysis and the severity of acute kidney injury. DGF: delayed graft function. eGFR: estimated glomerular filtration rate (4 variable MDRD equation in mls/min)*p<0.05, **p<0.01, ***, p<0.001

	Baseline	15 mins	2Hrs	8Hrs	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
No. of samples	39	39	37	37	39	36	39	38	39	38
DGF (1/0)	-0.04	-0.02	-0.03	-0.04	-0.21	0.08	0.22	0.27	0.32	0.47**
No. dialysis sessions	-0.11	-0.08	-0.14	0.10	-0.16	0.21	0.34*	0.38*	0.42**	0.54***
Time to last dialysis	-0.15	-0.12	-0.16	0.06	-0.19	0.18	0.31	0.36*	0.42**	0.52**
eGFR at day 7	-0.06	-0.09	-0.10	-0.17	-0.08	-0.32	-0.55***	-0.60***	-0.51***	-0.61***

7.3.15 Effect of EPO on the severity of acute kidney injury as measured by plasma KIM-1 levels

Plasma KIM-1 levels in the EPO and placebo group were compared at each time point until day 6 post-reperfusion (Figure 7.20). Between groups, there was no significant difference at any time point.

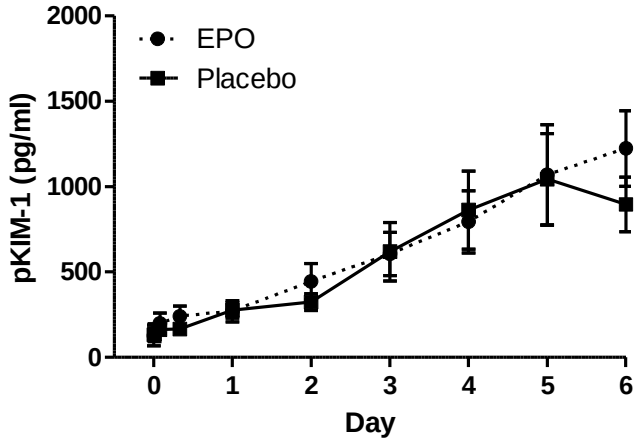


Figure 7-20: Plasma KIM-1 levels stratified by treatment group.
Plasma KIM-1 (pKIM-1) levels in EPO- and placebo-treated patients. There was no difference between the EPO and placebo groups at all time points.

7.3.16 Urine kidney injury molecule-1

7.3.16.1 Kidney injury molecule-1 urine levels in the study population

Kidney injury molecule-1 urine levels in the study population are shown in Table 7.12 and Figure 7.21. After a small decline in levels in the immediate post-reperfusion period, KIM-1 levels increased over time from baseline with statistical significance reached on day 3 (p=0.03, Mann-Whitney test). The highest urine KIM-1 levels occurred on day 6 in an ECD kidney recipient (DDF32) with delayed graft function and still requiring dialysis. Unfortunately, the patient was anuric and thus we did not have a pre-reperfusion control sample. Only 2/21 patients had no detectable KIM-1 in their pre-reperfusion urine sample.

Table 7-12: Urine KIM-1 levels (pg/mgCr) in all 39 study participants at each time point.

Urine KIM-1 (pg/mgCr)				
Time	**Mean ± SD**	**Median**	**Range**	**Sample No.**
0	2189 ± 2469	1440	0 – 9980	21
2hrs	1680 ± 1642	1047	0 – 6417	18
8hrs	1853 ± 2331	1233	0 – 11706	26
D1	2070 ± 1964	1653	0 – 9475	36
D2	2965 ± 3112	1691	0 – 14038	38
D3	3321 ± 2479	2766	124 – 11849	38
D4	3419 ± 2598	2997	171 – 12584	34
D5	5489 ± 5704	3452	0 – 27550	32
D6	7515 ± 9243	4584	315 – 51733	35

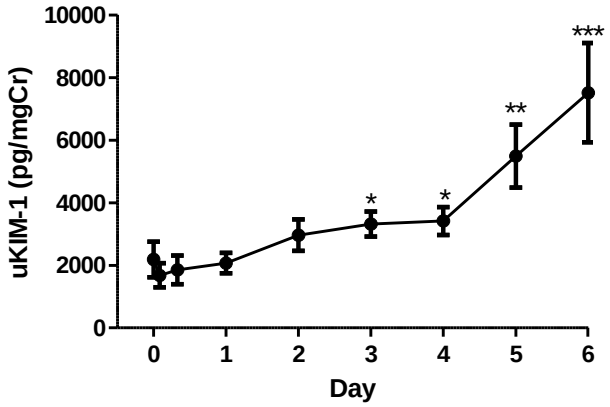


Figure 7-21: The natural history of urine KIM-1 in the study population.
Urine KIM-1 (uKIM-1) levels (pg/mgCr) in all 39 study participants plotted over time. Mean ± SEM shown. Mann Whitney test compared to baseline. *p<0.05, **p<0.01, ***p<0.001

7.3.16.2 Urine KIM-1 levels did not differentiate delayed graft function from immediate graft function.

Urine KIM-1 levels (pg/mgCr) do not differentiate DGF from IGF at any time point sampled, with levels increasing in both situations (Figure 7.22). Baseline levels were not different between the two groups. In keeping with this finding, urine KIM-1 levels did not correlate with delayed graft function.

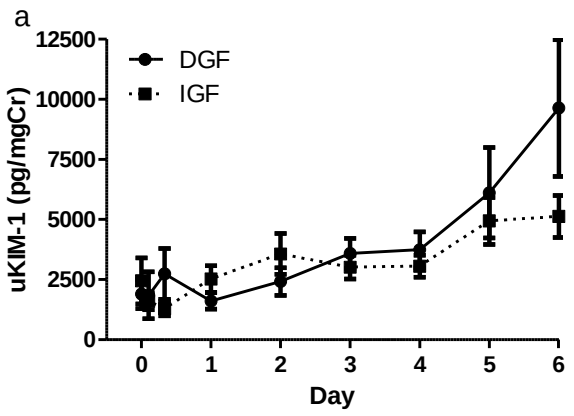


Figure 7-22: Urine KIM-1 levels over time stratified by graft function.
Urine KIM-1 (uKIM-1) levels do not separate delayed graft function (DGF) from immediate graft function (IGF) at any time point. Day 0 is pre-reperfusion. Mean ± SEM.

7.3.16.3 Urine KIM-1 levels are not markers of the severity of acute transplant injury

Urine KIM-1 levels did not correlate with the need for dialysis, the number of dialysis sessions, the duration of dialysis post-operatively and the eGFR at day 7.

7.3.16.4 Effect of EPO on the severity of acute kidney injury as measured by uKIM-1 levels

Urine KIM-1did not differentiate between EPO and placebo treatment (Figure 7.23). Small numbers of urine samples collected in the early post-operative period preclude a meaningful sub-group analysis of EPO (DGF vs IGF uKIM-1 levels) and placebo (DGF vs IGF uKIM-1 levels).

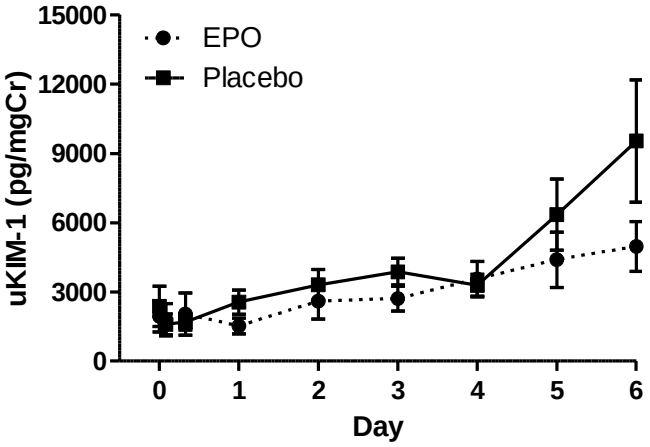


Figure 7-23: Urine KIM-1 levels over time stratified by treatment.
EPO-treatment had no effect on injury severity in the first 6 days post-reperfusion, as measured by urine KIM-1 levels. Mean ± SEM. Mann-Whitney test.

7.3.17 Biomarker gene expression in implant biopsies

7.3.17.1 General introduction

Probe set IDs for NGAL (212531_at), IL-18 (207052_at) and KIM-1 (207052_at) were identified at www.affymetrix.com and the signals for each probe set were identified from the implant biopsy microarrays. Only one probe set ID exists for each gene of interest. Signals were converted to fold change by dividing the biopsy probe set signal by the mean

nephrectomy probe set signal. Gene expression is shown as logarithmic transformed fold changes for each patient in the study and statistical tests were performed on the log transformed values. Nephrectomy samples are also shown for comparison.

7.3.17.2 Biomarker gene expression in the study population

There was no significant difference between NGAL and KIM-1 expression in comparison to nephrectomy (Table 7.13 and Figure 7.24). However, IL-18 gene expression was significantly higher in the study population compared to the nephrectomy group ($p<0.0001$). There was marked heterogeneity in the biomarker gene expression within both the study population and the nephrectomy population.

Table 7-13: Biomarker gene expression in the study population and nephrectomy samples.

Biomarker Gene Expression (Fold Change)				
Time	**Population**	**Mean ± SD**	**Median**	**Range**
KIM-1	EPO Study	0.95 ± 0.18	0.88	0.7 – 1.4
	Nephrectomy	1.00 ± 0.17	1.05	0.8 – 1.3
IL-18	EPO Study	1.17 ± 0.07	1.18	1.0 – 1.3
	Nephrectomy	1.00 ± 0.05	1.02	1.0 – 1.1
NGAL	EPO Study	0.99 ± 0.13	0.95	0.8 – 1.3
	Nephrectomy	1.00 ± 0.11	0.94	0.9 – 1.2

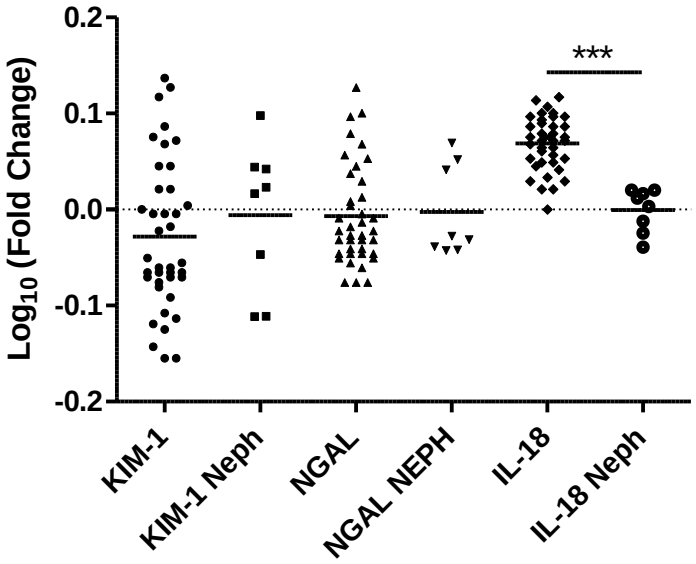


Figure 7-24: Biomarker gene expression in the study population (n=39).
Zero on the y-axis represents the mean nephrectomy value for each biomarker. Nephrectomy samples (n=8) are shown for comparison. ***p<0.0001, Mann-Whitney test.

7.3.17.3 Biomarker gene expression stratified by graft function

The study population was split into two groups, DGF and IGF, and biomarker gene expression was re-examined (Figure 7.25). The marked heterogeneity in NGAL and KIM-1 levels seen in the study population was not explained by graft function, with no significant difference between DGF and IGF populations for either biomarker. In contrast, IL-18 gene expression was higher in the DGF population (p=0.04, Mann-Whitney test). Patients with either DGF or IGF had significantly higher levels of IL-18 compared to the nephrectomy group.

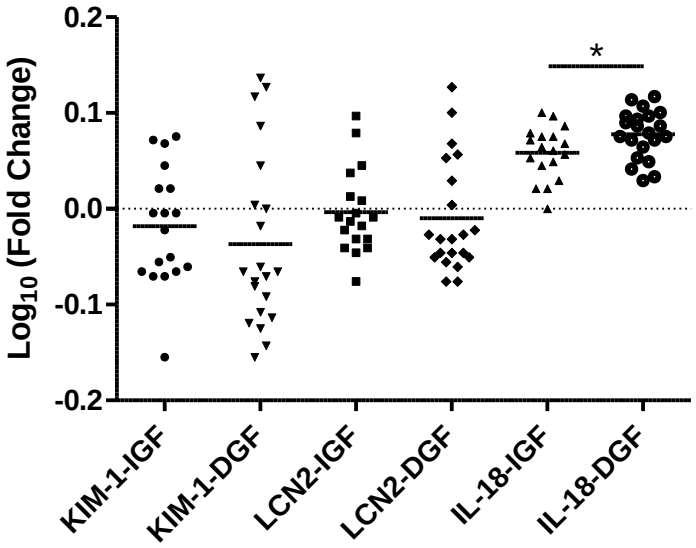


Figure 7-25: Biomarker expression stratified by graft function.
Zero on the y-axis represents the mean nephrectomy value for each biomarker. *p<0.05, Mann-Whitney test.

7.3.17.4 Biopsy gene expression does not reflect donor demographics, cold ischaemic time or graft function at 7 days

Biomarker gene expression did not correlate with donor age, donor serum creatinine at donation, donor type, cold ischaemic time or graft function at day 7 (Spearman correlation, p=ns).

7.3.17.5 Biopsy gene expression correlates with urine KIM-1 and IL-18 at 24 hours.

Spearman correlations were performed between individual biomarker gene expression values and plasma and urine levels of the biomarkers. There was no correlation with plasma biomarkers at any time point until day 6. However, urine KIM-1 and urine IL-18 in the 24 hour sample both correlated with KIM-1 and IL-18 gene expression ($r = 0.43$, $p=0.009$ and $r = 0.35$, $p=0.04$, respectively)

7.3.17.6 Class comparison reveals no difference in gene expression between the EPO group and the placebo group.

All probe sets were IQR filtered prior to performing a class comparison between the EPO and placebo groups. Correcting for multiple testing, no probe sets were significantly different between groups. Sub-group analysis in the NHBD and ECD groups comparing DGF with IGF cases also did not identify any significantly different probe sets.

7.3.17.7 IRITD3 and KT2 expression

IRITD3 expression ("injury-up") and KT2 expression ("injury-down") PBTs were compared between patients with IGF and those who went on to develop DGF (Figure 7.26). There was no difference between the groups.

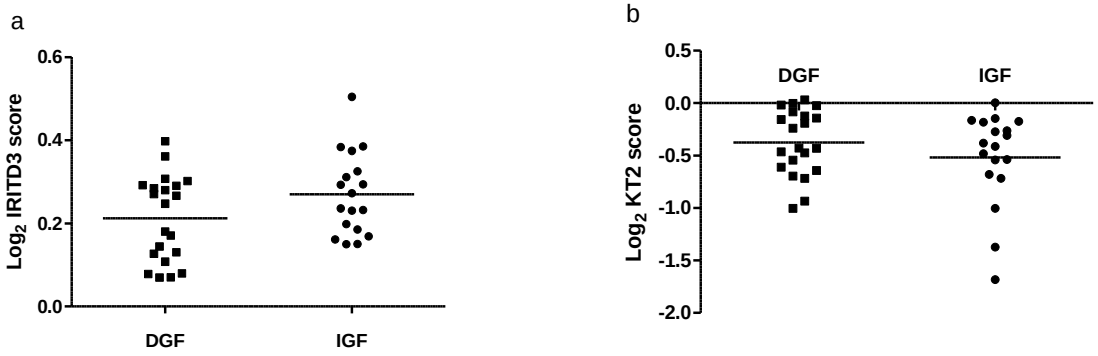


Figure 7-26: Injury-up and injury-down PBTs stratified by graft function.
Pathogenesis-based transcript scores were log transformed: (a) IRITD3 and (b) KT2. Zero on the y-axis represents the mean nephrectomy value for each biomarker. No difference between groups.

There was no difference in IRITD3 and KT2 expression between the trial drug groups (Figure 7.27).

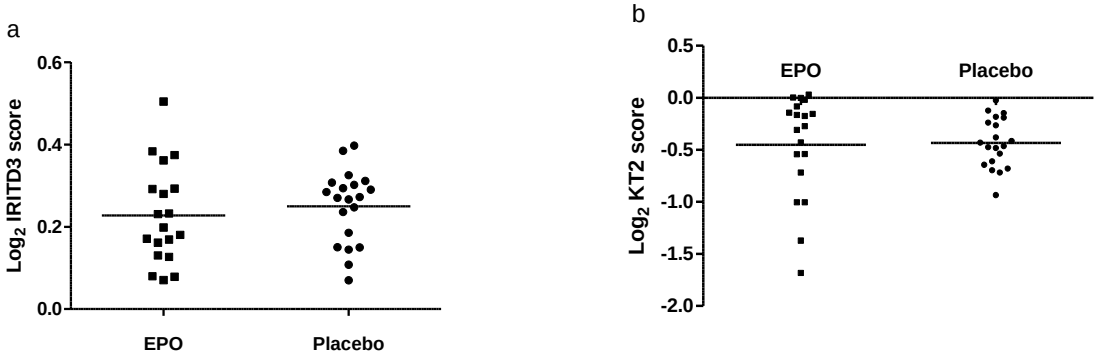


Figure 7-27: Injury-up and injury-down PBTs stratified by trial drug.
Pathogenesis-based transcript scores were log transformed: (a) IRITD3 and (b) KT2. Zero on the y-axis represents the mean nephrectomy value for each biomarker. No difference between groups.

7.4 Discussion

This is the most comprehensive study to date examining both plasma and urine biomarkers, in addition to gene expression, in recipients of kidneys that are seriously injured, in the case of non-heart-beating donors, or highly susceptible to injury, as is the case with extended criteria donors. The primary aim of this study was to identify potential differences between EPO- and placebo-treated kidney transplant recipients in terms of severity of injury following reperfusion, using the biomarkers NGAL, IL-18 and KIM-1 as well as microarray analysis. No difference was seen between the treatment groups at any time point, in keeping with the clinical data results. The secondary aim of this study was to investigate the natural history and potential benefits of these biomarkers in a kidney transplant population at high risk of severe injury and DGF. The ability of urine biomarkers to predict DGF was severely confounded by post-operative anuria preventing sample collection and high baseline native urine levels in those with residual renal function. Both plasma and urine NGAL were the best predictors of delayed graft function compared to KIM-1 and IL-18. However, none of the biomarkers were superior to the traditional methods of serum creatinine measurement and urine output monitoring, in detecting DGF. Gene expression in post-reperfusion biopsies did not identify patients at risk of DGF. The present study questions the reported benefits of NGAL, IL-18 and KIM-1 measurement in predicting delayed graft function in a kidney transplant population with a greater than 50% risk of delayed graft function. It also introduces a novel method of analysing the therapeutic effect.

The seminal paper of Parikh et al., which examined urine NGAL and IL-18 in kidney transplantation, found that at 24 hours, both had an AUC of 0.9 for the prediction of delayed graft function (109). In the EPO study however, neither biomarker achieved this AUC within 24 hours - a finding also shown in a later study from the same group (124). Although the studies had similar sizes, two major differences exist between the studies. The DGF rate in their population was 20% compared to >50% in the present study. Furthermore, they sampled the urine at any time within the first 24 hours, as opposed to set time collections. Interestingly, while urine NGAL levels were similar between the studies, IL-18 levels were markedly different (pg/mgCr vs ng/mgCr). From this EPO study, it would appear that biomarker levels do not change within the first 24 hours, even in the most severely injured kidneys, compared to pre-reperfusion levels, questioning the validity of either biomarker being a useful predictor of DGF at 24 hours.

The molecular mechanisms underlying the induction of NGAL and IL-18 following ischaemia-reperfusion injury in kidney transplantation are poorly understood. Experimental models have shown that NGAL mRNA expression is significantly increased with detectable protein in proliferating cell nuclear antigen-positive proximal tubule epithelial cells (96;425), suggesting a role for NGAL in the repair process. NGAL is also an iron transport protein and thus may be a player in cytoprotection during oxidative stress. Exogenous administration of NGAL has been shown to ameliorate ischaemia-reperfusion injury in experimental models (99). However, this latter finding is controversial as NGAL -/- mice exposed to IRI and given exogenous NGAL did not show a treatment benefit (102). In the EPO study, high baseline NGAL levels did not reduce the incidence or severity of DGF. IL-18, an inflammatory mediator, is activated by cleavage by caspase-1 and appears to be directly involved in the pathogenesis of ischaemia-reperfusion injury. Caspase-1 deficient mice had low levels of IL-18, were protected from acute kidney injury (126). Use of IL-18 anti-serum in ischaemic acute kidney injury also results in protection from injury (125;126). IL-18 has been also shown to increase cell adhesion molecule expression, leading to leukocyte infiltration, which is also known to play a pathogenic role in ischaemic injury (127).Thus, NGAL and IL-18 in addition to their biomarker status, play key roles in mediating injury or the injury-repair response.

Despite recent findings in acute kidney injury, KIM-1 in plasma or urine had no ability to differentiate between DGF and IGF. KIM-1 levels have been shown to be predictive of the

need for dialysis in hospitalised patients with acute kidney injury (117), with specificity for ischaemic injury and relatively unaffected by chronic kidney disease (121). In kidney transplantation, tissue KIM-1 expression and urinary KIM-1 was > 2 fold higher in brain dead donors when compared to living donors prior to transplantation (426) and is likely acting as a marker for acute kidney injury in the donor (123). However, two studies have also shown that KIM-1 was not predictive of DGF in the early post-operative period (123;427). During this post-operative period, energy is required for the generation and shedding of KIM1 into the urine, which cold storage and the resulting energy depletion will limit (112). Urine KIM-1 levels in the EPO study were similar to previous studies both in the transplant setting and in native acute kidney injury (124;398). Thus there is a discrepancy between KIM-1's sensitivity for detecting tubular injury (levels increase significantly over time post-reperfusion) and its ability to detect injury severity (the incidence and duration of DGF). KIM-1 appears to be important in repair following injury, facilitating the removal of debris and attenuating the pro-inflammatory response (114). There may be a ceiling effect where increasing severity of injury does not lead to greater production of KIM-1. Furthermore, renal function requires an intact nephron. Hypoxic injury may only damage the highly metabolically active Loop of Henle with shedding of KIM-1 in this area, yet the whole nephron will shut down, giving a disproportionate functional response to a limited area of injury.

Plasma biomarkers are a more reliable means of diagnosing DGF and quantifying it's severity than urinary biomarkers. There are many theoretical advantages to the use of urinary biomarkers, including non-invasive testing, a direct link to the site of injury and potentially large quantities of urine available for sampling. However, the greatest limitation of a urinary biomarker study is the inability to collect samples in anuric patients, as seen in this study with incomplete sample collections at all time points. It is also impossible to separate the contribution from the residual native renal function, which can impact on NGAL levels. Native kidney injury during hypotensive episodes intra-operatively or increased baseline levels due to chronic kidney disease or other chronic conditions (reviewed in (129)) may all serve to confound level interpretation. Finally, it is known that urine NGAL degrades significantly (21%) after 24 hours when stored at 4°C (428). The effect of storage in a urinary catheter bag at room temperature has never been assessed but is likely to be greater. Plasma levels can be collected and processed at any specified time point, particularly pre-reperfusion in anuric patients. This allows for the correction of the post-reperfusion levels to baseline

levels and allows the correct interpretation as to whether levels are changed as a result of reperfusion. Furthermore, of all the biomarkers tested, plasma NGAL had the best AUC at 24 hours, with a sensitivity of 85% and a specificity of 83%.

Microarray analysis offers a novel means of identifying tissue injury on a molecular level, offering both mechanistic insights and diagnostic potential. The identification of IL-18 as an early marker of injury is a novel finding, potentially predicting injury severity through separation of the DGF and IGF groups. This supports in vitro findings of increased IL-18 gene expression in RPTECs only exposed to cold injury. Also interesting is the correlation between gene expression and urine levels IL-18 and KIM-1 at 24 hours, but not earlier. Pre-anastamosis KIM-1 levels have previously been shown not to correlate with post-reperfusion levels until day 2 (123), supporting this finding. Furthermore, KIM-1 gene expression did not predict DGF, which again is a similar finding to this study. Class comparison did not identify any features differentiating the EPO from the placebo group, nor the DGF group from the IGF group. Only one study to date has shown that microarray analysis is capable of discriminating biopsies at increased risk of DGF from those with IGF (422). Specifically, they can identify living donors, from deceased donor kidneys, but also deceased donor kidneys with IGF from those with DGF. Methodology in both studies was similar with identical specimen preservation and the Edmonton group processing and analysing both sets of data. However, the populations were markedly different in terms of severity of injury, with a much lower incidence of DGF and no NHBD kidneys in the Edmonton study.

The increasing use of marginal donors, with their increased susceptibility to ischaemia-reperfusion injury, is leading to a dramatic increase in the rate of DGF, which has marked consequences both for the individual recipient and the transplanting centre. In this study, over 50% of patients had DGF, with an average time to last dialysis of greater than six days. Recognised co-morbidities associated with DGF include an increased risk of rejection, increased immunosuppression burden with its infective consequences and diminished nephron mass (429;430). The financial costs to the transplant centre are increased, with increased length of stay and greater dialysis requirement. Studies in this area are severely hampered by the multiple definitions of DGF and subjective nature on which they are based. Thus, biomarkers may provide an objective means of either re-defining DGF or assessing current definitions to improve diagnostics in this area. Furthermore, they have the potential to be a sensitive theranostic through which to assess new novel treatments.

There are also important design limitations to this study, including its small sample size, possible confounding effects of EPO therapy and the lack of formal post-transplant dialysis criteria. Biopsies were taken within 30 minutes of reperfusion, which may not have allowed sufficient time to elapse to enable alterations in gene expression to occur (the Edmonton study biopsied at 1 hour post-reperfusion (422)). Finally, the means of procuring the biopsy was left to the discretion of the operating surgeon. Thus, there was a mixture of wedge and core biopsies performed, with varying degrees of cortical sampling. If genes are specific to the proximal tubule, as KIM-1 is, this is likely to confound expression levels.

In summary, we have not demonstrated a significant effect of EPO on gene expression or on plasma and urine NGAL, IL-18 and KIM-1 levels. We have shown that plasma and urine NGAL are the best markers of delayed graft function, although at similar time points to traditionally used clinical parameters. KIM-1, in keeping with earlier studies, does not appear to have a role in the transplant setting. Finally, plasma biomarkers are a more reliable means of measuring biomarker levels, given frequent oligo-anuria in the early post-operative period. Microarray studies while offering mechanistic insights have yet to demonstrate their true potential as diagnostic and prognostic tools. Larger studies are needed to examine the potential roles of these biomarkers in kidney transplantation using marginal donors. It is likely that in these studies, a combination of techniques or biomarkers, will provide the sensitivity and specificity with which to accurately define severe ischaemia-reperfusion injury and assess novel treatment strategies.

CHAPTER 8 METABOLOMICS AND KIDNEY TRANSPLANTATION

8.1 Introduction

The disease phenotype is the end result of interactions between genetic and environmental factors, with the phenotype most often measured through gene transcripts, proteins and metabolites. While there has been much interest in proteomics and transcriptomics in the field of transplantation (423;431-433), little has been done examining the role of metabolites in terms of molecular pathophysiology or as potential biomarkers of acute kidney injury.

Metabolomics is the study of low molecular weight biochemicals or metabolites (molecular weight<1500Da) in biological systems (384). An estimated 7800 metabolites are present in the human metabolome including amino acids, sugars, lipids, nucleic acids and organic acids, arising from either endogenous metabolism or absorption from the external environment (food, drugs) and associated exogenous metabolism (http://www.hmdb.ca/) (434). Metabolomics applies both hypothesis generating strategies (discovery) and hypothesis testing strategies (validation) (435). Metabolomics is regularly applied to define a phenotype, be it at a cellular, tissue, organ or organism level. Its' advantage lies in that it examines the final downstream product of cell function (transcription and translation) and thus is potentially a closer reflection of the phenotype of the organism than transcriptomics or proteomics. Furthermore, the metabolome is a rapid indicator of system perturbations, with changes in metabolites (synthesis or consumption) occurring over seconds to minutes, as opposed to minutes to hours for mRNA and protein synthesis. These rapid intra-cellular changes are for the most part allosteric in nature, while changes in gene expression and protein levels are slower and part of the adaptive response. Most measured metabolites are involved in ubiquitous metabolic processes, such as glycolysis or lipid metabolism. However, quantitative differences in these universal metabolites, such as citrate or lactate or acetate, have been shown to reflect the apoptosis, hypoxia and acid/base dysregulation that occurs as a result of ischaemia-reperfusion injury (436).

To date, there have been few studies examining the metabolome in kidney transplantation, predominantly in experimental models with only a single human study (437). All have been

discovery phase studies. Animal models have identified a number of potential markers of ischaemia-reperfusion injury. Trimethylamine-N-oxide increases markedly in both serum and urine in response to damage to the renal medulla, a common finding in transplantation injury (436). Plasma allantoin, an oxidative product of uric acid that acts as a marker of oxidative stress, correlates with the duration of cold ischaemia and plasma TMAO levels (438). N-acetylcysteine pre-treatment for ischaemia-reperfusion injury has also been shown to reduce allantoin and TMAO levels (439). The single human study examined hypoxanthine and inosine levels, known markers of oxidative damage, directly in the transplant renal vein and identified significant increases over time (440). Thus there is potential to use metabolomics to assess ischaemia-reperfusion injury following kidney transplantation and indeed as a functional assay in testing a putatively tissue protective agent.

In this study, we report the use of discovery-phase (inductive) metabolomics in human kidney transplantation, applying Ultra Performance Liquid Chromatography – Mass Spectrometry (UPLC-MS). The primary goal of this study was to assess the longitudinal effects of EPO compared to placebo during renal transplantation. The high incidence of delayed graft function and poor urine output at 24 hours resulted in an insufficient sample size to study the urine metabolome in this pilot study. Thus, we examined serum alone taken pre-reperfusion, 24 hours post reperfusion and at 6 days to determine difference in the metabolome related to high dose NeoRecormon® therapy.

8.2 Methods

8.2.1 Collection of serum samples

Ethical approval for collection of serum samples was obtained as part of the EPO Study (LREC 07/Q1407/94; EudraCT No. 2006-005373-22). 10mls of whole blood was collected in serum collection tubes (BD Vacutainers®, Oxford, UK) prior to infusion of the trial drug (~20 minutes before reperfusion), 24 hours post-reperfusion and at 6 days. This was stored for a maximum period of 24 hours at 4°C prior to centrifugation and plasma collection. Aliquots were then transferred into 1ml centrifuge tubes and frozen and stored at -80°C. All 3 samples were collected for all 39 patients studied. From the study population, 22 male

patients were selected on the basis of similar age and BMI from both the EPO (n=10) and placebo (n=12) group (2 male placebo recipients were excluded due to their high BMI). Females were excluded to eliminate gender as a confounding variable.

8.2.2 Statistical analysis

Comparisons of clinical data between cases and controls were performed using the Student t-test, Mann–Whitney test, $\chi 2$ test or Fisher exact test, as appropriate. For each metabolite peak reproducibly detected in the discovery phase study, the null hypothesis that the means of the EPO and Placebo sample populations at each time-point were equal was tested using either the Mann–Whitney test or Student t-test, depending on data normality. The critical p-value for significance was set to 0.05. No correction for multiple comparisons was performed at this point, because the aim was to reduce the many thousands of detected features down to a subset of potentially "information-rich" peaks while keeping the number of probable false negatives (type II error) to a minimum, That said, it was important to be aware of the potential for a number of false positives to be reported in the univariate results. However, as this was a discovery phase study and the sample numbers were low it was more relevant to be inclusive rather than exclusive in the reporting of results at this point.

The metabolite data for each patient was then normalised to their pre-reperfusion levels to reduce the impact of transplantation as a confounding variable, thus reducing the data set to two measurements per patient, per metabolite, $\Delta 24$ & $\Delta 6$ (i.e. relative change in metabolite level from pre-reperfusion to 24 hours, and relative change in metabolite level from pre-reperfusion to 6 days). Univariate statistics were repeated looking for differences between EPO and Placebo. To uncover multivariate structure in the data, the significant features at each time point were combined into a single multivariate Principal Component Analysis (PCA) model(441). In this way correlated variance in the data could be assessed.

To model the data further, linear discriminant analysis was performed by way of Principal Component Canonical Variate Analysis (PC-CVA). This supervised statistical method is a multivariate equivalent of univariate hypothesis testing and is mathematically similar to MANOVA (multiple analyses of variance). It differs from MANOVA in that the initial Principal Component stage of the analysis projects the many thousands of detected features into a smaller "information rich" set principal component variables (typically 10-20) that are

then discriminately modelled using CVA(442). The resulting models demonstrate the significant differences in metabolite profile as a whole, rather than testing each metabolite individually. All supervised models were cross validated using K-fold methods.

8.2.3 Metabolite identification

A metabolite feature is defined by a unique accurate mass and/or retention time and a single metabolite can be detected as multiple different metabolite features, each with a different accurate mass and same retention time (443). Metabolite features were putatively identified using a metabolite identification pipeline operated in the Taverna Workflow Management System (444). The set of workflows annotate metabolite features and group metabolite features originating from the same metabolite (retention time error range, 3 seconds), match the accurate mass to all possible molecular formula within a given mass error range (+/- 3ppm) and then match the molecular formula to a reference file of metabolites (Manchester Metabolomics Database, MMD; (443;444). More than one metabolite can be assigned to a single metabolite feature because of similar accurate masses or the presence of chemical isomerism (for example, glucose and fructose are stereoisomer's with the same molecular formula and accurate mass). The Manchester Metabolomics Database is constructed with information from the Human Metabolome Database (http://www.hmdb.ca/), Lipidmaps (http://www.lipidmaps.org/) and metabolic reconstructions (e.g. [(445)]).

8.3 Results

8.3.1 Demographics

Donor and recipient demographics in addition to details specific to the transplantation process are shown in Table 8.1. There was no significant difference in donor or recipient age, sex and BMI between the two groups. All donors were white in ethnicity. Recipients in the EPO group were more likely to be white although this did not reach statistical significance (p=0.06). Prior to the first blood sample, all patients were fasting for 12 hours before undergoing general anaesthesia. Basiliximab and methylprednisolone were given prior to reperfusion in all patients. Maintenance immunosuppression was started post-reperfusion

with patients in the placebo group more likely to be on triple immunosuppression (p=ns). This reflects the transplant unit policy of triple immunosuppression in recipients with delayed graft function and higher rate of delayed graft function on day 1 in the placebo group. By day 6, similar numbers in both groups remained dialysis dependent. Interestingly, recipients with delayed graft function in the EPO group had quicker recovery of their renal function as evidenced by fewer dialysis episodes, although this did not reach statistical significance.

Table 8-1: Demographics of sub-population selected for the metabolomics arm of the EPO Study. Data shown are mean ± SD unless otherwise stated. AA: african-american; BMI: body mass index; RRF: residual renal function; ESRD: end stage renal disease; sCr: serum creatinine; ECD: extended criteria donor; NHBD: non-heart-beating donor; CIT: cold ischaemic time; DGF: delayed graft function; * one donor contributed two kidneys

Recipient Variable	**EPO**	**Placebo**	***p=***
Male gender (n=)	10	12	*ns*
Age (yrs)	48 ± 11	53 ± 14	*ns*
Race (White/AA/Asian) (n=)	5/4/1	11/1/0	*p=0.06*
BMI	25 3	26 4	*ns*
No. with RRF (n=)	7	7	*ns*
Cause of ESRD (n=)			
- **Glomerulonephritis**	6	6	*ns*
- **Other**	4	6	*ns*
Donor Variable			
Male gender (n=)	5	7	*ns*
Age (yrs)	51 ± 15	47 ± 12	*ns*
Race (White) (n=)	10	12	*ns*
BMI	26 ± 3	27 ± 5	*ns*
sCr (µmol/L)	65 ± 18	80 ± 48	*ns*
Donor type* (n=)			
- **ECD**	3	4	*ns*
- **NHBD**	7	7	*ns*
Transplant Specifics			
Immunosuppression (n=)			
- **Tacrolimus**	10	12	*ns*
- **Steroid**	7	12	*p=0.07*
- **Mycophenolate**	8	11	*ns*
CIT (mins)	1103 ± 344	1018 ± 279	*ns*
DGF on day 1 (n=)	5	9	*ns*
DGF on day 6 (n=)	4	4	*ns*
No. of dialysis episodes (n=)	2 ± 2	4 ± 5	*ns*

8.3.2 The effect of time and EPO on the metabolome

A PC-CVA discriminant model was built and cross-validated using the first 10 principal component scores (Figure 8.1). Dashed circles represent the 90% confidence intervals of the

group mean. Solid circles represent 95% confidence intervals of the group distribution. The quality control samples clustered together in a tight distribution demonstrating the high level of reproducibility of the sample preparation, the UPLC-MS analysis and data pre-processing steps, i.e. minimal experimental variation

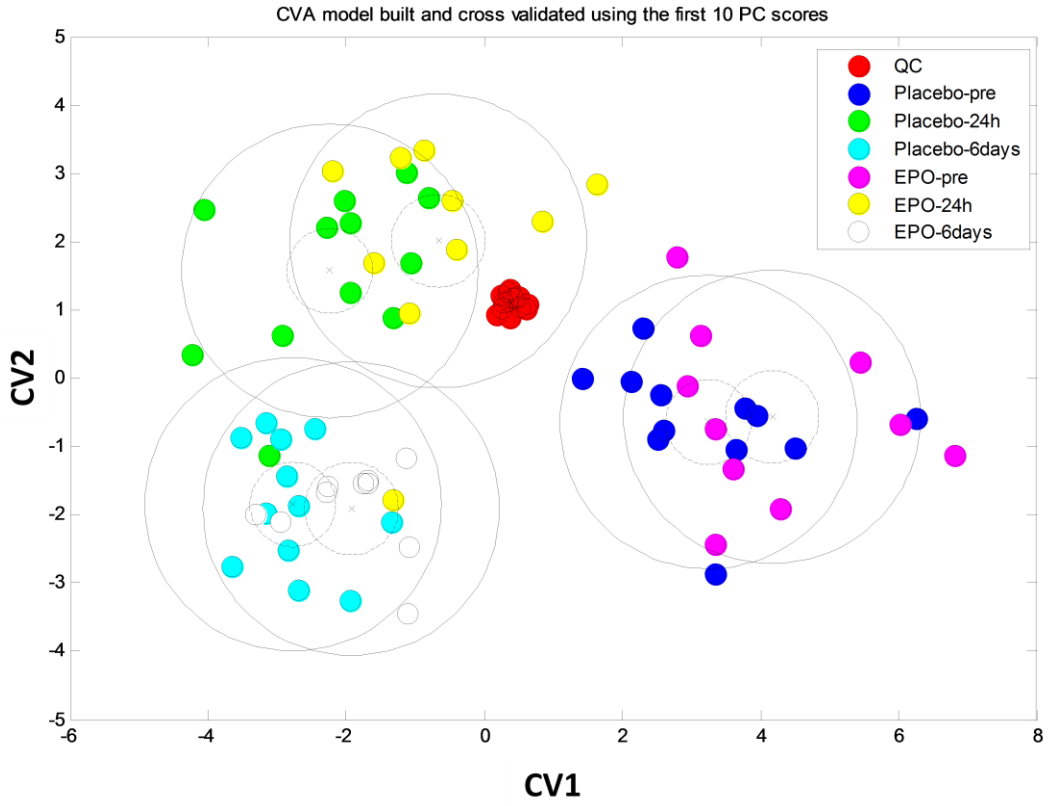


Figure 8-1: PC-CVA discriminant analysis of the metabolome over time.
This shows changes in the plasma metabolome due to transplantation and EPO treatment. Dashed circles represent 90% confidence intervals of the group mean. QC: quality control. Points that are close to each other are biologically similar. Transplantation and time have the greatest effects on the metabolome.

The largest changes in the metabolome were related to time of sampling, independent of EPO or placebo treatment, particularly between pre-reperfusion samples and post-reperfusion time points, as indicated by the leftward shift on the CV1 ($p<0.05$). Furthermore, the metabolome was also different between 24 hours and 6 days, but to a lesser degree, possibly reflecting elimination of anaesthetics or other medications, in addition to better renal function with removal of uraemic compounds ($p<0.05$). At 24hrs, only 7 (32%) of the study population were dialysis independent. This had increased up to 14 (64%) patients by day 6.

In the baseline samples taken prior to reperfusion, the confidence intervals of the mean overlap indicating that there was no significant difference between the two groups prior to receiving the trial drug and donor kidney, as is expected when applying a robust experimental design. At 24hrs, there is a statistical difference between the EPO and placebo groups which is then lost by 6 days. The difference between groups at 24 hours may be a reflection of the presence of trial drug compared to placebo or the effect of the EPO treatment on disease pathophysiology being reflected in the plasma metabolome. However, another possible explanation is that only 5 (50%) of the EPO group were dialysis dependent after day 1. In contrast, 9 (75%) of the placebo group were dialysis dependent. By day 6, dialysis dependency was similar in both groups at 4 (40%) of the EPO group and 4 (33%) of the placebo group – possibly explaining the PC-CVA results at day 6.

8.3.3 Metabolite features differentiating EPO from the placebo group at all time points

Out of a total of 4328 metabolite features, 599 (13%) differentiated the EPO group from the placebo group at a minimum of one of three time points by univariate analysis ($p<0.05$) (Figure 8.2). Examining this further, 188 features pre-reperfusion, 154 features at 24 hours and 199 features at day 6, independently identified patients in either group. The fact that 234 metabolite features differed between the EPO and placebo group ($p<0.05$) prior to the first infusion of the trial drug highlights the limitations of such a small study and the potential for both type I and type II errors.

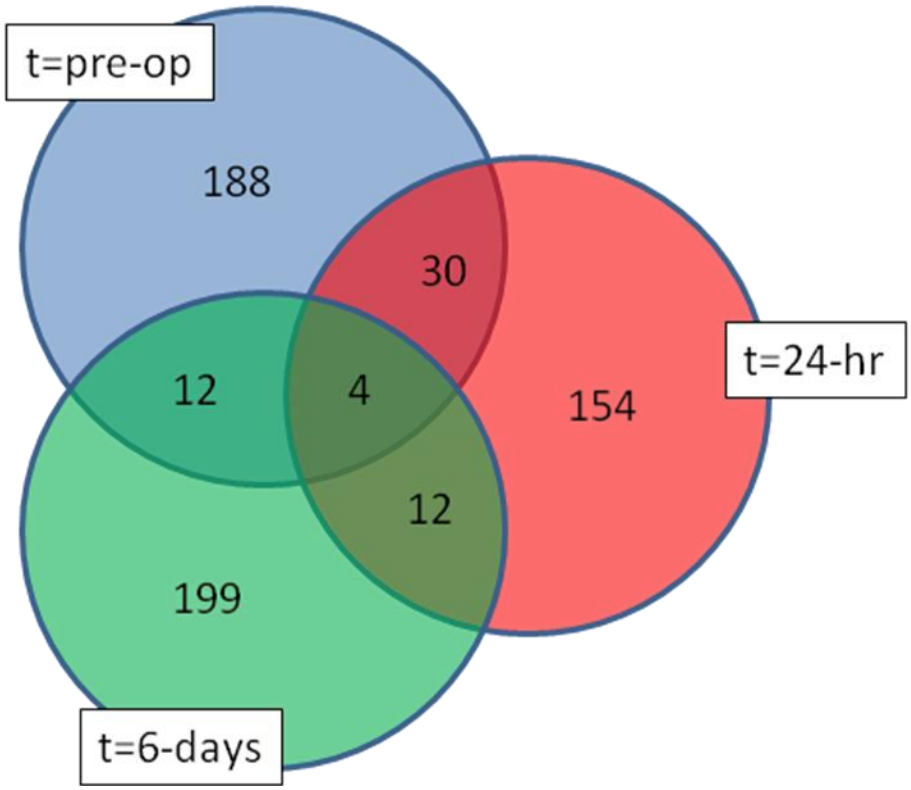


Figure 8-2: Metabolite features differentiating EPO from Placebo treatment at each time point. Univariate analysis ($p<0.05$)

8.3.4 Normalisation of the data to reduce the impact of transplantation as a confounding variable

To further separate the effects of transplantation from those of EPO or placebo treatment, all 24 hours and 6 day features were normalised to their pre-reperfusion sample, before performing PC-CVA (Figure 8.3). Again, time had the largest effect on the metabolome at 24 hours and 6 days. No effect of EPO was seen at either time point.

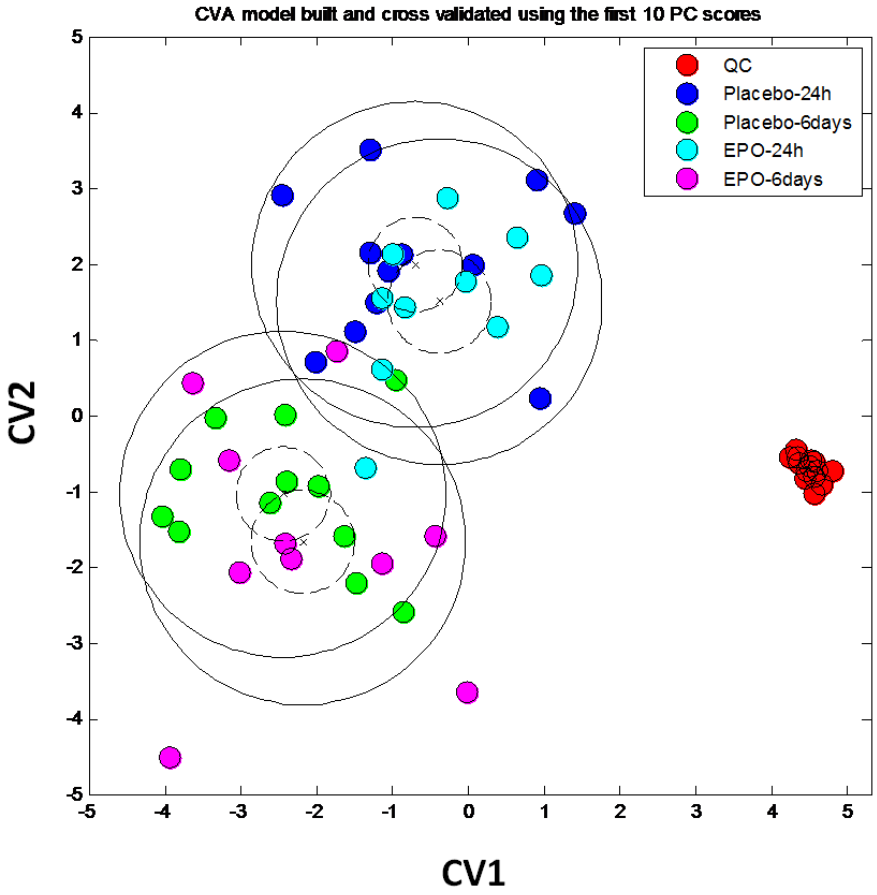


Figure 8-3: PC-CVA model of 24 hour and day 6 samples normalised against their pre-reperfusion samples.
This was done to eliminate the effect of transplantation on the analysis. QC: quality control or baseline point.

8.3.5 Metabolite features differentiating normalised treatment groups at 24 hours

To identify any potential differences between EPO and placebo groups, we focused on the normalised 24 hour and day 6 time points, screening for statistically significant peaks. Univariate analysis revealed that 81/3944 (2%) of metabolite features were significantly different between the two groups ($p<0.05$) at 24 hours. At 6 days, 131/3605 (3.6%) of metabolite features were significantly different ($p<0.05$). Combining both time points revealed an overlap of only 9 metabolite features, which is surprisingly small (Figure 8.4). It is likely that the trial drugs effect, given its pharmacokinetic profile(183), would extend out to day 6. However, recovery of renal function with increased clearance of uraemic toxins in addition to clearance of stopped drug metabolites may explain the lack of overlap.

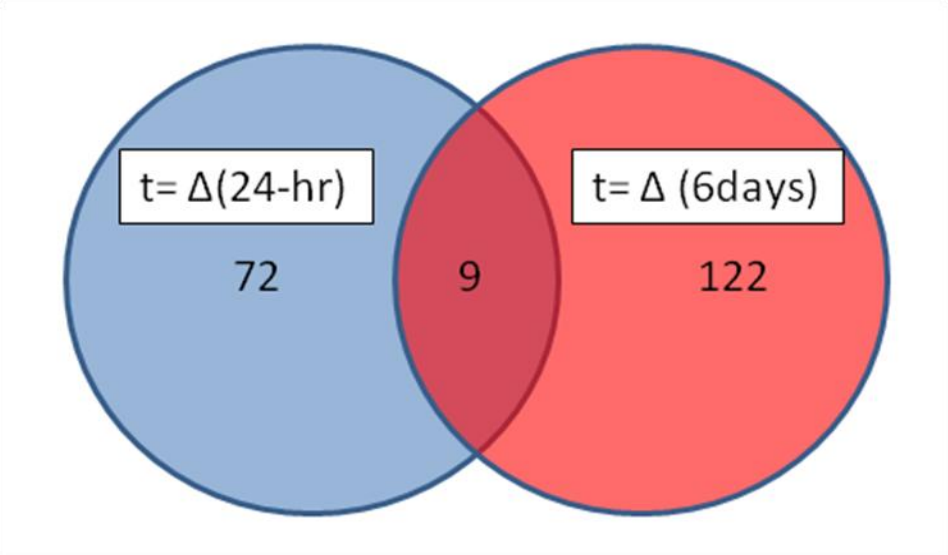


Figure 8-4: Venn diagram highlighting shared and differentiating metabolite features at 24 hours and 6 days in the normalised treatment group.

8.3.6 Principal Component Analysis in the normalised population at 24hrs and 6 days

To examine this potential difference between treatment groups at 24 hours, we performed Principal Components Analysis (PCA) in the patient population using the 81 metabolite features identified at 24 hours by univariate analysis in section 9.3.6 (Figure 8.5).

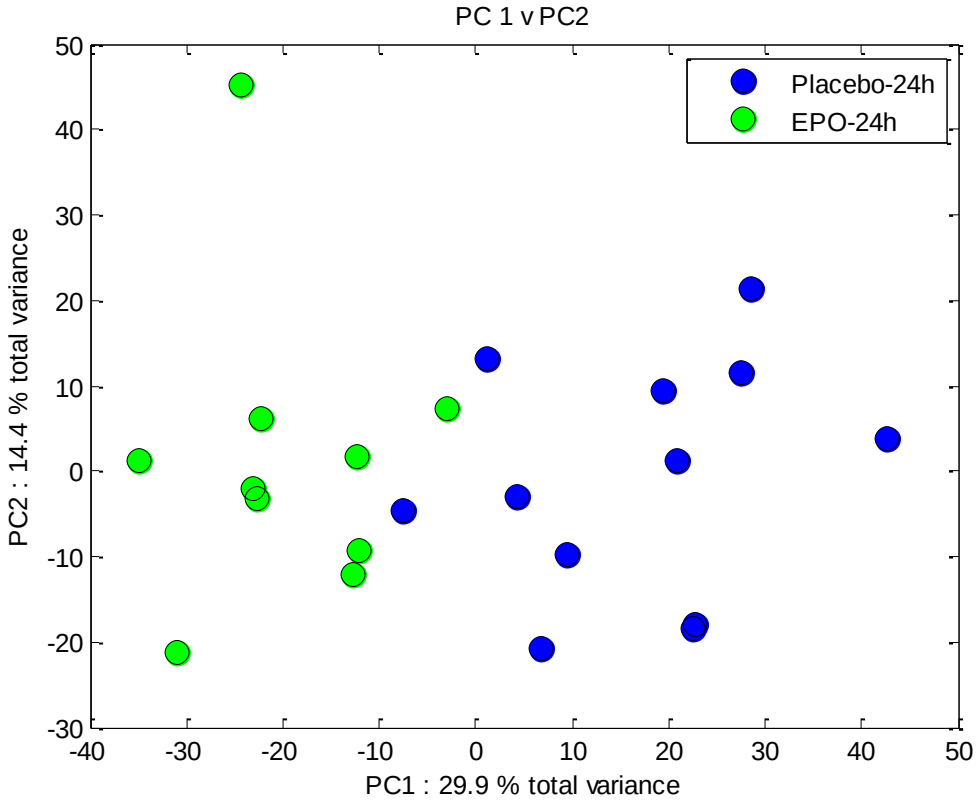


Figure 8-5: PCA plot using the 24 hours metabolites.
Principal component analysis in the study population using the 2% of metabolite features that were different at 24hrs, showing a clear differentiation between EPO and placebo.

Together PC1 and PC2 account for 44% of the total variance in the population, with PC1 clearly differentiating between EPO and placebo groups, thus confirming the potential significance of these 81 features. An example is shown below of one of the significant peaks (farnesyl diphosphate), which is split into treatment group and donor type (Figure 8.6). Zero on the y-axis is the pre-reperfusion sample mean for the metabolite. Farnesyl diphosphate, is significantly higher in the EPO group compared to the placebo group (p=0.001).

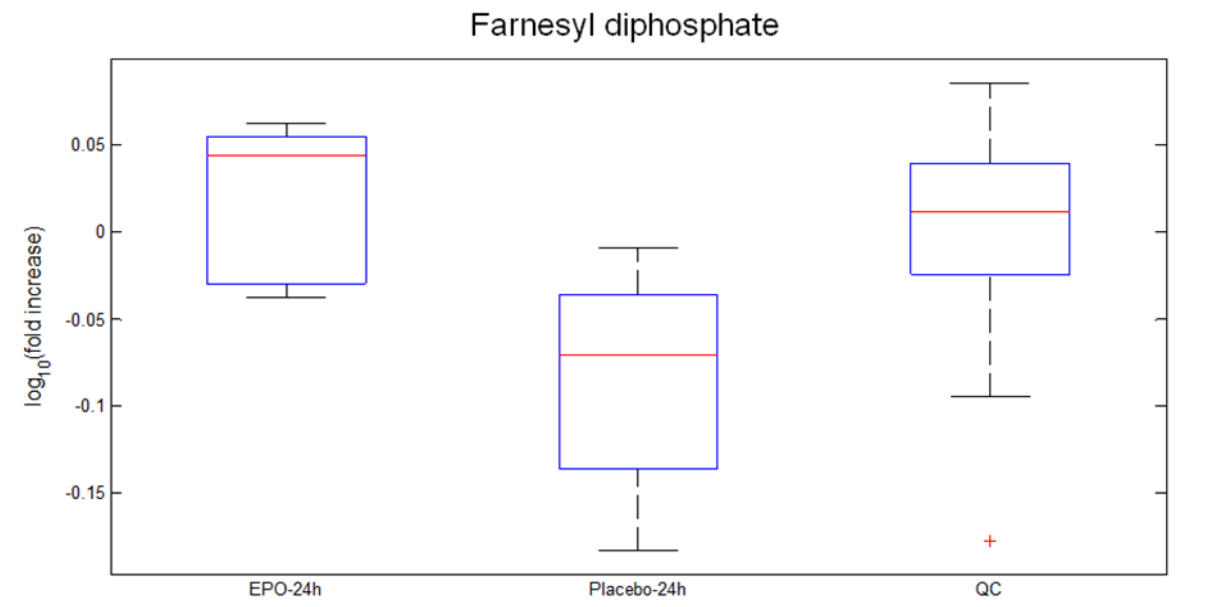


Figure 8-6: Farnesyl diphosphate differentiates EPO from placebo treatment.
Peak 4671, identified as farnesyl diphosphate, is an example metabolite feature stratified into treatment group that significantly differentiates EPO treatment from placebo treatment ($p<0.001$). QC: quality control.

Similarly, PCA was performed on the day 6 samples from the normalised population using the 131 metabolite features significant at day 6 ($p<0.05$) (Figure 8.7) as defined in section 1.3.5. Together, PC1 and PC2 accounted for almost 50% of the total variance, with PC1 accounting for the greatest difference between the treatment groups. Unsupervised PCA confirmed the ability of the peaks identified through univariate analysis in separating the two groups, particularly at 24hrs. We then focused specifically on this time point to identify the metabolite features.

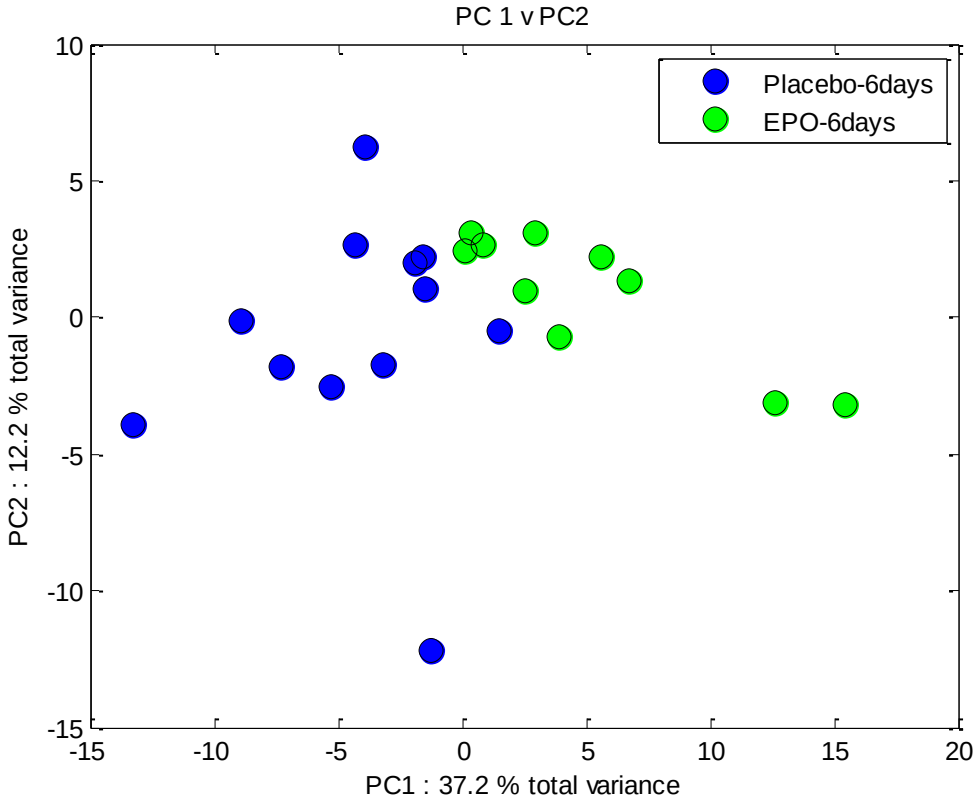


Figure 8-7: PCA using day 6 metabolites.
Principal component analysis in the study population using the 131 metabolite features that were significantly different at 6 days, showing a clear differentiation between EPO and placebo.

8.3.7 Specific metabolites have roles in inflammation and oxidative stress at 24 hours

Of the 81 features significantly different at 24 hours between EPO- and placebo-treated groups, 45 metabolites were putatively identified, on the basis of their mass to charge ratio and retention time. While many of these cannot been linked with specific biochemical processes related to ischemia-reperfusion, a number can be linked to vitamin D metabolism or oxidative stress. Interestingly, only one inflammatory metabolite, leukotriene C5, was identified in this analysis.

Vitamin D has multiple biochemical actions including anti-inflammatory and anti-fibrotic effects (446;447). The vitamin D_3 analogue, 1α, 25-dihydroxy-2α-(3-hydroxypropoxy)-19-norvitamin D_3, is present at a significantly higher concentration in EPO treated patients compared to the placebo group ($p<0.001$) (Figure 8.8). Concentrations in placebo patients are markedly diminished compared to the pre-reperfusion time point. This suggests that EPO

treatment maintains vitamin D levels, which have previously been shown to be important during oxidative stress.

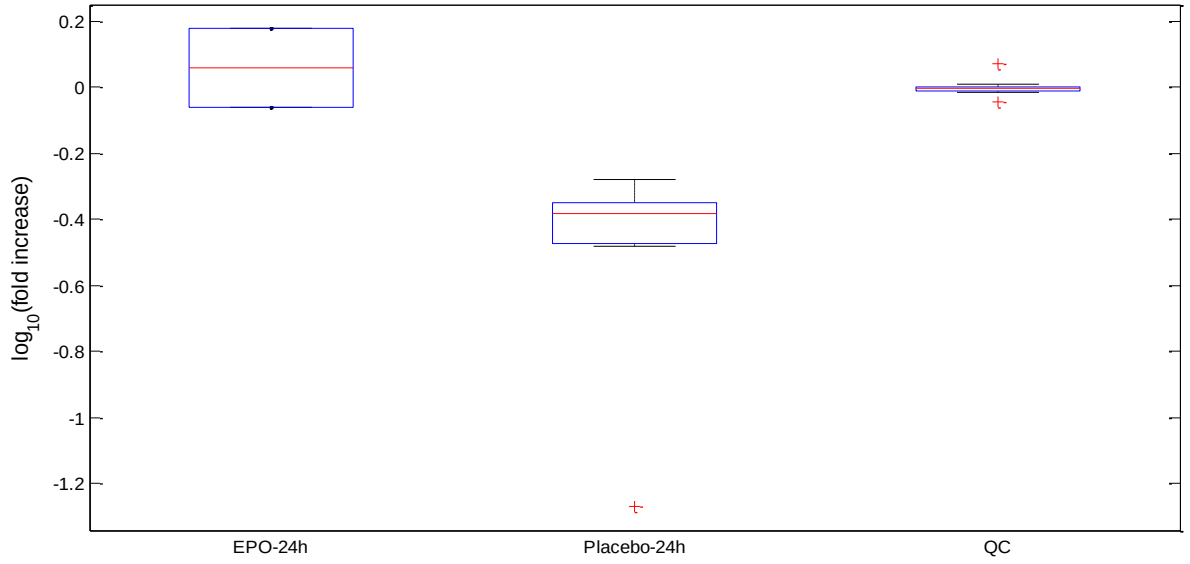


Figure 8-8: Vitamin D3 metabolite differentiating EPO from placebo treatment.
Vitamin D3 analogue metabolite stratified into treatment group and donor type that significantly differentiates EPO treatment from placebo treatment ($p<0.001$). QC: quality control or baseline level.

Leukotriene C5, an inflammatory metabolite with vasoconstrictive effects, was also significantly lower in EPO treated patients compared to placebo ($p=0.007$) (Figure 8.9).

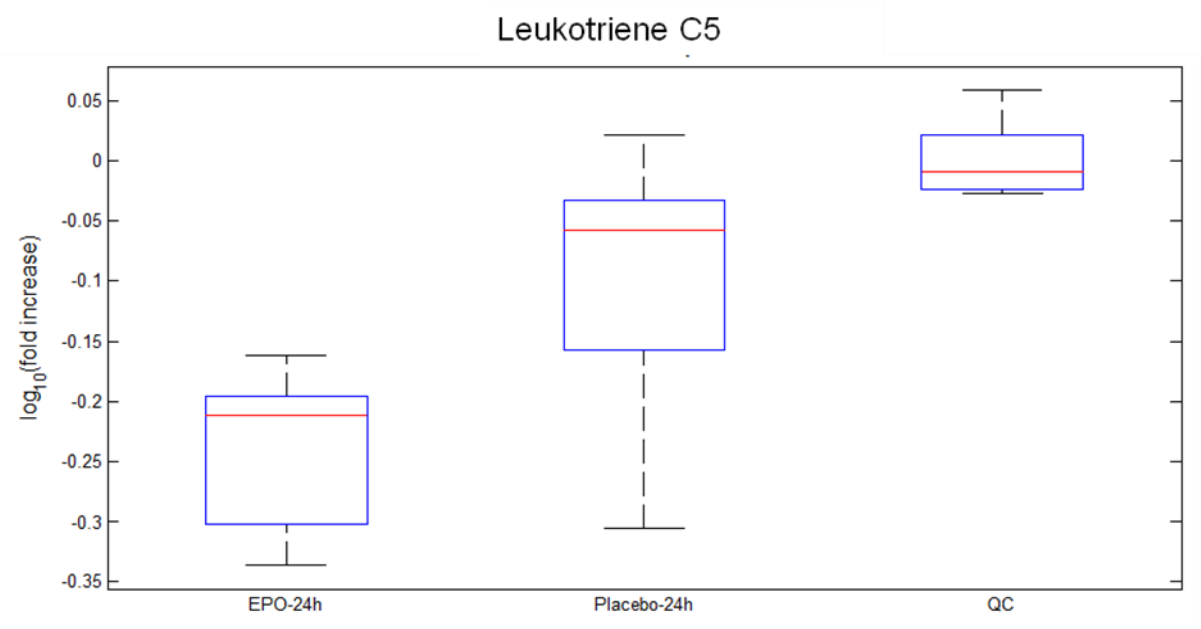


Figure 8-9: Leukotriene C5 differentiates EPO from placebo treatment.
Leukotriene C5 metabolite stratified into treatment group and donor type significantly differentiates EPO treatment from placebo treatment ($p<0.001$). QC: quality control or baseline level.

Spermidine and malonylcarnitine are metabolites which accumulate in disorders of fatty acid metabolism in the mitochondria such as in oxidative stress (Figure 8.10 and 8.11). Concentrations of both metabolites were lower in the EPO group suggesting a possible mechanism for EPO tissue protection.

Figure 8-10: Spermidine differentiates EPO from placebo treatment.
Spermidine stratified into treatment group significantly differentiates EPO treatment from placebo treatment (p=0.045). QC: quality control or baseline level.

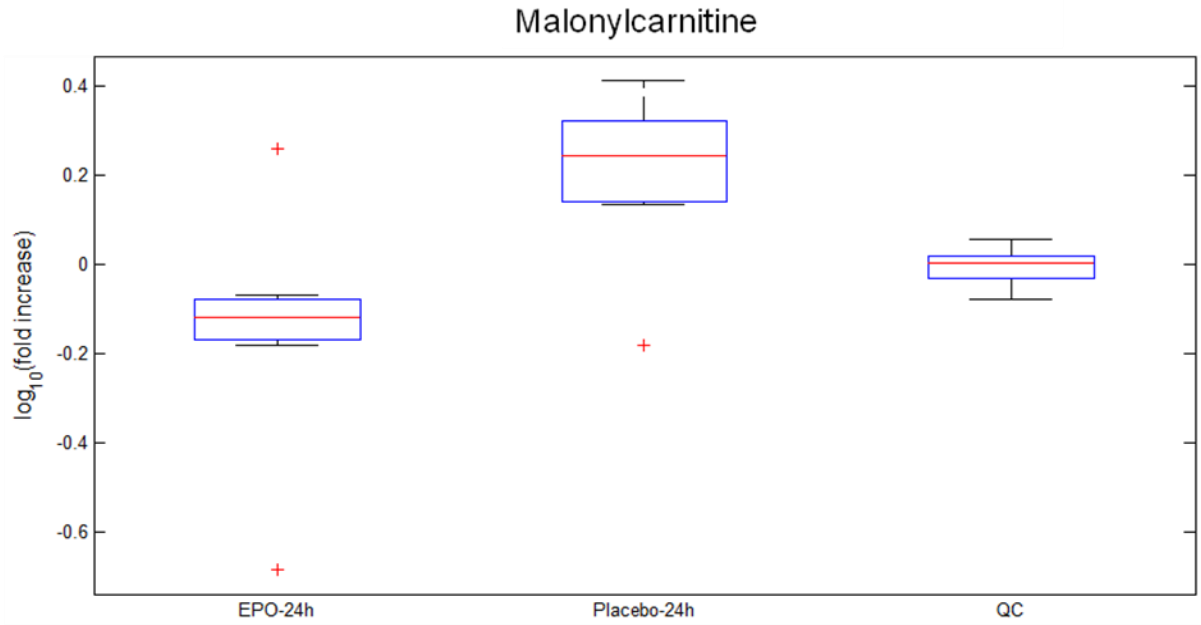


Figure 8-11: Malonylcarnitine differentiates EPO from placebo treatment.
Malonylcarnitine stratified into treatment group significantly differentiates EPO treatment from placebo treatment (p<0.019). QC: quality control or baseline level.

8.3.8 Subgroup analysis in NHBD and ECD recipients

There were too small a number in the EPO and placebo group, when stratified by donor type, to do any meaningful statistical analysis.

8.4 Discussion

This is the first proof of principle study in kidney transplant recipients, examining the impact of ischaemia-reperfusion on the metabolome in the early post-operative period. The most significant changes observed in the metabolic profiles were pre- and post-transplant, irrespective of EPO or placebo treatment. A smaller change in the metabolic profiles was observed when comparing EPO and placebo, of which the greatest changes were observed at 24 hours. Normalisation to pre-reperfusion samples (on a per subject basis) to eliminate the effect of transplantation, did not identify any differences between EPO and placebo in a multivariate PC-CVA model. Using a univariate approach to this discovery phase, diverse areas of metabolism were influenced by the administration of EPO, including vitamin D metabolism and oxidative stress. These data support the hypothesis that metabolomics has the potential to elucidate molecular pathophysiology involved in renal tissue injury post-transplant and to monitor the effects of a drug intervention.

As expected, the greatest influence on the metabolome is due to transplantation and time of sampling. This most likely represents a combination of anaesthesia, surgery, ischaemia-reperfusion injury, stoppage of unnecessary medication (e.g. all angiotensin II converting enzyme inhibitors and phosphate binders are stopped pre-theatre) and the addition of new medications (immunosuppression, infection prophylaxis). At 24 hours, recovery of renal function is unlikely to be a contributory factor as two thirds of the recipients were still dialysis dependent. In a multivariate and cross-validated PC-CVA model PC2 differentiated the metabolome at 24 hours and 6 days with two thirds of the population having recovered sufficient function to clear uraemic products and remain off dialysis. Again this difference may also be partly explained by further clearance of pre-operative drugs or anaesthesia, in addition to clearance of the study drug, EPO. Thus, it is possible to monitor graft progression in the early transplant period in human biofluids which may provide a novel biomarker or

panel of biomarkers to predict delayed graft function and the beginning of functional recovery.

EPO treatment significantly altered the metabolome compared to the placebo group at 24 hours with the difference lost by day 6, as shown in a PC-CVA model. This effect was lost once the post-reperfusion samples were normalised to the pre-reperfusion baseline samples, in keeping with the lack of clinical efficacy in the clinical arm of this study. In particular, the small numbers in this study increase the likelihood of both type I and type II errors in the analysis, which are limitations to this analysis. However, both univariate analysis followed by unsupervised Principal Components Analysis appear to identify a number of interesting changes in the metabolome as a result of treatment, at 24 hours, which may offer some insight into the putative tissue protective effects of EPO.

The identification of a vitamin D analogue, 1α,25-dihydroxy-2α-(3-hydroxypropoxy)-19-norvitamin D_3, is surprising, potentially reflecting an anti-apoptotic, anti-oxidant or anti-inflammatory mechanism of EPO protection. Most of the study population take 1α hydroxy vitamin D_3, as part of their maintenance treatment for hyperparathyroidism (this data was not collected). Thus it would be expected that the class comparison would eliminate this as a confounding variable. However, the EPO group have similar levels to the pre-reperfusion sample while the placebo group have a significantly lower level ($p<0.001$). Vitamin D is a pleiotropic hormone which has been shown to have anti-inflammatory, anti-fibrotic effects and tissue protective effects (448-450). In its haematopoietic role, EPO is required for its anti-apoptotic and proliferative effect on red cell precursors in the bone marrow. Enhancing vitamin D levels has been shown to have synergistic effects with EPO, reducing EPO dosing requirements needed to promote haematopoiesis (451). The finding in this study may therefore represent a novel mechanism through which EPO confers tissue protection through vitamin D dependent mechanisms.

The effect of EPO in decreasing the concentration of spermidine and malonylcarnitine is of great interest, given these are potential markers of ischaemia-reperfusion injury and oxidative stress. Spermidine is a polyamine which has multiple functions including cell cycle regulation. However, the expression of spermidine/spermine-N1-acetyl transferase has been shown to increase following ischaemia reperfusion injury, increasing polyamine back conversion to spermidine (452). Inhibition of this enzyme has been shown to reduce the

severity of experimental kidney injury following ischaemia-reperfusion or endotoxin injury (452;453). Furthermore, spermidine levels were directly correlated with the presence of cerebral ischaemia and the infarct size, suggesting they may be a useful biomarker of ischaemic injury(454). Malonylcarnitine is a marker of oxidative stress, produced when malonyl-CoA is degraded by malonyl-CoA decarboxylase (455). Lower malonyl-CoA levels result in an increase in mitochondrial fatty acid uptake for oxidative phosphorylation. In the heart, this has been shown to lead to acidosis and impairment of cardiac function both during and following ischaemia (456). Thus, increasing malonyl CoA or reducing its metabolism (to malonylcarnitine) may be a novel strategy to treat renal ischaemia-reperfusion injury.

Although this is a discovery phase study, it has several significant limitations. The sample size is small in part due to female recipients being excluded to remove gender as a confounding variable. The small effect size of the study increases the likelihood of both type I and type II errors. The latter are evident in the class comparisons with small numbers of features identified, similar to the numbers expected to be false positives ($p=0.05$). Urinary metabolites may be a better way to assess the kidney metabolome in response to injury and to EPO treatment, in addition to offering a validation strategy. However, delayed graft function may be complicated by anuria in the early post-transplant period - the time point of interest. Despite its' limitations, this study has been useful as a discovery tool, identifying a possible role for metabolomics in future larger studies and identifying novel mechanisms through which EPO may exert its tissue protective effects. Metabolomics has the potential to identify previously unrecognised factors released in the early post-transplant period as a result of injury, thereby providing new avenues for investigation including identifying potential biomarkers or assessing the impact of new treatments.

CHAPTER 9 CONCLUSIONS AND FUTURE WORK

9.1 In vitro model

There have been a number of in vitro models published examining the tissue protective effects of erythropoietin in acute kidney injury(248;319). However, none to date have used a model of kidney transplantation to examine the effect of EPO. Previous models of kidney transplantation have relied upon prolonged exposure to 4°C for periods up to 48 hours, prior to a re-warming phase to induce cell injury (413). In the setting of clinical transplantation, the kidney is exposed to cold storage generally for less than 16 hours. Thus, the model created in this study is a novel and robust in vitro model, mimicking the injurious stimuli received by the donor kidney peri-transplant. Storage of cells in University of Wisconsin at 4°C and 1% hypoxia mimics cold storage with nutrient deprivation. The optimum time for measuring LDH levels or cell viability, both of which can be done simultaneously and easily, has been determined to be 24 hours. For gene expression, shorter time periods are sufficient to detect increases in acute kidney injury biomarkers. In addition, re-warming for over 8 hours leads to mRNA degradation and is thus unsuitable for gene expression analysis.

The model is very dependent on the cell type used, with immortalised cells proving relatively resistant to injury in comparison to primary proximal tubulo-epithelial cells. EPO confers cytoprotection to RPTECs in a dose- and time- dependent manner, with 50U/ml given 1 hour prior to injury leading to the greatest protection, as measured by two different assays. There is no evidence for EPO toxicity at high doses or for a proliferative effect in this cell type.

The mechanism through which this protection occurs remains unclear. Tubulo-epithelial cells certainly have EPOR message and EPOR protein, but there is no evidence for CD131 in these cells with trace amounts of mRNA despite stimulation with cold hypoxia, and no protein demonstrable by flow cytometry, western blotting or immunfluorescence. This may reflect antibody sensitivity issues or simply absence of the protein, which has been shown in other cell lines (181). Thus, it is unclear whether the tissue protective signal occurs through an EPOR homodimer or an EPOR/CD131 heterodimer. In these cells, I have not been able to demonstrate protection from oxidative stress using hydrogen peroxide or from serum starvation, both of which are potential mechanisms of injury in the cell model. However, inducing necrosis using hydrogen peroxide and using LDH release as a marker of injury is

not a reliable model of oxidative stress. There is strong evidence that hydrogen peroxide denatures the purified protein making interpretation difficult.

Thus, with a working in vitro model of kidney transplantation in which EPO causes tissue protection in place, future work will focus on identifying the mechanism through which this protection is provided:

- Ischaemia-reperfusion injury affects all renal cell types. I would like to replicate the model, both in primary endothelial cells, particularly given the increasing evidence for EPO-inducing cardio-protective effects.

- I would also like to examine the new engineered EPO's which lack haematopoietic effects, but still convey tissue protection through the putative CD131/EPOR heterodimer. Carbamylated EPO or CEPO is the subject of phase II clinical studies and may offer some additional insights into EPO's actions.

- There is still controversy over whether CD131 is important to confer cytoprotection. An attempt will be made to demonstrate it using different antibodies to address specificity issues. Also, it may be possible to induce CD131 using interferon-gamma through inflammatory pathways, given its linkage with other interleukins.

- Microarrays on the in vitro model would offer an idea platform with which to interrogate the entire transcriptome and determine the effects of EPO or CEPO on gene transcription.

9.2 Clinical trial

The EPO Study is amongst the first clinical trials to examine the tissue protective effects of EPO in any organ, but is the first to do so with a systems biology approach. Clinical features, plasma and urine biomarker analysis, metabolomic profiling and finally microarray analysis of post-reperfusion biopsies have been combined to assess if EPO can reduce the effects of ischaemia-reperfusion injury in human kidney transplantation. Despite the size limitations of the study, it is clear that EPO in this study did not confer tissue protection, with no difference

demonstrable using clinical parameters, proteins, metabolites or transcripts. There are three potential explanations for this effect: the study was underpowered; the kidneys were too severely injured with DGF rates over 50% and thus not amenable to tissue protection; and finally, that not all results from experimental animal models are translatable into humans. Evidence for the last explanation comes from the IGF-1 trials, which were very successful at ameliorating acute kidney injury in the experimental models (418) but have not been shown to be efficacious in human kidney transplantation (419). Most importantly, high dose EPO appeared safe.

One interesting finding from the clinical study was that recipient renin-angiotensin blockade prior to transplantation may reduce rather than augment the incidence of DGF. This requires further exploration as these drugs are routinely stopped prior to engraftment.

The clinical trial also examined the feasibility of collecting samples in theatre and on the ward. This has been very successful, with all patients undergoing post-reperfusion biopsies with sufficient material for microarray analysis and plasma sample collection was almost complete. No bleeding events related to the biopsies were noted. Urine sample collection is difficult in the peri-operative period, due to delayed graft function, which questions the usefulness of urine biomarkers in establishing a diagnosis that are dependent on urine being produced – an uncommon finding in patients with DGF.

While the biomarkers may be useful in native kidney injury, their role in a transplant setting with a high incidence of DGF is less certain. None of the biomarkers appeared to predict DGF earlier than traditional markers such as the delta creatinine and urine output. None of the biomarkers increased significantly in the early post-operative period with the likely explanation being the high biomarker levels found in baseline plasma and urine samples acting as confounders. Of all the biomarkers testes, plasma and urine NGAL were the best predictors of DGF. However, the value of the biomarkers may be in prognosis rather than diagnosis, with higher levels associated with a greater number of dialysis episodes.

The post-reperfusion biopsies were probably performed too early to identify both static cold preservation-induced or recipient-induced injury. This may be a reason why the study differed from the other microarray paper examining DGF (422). The increase in IL-18 expression compared to expression in nephrectomy specimens is interesting with a suggestion

that high levels may indicate DGF as early as 30 minutes post-reperfusion. This was also seen in the in vitro model following cold hypoxia supporting this result. Finally, it is likely that post-operative events in the recipients play a key role in potentiating injury, such as hypotension or hypoperfusion, which cannot be predicted by the post-reperfusion biopsy. Microarray analysis of biopsies offers the potential of a real insight into molecular mechanisms with potential diagnostic applications, which will be the subject of future work.

Finally, the metabolomics analysis identified a number of novel mechanistic pathways through which EPO may exert a tissue protective effect. The systemic effects of vitamin D are of great current interest in the cardiovascular and renal literature.

Subsequent to this piece of work, I was invited over to the Halloran Lab at the University of Alberta, where I have become involved in the diagnostic applications of microarray technology, with a particular interest in the molecular phenotype of injury. This will form the basis of future work arising out of this study.

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APPENDIX A CLINICAL TRIAL PROTOCOL

A.1 *Study title*

Erythropoietin and delayed graft function in renal allografts from marginal donors: A pilot study.

A.2 Introduction

This project began by investigating potential drugs, which in addition to immunosuppression, could impact on delayed graft function and early graft injury. Initially interest focussed on the novel use of the spices curcumin and quercetin on ischaemia reperfusion injury in transplantation, after Dr Mike Picton attended a talk at the ASN in 2006 regarding their tissue protective properties (382). While examining the literature, it came to light that a more readily available drug that had been in use for over 20 years in renal patients, with an excellent safety profile, also had tissue protective properties.

From these beginnings the EPO Trial was developed.

The EPO clinical trial protocol is based on the suggested template for a clinical trials protocol developed by the British Transplantation Society. (www.bts.org.uk/clinicalresearch.htm)

A.3 Study details

Protocol Number:	10322
EudraCT Number:	2006-005373-22
Investigational Treatment:	Erythropoietin beta (NeoRecormon® Roche Pharmaceuticals)
Protocol Version	1.1

A.4 Investigators

Chief Investigator:	Dr Michael Picton
Co-investigators:	Dr Declan de Freitas,
	Ms Beatrice Coupes,
	Mr Hany Riad,
	Dr Lorna McWilliam
	Dr Ian Read
	Professor Paul Brenchley
Chief Investigators Address:	Department of Renal Medicine, Manchester Royal Infirmary, Oxford Rd, M13 9WL
Telephone:	0161 2764290 (Hospital)
	0161 2766748 (Secretary)
Study Location:	Single centre study
Study Sponsor:	CMMCUH NHS Trust R&D
Medical Contact:	Dr Declan de Freitas
Data / Safety Monitor:	Dr Alastair Hutchison
Pharmacovigilance:	PDMS Roche Products Limited

A.5 Study synopsis

Title of clinical trial	Erythropoietin (EPO) and Delayed Graft Function in Renal Allografts from Marginal Donors
Sponsor name	CMMCUH NHS Trust R&D
Eudract number	2006-005373-22
Sponsor Protocol Number	10322
Condition under investigation	Reperfusion injury following kidney transplantation
Purpose of clinical trial	Evaluate the tissue protective effects of EPO given immediately prior to reperfusion in marginal kidneys
Primary objective	• To evaluate the effects of EPO on: (a) biomarkers of acute kidney injury; and (b) gene expression.
Secondary objectives	• To compare the incidence and severity of delayed graft function and acute rejection between the 2 groups. • To monitor and compare kidney function using standard clinical parameters at 3, 6, 9 and 12 months
Study design	Randomised, double blind, placebo controlled trial
Study endpoints	The Primary Endpoint is: - compare the effect of EPO on biomarkers levels (NGAL, KIM-1 and IL-18) and gene expression. The Secondary Endpoints (measured at 0, 3, 6, 9 and 12 months where appropriate) are: - Delayed graft function incidence - Time to last dialysis post transplantation - Creatinine reduction ratio day 0 to 7 < 30% - Renal function measured using a calculated GFR - Acute rejection rate - Patient survival - Graft survival - Safety of high dose EPO peri- transplantation - Feasibility of sample collection in theatre - Data to enable a power calculation for a multi-centre study
Sample size	40 patients – Pilot Study
Summary of eligibility	Adult recipients of a kidney transplant from non-heart-beating and extended criteria donors.
Investigational product	NeoRecormon® Roche Pharmaceuticals
Active comparator	Saline
Maximum duration of treatment of a subject	3 days

Procedures: **Screening and enrolment**	All non-heart beating and extended criteria donor kidneys will be eligible. Patients are excluded if their haemoglobin is >15g/dl** or are taking an investigational medicinal product within 30days of enrolment. Paired kidneys in non-enrolled patients will be included as controls*.
Baseline	Baseline donor and recipient data will be recorded.
Treatment Period	From time of surgery until day 3 post operatively
End of Study	Follow up of recipients will continue for one year following transplantation
Procedures for safety monitoring during the trial	Adverse Event and serious adverse event reports (expected and unexpected) will be forwarded to Roche Pharmaceuticals and the sponsor.
Criteria for withdrawal of patients on safety grounds	Patient treatment will be unblinded if the data monitor feels there is a safety issue and they will make a decision whether to withdraw the patient or not on safety grounds.
Regulatory submissions on safety grounds	A pharmacovigilance agreement exists between the sponsor and Roche Pharmaceuticals as described above.

A.6 Rationale for study

Extended criteria and non-heart-beating donors are increasingly used as a source of organs in kidney transplantation as the number of standard criteria donors diminishes. These organs are more sensitive to ischaemia-reperfusion injury, manifest as delayed graft function in the early post-transplant period. There is a body of evidence to suggest that high dose EPO attenuates ischaemia-reperfusion injury in experimental acute kidney injury and thus may have a role in protecting the kidney transplant peri-operatively. This has not been studied in humans and this study sets out to determine whether EPO confers tissue protection at the time of transplantation.

A.7 Trial design

A.7.1 Statement of design

This is a randomised, double blind, parallel-group, placebo controlled trial. Patients undergoing cadaveric renal transplantation from extended criteria or non-heart-beating donor kidneys or kidneys with a cold ischaemic time > 24hrs will be invited to participate. The patient will be randomised to either receive EPO or placebo, prior to going to theatre. Patients are otherwise treated as per the unit protocol.

A.7.2 Number of centres

This is a single centre study.

A.7.3 Number of subjects

40 patients will be recruited to this study. Subjects will numbered sequentially. Each subject will be assigned a unique subject number and will keep that number throughout the study. The MRI transplant trials unit will maintain a subject master log.

A.7.4 Sample size determination

This is a pilot study designed to determine the logistics of performing a national randomised controlled trial, with respect to both feasibility and clinical end points. It is powered to show a difference in biomarkers at $p<0.05$, with a greater than 80% confidence interval, allowing no correction for multiple analyses.

A.7.5 Randomisation and blinding

Subjects who meet eligibility criteria will be randomly assigned in a 1:1 ratio to receive either EPO or placebo.

The Trials Pharmacist at Manchester Royal Infirmary (MRI) will provide the NeoRecormon® powder with solvent in sealed boxes, anonymised, with the randomisation code. Placebo will be provided in identical packaging. On demand, equivalent volumes of

EPO or placebo (saline) will be prepared by un-blinded reconstitution and handed as a re-blinded syringe to the anaesthetist in theatre for administration. The used packaging will be returned to the pharmacy department. The operator who carries out the un-blinded reconstitution will not be involved in the trial. A record of randomisation codes will be kept in both the clinical trials office and in the MRI pharmacy. The record will consist of sealed consecutively numbered envelopes naming either EPO or saline. In case of a medical emergency, either pharmacy or designated clinical trials office unit staff can make a rapid identification of the medication.

A.7.6 Study duration

Subjects will participate in the interventional phase (implantation to post-operative discharge) for approximately 2 weeks. The end of the intervention phase of the trial will be the date of the last sample collection from the last patient. The remainder of the study period (the follow up period for study purposes will be 1 year) will be non-interventional, and will not include any trial-specific tests. On this basis, the study recruitment duration is expected to be 1.5-2 years.

A.7.7 Study endpoints

A.7.7.1 Primary endpoint

The primary endpoint of this study is to investigate whether giving erythropoietin (EPO) to recipients at the time of kidney transplantation will significantly alter the gene expression and protein levels of known biomarkers of ischaemia/reperfusion injury (NGAL, KIM-1 and IL-18) compared to patients receiving placebo.

A.7.7.2 Secondary endpoints

Between group comparisons of the following:

- Delayed graft function incidence
- Time to last dialysis post transplantation
- Creatinine reduction ratio day 0 to 7 < 30%
- Renal function measured using a calculated GFR
- Acute rejection rate

- Patient survival
- Graft survival
- Safety of high dose EPO administered peri- transplantation
- Feasibility of sample collection in theatre
- Data to enable a power calculation for a national multi-centre study

A.7.8 Conduct of the study

Delayed graft function will be defined as the need for dialysis in the first week post transplantation.

The person deciding on the need for dialysis will not be a member of the study team.

A.8 Selection and withdrawal of subjects

A.8.1 Inclusion criteria:

1. Men and women aged = or > 18 years.

2. The subject is willing to provide signed written informed consent.

3. The subject is the recipient of a non-heart-beating donor kidney: Maastricht category III (awaiting cardiocirculatory death after withdrawal of treatment) or Maastricht category IV (cardiocirculatory death in a brain dead donor).

4. The deceased heart beating donor and/or donor kidney meet at least one of the following extended criteria for organ donation from either (a) or (b) as described below:

(a)

Donor condition	Donor age categories	
	50-59 years	>or=60 years
CVA + HTN + sCr >133	Eligible	Eligible
CVA + HTN	Eligible	Eligible
CVA + sCr>133	Eligible	Eligible
HTN + sCr>133	Eligible	Eligible
CVA	No	Eligible
HTN	No	Eligible
sCr>133	No	Eligible
None	No	Eligible

Extended Donor Criteria: (CVA: cerebrovascular accident; HTN: hypertension; sCr: serum creatinine)

b) cold ischaemic time (CIT) ≥ 24hrs

A.8.2 Exclusion criteria:

1. Women who are pregnant or breastfeeding.

2. Women with a positive pregnancy test on enrolment.

3. Subjects with any active infection that would normally exclude transplantation.

4. Subjects who have used any other investigational drug within 30 days prior to transplantation.

5. Subjects with a haemoglobin level = or > 15g/dl*

6. Subjects with a diastolic blood pressure > 100 mm/Hg pre-transplantation.

7. Subjects previously intolerant of NeoRecormon®.

*At the start of the trial, the haemoglobin exclusion criterion was 13g/dl. It rapidly became apparent that we were excluding nearly 1/3rd of potential recruits due to this and a full review of the pre-op haemoglobin levels in all adults transplanted at this centre in 2007 was performed. The mean pre-op haemoglobin was 12.2g/dl with a range of 7.4-17.7g/dl and mean decrease of 2.4g/dl within 24hours of surgery. As a result, the haemoglobin exclusion criterion was set to ≥15g/dl, following LREC and MHRA approval.

A.8.3 Screen failures

There will not be any screen failures as subjects will only enter the trial following fulfilment of the inclusion/exclusion criteria and agreement to participate.

A.8.4 Subject withdrawal from the trial

Reasons why a subject may discontinue or be withdrawn from the study include, but are not limited to, adverse events, subject request, protocol violation and study termination by the sponsor. When a subject discontinues or is withdrawn the investigator will notify the sponsor. Treatment of the subject will continue as per standard clinical care. If the trial is prematurely terminated or suspended the sponsor will promptly inform the regulatory authority and the LREC, and provide the reason(s) for the termination.

Patients who request to withdraw from the study are given the option of having their information removed from the trial database and having their samples destroyed or allowing the investigators to continue to use this material as part of the study.

A.8.5 Replacement of withdrawn subjects

If a subject withdraws from the study or does not receive the kidney due to surgical reasons, they will not be replaced.

A.8.6 Follow up of subjects withdrawing from the study

All patients participating in the study will undergo routine post transplant follow up. Patients withdrawing from the trial will continue to be followed up in this manner. If they have agreed to allow continued collection of their data, this will occur as per the trial protocol.

A.9 Treatment regimens

A.9.1 Placebo

Patients will receive 50mls of 0.9% saline immediately prior to reperfusion, at 24 hours and 48 hours.

A.9.2 Investigational medicinal product - Erythropoietin

NeoRecormon® will be presented as a multi-dose powder and solvent, 100,000iu per vial. The powder (100,000iu) is dissolved in solvent as per instruction leaflet. 1/3rd of the volume (33,000iu) is aspirated and added to 100mls of 0.9% saline. This is given as an infusion over 15 minutes at the time the venous anastamosis is performed (approximately 15min before reperfusion commences). The second infusion is given the following morning, unless the first infusion was given after midnight, in which case it was given on the next morning. The third infusion was given 24hours later.

A.9.3 Immunosuppressive therapy

A.9.3.1 Cd25 monoclonal antibody Basiliximab (Simulect)

All patients will receive Basiliximab 20mg IV on day 0 and 4 as per NICE guidelines.

A.9.3.2 Tacrolimus

All patients will receive Tacrolimus 0.1mg/kg/day in divided doses from day 0

- Aim for drug levels of 10-15ng/ml for first 3 months

- After 3 months, aim for levels 5-10ng/ml
- If DGF, aim for levels of 5.0-7.5ng/ml

A.9.3.3 Prednisolone

All patients will receive 500mg of methylprednisolone IV prior to reperfusion and subsequently 20mg prednisolone orally. Steroid withdrawal will occur according to unit practice.

A.9.3.4 Mycophenolate Mofetil (MMF) (Cellcept)

All patients will receive MMF 1g BD from day 0 as per unit protocol.

A.9.3.5 Anti-microbial and anti-thrombotic prophylaxis

Anti-microbial and anti-thrombotic prophylaxis will be in accordance with local practice. Subjects will receive prophylaxis against pneumocystis pneumonia and cytomegalovirus (donor positive/recipient negative scenario).

A.9.3.6 Concomitant treatment: Erythropoietin

Patients may remain on pre-transplant erythropoietin regimens as directed by their responsible clinician.

A.9.3.7 Cold storage

Kidneys retrieved by our site undergo standard cold-storage at 4°C in University of Wisconsin or Marshalls Solution. After an initial vascular washout with preservation solution, kidneys are submerged in preservation solution and stored on melting ice, according to established national protocols. If kidneys are retrieved elsewhere and received machine perfusion, this will be recorded.

A.10 Study procedure and assessments

A.10.1 Randomisation

NeoRecormon® and placebo will be randomly assigned and placed in sealed boxes labelled 1 to 40 in the MRI pharmacy department. Sequential recipients consenting to the trial will then receive sequential boxes, from 1 to 40, containing the experimental drug.

A.10.2 Informed consent

A member of the study team will begin the consent process when the recipient is in the hospital and has been identified as fulfilling all of the inclusion/exclusion criteria. It will be explained that the donor kidney is from a marginal donor with an increased risk of delayed graft function of approximately 50% and may need dialysis post operatively. The rationale and evidence for using EPO in renal transplant recipients will be explained including potential expected adverse events and the safety evidence available both from the Stroke Trial and published EPO trials in renal patients. The randomised double blind design of the trial will be explained including that this is an experimental treatment in this group of patients for this indication. Finally sample procurement will be explained including the non-standard practice second biopsy 15 minutes post-reperfusion. They will then be invited to participate in the study and will be given a suitable period of time to review the study information sheet and ask any questions they may have. If they are then agreeable, they will be invited to give informed consent. Consent forms have been approved by an independent ethics committee.

A.10.3 Baseline data

A.10.3.1 Patient data

All patients will have a full medical history and a clinical examination. The following are to be recorded:

i. Name
ii. Date of birth
iii. Ethnicity
iv. Medical history (Diabetes / Hypertension / Hyperlipidaemia / Smoking)

v. Cause of end stage renal disease
vi. Date when started dialysis (or creatinine if pre-dialysis)
vii. Type of dialysis at time of admission: Pre-dialysis, haemo- or peritoneal dialysis
viii. EPO usage (type, dose and date of last dose)
ix. Current medication
x. Haemoglobin, haematocrit, platelet count, PT, aPTT, creatinine, urea pre-transplant
xi. Calculated GFR (4vMDRD)
xii. Height and Weight
xiii. Number of previous transplants
xiv. Sensitisation status recorded by NHSBT
xv. HLA mismatch of graft

A.10.3.2 Donor data

The following donor information will be recorded:

i. Gender
ii. Date of birth
iii. Ethnicity
iv. Primary cause of death
v. Cytomegalovirus (CMV) serology status
vi. Diabetes (Y/N)
vii. Type of donation (extended criteria / NHBD)
viii. Creatinine value prior to organ retrieval
ix. Total urine output for 24 hours prior to organ retrieval

A.10.3.3 Day of transplant (Day 0)

The following will be recorded:

i. BP before, during and after trial drug infusion
ii. Number of vessels

iii. Any sacrificed vessels
iv. Kidney colour after reperfusion
v. Biopsies performed (Y/N)
vi. Mannitol infusion
vii. Frusemide infusion
viii. Anti-coagulation given
ix. Cold ischaemic time
x. Dialysis (indication / type / duration)

A.10.3.4 Day 1 and 2 post transplantation

i. BP before, during and after trial drug infusion
ii. Haemoglobin, haematocrit, platelet count, PT, aPTT, creatinine (first value of the day), urea
iii. Calculated GFR (4vMDRD)
iv. Dialysis (indication / type / duration)
v. Need for blood pressure medication

A.10.3.5 Day 3 until discharge

i. Blood pressure
ii. Haemoglobin, haematocrit, platelet count, PT, aPTT, creatinine, urea
iii. Calculated GFR (MDRD)
iv. Dialysis (indication / type / duration)
v. Need for blood pressure medication

A.10.3.6 Month 3, 6, 9 and 12

i. Creatinine, haemoglobin, haematocrit
ii. Calculated GFR (MDRD)
iii. Graft loss (date and cause)

iv. Patient death (date and cause)

A.10.3.7 Subsequent follow up

Subjects will be followed up at their base hospital as per local protocol after one year. There are no additional requirements for study visits.

A.11 Sample collection

A.11.1 Kidney tissue

Two biopsies of the transplant kidney will be taken at the time of surgery:

1. As part of standard clinical practice, a tissue biopsy will be taken from the donor kidney while still on ice prior to surgery, and will be split to be used for diagnostic histopathology and research purposes. If tissue surplus to diagnostic requirements remains, research specific histology will be performed to identify trial-specific biomarkers in the tissue. Molecular studies will be performed on samples stored for research purposes.

2. Approximately 15-20 minutes following reperfusion, a second biopsy will be collected from the kidney (the post-reperfusion biopsy) for molecular studies.

In those subjects where the kidney fails to function post-operatively, a biopsy is routinely indicated on or around day 7. Tissue which is surplus to diagnostic requirements will be used for research molecular investigations and biomarker staining.

A.11.2 Blood sampling

A.11.2.1 Intra-operative sampling

During implantation of the kidney, prior to reperfusion of the kidney, a 20ml systemic blood sample will be collected via the central line from the patient (the pre-reperfusion sample). A second 20ml blood sample will be collected approximately 15 minutes after reperfusion (the post-reperfusion sample).

A.11.2.2 Post-operative sampling

10mls of blood will be collected at 2, 8 and 24 hrs and daily for the first week post-operatively, coinciding with venepuncture for routine care where possible.

A 10ml blood sample taken on the day of a protocol biopsy to out-rule rejection in the presence of delayed graft function will be used for biomarker assay.

A.11.3 Urine sampling

A.11.3.1 Pre-operative sampling

Up to 10mls of urine will be collected from subjects who continue to pass urine prior to surgery for biomarker assay.

A.11.3.2 Post-operative sampling

Urine samples will be collected daily until discharge for biomarker assay.

A.12 Laboratory methodology

Known and suspected biomarkers of ischaemia-reperfusion injury will be compared between the Erythropoietin and placebo treated groups with respect to plasma and urine protein levels and gene expression. The biomarkers will include key proteins (NGAL, KIM-1 and IL-18) and genes in the injury and inflammation pathways. Blood samples will be used to prepare plasma and DNA (Qiamp, Qiagen) for immunoassay of biomarkers and future genotype

studies respectively. Genotyping for SNPs may be performed in the future. Urine will be prepared for biomarker assay and metabolomics screening.

All trial specific laboratory investigations will be carried out in the Renal Research lab at the MRI, with the exception of the Affymetrix, which will be performed in the University of Manchester Core Technology Facility. All patient material will be anonymous at this stage.

Subject to patient consent, samples will be stored in the MINT BioBank for future studies, to determine the proteomic and metabolomic patterns of ischaemia-reperfusion injury. The MINT BioBank has ethical approval (North Manchester Research Ethics Committee (ref: 06/q1406/38).

A.12.1Biomarker assay methodology

All biomarkers will be assayed by immunoassay, developed with a TMB substrate and read as optical densities (OD). Briefly, 96-well Immulon 2 HB plates (Thermo, UK) will be coated with a capture antibody. Plasma or urine samples, in addition to standard dilutions of recombinant protein, will be added in 100µL volumes to the wells and incubated overnight. After washing, the detection antibody will be added and incubated overnight. The plates will be then washed and developed colorimetrically using a TMB substrate, and the optical density read on a Spectromax 340PC microplate spectrophotometer (Molecular Devices Ca 94089). A standard curve will be constructed from the standards and sample concentrations calculated from the 4-parameter curve (SOFTmax PRO v4 software, Molecular Devices, Ca 94089). Biomarker concentrations in urine will be corrected for creatinine concentration in the sample to compensate for dilution, and expressed as units/mg creatinine (units/mgCr). Where WHO standards were available, these will be included as internal controls (NGAL and IL-18), confirming the inter-assay coefficient of variation (CV) at <20%. Intra-assay variation is acceptable if <10%. The KIM-1 duoset has been fully evaluated elsewhere(116).

A.12.2 Microarray methodology

Biopsies will be obtained and processed for microarray as described in detail in (383). Microarray data files were processed as described previously. Expression values for transcripts will be calculated as fold change versus eight nephrectomies that serve as controls.

A.12.3 Metabolomics methodology

Serum samples will be prepared for UPLC-MS positive ion mode (UPLC-MS +) metabolomic analysis by deproteinisation and lyophilisation. Samples will be thawed on ice followed by addition of 300µL of methanol to 100µL of serum followed by vortex mixing (15 seconds) and centrifugation (15 minutes, 13,865 g). The supernatant will be transferred to a new centrifuge tube and lyophilised (HETO VR MAXI vacuum centrifuge attached to a Thermo Svart RVT 4104 refrigerated vapour trap; Thermo Life Sciences, Basingstoke, UK). Lyophilised samples were stored at -80°C prior to analysis. The procedure will be repeated to prepare samples for UPLC-MS negative ion mode (UPLC-MS -) analysis.

A pooled QC sample will be prepared to perform quality assurance and quality control procedures and ensure appropriate data quality after sample preparation, data acquisition and data pre-processing (384). 100µL aliquots of each sample studied (each subject and time point) will be combined to prepare the pooled QC sample. 100µL aliquots will be prepared and stored as described above.

Subject and QC samples will be reconstituted in 100µl of water prior to analysis followed by vortex mixing (15 seconds) and centrifugation (15 minutes, 13 865 g). All subject and QC samples will be analysed in a single analytical batch and in a random order and within 48 hours of reconstitution. The two sets of prepared samples will be analysed separately in UPLC-MS + and UPLC-MS – ion modes. The first ten injections will be QC samples and QC samples will be analysed every 7^{th} injection throughout the analytical run. Samples analysis will be performed on an Ultra Performance Liquid Chromatography system (Waters Acquity, Elstree, UK) interfaced to an electrospray LTQ-Orbitrap XL hybrid mass spectrometer system (ThermoFisher Scientific, Bremen, Germany). Chromatographic separation will be performed using an Acquity UPLC BEH C_{18} column (2.1mm i.d., 1.7µm particle size; Waters, Elstree, UK) at flow rates of 0.36 and 0.40 $mL.min^{-1}$ in negative and positive ion modes respectively. A binary solvent system will be applied using water + 0.1% formic acid (solvent A) and methanol + 0.1% formic acid (solvent B). The gradient conditions have been described previously and are different for positive ion and negative ion operating modes (385). The autosampler and column will be operated at temperatures of 4 and 50°C, respectively and a 10µL sample injected. Fifty percent of the column effluent will be continuously introduced to the mass spectrometer and centroid data will be collected in

accurate mass mode in the Orbitrap mass analyser in the mass range 50-1000 Th at a scan rate of 0.4scans.sec^{-1}. The Orbitrap mass analyser is operated with a mass resolution of 30 000 (FWHM at 400 Th). Mass calibration will be performed according to the manufacturer's guidelines using a manufacturer defined mixture of sodium dodecyl sulphate, sodium taurocholate, the tetra-peptide MRFA and Ultramark 1621.

Raw data files (.RAW) will be converted to the NetCDF format using the File converter program in XCalibur (ThermoFisher Scientific, Bremen, Germany). Deconvolution of data will be performed using XCMS as described previously (386).

A.13 Evaluation of results

A.13.1 Response criteria

A.13.1.1 Delayed graft function

Delayed graft function is defined as the need for dialysis within the first seven days of transplantation.

A.13.1.2 Graft function

Calculated GFR (eGFR) will be calculated based on a 4 variable MDRD equation including creatinine, race, sex and age (387).

A.13.1.3 Survival (patient and graft)

These will be measured from the date of transplant and will be recorded for all deaths and graft loss due to all causes.

A.13.1.4 Renal biopsies

In addition to standard histopathology, the pre-transplant biopsy, immediate post transplant biopsy and any subsequent biopsies will be stored for subsequent analysis of biomarkers, if sufficient tissue is available surplus to diagnostic requirement.

A.14Assessment of safety

A.14.1 Definitions

A.14.1.1 Adverse event (AE)

An adverse event is any untoward medical occurrence in a patient or kidney in the first week following trial drug treatment which does not necessarily have a causal relationship with EPO.

An adverse event can be any unfavourable and unintended symptom, sign, diagnosis or laboratory finding temporally associated with the randomised infusion, whether or not it is considered related to the infusion.

A.14.1.2 Unexpected adverse event

An adverse reaction, the nature or severity of which is not consistent with an expected consequence of the randomised treatment. The term “severe” is often used to describe the intensity (severity) of a specific event. This is not the same as “serious”, which is based on patient/event outcome or action criteria.

A.14.1.3 Serious adverse events (SAE)

Any untoward medical occurrence or effect that:

- results in death
- is life-threatening

- requires hospitalization or prolongation of existing in-patient hospitalization
- results in significant or persistent disability or incapacity
- in a congenital anomaly or birth defect

Life threatening in the definition of a SAE refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

A.14.1.4 Events not considered to be SAE's for the purpose of the study

For the purposes of this study, the following events are not considered to be SAE's:

- delayed graft function
- admission for removal of ureteric stents
- admission for removal of a PD or HD catheter
- admission for biopsies of kidneys with suspected rejection
- haemodialysis or peritoneal dialysis

A.14.1.5 Events not considered to be SUSAR's for the purpose of the study

- occurrence and treatment of wound infection or dehiscence
- occurrence and treatment of a ureteric stricture/leak
- occurrence and treatment of a lymphocoele

A.14.2 Expected adverse events

There is a wealth of data to support the safety of EPO treatment and transplantation in patients with end stage renal failure.

A comprehensive description of possible undesirable effects can be found in the SmPC for NeoRecormon®. Based on clinical trials including 1725 patients, approximately 8% of patients treated with NeoRecormon® are expected to experience adverse events. Undesirable effects are observed predominantly in patients with chronic renal failure and underlying malignancies and are most commonly an increase in blood pressure or a deterioration in

blood pressure control. The therapeutic margin of NeoRecormon® is very wide. At high serum levels, no symptoms of toxicity have been reported. The following will be measured at designated intervals for safety evaluation:

a) Mean systolic and diastolic blood pressure will be measured daily.
b) Haemoglobin and haematocrit levels will be measured daily.

Recognized complications of EPO treatment in the context of transplantation include:

1. Hypertension. Hypertension is a well established complication of recombinant human EPO therapy, with approximately 25% of renal patients developing new onset hypertension or worsening of their blood pressure control.
2. Thrombotic events (most evidence relates to thrombotic events in renal patients with haemoglobins >13.5g/dl and seem to be related to haematocrit level and vascular access thrombosis.

Recognised complications of renal transplantation include:

1. Delayed graft function
2. Acute rejection
3. Renal artery thrombosis
4. Renal vein thrombosis
5. Infection
6. 1yr mortality 2-5%
7. 1yr graft loss <15%

Immunosuppressive agents are associated with a number of adverse events as quoted in their individual SmPC's.

A.14.3 Recording and evaluation of adverse events

Individual adverse events will be evaluated by the investigator, particularly with respect to causality between the treatment and the adverse event.

The MRI trials unit will keep a detailed record of AE's and perform an evaluation with respect to seriousness, causality and expectedness.

A.14.3.1 Assessment of seriousness

Mild: The subject is aware of the event or symptom, but the event or symptom is easily tolerated.

Moderate: The subject experiences sufficient discomfort to interfere with or reduce his or her usual activity.

Severe: Significant impairment of functioning; the subject is unable to carry out usual activities and/or the subject is at risk from the event.

A.14.3.2 Assessment of causality

Probable: A causal relationship is clinically / biologically highly plausible and there is a plausible time sequence between onset of the AE and the investigational treatment.

Possible: A causal relationship is clinically / biologically plausible and there is a plausible time sequence between the onset of the AE and the investigational treatment.

Unlikely: A causal relationship is improbable and another documented cause of the AE is most plausible.

Unrelated: A causal relationship can be definitely excluded and another documented cause of the AE is most plausible.

A Pharmacovigilance Agreement has been put in place between Roche Pharmaceuticals and the sponsor. Under the terms of this agreement, the chief investigator, delegated by the sponsor, is responsible for collecting study SAE and pregnancy reports and sending these to Roche. Roche will process and evaluate these reports, maintain a safety database and report safety reports to the regulatory authority. The sponsor will distribute investigator letters

provided by Roche, report safety reports to the ethics committee and prepare an annual safety report for the study.

All AE and SAE for subjects who are not screen failures will be recorded on CRFs. The chief investigator will follow up on all AE's and SAEs and other reportable information until the events have subsided or returned to baseline.

All SAE's and SUSARs and follow up information will be reported within one business day of learning of the events by faxing a completed SAE / SUSAR form to Roche Pharmaceuticals on their emergency contact number, and confirming receipt by email or phone.

In the case of an SAE that a clinician believes to be related to the medication administered, the doctor involved will contact the consultant nephrologist/transplant surgeon in charge of the care of the patient. In addition, this will be brought to the attention of the data and safety monitoring clinician, Dr Alastair Hutchison, by telephone or fax within 24 hours, and the principal investigator, Dr Michael Picton. If there is a causal effect found by him or the independent clinician, the SAE will be reported to Roche Products. Full details of the SAE will be documented on the medical records and CRF. In addition, the investigator will complete the sponsor's Serious Adverse Event Report, containing all the information that is required by the sponsor. All correspondence for an SAE will be marked urgent. The following minimum information is required:

a) Study Number
b) Subjects identification, initials, date of birth
c) The name and address of the reporting person (in confidence)
d) A description of the SAE
e) Study drug details (start and stop date, information on dosage)

A.15 Statistics

A.15.1 Statistical methods to be employed

The statistical methods have been discussed with the CMMC Trust statistician, Dr Steven Roberts, Senior Lecturer in Medical Statistics, University of Manchester, Dr David

Broadhurst, Associate Professor, University of Alberta and with Dr Jeff Reeve, Principal Statistician, ATAGC, University of Alberta.

Data will be presented as mean ± SEM, median ± IQR (range from the 25^{th} to the 75^{th} percentile) or as frequencies for categorical variables. Data analysis will include Student's t test or Mann-Whitney. A significance level for a two tailed test will be set at a p value ≤ 0.05. The survival times of the grafts and patients will be summarised using Kaplan-Meier estimates of the survivor function for EPO and placebo.

The anonymous microarray analysis will be performed by a bioinformatician in conjunction with the University of Alberta.

Biomarker levels in the pre- and post-reperfusion blood and urine samples will be compared using a paired t test or Wilcoxon signed rank test or Mann-Whitney test (depending on whether the samples are normally distributed).

A.15.2 Interim analyses

An interim analysis will not be performed.

A.15.3 Number of subjects to be enrolled

40 atients will be recruited.

A.15.4 Expected time to full recruitment

Recruitment is expected to be complete by 2 years from 1^{st} September 2007 based on MRI performing 23 NHBD in 2007 and 18 NHBD in 2006.

A.15.5 Criteria for termination of the trial

Recruitment to the study will be terminated early when one of the following criteria is met:

a) Significant concerns regarding the safety of the study have been raised, and agreed by the study steering group or the independent safety monitor.

b) Recruitment is slower than expected and the 2 year period is complete.

A.15.6 Study steering group

The Study steering group comprises of Dr Declan de Freitas, Dr Michael Picton, Miss Beatrice Coupes, Professor Paul Brenchley, Mr Hany Riad and Dr Clare Hamer.

A.15.7 Data and safety monitoring committee

A data and safety monitoring protocol under the auspices of Dr Alastair Hutchison, Clinical Director of MRI Renal Unit, has been established by the sponsor to assess at intervals, or when required , the progress of the clinical trial, the safety data, and the critical efficacy end points. He will recommend to the sponsor whether to continue, modify or stop the trial. Reports will be forwarded to the regional ethics committee which reviews the trial.

A.16 Direct access to source data / documents

The chief investigator shall ensure that all study data is available for trial related monitoring, audits, REC review and regulatory inspections.

A.17 Regulatory body review

A.17.1 MHRA review

The MHRA has reviewed the study protocol and given a favourable opinion. A copy of the letter of approval is filed in the MRI Transplant Unit trials office.

A.18 Ethical considerations

A.18.1 Ethical committee review

The local regional ethics committee has reviewed the study protocol and given a favourable opinion. A copy of the letter of approval is filed in the MRI Transplant Unit trials office.

A.18.2 Declaration of Helsinki and ICH Good Clinical Practice

This study will be carried out in conformation with the spirit and the letter of the Declaration of Helsinki, and in accord with the ICH Good Clinical Practice Guidelines.

A.19 Data handling and record keeping

The master randomization list, the pre-trial monitoring report, the trial initiation monitoring report, the subject screening log, the subject ID code list, the subject enrolment log, a copy of the signature sheet and a copy of the retained patient samples list will be held in the transplant unit trials office at the MRI. The CRFs will be hard copy forms held in the subjects file in the transplant unit trials office. Entries into the CRF will be hand written and initialled by the designated personnel. Errors will be scored through and initialled.

Source documents will include medical notes and the subject file containing the CRF. All trial related clinical data required for the research analysis of the clinical trial will be entered into a secure (password protected) electronic database and will be available only to those individuals named on the ethics application. All data shall be accompanied by the date of the record and the name of the person entering the data. Trial related data should be kept for 10 years following completion of the study. The use of data from the study will be controlled by the principal investigator in consultation with the study steering group.

The master list of retained samples will be held in the Renal Research laboratory at the MRI. A copy of the signature sheet will be held in the Renal Research laboratory at the MRI. Access to source data outside the clinical care team will be limited to the research investigators as indicated on the patient consent form.

IMP accountability at the site will be the responsibility of the Pharmacy Dept at the MRI.

A.20 Financial and insurance

An unrestricted grant of £15,000 to support the study has been given by Roche Pharmaceuticals. The research team are fully funded staff members employed by the CMMCUH NHS Trust.

Centres will be covered by NHS indemnity for negligent harm providing researchers hold a contract of employment within the NHS, including honorary contracts held by academic staff. Medical co-investigators will also be covered by their own medical defence insurance for non-negligent harm. Ex-gratia payments may be considered.

A.21 Publications policy

It is the intention to disseminate the results of this study as widely as possible. This is likely to be through a publication in a peer reviewed transplant journal, as well as through presentations at national and international transplant conferences. Publications will follow the CONSORT guidelines and authorship will follow international guidelines.