Study of Eryptosis in Patients with Renal Disease

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Statement of originality

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Abstract

Eryptosis (erythrocyte programmed cell death) is postulated to be related to the efficacy of erythropoietin (Epo) treatment in chronic kidney disease (CKD) patients. This project was undertaken to determine whether this is the case and if any other factors correlate with eryptosis levels. Red cell microparticle (RCMP) numbers in whole blood were determined as a measure of eryptosis, using flow cytometry adapted from previous methods. Further investigation was performed using a flow loop model involving perfusion of whole blood through intact tumour necrosis factor (TNF) treated and untreated human umbilical arteries.

Contrary to expectations, fewer RCMP were observed in CKD patients compared to controls ($p=0.0167$). Weak positive correlations were found between RCMP numbers and both CRP levels ($p=0.0362$) and Epo dose ($p=0.0014$) in the patient group. These results suggest erythrocytes in CKD patients undergo less eryptosis than in control subjects. *In vitro* investigations imply that erythrocytes in patients are less susceptible to the impact of flow stress and TNF treated endothelium than controls, corroborating this argument. When patients receiving Epo were compared to patients not receiving Epo there were no significant differences in RCMP numbers under all flow conditions. Further investigations are required, including the recruitment of more Epo hyporesponsive patients.

This study highlighted the need for better standardisation of methods for measuring not only RCMP, but eryptosis in general, as well as better definitions of RCMP populations.

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Abbreviations

2,3-DPG	2,3-diphosphoglycerate
51Cr	Chromium 51
ABB	Annexin binding buffer
ACE	Angiotensin converting enzyme
AFM	Atomic force microscopy
Akt	Protein kinase b
ALA	δ-Aminolevulinic acid
APC	Allophycocyanin
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosinetriphosphate
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2 protein
Bcl-xL	B-cell lymphoma-extra large
BFU-E	Burst forming unit – erythroid
BMI	Body mass index
CAPD	Continuous ambulatory peritoneal dialysis
CERA	Continuous erythropoietin receptor activator
CFU-E	Colony forming unit – erythroid
CHO	Chinese hamster ovary
CHOIR	Correction of Haemoglobin and Outcomes in Renal Insufficiency
CKD	Chronic Kidney Disease
CREATE	Cardiovascular Risk Reduction by Early Anemia Treatment with Epoetin Beta
CRF	Chronic renal failure
CRP	C reactive protein
CST	Cytometer setup and tracking
CTAD	Citrate, theophylline, adenosine and dipyridamole
CVD	Cardivascular disease
DNA	Deoxyribonucleic acid
DOQI	Disease outcomes quality initiative
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate

Epo	Erythropoietin
EpoR	Erythropoietin receptor
ESA	Erythropoiesis stimulating agent
ESRD	End stage renal disease
ESRF	End stage renal failure
EV	Extracellular vesicles
FBC	Full blood count
FBS	Foetal bovine serum
FCM	Flow cytometry
FDA	US food and drug administration
FF-TEM	Freeze fracture transmission electron microscopy
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GATA	GATA binding factor
GFR	Glomerular filtration rate
GM-CSF	Granulocyte-macrophage colony stimulating factor
GSK-3β	Glycogen synthase kinase-3β
Hb	Haemoglobin
HbA1c	Glycated haemoglobin
HBSS	Hanks balanced salts solution
Hct	Haematocrit
HD	Haemodialysis
HIF	Hypoxia inducible factor
HRE	Hypoxia response element
HRQOL	Health related quality of life
HSC	Haematopoietic stem cell
Hsp70	70 kilodalton heat shock protein
IAP	Inhibitors of apoptosis
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IQR	Interquartile range

ISTH	International Society on Thrombosis and Haemostasis
IV	Intravenous
Jak2	Janus activated kinase 2
JNK	c-Jun N-terminal kinases
kDa	Kilodaltons
Kt/v	Dialysis index
L-NMMA	NO-monomethyl-L-arginine
MAP	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
miRNA	Micro RNA
MP	Microparticle
mRNA	Messenger RNA
NESP	Novel erythropoiesis stimulating protein
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHANES	National Health and Nutrition Examination Survey
NHSBT	NHS Blood and Transplant
NKF	National Kidney Foundation
NO	Nitric oxide
NTA	Nanoparticle tracking analysis
PBS	Phosphate buffered saline
PCD	Programmed cell death
PD	Peritoneal dialysis
PE	Phycoerythrin
PFP	Platelet free plasma
PI	Principal Investigator
PI3K	Phosphatidylinositol 3-kinase
PIP	Phosphatidylinositol phosphate
PIP_2	Phosphatidylinositol 4,5-biphosphate
PMP	Per million population
PMP	Platelet microparticle
PPP	Platelet poor plasma
PS	Phosphatidylserine
RBC	Red blood cell (erythrocyte)
RCMP	Red cell microparticle

rHuEpo	Recombinant human erythropoietin
ROS	Reactive oxygen species
RPS	Resistive pulse sensing
RRT	Renal replacement therapy
SAXS	Small-angle X-ray scattering
SC	Subcutaenous
SD	Standard deviation
SEC-DLS	Size exclusion chromatography coupled with on-line dynamic light scattering detection
SH2	Src homology 2
SSC	Side scatter
STAT-5	Signal transducer and activator of transcription 5
TF	Tissue factor
TNF	Tumour necrosis factor
uEpo	Urinary erythropoietin
URR	Urinary retention rate
VCAM-1	Vascular cell adhesion molecule 1
WBC	White blood cell
WHO	World Health Organisation
δ-ALA	δ -Aminolevulinic acid synthase

Chapter 1: Introduction

Chronic kidney disease (CKD) is associated with a significant increase in morbidity and mortality with many related medical problems. A major clinical finding is the development of anaemia which occurs early and contributes to a poor quality of life. It has also been shown to be strongly predictive of outcome (Collins *et al.*, 1998), with an increasing prevalence seen with decreasing renal function. The primary cause of this renal anaemia is inappropriately low erythropoietin (Epo) levels.

Prior to the development of recombinant human erythropoietin (rHuEpo) as a therapeutic agent, treatment required frequent transfusions. This brought the associated risks of iron overload, infection with HIV or viral hepatitis and the development of antibodies, limiting transplant options. Development of rHuEpo in the 1980s produced major advances in the management of CKD patients and drastically altered renal anaemia treatment. However, there is evidence that the high doses of rHuEpo required by some patients may adversely affect morbidity and mortality (Singh *et al.*, 2006).

Investigating the process of eryptosis may give an insight into the mechanisms of renal anaemia and therefore possibly help to explain rHuEpo hyporesponsiveness.

1.1 Chronic Kidney Disease

The kidney functions to excrete toxic substances via the urine and optimally regulate blood solute concentrations, as well as performing a range of metabolic processes and producing hormones (Besarab, 1997). It maintains a stable extracellular environment, balancing water and ionic components and supporting the function of all cells.

Chronic kidney disease (CKD) describes a heterogeneous range of disorders of kidney structure and function (Levey *et al.*, 2011). CKD is a progressive and irreversible loss of renal function (Nahas, 2005). It can progress to Stage 5 CKD when it is known as end stage renal failure (ESRF), which is irreversible and

requires renal replacement therapy (RRT) (Barratt *et al.*, 2009). CKD is associated with a range of disorders resulting from the effects of solute retention and the absence of hormones produced by the kidneys, especially Epo (Besarab, 1997).

The 2002 Kidney Disease Outcomes Quality Initiative Clinical Practice Guidelines define CKD as the presence of kidney damage for greater than 3 months (either structural or functional abnormalities) or a glomerular filtration rate (GFR) less than 60ml/min/1.73m^2 for more than 3 months, with or without kidney damage (National Kidney Foundation, 2002). CKD is then classified, according to the level of GFR, into 5 stages (see Table 1.1) (Barratt *et al.*, 2009).

CKD Stage	**Description**	**GFR (ml/min/1.73m^2)**
1	Kidney damage (such as microalbuminuria/proteinuria, haematuria or histological changes) with normal or increased GFR	>90
2	Kidney damage with mild decrease in GFR	60-89
3	Moderate decrease in GFR	30-59
4	Severe decrease in GFR	15-29
5	Kidney failure	<15 (or dialysis)

Table 1.1: Classification of CKD Based on recommendations from the National Kidney Foundation Kidney Disease Outcomes Quality Initiative Guidelines 2002 (National Kidney Foundation, 2002). GFR gives an indication of the kidneys ability to filter waste products from the blood, showing the volume of water and other solutes filtered out of the blood in the glomeruli within a given period of time (Burrows-Hudson, 2005). It is used to grade CKD and monitor progression prior to ESRF.

Once a patient reaches ESRF the GFR is no longer a useful measure. Using pharmacokinetic models of dialysis a more accurate quantification method was developed, the dialysis index - Kt/V (Cambi *et al.*, 2005). This gives an indication of the normalised whole body urea clearance, and therefore the dialysis dose/adequacy.

It is calculated as follows:
K = dialyser clearance of urea, the rate at which blood passes through the dialyser.
t = dialysis time

V = volume of distribution of water (approximately equal to the patients total body water)

Kt represents the volume of fluid cleared of urea during a single dialysis session, and V is approximately equal to the patients total body water, therefore Kt/V compares the amount of fluid that passes through the dialyser with the amount of fluid in the patient's body.

The UK Renal Association Guidelines state that in haemodialysis the Kt/V target is > 1.3, and in peritoneal dialysis the target is ≥ 1.7/week (Mactier *et al.*, 2009; Woodrow and Davies, 2010).

The urea reduction ratio (URR) is another measure which shows the reduction in urea after dialysis and is also used to determine dialysis efficiency. It gives a dimensionless number, often expressed as a percentage.

$$URR = \frac{U_{pre} - U_{post}}{U_{pre}} \times 100\%$$

Where:

- U_{pre} is the pre-dialysis urea level
- U_{post} is the post-dialysis urea level

The target dose for HD 3 times a week is URR of 70% (National Kidney Foundation, 2006).

Patients with stage 1-3 CKD rarely have any symptoms, these do not develop until ESRF is reached. Clinical signs include fluid retention, presenting as ankle swelling or breathlessness, pallor and raised blood pressure, and poor growth and development in children, accompanied by falling haemoglobin levels and abnormality of several biochemical indices (including serum urea, creatinine and potassium), while the patient may become tired, nauseated, lose their appetite and be less able to cope with life mentally and physically (Department of Health, 2004).

Studies examining the true prevalence of CKD have shown that in the South East of England there are 5,554 cases per million population (PMP) (John *et al.*, 2004), while in the US as many as 5% of adults may have CKD stage 1-2 and another 5%

stages 3-5 (Coresh *et al.*, 2003). Despite its prevalence, less than 1 in 10 people with CKD ever require dialysis or a transplant.

The number of new patients starting treatment and the total number of patients receiving treatment increased from 65 PMP and 396 PMP respectively in 1992 to 91 PMP and 547 PMP in 2001. More than 27,000 people were receiving renal replacement therapy (RRT) in England in 2001. Around half of these had a functioning transplant; the remainder were on dialysis (Department of Health, 2004). In 2009 the incidence rate had increased further to 109 PMP (Caskey *et al.,* 2011). This is expected to rise, as in other developed countries, at an annual rate of 5-8% (Lysaght, 2002), due to two factors. Firstly to the aging population; CKD is more common in elderly people. The United States Renal Data System (2003) showed the incidence of ESRF in over 65s to be greater than 1200/PMP/yr. Secondly, the number of people worldwide with type II diabetes in 2011 was estimated to be 366 million (International Diabetes Federation, 2013). This is predicted to increase to over 550 million by 2030 and as diabetes is the most common cause of CKD this is likely to impact upon renal failure rates.

Data from the three largest renal dialysis and transplant registries, in Europe, the US and Australia and New Zealand, show the commonest causes of ESRF to be glomerulonephritis, diabetes and hypertension. Renovascular disease, infective or obstructive nephropathies and hereditary diseases also have an impact (Levy *et al.*, 2004). Diabetic renal disease is the most common cause of renal failure in the UK, accounting for 25% of all cases (Caskey *et al.,* 2011).

Diabetes is monitored using the haemoglobin A1c (HbA1c) assay, which measures a type of glycosylated haemoglobin and indicates the level of glucose control. Analysis of the data from the National Health and Nutrition Examination Survey (NHANES) found that of those with diabetes only 37% achieved recommended HbA1c levels (Saydah *et al.*, 2004). Such a lack of control leads to organ damage, including the kidneys (Burrows-Hudson, 2005). High glucose levels in the blood damages vessels within the kidneys. Diabetes can also cause damage to nerves which means the bladder cannot signal when it is full, putting extra pressure on the kidneys. The combination of urine spending excessive time in the bladder and high sugar levels can lead to infection which may spread to the kidneys (National Kidney Foundation, 2007).

There are clear race related differences in susceptibility and outcome of nephropathies. The Multiple Risk Factor Interventional Trial demonstrated a higher incidence of ESRD for all causes in African-Americans compared with the white population (Klag *et al.*, 1997). Pazianas *et al.* carried out a study in the UK, concluding that there is a higher rate of diabetic nephropathy in patients from the Indian subcontinent and more hypertensive renal disease in individuals of Caribbean and African descent (Pazianas *et al.*, 1991). It has been questioned whether there is a genetic component for this difference.

1.1.1 Renal Replacement Therapy

A 2005 survey using the Fresenius medical care network data showed that worldwide there were 1.3 million people on renal replacement therapy (RRT), 89% using HD and 11% using PD (Grassman *et al.*, 2005). Dialysis is the default therapy for CKD; it provides incomplete replacement of lost renal excretory function and is a compromise between the outcome for the patient and the cost and inconvenience (Cambi *et al.*, 2005). Renal transplantation is the ideal treatment for ESRF as it replaces the lost renal function completely. The goal for future treatment is an artificial kidney which encompasses a small affordable device to deliver safe and effective RRT including metabolic and endocrine functions.

Treatment for ESRF is costly; it is estimated that 3% of the NHS budget is spent on this group of patients; however they only comprise 0.05% of the total population. The average cost of dialysis is £30,800 per patient per year; on 1 April 2009 there were 6,920 patients waiting for a transplant of which the majority were on dialysis, costing around £193m per year. The cost benefit of a transplant compared to dialysis is £24,100 per year for each year that the patient has a functioning transplanted kidney (NHS Blood and Transplant, 2009).

1.1.1.1 Dialysis

Dialysis is initiated upon the appearance of symptoms of uraemia. Due to the very slow progression of renal failure the patient adapts well to changes in metabolic state and can still feel well despite very low level residual renal function (Cambi *et al.*, 2005). Dialysis is not the ideal treatment for ESRF; the morbidity and mortality rates are very high, quality of life is suboptimal, the equivalent clearance is low and

the cost can be restrictive (Rastogi and Nissenson, 2009). There are two main dialysis options, haemodialysis (HD) or peritoneal dialysis (PD), the choice of which depends upon many factors. Comorbidities are taken into consideration as well as the home situation of the patient. On average patients on PD are 6 years younger than HD and have more chance of being white (Xue *et al.*, 2002a). Underweight patients have a lower chance of initiating on PD than those of a healthy weight (Xue *et al.*, 2002a). PD patients have a higher initial serum albumin and Hct and lower initial serum creatinine and blood nitrogen levels (Xue *et al.*, 2002a). Unequal clinical conditions between the groups make the determination of mortality rates between the two groups difficult to assess; patients on PD are generally in a more favourable condition on initiation of dialysis than HD patients (Xue *et al.*, 2002).

1.1.1.1.1 Haemodialysis

The fundamental process of separating solutes using semipermeable membranes *in vitro* was invented by Thomas Graham in 1854, and described as "dialysis" (Graham, 1854). Dialysis using an "artificial kidney" was first carried out on dogs at Johns Hopkins University in 1913 (Abel *et al.*, 1914). By 1944, the development of cellophane, antibiotics and heparin as an anticoagulant meant that Willem Kolff's method of extracorporeal dialysis was a success and is the basis of the methods used today (Kolff *et al.*, 1944; Kolff, 1965). The improvements in vascular access developed by Scribner *et al.* (Quinton *et al.*, 1960) allowed for repeated dialysis over many years as a long term treatment.

Haemodialysis is based on a very simple principle; blood flows on one side of a semipermeable membrane, with dialysis fluid on the other. Dialysis fluid consists of an osmotically balanced solution of electrolytes, buffer and glucose. This allows the passage of water molecules and low molecular weight solutes into the dialysate but larger solutes such as proteins and blood cells are retained. This mostly occurs by diffusion, the rate of passage depending on the concentration gradient. To maintain this concentration gradient the blood and dialysis fluid flow in opposite directions through the machine (Cambi *et al.*, 2005). Standard haemodialysis is performed three times a week, for 4-5 hours each time (Cambi *et al.*, 2005).

Excellent access is required for HD to be successful; this is usually obtained from a fistula created between a peripheral artery and vein or a permanent plastic catheter into a vein. Access problems are a major cause of morbidity. HD can be carried out in a main hospital, a satellite unit, or in the patient's home. Home HD generally provides the best quality of life but requires a trained helper as well a suitable space. It is likely that HD patients have more comorbidities upon initiation of dialysis which may affect dialysis efficiency and Epo responsiveness (Snyder *et al.*, 2004).

1.1.1.1.2 Peritoneal dialysis

During experiments on cats receiving PD in 1923 it was noted that the peritoneum was a "living dialyser" (Putnam, 1923). The concept of modern continuous ambulatory peritoneal dialysis (CAPD) was first described in 1976 and extensive developments have occurred to make it viable in humans for the treatment of ESRF (Popovich *et al.*, 1976). Peritoneal dialysis relies on the exchange of solutes and fluid between the peritoneal capillary blood and a dialysis solution in the peritoneal cavity (Gokal, 2005), which is the space between the abdominal viscera and the abdominal wall (Barratt *et al.*, 2009). This occurs across the semipermeable peritoneal membrane (Barratt *et al.*, 2009). Solutes move by diffusion and convective transport whilst fluids move by osmosis (Gokal, 2005). The kinetics of solute and fluid transport across the peritoneal membrane during PD remains incompletely understood (Gokal, 2005).

The peritoneum is a complex structure of living tissue which will vary between patients affecting the transport kinetics of PD as well as the dialysis efficiency. The transport characteristics may also change over time due to the dialysis procedure, effects of drugs or various physiologic reactions.

PD requires the insertion of a catheter into the patient's peritoneum which remains permanently and through which dialysate is infused. Safe and reliable access to the catheter is vital. PD is carried out by the patient at home or work and so allows a lot of independence. Contraindications for PD include intra-abdominal adhesions, abdominal wall stoma, obesity, intestinal disease, respiratory disease and hernias. Peritonitis is the most important cause of technique failure in PD, and complications can lead to death. PD provides a variety of modalities to allow

treatment to be tailored to each individual patient's needs (Barratt *et al.*, 2009). Few trials have been carried out comparing the clinical outcomes of these, but the small studies so far demonstrate no differences in mortality, dialysis related hypotension and hospitalisation (Rabindranath *et al.*, 2008).

1.1.1.2 Transplantation

The most clinically and cost effective treatment for many patients with ESRF is a successful kidney transplant. The first successful procedure was carried out on identical twins by Dr Joseph Murray in 1954 (Murray *et al.*, 1956). In 2008-09, 2,497 people in the UK received a kidney transplant (NHS Blood and Transplant, 2009) and at the end of March 2009, the UK Transplant Registry had records of over 23,000 people in the United Kingdom with a functioning kidney transplant (NHS Blood and Transplant, 2009). A transplant can come from a cadaveric donor, a living relative or an unrelated living donor. Over 90% of transplants should be working one year after surgery. A cadaveric transplant has a mean survival of 15 years and a living transplant 18-20 years. To make this an option for everyone who could benefit, a considerable increase in the number of kidneys donated is required, especially from black and ethnic minority populations (Department of Health, 2004).

1.1.2 Inflammation in chronic kidney disease

It appears that kidney disease is a pathological state of chronic inflammation. This causes damage to vascular walls, altering the expression of adhesion molecules on the endothelium and platelets and activating leukocytes which enhance the inflammatory state (Sharain *et al.*, 2013). The presence of inflammation in CKD is a strong predictor of outcome (Reddan *et al.*, 2003; Stenvinkel *et al.*, 2002). C reactive protein (CRP) is a plasma protein, levels of which increase during systemic inflammation. A European study found CRP to be increased in CKD patients, with about 75% of them having a CRP > 3.4mg/L (no CRP should be detectable) (Steinvinkel *et al.*, 2002). ESRF is associated with an increase in proinflammatory cytokines, with IL-1, IL-6 and TNFα 8-10 fold higher than in controls (Kimmel *et al.*, 1998). Yeun *et al.* concluded that CRP predicts mortality and is a significant risk factor for CVD in ESRD (Yeun *et al.,*2000); whilst another study found patients with

raised tumour necrosis factor 1 (TNF1) had increased chance of a non-fatal myocardial infarction or death as a result of coronary disease (Knight *et al.,* 2004).

Shlipak *et al.* found increased levels of all the inflammatory and procoagulant biomarkers they tested in elderly patients with renal insufficiency (Shlipak *et al.*, 2003). They state that inflammation appears to be found early in renal disease and could explain the high incidence of CVD in those with ESRF. Significantly they found that these inflammatory markers increased in a predictable fashion when progressing from control to CKD to ESRF.

As well as the general systemic inflammation often found in CKD there are many potential reversible causes, such as chronic obstructive uropathies, vasculitis, infection, biofilms in haemodialysis and *in situ* failed kidney grafts (Elewa *et al.*, 2012). Uraemia, atherosclerosis and obesity may also contribute to the inflammatory state (Meuwese *et al.*, 2011). Inflammatory mediators cause endothelial cell dysfunction and apoptosis, vascular smooth muscle proliferation, recruitment of leukocytes to the vascular wall, vascular calcification, plaque destabilisation and thrombosis (Schiffrin *et al.*, 2007; Elewa *et al.*, 2012). C reactive protein (CRP) is an acute phase reactant which is released in the liver in response to inflammation; it is a prominent marker of systemic inflammation as well as mortality in the general population and specifically CKD patients (Cachofeiro *et al.*, 2008; Weiner *et al.*, 2008).

Up to half of all CKD patients have increased serum levels of inflammatory markers such as C-reactive protein, fibrinogen, interleukin-6, tumour necrosis factor, factor VIIc, factor VIIIc, plasmin-antiplasmin complex, D-dimer, and the adhesion molecules E-selectin, VCAM-1 and ICAM-1 (Vaziri *et al.*, 1998; Iseki *et al.*, 1999; Oberg *et al.*, 2004; Nagesh and Pfeffer, 2004; Jofre *et al.*, 2006). The production of increased inflammatory mediators has been linked to increased oxidative stress, accumulation of postsynthetically modified proteins, advanced glycation end products and other substances normally cleared by the kidney. Causes of inflammation may be comorbidities, oxidative stress, infections and haemodialysis related factors such as bioincompatability and catheter use (Himmelfarb *et al.*, 2002). Deterioration of renal function may lead to dyslipidaemia and accumulation of uraemic toxins which can stimulate oxidative stress and inflammation (Schiffrin *et al.*, 2007). Erythropoiesis appears to be inhibited by cytokines such as TNF, IL-1

and interferon γ by supressing stem cell proliferation and erythroid colony formation (Kwack and Balakrishnan, 2006). Several studies have found that markers of inflammation are linked with a decreased response to Epo, and that these cytokines can increase their effect by disrupting iron metabolism (Stenvinkel, 2001; Drüeke, 2001)

1.2 Erythrocytes and haemoglobin

Red blood cells were first noted microscopically by Jan Swammerdam and described by van Leeuwenhoek in 1695 (van Leeuwenhoek, 1695). However these cells were not thought to be important until William Hewson in the 18th century concluded they must be vital due to their abundance (Dameshek, 1963). Knowledge of these cells was of little diagnostic value until 1879 when Paul Ehrlich developed stains that allowed a clear distinction between the different types of cells in the blood (Kasten, 1996).

Erythrocytes are the most numerous of the blood cells, responsible for delivering oxygen to tissues, and removing waste carbon dioxide. The cells develop in the bone marrow and circulate for around 120 days, covering about 300 miles in this time. Humans have 20-30x10^{13} red cells, making up about a quarter of the cells in the body. They are approximately 8μm in diameter, flexible biconcave disks and to fulfil their purpose effectively erythrocyte precursors lose most organelles leaving a mature red cell containing around 640 million haemoglobin molecules (Hoffbrand *et al*., 2006).

Haemoglobin (Hb) is a 64.4 kDa metalloprotein tetramer. Each molecule of normal adult HbA is made up of four polypeptide chains, two α and two β, each covalently bound to a haem group. The haem group is formed of a protoporphyrin molecule bound to a single ferrous ion (Fe^{2+}). The Hb tetramer is in equilibrium between a relaxed (R) structure and a tense (T) structure. Deoxygenated Hb is stabilised by inter and intra subunit bonds forming the T structure. When oxygen is unloaded, the β chains are pulled apart allowing 2,3-diphosphoglycerate (2,3-DPG) to enter, leading to lower oxygen affinity. As the molecule becomes saturated with oxygen these bonds break shifting to the R conformation. The tense form of Hb has low oxygen affinity and is favoured by increased H^+ or CO_2 concentration, allowing

oxygen to be given up more easily. In the lung capillaries the opposite conditions favour the relaxed form to bind oxygen (Hoffbrand *et al.,* 2006).

Haem synthesis occurs mostly in the mitochondria via a series of biochemical reactions. It begins with the condensation of glycine and succinyl coenzyme A by δ-aminolaevulinic acid (ALA), in a rate limiting step which requires the coenzyme pyridoxal phosphate (vitamin B6) and stimulation from erythropoietin. Eventually protoporphyrin combines with Fe2+ to form haem, a molecule of which then combines with a globin chain; four of these form a tetramer to create a haemoglobin molecule.

The red cell membrane contains an asymmetric phospholipid bilayer. The outer layer is formed of mostly choline-containing phospholipids, such as sphingomyelin, while the amine-containing phospholipids are mostly on the inner leaflet, apart from phosphatidylserine (PS) which is found exclusively on the inner layer. The membrane skeleton contains structural proteins such as α and β spectrin, ankyrin and actin which form a lattice on the internal membrane surface and help maintain the shape of the cell.

The membrane skeleton, composed of an intricately interwoven meshwork of proteins interacts with both integral membrane proteins and the lipid bilayer. The major proteins of the membrane skeleton include spectrin, actin, ankyrin, protein 4.1 and protein 4.2. α and β spectrin chains intertwine to form heterodimers which associate with other αβ-spectrin heterodimers to form heterotetramers, the functional spectrin subunit in the erythrocyte. These link to the plasma membrane via binding of ankyrin which binds the integral protein band 3. Protein 4.2 binds to band 3 and ankyrin, promoting their interaction. Protein 4.1 interacts with both spectrin and actin, as well as other proteins, including band 3 and glycophorin C, and the plasma membrane (Gallagher and Glader, 2013). See Figure 1.1.

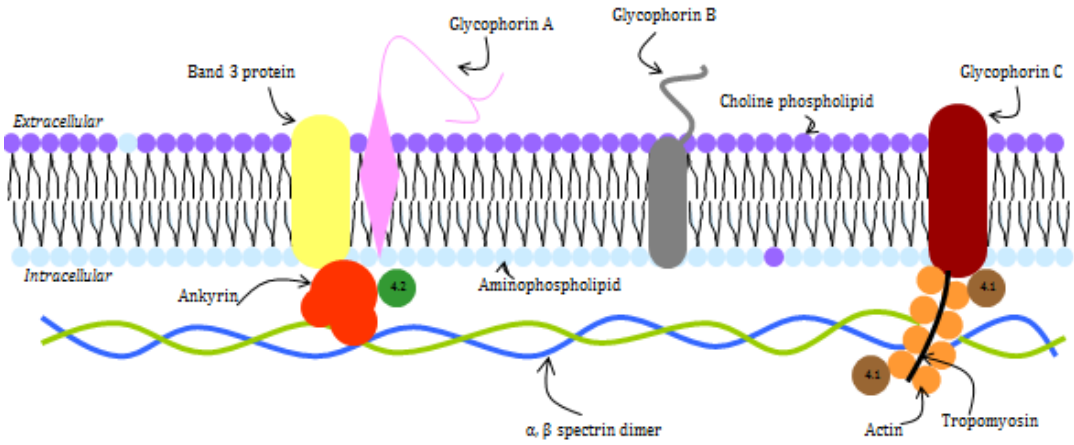


Figure 1.1 A representation of the red cell membrane structure. The lipid bilayer contains integral membrane proteins, which may have an external component. The membrane skeleton is formed by structural proteins which include α and β spectrin, ankyrin, protein 4.1 and actin. These form a lattice on the internal side of the membrane.

The process of maintaining this asymmetry is of major physiological importance to the cell and requires a large amount of energy. It is facilitated by a number of enzymes including aminophospholipidtranslocase, floppase, scramblase and calpain (Bevers *et al.*, 1999; Piccin *et al.*, 2007).

1.2.1 Erythropoiesis

Erythropoiesis is the process of red blood cell production. It first occurs in the foetal yolk sac and by day 40 moves to the foetal liver and spleen. During the last three months of foetal life erythropoiesis is established in the bone marrow and continues to occur in all bones until the age of 5. By the age of about 20 most long bones lose their erythropoietic ability and so the process almost exclusively occurs in the vertebrae, sternum, ribs and pelvis.

Healthy erythrocytes survive for about 120 days in the circulation and so there is a constant need to replace these senescent cells. Erythropoiesis is tightly regulated but can be rapidly increased when required, for example after blood loss or increased destruction. At the end of their lifespan changes in the plasma membrane lead to destruction by the reticuloendothelial system.

1.2.2 Regulation of erythropoiesis

The process of erythropoiesis is complex and influenced by many factors. These include growth factors such as erythropoietin (Epo), interleukin-3 (IL-3) and

granulocyte macrophage colony-stimulating factor (GM-CSF) and transcription factors including GATA-1 and 2 (Chateauvieux *et al.,* 2011). The anaemia found in renal patients appears to be most closely related to Epo so this thesis will concentrate on its role in erythropoiesis.

1.2.2.1 Erythropoietin

The existence of erythropoietin (Epo) was first suggested in 1906 by Carnot and Deflandre, as an intermediary factor that regulates erythrocyte production according to blood oxygen levels. They removed around 30ml of blood from anaemic rabbits, then took another sample the next day and injected that serum into normal rabbits. Within 1-2 days the concentration of erythrocytes in the recipients increased by 20-40% (Carnot and Deflandre, 1906a; Carnot and Deflandre, 1906b). This was further demonstrated in 1953 by Erslev and his team, establishing that this factor was responsible for regulating erythropoiesis. Large volumes of plasma were transfused from anaemic rabbits into normal rabbits which responded with significant reticulocytosis and increased haematocrit values (Erslev *et al.*, 1953). This substance was named erythropoietin by Bonsdorff and Jalavisto in 1948.

Epo has a short plasma half-life of 6-9 hours allowing a relatively rapid response to hypoxia. Epo levels increase in a range of situations reflecting changes in oxygen delivery to tissues. This gives a compensatory response to anaemia by increasing erythropoiesis, as there should be an inverse correlation with Epo concentration and Hb levels (Caro *et al.*, 1979).

1.2.2.1.1 Site of Production

In 1957 it was reported that Epo was released by the kidneys in response to anaemia (Jacobson *et al.*, 1957). Animal experiments further demonstrated this, as a bilateral nephrectomy in a dog suppressed erythropoiesis and prevented red cell production irrespective of the Hb level (Naets, 1960). The bone marrow was rapidly depleted of erythroblasts, often with none found after 72 hours. Administration of erythropoietic factor led to normal erythroblast production, demonstrating that the bone marrow can respond despite the uraemia and absence of the kidneys (Naets, 1960). The role of the kidney in Epo production

was confirmed using isolated kidneys, which responded to hypoxia by producing Epo (Erslev, 1975).

Later human studies also supported these findings. Demonstration of a low basal level of erythropoiesis in an anephric patient showed that the kidney was the primary production site of Epo (Nathan *et al.*, 1964). Patients with end stage renal failure were found to have low levels of serum Epo. After transplantation normal levels of Epo were restored (Denny *et al.*, 1966).

Cloning of the mouse Epo gene (Lin *et al.*, 1985) allowed the study of Epo mRNA following bleeding. In mice, blood loss resulted in a huge increase in Epo mRNA in the kidney, and a slight increase in the liver (Bondurant and Koury, 1986). The increase in serum Epo levels paralleled this increase, supporting the hypothesis that bleeding induces *de novo* synthesis of Epo, rather than release from storage (Koury *et al.*, 1989). Initial studies demonstrated that mRNA can be found in interstitial cortical cells near the base of the proximal tubular cells (Koury *et al.*, 1988) and it was later proven that most Epo is produced by the peritubular interstitial cells in the renal cortex (Lacombe and Mayeux, 1998). Using transgenic mice, cell type specific and hypoxia inducible expression of the human Epo gene have been demonstrated (Semenza *et al.*, 1991).

It is now known that around 90% of Epo is made in the kidney but it is also produced in small amounts by the liver, spleen, lung and testis and in macrophages. There are no preformed stores of Epo and it has a short plasma half-life, allowing relatively fast responses to changes in the tissues. The rate of production is inversely proportional to the oxygen capacity of the blood, and the vascular structure within in the kidneys makes them very sensitive to changes in oxygen levels, allowing the negative feedback mechanism to work effectively (Jelkmann, 2007).

1.2.2.1.2. Epo Structure

Epo is a 166 amino acid glycoprotein of 30.4kDa which acts as a hormone, cytokine and growth factor. Following translation, the molecule is heavily glycosylated, which is essential for its biological activity *in vivo*. It also contains three N-linked and one O-linked acidic oligosaccharide side chains, as well as 4 cysteine residues that form 2 disulphide bridges (Lai *et al.*, 1986).

1.2.2.1.3. Control of Epo gene expression

The human Epo gene is on the long arm of chromosome 7 (q11-q22) (Law *et al.*, 1986) and contains five exons and four introns (Lin *et al.*, 1985). The Epo gene encodes a 166 amino acid protein, which is biologically active, and a 26 amino acid leader sequence (Lin *et al.*, 1985; Jacobs *et al.*, 1985).

Epo levels vary according to the tissue availability of oxygen, with hypoxia being the main stimulus to Epo production (Adamson, 1988). This is detected in the kidney via the intracellular oxygen sensor hypoxia inducible factor (HIF) (Wang *et al*., 1995). HIF is a transcription factor which controls the rate of Epo production as well as affecting a large number of other hypoxia induced genes such as vascular endothelial growth factor and platelet derived growth factor (Wang and Semenza, 1993). Its degradation is inhibited by hypoxic conditions.

HIF is a heterodimer containing one of three α subunits (HIF1-α, HIF2-α or HIF3-α) bound to the aryl hydrocarbon receptor nuclear translocator (ARNT; also known as HIF1-β). The HIF complex is a member of a large family of transcription factors which contain a basic helix-loop-helix region, allowing unit dimerisation and the binding of hypoxia response elements (HREs) within DNA. HIF1-β is constitutively expressed, however hypoxia causes increased levels of HIF1- α by increasing protein stability (Jelkmann, 2013).

Ferrous iron prolyl hydroxylase is thought to be the oxygen sensor involved in this process (Bruick and McKnight, 2001). It requires oxygen as a co-substrate to hydroxylate conserved prolyl residues in the α-subunits of HIF, leading to ubiquitination by von Hippel-Lindau protein (Maxwell *et al.*, 1999; Ohh *et al.*, 2000). The molecules are then targeted for proteosomal degradation. Hydroxylation of an asparaginyl residue in the C terminal transactivation domain adds to the effect by blocking the formation of an active transcription complex in the cell (Maxwell, 2005). When exposed to hypoxia the α subunits are no longer hydroxylated and destroyed, leading to heterodimerization of HIF1-α and HIF1-β subunits and formation of an active transcription complex. Accumulated HIF can then bind to key sequences in several regulatory DNA regions, known as the hypoxia response element (HRE), which activates Epo transcription (Nangaku and Eckardt, 2006).

Tissue-specific expression of the Epo gene depends on distinct upstream (5') DNA sequences (Semenza *et al.*, 1991). One of the most important regulatory DNA sequences found near the Epo gene is the hypoxia response element (HRE). The active HIF transcription factor binds the HRE leading to enhanced Epo expression (Peyssonnaux *et al.*, 2007). HIF1-α may also affect hepcidin levels by negatively transactivating the hepicidin promoter, causing mobilisation of iron for red blood cell production (Peyssonnaux *et al.*, 2007).

Epo expression can be modulated by several other transcription factors. The 5'-promoter possesses GATA-binding sites (Jelkmann, 2007). GATA-4 is thought to recruit chromatin-modifying activity, promoting the expression of the Epo gene, while NF-κB and GATA-2 appear to inhibit Epo gene expression (Imagawa *et al.*, 1994). The nitric oxide synthase inhibitor, NO-monomethyl-L-arginine (L-NMMA) increases GATA-2 binding, thereby reducing Epo production. L-NMMA has been found to be markedly increased in uraemic patients, and has been implicated as a suppressor of Epo synthesis in CKD (Ribero *et al.*, 1996).

The proinflammatory cytokines IL-1 and TNFα activate GATA-2 and NFκB, contributing to the anaemia of chronic disease caused by Epo suppression (Jelkmann, 1998). Experiments have shown that a GATA specific inhibitor reversed the inhibitor effects of IL-1, TNF-α and L-NMMA on Epo production in hepatoma cell cultures (Imagawa *et al.*, 2003). Therefore, one common pathogenesis of anaemia of chronic disease and anaemia with renal disease appears to be via the stimulation of GATA binding activity by IL-1β, TNF-α, or L-NMMA (Imagawa *et al.*, 2003).

1.2.2.1.4. The Epo receptor

A single high affinity receptor expressed at low levels on erythrocyte precursors allows Epo to control red cell development (D'Andrea and Zon, 1990; Wognum *et al.*, 1990). The Epo receptor (EpoR) is a 484 amino acid glycoprotein which is part of the class I cytokine receptor family (D'Andrea and Zon, 1990). It is formed of a hydrophobic transmembrane sequence, a variable cytoplasmic domain and an extracellular domain with conserved cysteines and a WSXWS-motif (Youssoufian *et al.*, 1993). Upon binding of Epo, a p66 chain is dimerised causing JAK2 transphosphorylation (Lacombe and Mayeux, 1998). This in turn phosphorylates

specific tyrosine residues in the EpoR creating docking sites for several intracellular proteins with SH2 domains. Once docked, these proteins are activated, beginning several signal transduction pathways, including PI3K, JAK2, STAT5, MAP kinase and protein kinase C (Wang *et al.*, 1993; Damen and Krystal, 1996).

The EpoR is highly expressed on colony forming units-erythroid (CFU-E). They proliferate and differentiate in response to Epo, eventually producing mature erythrocytes. The expression of EpoR declines as the cell matures (Youssoufian *et al.*, 1993). Reticulocytes and mature erythrocytes do not express EpoRs (Sawyer and Koury, 1987).

A lack of Epo to bind to erythroid progenitors leads to caspase induced cleavage of GATA-1. This is crucial for progenitor maturation and survival and so Epo levels must be controlled to allow normal erythroid development (De Maria *et al.*, 1999).

1.2.2.1.5. Anti-apoptotic effects of Epo

Programmed cell death eliminates damaged or non-functional cells and controls the balance between removal of these cells and protection of the progenitors to maintain the red cell lineage. The fate of a cell is determined by its microenvironment which contains various cytokines and cell surface interactions to promote survival, proliferation and differentiation.

Several of the signal transduction pathways stimulated by Epo have anti-apoptotic effects. Phosphorylation of protein kinase b (Akt) has multiple effects on cell survival by maintaining mitochondrial integrity, inhibiting pro-apoptotic mediators such as glycogen synthase kinase-3β (GSK-3β) and caspase-9 and inhibiting activation of c-Jun N terminal kinases (JNK) (Kashii *et al.*, 2000; Sharples *et al.*, 2004). NFκB is dependent on JAK2 activation and causes expression of X-linked inhibitors of apoptosis (IAP) which inhibit caspase-3, -7 and -9, as well as enhancing expression of Bcl-xL which interacts with the pro-apoptotic Bcl-2-associated X protein (Bax). JAK2 phosphorylation by the EpoR also activates the STAT-5 family of transcription factors, leading to transcription of several genes, including an anti-apoptotic molecule of the Bcl-2 family (Silva *et al.*, 1999).

Involvement with molecular chaperones provides further anti-apoptotic mechanisms for Epo. Molecular chaperones guide folding, transport and assembly of proteins (Garrido *et al.*, 2003) but are not associated with the final product or function (Weiss and dos Santos, 2009). Epo stimulates nuclear translocation of Hsp70 which can rescue cells from apoptosis downstream of caspase activation (Jaattela *et al.*, 1998).

1.2.2.1.6 Extra haematopoietic effects of Epo

EpoR mRNA is expressed in a range of non-haematopoietic tissues such as endothelium, neuronal cells and the placenta (D'Andrea and Zon, 1990). Epo has been shown to improve outcomes in ischaemia and stroke (Ehrenreich *et al.*, 2002; Cai *et al.*, 2003; Joyeux-Faure *et al.*, 2006; Ramond *et al.*, 2007). rHuEpo induced protection is associated with a decrease in apoptosis in models of neurone and cardiac ischaemia (Celik *et al.*, 2002; Cai *et al.*, 2003; Cavillo *et al.*, 2003). It also appears to reduce apoptosis in proximal tubule cells (Sharples *et al.*, 2004) and endothelial cells (Chong *et al.*, 2002). Epo has been shown to reduce inflammation (Brines *et al.*, 2000; Liu, 2006 *et al.*) and have a protective effect on vascular endothelium (Chong *et al.*, 2002).

1.2.3 Erythrocyte differentiation in the bone marrow

All blood cells differentiate from a common progenitor in the bone marrow; the haematopoietic stem cell (HSC) (Kondo *et al.*, 2003). HSCs have the ability to self-renew and give rise to multipotent stem cells (Morrison *et al.*, 1997). Multipotent stem cells differentiate into myeloid or lymphoid lineages. These terminally differentiated cells cannot self-renew so must be constantly replenished (Opferman, 2007).

Figure 1.2 illustrates the development of erythrocytes. Pluripotent haematopoietic stem cells in the foetal liver and adult bone marrow generate erythroid progenitors. The earliest identified precursors are burst forming unit- erythroid (BFU-E) which proliferate slowly and do not express EpoR and are therefore unresponsive to Epo (Youssoufian *et al.*, 1993). After 48 to 72 hours in the presence of IL-3 or granulocyte-macrophage colony stimulating factor (GM-CSF), more mature BFU-E, which express EpoR, develop (Emerson *et al.*, 1985; Sawada *et al.*, 1990). Culture for another 4-5 days produces colony forming units-erythroid

(CFU-E) which are highly responsive to Epo and give rise to erythroblast colonies in around 7 days (Gregory and Eaves, 1977). Beyond the erythroblast stage erythroid cells are no longer dependent on Epo and so the EpoR levels decrease with maturation (Youssourian *et al.*, 1993).

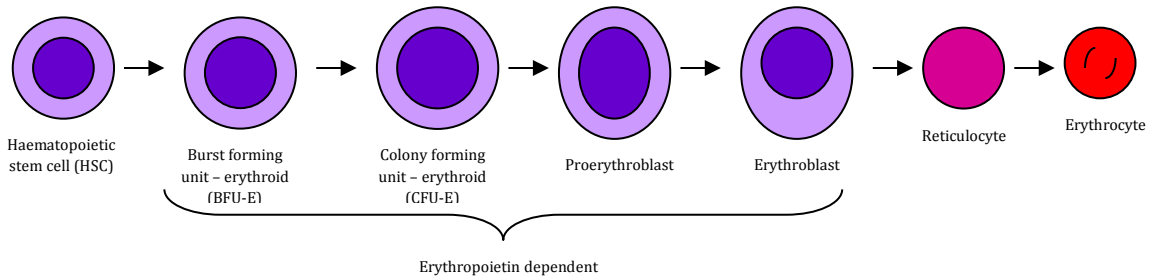


Figure 1.2 The maturation sequence in the development of mature erythrocytes from the haematopoietic stem cell.

It has been suggested that Epo promotes the survival of erythrocyte progenitor cells by retarding DNA cleavage (Koury *et al.,* 1990). A model predicted that normal levels of endogenous Epo are insufficient for the survival of most Epo dependent progenitors. An increase would therefore permit maturation of many more progenitors, thereby increasing erythrocyte production.

Studies in transgenic mice overexpressing Epo found that circulating erythrocytes in these conditions were sensitised to triggers of eryptosis but more resistant to osmotic lysis (Foller *et al.*, 2007). Epo inhibits apoptosis of erythroid progenitors, resulting in increased numbers of mature cells (Maiese *et al.*, 2005). Epo inhibits the cation channel function, therefore making the cell more resistant to eryptosis triggered by Ca2+ entry (Myssina *et al.*, 2003). However during production of red cells under conditions of high Epo, upregulation of proeryptotic factors may occur, allowing rapid removal of excess erythrocytes when Epo levels fall (Foller *et al.*, 2007).

When situations occur resulting in excess erythrocytes there is selective lysis of the youngest circulating red cells. This process is known as neocytolysis (Alfrey *et al.*, 1997). Rice *et al.* theorised that the process is initiated by a decrease in Epo to below a certain threshold (Rice *et al.,* 1999). They examined the process in CRF to see if it led to renal anaemia. Erythrocyte survival curves show increased cell death in the first 9 days after Epo withdrawal, consistent with neocytolysis. They suggested this process as an explanation for the better results seen with

subcutaneous than intravenous administration of Epo, due to smaller fluctuations in the levels of Epo occurring after subcutaneous administration. Neocytolysis appears to be required to allow control of excess red cell mass (Rice and Alfrey, 2005); if the decline was dependent upon only apoptosis of progenitors the fall would be very slow.

1.3 Anaemia in chronic kidney disease

Anaemia as a symptom of CKD was recognised by Richard Bright in 1836 when he noted the fading of the "healthy colours of countenance" of his renal patients. It is still considered as such, with anaemia an independent risk factor for the increased mortality and morbidity of CKD patients (Pereira *et al.*, 2010).

The world health organisation definition of anaemia is a Hb level < 13 g/dL for men and < 12 g/dL in women (WHO, 2009), however this was not designed to be taken as an international standard (Beutler and Waalen, 2006). Data from the Scripps-Kaiser database and the National Health and Nutrition Examination Survey (NHANES) studies suggest that a Hb lower than 13.7 g/dL in a white man aged between 20 and 60 years would indicate anaemia, with 12.2 g/dL being the corresponding value for a woman (Beutler and Waalen, 2006). The National Kidney Foundation (NKF) defines anaemia in CKD patients as a Hb level <13.5 g/dl in men and 12.0 g/dl in women (Macdougall et al., 2008a).

Anaemia is common in CKD patients, with the incidence and severity increasing with declining glomerular filtration rate (Astor *et al.*, 2002). Population studies demonstrate the incidence of anaemia to be less than 10% in CKD stages 1 and 2, 20-40% in stage 3, 50-60% in stage 4 and more than 70% in stage 5 (Hsu, 2002 *et al.*; Astor *et al.*, 2002).

The anaemia of CRF is normocytic (red cells are normally sized) and normochromic (red cells contain normal amounts of Hb) (Eschbach *et al.*, 1992). In anaemia, Epo levels are generally inversely proportional to Hb levels. However in renal failure the Epo levels are inappropriately low, because Epo deficiency is the primary cause of the anaemia (Spivak, 2000). It is generally accepted that a shortened erythrocyte lifespan is also partially responsible for the anaemia in

renal patients. However the cells are intrinsically normal, as they survive normally when transfused into a healthy person (Erslev and Besarab, 1997). A defect in a red cell metabolic pathway or a toxin found in uraemic plasma has been suggested as the cause of premature destruction (Erslev and Besarab, 1997). However whatever the cause it appears to be overcome by dialysis as the red cell lifespan in successfully managed patients is near normal (Erslev and Besarab, 1995). The anaemia caused by Epo deficiency is often compounded by iron and other vitamin deficiencies, inflammation, hyperparathyroidism, blood loss from haemodialysis, decreased erythrocyte life span and chronic gastro-intestinal blood loss. The illness causing the renal failure may also contribute to the anaemic state and diabetic patients seem to present earlier and with more severe anaemia (Bosman *et al.*, 2001).

Due to the slow progression of anaemia and CKD many patients adjust well to the fall in haemoglobin, however after treatment with rHuEpo there is generally a noticeable improvement in energy and vitality (Erslev and Besarab, 1997). Renal anaemia leads to a decreased quality of life often including the inability to work, fatigue and poor exercise tolerance. A requirement for transfusions comes with the associated risks (Parfrey and Wish, 2010). The Third National Health and Nutrition Examination Survey in the US found anaemia was more common in non-Hispanic black participants than non-Hispanic white people (Astor *et al.*, 2002).

Zeigler *et al.* developed a mouse model of Epo deficient anaemia to allow pre-clinical studies of this condition. Lack of a functional Epo gene causes embryonic lethality, but postnatal ablation using Cre recombinase allowed the gene to be silenced, leading to development of Epo deficiency and therefore chronic, normocytic, normochromic anaemia (Zeigler *et al.,* 2010).

Untreated anaemia places patients at increased risk of cardiovascular events, more rapid progression of CKD and significantly decreased quality of life (Lankhorst and Wish, 2010). A low haematocrit (Hct) (<30%) is associated with a significantly increased risk of death in patients with ESRD (Collins *et al.*, 1998).

Results from a study of patients with CRF and healthy people showed that serum Epo levels in normal individuals range between 1 and 27mu/ml (mean 6.2, n=53) whilst in CRF this increases to between 4.2 and 102mu/ml (mean 29.5, n=36)

(Garcia *et al.*, 1990). It has been suggested that even though the Epo deficiency is the primary cause of renal anaemia the uraemic state may blunt the bone marrow response to Epo (Fisher, 2003). The response of bone marrow cultures to Epo in the presence of plasma from patients with anaemia has been shown to be reduced compared to healthy individuals (McGonigle *et al.*, 1985; Radtke *et al.*, 1980). It may therefore be the case that pharmacological doses of Epo given to renal anaemia patients may correct the Epo deficiency but also overwhelm and suppress the bone marrow response (Fisher, 2003).

1.3.1 Use of erythropoiesis stimulating agents

Hegstrom *et al.* first predicted the requirement for recombinant human Epo (rHuEpo), stating "supplementary erythropoietin, were it available, might reduce significantly the transfusion requirement" (Hegstrom *et al.,* 1961). Prior to the introduction of rHuEpo therapeutically, red cell transfusions were used routinely when the symptoms of renal anaemia could not be sufficiently controlled by iron and anabolic steroids (Kliger *et al.*, 2012). The introduction of rHuEpo led to a huge decline in the number of transfusions required, however currently patients who have symptomatic anaemia requiring immediate treatment or those refractory to Epo may still receive red cells. Associated with these transfusions are the risk of viral infection, haemolytic transfusion reactions, iron overload and alloimmunisation (making finding a suitable donor more difficult) (Tanhehco and Berns, 2012).

Epo was initially extracted from 2500 litres of urine from patients with aplastic anaemia (uEPO) (Miyake *et al.*, 1977); however this method did not yield much product for therapeutic use or research. A small amount of amino acid sequence information was derived from the uEpo, allowing oligonucleotide probes to be used in a human foetal liver genomic library. Once the gene was found, it was spliced into a plasmid vector and transfected into Chinese hamster ovary (CHO) cells (Lin *et al.*, 1985; Jacobs *et al.*, 1985). Using mammalian cells leads to post translational glycosylation of the rHuEpo, which is secreted into the surrounding medium in an *in vivo* functional form. This produced a hormone fully active *in vivo* and physiochemically and biologically indistinguishable from uEpo (Imai *et al.*, 1990). Recombinant human erythropoietin (rHuEpo) has now become a standard treatment for the anaemia of CKD (Eschbach *et al.*, 1989).

The rHuEpo epoetin alpha was approved by the FDA in 1989 for use in dialysis patients (Lim *et al.*, 1989). It is identical in structure to endogenous Epo and administered by injection, but only has a half-life of 6 hours so must be given two or three times a week (Chateauvieux *et al.*, 2011). A multicentre study to determine dose regimen and safety profile of epoetin alpha concluded that 200-225U/kg/week, as two or three intravenous injections, is sufficient to maintain a Hb of 10-10.5g/dl over a one year period (Sundal *et al.*, 1991). They found the drug to be safe and well tolerated when used according to guidelines, with easily managed predictable side effects (Sundal *et al.*, 1991).

Darbopoietin was developed after the discovery that isoforms of Epo with more sialic acid residues have a longer half-life *in vivo* (Egrie *et al.*, 1993). Using site directed mutagenesis, the amino acid sequence at non-binding sites was altered to allow the incorporation of 22 sialic acid residues rather than 14 in endogenous Epo (Macdougall, 2008). This gives a half-life approximately three times longer than epoetin alpha, allowing less frequent dosing (Chateauvieux *et al.*, 2011). This novel erythropoiesis stimulating protein (NESP) appeared to work as effectively as epoetin at maintaining target Hb levels (Navaneethan *et al.*, 2011).

Continuous erythropoietin receptor activator (CERA) is composed of an Epo molecule with a large methoxy-polyethyleneglycol polymer chain integrated via amide bonds, leaving a molecule with double its original mass (Macdougall, 2008). After intravenous or subcutaneous injection it takes 135 hours to be eliminated from the circulation, vastly increasing the half-life of Epo (Chateauvieux *et al.*, 2011).

The response to rHuEpo is dose dependent, but a large variance is seen between individuals (Erslev and Besarab, 1997). The target Hb should be personalised to each patient depending upon their symptoms, comorbidities and Epo responsiveness within the range specified by guidelines (Lankhorst and Wish, 2010). The anaemia of CRF can be corrected in almost every patient provided they are not iron deficient (Pollock *et al.*, 2008). Eschbach *et al.* (1992) investigated the difference in rHuEpo response between normal and uraemic subjects and concluded that chronic uraemia does not alter the *in vivo* responsiveness to rHuEpo. These authors suggested that the anaemia of CRF is caused primarily by a hormone deficiency.

Analysis has not been conclusive regarding the risk of death by cardiovascular events in CKD patients on rHuEpo treatment (Mehdi and Toto, 2009); however there is some evidence that high doses of rHuEpo would be best avoided (Singh *et al.*, 2006). A study of 1233 HD patients with heart disease found that the group with target haematocrit (Hct) levels within the normal range (42±3%) had significantly higher mortality rates than controls (Hct 30±3%) (Besarab *et al.*, 1998). This led to suspension of the study and the suggestion that the Hct should not be increased to normal levels in HD cardiac patients. However Ma *et al.* analysed Hct and mortality in HD patients and found an increased risk of death with a Hct of <30% rather than 30-33% (Ma *et al.*, 1999). Examination of records from 71,717 dialysis patients showed a lesser probability of hospitalisation with a Hct of 33-36% than with a lower Hct (Xia *et al.*, 1999). In 2000 a further study concluded that dialysis patients without diabetes aged under 65 with no significant comorbidities should aim for a normal Hct as this increases quality of life and decreases mortality rates (Moreno *et al.,* 2000).

The specific mechanisms by which high doses of Epo may be associated with adverse events is also unclear, however Epo receptors have been found in a variety of tissues (Szczech *et al.*, 2008). Therapy with large rHuEpo doses does not reflect normal Epo biology and therefore the effect on Epo receptors is unknown (Fishbane, 2006).

Dosage requirements also appear to vary dependent upon the route of administration; rHuEpo via the subcutaneous route requires 15-50% less than the intravenous route (Uehlinger *et al.*, 1992). Snyder *et al.* studied over 100,000 dialysis patients aged at least 65 in the USA and concluded that those on peritoneal dialysis were far less likely to require Epo than those on haemodialysis (Snyder *et al.*, 2004). The Hb levels achieved with Epo treatment were similar in both groups but the dosage required in HD patients was approximately double that of those on PD. In the US Epo is usually administered intravenously to HD patients and subcutaneously to PD patients, however some studies have shown that in terms of the Hb level achieved, subcutaneous administration is more efficacious than intravenous (Kaufman *et al.*, 1998; Besarab *et al.*, 2002). The Veterans Affairs Cooperative Study Group randomly assigned haemodialysis patients to either intravenous or subcutaneous Epo and found the subcutaneous route required a

32% lower dose (Kaufman *et al.*, 1998). A pilot study suggested that the subcutaneous route can reduce the required Epo dose compared with intravenous administration (Bommer *et al.,* 1988). Eidmak *et al.* found that in HD and CAPD the target Hb was reached faster with subcutaneous than intravenous administration of Epo and that lower doses were required, leading to the conclusion that there is an increased efficacy of subcutaneous Epo over intravenous (Eidmak *et al.*, 2002).

1.3.2 Benefits of erythropoiesis stimulating agents

The use of erythropoiesis stimulating agents (ESAs) has significantly reduced the requirement for transfusion in dialysis patients to maintain a suitable Hb. Transfusion events in HD patients decreased during the period from 1992 to 2005, mostly in the 5 years after rHuEpo was introduced (Ibrahim *et al.*, 2008). Goodnough *et al.* stated "the availability of Epo in 1989 was accompanied by a significant reduction in the frequency of red cell exposure in patients undergoing dialysis from 1988". In 1988 the transfusion rate was 13.4/100 and this decreased to just 4.1/100 by 1991 (Goodnough *et al.*, 1994).

One of the most important perceived benefits of Epo treatment is the improved health related quality of life (HRQOL) however the evidence for this is poor. There are many limitations to the data analysis, such as the heterogeneity of study groups, the range of available HRQOL measures as well as the fact many anaemia studies are open label (potentially confounding the results) and are powered to assess other outcomes (Clement *et al.*, 2009). The Cochrane review (Strippoli *et al.*, 2006) could not perform meta-analysis due to these limitations but they did conclude that HRQOL is very likely to improve when higher Hb levels are targeted. Despite these issues there do appear to be patterns emerging. Leaf and Goldfan (2009) examined many studies and found that the best improvements are in vitality, energy and performance with modest improvements in social functioning and mental health but little improvement in emotional function and pain. They concluded that correction of anaemia in CKD leads to clinically relevant improvements in HRQOL. The maximum increase in HRQOL per incremental increase in Hb appears to occur between 10-12g/dL (Lefebure *et al.*, 2006).

Since the implementation of Epo therapy, mortality rates in dialysis patients have fallen, with a reduction in standardised mortality ratios of 17% shown between

1985 and 2002 (Wolfe *et al.*, 2005). Despite the widespread and routine use of ESAs very few randomised controlled trials assessing clinical outcomes with greater than 100 participants have been performed in the dialysis or CKD population.

Partial correction of anaemia with low doses of Epo was reported to slow the progression rate to ESRD in a group of CKD patients (Gouva *et al.*, 2004). However the CREATE (Cardiovascular Risk Reduction by Early Anemia Treatment with Epoetin Beta) study found that early treatment with Epo increased the likelihood of starting dialysis and the CHOIR (Correction of Hemogloblin and Outcomes in Renal Insufficiency) study found no reduction in the rate of progression of CKD in patients in the higher Epo dose group compared to those on the lower dose (Drueke *et al.*, 2006; Singh *et al.*, 2006)

Jungers *et al.* (2001) analysed the rate of decline of renal function and duration of the predialysis period in patients treated with rHuEpo. They compared this with the results from patients with the same degree of renal impairment but less degree of anaemia, not requiring rHuEpo. They found that moderate doses of rHuEpo in predialysis CRF patients which corrected the anaemia, resulted in a substantial delay in the need for renal replacement therapy (RRT) in half the patients studied.

1.3.3 Epo hyporesponsiveness

The introduction of rHuEpo has allowed the correction of anaemia in the majority of CKD patients, however around 25% of them require high doses and 5-10% do not respond to therapy (Macdougall, 1995). The need for large doses of rHuEpo to correct anaemia in certain patients was recognised soon after the treatment came into routine usage, but the reason was unknown (Adamson *et al.*, 1990). This phenomenon was called Epo resistance. A study of refractoriness to rHuEpo in 6 medically stable HD patients did not show any correlation with erythroid marrow responsiveness, red cell survival, rHuEpo pharmokinetics, hyperparathyroidism or aluminium excess (Adamson *et al.*, 1990). They concluded the reason for such large rHuEpo doses in HD patients was unknown.

rHuEpo resistance is a failure to achieve a Hb greater than 11g/dL with an epoetin alpha dose greater than 500units/kg/week (or equivalent of other rHuEpo) (Lankhorst and Wish, 2010). European best practice guidelines define an

inadequate response to rHuEpo as >300iu/kg/week subcutaneous or 400iu/kg/week intravenous (Hörl *et al.*, 2000). US guidelines require a "failure, in the presence of adequate iron stores, to achieve and maintain the target Hb level with 450iu/kg/wk intravenous or 300iu/kg/wk subcutaneous erythropoeitin" (National Kidney Foundation, 1997). The major finding of one study was that albumin is an important predictor of baseline Hb and Epo sensitivity in a representative sample of chronic HD patients (Agarwal *et al.,* 2008). The most common cause of an inadequate response to rHuEpo is iron deficiency (Hörl *et al.*, 2000). All patients must be iron replete to achieve and maintain an optimum Hb with minimum doses of rHuEpo, with intravenous iron used if required (Macdougall *et al.*, 1996). It has been suggested that inflammation and proinflammatory cytokines may play a role in Epo resistance (Drüeke *et al.*, 2001). Erythropoiesis is inhibited by cytokines such as TNFα, IL-1 and IFNγ. Markers of inflammation are associated with a decreased response to rHuEpo, especially CRP (Barany *et al.*, 1997).

Dialysis adequacy also appears to affect rHuEpo response. A US study found that rHuEpo dose decreased with increasing urea reduction rate (URR) and Hct increased with increased URR (McLennan *et al.*, 2000). Movilli *et al.* also found that rHuEpo dose decreased with increasing Kt/V Movilli *et al.* (2001). Less common causes of Epo hyporesponsiveness include blood loss, hyperthyroidism, aluminium toxicity, B12/folate deficiency, haemolysis, marrow dysfunction, haemaglobinopathy, ACE inhibitor therapy, carnitine therapy and antibodies against Epo (Macdougall and Cooper, 2002).

A study in 1990 involved giving rHuEpo to 8 patients with CRF and uraemia for a year whilst analysing red cell survival (Schwartz *et al,* 1990). They found that rHuEpo maintains a higher haematocrit in patients with progressive CRF by increasing the length of RBC survival. They suggested that rHuEpo has an effect on the bone marrow, producing red cells which resist the toxic uraemia haemolytic effect. It has been demonstrated that red cells formed in response to Epo or in conditions of severe anaemia have a shortened lifespan. Stohlman suggested that the rapid response in these conditions may account for these changes. The differentiated cells may skip division or the generation times may be shortened, in what was referred to as a "panic mechanism" (Stohlman, 1959; Stohlman, 1961). It

was suggested that reticulocytes produced under high Epo conditions may have an intrinsic membrane defect. However Gordon *et al.* found that erythrocytes produced in response to Epo have a normal pattern of haemoglobin electrophoresis, normal osmotic fragility and a normal oxygen carrying capacity (Gordon *et al.*, 1959).

1.4 Apoptosis and eryptosis

"Apoptosis" was first defined in 1972 to describe a morphologically distinct form of cell death (Kerr *et al*., 1972). The process occurs during normal development and aging as a homeostatic mechanism to maintain cell populations, as well as a defence mechanism in situations such as immune reactions or cell damage by disease (Norbury and Hickson, 2001). It appears that the majority of pro apoptotic stimuli require a mitochondrion dependent step, and so it was thought that mitochondria played an important role in programmed cell death (PCD) (Desagher and Martinou, 2000). Studies of apoptosis in mammalian cells found the only cells not to undergo apoptosis when treated with the protein kinase inhibitor staurosporine and protein synthesis inhibitor cycloheximide were erythrocytes (Weil *et al.,* 1996). They were therefore considered to be lacking the capability to undergo PCD. Thus PCD was only investigated in mitochondria containing cells, until Bratosin *et al.* examined the process in erythrocytes (Bratosin *et al.,* 2001). They found a regulated form of self-destruction could be induced, which shared several features with apoptosis. This indicated that erythrocytes express death machinery which can induce cytoplasmic and membrane changes associated with apoptosis.

The term eryptosis was first proposed by Lang *et al.* (Lang *et al.,* 2005). They noted the similarities between apoptosis and eryptosis (see Table 1.2), however they also acknowledged that erythrocytes are lacking certain mechanisms which are considered to be required for apoptosis. They stated "To distinguish the death of erythrocytes from apoptosis of nucleated cells, we do suggest the term 'eryptosis'" (Lang *et al.*, 2005).

Apoptosis	Eryptosis
Nuclear condensation, DNA fragmentation	No equivalent
Loss of mitochondrial membrane potential	No equivalent
Cell shrinkage	Cell shrinkage
Apoptotic body formation	Vesiculation
Activation of caspases	Generally caspase independent. Activation of μ-calpain
Loss of cell membrane asymmetry and therefore PS exposure	Loss of cell membrane asymmetry and therefore PS exposure
Death receptor expression	CD95 expression
Sphingomyelinase mediated sphingomyelin breakdown or excess ceramide synthesis causing ceramide accumulation	Sphingomyelinase induced ceramide formation
Release of Ca^{2+} from endoplasmic reticulum causing increased intracellular levels	Activation of cell membrane Ca^{2+} permeable cation channels

Table 1.2 Comparison of apoptosis and eryptosis. Based on table from Lang et al., 2006a. Apoptosis is characterised by loss of cellular K+ leading to cell shrinkage, nuclear condensation, DNA fragmentation, mitochondrial depolarisation, cell membrane blebbling and breakdown of plasma membrane asymmetry (Gulbins et al., 2000). Some of these features, cell shrinkage, membrane blebbing and PS exposure also occur in eryptosis (Bratosin et al., 2001).

A typical mature erythrocyte lives for about 120 days in the circulation. Since these cells lack a nucleus, they cannot divide or synthesize new cellular components. As a result, the cells degenerate due to aging or damage in a process of senescence (Bratosin *et al.*, 2001). The process of eryptosis is an alternative to haemolysis which has negative effects within the body. Rupture of erythrocyte cell membranes releases haemoglobin to the extracellular fluid, this may be filtered in the glomerula of the kidney and precipitate in the acid lumen of the tubules, obliterating the tubules and causing renal failure (Lang *et al.*, 2005). Erythrocytes therefore require a disposal mechanism which does not lead to the release of intracellular components. The process of eryptosis leads to the production of red cell microparticles (RCMPs).

Excessive eryptosis has been observed in a wide variety of diseases or clinical conditions, including haemolytic uremic syndrome (Lang *et al.*, 2006a) and renal insufficiency (Myssina *et al.*, 2003), as well as related to a range of drugs, environmental pollutants and endogenous substances (Lang *et al.*, 2008). Further studies have shown eryptosis may be inhibited under other conditions (Lang *et al.*, 2008), most significantly by erythropoietin (Myssina *et al.*, 2003).

Neocytolysis is another process postulated to be related to eryptosis. It is the selective destruction of young circulating erythrocytes when red cell mass becomes excessive for the environment (Alfrey and Fishbane, 2007). It is not clear whether neocytolysis occurs by an identical mechanism to eryptosis, but it has been speculated that stimulation of progenitor cell survival by excessive Epo leads to red cells overly susceptible to eryptosis (Lang *et al.,* 2012a). In response to a rapid decrease in already elevated levels of serum Epo, neocytolysis is triggered to increase from basal levels. The process is thought to begin within 24 hours and can reduce red cell mass by 10–15% within 7–10 days (Alfrey and Fishbane, 2007). This may accelerate the otherwise slow negative feedback loop (Lang *et al.*, 2012a).

1.4.1 The process of eryptosis

MPs are formed in response to activation or apoptosis (Lacroix *et al.*, 2011). Activation of caspases and calpains during apoptosis result in activation of the MP pathway (Piccin *et al.*, 2007). Current knowledge of microparticle (MP) formation derives mainly from experiments performed in isolated or cultured cells (Leroyer *et al.*, 2008). The mediators and mechanisms involved *in vivo* are mostly unknown. Most studies and therefore knowledge on MP formation are based on platelets or endothelial microparticles. Whether all of the details translate to red cell microparticles is yet to be established.

Studies on platelets have given indications of the mechanism of microparticle formation. It is thought that microparticle formation occurs due to the disruption of the asymmetric distribution of phospholipids in the cell membrane. Phospholipid expression is conserved throughout all cell types, with phosphatidylcholine and sphingomyelin expressed externally and phosphatidylserine and phosphatidyl-ethanolamine found on the internal cell

membrane. The distribution is maintained in a dynamic steady state by a number of enzymes including aminophospholipid translocase, floppase, scramblase and calpain (Bevers *et al.,* 1999; Piccin *et al.*, 2007) as described previously.

The activity of aminophospholipid translocase is dependent on ATP and it functions to transport aminophospholipids (phosphatidylserine and phosphatidyl-ethanolamine) from the surface to the inner layer of the cell membrane. It is inhibited in the presence of calcium. Floppase works in a similar ATP dependent fashion, but exports lipids from the inside to the outside of the cell. Scramblase also facilitates the movement of lipids, but is usually inactive (Bevers *et al.*, 1999). These three enzymes are all membrane bound. The cytoplasmic enzyme calpain is also usually inactive, but upon activation it cleaves long actin filaments leading to cytoskeleton disruption. It also cleaves phosphatidylinositol phosphate (PIP) kinases, which usually catalyse the conversion of PIP to phosphatidylinositol 4,5-biphosphate (PIP_2) (O'Connell *et al.*, 2005). An increase in PIP_2 within the cell membrane increases the membrane-cytoskeleton adhesion energy (Raucher *et al.*, 2000). Therefore calpain acts in a twofold fashion by destabilising the cytoskeleton as well as preventing its reinforcement via inhibition of PIP_2 formation.

Microparticle formation is generally triggered by cellular activation, apoptosis/eryptosis or necrosis, all of which involve the release of calcium ions from the endoplasmic reticulum. This calcium influx inhibits aminophospholipid translocase and activates scramblase and calpain. This causes a loss of lipid symmetry in the cell membrane and the surface exposure of phosphatidylserine (Lang *et al.,* 2005; Lang *et al.*, 2003). The disruption of the cytoskeleton mediated by these enzymes after calcium activation is the key to microparticle formation. The lack of attachment of the membrane and cytoskeleton allows budding from the surface, eventually producing detached microparticles which expose phosphatidylserine on their cell surface. Phosphatidylserine at the erythrocyte surface is recognised by macrophages which engulf and degrade the affected cells (Lang *et al.*, 2005).

1.4.2 Microparticles

Platelet microparticles were first reported by Peter Wolf in 1967 as "cell dust". Microparticles are vesicles formed from the budding of cell membranes from a

variety of cell types, including platelets, endothelial cells, monocytes, granulocytes and erythrocytes. They are heterogeneous (Mause and Weber, 2010) and generally 0.2-2μm in diameter but red cell microparticles (RCMP) are typically smaller, with a diameter of approximately 0.15 μm (Piccin *et al.*, 2007). In the blood of a healthy individual the majority of microparticles are derived from platelets (Horstman *et al.*, 1999), however there are variations in the reported figures, from 56% (Berckmans *et al.*, 2001) to greater than 90% (Horstman *et al.*, 1999). The contribution of erythrocytes and leukocytes to the overall pool of circulating microparticles is much lower, reported in one study as 7% RCMP in whole blood (Xiong *et al.*, 2012).

Most studies involving MPs concentrate on platelets, as these are the most numerous. Based on this population a commonly used definition is particles less than 1μm in diameter, exposing PS and surface proteins of the cell of origin (Distler *et al.*, 2005a). Whether this is a robust definition which can be applied to MPs derived from any cell type is yet to be established.

It is also thought MPs carry a cargo of cytokines, signalling proteins, mRNA and microRNA (miRNA) which can act in signalling pathways and are involved in intercellular communication (Mause and Weber, 2010). It has been suggested that microparticles act as transport vesicles for miRNA, protecting them from degradation (Diehl *et al.,* 2012). Significant differences between the miRNA profile of patients with stable and unstable coronary artery disease were noted, all of which were different again from the cell of origin. The findings describe an interesting mechanism for transferring gene-regulatory function from microparticle releasing cells to target cells via circulating microparticles.

It has been postulated that microparticles may play a role in the regulation of inflammation (Distler *et al.*, 2006). Microparticles can both induce and amplify inflammation, and depending on the cell type affected and the local microenvironment different mechanisms may be involved, including the transfer of surface receptors, activation of complement and stimulation of cytokine release (Distler *et al.*, 2006). Both pro- and anticoagulant proteins have been detected in platelet microparticles (Tans *et al.*, 1991). It has been shown that monocytes shed microvesicles of <0.5μm diameter from their plasma membrane after activation, which contain bioactive IL-1β. T cell microparticles can induce macrophages to

undergo apoptosis, potentially impairing a key element of the immune system and triggering MP release from the dying macrophage to augment other immunological and vascular events (Distler *et al.,* 2005a).

As shown in *in vitro* studies, microparticles display a variety of proinflammatory activities that could contribute to the pathogenesis of inflammatory disease. Thus, these particles can promote adhesion and rolling of leukocytes, contain proinflammatory cytokines, and trigger the release of microparticles from various cell types *in vitro* (Aupeix *et al.*, 1997; Nieuwland *et al.*, 2000; Minagar *et al.*, 2001; Brogan *et al.*, 2004). Microparticles could trigger inflammation by activating the complement cascade. It has been found that the recognition unit of the classical complement pathway, C1q, binds to microparticles released from apoptotic Jurkat T cells. C1q bound to microparticles can activate the classical complement pathway, as demonstrated by deposition of C3 and C4 on the surface of microparticles (Nauta *et al.*, 2002)

There is evidence of elevated microparticle levels in a number of systemic and chronic conditions such as diabetes mellitus (Diamant *et al.*, 2002), renal failure (Faure *et al.*, 2006) and coronary artery disease (Bernal-Mizrachi *et al.*, 2003), however none of these studies investigated red cell microparticles. Daniel *et al.* (2008) state that "elevated levels of MP have been detected throughout the entire process of vascular damage associated with renal diseases". They suggest that microparticles relate to the cardiovascular risk associated with CKD and they have a deleterious effect on renal patients. Studies of MP found similar numbers of cell-derived MP in asymptomatic patients with well-controlled, uncomplicated type II diabetes and in controls (Diamant *et al.,* 2002). However they did find higher proportions of MP derived from T-helper cells, granulocytes, and platelets which exposed tissue factor (TF) in patients. TF is the major *in vivo* initiator of coagulation and so increased expression of TF may promote thrombotic events.

Increased endothelial MPs have been found in chronic renal failure patients, both those on HD and pre dialysis, possibly attributing to the higher risk of cardiovascular events and endothelial dysfunction, a critical element in the pathogenesis of atherosclerosis (Faure *et al.,* 2005). Studies of patients with chronic arterial disease found increased endothelial MPs compared with control subjects (Bernal-Mizrachi *et al.*, 2003).

Erythrocytes of patients with CRF are often found to have membrane structural and functional abnormalities, such as an increase in PS exposure in uraemic patients (Bonomini *et al.,* 1999). This is thought to be due to a failure of the transport mechanism which internalises PS from the outer leaflet of the erythrocyte membrane (Bonomini *et al.*, 1999). It has also been demonstrated that upon exposure to uraemic plasma, increased PS externalisation occurs (Bonomini *et al.*, 2004). Exposure of PS leads to recognition and destruction by macrophages, so the abnormal exposure found in uraemic RBCs may explain the shortened life span seen in uraemia (Bonomimi *et al.*, 2004). Patients with CKD often experience associated vascular disease; this may be explained by the increased PS exposure on MP promoting adhesion to the vascular endothelial cells, leading to vascular inflammation and/or leucocyte adhesion (Bonomini *et al.*, 2002).

It has been shown that hyperglycaemia can induce a loss of phospholipid asymmetry in human erythrocytes, an effect which can be seen in diabetes (Wilson *et al.*, 1993). With diabetes being such a common cause of CKD this could influence the patterns seen in renal patients. Willekens *et al.* concluded that "vesiculation constitutes a mechanism for the removal of erythrocyte membrane patches containing removal molecules, thereby postponing the untimely elimination of otherwise healthy erythrocytes" (Willekens *et al.,* 2008). Consequently, these same removal molecules mediate the rapid removal of erythrocyte-derived vesicles from the circulation." Similarly, it has been speculated that MPs could be a means for erythrocytes to prevent a premature removal from circulation when they are still functional or when lesions are reversible (Solheim *et al.*, 2004). If this was correct it would allow erythrocytes to clear non-functional molecules that would otherwise cause eryptosis or remove IgG binding senescent erythrocytes via macrophages (Kriebardis *et al.*, 2007; Bosman *et al.*, 2008).

1.4.3 Measurement of eryptosis

Previous studies have used a variety of measures of eryptosis, including cell size, annexin V binding, Ca^{2+} activity and ceramide formation. Phosphatidylserine exposure measured via annexin V binding with no size discrimination has been used (Calderón –Salinas *et al.*, 2011), and the same method but considering only annexin V events with a mean fluorescence greater than 1.0 as positive (Bonomini *et al.*, 1999). Another study measured glycophorin A and annexin V dual positivity,

similarly with no sizing (Biro *et al.*, 2004). Shet *et al.* imposed a strict definition of microparticles, which had to fulfil the criteria: (i) size = 1.0μm; (ii) ability to be isolated from platelet-free plasma by ultracentrifugation; and (iii) surface expression of phosphatidylserine, as shown by annexin V binding (Shet *et al.*, 2004). Studies by Xiong *et al.* defined red cell microparticles as annexin V+ glycophorin A+ events between 0.5 and 0.9μm (Xiong *et al.*, 2011; 2012). Multiple studies have measured all of; cytosolic calcium activity (using Flou3 fluorescence), cell volume (forward scatter), phosphatidylserine exposure (annexin V binding) and ceramide formation (anti ceramide antibody) to determine eryptosis levels (Lang *et al.*, 2005; Niemoeller *et al.*, 2006; Kempe *et al.*, 2007; Kiedaisch *et al.*, 2008; Ahmed *et al.*, 2013).

1.5 Aims and thesis plan

The process of eryptosis, leading to the production of red cell microparticles has not previously been studied in ESRF patients on dialysis. This study aims to assess the levels of microparticles in these patients as a measure of eryptosis. Determination of associations between clinical factors and numbers of eryptotic cells will provide valuable information on the pathogenesis and drivers of the response.

Currently, the anaemia associated with renal failure is treated with supraphysiological doses of Epo. There is some evidence that giving very high doses of this hormone is associated with morbidity and mortality (irrespective of the haemoglobin level). Investigating the process of eryptosis may provide insight into the mechanisms of renal anaemia and therefore possibly help to explain rHuEpo hyporesponsiveness. If eryptosis plays a significant role in causing renal anaemia, then this would permit exploration of other ways of treating these patients.

The objectives for this study were to determine:

- Does the degree of renal failure affect the concentration of eryptotic cells?
- Does this exacerbate anaemia?

- Does dialysis modality affect eryptosis levels?
- Are there other factors which correlate with eryptosis levels eg inflammation, co morbidity (especially diabetes), race, Epo requirements?

The subsequent chapters will address these aims:

Chapter 2 – Method development

In order to fulfil these aims, this thesis will describe the development of a flow cytometric method for the measurement of red cell microparticles, as an indication of the level of eryptosis occurring *in vivo*.

Chapter 3 – Flow cytometric analysis of red cell microparticles

Samples from dialysis patients will be analysed using this method, and the microparticle levels compared with available patient demographics and other pathology results. Any associations will be investigated further.

Chapter 4 – *In vitro* flow model

Further investigations into the link with inflammation and erythropoietin will be undertaken using an *in vitro* whole umbilical artery continuous perfusion loop.

Chapter 2: Development of a flow cytometric method for the analysis of red cell microparticles

2.1 Introduction

This chapter describes the development of a flow cytometric method suitable for the analysis of RCMP. The methods within this project have been used previously to analyse MPs derived from a range of cell types (Macey *et al.*, 2011; Macey *et al.*, 2010). In order to refine them solely for RCMP analysis and to ensure suitability for the purpose of measuring eryptosis, method development was undertaken prior to the receipt of any patient samples.

There is no standardised method to enumerate RCMPs (Shah *et al.*, 2008; Rubin *et al.*, 2008; Ayers *et al.*, 2011; Lacroix *et al.,* 2012; Xiong *et al.*, 2012). However the primary method for microparticle detection is to use flow cytometry (FCM) of purified MP using one or two colour fluorescence (Christersson *et al.*, 2013). This gives the ability to quantitate as well as provide data on the surface antigens of the particles. There are a range of technical issues relating to the methodology as well as a lack of consensus on the definition of a microparticle. The major sources of variability are differences in pre analytic processing, the definition of MP used and the difference in flow cytometer settings and MP analysis (Shah *et al.*, 2008; Rubin *et al.*, 2008; Robert *et al.*, 2009; Ayers *et al.*, 2011; Lacroix *et al.*, 2012). There have been a range of approaches cited in the literature, mostly dealing with platelet MPs (Rubin *et al.*, 2008).

Technical issues include the detection limits of the chosen flow cytometer, whether to use plasma or whole blood, the preparation process, which anticoagulant to use and the choice of antibodies. The definition of a microparticle is poorly standardised between laboratories, with regard the antigenic characteristics and the sizing. Some studies have reported that the detection limits of flow cytometry mean that small events are excluded from analysis and therefore totals are underestimated (Jy *et al.*, 1995). However the limit of detection of the instrument in the present study was previously demonstrated to be between 0.6 and 0.1μm when using side scatter but not forward scatter (Macey *et al.*, 2011).

Microparticles are too small to be seen with light microscopy but can be detected by electron microscopy. Their morphology has been reported to be "heterogeneous" (Horstman *et al.,* 2004) with relation to size and density and the presence of multiple phenotypes reported dependent upon the conditions under which they are produced (Jimenez *et al.*, 2003).

A major problem in enumerating microparticles is cellular activation, which can occur during venesection or *in vitro* prior to analysis. Artefactual cellular activation can be minimised by adequate blood collection procedures but an appropriate anticoagulant and storage temperature must also be used. None of the commonly used anticoagulants (citrate, EDTA or heparin) can prevent platelet or neutrophil activation (McCarthy, 2001; Macey *et al.*, 2002; Macey *et al.*, 2003) but the effect of anticoagulants on red cells has not been investigated.

Most studies have used frozen and thawed platelet poor plasma (PPP), and prepared cell free plasma employing different centrifugation steps, including high speed centrifugation (Shah *et al.*, 2008; van Ierssel *et al.*, 2010). The preparation steps, of centrifugation and thawing frozen samples, influence the amount of MPs. Recommendations for pre-analytical procedures have been published (Lacroix *et al.*, 2011), however there is still a lack of standardisation. A method has been developed using whole blood, to reduce *in vitro* activation and negate the influence of centrifugation on MP numbers (Christersson *et al.*, 2013).

The definition of RCMPs is unclear. Using them as a measure for eryptosis causes further confusion. Previous studies have used a variety of measures of eryptosis, including cell size, annexin V binding, Ca^{2+} activity and ceramide formation (Lang *et al.*, 2005; Niemoeller *et al.*, 2006; Kempe *et al.*, 2007; Kiedaisch *et al.*, 2008; Ahmed *et al.*, 2013).

Platelet microparticles are still the most widely studied group of MPs. Based on this, the definition of a MP is determined by the presence of PS on the surface, surface antigens representative of the cell of origin and some sizing parameters. However whether this can be universally applied to all MP populations is debatable.

In this study, RCMPs were initially defined as glycophorin A+ annexin V+ events as described previously (Xiong *et al.,* 2012). However this is not always the definition of choice as MP may not always express PS or bind annexin V.

2.2 Materials

1x Annexin V binding buffer (ABB: 10 mMHepes/NaOH (pH 7.4) 140 mMNaCl, 2.5 mM $CaCl_2$) passed through a 0.22µm diameter filter (Merck-Millipore, Billerica, USA).	BD Biosciences, Oxford, UK
1.1µm diameter latex polystyrene beads diluted 1µl in 1ml filtered ABB	Sigma, Dorset, UK
SpheroTech AccuCount Fluorescent Particles 10.1µm ~$1x10^6$ particles/mL	SpheroTech Inc, Illinois, USA (from Saxon Europe, Scottish Borders, UK)
Monoclonal mouse anti-human glycophorin A (CD235a) Fluorescein Isothiocyanate (FITC) Clone JC159	Dako, Glostrup, Denmark (from Alere, Cheshire, UK)
Annexin V phycoerythrin (PE)	BD Pharmingen, Oxford, UK
Anti-human CD45 Allophycocyanin (APC) clone 2D1	eBioscience, Hatfield, UK
Cytometer Setup and Tracking (CST) Beads	BD Biosciences, Oxford UK
Tripotassium Ethylenediaminetetraacetic acid (K_3 EDTA) vacutainers	BD Biosciences, Oxford UK
Citrate, theophylline, adenosine and dipyridamole (CTAD) vacutainers	BD Biosciences, Oxford UK

Table 2.1 Reagents for FCM analysis of RCMPs

2.3 Samples

The development of the assay was carried out on samples from healthy controls, who all gave written informed consent as stipulated by the protocol submitted to City Road & Hampstead Research Ethic Committee (ref: 11/LO/0816) . Whole

blood was obtained by clean venepuncture from the antecubital vein using the BDVacutainer® Safety-Lok™ Blood Collection Set, into 4.5ml EDTA tubes.

2.4 General methodology

The basic methods for this study have been employed previously (Macey *et al.*, 2010; Holtom *et al.*, 2012). Flow cytometric analysis involved incubation of the sample with antibodies, annexin V binding buffer, 10μm counting beads and 1μm sizing beads. Events gated on the annexin V/glycophorin A dual positive population were enumerated using 10μm standardised beads. The method was varied to allow comparisons of different variables and the final experimental method described in Chapter 3.

2.5 Reagents

Antibodies conjugated to fluorescent dyes can be used to detect the presence of antigens on the surface of microparticles using flow cytometry. Cell specificity, abundance of the antigen on parent cells and microparticles and stability and availability of monoclonal fluorescently conjugated antibodies are important factors in choosing a target antigen. Glycophorin A was chosen to allow detection of microparticles expressing red cell antigens (Simak and Gelderman, 2006).

Microparticle phosphatidylserine exposure can be detected *in vitro* by labelling with fluorochrome conjugated annexin V (Thiagarajan *et al.*, 1990; Vermes *et al.*, 1995). Annexin V is a phosphatidylserine (PS) binding protein derived from placenta which binds PS at physiological calcium concentrations (Reutelingsperger *et al.*, 1985; Funakoshi *et al.*, 1987). It has been used to detect PS exposure on the surface of aged erythrocytes (Boas *et al.*, 1998), circulating microparticles (Dachary-Prigent *et al.*, 1993), activated platelets (Thiagarajan *et al.*, 1990) and apoptotic leukocytes (Vermes *et al.*, 1995).

PS exposing platelets have a significant role in coagulation; however the role of PS exposing RCMP is unclear. The ability to express PS suggests that erythrocytes should have a role in clotting (Andrews and Low, 1999) and evidence shows that the increased PS exposure may contribute to the increased thrombotic risk in

paroxysmal nocturnal haemaglobinurea and polycythaemia vera (Ninomiya *et al.*, 1999; Tan *et al.*, 2013).

However it appears that not all microparticles expose PS. Freyssinet (2003) proposed that all microparticles express PS and therefore bind annexin V, however this was disputed, with evidence presented of endothelial derived microparticles which do not bind annexin V (Jy *et al.*, 2004). Further studies have found a discrepancy in annexin V binding of platelet derived microparticles in thalassaemic patients, with only 7% of them capable of binding annexin V (Pattanapanyasat *et al.*, 2007). There has been little research into the binding of annexin V to red cell microparticles.

2.6 Methodology variations

2.6.1 Antibody titration

Antibody titrations were used to determine the optimum antibody volume for each experiment without unnecessarily wasting antibody. Also nonspecific antibody binding may cause erroneous and difficult to interpret results. This can be eliminated by optimising the amount of antibody used, to a volume which gives the highest signal of the positive population and the lowest signal of negative population (Hulspas, 2010).

A titration curve was created by using serial dilutions of antibody covering a range above and below the manufacturers' recommendation (which varies between products). Six dilutions were used and mixed with 10µl of whole blood, as in the original method, then incubated for 10 minutes. The samples were then analysed on the flow cytometer. This was carried out for both glycophorin A and annexin V.

2.6.2 CTAD-EDTA v EDTA

There is a lack of consensus with regard to the choice of anticoagulant for microparticle enumeration. Platelet derived microparticles are the most commonly studied and these generally involve the use of the anticoagulant sodium citrate. However EDTA is the general anticoagulant of choice for the study of red blood cells, and so may be more appropriate for RCMP enumeration. Studies have shown

increased stability of platelets in CTAD-EDTA, compared to sodium citrate (Mody *et al.*, 1999; Macey *et al.*, 2003), and so this is often used. The lack of consensus limits the standardisation of microparticle analysis methods.

CTAD contains sodium citrate and citric acid as anticoagulants, and theophylline, adenosine and dipyridamole as inhibitors of platelet activation. These inhibitors prevent *in vitro* spontaneous activation of platelets by increasing cytosolic AMP concentration (Contant *et al.*, 1983). When taken into CTAD, platelet MP increased with time, but to a lesser extent than when taken into sodium citrate (Kim *et al.*, 2002). CTAD was first developed for coagulation tests to eliminate coexisting platelet effects in platelet-poor plasma. Reinhart *et al.* first tried to use CTAD anticoagulated blood for haematological testing in a relatively small number of cases, and reported no difference in haematological data produced by electronic particle counters between blood anticoagulated with EDTA and CTAD (Reinhart *et al.*, 1990). Tsuda *et al.* also used CTAD blood for complete blood counts and automated white cell differentials to prove its efficacy for haematological analysis (Tsuda *et al.*, 2000). In this study, large numbers of samples were investigated with two automated analyser systems based on different methods. The data for parameters related to RBC were similar to those in EDTA blood and CTAD blood. This has been confirmed by Macey (2001, unpublished data).

The use of CTAD-EDTA requires the blood samples to be taken into an EDTA vacutainer, mixed, and then immediately decanted into a CTAD vacutainer. This was carried out with bloods from four controls, with EDTA samples taken at the same time. They were analysed on the flow cytometer and the number of RCMP in the two differently anticoagulated bloods compared.

2.6.3 Time delay

Whole blood samples are known to degrade over time, however the effect on microparticle formation is unknown. Lacroix *et al.* found an increase in total microparticles over 4 hours in platelet free plasma (PFP) but no change in erythrocyte microparticles (Lacroix *et al.*, 2011). Ayers *et al.* found the number of microparticles increases with increased time between venepuncture and processing. They found that total microparticle number (annexin V+) doubled after two hours, however they did not analyse red cell microparticles separately (Ayers

et al., 2011). Studies of erythrocyte concentrates destined for transfusion found an increase in the number of RCMP, around 20 fold after 50 days at 4°C; however there was a huge variation amongst samples (Rubin *et al.*, 2008). This suggests that the effect of time delay may vary from sample to sample.

To investigate this, aliquots (10µl) of whole blood taken into EDTA from four controls were analysed at 0, 2, 4, 6 and 24 hours after venesection.

2.6.4 Plasma and whole blood microparticles

Either plasma or whole blood can be used to examine microparticles, each have advantages and disadvantages. Whole blood is more physiologically relevant, avoiding handling issues related to generation of plasma and requires smaller sample volumes; however the presence of a large number of erythrocytes may affect antibody binding. Also samples cannot be stored, whereas plasma samples can be frozen and processed in batches. Preparation of plasma may lead to artifactual increases in microparticle levels and centrifugation has been shown to cause increases in MPs (Rubin *et al.*, 2008). Freezing plasma samples prior to analysis also appears to generate artifactual MPs (Simak and Gelderman, 2006).

Therefore the difference in red cell microparticle levels in whole blood and plasma was analysed in 20 patients, using either 50µl of plasma or 10µl of whole blood.

2.6.5 Temperature

The effect of storage temperature on *in vitro* microparticle levels is unknown. It has been suggested that cooling or heating of samples may induce microparticle release from blood cells (Simak and Gelderman, 2006). Generally samples for analysis by flow cytometry are not refrigerated due to the degradation effect this may have on antigenic expression. However some studies advocate maintaining samples at 4°C prior to analysis (Macey *et al.,* 2003).

Four control samples were taken into EDTA and each immediately divided into two aliquots. One was kept at 4°C and one at room temperature. Aliquots of blood were then analysed at intervals up until 6 hours.

2.7 Statistics

Statistical analysis was performed using GraphPad Prism software (version 6.01). Tests of normality found much of the data to be non normally distributed, with transformation unsuccessful, so all analysis of data used non parametric methods. Comparisons were made using a Mann Whitney test, correlations were assessed with Spearman Rank and multiple comparisons with a Kruskal-Wallis test and Dunn's post analysis test. Paired comparisons used the Wilcoxon test. All results, unless otherwise stated, are expressed as the median ± IQR.

2.8 Results

2.8.1 Antibody titrations

The antibodies were titrated according to the method described in section 2.6.1. Glycophorin A was titrated to 2.5µl and Annexin V to 5µl (see Figure 2.1). These volumes were used throughout the project.

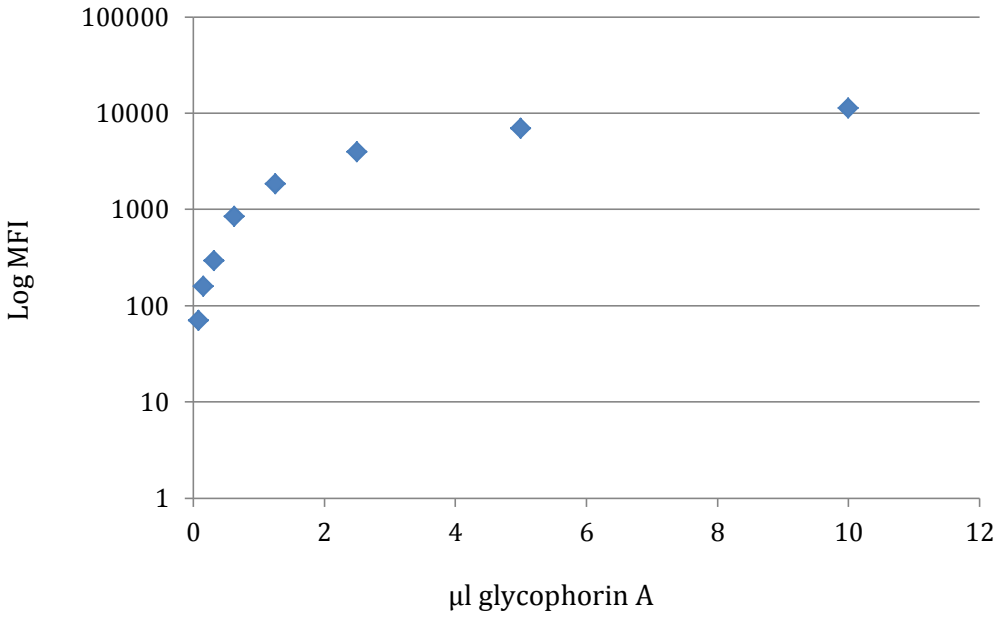


Figure 2.1a Titration of glycophorin A.

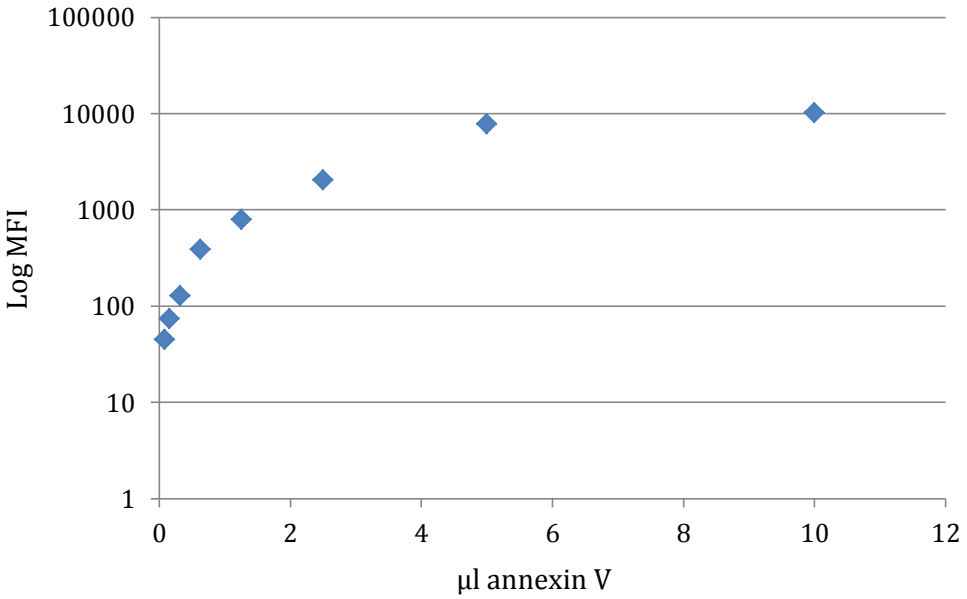


Figure 2.1b Titration of annexin V.

2.8.2 Time delay

As the clinical samples were taken in the Renal Unit they sometimes did not reach the laboratory until a significant amount of time had elapsed. There are reports (Macey *et al.*, 2003) that microparticle production increases over time in anticoagulated blood. Therefore this was tested to determine a cut off time for the acceptance of samples for processing.

Control bloods were labelled with mAbs and analysed at intervals over 24 hours. A significant increase in the numbers of RCMP was found to occur between 1 and 24 hours ($p=0.0325$) post venesection, but no significant difference was found between 1 and 6 hours ($p=0.3502$) (see Figure 2.2). All subsequent samples were analysed within 6 hours of venesection.

There was a small increase over 6 hours and so ideally all samples should be processed at the same time after venepuncture. However this is logistically challenging due to the nature of the samples available and the transportation of the bloods to the laboratory.

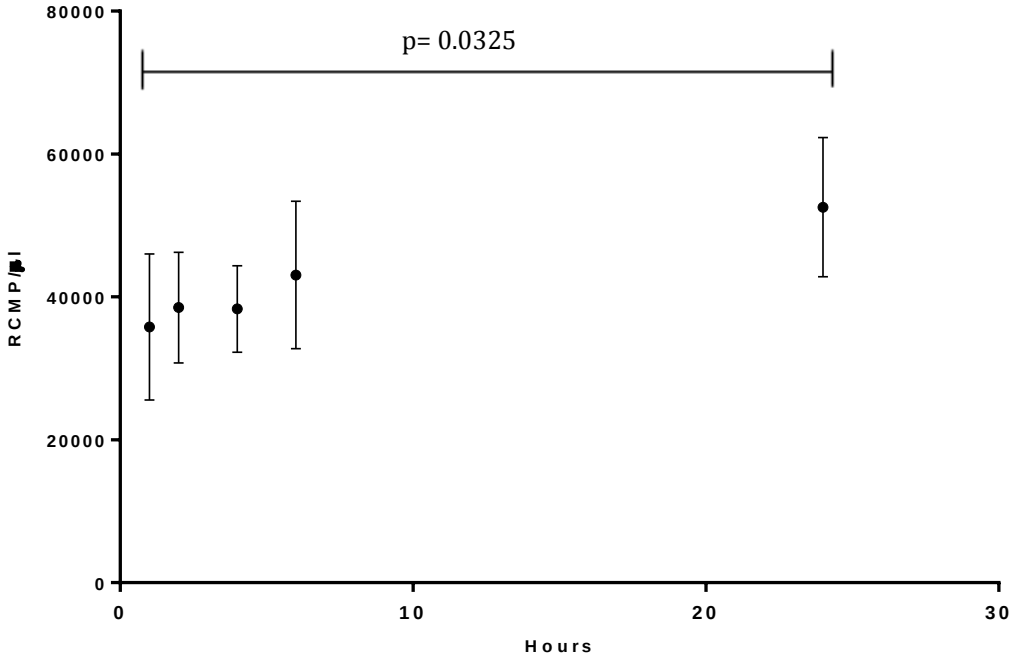


Figure 2.2 Increase in red cell microparticle levels in control samples over a 24 hour period. There was a significant increase from time 1 hr to 24 hours (p= 0.0325), but not over the first 6 hours (p=0.3502) (n=4).

2.8.3 CTAD-EDTA v EDTA

Samples from four controls were taken into EDTA and CTAD-EDTA and tested after 6 hours at RT, as this was determined to be the cut off for sample processing in section 2.6.2. No statistically significant difference (P=0.0571) was found between the two anticoagulants at 6 hours.

As the ethical approval for this project only permits the use of "left over" samples the use of CTAD would be quite difficult to implement as samples are taken into EDTA on the ward and would not be immediately available to transfer into CTAD. The amount of time between venepuncture and transfer into CTAD would also vary from patient to patient which could also affect the results. It was therefore decided on a practical basis that all samples would be processed in EDTA.

2.8.4 Plasma and whole blood

Platelet poor or platelet free plasma (PPP/PFP) appears to be the most commonly used source of microparticles, however the majority of studies have investigated platelet microparticles for which plasma is the obvious choice. Whole blood is more physiologically relevant but has its own disadvantages so the two were compared.

Whole blood samples were analysed on day of collection. Plasma was stored frozen for one month then analysed. The analysis of RCMPs in whole blood and stored plasma samples was compared. If plasma gave similar results to whole blood it would make batch testing possible which would be more practical.

Figure 2.3 shows a significant difference in RCMP levels (p=0.0017) in whole blood and plasma. Clearly part of the processing caused an increase in RCMP. The results also show much greater variability in the levels in plasma (whole blood 13511 ± 8256 RCMP/µl; Plasma 44267 ± 41089 RCMP/µl). However the process of plasma production leads to a reduction in volume by around a half, which will impact upon the concentration of MPs in the samples.

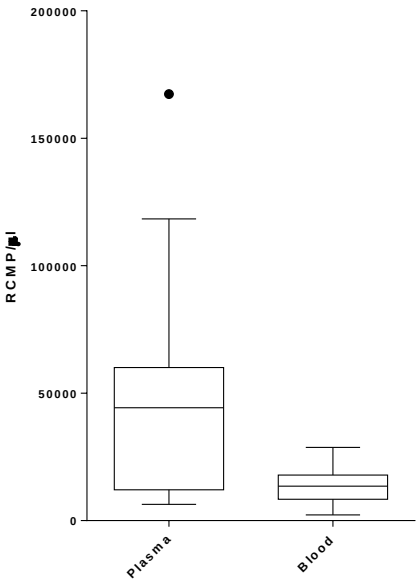


Figure 2.3 Comparison of microparticle levels in plasma and whole blood. Showing a significant increase in plasma samples compared to whole blood (p=0.0017), as well as increased variance between samples.

It was therefore decided that whole blood would be used throughout the study.

2.8.5 Temperature

Storing samples taken into EDTA at either room temperature or 4°C gave no significant difference at 6 hours (p=0.3429).

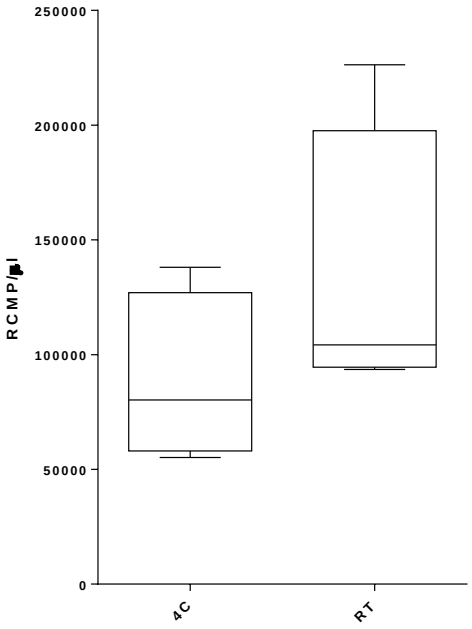


Figure 2.4 Difference in RCMP levels between samples stored at room temperature or 4°C for 6 hours prior to processing was not shown to be significant (p=0.3429).

The samples for this project were from patients located on the dialysis ward and transported to the laboratory via normal portering services. Therefore the immediate storage of samples at 4°C would be logistically challenging. As there appears to be no statistical difference between the two conditions it was decided that samples should be stored at room temperature prior to analysis.

2.9 Final Methodology

2.9.1 Agitation/transportation

The effect of agitation on microparticle formation has been examined previously (Lacroix *et al.*, 2011). Moderate agitation did not have a significant impact on erythrocyte microparticle numbers measured in PFP. The patient samples for this project were delivered from the ward via porters and so experienced some unavoidable agitation however previoud work suggested this would not impact

significantly on the results. However it was also found that samples transported vertically produced less MP that those transported horizontally (Lacroix *et al.*, 2011). The investigators had no control over specimen transport and so this was not controlled in this study.

2.9.2 Phlebotomy

The sampling of patients and controls was carried out under slightly different conditions due to cannulation on the ward, however it has previously been established that the use of different gauge needles has no effect on microparticle levels (Shah *et al.* 2008). Also no significant difference was observed in MP levels between a light or strong tourniquet and a straight needle or butterfly device for blood collection (Larcoix *et al.,* 2011).

2.9.3 Methodology used

From these preliminary experiments a protocol was developed to enumerate red cell microparticles. The study of red cell microparticles does not have a standardised method, and there are few previous examples of the process. These preliminary experiments should provide accurate results using the methods described below.

2.9.3.1 Flow cytometry

The flow cytometric analysis of red cell microparticles was performed on a BD Canto II flow cytometer using Diva software version 6.1. The instrument was calibrated daily with Cell Setup and Tracker (CST) Beads (BD). The instrument had three lasers and a standard optical filter setup. Compensation was performed using Comp Beads (BD) and photomultiplier voltages suitable for cellular analysis as determined from the CST beads.

2.9.3.2 Immunolabelling

To measure red cell microparticle levels, 10µl of EDTA anticogulated blood was incubated for 15 minutes in the dark with 2.5µl of glycophorin A FITC and 5µl of Annexin V PE, plus 50µl of Annexin V binding buffer (ABB). After incubation, 50µl of AccuCount 10µm enumeration beads, plus 1µl of 1µm sizing beads were added and the samples diluted to 1ml with ABB.

2.9.3.3 Flow cytometric analysis

The microparticle/platelet cloud was first identified on a plot of forward scatter versus side scatter. The 1μm sizing beads were gated to allow identification of microparticles smaller and greater than 1μm. A plot of glycophorin A versus annexin V allowed the identification of dual positive events, which were gated to give an absolute count. The 10μm enumeration beads were used to provide a stopping gate; once 1500 bead events were acquired data collection was stopped. This then allowed calculation of the red cell microparticles present per μl of original sample. Refer to Figure 2.5 for an illustration of this.

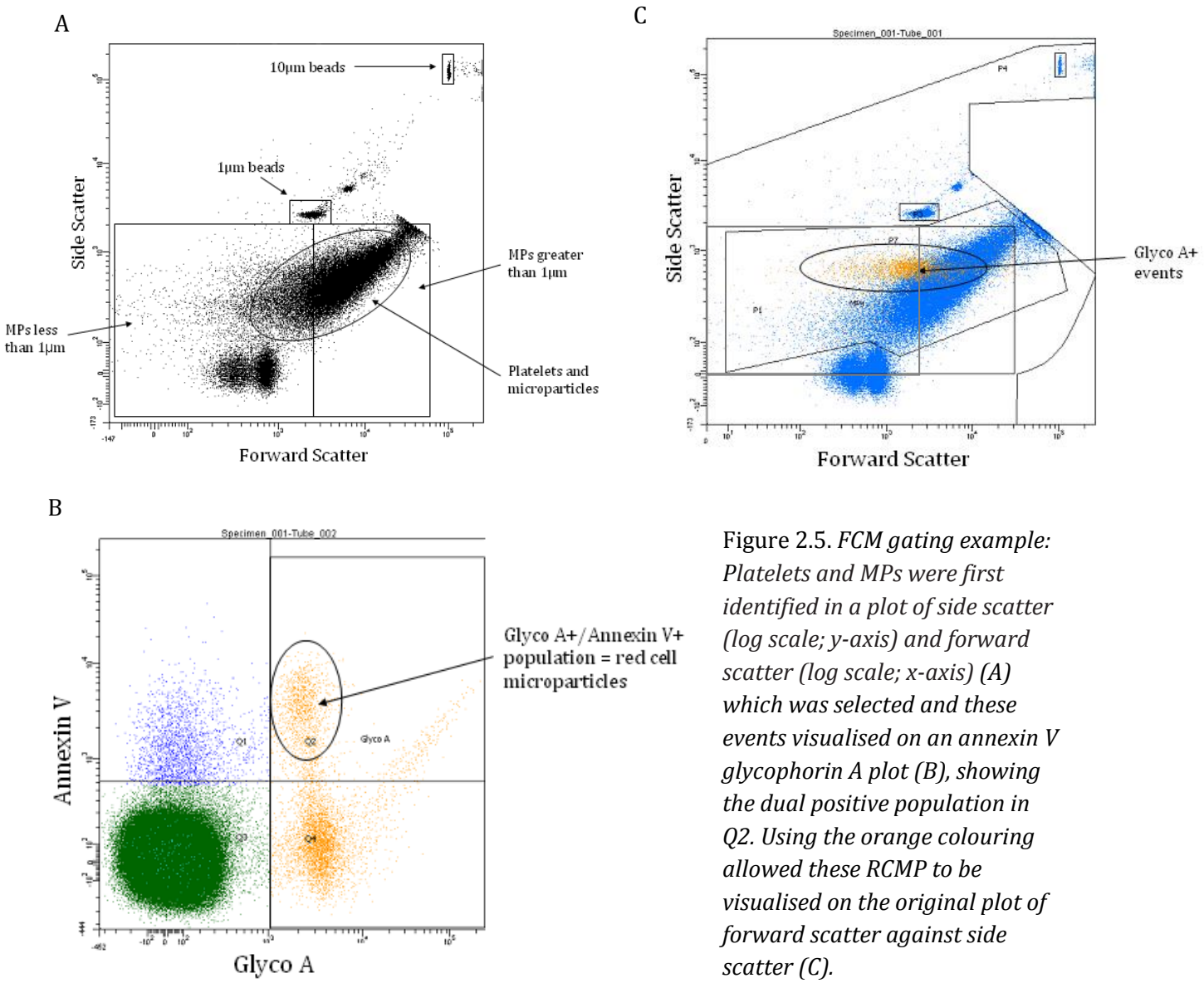


Figure 2.5. *FCM gating example: Platelets and MPs were first identified in a plot of side scatter (log scale; y-axis) and forward scatter (log scale; x-axis) (A) which was selected and these events visualised on an annexin V glycophorin A plot (B), showing the dual positive population in Q2. Using the orange colouring allowed these RCMP to be visualised on the original plot of forward scatter against side scatter (C).*

2.10 Discussion

The individual flow cytometer may be affected by a variety of factors, such as the machine age, laser strengths and detection capabilities. The use of effective cleaning protocols, filtration of buffers and the purity of antibodies are also important issues for accurate microparticle detection.

The topic of standardisation for microparticle enumeration was addressed in a published forum (Jy *et al.,* 2004) and has been the focus of Working Group meetings of the International Society of Thrombosis and Haemostasis (Lacroix *et al.*, 2010). These attempts mostly relate to platelet microparticles but may equally be applied to red cell microparticles. The definition of a red cell microparticle is still not entirely standardised and therefore neither are the methods used to detect

them. This makes comparisons between studies challenging and the decision of the methodology to use difficult.

During initial experimentation it was noted that there are events which may reflect different populations of red cell microparticles. These will be investigated in the next chapter as well as the initially defined RCMP population.

Chapter 3: Flow cytometric analysis of red cell microparticles in patients with chronic kidney disease

3.1 Introduction

The previous chapter described development of a method for the flow cytometric analysis of red cell microparticles. This method was used to quantify red cell microparticles in patients with ESRF on dialysis, as an indicator of levels of eryptosis occurring *in vivo*. These values were compared with healthy controls and differences between the patient groups analysed to determine if there were any factors influencing the level of microparticles observed.

The aim of this part of the study was to test the hypothesis that eryptosis levels vary between healthy controls and ESRF patients on dialysis. Investigating the process of eryptosis may give an insight into the mechanisms of renal anaemia and therefore possibly help to explain rHuEpo hyporesponsiveness.

3.2 Materials

See Table 2.1, page 50.

3.3 Control samples

Healthy controls were recruited. A poster (see Appendix 2) was displayed explaining the study. All controls were consented and samples coded to include details of age and gender, with no other identifiable details stored. A full blood count was carried out on all controls, but no other analysis. Exclusion criteria included know serious illnesses and medication.

3.4 Patient samples

Patients from the Renal Unit at the Royal London Hospital were consented upon initiation of treatment (see Appendix 2). They agreed to the use of any leftover

clinical samples for ethically approved research projects. Any patients who signed the consent form were eligible to take part in the current study.

This project was specifically study to patients in CKD stage 5, undergoing regular haemodialysis or peritoneal dialysis. All dialysis patients were monitored monthly for routine haematology and biochemistry tests, the samples for this project were those left over from these analyses, as stipulated in the ethics approval.

Phlebotomy was performed by the ward nurses for patients and by the principal investigator (PI) for controls.

Patients also gave permission for their health records to be used by authorised members of staff who are not directly involved in their care if this was used for research as approved by research ethics committee.

3.5 Ethics

Ethical approval for the study was granted by City Road & Hampstead Research Ethic Committee (ref: 11/LO/0816). The trial was sponsored by Barts and the London NHS Trust. The patients were recruited from the Renal Department, Royal London Hospital, Whitechapel, London, E1 1BB, United Kingdom. Full, written consent was obtained from all participants. (See Appendix 2 for details.)

3.5.1 Confidentiality

Documentation was made in the Renal Units' database Filemaker Pro which acts as source data. The patient's participation in the trial was indicated in a specific study documentation screen. All data collected and results were then placed into the Study Spreadsheet (Microsoft Excel 2003). FileMaker Pro is password protected and all computers used during data collection were also password protected. Data was kept on Trust computers or Trust encrypted USBs.

3.6 Sample collection

Patient bloods were taken via a cannula straight into EDTA vacutainers. The phlebotomy was carried out on the dialysis unit by the nurses. HD patient samples were taken pre dialysis, before any part of the dialysis procedure had begun. PD patients were in a steady state due to the nature of their dialysis regime and so bloods were not taken at a specific time in the cycle.

Control bloods were taken using a BD Vacutainer® Safety-Lok™ Blood Collection Set needle straight into EDTA vacutainers.

All samples were stored at room temperature and processed within 6 hours of venepuncture.

3.7 Sample analysis

All samples were processed by the same operator.

3.7.1 Flow cytometric analysis

10µl of whole blood was incubated in the dark for 15 minutes with 2.5µl glycophorin A FITC, 5µl annexin V PE and 50µl filtered annexin binding buffer (ABB). Prior to analysis, 50µl 10.0µm AccuCount fluorescent particles and 1µl of 1:1000 1.1µm latex beads were added to each tube and the volume made up to 1ml with ABB.

To allow for additional data analysis, 2.5µl of CD45 APC was also added to each tube prior to incubation. The plots produced represent white cell MP events and were treated in the same way as RCMP data, see Figure 3.1b.

Samples were analysed by flow cytometry on a FACSCanto II (BD, Oxford, UK) flow cytometer equipped with FACSDiva version 6.1 software. The instrument was calibrated and standardised daily prior to use with set up and tracking beads (BD, Oxford, UK) and cleaned after use. The instrument had three lasers and a standard optical filter setup with forward scatter (FSC) and side scatter (SSC) thresholds set low. Compensation was performed using anti mouse Ig κ/negative control compensation particle set (Comp Beads, BD, Oxford, UK). The limit of detection of

the instrument was previously demonstrated to be below 0.6µm (Macey *et al.*, 2011).

The plots produced allowed enumeration of the red cell microparticles per µl of original sample.

As discussed previously there is no standard definition of a red cell microparticle. Therefore all potential RCMP populations were analysed in this study, for example Glycophorin A+ Annexin V+ <1µm, Glycophorin A+ Annexin V- >1µm, by gating the plots produced. An example of this is shown in Figure 3.1.

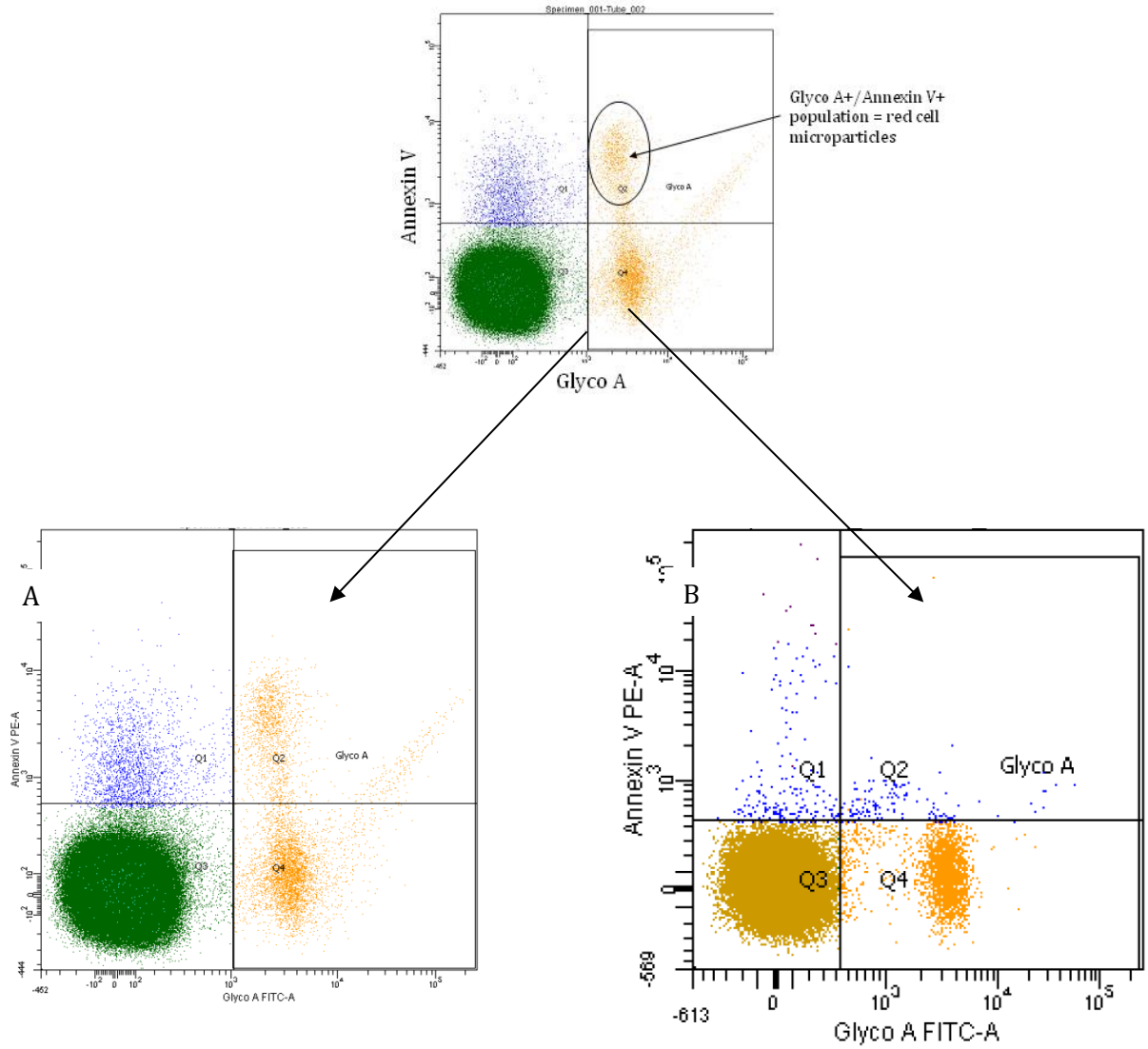


Figure 3.1a Example flow cytometric plots of results. These plots demonstrate the different results seen in different samples. Initial gating on the FSC/SSC plot gave a MP/platelet cloud which was then used to plot glycophorin A/annexin V. Plot A clearly shows both a dual positive annexin V+ glycophorin A+ population and a glycophorin A+ annexin V- population, whilst plot B only shows annexin V- glycophorin A+ MP. Using the sizing beads from the FSC/SSC plot these populations were also split by MP size.

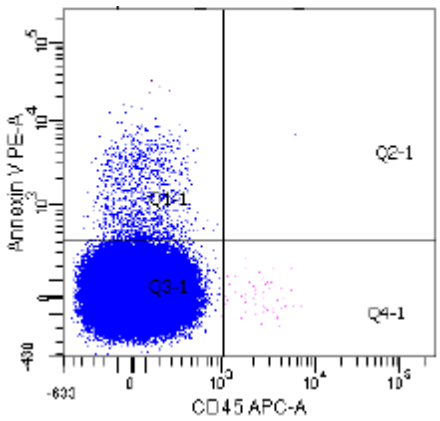


Figure 3.1b Example flow cytometric plot of results for determination of CD45+ populations. The same gating strategy is used initially to separate the microparticle population, with a plot of CD45/Annexin V used to quantify dual positive CD45+ annexin V+ events and CD45+ annexin V- events. There are far fewer CD45+ events than glycophorin A+.

The absolute value was then used to calculate the concentration of MPs/µl of original sample:

$$\text{MPs/µl} = \frac{\text{Number of MPs}}{\text{Number of bead events (1500)}} \times \frac{\text{Number of beads per 50µl (~500,000)}}{\text{Volume of test sample used (10µl)}}$$

Each sample was processed in triplicate and the mean of the three MP/µl results used for all analysis.

3.7.2 Haematology

All haematological indices were measured in the Haematology laboratories of the Royal London Hospital on a Sysmex XE-2100 Haematology Analyser (Sysmex Ltd, Milton Keynes, UK). This analyser uses fluorescence flow cytometry to give a WBC differential, nucleated red cell count and reticulocyte count and impedance counting for platelet and red cell counts and haematocrit. The cyanide-free sodium lauryl sulphate method was used for Hb measurement.

3.7.3 Biochemistry

All biochemistry was performed in the Biochemistry laboratory at the Royal London Hospital. CRP, serum albumin and urine creatinine were measured on the Roche Modular Analytics P-Unit (Roche, Burgess Hill, UK).

An *in vitro* immunoturbidimetric assay was used to determine C reactive protein (CRP) levels in human serum, by adding anti human CRP antibodies which bound CRP to create an insoluble aggregate causing turbidity which was measured spectrophotometrically, and was proportional to the amount of CRP in the sample. The results of this assay are not very sensitive, with the lowest result produced being "<5mg/L". However as the normal range was less than 5mg/L, any patient with this result is within the normal range and so the exact result was less important. For the purpose of data analysis all results given as less than 5 were treated as being equal to 1mg/L.

Serum creatinine was measured with a kinetic colorimetric assay. Alkaline creatinine forms a yellow-orange complex when mixed with picric acid which can be measured photometrically and is directly proportional to the initial creatinine concentration.

Serum albumin was measured using bromocresol green, which binds albumin forming a complex that can be measured spectrophotometrically. This value is proportional to the serum in the original sample.

3.7.4 Renal Function

Kt/V is a measure of dialysis adequacy. eGFR (estimated glomerular filtration rate) is used to give an indication of renal function in CRF patients, however once ESRF is reached this is no longer appropriate as there is very little or no residual renal function. Once dialysis treatment begins Kt/V is then used to determine its effectiveness.

Kt represents the volume of fluid cleared of urea during a single dialysis session, and V is approximately equal to the patients total body water, therefore Kt/V compares the amount of fluid that passes through the dialyser with the amount of fluid in the patient's body.

Other data used in this project was collected from the renal database, as permitted by ethics approval.

3.8 Statistics

All samples were tested in triplicate and the raw data converted to MP/μl. The mean of the three values was then used for all analysis. Statistical analysis was performed using GraphPad Prism software (version 6.01). Tests of normality found much of the data to be non normally distributed, with transformation unsuccessful, so all analysis carried out was using non parametric methods. Comparisons were made using a Mann Whitney test, correlations were assessed with Spearman Rank and multiple comparisons with a Kruskal-Wallis test and Dunn's post analysis. Paired comparisons used the Wilcoxon test. All results, unless otherwise stated, are expressed as the median ± IQR. All box and whisker plots are plotted using the Tukey method (Tukey, 1977).

3.9 Results

Samples were analysed from 83 patients and 19 controls, demographics are shown in Table 3.1

	Patients	**Controls**
Gender (% male)	58	58
Mean age (range)	58.2 (24-86)	50.7 (25-63)
Mean Hb	11.1	14.6
Dialysis modality (% HD)	58	-
Diabetic status (% diabetic)	42	-
Epo status (% on Epo)	86	-
Mean time on dialysis (range) (days)	980 (26-4293)	-
eGFR (range)	7.4 (3.0-29.73)	-

Table 3.1 Demographics of patients and controls.

All the patients enrolled on the study had a range of comorbidities and therefore were on a selection of medications. Due to the small sample size it was decided that splitting the groups further by any other measure would give such small subgoups as to make any statistical analysis impossible. It was noted that transplant history, hypertension, heart disease, malignancy, smoking, medications,

BMI and blood pressure may influence the process of eryptosis, however due to limitations of this project they were not analysed.

Age and sex matching of the patients and controls was attempted but due to the greater age of the general dialysis population it was not possible to recruit sufficient controls of a suitable age.

Preliminary experiments demonstrated the presence of microparticle populations other than the Annexin V+ GlycoA+ <1µm events. These events were counted to determine if these populations could provide further insight into red cell microparticles. Analysis was carried out to determine if there was a difference between the patient and control groups.

Population	Patients		Controls		p	Comment
	Median	IQR	Median	IQR		
All Annexin V <1µm	36350	34791	44944	38852	**0.0692**	Fewer all Annexin V+ MP in patients than controls, but no difference when split
All Annexin V >1µm	14911	10266	18377	15980	**0.1015**	
All Annexin V+	52155	40169	63172	60172	**0.0496**	
All Glyco A+	122011	59366	202894	109308	**0.0001**	Fewer Glyco A+ MP in patients than controls, when comparing all MP and those >1µm, but not <1µm
All Glyco A<1 µm	86511	41044	86327	18036	**0.7959**	
All Glyco A>1µm	43900	25966	50377	29127	**0.0001**	
All MP	5477206	2806064	9151322	2835494	**0.0001**	Fewer MP in patients than controls
All MP<1µm	1806917	1714672	4112206	1786569	**0.0001**	
All MP>1µm	3607061	1922533	5549911	1444625	**0.0001**	
Glyco A- Annexin V+ <1µm	25400	28652	32783	16630	**0.0825**	No difference between patients and controls
Glyco A- Annexin V+ >1µm	12500	8550	16100	7388	**0.0582**	
Glyco A+ Annexin V- > 1µm	41061	29116	43533	21602	**0.2672**	Fewer Glyco A+ Annexin V– MP <1µm in patients compared to controls but not >1µm
Glyco A+ Annexin V- <1µm	69722	46780	130611	67213	**0.0001**	
All Glyco A+ Annexin V+	19494	15616	30900	25230	**<0.0001**	Fewer Glyco A+ Annexin V+ MP in patients than controls
Glyco A+ Annexin V+ <1µm	15350	12883	22067	22980	**0.0167**	
Glyco A+ Annexin V+ >1µm	3672	3886	7666	8955	**0.0013**	

Table 3.2 Comparisons of patients and controls using varying definitions of RCMPs.

Analysis found there to be statistically significantly ($p < 0.05$) more microparticles per µl in controls than patients in the following populations:

All MP< 1µm, GlycoA+ AnnexinV+ < 1 µm, All MP> 1 µm, All GlycoA> 1 µm, GlycoA+ AnnexinV+ > 1 µm, All MP, All GlycoA+, All Annexin V+.

Gating on all microparticles, those >1µm, <1µm and all events, showed a significant difference in numbers between patients and controls with patients having significantly less. This gate is only based upon size, and so may not be an accurate representation of microparticle numbers. Platelets, red cell fragments, microvesicles and fragments of other cells may be included in this count.

Analysis of Glycophorin A+ events within the microparticle gate shows significantly more events in controls than patients in MP > 1µm, and in all events but not in MP <1µm. This is interesting as it could imply that the larger events include some intact red cells, which are decreased in patients due to anaemia. The smaller events are likely to be microparticles and possibly fragments and this suggests the levels of these are equivocal in both groups.

Gating on Annexin V+ events did not show a significant difference in values between patients and controls when split into < and > than 1µm events, however analysis of all annexin V+ events did show a significant difference ($p=0.0496$). This demonstrates either a decreased level of PS expression on these MPs in patients, or just a lack of MPs expressing PS.

Fewer GlycoA+ AnnexinV – MP<1µm were found in patients but not > 1µm. These could represent annexin V- microparticles, or other red cell debris.

GlycoA- AnnexinV+ events showed no significant difference between patients and controls.

Analysis of GlycoA+ AnnexinV+ events showed fewer in patients < 1µm,> 1µm and all events. All three are statistically significant.

This data shows inconsistencies between populations, demonstrating the requirement for a robust definition of red cell microparticles. A commonly used definition is sized between 0.1 and 1µm and expressing PS and antigens representative of cellular origin. Therefore further analysis was carried out on the

glycophorinA+ annexinV+ events <1µm as these gave a statistically significant difference between patients and controls and may be true red cell microparticles. The data was analysed to see if any correlations or relationships could be established to possibly explain the differences between patients and controls.

3.9.1 Patients v controls

As stated previously there were significantly more red cell microparticles observed in patients than in controls. See Figure 3.2.

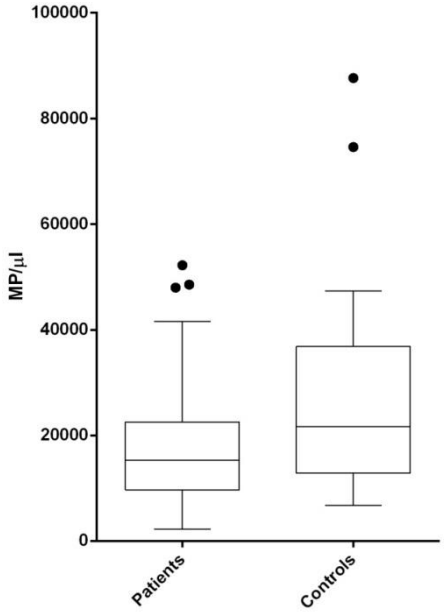


Figure 3.2 RCMP in patients v controls. There was a significant lower number of RCMPs in patients compared to controls (p= 0.0167).

3.9.2 Patient demographics

When comparing the demographics of the patients (see Figure 3.3) it was found there were significantly more RCMP in haemodialysis patients than peritoneal dialysis patients. No difference was observed with relation to diabetic status or between male and female patients, or between those patients receiving Epo treatment and those not. No correlation was observed between patient age and MP levels.

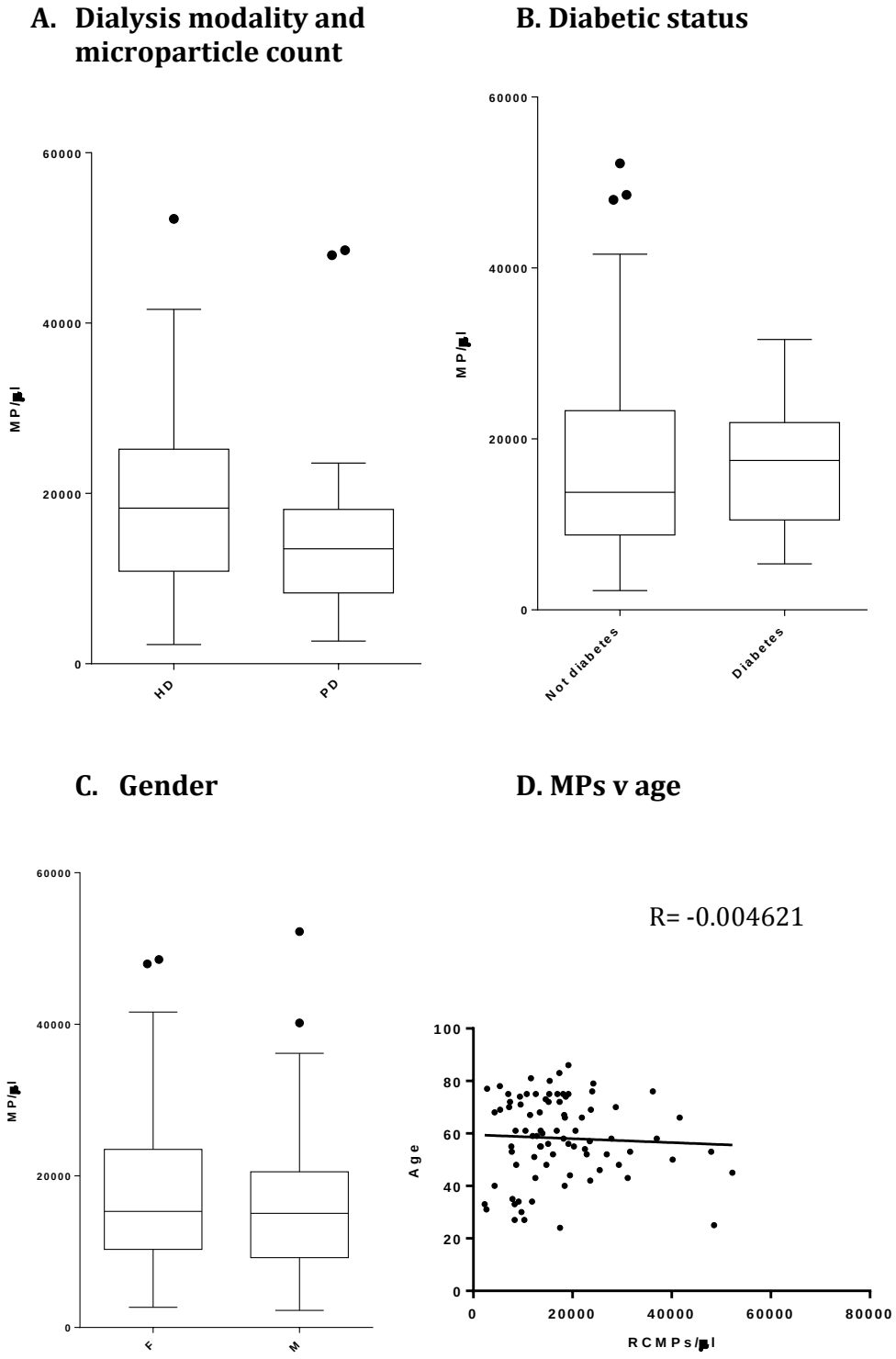


Figure 3.3 RCMP levels and patient demographic comparisons. ***A.*** *There were significantly more red cell microparticles in haemodialysis patients compared with peritoneal dialysis patients (p=0.0128).* ***B.*** *There was no significant difference in red cell microparticle counts between patients who are diabetic and those who are not (p= 0.4021).* ***C.*** *No significant difference was observed between male patients and female patients (p= 0.5103).* ***D.*** *No correlation was observed between patient age and RCMP levels (R=-0.004621, p=0.9669).*

3.9.3 Investigation of RCMP relationships with other parameters

3.9.3.1 Dialysis adequacy

There were no statistically significant correlations found of RCMPs with kt/v or time on dialysis, suggesting that dialysis adequacy and stability have no impact upon microparticle formation *in vivo* (see Figure 3.4).

A MPs v time on dialysis **B MPs v kt/v**

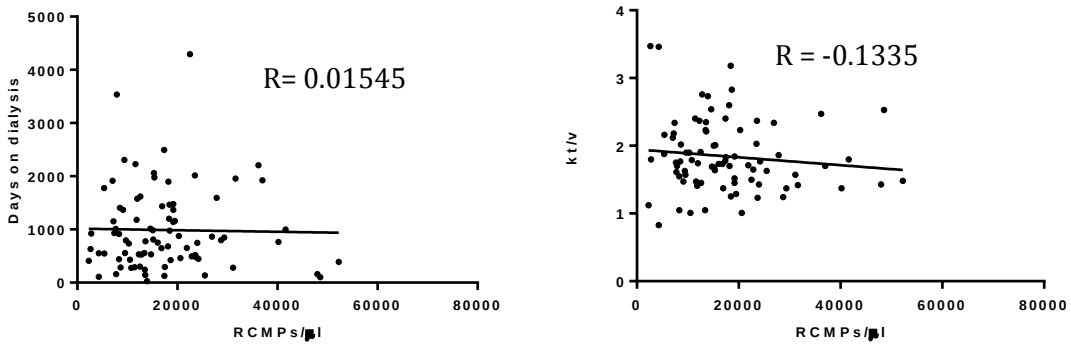


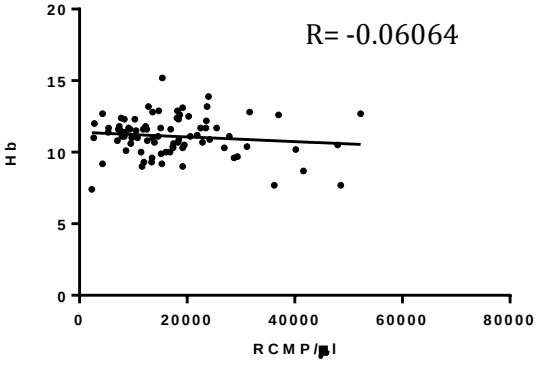
Figure 3.4 RCMP levels and dialysis adequacy. ***A.*** *No correlation was observed between the time on dialysis and RCMP/µl (r=0.01545. p*=0.8898). ***B.*** *No correlation was observed between kt/V as a measure of dialysis adequacy and RCMP/µl (r=-0.1335, p*=0.2288).

3.9.3.2 Red cell indices

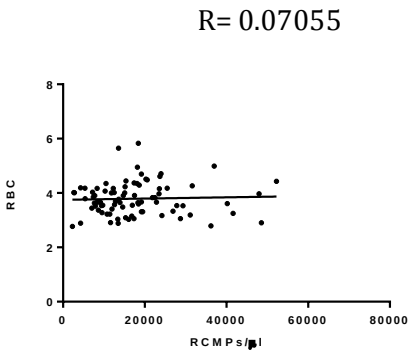
Hb and RBC showed no relationship with MP numbers per µl of blood. This implies red cell indices are not related to the RCMP levels. Also no significant correlation was observed between reticulocyte counts and MP levels. Reticulocyte counts were performed on very few patients (n=8). All were in the normal range.

The RCMP data was also analysed with respect to the RCMPs/RBC, giving a ratio of RCMPs per red cell in the circulation. Comparing this data between patients and controls gave the same pattern as just RCMP, implying the difference is not related to the lack of red cells in renal patients. See Figure 3.5.

A MPs v Hb



B MPs v RBC



C Reticulocytes

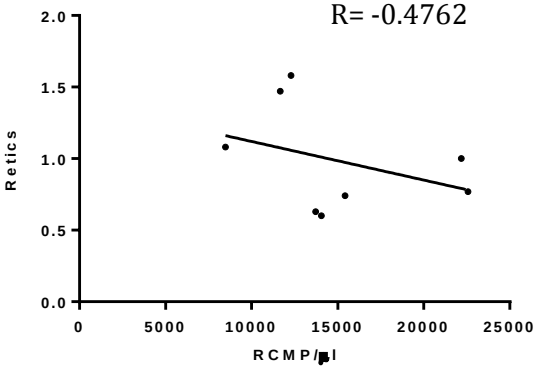


Figure 3.5 RCMP level and red cell indices. ***A****. No correlation was observed between haemoglobin and RCMP (r=-0.06064, p=0.5860).* ***B****. No correlation was observed between red cell count and RCMP (r=0.07055, p=0.5262).* ***C*** *.No correlation was observed between reticulocyte count and RCMP (r=-0.4762, p=0.2431, n=8).*

3.9.3.3 Inflammation

A statistically significant weak positive relationship was found between RCMPs and CRP; however there was no correlation with serum albumin measurements (see Figure 3.6). CRP is a more reliable measure of inflammation than albumin and so this analysis warrants further investigation.

A MPs v CRP **B MPs v serum albumin**

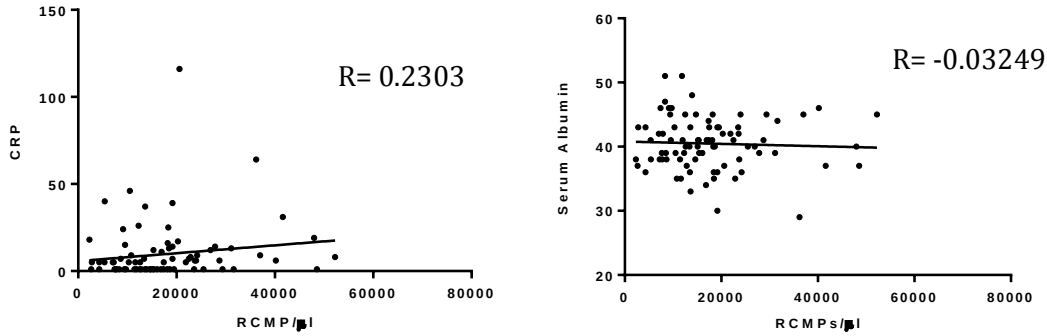
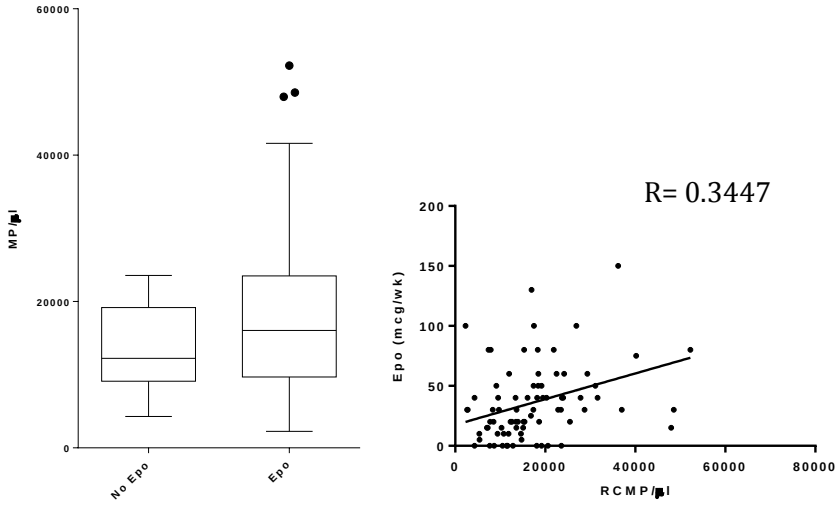


Figure 3.6 RCMP levels and inflammation. ***A****. A weak statistically significant correlation was found between CRP and RCMP (r=0.2303, p=0.0362).* ***B****. No correlation was found between serum albumin and RCMP (r=-0.03249, p=0.7706).*

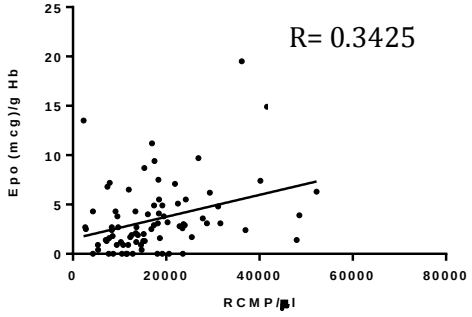
3.9.4 Erythropoietin

A weak statistically significant correlation was found between Epo dose and RCMPs. Further investigation found this was upheld when compared to the Epo dose per g of Hb. Dividing the patients in half to give a "high" Epo dose and "low" Epo dose group showed a statistically significant difference between the two. Using the definition of Epo resistance as requiring more than 15000IU of rHuEpo/wk to maintain target Hb the patients were split into Epo resistant and non-resistant. Comparison of these groups did not show any difference. However there are only 4 patients in this study who met the criteria for "Epo resistant". There was no significant difference observed between patients treated with Epo and those not (p= 0.3184). See Figure 3.7.

A Epo treatment status **B MPs v Epo**



C Epo/Hb (mcg/wk/g)



D High Epo dose v low Epo dose **E "Epo resistant" v not resistant**

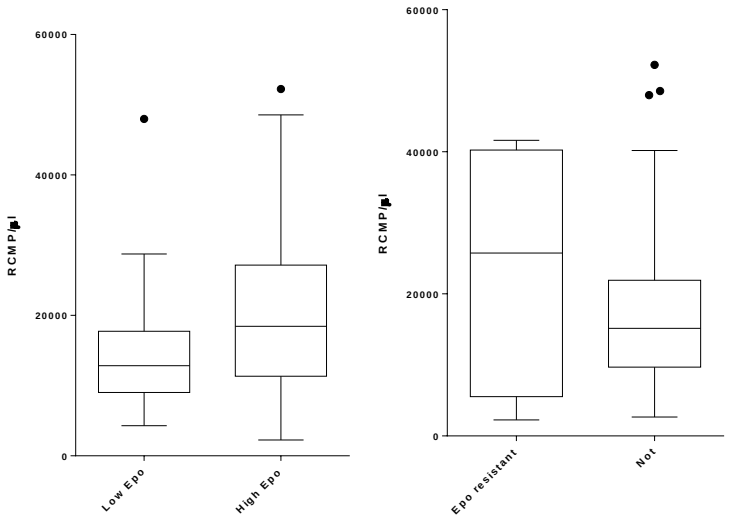


Figure 3.7 RCMP levels and erythropoietin. ***A****. No difference was observed between RCMP levels in those patients on Epo treatment and those not (p= 0.3184).* ***B****. A*

statistically significant weak positive correlation was found between Epo dose and RCMP (r=0.3447, p=0.0014). ***C****. A weak statistically significant correlation was seen between RCMP and Epo/Hb, a measure of the amount of Epo required to maintain target Hb (r= 0.3425, P= 0.0015).* ***D****. A statistically significant increase in RCMP was seen in patients on a high dose of Epo compared to a low dose (p=0.0075).* ***E****. No significant difference was seen between Epo resistant patients (defined as requiring more than 15000IU of rHuEpo/wk to maintain target Hb) and not (p=0.5163).*

3.9.5 White cell microparticles

Further analysis was carried out to enumerate the CD45+ white blood cell microparticles in these subjects. Comparing patients and controls in 4 different subpopulations of white cell microparticles showed no significant difference between any of them (see Table 3.3).

Patients v controls for WBC MP populations:		Comment
CD45+ AnnexinV+ >1	p=0.6416	No significant difference in WBC MPs between pts and controls
CD45+ AnnexinV+ <1	p=0.1928	No significant difference in WBC MPs between pts and controls
CD45+ AnnexinV- >1	p=0.4721	No significant difference in WBC MPs between pts and controls
CD45+ AnnexinV- <1	p=0.8363	No significant difference in WBC MPs between pts and controls

Table 3.3 Comparison of patients and controls using differing definitions of white cell MPs.

These results were then correlated with RCMP data to determine if they demonstrate a similar pattern. No correlation was found with any population (see Table 3.4).

	Spearman rank correlation (WBCMP v RCMP)	
	r	p
AnnexinV+ <1 CD45+ v Annexin V+ <1 GlycoA+	0.1736342	0.1142071
AnnexinV- <1 CD45+ v AnnexinV- <1 GlycoA+	0.07864	0.477
AnnexinV+ >1 CD45+ v AnnexinV+ >1 GlycoA+	0.0377	0.7335
AnnexinV->1 CD45+ v AnnexinV->1 GlycoA+	0.00809	0.9418

Table 3.4 Comparison of RCMP and WBC MP populations in patients to determine if any correlation was present.

Previous work has focused on PMPs, and therefore many generalisations regarding MPs are based on these observations. However in this study, analysis of WBC MPs

showed that they do not display the same characteristics as RCMPs. This suggests that MP may more heterogeneous than previously thought, with differences dependent upon the cell of origin. Findings relating to one specific population of MPs may therefore only relate MP derived from that source, and not to MP in general.

3.10 Conclusions

The method described in Chapter 2 allowed the enumeration of red cell microparticles.

Preliminary experiments highlighted the issues with RCMP definitions, as it was observed that a number of populations of cells could represent microparticle populations. These were analysed initially, comparing patients with controls. It was found that there are statistically significantly more microparticles per μl in controls than patients in several of the populations studied.

Previous studies have used a variety of measures of eryptosis, including cell size, annexin V binding, Ca^{2+} activity and ceramide formation. The International Society on Thrombosis and Haemostasis (ISTH) Scientific and Standardisation Subcommittees define platelet microparticles by the presence of cell specific antigens and surface PS using annexin V (Lacroix *et al.*, 2011). Whether this definition can be used across microparticles derived from other cell types is yet to be determined.

However, it is also clear there is a population of red cell microparticles which do not express annexin V. Horstman *et al.* demonstrated that only a fraction of MPs are positive for annexin V (Horstman *et al*., 2004). They found that 35 times more endothelial microparticles were identified using anti-CD62 than annexin V binding. A similar situation has been observed in platelet microparticles, with 80% of them not binding annexin V, which seems to be dependent upon the agonist inducing MP formation (Connor, 2010).

Comparing the mean of all MP and mean annexin V+ events in this study found approximately a 100 fold difference. In populations <1μm approximately 2% of all

MP are annexin V+, and in events >1µm this falls to 0.5%. Some of these events may not be true microparticles but this clearly shows there are many other particles present which are not counted as microparticles if annexin V positivity is part of the definition.

This study found annexin V- glycophorin A+ populations both <and > than 1µm in size. Whether these populations are different to those which are annexin V positive is yet to be determined.

This demonstrates some of the issues involved with MP analysis, as the definition used affects the results. In order to analyse the data further it was necessary to specify a population to be defined as red cell microparticles in this study. Previous studies have used the definition of annexin V+ glycophorin A+ <1µ and this group gave a statistically significant difference between patients and controls and was our initial definition. It is noted that this may not be a definitive use of the term RCMP but for the purposes of this study it was used.

Contrary to expectations, statistically significantly more RCMP were found in controls than patients ($p=0.0167$). When adjusted to account for the anaemia found in the patient group, by measuring RCMP per g/Hb or in relation to the absolute red cell count, the correlation remained. The patient data was then correlated with a selection of other parameters.

No difference was found between diabetic and non-diabetic patients in this study. Previously annexin V binding was observed to be higher in CKD patients with type II diabetes (Calderón-Salinas e*t al.*, 2011), using washed red cells with no size discrimination. Caspase-3 activation, an effector mechanism of eryptosis, has been found to be higher in diabetic erythrocytes (Maellaro *et al.*, 2013) but whether this translates to microparticle production has yet to be established.

The prevalence of CKD is much greater in men however this predisposition did not appear to have an effect on the RCMP levels in this study. Previously it has been found that healthy women have increased levels of platelet and endothelial microparticles, compared to healthy men (Toth *et al.*, 2007). This may suggest RCMPs do not act in the same way as other, more commonly studied MP populations.

No correlation was observed between microparticle levels and age in this population. Previously, decreased endothelial microparticles have been found in stable elderly patients, but no corresponding change in red cell or platelet microparticles (Forest *et al.*, 2010).

This study found significantly more red cell microparticles in haemodialysis patients compared to peritoneal dialysis patients (p=0.0128). This could be related to the sheer stress (Boulanger *et al*, 2007) or increased systemic inflammation in HD. Further investigation into this is required.

It is very difficult to measure the severity of CKD once the patient has initiated upon RRT. Kt/V may be used to determine dialysis adequacy, however this result can be inaccurate due to any residual renal function the patient has. There was no correlation found between Kt/V and red cell microparticle levels. Also no correlation was found with time on dialysis and microparticle levels; this suggests that stability of dialysis has no impact on RCMP formation and eryptosis.

It was found that there was no correlation between microparticle levels and Hb or red cell count. Reticulocyte counts also demonstrated no relationship with microparticles. This lack of association with any measured red cell indices suggests that these factors have little impact upon the process of eryptosis, suggesting microparticle levels are unrelated to the process of anaemia.

CKD is a chronic inflammatory state and so the relationship between inflammation and microparticle levels was analysed. CRP is a standard marker for inflammation and is routinely monitored in CKD patients. A weak but statistically significant correlation was found between CRP and red cell microparticle levels. However serum albumin, another indicator of inflammation did not give a correlation. This is a less relevant marker but this adds to the weak nature of the correlation.

Comparing the CRP levels between HD and PD patients gave a median level of 7 to 1 mg/L. This is statistically significant (p=0.0036) showing a considerable difference in inflammation found in patients with HD compared to PD. The difference in the physical methods of dialysis clearly has a large impact on systemic inflammation levels.

A weak, statistically significant, correlation was found between red cell microparticle levels and Epo dose. Dividing the patient group in half into "high Epo dose" and "low Epo dose" gave a significant difference; however comparing patients receiving Epo with those not receiving Epo did not. Using Epo/g Hb, as an indicator of the amount of Epo required to maintain an adequate Hb level also gave a significant correlation with microparticle levels.

Further analysis of this data showed that comparing the Epo resistant group (defined as requiring more than 15000IU of rHuEpo/wk to maintain target Hb) with the remaining patients receiving Epo showed no difference. However the resistant group was very small and the range was wide, indicating a lack of consistency. A difference would be expected between these groups and so further investigations are required with more Epo resistant patients to confirm this hypothesis.

The reticulocyte count was correlated with microparticle levels. This did not give a significant correlation, however there were very few patients for whom a reticulocyte count was performed. Also all the results are in the normal range, which is unexpected with those on such high Epo doses.

From these investigations is it clear that both erythropoietin dose and inflammation may impact upon microparticle levels and therefore the process of eryptosis. Further work into the effect of erythropoietin dosage was not within the scope of this project, due to the logistical challenges of collecting samples from the relevant patients. It was therefore decided to carry out some *in vitro* experiments to try to further characterise the nature and cause of the inflammation in HD patients.

Chapter 4: *In vitro* flow model using whole umbilical artery continuous perfusion loops

4.1 Introduction

The results from Chapter 3 suggested that there may be a link between eryptosis, Epo dose and CRP. It has previously been established that patients with a high CRP require greater doses of Epo to achieve similar Hb levels (Barany *et al.*, 1997; Bradbury *et al.*, 2009). However the link of Epo with eryptosis is novel. As a marker of inflammation, CRP is increased in a range of acute and chronic conditions, and so would be expected to be elevated in ESRF. This chronic inflammatory state may influence eryptosis levels.

The experiments described in this chapter were designed to compare the effect of normal blood with that from CKD patients on inflamed and normal endothelium. The flow model used was based upon an *ex vivo* model developed by Baumgartner and Haudenschild in the 1970s using rabbit aortas perfused with blood to measure platelet activation and aggregation (Baumgartner and Haudenschild, 1972). The model has been recently modified (Holtom *et al.*, 2012), to allow investigation of interactions between the vascular endothelium and blood cells whilst retaining the 3D vascular anatomy. It involves using intact umbilical arteries isolated from human umbilical cords as part of an *ex vivo* flow loop (Holtom *et al.*, 2012). Previous studies used blood from healthy subjects to analyse the difference in microparticle production resulting from flow over TNF treated and normal endothelium (Holtom *et al.*, 2012).

Two loops were set up in parallel (see below); one was treated with TNF to cause an inflammatory response in the endothelial cells. The other remained untreated. TNF causes vascular endothelial cells to undergo a range of proinflammatory changes leading to increased leukocyte adhesion, transendothelial migration and vascular leak (Bradley *et al.*, 2008). Different combinations and patterns of adhesion molecules are expressed, including E-selectin, ICAM-1 and VCAM-1 (Pober *et al.*, 1986; Munro *et al.*, 1989). The release of chemokines, such as IL-8 and monocyte chemoattractant protein-1 (MCP-1) (Rollins *et al.*, 1990), together with the adhesion molecule expression leads to recruitment of leukocytes. TNF

causes an increase in inducible nitric oxide synthase (iNOS) expression in a range of cells, leading to increased nitric oxide (NO) production (Miyasaka and Hirata, 1997).

The TNF treated loop was therefore used as a model for the endothelium found in HD patients and untreated endothelium used as a model for healthy endothelium. Blood from healthy controls and HD patients was allowed to flow over both treated and untreated endothelium.

4.2 Reagents

All general laboratory reagents were from Sigma (Poole, Dorset, UK) unless otherwise stated. All tubing and connectors were from Cole Parmer (London, UK). Reagents for flow cytometry were used as described in Table 2.1.

4.3 Samples

A subgroup of patients and controls from the initial study were used again for these *in vitro* studies. Only HD patients were used in this study, due to their generally increased CRP levels. All the same criteria and ethical approval applied. All venepuncture occurred at the Royal London Hospital; samples were transferred to the Royal Veterinary College for flow loop analysis, which involved dilution of anticoagulated blood 1 in 10 in PBS with 0.2% EDTA and then transported on ice back to the Royal London Hospital for flow cytometric analysis.

4.4 Ethics

The use of left over blood samples was covered by the ethics approval as stated previously. Umbilical cords were collected from the Labour Ward of the Royal London Hospital with informed consent from the pregnant women and under conditions approved by the East London Local Research Ethics Committee (ref 04/Q0604/4).

4.5 Methods

4.5.1 Umbilical Artery Preparation

Umbilical cords were collected as above. They were stored in Na_2HCO_3 buffered Hanks balanced salts solution (HBSS) with penicillin/streptomycin and gentamycin at 4°C. The cords were dissected to excise sections of artery around 10cm in length, removing as much fat as possible but not damaging the artery wall. 1/16" to female luer adaptors (Cole Parmer, London, UK) were inserted into each end and attached securely with strong embroidery thread. Each vessel was gently flushed with 1xPBS to remove clots and ensure the viability of the artery.

4.5.2 Flow Loop

A flow loop, as shown in Figure 4.1 was set up in a 37°C tent. The vessels were kept in a bath of warmed 1x PBS. All tubing was Tygone silicone 1/16" internal diameter (Cole Parmer) connected with polystyrene luer adaptors (Cole Parmer), sterilised by autoclaving prior to use. A peristaltic pump was used to provide a continuous flow and a flexible capacitance reservoir was used to dampen the pulsatile flow.

Two artery segments were set up for each experiment and acclimatised to the loop containing flow medium (M199, 10% foetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, amphotericin B) for an hour. One circuit then had 10ng/ml human recombinant TNF (Insight Ltd, London, UK) added and left for 18 hours. 1x PBS with 0.2% EDTA was used to wash out the loop without disrupting the flow, and repeated three times. Diluted whole blood (1 in 10 in PBS with 0.2% EDTA) was added to each reservoir and run through the loop continuously for 30 minutes, then collected and held on ice until flow cytometric analysis. Further aliquots of diluted blood were used as controls; one was incubated at 37°C for the length of the experiment and one was circulated through a flow loop without any umbilical artery for 30 minutes and collected as above. This was carried out with 6 patients and 6 controls.

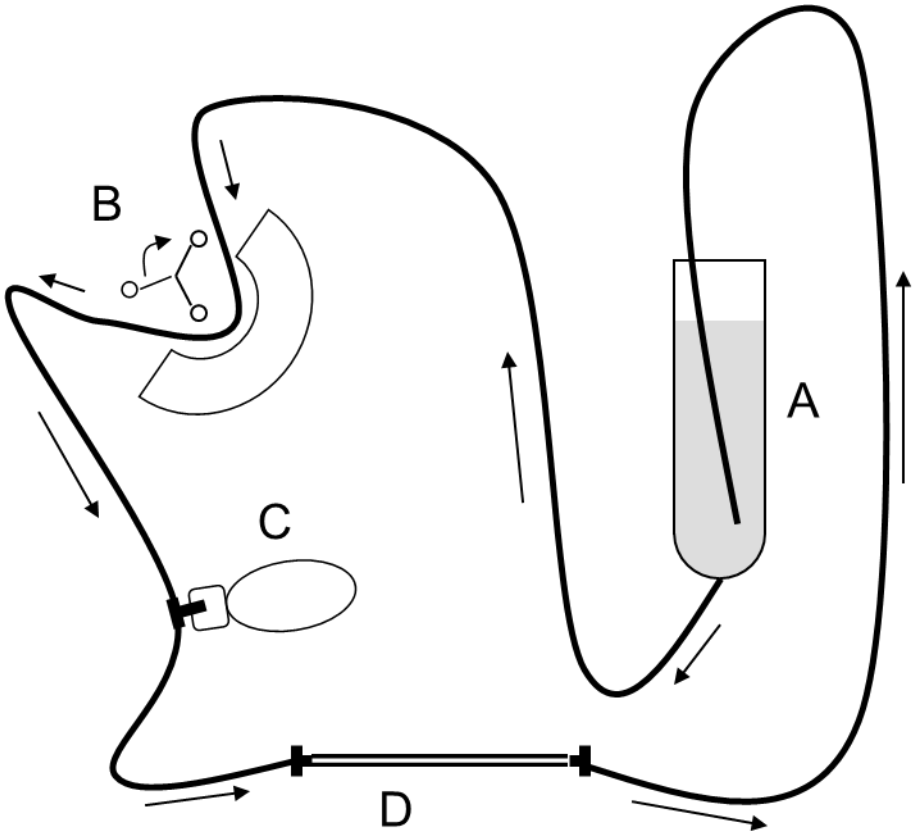


Figure 4.1 Simplified schematic of in vitro whole umbilical artery continuous perfusion loop. A: Reservoir containing diluted anticoagulated whole blood. B: Peristaltic pump. C: Compliance reservoir to remove pulses from peristaltic pump. D: Dissected human umbilical artery cannulated with luer adaptors. All tubing is silicone 3/16" internal diameter with polystyrene luer attachments (adapted from Holtom et al., 2012).

4.5.3 Flow cytometric analysis

Flow cytometric analysis was carried out as described in Chapter 2; however 100µl of diluted blood was used.

4.6 Statistics

See section 3.8.

Multiple comparisons, between each condition in the flow loop, were done using the Kruskal Wallis test with Dunns post-test analysis.

4.7 Results

4.7.1Patients v controls

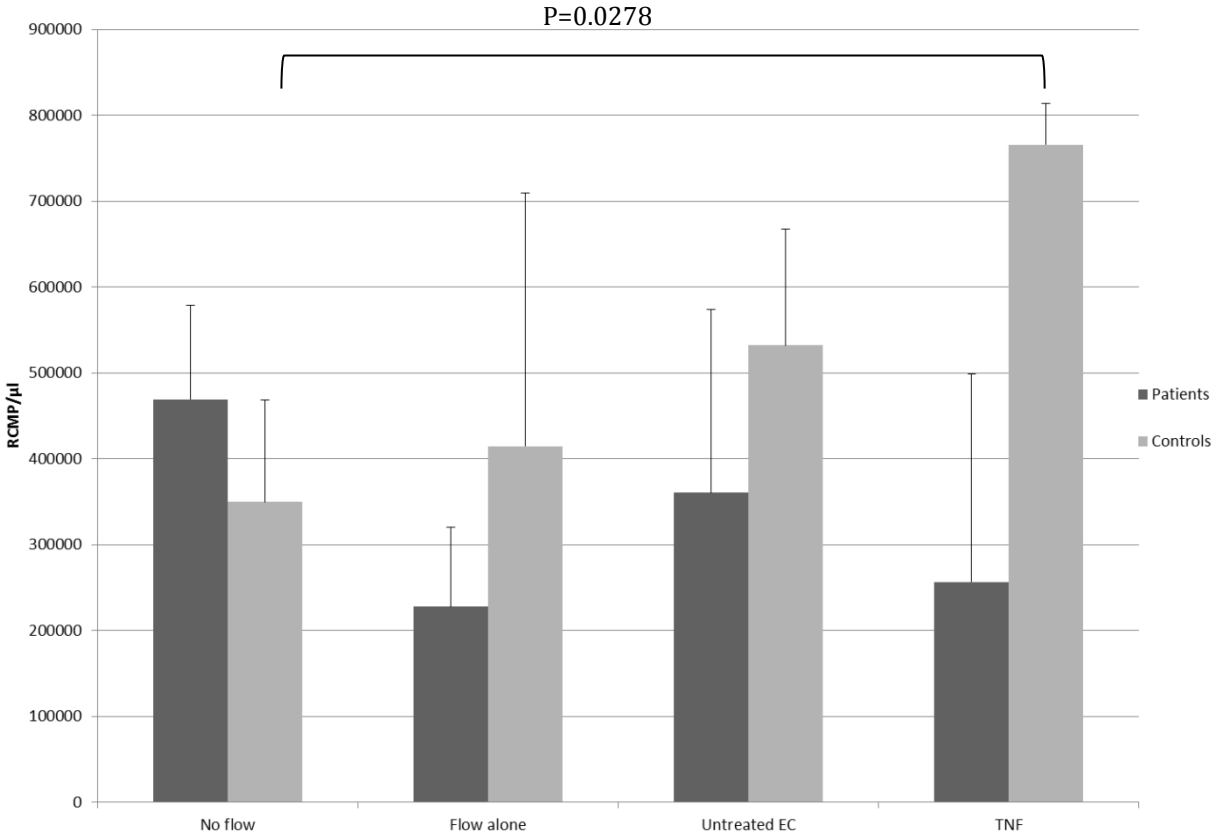


Figure 4.2 Medians +IQR of results from all patients and all controls under flow loop conditions. Diluted samples held in a tube for the duration of the experiment were classed as 'no flow', bloods passed through the apparatus with no endothelium were 'flow alone', bloods passed through the apparatus with a portion of umbilical artery were 'untreated EC' and bloods passed through the loop with a portion of umbilical artery pretreated with TNF were 'TNF'.

These results show that initially the patient and control bloods do not contain differing numbers of red cell microparticles. When not exposed to flow conditions the behaviour of the bloods remain the same (p= 0.1255). However once exposed to flow, endothelium and TNF treated endothelium, the RCMP concentration in the controls increases (p=0.0410). A statistically significant difference is seen between no flow and TNF treated endothelium (p=0.0278). In contrast the red cell microparticle levels in the patient samples did not vary significantly with any of the conditions imposed (p=0.1406).

However compared to the experiments in Chapter 3 the absolute values were much greater, the average of the control blood samples not undergoing flow was 1,1815,741MP/µl compared to 21,656MP/µl in experiments from the previous

chapter. The numbers found for the patients and controls are more comparable in this experiment, rather than the almost two fold difference seen in the previous chapter.

4.7.2 Epo v no Epo

Further data analysis was carried out, splitting the patient group into those being treated with Epo and those not, to determine if the impact of both inflammation and Epo may have an effect on eryptosis.

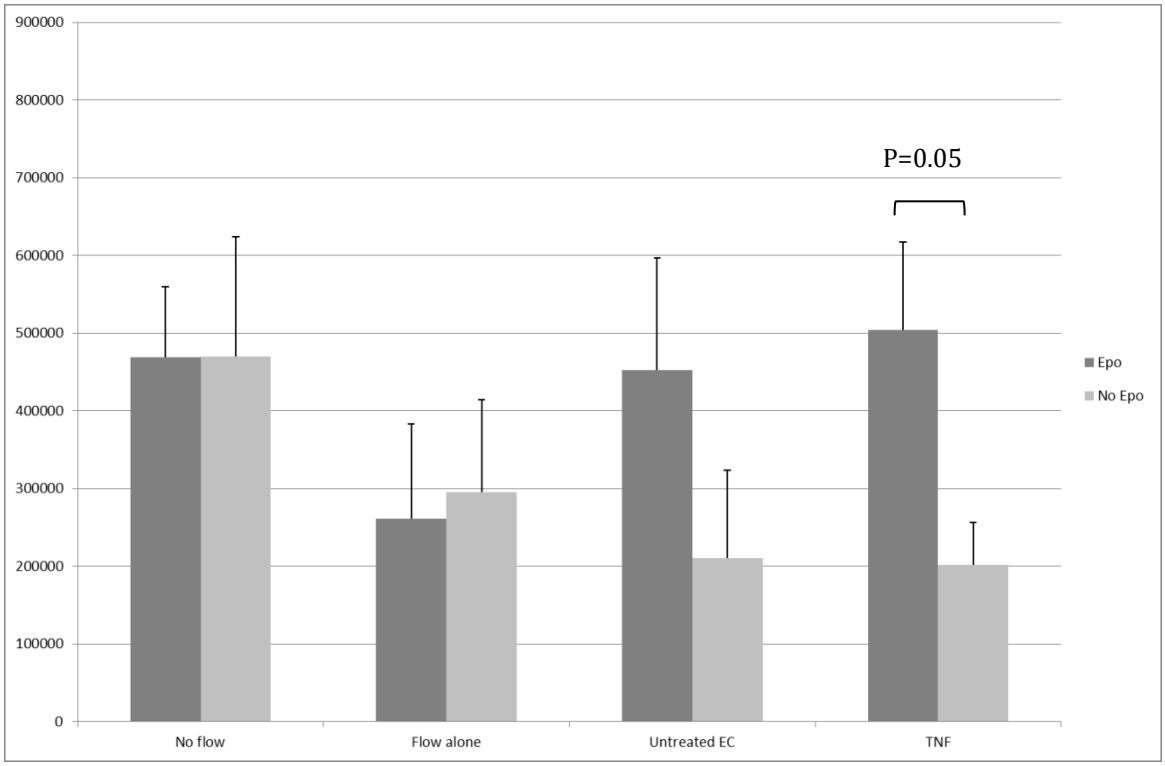


Figure 4.3 Comparison of patients who are being treated with Epo and those who are not, using an in vitro flow loop to assess the impact of inflammation on endothelium.

Statistical analysis of this data shows no significant differences between any of the experimental conditions in either patient group (Epo p=0.2833, No Epo p=0.1606). Comparison of blood from the two groups when flowed through untreated endothelium did not show a significant difference (p=0.275). However, treating the endothelium with TNF and repeating the flow loop experiment demonstrated an almost statistically significant difference (p=0.05) between the Epo treated and Epo not treated groups. However this cohort is very small, with only 3 patients in each group.

4.8 Conclusions

These experiments gave inconclusive results which require more investigations to fully understand. They show that the number of red cell microparticles was not increased when CKD patient whole blood was exposed to flow, vascular endothelium under flow or TNF treated vascular endothelium under flow. However the same conditions with healthy control blood gave increasing microparticle levels, as flow was introduced and inflammation increased. A statistically significant increase was observed between blood not undergoing flow and TNF treated endothelium under flow conditions in controls.

The patient population was further divided into those receiving Epo and those not. Multiple comparisons showed there was no significant difference between any of the experimental treatments in either group. However comparing the MP levels in each group when flowed through TNF treated endothelium, showed an almost significant increase in RCMP numbers in Epo treated patients ($p=0.05$). This may suggest that there is a difference in the level of eryptosis during inflammation between patients treated with Epo and not. However these cohorts were very small and would benefit from a repeat experiment with more patients.

This set of experiments also have hugely increased absolute red cell microparticle counts, around 50 fold that of the previous experiments. This may have impacted upon the results. This is possibly due to the increased time involved in the methodology, particularly prior to analysis.

Chapter 5: Discussion

Eryptosis is a process analogous to apoptosis which leads to production of red cell microparticles. The hypothesis for this study was that this would be upregulated in patients with CKD; however this study has found lower red cell microparticles levels in patients compared to controls. Further investigations were undertaken to attempt to explain this and it is hypothesised that erythrocytes in CKD patients undergo less eryptosis than those in controls.

Each chapter contains a summary of the results. This chapter aims to discuss the relevance of the data and potential limitations, as well as further work which could be done to answer questions which have arisen from this study.

5.1 Development of a flow cytometric method for the analysis of red cell microparticles

There is no standardised method to enumerate RBC MPs (Shah *et al.*, 2008; Rubin *et al.*, 2008; Ayers *et al.*, 2011; Xiong *et al.*, 2012; Lacroix *et al.,* 2012). However the primary method for microparticle detection is to use flow cytometry of purified MP using one or two colours (Christersson *et al.*, 2013).

The methods within this project have been used previously to analyse MPs derived from a range of cell types (Macey *et al.*, 2011; Macey *et al.*, 2012). In order to refine them solely for RCMP analysis and to ensure suitability for the purpose of measuring eryptosis, method development was undertaken prior to the receipt of any patient samples.

The preliminary experiments outlined in Chapter 2 led to the development of a protocol to enumerate RCMPs. This involved flow cytometric analysis of red cell microparticles using a BD Canto II flow cytometer with Diva software version 6.1. EDTA anticoagulated blood was stained with glycophorin A FITC and annexin V PE, and after incubation, 10μm enumeration beads and 1μm sizing beads added. The microparticle/platelet cloud was first identified on a plot of forward scatter-side scatter. The 1μm sizing beads were gated to allow differentiation of microparticles by size. A plot of glycophorin A-annexin V allowed the identification of events

positive for each or both antigens, which were gated to give an absolute count. The 10μm enumeration beads were used to provide a stopping gate; once 1500 events were acquired data collection was stopped. This then allowed calculation of the red cell microparticles present per μl of original sample.

In this part of the study it was also noted there are multiple populations which could be classified as RCMP. These vary with their expression of annexin V and the sizing limits imposed. For example the definition used in the initial stages of this project was annexin V+ glycophorin A+ <1μl, however it could also be stated that there is no need to split the MP cloud by size and so gating on annexin V+ glycophorin A+ events could be classified as RCMP. Similarly there is evidence that not all MP bind annexin V and so simply glycophorin A+ events from within the MP cloud could be defined as RCMP. Comparisons of the different populations between patients and controls gave varying results. Some demonstrated highly significant differences, whilst others showed no difference, demonstrating heterogeneity amongst the populations. This highlights the issues involved in defining a suitable population as red cell microparticles.

Previous studies have used a variety of measures of eryptosis, including cell size, annexin V binding, Ca^{2+} activity and ceramide formation. Phosphatidylserine exposure measured via annexin V binding with no size discrimination has been used (Calderón-Salinas *et al.*, 2011), and the same method but considering only annexin V events with a mean fluorescence greater than 1.0 as positive (Bonomini *et al.*, 1999). Another study measured glycophorin A and annexin V dual positivity, similarly with no sizing (Jy *et al.*, 2004). Others have imposed a strict definition of microparticles, which had to fulfil the criteria: (i) size = 1.0μm; (ii) ability to be isolated from platelet-free plasma by ultracentrifugation; and (iii) surface expression of phosphatidylserine, as shown by annexin V binding (Jy *et al.,* 2004). Closest to the definition initially used in this project, studies by Xiong *et al*. defined red cell microparticles as annexin V+ glycophorin A+ events between 0.5 and 0.9μm (Xiong *et al*., 2011; 2012). Multiple studies have measured all of; cytosolic calcium activity (using Flou3 fluorescence), cell volume (forward scatter), phosphatidylserine exposure (annexin V binding) and ceramide formation (anti ceramide antibody) to determine eryptosis levels (Lang *et al.*, 2005; Niemoeller *et al.*, 2006; Kempe *et al.*, 2007; Kiedaisch *et al.*, 2008; Ahmed *et al.*, 2013). Sample

processing also appears to have a large impact upon microparticle levels (Lacroix *et al.,* 2012).

5.2 Flow cytometric analysis of red cell microparticles in patients with chronic kidney disease

The aim of this project was to determine whether eryptosis was related to renal anaemia and could therefore help to explain Epo hyporesponsiveness. The results do not seem to support the hypothesis that eryptosis is related to anaemia directly, as there was no correlation with microparticle levels and Hb, or any other red cell indices. However there may be a correlation with Epo treatment. The significance of this is yet to be determined. There was also a weak correlation with microparticle numbers and the level of CRP, which may be used clinically as an indicator of inflammation.

Contrary to expectations, more red cell microparticles were found in the blood from controls than from patients. Previous studies have found increased endothelial microparticles in uraemia (using PFP) (Faure *et al.*, 2006). Amabile *et al.* (2005) found an increase in red cell microparticles (defined as CD235a+ and between 0.1 and 1μm), in ESRF patients compared with healthy controls, using washed freeze-thawed MP pellets. However Trappenburg *et al.* found no difference in red cell microparticle (glycophorin A+ annexin V+) levels between controls and CRF patients, and no difference between those on HD or PD (Trappenburg *et al.,* 2012).

Analysis of other data collected from these patients was undertaken to try and determine any factors which may influence the microparticle levels.

It could be the case that the membranes involved in haemodialysis are providing a surface to which microparticles adhere, thereby reducing the numbers circulating *in vivo*. Analysis of the membranes post dialysis would confirm or rebuke this theory, whether it would be possible to wash them to remove all adherent particles is unclear. However analysing the microparticle enumeration data from HD and PD patients does not support this; in fact it shows increased microparticle levels in HD patients. There are no artificial membranes involved in PD and so this

hypothesis would suggest more microparticles should be expected in PD patients. However PD is a more physiological process and this would therefore lead to reduced interference and inflammation, potentially confounding the effect. PD patients are more likely to suffer infections and therefore the effects of inflammation, this may impact upon the microparticles. The membranes involved in HD may however cause an increase in inflammation thereby exacerbating the proinflammatory phenotype seen with these patients. As described in Chapter 4 this may have an impact upon microparticle production. TNF treated endothelium was shown to cause an increase in RCMP production in control blood but not in patient blood, but the impact of artificial membranes is unknown.

Diabetes is a common cause of CKD, due to the effects of glucose damage to the vasculature and nervous system and this may be related to the increase in eryptosis previously described in these patients. Manodori *et al.* (2002) found that a population of erythrocytes in diabetic patients expose PS, and the size of this population is related to blood glucose levels. These PS exposing red cells are abnormally adherent to each other and endothelial cells, especially the vascular endothelium (Wautier *et al.*, 1981). Increased MP have been observed in type II diabetes, derived from platelets (Zhang *et al.,* 2013), endothelium (Tramontano *et al.*, 2010) and lymphocytes, as well as annexin V+MPs (Chen *et al.*, 2012). However no such correlation has been observed with erythrocyte derived MP. Interestingly no correlation was found with RCMP and HbA1c levels in type II diabetes patients (Alkhatatbeh *et al.*, 2013), suggesting that potentially glucose levels do not affect RCMP, only intact erythrocytes. No difference in RCMP levels was found between diabetic and non-diabetic patients in this study, suggesting this common comorbidity has no impact upon eryptosis.

Epo status was analysed to determine its effect on eryptosis, with no difference in RCMP numbers being found between those on Epo and those not. However the RCMP range within the Epo treatment group was very large and the dosage range was also very large, making it difficult to assess this accurately. Due to regular monitoring and adjustment of Epo therapy very few patients were on a consistent dose of Epo. The dose data used was that for the date of the sample provided, however the previous 3 months Epo dose would be likely to have an effect on the behaviour of the red blood cells.

Monitoring dialysis adequacy is challenging, especially in patients who have some residual renal function remaining. Kt/V gives some indication but is not very reliable. No correlation was found between Kt/V and red cell microparticle levels, suggesting that their production is not dependent upon factors within the uraemic plasma, as these would be removed during effective dialysis. It would be interesting to analyse HD patient blood before and immediately after a dialysis session to determine how the process impacts upon RCMP production. No correlation was found between microparticles and time on dialysis, further implying no link between eryptosis and the process of dialysis.

It was anticipated that the levels of RCMP would correlate with haemoglobin levels, in order to help explain the anaemia found in renal patients. However this was not the case. No correlation was found, implying that the anaemia is not influenced by microparticle levels. The red cell count did not correlate with red cell microparticle levels; further supporting the argument that eryptosis does not affect anaemia.

No correlation was found between the reticulocyte concentration and the numbers of RCMP. If the process was related to red cell age this would have been expected. However all the patients had a reticulocyte count within the normal range and so the effect may not have been as noticeable as expected. There are other methods to more accurately determine red cell age which could be employed in the future. The patient group in this study were all anaemic; studying eryptosis in conditions of excess erythrocytes, polycythaemia, may give more clues about the mechanisms involved.

CRP is an indicator of systemic inflammation within the patient. A weak positive correlation was found with red cell microparticles and CRP levels. This suggests that inflammation in some way impacts upon the process of eryptosis. Analysis of serum albumin, also an indicator of inflammation gave no correlation. This suggests there may be a weak relationship, which was investigated further.

The most promising correlation found was with Epo treatment. There was a statistically significant positive correlation found between rHuEpo dose and RCMP levels. Comparing those on a high dose of Epo to those on a low dose gave a statistically significant difference. However, comparing red cell microparticles

from those who are classified as Epo resistant and those who are not, failed to demonstrate any difference. Within this cohort there were only 4 patients who were classified as Epo resistant, giving little weight to this analysis, and suggesting the need for recruitment of more patients. These results suggest that Epo may have an impact upon red cells which affects their ability to undergo eryptosis.

Overall these results suggest that red cells in patients are less likely to undergo eryptosis than in controls. There appears to be a link with Epo treatment and possibly with inflammation.

Further analysis, including other possible RCMP populations and white cell microparticles (CD45+) was carried out. This did not demonstrate any consistent pattern, or any correlation between red and white cell MPs. This implies both that the definition chosen is very important, and that this phenomenon may not apply to microparticles derived from all cell types. Comparing studies is therefore made more challenging, as they may not really be assessing the same populations.

5.3 *In vitro* flow model using whole umbilical artery continuous perfusion loops

Further investigations were carried out using an *in vitro* model of TNF treated endothelium. The aim of this part of the study was to determine whether the weak correlation seen with CRP and microparticle levels in the initial study may have an impact upon red cell microparticle production.

This part of the study used human umbilical arteries, which were assembled into a flow loop. Two flow loops were set up in parallel with one treated with TNF to induce inflammation. Diluted blood was then passed through the loop and analysed by FCM to determine the RCMP concentration, as described previously.

The methods used were based on those employed previously (Holtom *et al.*, 2012). They found an increase in red cell microparticles in control blood after exposure to the TNF activated endothelium. The behaviour of patient blood was investigated to determine the mechanism of red cell microparticle formation in renal patients.

The results from the controls confirm those found previously and showed an increase in red cell microparticles after incubation with TNF treated umbilical arteries (Holtom *et al.,* 2012). A statistically significant difference was observed between no flow and TNF treated endothelium under flow conditions with control blood.

However the patient samples demonstrated no significant difference in RCMP levels between any of the flow conditions. Further subdivision of the patient group into those treated with Epo or without prior Epo treatment demonstrated no significant difference between any of the flow conditions. However the TNF treated endothelium flow loop gave an almost significant difference ($p=0.05$) between the Epo and not Epo treated patients.

These results back up the finding from Chapter 3 that erythrocytes in renal patients are undergoing less eryptosis, as MP numbers do not appear to increase with increased flow/inflammatory insult, as in controls. The analysis of the TNF treated model demonstrated a difference ($p=0.05$) between those patients being treated with Epo and those not. This suggests there may be interplay between inflammation and Epo treatment which may impact upon RCMP levels.

This study suggests that red cells in patients with ESRF are undergoing eryptosis less readily and producing less RCMP. The reasons for this are yet to be elucidated, however this section will present relevant information which may suggest the processes involved.

The lack of eryptosis in CKD could be due to another process occurring, which removes intact erythrocytes prior to microparticle formation. Erythrophagocytosis occurs to engulf and remove erythrocytes which are exposing PS, generally by macrophages. An increase in macrophage number or activity could make them more efficient at removal of erythrocytes, leaving fewer red cells to undergo eryptosis. Increased PS exposure has been previously observed on erythrocytes in conditions of uraemia (Sakthivel, *et al.*, 2007). It was also found that patients with CKD had increased PS exposure on RBC, no matter the dialysis treatment, which was caused by a factor within uraemic plasma (Bonomini *et al.*, 1999). This could lead to erythrophagocyctosis and engulfment of whole red cells before the opportunity for eryptosis occurs. An increase in specific monocyte populations

have been found in HD patients, with further increases seen in chronic infection (Nockher and Scherberich, 1998). It has been suggested this is due to an increase in inflammatory cytokines, particularly TNF (Guidi and Santonastaso, 2010). These experiments could be carried out on the populations in this study, measuring PS exposure on whole erythrocytes to determine if this could be leading to premature destruction. As demonstrated previously (Manodori, 2002) this could also provide a link between diabetes and CKD.

The results seen could also be explained by the increased adherence of microparticles derived from patient cells. In initial experiments this would occur during dialysis itself, with microparticles adhering to the dialysis membranes and equipment. This would also be observed in the flow loop model, where they could be present in similar numbers to in the controls, however adhering to the tubing, adhering more to the endothelium and adhering even more to the inflamed endothelium. Previous reports show that the induction of eryptosis leads to a significant increase in the number of erythrocytes adhering to the vascular endothelium under flow (Borst *et al.*, 2012). It appears that TNF causes upregulation of CXCL16 on endothelial cells, to which the upregulated PS binds (Borst *et al.*, 2012). This could be investigated further by washing the dialysis apparatus and flow loops to remove the adhered microparticles and measuring the levels.

Enhanced PS exposure on the cell surface of erythrocytes has been linked to abnormal adhesion to endothelial cells in chronic uraemia (Bonomini *et al.*, 2002). The role of PS in the adhesion has not been elucidated however there appear to be specific interactions with the endothelial matrix (Yang *et al*., 2010). Schlegel *et al*. found that red cells with an asymmetric bilayer do not bind to endothelial cells, but those with a symmetric bilayer do (Schlegel *et al*.**,** 1985); suggesting that bilayer rearrangement influences the behaviour of erythrocytes and may contribute to microvascular occlusion formation. This may reflect the situation in other pathogenic states which also demonstrate increased adhesive properties. It is postulated that extravascular haemolysis and the related upregulation of erythrocyte production may contribute to increased red cell adhesion in sickle cell disease (Sakamoto *et al.,* 2013). *Plasmodium falciparum* infection leads to erythrocyte adhesion to the vascular endothelium to allow parasite dissemination

and in diabetes glycated band 3 protein leads to interaction with the endothelium (Wautier and Wautier, 2013). These mechanisms may also be involved in CKD and should be explored further.

The samples used in this set of experiments were whole blood, meaning there were many other cell type and microparticles derived from these cells present. There is evidence that platelet and leukocyte microparticles cause release of cytokines which lead to adhesion onto endothelial cells (VanWijk *et al.*, 2003). Platelet derived microparticles may also initiate inflammation and bind to already activated endothelium, further exacerbating the already inflamed endothelium. There is also evidence that microparticles produced in response to an inflammatory stimulus cause endothelial expression of adhesion molecules *in vitro* (Mesri and Altieri, 1999). This could lead to adhesion of red cell microparticles.

However the initial experiments do not back up this theory as they demonstrated significantly more RCMP in HD than PD. The extra mechanical devices involved in HD would be expected to cause greater adhesion and therefore fewer detectable MP. The flow loop experiments do appear to demonstrate that control blood may follow this pattern. This further demonstrates the robust nature of the patient blood, as they do not seem to be affected by the increasing inflammation.

There is evidence that CKD has an impact upon the structure of erythrocytes (Gwozdzinski *et al.*, 1997; Brzeszczynska *et al.*, 2008; Costa *et al.*, 2008; Antonelou *et al.*, 2011), however whether there could be a factor involved to make the cells less susceptible to eryptosis is unclear. The relationship with inflammation is also unclear.

Results from the flow loop appear to show that control blood is more influenced by inflammation than patient blood. It is postulated that this may be due to the chronic inflammation in CKD patients, whereas controls are less likely to be desensitised to chronic inflammatory signals to these conditions and react strongly to them.

Previous investigations showed that microparticles derived from co-culture with TNF treated endothelium induced significantly enhanced levels of reactive oxygen species (ROS) (Holtom *et al.*, 2012). These data suggest that the presence of TNF treated endothelium caused release of pro-inflammatory microparticles from

circulating blood cells, which could contribute to prolonged endothelial activation. This process may be occurring in CKD patients, so the red cells do not react to the TNF treated endothelium in the flow model.

The process of eryptosis may be inhibited by Epo, in both progenitors and mature red cells, but it has also been suggested that in a mechanism similar to neocytolysis, high Epo doses may lead to the production of erythrocytes which are more susceptible to eryptosis.

It has been noted that erythropoietin has a direct antiapoptotic effect on mature erythrocytes, which significantly contributes to the enhanced erythrocyte survival observed in erythropoietin treated patients (Myssina *et al.*, 2003). This direct regulatory effect of erythropoietin is thought to be due to inactivation of calcium-permeable cation channels.

It was also found that RBC survival was prolonged by the action of Epo on erythroid progenitors, resulting in the production of RBC with improved viability (Polenakovic and Sikole, 1996). Erythropoietin inhibits apoptosis of erythrocyte progenitor cells as well as suicidal death of mature erythrocytes. The hormone is effective through inhibition of the Ca2+-permeable cation channels (Lang *et al.*, 2006).

However Foller *et al.* found that erythrocytes drawn from Epo-overexpressing transgenic mice were significantly more resistant to osmotically-induced lysis than wild type erythrocytes but more sensitive to the eryptotic effects of Cl-removal and exposure to the Ca^{2+} ionophore ionomycin (Foller *et al.*, 2007). Those observations prompted the hypothesis that erythropoietin treatment leads to the generation of erythrocytes expressing genes which render the erythrocytes more sensitive to eryptosis. The generation of susceptible erythrocytes under the influence of high erythropoietin concentrations is expected to trigger removal of excessive erythrocytes as soon as the enhanced erythrocyte concentration is no longer needed and the plasma erythropoietin concentration declines (Foller *et al.*, 2007). The influence of erythropoietin on proeryptotic genes would thus shorten negative feedback regulation of the circulating erythrocyte number, which otherwise would take 120 days. According to this view, the accelerated death of young erythrocytes following a limited exposure to high altitude or space flight (Rice and Alfrey, 2005)

may reflect the death of those erythrocytes that have been generated under high erythropoietin concentrations and are thus more vulnerable to eryptosis (Foller *et al*., 2007).

This theory is based on the principles of neocytolysis; the selective destruction of neocytes. It is an adaptive physiologic process which controls red cell mass by initiating neocyte removal when the red cell mass becomes excessive for the environment. This is detected by a decrease in previously elevated levels of Epo (Alfrey and Fishbane, 2007). Fluctuating circulating Epo levels could cause the initiation of neocytolysis.

5.4 Evaluation of study

The methods used in the study were based upon those used previously (Macey *et al.,* 2010; Holtom *et al.,* 2012) but have been adapted for the analysis specifically of red cell microparticles. Measurement of microparticles has been performed by many groups but there is little method standardisation. Due to the heterogeneity of the populations it is difficult to compare methods used for microparticles derived from different cell types. The methods used are therefore not necessarily comparable to previous studies. From the preliminary experiments it was decided to use whole blood due to its physiological relevance which is in contrast to most other microparticle studies. A recent paper described the development of a method to measure microparticle populations in whole blood, however they only stained with annexin V and antibodies to tissue factor (TF), platelets (CD41 and CD62P), monocyte (CD14) and endothelial cells (CD144) (Christersson *et al*., 2013). This multicolour flow cytometry assay in whole blood mimics the *in vivo* situation by avoiding several procedure steps interfering with the MP count. The steps involved in producing the plasma, as used in many previous studies, may lead to the *ex vivo* stimulation of erythrocytes, falsely elevating microparticle levels. The majority of previous work has been carried out with platelet microparticles, using plasma, and there are now accepted recommendations relating to their analysis (Lacroix *et al.*, 2010).

The study population used in this project also imposed limitations for data analysis. There were fewer patients available than ideally required due to the need

to analyse samples within 6 hours of venesection. Due to the small number of patients available it was not possible to analyse patients based upon comorbidity or drugs or cause of CKD. Splitting patients into smaller groups was also impractical and would have made any statistical analysis invalid and so there are many other possible reasons and correlations which will not have been detected in this study. Especially with the *in vitro* work this became an issue as the unexpected results could have been genuine or due to the individual patients who happened to be selected for this part of the study.

Practicalities made the flow loop experiments challenging and certain aspects appear to have affected the results. The samples had to be taken on the dialysis unit at the Royal London Hospital and the flow loop equipment was all situated at the Royal Veterinary College, meaning transport was required between the two sites. The flow cytometer was also located at the Royal London Hospital meaning samples had to be transported back after the experiments. It is suspected that this transport, as well as the additional time it therefore took, affected the absolute numbers of red cell microparticles found in the samples, as this was considerably higher than the initial experiments. The use of human umbilical cords added further variability to the system, as the nature of the vascular endothelium could be altered dependent upon many factors.

An important point to note with the results from Chapter 4 compared to those from the initial experiments in Chapter 3 is the change in absolute values of red cell microparticles found. They are increased, on average, around 50 fold. The reason for this increase is unclear; it could be related to the transport of the samples from the Royal London Hospital to the Royal Veterinary College and back again. The samples were kept on ice due to the increased transport time, but this could also have had an effect. The samples were not analysed within 6 hours of venepuncture as previously because of the length of time required for travel and processing. The microparticle numbers were shown to increase with time in Chapter 2, however the differences were not as great as seen here. The samples were also diluted in EDTA buffer prior to being added to the flow loop; this has an unknown effect. A small investigation was done into this; one control sample had an extra EDTA sample taken, half of which was left at the Royal London Hospital and half transported to the Royal Veterinary College, but not used for any

experimentation. The sample left at the Royal London gave a tenfold increase compared with the Chapter 3 baseline and that which was transported demonstrated similar results to the diluted samples not subjected to flow. This suggests a combination of the time delay and the agitation caused by transport of the samples led to the hugely increased microparticle counts. Transporting samples vertically has been shown to be beneficial in producing results as similar to *in vitro* as possible (Lacroix *et al.,* 2011). Therefore the samples in this study were carried upright whilst travelling.

The absolute numbers are also more similar in patients and controls for the baseline conditions which is dissimilar to the initial findings. This may just be due to the huge numbers of MP measured due to handling and time constraints outlined above.

There have been issues raised over the use of annexin V for microparticle enumeration (Jy *et al*., 2004; Boulanger *et al.*, 2006), because it has been shown that not all microparticles bind annexin V. Jimenez *et al.* (2003) reported that only a small proportion of microparticles bind annexin V and that relying only on annexin V for microparticle detection is likely to give inaccurate results. It is possible that a lack of annexin V binding may result from sub optimal binding conditions, the presence of inhibitors or insufficient PS exposure (Connor, 2007).

In agreement with previous work (Jimenez *et al.*, 2003; Jy *et al.*, 2004) in this study it was also observed that not all red cell microparticles bind annexin V. Comparing the mean of all MP and mean annexin V+ events in this study found approximately a 100 fold difference. In populations <1μm approximately 2% of all MP are annexin V+, and in events >1μm this falls to 0.5%. Whether the "all MP" population truly represents just microparticles is uncertain, however there are clearly a proportion of microparticles which do not bind annexin V.

Studies by Dasgupta *et al*. found lactadherin to be more sensitive than annexin V for detection of both erythrocytes and red cell microparticles (Dasgupta *et al*., 2006). Albanyan *et al.* studied PS exposure in platelets and found that lactadherin is more sensitive than annexin V at detecting low levels of PS (Albanyan *et al.*, 2009). They concluded that due to their small surface area, it is expected that MPs express a limited number of PS molecules that are below the binding threshold of

annexin V, suggesting that lactadherin is likely to be more sensitive at detecting PS exposing platelet MPs. It has been shown using synthetic membranes that lactadherin detects low levels of PS (0.5%) independently of phosphatidylethanolamine and calcium content (Shi *et al.*, 2008; 2006; Waehrens *et al.*, 2009). In contrast, annexin V binding was detected only when PS was 8 percent and in the presence of 2 percent phosphatidylethanolamine (Shi *et al.*, 2006). Furthermore, the actual binding properties of lactadherin and annexin V appear to be different because lactadherin, but not annexin V, preferentially binds to highly curved membranes (Shi *et al.*, 2004).

5.5 Recommendations for further research

5.5.1 More erythropoietin resistant patients

The results of this study suggest that Epo dose may have an effect upon eryptosis, but further investigation is required to confirm this.

Due to the requirement for using only left over blood, this restricted the samples which could be collected. Recruiting more patients who are resistant to Epo would be of benefit in investigating the effect of Epo resistance upon microparticle production.

Epo is speculated to have an impact upon eryptosis and recruitment of patients on a consistent dose of therapy would be beneficial to truly determine the effect. In this study the Epo dosage data was provided for that specific blood test date, however monthly reviews of Hb levels lead to regular review of Epo dose. As red cells generally exist in the circulation for 120 days the treatment for the last 3 months could well affect the results, so controlling this would give more of an insight into the process involved.

5.5.2 Improved methods for measuring phosphatidylserine exposure

As discussed earlier the reliance of annexin V as a measure of PS exposure has been called into question (Horstman *et al.,* 2004; Piccin *et al.*, 2007). The flow cytometric analysis could therefore be repeated using lactadherin to ensure all PS exposure is detected.

Annexin V binding counts demonstrate the number of microparticles capable of binding annexin V and do not relate to the number of membrane site exposing PS.

5.5.3 Other ways to measure eryptosis

It is apparent that microparticles are a heterogeneous population (Jy *et al.*, 1995; Abid Hussein *et al.*, 2003; Freyssinet, 2003) and therefore vary in their size, protein composition and lipid content. Endothelial microparticles vary in their antigenic characteristics and PS exposure, which has been attributed to the mechanism by which they are generated (Abid Hussein *et al.*, 2003). It is likely that a similar mechanism will apply to red cell microparticles. There is no standardised method to enumerate RCMPs by flow cytometry (Shah *et al.*, 2008; Rubin *et al.*, 2008; Ayers *et al.*, 2011; Xiong *et al.*, 2012; Lacroix *et al.,* 2012). Many previous studies do not use dual positivity to define microparticles, but instead analyse annexin V positive events separately (Faure *et al.*, 2006; Trappenburg *et al.,* 2012).

Using strict definitions and flow cytometry may therefore not be the most effective way to determine eryptosis levels. Previous studies have used entirely different methods to measure eryptosis. This could be employed in conjunction with flow cytometry to give a more accurate picture of the process. Multiple studies have measured all of; cytosolic calcium activity (using Flou3 fluorescence), cell volume (forward scatter), phosphatidylserine exposure (annexin V binding) and ceramide formation (anti ceramide antibody) to determine eryptosis levels (Lang *et al.*, 2005; Niemoeller *et al.*, 2006; Kempe e*t al.*, 2007; Kiedaisch *et al.*, 2008; Ahmed *et al.*, 2013).

Flow cytometry was designed to measure events greater than 3μm in diameter and is therefore unable to detect the smallest microparticles, limiting its accuracy. It is also possible for multiple microparticles to pass through the aperture at once, leading to an underestimation of numbers and an overestimation of size. Also the binding of antibodies may be restricted in very small particles, as a limited surface area will inhibit available epitopes. Currently there are companies working towards developing new flow cytometry technology which can detect microparticles.

There are other methods which may be employed to assess microparticles further, all of which have their own benefits and limitations.

Resistive pulse sensing is able to detect very small particles, and therefore the smallest microparticles which cannot be identified by flow cytometry. It is based on the technology developed by Coulter, that when a particle passes through a thin channel filled with aqueous electrolyte there is a change in the ionic resistance known as a resistive pulse (Coulter, 1953). This is valuable for identification of small particles, but has no phenotyping capabilities and so would require combination with a pre-sorting stage to separate RCMP from other cells and MPs (Willmott *et al.*, 2012).

Microparticles can also be detected using enzyme-linked immunosorbent assays (ELISAs), capturing them with immobilized annexin V or cell-specific antibodies (Jy *et al*., 2004). The MP are then quantified via measuring the concentration of negatively charged phospholipids. However this method does require that the MP expose PS on their surface and does not give information about the numbers of MPs (Enjeti *et al*., 2007).

Atomic force microscopy (AFM) allows high-resolution topography imaging of particles with a resolution down to the subnanometer range. AFM has been used to detect CD41+ MPs, demonstrating 1000-fold more than with flow cytometry, the majority of which were below the size detection limits of conventional flow cytometry (Yuana *et al*., 2010). This could be valuable for assessing the smallest RCMP and achieving more accurate counts, however this technique is highly labour intensive.

Proteomics has been used to characterise populations of MPs, including platelets and leukocyte derived MP from atherosclerotic plaques (Mayr *et al.,* 2005; Garcia *et al*., 2009). Garcia *et al.* found proteins characteristic of platelets as well as other proteins, suspected to be related to the formation of MPs (Garcia *et al.*, 2005). This method requires purified preparations but would allow for detailed study of the protein characteristics of RCMPs.

Nanoparticle tracking analysis (NTA) has been developed for direct real time visualisation and analysis of nanoparticles in liquids by relating the rate of Brownian motion to particle size. This technology has been combined with antibody-conjugated quantum dots to measure placental vesicles as small as

~50nm (Dragovic *et al.*, 2011). This combines the ability to identify small particles with phenotyping, providing benefits over flow cytometry.

Varga *et al.* used five methods to determine the size distribution of erythrocyte derived extracellular vesicles (EVs) (Varga *et al.*, 2014). These were small-angle X-ray scattering (SAXS), size exclusion chromatography coupled with on-line dynamic light scattering detection (SEC-DLS), freeze fracture transmission electron microscopy (FF-TEM), nanoparticle tracking analysis (NTA) and resistive pulse sensing (RPS). They concluded that "coupling of SAXS to SEC may represent a promising way towards traceable size determination of EVs, which, together with the development of reliable reference materials with similar properties to EVs, may facilitate standardisation in the near future". There is still some way to go to find a reliable and standardised method for the detection of RCMPs.

5.5.4 General methodology improvements

It appears that the transport of samples and the extra time involved in processing during the flow loop experiments had a significant impact upon the results. Therefore repeating these experiments with the flow loop equipment on the same site as the patients would be advantageous. It would also prove whether the huge increases in absolute microparticle numbers is related to the conditions in the model or simply the differing transport/storage/timings. Further investigations could also be carried out into the impact of transportation and time before analysis on RCMP production in patient samples.

In order to draw meaningful conclusions this study should be continued with more HD and PD patients, a power calculation based on the data from this study would give an indication the numbers required. The ability to either recruit dependent upon certain factors or to have enough patients to split them into groups would allow for a much more effective analysis. There are a large number of comorbidities associated with CKD and therefore many drug options and the cause of the renal failure may also impact upon the disease progression.

The measurement of CRP by current clinical methodology is only sensitive to 5mg/L. This is adequate for routine analysis as this demonstrates whether the patient is within the normal range or not. However, recently high sensitivity CRP testing has demonstrated accuracy down to 0.3 mg/L (Pearson et al., 2003; Rudolf

and Lewandrowski, 2014). It has been used as a prognostic marker in cardiac disease, and may also be applicable for detecting early changes in CRP in CKD patients. Using this measure on the cohort from this study may give a more robust correlation with RCMPs.

5.5.4 Measurement of cell age

Using reticulocyte counts to determine cell age was inconclusive in this study, as all patients were within the reference range. A better measure of cell age would be useful in order to ascertain whether this impacts upon eryptosis. Possibly the red cells within the renal patients are generally younger than those in healthy controls and so cannot undergo eryptosis. It has been shown that susceptibility to eryptosis increases with erythrocyte age, at least partially due to increased sensitivity of oxidative stress (Ghashghaeinia *et al.*, 2012).

Most, but not all, techniques to estimate RBC survival require that a label be placed on the cells that can be followed while the RBC age in the circulation (Franco, 2009). Determining the RBC life span in healthy populations and patients with disease states is restricted by the lack of a simple and reliable method. The technique most widely used is the random-labelling method in which RBCs are tagged with radioactive chromium (51Cr), such that disappearance of the radioactivity reflects loss of RBCs. This method provides data that often are confounded with other processes, such dissociation of 51Cr from the haemoglobin, and potential loss of 51Cr bound haemoglobin due to RBC vesiculation. The extent of elution varies depending on the labelling technique used. Consequently, methods used previously provide only relative RBC survival and the survival rate obtained is comparable to only other survival rates measured in the same study (Vos *et al.,* 2011).

As the red cell ages its density progressively increases therefore separation on the basis of age may be done by centrifugation through a density gradient (Danon and Marikovsky, 1964; Wilson and Peterson, 1988). Elutriation followed by a fractionation according to cell density using discontinuous Percoll gradients can also be carried out (Bosch *et al.*, 1992). Comparison of the characteristics of the differing populations in both healthy people and CKD patients may give an insight into the process of eryptosis.

5.6 Conclusions

This study has shown that erythrocytes in CKD patients appear to be less susceptible to eryptosis. The reasons for this are unclear. There could be a relationship between the process and Epo treatment or chronic inflammation. The age of erythrocytes in these patients may have an effect, or another process may be occurring to replace eryptosis.

The hypothesis examined in this thesis needs to be tested further. The susceptibility of erythrocytes in CKD patients and controls to undergo eryptosis in response to inflammatory conditions, Epo and aging should be assessed using further methods.

It has also become clear that the behaviour of populations of MP varies depending on the cell of origin, and the definition of MP which is used. Therefore use of the generalised term “microparticle” may not be relevant.

Previous studies have demonstrated varying results, but also used a wide range of methods and employed inconsistent definitions of red cell microparticles. There is a requirement for standardisation of methodology and definitions. This thesis has provided a possible method that could be developed for use by other groups to further investigate red cell microparticles. Further validation and standardisation would be required to achieve this.

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Appendix 1: Data tables

Chapter 3

Patient data

ID	Age	Sex	Dialysis modality	Time on dialysis (days)	Diabetes?	Epo?	Epo drug	Mode of Admin	Epo dose (equivalent mcg/wk)	Hb	eGFR	Kt/V	Serum albumin (g/L)	CRP	RCMP/µl <1µm*	SD
001	72	F	PD	128	N	Y	Mircera (pre-filled syringe)	SC	50	10.3	9.24	2.4	41	5	17400	1560
002	25	F	PD	100	N	Y	Darbepoetin (syringe)/Aranesp	SC	30	7.7	3.7	2.53	37	1	48544	28949
003	33	F	HD	916	N	Y	NeoRecormon - syringe	IV	30	12.3	3.81	1.05	47	1	8322	1588
004	48	M	HD	531	N	Y	Darbepoetin (syringe)/Aranesp	IV	5	12.9	6.14	1.69	45	1	14733	12042
005	27	F	HD	737	Y	Y	Darbepoetin (syringe)/Aranesp	IV	15	12.3	6.23	1.9	43	5	10311	1782
006	34	M	HD	1181	N	Y	Darbepoetin (syringe)/Aranesp	IV	10	11.6	4.97	1.41	51	1	11856	84
007	30	F	HD	793	N	Y	Darbepoetin (syringe)/Aranesp	IV	30	11.0	5.79	1.9	46	1	9689	1641
008	77	M	HD	921	N	Y	Darbepoetin (syringe)/Aranesp	IV	30	12.0	8.62	1.8	43	5	2756	195
009	71	M	HD	558	N	Y	Darbepoetin (syringe)/Aranesp	IV	40	10.6	8.7	1.57	41	15	9522	10770
010	72	F	HD	932	Y	Y	Darbepoetin (syringe)/Aranesp	IV	80	11.8	4.81	2.34	46	1	7411	1036
011	61	M	PD	1408	N	Y	Darbepoetin (syringe)/Aranesp	SC	20	11.4	9.16	1.77	39	1	8533	3979
012	34	M	HD	1370	N	Y	Darbepoetin (syringe)/Aranesp	IV	50	11.7	4.88	1.47	46	24	9144	3458

ID	Age	Sex	Dialysis modality	Time on dialysis (days)	Diabetes?	Epo?	Epo drug	Mode of Admin	Epo dose (equivalent mcg/wk)	Hb	eGFR	Kt/V	Serum albumin (g/L)	CRP	RCMP/µl <1µm*	SD
013	66	F	HD	998	N	Y	Darbepoetin (syringe)/Aranesp	IV	130	8.7	5.09	1.8	37	31	41611	1309
014	79	F	HD	448	N	Y	Darbepoetin (syringe)/Aranesp	IV	60	10.9	6.51	1.77	36	9	24211	2269
015	55	M	PD	1012	N	N	None	SC	0	11.5	5.07	1.75	39	1	7678	1022
016	33	M	HD	411	N	Y	Darbepoetin (syringe)/Aranesp	IV	100	7.4	5.73	1.12	38	18	2267	233
017	61	M	PD	145	Y	Y	Darbepoetin (syringe)/Aranesp	SC	15	12.8	8.24	2.35	43	1	13556	8298
018	53	M	PD	161	Y	Y	Darbepoetin (syringe)/Aranesp	SC	20	12.4	8.66	1.61	38	1	7756	1202
019	75	F	PD	1914	Y	Y	NeoRecormon - syringe	SC	15	10.8	8.17	2.12	42	5	7033	233
020	68	M	PD	553	N	N	None	SC	0	12.7	13.59	3.46	36	1	4289	2135
021	31	F	PD	632	N	Y	Darbepoetin (syringe)/Aranesp	SC	30	11.0	9.37	3.47	37	1	2656	280
022	55	F	PD	244	N	Y	Darbepoetin (syringe)/Aranesp	SC	20	9.6	6.81	2.23	36	1	13511	5412
023	78	M	PD	1779	N	Y	NeoRecormon - syringe	SC	10	11.4	5.52	1.88	41	5	5367	384
024	75	M	PD	279	N	Y	NeoRecormon - syringe	SC	10	11.0	6.76	1.79	35	9	10756	5861
025	40	M	HD	112	N	Y	Darbepoetin (syringe)/Aranesp	IV	40	9.2	5.51	0.83	43	5	4278	674
026	74	M	PD	423	Y	Y	Darbepoetin (syringe)/Aranesp	SC	20	12.6	10.84	2.83	40	1	18656	20101
027	69	M	PD	548	Y	Y	Darbepoetin (syringe)/Aranesp	SC	5	11.7	7.65	2.16	38	40	5389	482
028	48	F	PD	285	N	N	None	SC	0	10.1	7.21	2.02	38	7	8644	1075

ID	Age	Sex	Dialysis modality	Time on dialysis (days)	Diabetes?	Epo?	Epo drug	Mode of Admin	Epo dose (equivalent mcg/wk)	Hb	eGFR	Kt/V	Serum albumin (g/L)	CRP	RCMP/µl <1µm*	SD
029	58	F	HD	1897	N	Y	Darbepoetin (syringe)/Aranesp	IV	40	12.9	8.6	1.7	45	16	18211	5176
030	35	F	HD	3537	N	Y	Darbepoetin (syringe)/Aranesp	IV	80	11.1	4.41	1.7	42	1	7900	862
031	60	F	PD	26	N	Y	Darbepoetin (syringe)/Aranesp	SC	20	10.7	6.21	2.73	48	1	13922	832
032	66	F	PD	654	Y	Y	Mircera (pre-filled syringe)	SC	80	11.2	5.48	1.71	42	5	21911	6236
033	55	M	PD	878	Y	Y	Darbepoetin (syringe)/Aranesp	SC	40	12.5	8.44	2.23	42	17	20311	3351
034	80	M	PD	1977	N	Y	Darbepoetin (syringe)/Aranesp	SC	20	15.2	7.21	2.01	39	1	15378	6767
035	76	M	HD	2206	N	Y	Darbepoetin (syringe)/Aranesp	IV	150	7.7	10.79	2.47	29	64	36178	28627
036	70	M	HD	798	N	Y	NeoRecormon - syringe	IV	30	9.6	7.08	1.24	41	6	28744	9297
037	61	M	HD	431	Y	N	None	IV	0	11.5	9.59	1.01	39	46	10533	145
038	72	F	HD	809	Y	Y	Darbepoetin (syringe)/Aranesp	IV	20	9.9	5.26	1.68	41	1	15156	3136
039	74	M	HD	2308	Y	Y	Darbepoetin (syringe)/Aranesp	IV	10	11.6	5.73	1.63	45	1	9400	694
040	66	M	HD	978	Y	Y	Darbepoetin (syringe)/Aranesp	IV	60	10.9	8.63	1.25	35	13	18467	7060
041	83	F	HD	2494	N	Y	Darbepoetin (syringe)/Aranesp	IV	30	10.4	6.69	1.78	44	1	17367	5472
042	75	M	HD	1618	N	Y	Darbepoetin (syringe)/Aranesp	IV	20	10.8	7.86	1.45	40	5	12611	2040
043	58	F	HD	1925	N	Y	Darbepoetin (syringe)/Aranesp	IV	30	12.6	8.2	1.7	45	9	37000	28354
044	50	M	HD	763	Y	Y	NeoRecormon - syringe	IV	75	10.2	4.55	1.37	46	6	40189	43707

ID	Age	Sex	Dialysis modality	Time on dialysis (days)	Diabetes?	Epo?	Epo drug	Mode of Admin	Epo dose (equivalent mcg/wk)	Hb	eGFR	Kt/V	Serum albumin (g/L)	CRP	RCMP/µl <1µm*	SD
045	86	M	HD	1367	N	Y	NeoRecormon - syringe	IV	50	10.3	10.12	1.52	36	14	19189	10431
046	46	F	HD	134	N	Y	Darbepoetin (syringe)/Aranesp	IV	20	11.7	6.94	1.63	40	1	25511	21959
047	52	F	HD	866	N	Y	Darbepoetin (syringe)/Aranesp	IV	100	10.3	4.06	2.34	40	12	26933	11566
048	67	M	HD	1201	Y	Y	Darbepoetin (syringe)/Aranesp	IV	80	10.7	10.97	1.7	40	25	18333	6954
049	75	F	HD	2060	Y	Y	Darbepoetin (syringe)/Aranesp	IV	80	9.2	6.91	1.64	41	12	15322	2200
050	59	M	PD	527	N	N	None	SC	0	13.2	12.73	2.76	37	1	12833	1206
051	61	M	PD	646	Y	Y	Darbepoetin (syringe)/Aranesp	SC	25	10.0	6.6	1.73	34	1	16800	2411
052	61	M	HD	459	Y	N	None	IV	0	11.1	8.28	1.01	37	116	20611	4802
053	55	F	HD	776	N	Y	NeoRecormon - syringe	IV	30	11.0	29.73	2.21	33	37	13600	5916
054	27	M	PD	441	N	Y	Darbepoetin (syringe)/Aranesp	SC	30	11.1	8.68	1.55	51	1	8322	782
055	70	F	PD	1152	Y	Y	NeoRecormon - syringe	SC	15	11.6	10.37	2.18	38	5	7233	2174
056	58	M	HD	1595	Y	Y	Darbepoetin (syringe)/Aranesp	IV	40	11.1	7.2	1.86	39	14	27833	10408
057	75	M	PD	683	Y	N	None	SC	0	12.4	12.58	2.6	41	1	18133	10616
058	56	F	PD	987	N	Y	Mircera (pre-filled syringe)	SC	15	11.7	8.99	2	40	1	15067	6073
059	59	M	PD	1577	Y	Y	Mircera (pre-filled syringe)	SC	60	9.3	6.63	1.74	41	1	11956	1336
060	51	F	PD	532	Y	Y	Darbepoetin (syringe)/Aranesp	SC	20	11.8	5.2	2.37	39	26	12311	1707

ID	Age	Sex	Dialysis modality	Time on dialysis (days)	Diabetes?	Epo?	Epo drug	Mode of Admin	Epo dose (equivalent mcg/wk)	Hb	eGFR	Kt/V	Serum albumin (g/L)	CRP	RCMP/µl <1µm*	SD
061	69	M	PD	218	Y	Y	Darbepoetin (syringe)/Aranesp	SC	20	13.5	5.69	1.91	41	1	120711	7959
062	67	M	PD	288	N	N	None	SC	0	10.0	8.51	2.4	38	1	11456	1628
063	73	M	PD	1014	N	Y	Darbepoetin (syringe)/Aranesp	SC	10	11.1	9.03	2.54	38	1	14622	1884
064	53	F	PD	159	N	Y	Darbepoetin (syringe)/Aranesp	SC	15	10.5	7.54	1.43	40	19	47967	819
065	42	M	PD	517	N	N	None	SC	0	12.2	6.02	2.37	42	1	23567	6094
066	75	M	PD	1476	Y	N	None	SC	0	9.0	8.01	1.84	30	39	19189	9307
067	48	M	HD	847	Y	Y	Darbepoetin (syringe)/Aranesp	IV	60	9.7	4.94	1.37	45	1	29356	1309
068	40	F	PD	1465	N	Y	Darbepoetin (syringe)/Aranesp	SC	50	12.3	3.02	3.18	36	1	18411	681
069	52	M	PD	752	Y	Y	Mircera (pre-filled syringe)	SC	40	10.0	5.96	1.73	39	1	16056	1946
070	44	F	HD	1161	N	Y	Darbepoetin (syringe)/Aranesp	IV	40	10.5	10.15	1.29	43	1	19489	3401
071	53	M	HD	1958	Y	Y	Darbepoetin (syringe)/Aranesp	IV	40	12.8	5.24	1.42	44	1	31644	2533
072	75	F	HD	1436	Y	Y	Darbepoetin (syringe)/Aranesp	IV	130	11.6	5.76	1.37	41	11	16944	1039
073	68	F	HD	558	N	Y	Darbepoetin (syringe)/Aranesp	IV	40	9.3	5.85	1.05	40	7	13422	4665
074	43	F	HD	304	N	Y	Darbepoetin (syringe)/Aranesp	IV	20	11.6	8.41	1.91	45	1	12522	1858
075	54	M	HD	4293	N	Y	Darbepoetin (syringe)/Aranesp	IV	60	11.7	5.68	1.5	41	7	22533	2450
076	45	M	HD	388	N	Y	Darbepoetin (syringe)/Aranesp	IV	80	12.7	5.92	1.48	45	8	52244	3039

ID	Age	Sex	Dialysis modality	Time on dialysis (days)	Diabetes?	Epo?	Epo drug	Mode of Admin	Epo dose (equivalent mcg/wk)	Hb	eGFR	Kt/V	Serum albumin (g/L)	CRP	RCMP/µl <1µm*	SD
077	69	M	HD	478	Y	Y	Darbepoetin (syringe)/Aranesp	IV	40	13.2	8	1.23	38	6	23722	3415
078	76	M	HD	749	Y	Y	Darbepoetin (syringe)/Aranesp	IV	40	13.9	3	1.43	45	6	23956	17302
079	43	F	HD	282	N	Y	Darbepoetin (syringe)/Aranesp	IV	50	10.4	6.77	1.57	39	13	31122	3664
080	57	F	HD	2014	Y	Y	Darbepoetin (syringe)/Aranesp	IV	30	11.7	7.21	2.03	43	1	23500	5467
081	56	M	HD	1140	Y	N	None	IV	0	13.1	6.49	1.45	43	7	19167	4521
082	52	F	HD	494	Y	Y	Darbepoetin (syringe)/Aranesp	IV	30	10.7	7.25	1.65	35	8	22867	1538
083	81	M	PD	2229	Y	N	None	SC	0	9.0	6.71	1.47	35	5	11644	1578
084	24	M	HD	294	Y	Y	Darbepoetin (syringe)/Aranesp	IV	100	10.6	7.04	1.83	43	1	17489	3430

* These are the mean of the triplicate repeats for each subject, calculated from the absolute counts as described in the methods.

Control data

ID	Age	Sex	Diabetic?	Hb	RCMP/µl <1µm*	SD
C01	25	F	N	14.4	6744	3067
C02	32	F	N	14.1	15167	65
C03	39	M	N	15.6	33500	14700
C04	60	M	N	13.7	7789	71
C05	47	M	N	15.7	12867	1519
C06	42	M	N	15.4	17478	128
C07	58	M	N	14.9	21522	7809
C08	47	F	N	13.1	34989	122
C09	57	F	N	14.2	39789	11210
C10	58	F	N	13.9	12844	82
C11	60	F	N	13.1	19033	6365
C12	58	M	N	15.6	47378	204
C13	49	M	N	14.5	36833	6967
C14	54	M	N	16.8	248622	677
C15	44	F	N	14.4	21656	4752
C16	58	M	N	15.2	36833	382
C17	62	F	N	12.7	74622	13030
C18	63	M	N	15.0	87678	419
C19	44	M	N	15.1	11267	7499
C20	61	M	N	15.2	22478	107

* These are the mean of the triplicate repeats for each subject, calculated from the absolute counts as described in the methods.

MP populations using different definitions

ID	MP/µl for each population*									
	All Annexin V <1µl	All Annexin V >1µl	All Annexin V+	All Glyco A+	All Glyco A<1 µl	All Glyco A>1µl	All MP	All MP<1µl	All MP>1µl	Glyco A- Annexin V+ <1µl
001	37044	14800	47789	112522	88022	72522	1142089	340778	801311	21989
002	120256	75856	197422	121889	144711	73344	6880744	1541589	5339156	108456
003	11111	9578	19989	66711	44222	28389	3295144	633333	2661811	5367
004	34111	21722	33422	53089	38233	15089	2044578	203478	1841100	8511
005	25044	12200	35656	44422	29833	13022	4679100	898589	3780511	16978
006	20244	16789	32167	74922	48111	29589	1842833	234667	1608167	9311
007	18389	10567	27567	78800	59722	24389	3026356	566567	2459789	11133
008	3567	3544	7711	43322	20833	24400	2552978	543511	2009467	2233
009	7256	6000	11078	48800	27589	20111	4172411	789367	3383044	3789
010	10800	4644	15489	89144	62189	31100	1973978	308589	1665389	3867
011	19011	19300	35300	144822	71878	73178	6569722	1129711	5440011	12244
012	13933	11222	29100	115667	75756	39756	5730322	1527767	4202556	9189
013	75011	36700	110078	127956	88067	45056	5990644	2221022	3769622	38400
014	33256	19089	51844	123300	76467	55511	3822822	1361333	2461489	12811
015	21267	20356	38744	74156	47111	33367	6572211	1813400	4758811	14978
016	3644	3178	6889	74756	32600	28856	2649200	1011267	1637933	2689
017	166378	69589	232700	137300	95189	48733	3447956	1143989	2303967	155633
018	16378	12911	29433	64567	45422	23844	3392956	715722	2677233	10122

ID	MP/µl for each population*									
	All Annexin V <1µl	All Annexin V >1µl	All Annexin V+	All Glyco A+	All Glyco A<1 µl	All Glyco A>1µl	All MP	All MP<1µl	All MP>1µl	Glyco A- Annexin V+ <1µl
019	15322	10622	23100	37233	27867	13556	4746944	1352200	3394744	9411
020	12422	13656	25944	32967	18456	18744	4704489	1052667	3651822	9233
021	4156	4900	8311	55233	27778	27578	4388233	1310511	3077722	5400
022	11389	5411	16178	119767	96711	43900	5044289	1718211	3326078	7811
023	11744	10567	36178	84367	35578	51200	4894233	1903022	2991211	12300
024	26644	28578	57700	126144	92167	43900	9655367	3333600	6321767	19033
025	5044	7289	13256	92622	59900	43867	5307822	1046411	4261411	6467
026	21067	17511	37411	154033	83656	50333	4271633	2060511	2211122	20700
027	8811	9533	19222	151611	102222	58056	4867000	1803122	3063878	5678
028	25989	15011	32600	138333	95211	47622	9152156	4206622	4945533	15611
029	40267	23889	67967	235500	129689	122500	5763433	2801678	2961756	26556
030	15700	8822	22778	100278	78189	31578	6364178	3026822	3337356	8544
031	50511	29822	65456	217144	136000	89744	11482911	6879389	4603522	37200
032	51644	25878	72889	160300	105256	72589	7304111	2080856	5223256	42767
033	49689	31589	78878	157144	120856	45400	5439733	1929356	3510378	63667
034	42289	25422	61467	149289	110633	59767	6592867	1715267	4877600	33411
035	88867	65922	144889	155233	115000	47889	5562244	1735222	3827022	66656
036	54089	33300	80778	153300	90756	43533	3384800	1380367	2004433	36989
037	21056	12800	25867	97856	52067	51544	2868856	995456	1873400	12022
038	24344	13467	37433	154456	81389	81311	3504256	1513511	1990744	11544

ID	MP/µl for each population*									
	All Annexin V <1µl	All Annexin V >1µl	All Annexin V+	All Glyco A+	All Glyco A<1 µl	All Glyco A>1µl	All MP	All MP<1µl	All MP>1µl	Glyco A- Annexin V+ <1µl
039	16622	8756	18778	40867	27700	18244	2463500	1161178	1302322	10644
040	26689	14811	43400	116322	80756	45211	5340333	1915178	3425156	17667
041	22967	13944	36556	63489	42156	19156	4330622	1510811	2819811	12667
042	22022	12344	32211	53089	35478	25133	4486056	1702900	2783156	13122
043	71800	35478	108222	144356	83700	50722	4774022	1860622	2913400	56967
044	65100	24300	83678	103644	68556	36867	5064556	2268356	2796200	36722
045	36322	15233	48911	135856	91900	46700	8069889	3489878	4580011	28467
046	58589	23089	70100	158178	97711	62944	8666789	4192933	4473856	37500
047	47744	27522	62778	107778	73522	42967	7988989	3288567	4700422	32400
048	30611	12756	42878	113167	83611	39222	6480511	3165700	3314811	19956
049	24422	10711	32289	121244	81456	49322	6536867	3258267	3278600	16811
050	23178	23300	38156	147644	107589	50122	9287689	3623833	5663856	20189
051	25867	9578	33656	96167	77000	23078	4851756	2130289	2721467	14133
052	30033	15500	49256	187578	136100	62144	3180811	1082833	2097978	9778
053	43111	16211	59267	119933	98500	24889	8819733	3353333	5466400	32244
054	13367	3867	19433	97978	72956	27956	3973756	1692667	2281089	9689
055	22400	9922	31856	233600	104567	79033	5015733	1493111	3522622	11444
056	46100	14022	61456	156267	100222	59211	10039278	5052044	4987233	24644
057	53911	18022	70467	119422	86511	33167	12242489	5540656	6701833	40522
058	35656	9333	46411	108489	79533	29933	7360933	1589178	5771756	23956

ID	MP/µl for each population*									
	All Annexin V <1µl	All Annexin V >1µl	All Annexin V+	All Glyco A+	All Glyco A<1 µl	All Glyco A>1µl	All MP	All MP<1µl	All MP>1µl	Glyco A- Annexin V+ <1µl
059	33833	11544	46244	103389	77611	25300	5038522	1140356	3898167	26356
060	36378	13956	52467	105256	86333	21833	9737156	3098744	6638411	28944
061	142256	21889	165856	309556		53944	7953078	2919589	5033489	35789
062	47467	13067	58822	142678	97922	47156	11846811	5014967	6831844	36678
063	15022	12233	60044	154222	125511	27311	9052689	3942356	5110333	10756
064	90767	25522	114922	215344	151844	65189	11269267	4388178	6881089	48533
065	50467	17300	65333	208844	145889	70033	9669056	4421111	5247944	39378
066	41511	10622	44700	102156	94022	27978	6628900	3577878	3051022	26156
067	90611	23400	131378	248867	154167	74511	10723911	4725911	5998000	81511
068	66244	17256	58833	191356	136111	63333	5833100	2253878	3579222	52167
069	57589	23267	74244	122133	98000	26844	4358200	1396922	2961278	46389
070	60467	13744	59689	138978	117678	22733	5514678	1658844	3855833	44300
071	71467	14656	78811	183633	144967	47367	5351122	1193322	4157800	39067
072	45189	14489	71478	205467	138944	77944	4885533	1250633	3634900	39700
073	44256	11289	58367	172244	122989	49800	7913322	2171011	5742311	38222
074	48078	19100	56278	94467	75411	22489	6480922	1815456	4665467	37778
075	58844	17656	75356	111956	80356	25033	5835944	1687467	4148478	41511
076	109956	40911	139144	263978	167389	100122	6283656	2595544	3688111	84400
077	50889	12278	69044	165411	134689	36522	5206344	2041111	3165233	44778
078	61411	12244	83322	204778	154078	50033	5762422	149578	5612844	42800

ID	MP/µl for each population*									
	All Annexin V <1µl	All Annexin V >1µl	All Annexin V+	All Glyco A+	All Glyco A<1 µl	All Glyco A>1µl	All MP	All MP<1µl	All MP>1µl	Glyco A- Annexin V+ <1µl
079	80211	19678	96089	127144	101489	24022	8688822	3782922	4905900	77022
080	57822	16478	89956	209933	173956	44478	6743767	2143644	4600122	51189
081	56000	15078	76611	197822	135033	61478	5288211	1810711	3477500	45233
082	55400	19922	77744	102978	79089	27789	3389978	1567544	1822433	44633
083	56933	19722	68556	66800	52078	15333	6615356	2098411	4516944	51211
084	51678	18522	57667	173167	114000	45444	6596644	2959311	3637333	36522
C01	18267	11300	26989	97844	104364	40367	12378844	6888678	5490167	10689
C02	31167	11600	43578	113867	102465	35522	14514678	8349378	6165300	27267
C03	34989	11578	43744	221500	100566	63644	10358867	5519978	4838889	23222
C04	26933	14611	45944	227311	98668	51511	4779122	1197144	3581978	18100
C05	65244	29511	93356	156700	96769	32511	5209044	949622	4259422	36167
C06	64567	26933	96011	137133	94871	26744	6503778	1345833	5157944	35211
C07	55689	21478	76133	251089	92972	82489	8971522	4188000	4783522	31333
C08	20778	8833	28656	132267	91074	38378	7923356	4412756	6431844	16400
C09	43544	18033	54744	163422	89175	41967	9822189	3603211	6218978	34233
C10	32878	17033	53356	138022	87276	33267	9041000	3972733	5068267	24978
C11	39533	15089	53100	226200	85378	63922	8305922	2696267	5609656	27967
C12	79078	30011	134233	249122	83479	58533	7374522	2394911	4979611	53644
C13	68711	14189	64044	212111	81581	63011	9261644	4122811	7735778	35689
C14	373500	91511	467589	1508378	79682	123722	8895211	4101600	4793611	120567

ID	MP/µl for each population*									
	All Annexin V <1µl	All Annexin V >1µl	All Annexin V+	All Glyco A+	All Glyco A<1 µl	All Glyco A>1µl	All MP	All MP<1µl	All MP>1µl	Glyco A- Annexin V+ <1µl
C15	40411	18722	69311	171567	77783	41456	5414989	1486922	3928067	37011
C16	113144	29556	155467	349756	75885	91344	11409967	4860911	6549056	107456
C17	178267	67567	249900	296956	73986	78322	14004067	4405744	9598322	125533
C18	186411	58267	227533	357289	72088	84878	13337044	4043278	9293767	125900
C19	25911	9722	29500	193678	70189	49244	10179100	4241511	5937589	19944
C20	46344	19811	62300	141067	68290	38400	10009056	4367389	5641667	29411

* These are the mean of the triplicate repeats for each subject, calculated from the absolute counts as described in the methods.

MP populations using different definitions (cont.)

ID	MP/µl for each population*									
	Glyco A- Annexin+ >1µl	Glyco A+ Annexin- > 1µl	Glyco A+ Annexin V- <1µl	All Glyco A+ Annexin V+	Glyco A+ Annexin V+ <1µl	Glyco A+ Annexin V+ >1µl	CD45+ AnnexinV+ >1	CD45+ AnnexinV+ <1	CD45+ AnnexinV- >1	CD45+ AnnexinV- <1
001	9967	287100	68611	31267	17400	13867	1233	3800	5233	7489
002	68189	40867	50600	51433	48544	2889	2711	5800	11278	14578
003	7556	25411	36811	11533	8322	3211	133	133	9911	1833
004	10722	17767	24678	16922	14733	2189	56	200	4811	1111
005	11167	12289	23956	11967	10311	1656	144	133	8356	1822
006	12056	27700	38156	14622	11856	2767	178	167	12944	2344
007	7722	8411	11122	12056	9689	2367	89	167	8822	1278

ID	MP/µl for each population*									
	Glyco A- Annexin+ >1µl	Glyco A+ Annexin- > 1µl	Glyco A+ Annexin V- <1µl	All Glyco A+ Annexin V+	Glyco A+ Annexin V+ <1µl	Glyco A+ Annexin V+ >1µl	CD45+ AnnexinV+ >1	CD45+ AnnexinV+ <1	CD45+ AnnexinV- >1	CD45+ AnnexinV- <1
008	3356	23556	18867	3256	2756	500	67	133	6811	1367
009	5633	19167	28778	9767	9522	244	56	156	15000	1800
010	7422	30456	58367	8111	7411	700	522	911	12222	5322
011	15389	69089	65878	12800	8533	4267	267	389	24211	3156
012	10800	38178	68511	11578	9144	2433	300	589	17000	4411
013	9956	34533	47833	51722	41611	10111	711	667	22967	5167
014	12144	48533	53789	30911	24211	6700	356	422	13211	3656
015	18711	31700	39544	9300	7678	1622	133	400	12144	2478
016	2811	28733	32122	2433	2267	167	56	211	3444	1211
017	65911	71611	80044	16633	13556	3078	1667	1378	34378	5244
018	11300	22367	39000	9622	7756	1867	156	400	13333	2556
019	8867	11756	21033	8089	7033	1056	144	144	9733	1989
020	13211	17122	12400	5856	4289	1567	78	122	10689	2100
021	12700	27178	24478	4022	2656	1367	167	89	6989	1844
022	3200	41444	67556	16022	13511	2511	78	211	16156	3600
023	17189	49744	29000	7378	5367	2011	89	144	7644	3167
024	27244	58278	80811	13433	10756	2678	322	233	17978	5033
025	21322	42844	53978	5289	4278	1011	244	156	29733	2622
026	14789	47278	88511	20989	18656	2333	100	189	7667	3178
027	8367	55689	96044	6956	5389	1567	78	144	7911	3167
028	11133	51411	88256	11278	8644	2633	22	33	1056	1333

ID	MP/µl for each population*									
	Glyco A- Annexin+ >1µl	Glyco A+ Annexin- > 1µl	Glyco A+ Annexin V- <1µl	All Glyco A+ Annexin V+	Glyco A+ Annexin V+ <1µl	Glyco A+ Annexin V+ >1µl	CD45+ AnnexinV+ >1	CD45+ AnnexinV+ <1	CD45+ AnnexinV- >1	CD45+ AnnexinV- <1
029	21700	110178	107433	25800	18211	7589	44	111	1811	2467
030	8344	29922	70733	9989	7900	2089	11	67	978	1200
031	24900	87022	123344	17333	13922	3411	122	89	3067	2678
032	20200	66844	83378	26267	21911	4356	222	256	3422	3233
033	33567	49867	100522	27344	20311	7033	111	133	1844	1878
034	26600	54922	101367	17944	15378	2567	78	56	1367	1644
035	70611	44600	82600	42256	36178	6078	311	533	2378	2133
036	23444	48056	82778	43978	28744	15233	100	100	967	600
037	8133	50622	42911	14833	10533	4300	56	89	1033	1656
038	9122	82611	71167	19256	15156	4100	89	100	1356	1556
039	9289	15456	23289	12289	9400	2889	56	56	978	1078
040	13100	52678	65444	25178	18467	6711	111	144	1822	2144
041	9878	14033	26644	25711	17367	8344	67	144	700	989
042	8344	23578	24522	18167	12611	5556	56	78	989	1078
043	29700	47189	55567	49889	37000	12889	156	244	2489	2789
044	18022	39478	37789	51289	40189	11100	122	289	1244	1856
045	16967	45856	74411	23178	19189	3989	44	100	1144	722
046	21222	66822	72878	30333	25511	4822	100	211	2389	2400
047	19189	37678	48322	34511	26933	7578	144	222	1733	1644
048	12578	40978	64322	24656	18333	6322	56	167	833	911
049	10467	47911	65300	20522	15322	5200	11	56	733	1144

ID	MP/µl for each population*									
	Glyco A- Annexin+ >1µl	Glyco A+ Annexin- > 1µl	Glyco A+ Annexin V- <1µl	All Glyco A+ Annexin V+	Glyco A+ Annexin V+ <1µl	Glyco A+ Annexin V+ >1µl	CD45+ AnnexinV+ >1	CD45+ AnnexinV+ <1	CD45+ AnnexinV- >1	CD45+ AnnexinV- <1
050	10056	43556	87367	15722	12833	2889	167	167	4411	1133
051	6178	18567	58533	21522	16800	4722	122	222	1956	2778
052	8800	55089	115900	29133	20611	8522	67	67	1189	1311
053	12389	21822	87278	17367	13600	3767	0	22	2033	567
054	4522	26256	64400	9944	8322	1622	11	22	411	844
055	7767	74156	146156	10211	7233	2978	0	33	967	1111
056	11978	58411	74522	32422	27833	4589	67	178	2467	2078
057	19944	31556	69200	21800	18133	3667	44	144	2389	1189
058	8644	29922	65578	16522	15067	1456	0	33	1622	1611
059	13300	25089	66356	13800	11956	1844	33	22	2856	1133
060	14511	24589	73878	13978	12311	1667	56	100	2200	1922
061	17533	64444	129700	132133	120711	11422	33	122	2744	2322
062	10444	31822	87311	13322	11456	1867	189	133	2444	2744
063	6122	30144	118922	16056	14622	1433	722	156	1267	1100
064	25078	59800	106244	56011	47967	8044	78	378	2233	2122
065	17878	69244	124767	26678	23567	3111	67	78	2367	2511
066	11222	25544	75778	22056	19189	2867	78	44	2022	2144
067	21933	51189	124367	35211	29356	5856	133	122	2633	2711
068	17789	68533	118144	23544	18411	5133	56	89	2256	3356
069	20444	22300	83000	19733	16056	3678	22	100	911	2044
070	13522	20311	97878	24400	19489	4911	200	300	1333	1978

ID	MP/µl for each population*									
	Glyco A- Annexin+ >1µl	Glyco A+ Annexin- > 1µl	Glyco A+ Annexin V- <1µl	All Glyco A+ Annexin V+	Glyco A+ Annexin V+ <1µl	Glyco A+ Annexin V+ >1µl	CD45+ AnnexinV+ >1	CD45+ AnnexinV+ <1	CD45+ AnnexinV- >1	CD45+ AnnexinV- <1
071	13611	41644	106667	39789	31644	8144	89	156	1867	2444
072	13511	74756	122478	21700	16944	4756	56	189	1878	2733
073	10967	47456	111211	16278	13422	2856	22	33	889	1244
074	15444	21622	62522	15256	12522	2733	22	156	1511	2311
075	14067	25144	60233	28811	22533	6278	111	267	2422	2200
076	42533	97556	113600	60400	52244	8156	200	244	2267	3078
077	10911	33700	109622	28567	23722	4844	22	144	1456	1589
078	11111	51411	131400	27822	23956	3867	33	56	978	1267
079	17344	23189	70244	38611	31122	7489	133	100	1033	844
080	12422	41144	146278	28489	23500	4989	22	78	1233	1567
081	12389	60300	117533	24389	19167	5222	22	22	1689	2311
082	13633	21789	57867	30778	22867	7911	33	89	1533	2156
083	15378	13100	41033	14744	11644	3100	44	78	1200	1189
084	9000	42444	101967	30033	17489	12544	89	200	1044	1189
C01	8400	39533	51433	9467	6744	2722	11	33	1078	1011
C02	13100	30756	62389	20922	15167	5756	22	89	1367	1744
C03	9789	62778	151356	36256	33500	2756	56	22	2656	2556
C04	12822	52122	174478	12111	7789	4322	33	133	1633	2200
C05	19667	26833	87100	27411	12867	14544	189	389	2911	3233
C06	19100	19144	72733	29978	17478	12500	111	133	2911	1756
C07	17911	74567	146022	30711	21522	9189	144	89	2278	2367

ID	MP/µl for each population*									
	Glyco A- Annexin+ >1µl	Glyco A+ Annexin- > 1µl	Glyco A+ Annexin V- <1µl	All Glyco A+ Annexin V+	Glyco A+ Annexin V+ <1µl	Glyco A+ Annexin V+ >1µl	CD45+ AnnexinV+ >1	CD45+ AnnexinV+ <1	CD45+ AnnexinV- >1	CD45+ AnnexinV- <1
C08	7778	39411	91911	36144	34989	1156	11	33	2544	3122
C09	13500	40233	115811	43044	39789	3256	56	67	1300	1656
C10	16067	28156	95044	17967	12844	5122	78	78	1022	1044
C11	13056	58800	157867	25756	19033	6722	44	78	1311	1478
C12	25733	48644	158089	62633	47378	15256	211	267	2344	2011
C13	17233	40944	118111	52678	36833	15844	44	144	3956	1378
C14	84167	114978	1156711	257733	248622	9111	811	689	3844	2556
C15	16133	34744	113189	28367	21656	6711	111	189	1756	1289
C16	22544	81033	221233	48811	36833	11978	100	111	1533	1400
C17	47944	46122	144811	110756	74622	36133	144	244	2222	2189
C18	34889	54200	189289	126378	87678	38700	167	144	3456	1833
C19	10778	48400	143111	12778	11267	1511	89	67	1678	2833
C20	15367	19667	85022	31089	22478	8611	33	100	1778	1844

* These are the mean of the triplicate repeats for each subject, calculated from the absolute counts as described in the methods.

Chapter 4

Patients/Control demongraphics

ID	Age	Sex	Dialysis modality	Time on dialysis (days)	Diabetes?	Epo?	Epo drug	Mode of Admin	Epo dose (equivalent mcg/wk)	Hb	eGFR	Kt/V	Serum albumin (g/L)	CRP
P01	77	M	HD	943	N	Y	Darbepoetin (syringe)/Aranesp	iv	30	11.0	8.63	1.7	45	5
P02	43	F	HD	341	N	N	-	-	-	12.3	8.51	1.76	41	1
P03	56	M	HD	1159	Y	Y	Darbepoetin (syringe)/Aranesp	iv	20	12.4	6.51	1.43	44	8
P04	48	M	HD	571	N	N	-	-	-	12.0	6.19	1.71	46	1
P05	33	F	HD	938	N	N	-	-	-	9.7	3.79	1.02	42	1
P06	67	F	HD	1914	Y	Y	NeoRecormon - syringe	iv	30	11.6	7.11	1.63	43	5
C01	32	M	-	-	N	-	-	-	-	14.6	-	-	-	-
C02	31	F	-	-	N	-	-	-	-	12.8	-	-	-	-
C03	30	F	-	-	N	-	-	-	-	13.5	-	-	-	-
C04	29	M	-	-	N	-	-	-	-	14.1	-	-	-	-
C05	52	M	-	-	N	-	-	-	-	13.8	-	-	-	-
C06	43	M	-	-	N	-	-	-	-	14.3	-	-	-	-

Patient RCMP/µl for each part of the *in vitro* model

	RCMP/µl <1µm* (±SD)					
	P01	P02	P03	P04	P05	P06
No flow	536556 (±71064)	469444 (±46684)	573444 (±63600)	468889 (±51548)	226889 (±14416)	391222 (±19825)
Flow alone	287222 (±35586)	60889 (±15763)	467222 (±68699)	179667 (±82289)	210222 (±33128)	452556 (±83060)
Untreated EC	295444 (±111742)	424778 (±114135)	437556 (±61707)	194111 (±28253)	186667(±7937)	261111 (±58705)
TNF	116000 (±48439)	201556 (±19004)	287556 (±111341)	514556 (±111277)	225444 (±49055)	504000 (±57159)

* These are the mean of the triplicate repeats for each subject, calculated from the absolute counts as described in the methods.

Control RCMP/µl for each part of the *in vitro* model

	RCMP/µl <1µm* (±SD)					
	C01	C02	C03	C04	C05	C06
No flow	202333 (±75675)	349778 (±152698)	1413111 (±143133)	413556 (±69360)	295111 (±39363)	436333 (±57197)
Flow alone	432222 (±34022)	687444 (±105550)	414111 (±146716)	567444 (±110976)	304778 (±9674)	532222 (±64633)
Untreated EC	586333 (±33451)	1260667 (±205060)	795556 (±418967)	292000 (±103026)	414667 (±13642)	266333 (±44886)
TNF	450111 (±47619)	1233000 (±358345)	259778 (±43656)	765556 (±58989)	761667 (±191087)	809778 (±44883)

* These are the mean of the triplicate repeats for each subject, calculated from the absolute counts as described in the methods.

Appendix 2: Ethics approval documentation

Protocol

Full title of the research: Study of Eryptosis in Patients with Renal Disease

Short title: Eryptosis in Renal Failure

Chief Investigator: Prof Marion Macey, Department of Haematology

Co-Investigators: Prof Magdi Yaqoob and Dr Stanley Fan, Renal Unit

Summary of the study

Historically, the cause for anaemia (low red cells in the blood) in patients with renal failure is thought to be because of reduced levels of a hormone called erythropoietin (Epo) that is produced in the kidneys.

However, this would not explain several observations:

- The doses of Epo used to treat patients with renal failure are much larger than "physiological" concentrations.
- The mean dose of Epo required for patients on different dialysis modalities are different.

We now know that red cells can undergo a process of programmed suicidal death (called eryptosis; analogous to a process called apoptosis but erythrocytes are devoid of nuclei and mitochondria).

Very little is known about eryptosis in patients with renal failure. We would like to study the amount of eryptotic cells in patients with renal failure.

Aims would include:

- Does the degree of renal failure affect the concentration of eryptotic cells? (thereby exacerbating anaemia)?

- Does dialysis modality (Peritoneal Dialysis vs Haemodialysis) influence concentration of eryptotic cells?
- Can we find factors that might be associated with the concentration of eryptotic cells (e.g. does the degree of inflammation or comorbidity affect this process)?

Summary of main Ethical issues

We regularly monitor patients with renal failure by blood tests. We have their signed consent allowing us to store left over samples for research. We also have their agreement that authorised members of staff who are not directly involved in their care may access their health records for research approved by a research ethics committee (refer to the end of this document for a copy of the consent form). We therefore do not intend to go back to the patients for permission to use their surplus blood. However, we shall seek consent from health volunteers for a control group (see information sheet and consent form).

Primary Aim:

Does the degree of renal failure affect the concentration of eryptotic cells? (thereby exacerbating anaemia)?

Secondary Aims:

Does dialysis modality (Peritoneal Dialysis vs Haemodialysis) influence concentration of eryptotic cells?

Can we find factors that might be associated with the concentration of eryptotic cells (eg does the degree of inflammation or comorbidity affect this process)?

What is the scientific justification for the research?

At the moment, we treat the anaemia associated with renal failure by giving supra-physiological doses of a hormone called EPO. There is some evidence that giving very high doses of this hormone is associated with morbidity and mortality (irrespective of the haemoglobin level).

If eryptosis plays a significant role in causing the anaemia that is associated with renal failure, then this would permit us to explore other ways of treating these patients.

If we can find association between clinical factors and the amount of eryptotic cells in blood, this will give us a clue about the pathogenesis and drivers of this process.

Methodology

Flow cytometry for analysis of microparticle formation

The immunolabelling and flow cytometric analysis of MP in whole blood will be performed as described previously (1, 2) using a Canto II flow cytometer (BD, Oxford UK) with Diva Software version 6.1. The instrument will be calibrated daily. The instrument will have a standard set up and be suitable for cellular analysis.

For the analysis of platelets and microparticles anticoagulated blood (50µl) will be labelled with fluorescent antibodies to cell surface proteins. 1.09µm latex beads from a manufacturers stock solution (Sigma) diluted 1:1000 will be added to each sample to allow detection of particles less than and greater than 1µm. For enumeration 10µm AccuCount beads (from Spherotech, Glasgow UK) will be added to each sample after labelling. Samples will be diluted to 1 ml with filtered buffer solution and analysed immediately by flow cytometry as described previously (3, 4). Changes in the number of microparticles will be recorded relative to total gated microparticles. The number of microparticles per microlitre carrying cell surface molecules will be counted on at least 10,000 microparticle events

References

1. Macey MG, Enniks N, Bevan S. Flow cytometric analysis of microparticle phenotype and their role in thrombin generation. Cytometry: Part B Clin Cytom. 2010;

2. Macey MG, Wolf SI, Lawson C. Microparticle formation after exposure of blood to activated endothelium under flow. Cytometry A. 2010;77:761-8.

3. Macey M, McCarthy D, Azam U, Milne T, Golledge P, Newland A. Ethylenediaminetetraacetic acid plus citrate-theophylline-adenosine-dipyridamole (edta-ctad): A novel anticoagulant for the flow cytometric assessment of platelet and neutrophil activation ex vivo in whole blood. Cytometry B Clin Cytom. 2003;51:30-40.

4. McCarthy DA, Macey MG, Allen PD. A simple, novel, procedure for monitoring membrane scrambling and permeability in microparticles, platelets, and leukocytes in whole blood samples. Exp Hematol. 2008;36:909-21.

Study 1

To determine stability of eryptotic cells in blood samples at room temperature.

We shall use "fresh" surplus samples from patients that have consented. These samples shall be divided into aliquots that will be left at room temperature for varying duration. This will simulate the different times it might take for blood samples to reach the laboratory from different dialysis sites. We shall determine the variability in the concentration of eryptotic cells measured in the aliquots

Study 2

Depending on how stable eryptotic cells are in the blood samples, we shall measure the concentration of eryptotic cells in patients with renal failure. If eryptotic cells are stable, then we can use blood samples from patients that have consented even if they are collected at "satellite" haemodialysis units. Otherwise the study would be restricted to dialysis units that can arrange rapid transport of the surplus blood samples to the laboratory.

We shall study the concentration of eryptotic cells in patients with chronic renal failure (not on dialysis) and patients on peritoneal dialysis and haemodialysis.

Secondary analysis: We shall correlate the concentration of eryptotic cells with various biochemical and comorbidity factors (including but not restricted to: age, diabetic status, dialysis modality, level of renal function, serum CRP, serum albumin).

Study numbers

We have approximately 800 HD patients, 200 PD patients and 200 patients with severe chronic renal failure. We shall aim to use “left over” blood from 100 HD and 20 PD patients who have signed consent permitting “left over” blood to be used for research.

We shall also compare blood from the study groups against 20 normal controls (healthy volunteers within the hospital).

Statistical analysis

Unfortunately, because eryptosis has not been studied in this patient group, we are unable to perform a power calculation to determine the numbers we hope to recruit will be sufficient for either the primary or secondary analysis. In effect, this study is a pilot to help determine the size of future studies.

Inclusion criteria

All patients with renal failure who have given consent for their blood to be stored and used for research. We are particularly interested in patients who are EPO unresponsive and will target these patients until we have acquired 20 patients. Patients must also have given permission for their health records to be used by authorised members of staff who are not directly involved in their care for research approved by research ethics committee.

Healthy volunteers will be consented if they are unknown to have any serious illnesses (in the opinion of the investigators). Their samples will be coded and include details of age and gender. No other identifiable details will be kept.

Exclusion criteria

Nil. This is a non-interventional, observational study using surplus blood

How will potential participants, records or samples be identified? Who will carry this out and what resources will be used?

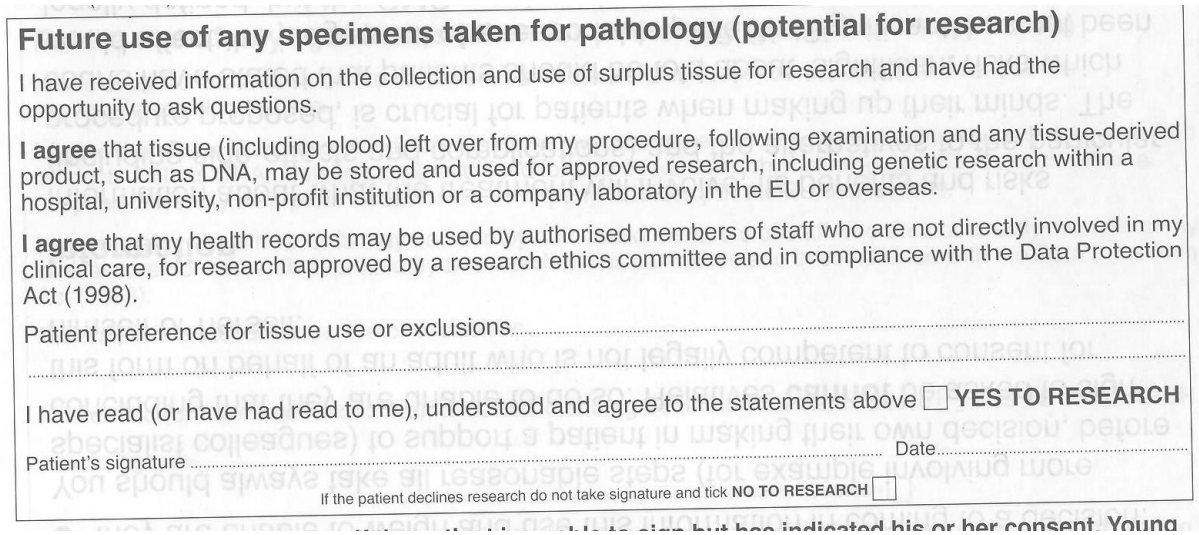
Patients with Renal Disease: The renal database has a record of patients that have consented for their blood to be stored and used for approved research. When these patients have routine blood tests, the surplus blood is automatically flagged for storage. We shall use this surplus blood. The “clinical” sample result will allow us to identify suitable patients (eg EPO unresponsive or responsive patients).

Healthy volunteers: This will be recruited by word of mouth and using a poster asking for a 3mL blood sample (see poster). Information Sheet and Consent for the healthy volunteers are attached.

Study Size

We already have consent from almost 100 PD patients and we have 800 HD patients. We are in the process of obtaining consent (as part of standard clinical practice). We would hope to collect 100 surplus samples during the study period. We aim to collect 20 healthy controls.

Consent for future use of any specimens taken for pathology (potential research)



Future use of any specimens taken for pathology (potential for research)

I have received information on the collection and use of surplus tissue for research and have had the opportunity to ask questions.

I agree that tissue (including blood) left over from my procedure, following examination and any tissue-derived product, such as DNA, may be stored and used for approved research, including genetic research within a hospital, university, non-profit institution or a company laboratory in the EU or overseas.

I agree that my health records may be used by authorised members of staff who are not directly involved in my clinical care, for research approved by a research ethics committee and in compliance with the Data Protection Act (1998).

Patient preference for tissue use or exclusions.....

.....

I have read (or have had read to me), understood and agree to the statements above ☐ **YES TO RESEARCH**

Patient's signature ..... Date.....

If the patient declines research do not take signature and tick **NO TO RESEARCH** ☐

Healthy Volunteers Wanted

Research into “Eryptosis in Patients with Renal failure”

Prof Marion Macey, Prof Magdi Yaqoob and Dr Stanley Fan

We wish to study the phenomenon of eryptosis (equivalent of apoptosis in red cells) in patients with renal failure. We wish to compare the amount of eryptotic cells in patients with renal failure with normal healthy volunteers.

We require 3mLs of blood from 20 healthy volunteers.

If you do not take any regular medication and do not have any serious medical illnesses, would you volunteer?

We only want a single blood test. The sample will be anonymised immediately and your result will not be identifiable in any way.

If you are agreeable, please contact:

Prof Marion Macey:
Professor of Haematology, BLT
marion.macey@bartsandthelondon.nhs.uk
Tel: 0203 246 0228

Dr Stanley Fan:
Consultant Nephrologist, BLT
fan.stanley@bartsandthelondon.nhs.uk
Tel: 020 7377 7480 or Extn 2861

Healthy Volunteer Information

Eryptosis in Patients with Renal Failure

Investigators: Prof Marion Macey, Prof Magdi Yaqoob and Dr Stanley Fan, Barts and The London NHS Trust

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of the study?

Patients with renal failure have anaemia. Historically this is thought to be due to the lack of a hormone called Erythropoeitin (EPO) that is made by the kidneys.

However, the dose of EPO that is required to maintain patients' haemoglobin levels is much higher than expected. So there may be other reasons for patients having anaemia.

Cells are known to undergo a process of programmed cell deaths. This is commonly known as apoptosis. Because red blood cells do not have nuclei, this process is called eryptosis. It has only been described very recently and we do not know if the rate of eryptosis is higher in renal failure patients.

Research Study Question

We would like to compare the concentration of eryptotic cells in patients with renal failure with normal healthy controls.

Why have I been chosen?

We have invited you because we believe you do not have any serious illnesses and are not taking any medications. If you are, please let us know.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the relationship you may have with anyone involved with this research project.

What will happen to me if I take part?

If you agree to take part, we will ask you to sign a consent form. We shall then take a 3mL blood sample under sterile technique in a place and time of your convenience.

Expenses and payments

We do not think this study will cost you any money so we are not making any payments.

What do I have to do?

If you agree to this study, we shall ask that you confirm that:

You are not pregnant (if applicable)

You do not have any serious illnesses (if in doubt tell us of any illnesses)

You are not taking any drugs

Your details are not recorded anywhere. We shall only label your blood sample with a number, your age and gender. We shall not be able to match your blood or the results with you.

What is the treatment that is being tested?

None. We want to know if the concentration of eryptotic cells are higher in patients with renal failure.

What are the other possible disadvantages and risks of taking part?

We think the risks of having 3mL blood sample taken are negligible. There may be temporary pain involved with the phlebotomy.

What are the possible benefits of taking part?

The study will not help you but the information we get might help improve the treatment of people with renal failure.

What happens when the research study stops?

At the end of the study, your blood sample will be discarded.

What if there is a problem? Who can I contact?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

If you have any complaints or have any questions, please contact:

Dr Stanley Fan or Prof Marion Macey.

Tel: 020 7377 7480 (Direct Line to Dr Fan's Secretary)

Tel: 020 7377 7000 Extn: 14-60228 (Prof Macey)

Alternatively, you can contact:

Patient Advice and Liaison Service (PALS)

Telephone: 020 7943 1335, Minicom: 020 7943 1350

E-mail: pals@bartsandthelondon.nhs.uk

Will my taking part in the study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. If you consent to take part in the research the people conducting the study will abide by the Data Protection Act 1988, and the rights you have under this Act.

All the information about your participation in this study will be kept confidential. The details are included in Part 2.

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

What if relevant new information becomes available?

Not applicable as your participation lasts only for the duration of the blood test.

What will happen if I don't want to carry on with the study?

Not applicable as your participation lasts only for the duration of the blood test.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

Harm

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against Barts and The London, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate). NHS Indemnity does not offer no-fault compensation i.e. for non-negligent harm and NHS bodies are unable to agree in advance to pay compensation for non-negligent harm. They are able to consider an ex-gratia payment in the case of a claim.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. If you consent to take part in the research the people conducting the study will abide by the Data Protection Act 1988, and the rights you have under this Act.

Your blood sample will only be labelled with your age, gender and a study number. Details about you will not be kept (only the consent form will be kept).

Involvement of the General Practitioner/Family doctor (GP)

We will not inform your GP.

What will happen to the results of the research study?

We hope the results of our study will be important to all doctors looking after patients with renal failure. We will therefore publish the results and present the data at various meetings. However, at all times, your confidentiality will be protected. You will NOT be identified in any report/publication unless we ask you for specific permission.

Who is organising and funding the research?

Barts and The London is organising and running this study.

Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by the East London and The City Research Ethics Committee. The detailed study has also been carefully considered by an independent internal research committee of the Renal Unit at BLT.

You may wish to thank your participant for considering taking part or taking time to read this sheet.

Control consent form

Centre Number: Patient ID:

Title of Project: Eryptosis in Patients with Renal Failure

Name of Researcher: Prof M Macey

Please initial boxes

1. I confirm that I have read and understand the information sheet dated 1 April 2011 (version 1) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I agree to take part in the above study. ☐

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