

Predictors of response to iron and erythropoietin stimulating  
agents in haemodialysis patients

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## Abstract

Renal anaemia affects a large proportion of haemodialysis patients. Treatment for this is with erythropoietin stimulating agents (ESA) and intravenous iron, response to which is unpredictable. These medications are titrated according to haemoglobin and ferritin levels which are measured monthly in these individuals. This study aims to evaluate the role of alternate biomarkers in predicting a treatment response.

### Method

This study randomised individuals with a haemoglobin (Hb) between 90 and 104g/l and ferritin between 100 and 800ng/l to either an incremental increase in ESA dose or a course of intravenous iron sucrose, with bloods for biomarkers taken prior to the receipt of this medication. A positive treatment response was defined as an increase in haemoglobin of 5g/l within two months. Non-responders received the alternate treatment with response re-assessed.

### Results

Over 2423 patient months amongst 196 individuals, there were 160 completed randomisation episodes. Response to ESA was seen in 61/84 (72.6%) and to iron in 53/76 (69.7%). C-reactive protein (CRP) was the strongest predictor of a positive treatment response (13.46mg/l versus 28.59mg/l in non-responders,  $p=0.038$ ) with a lesser impact of a lower baseline ESA dose (2529.5 versus 2913.0 units/session,  $p=0.243$ ). Successful iron treatment was associated with lower reticulocyte haemoglobin (33.8 vs 35.5pg,  $p=0.047$ ), lower hepcidin (91.4 vs 131.0ug/ml,  $p=0.021$ ) and higher C-reactive protein (29.5 vs 12.6mg/l,  $p=0.085$ ). Interaction modelling indicated that CRP ( $p=0.08$ ) and ESA dose ( $p=0.058$ ) were the strongest differentiators of response to the two different treatments.

### Discussion

Increased ESA and iron are equally effective, though treatment failure occurs in almost 30%. Baseline variables can predict treatment response, and a four-variable score shows promise in allowing directed treatment with improved response rates.

## Statement of work

The original concept for the study was provided by Dr Damien Ashby. The author developed the protocol, and obtained all the necessary regulatory approval, under the guidance of Dr Damien Ashby. The running of the study was conducted in its entirety by the author. The author performed a limited number of the hepcidin ELISA assays, under the guidance of Dr Mark Busbridge, who conducted the rest.

I declare that this thesis has been solely composed by myself and it has not been submitted previously, in whole or in part, for any previous application for a degree.

## Acknowledgements

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## Abbreviations

|                  |                                                                                 |
|------------------|---------------------------------------------------------------------------------|
| AE               | Adverse event                                                                   |
| ANOVA            | Analysis of variance                                                            |
| ATP              | Adenosine triphosphate                                                          |
| BFU-E            | Blast forming unit-erythroid                                                    |
| BMP6             | Bone morphogenetic protein 6                                                    |
| CFU-E            | Colony forming unit-erythroid                                                   |
| CHOIR            | Correction of Anemia with Epoetin Alfa in Chronic Kidney Disease trial          |
| CHr              | Reticulocyte haemoglobin content                                                |
| CREATE           | Cardiovascular Risk Reduction by Early Anemia Treatment with Epoetin Beta trial |
| CRP              | C-reactive protein                                                              |
| Dcytb            | Duodenal cytochrome b                                                           |
| DMT1             | Divalent metal transporter 1                                                    |
| DNA              | Deoxyribonucleic acid                                                           |
| DRIVE            | Dialysis patients' response to intravenous iron with elevated ferritin          |
| ELISA            | Enzyme-linked immunosorbent assay                                               |
| EpoR             | Erythropoietin receptor                                                         |
| ESA              | Erythropoietin stimulating agent                                                |
| FDA              | Food and Drug Administration                                                    |
| Fe <sup>2+</sup> | Ferrous                                                                         |

|                  |                                            |
|------------------|--------------------------------------------|
| Fe <sup>3+</sup> | Ferric                                     |
| FPN              | Ferroportin                                |
| GDF              | Growth differentiation factor              |
| H                | Heavy                                      |
| Hb               | Haemoglobin                                |
| HCP1             | Haem carrier protein 1                     |
| HD               | Haemodialysis                              |
| HIF              | Hypoxia inducible factor                   |
| HIV              | Human immunodeficiency virus               |
| HLA              | Human leucocyte antigen                    |
| HRE              | Hypoxia response element                   |
| holoTC           | Holotranscobolamin                         |
| HRC              | Hypochromic red cell percentage            |
| IHD              | Ischaemic heart disease                    |
| IL               | Interleukin                                |
| IRE              | Iron response element                      |
| IRP              | Iron regulatory protein                    |
| JRCO             | Joint Research Compliance Office           |
| KDIGO            | Kidney Disease: Improving Global Outcomes  |
| KDOQI            | Kidney Disease Outcomes Quality Initiative |

|         |                                                                    |
|---------|--------------------------------------------------------------------|
| kg      | Kilogram                                                           |
| l       | litre                                                              |
| L       | Light                                                              |
| LPS     | Lipopolysaccharide                                                 |
| MCV     | Mean corpuscular volume                                            |
| mg      | Milligram                                                          |
| MHRA    | Medicines and Healthcare products Regulatory Authority             |
| MMA     | Methylmethalonic acid                                              |
| NEJM    | New England Journal of Medicine                                    |
| ng      | Nanogram                                                           |
| NICE    | National Institute of Clinical Excellence                          |
| OR      | Odds ratio                                                         |
| pg      | picogram                                                           |
| PIVOTAL | Proactive intravenous iron therapy in haemodialysis patients trial |
| pmol    | picomolar                                                          |
| PRCA    | Pure red cell aplasia                                              |
| PTH     | Parathyroid hormone                                                |
| PVD     | Peripheral vascular disease                                        |
| RA      | Renal Association                                                  |
| RR      | Relative risk                                                      |

|        |                                                            |
|--------|------------------------------------------------------------|
| Ret-He | Reticulocyte haemoglobin equivalent                        |
| SAE    | Serious adverse event                                      |
| STAT5  | Signal transducer and activator of transcription 5         |
| Tf     | Transferrin                                                |
| TfR-1  | Transferrin receptor-1                                     |
| TfR-2  | Transferrin receptor-2                                     |
| TREAT  | Trial to Reduce Cardiovascular Events with Aranesp Therapy |
| TSat   | Transferrin saturation                                     |
| UK     | United Kingdom                                             |
| USA    | United States of America                                   |
| USF    | Upstream transcription factor                              |
| µg     | microgram                                                  |
| µmol   | Micromolar                                                 |

# 1 Introduction

Research into iron regulation within the renal system and its interplay with anaemia has developed along parallel tracks; which this literature review will explore and draw together.

One path for research has focused specifically on the pathophysiology of anaemia in patients with renal disease. In this context, the improved understanding that has developed over the course of time has highlighted the important function of erythropoietin. This has resulted in a revolution in treatment from a situation where blood transfusions were liberally used prior to the 1980s – with its attendant complications - to one in which erythropoietin stimulating agents (ESA) and iron treatment used in conjunction, have dramatically improved mortality rates. Sections 1.1-1.4 will explore these developments.

However, it will be contended in section 1.5 that the limitations of this research taken in isolation – focusing on erythropoietin as the pathophysiological basis of renal anaemia - has meant the effectiveness of the current treatments have not progressed to the point where they provide predictable outcomes for patients, or allow effective guidelines for their use. The empirical studies cited demonstrate that these treatments are not universally effective. Consequently, progress is required to either devise innovative treatments or provide better indicators for the optimal use of the current ones.

The other thread of research discussed in section 1.6 has focused on iron physiology and metabolism more broadly. A greater understanding of the normal systemic control of iron has led to the development of biomarkers (such as ferritin, transferrin and, more recently, hepcidin) to assess iron status. The application of this research to renal anaemia is evident as hepcidin levels have been found to be elevated in those with renal disease. Even though this has led to a revised theoretical model of renal anaemia (section 1.7), it is less clear how this can be used in clinical practice (section 1.9).

This study will bring this into sharper focus by exploring how well these biomarkers predict a successful response to ESA and iron treatment.

### *Background*

Anaemia is the pathophysiological condition in which there is a reduction in the number of red blood cells transporting oxygen around the body. Red blood cells contain haemoglobin which enable it to bind oxygen. The haemoglobin can be measured to assess anaemic status. The World Health Organisation defines anaemia as a haemoglobin of less than 120g/l in women and 130g/l in men. <sup>1</sup>

The United Kingdom (UK) Renal Registry, which collates information annually about those in receipt of received renal replacement therapy, highlights the prevalence of anaemia within this population. Of those established on haemodialysis for greater than 3 months, there is a median haemoglobin of 111g/l and only 81% have a haemoglobin greater than 100g/l.<sup>2</sup>

## 1.1 A brief history of renal anaemia

As far back as in 1836, the association between renal disease and anaemia was first reported in the Guy's Hospital Reports. Richard Bright described the "healthy colour of the countenance fades" with renal disease.<sup>3</sup> Three years later, Robert Christison, in a talk about renal disease described "no natural disease...which so closely approaches haemorrhage in its power of impoverishing red particles of the blood".<sup>4</sup>

### 1.1.1 The role of a circulating factor

It was not until the following century that some progress was made in understanding the link between anaemia and renal disease. In 1906, Carnot and Deflandre injected the serum from anaemic rabbits into non-anaemic rabbits. This induced polycythaemia in the recipients. The researchers proposed that a circulating factor, present within serum, is upregulated in response to anaemia. Following the injection of serum from the anaemic rabbit, there is a transfer of this upregulated circulating factor encouraging a supraphysiological rate of generation of new blood cells. This circulating factor was

initially called 'hemopoietine' as it was believed to upregulate all blood cell lines.<sup>5</sup> Once it was realised, in 1948, that it only upregulated the red blood cell line, it was renamed erythropoietin.<sup>6</sup>

## 1.2 Erythropoietin

### 1.2.1 The isolation of erythropoietin

Isolation of erythropoietin was difficult due to the very low levels present in serum. Human erythropoietin was initially extracted from urine of patients with aplastic anaemia, as these patients would have elevated erythropoietin levels. By the early methods of purification of erythropoietin, it was calculated that 3,250 litres of urine would be required to extract 10 milligrams (mg) of erythropoietin.<sup>7</sup> In 1977, Miyake et al first reported the isolation of purified human erythropoietin from 2,550 litres of urine from patients with aplastic anaemia following a seven stage purification process.<sup>8</sup>

The purified form of erythropoietin derived from urine allowed work to progress on development of an accurate antibody assay to measure erythropoietin.<sup>9</sup> Recombinant biotechnology, which allowed mass production of erythropoietin from a small sample, enabled the distribution of erythropoietin across multiple research laboratories. By 1990, Mason-Garcia et al reported on the successful production of an accurate radioimmunoassay to measure serum erythropoietin. This progress accelerated the furthering of the understanding and role of erythropoietin.<sup>10 11</sup>

### 1.2.2 Site of production

The role of the kidney as the primary site of erythropoietin was first discovered in 1957. Following selective organ removal in rats, researchers then either phlebotomized the animals or injected intravenous cobalt to stimulate erythropoiesis. There was an absence of the expected accelerated erythropoiesis following nephrectomy which remained present following the removal of other organs.<sup>12</sup> The site of production was confirmed following an experiment that isolated kidneys of rabbits and maintained these in serum-free perfusion. In those kidneys that had been exposed to hypoxic conditions, which would cause upregulation of erythropoietin, measurable amounts of the erythropoietin hormone were detected in the perfusate.<sup>13</sup>

In 1972, following the use of fluorescent antibody techniques, it was proposed that the glomerulus was the site of synthesis of erythropoietin.<sup>14</sup> By 1988, the site of erythropoietin production was revised. Labelled messenger ribonucleic acid (mRNA) was exposed to the mRNA of erythropoietin which localised the site of erythropoietin production to the peritubular interstitial fibroblasts.<sup>15</sup> Antenatally, the liver is the primary site of erythropoietin production and switches to the kidney in the postnatal period.<sup>16</sup>

### 1.2.3 Function and mode of action of erythropoietin

Erythropoiesis is the process by which the haematopoietic pluripotent stem cells, located within the bone marrow, differentiate down the erythroid cell line. Once they have become mature red cells, known as erythrocytes, they are expelled into the systemic circulation. Here, the haemoglobin contained within is able to bind and then transport oxygen.

Erythropoietin interacts with the erythropoietin receptor (EpoR) which is located on the developing red cell. This interaction is an important regulator of the rate of erythropoiesis.<sup>17</sup> Figure 1 shows the pathway of red cell generation from the pluripotent stem cell to the erythrocyte with the role, with the influence of erythropoietin on this process highlighted.

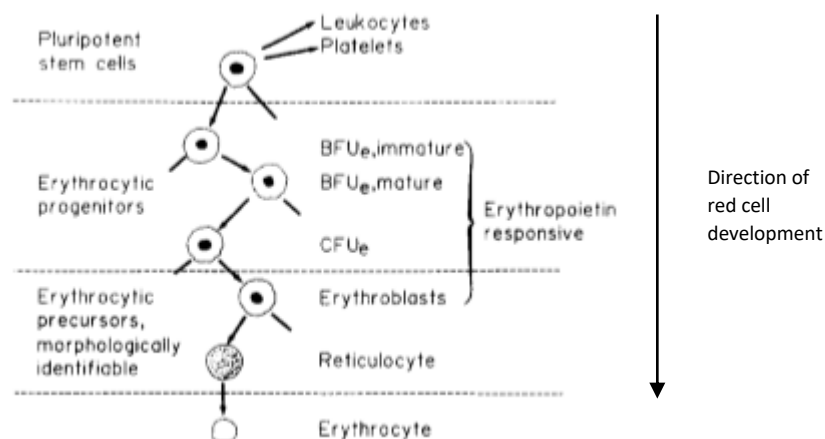


Image taken from Jelkmann, 1986.<sup>9</sup>

*Figure 1. Red cell development. This figure demonstrates the differentiation of the pluripotent stem cell into the developed circulating erythrocyte. The erythrocyte progenitors, the burst forming unit-erythroid (BFU-E) and colony forming units-erythroid (CFU-E) are erythropoietin responsive as they*

*have erythropoietin receptors located on their surface. Under the influence of erythropoietin, these cells mature. They become erythroblasts then reticulocytes in the bone marrow. The nucleus is then extruded and they are released into the circulation as erythrocytes.*

Erythropoietin receptors are found in low concentrations on the mature blast forming blast form unit-erythroid (BFU-E). Interaction between these and erythropoietin promotes the onward differentiation of the developing red cell. In contrast, the EpoR is highly prevalent on the colony forming unit-erythroid (CFU-E). Activation of this receptor is essential to prevent apoptosis of the developing red cell.<sup>18 19</sup>

In order for the EpoR to be activated, circulating erythropoietin has to bind to two adjacent receptors. This causes dimerization of the EpoR which activates an intracellular tyrosine kinase cascade. The activated tyrosines serve as docking stations for a variety of intracellular proteins, including Signal Transducer and Activator of Transcription 5 (STAT5). STAT5 activates the promotor of the erythroid transcriptor gene within the nucleus which allows onward cellular development.<sup>20</sup>

#### 1.2.4 Non-haematological effects of erythropoietin

Outside the haematopoietic system, EpoRs have been identified throughout the body thus indicating a wider role for erythropoietin. In endothelial cells, erythropoietin-EpoR interaction promotes angiogenesis. This likely underlies its neuroprotective role in the central nervous system and its preventative role in ischaemic-reperfusion injury in the heart. Within the urogynaecological system, erythropoietin has a local regulatory role in the menstrual cycle. The role of erythropoietin within the gastrointestinal system remains uncertain despite EpoR being identified there. Erythropoietin may have a pathological role in malignancy by promoting cellular proliferation.<sup>21</sup>

#### 1.2.5 Regulation of erythropoietin

Hypoxia is the primary regulator of the rate of erythropoietin production. Upregulation of erythropoietin occurs in response to altitude and cyanotic heart disease; resulting in polycythaemia in affected individuals. Experimentally, this has been demonstrated in animals where exposure to environments with low partial pressures of oxygen results in upregulation of erythropoietin.<sup>22-25</sup>

In the setting of hypoxia, there is a reduction in oxygen delivery to the peritubular cells. This causes an upregulation of hypoxia-inducible transcription factors (HIF). These are able to bind hypoxia response elements (HRE) present on the promotor region of the erythropoietin gene. This transcription of the gene is then upregulated with a resultant increase in the rate of erythropoietin synthesis.<sup>16</sup> Figure 2 provides a diagrammatic illustration of the feedback mechanism responsible for erythropoietin regulation.

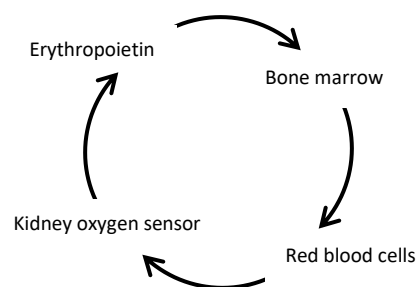


Figure adapted from Erslev et al, 1980.<sup>26</sup>

*Figure 2. The feedback mechanism of erythropoietin regulation. Oxygenated red blood cells are sensed by the kidney. If there are insufficient amounts detected, this results in an increase in erythropoietin production. This increase in erythropoietin is detected by the developing red cells promoting onwards differentiation, increasing the number of red cells. Increased red cell numbers increase cellular oxygen delivery within the kidney and thereby switch off the upregulated HIF. This feedback mechanism maintains erythropoietin and red blood cell concentration within a physiological range.*

### 1.2.6 Summary

The isolation of erythropoietin was fundamental in facilitation of further experiments on both the function and role of this circulating hormone. The oxygen-sensing capacity of the peritubular cells of the kidney are able to regulate levels of erythropoietin production, and identification of this physiological role of erythropoietin underlies the more recent understanding of how this tightly regulated system of erythropoietin production is affected by renal disease.

### 1.3 The first model of renal anaemia

#### 1.3.1 Central role of erythropoietin

In anaemic patients with normal renal function, erythropoietin levels are increased proportionally to the degree of anaemia.<sup>27</sup> In contrast, in those with renal impairment, as shown in Figure 3, there is no apparent relationship between degree of renal impairment and erythropoietin levels. This is despite the progression in anaemia as renal function declines, as shown in Figure 4. Figure 5 and Figure 6 emphasise this lack of correlation between erythropoietin and both haemoglobin and renal function.

28-31

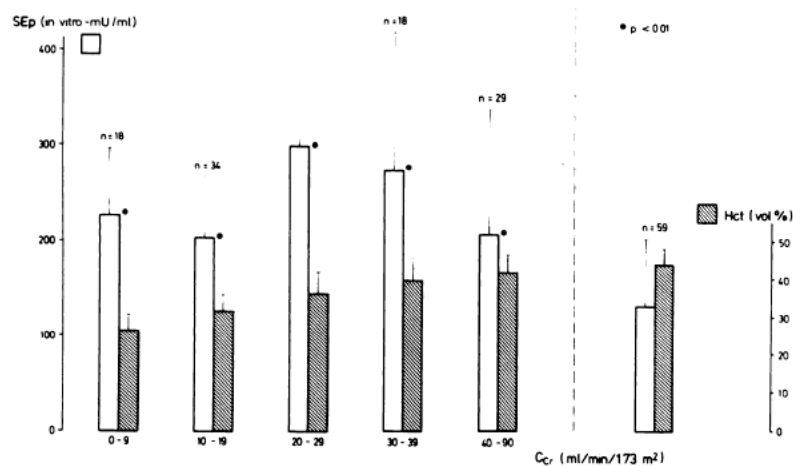


Figure from Radtke et al, 1979.<sup>32</sup>

Figure 3. Changing serum erythropoietin levels with decline in renal function and falling haematocrit. Serum erythropoietin levels, demonstrated by the white bars, are elevated in those with renal impairment, as shown on the left hand side of the graph, in comparison to the normal population, shown on the right of the diagram. The haematocrit, shown in the grey bar, decreases as renal function decline, going left along the x-axis. There however is no correlation between haematocrit and serum erythropoietin levels with peak erythropoietin levels seen with creatinine clearance between 20-29ml/min. Of note, nowadays, anaemia is normally defined according to haemoglobin although haematocrit measurement has been used widely in the past and the principles are transferable between the two different measurements.

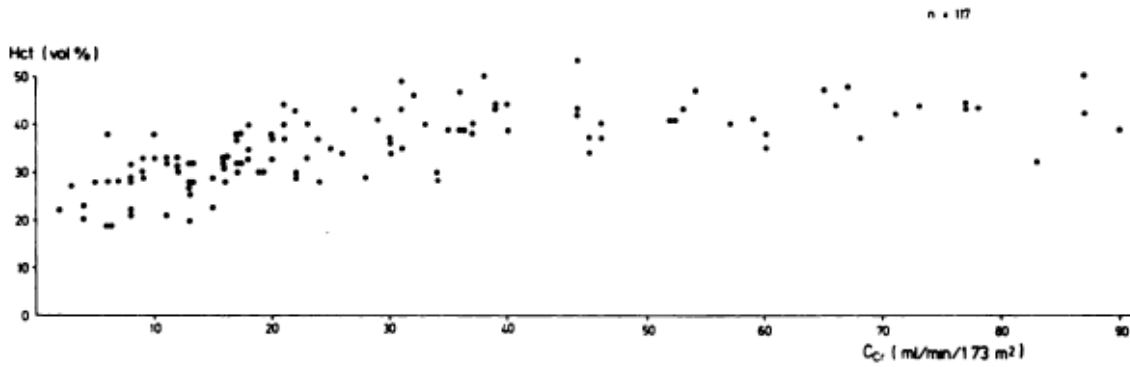


Figure from Radtke et al, 1979.<sup>32</sup>

Figure 4. Correlation between haematocrit (y-axis) and creatinine clearance (x-axis). As renal function declines, with reducing creatinine clearance from right to left on the x-axis, there is a corresponding decline in haematocrit. With creatinine clearance falling to less than 40ml/min, there is a significant correlation with the declining haematocrit ( $r=0.69$ ,  $p<0.01$ ).

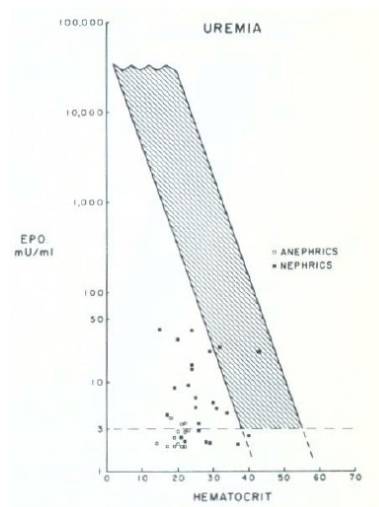


Figure from Erslev et al, 1980.<sup>33</sup>

Figure 5. Correlation between erythropoietin levels (y axis) and haematocrit (x axis) in those with renal dysfunction. In patients with known advanced renal disease, there is no correlation seen between haematocrit and erythropoietin levels both in patients with or without their kidneys in situ as demonstrated by the single points. The hatched area, which is superimposed from another graph, approximates the correlation between haematocrit and erythropoietin levels seen in a healthy

cohort with normal renal function and hence highlights the differences between the healthy group and those with renal disease.

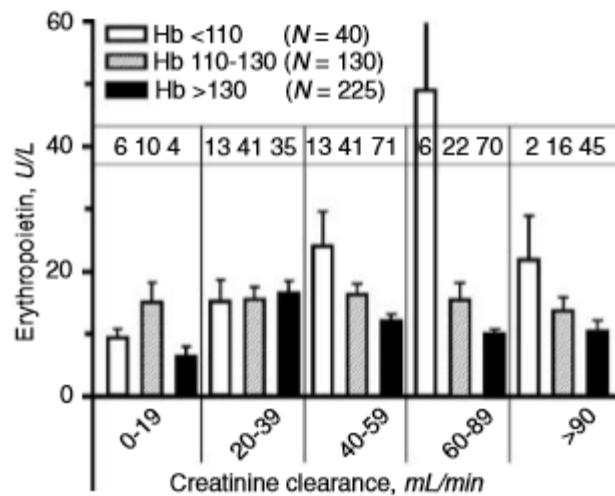


Figure from Fehr et al, 2004. <sup>31</sup>

Figure 6. Erythropoietin levels and creatinine clearance stratified according to haemoglobin. There is no correlation between erythropoietin levels and haemoglobin in those with advanced renal disease. Once the creatinine clearance is above 40ml/min, those with the lower haemoglobin have the highest erythropoietin levels.

In summary, patients with renal impairment have an abnormal erythropoietin response to anaemia. This relative erythropoietin deficiency was long thought to be the primary mechanism of anaemia in renal disease.

### 1.3.2 Bone marrow insensitivity to erythropoietin

One hypothesis of renal anaemia is that there is an insensitivity of the developing red cell to the effects of erythropoietin. Experimentally this has been demonstrated through assessment of the rate of erythropoiesis in bone marrow of dogs exposed to serum from healthy volunteers, renal impairment and those with other chronic diseases. The rate of incorporation of labelled iron into bone marrow was significantly lower in those who were exposed to serum from those with renal impairment compared to both healthy volunteers and those with chronic disease. <sup>34</sup>

The mechanism for the anaemia was thought to be secondary to a circulating factor which reduced the number of CFU-E in patients with renal impairment, the step of erythropoiesis modulated by erythropoietin.<sup>35 36</sup> The hypothesis of a circulating factor is supported by the reduction of renal impairment on CFU-E formation following dialysis.<sup>37</sup>

### 1.3.3 Anaemia in haemodialysis

The anaemia in haemodialysis patients is the result of a combination of those factors common to renal anaemia, described in the section above and additional iatrogenic factors that occur as the direct result of haemodialysis.

#### *Blood loss*

When haemodialysis was first becoming more commonplace, estimates of annual blood loss through the dialysis circuit was up to 7.9 litres.<sup>38</sup> Blood loss was multifactorial. Blood was left in the haemodialysis circuit at the end of each session. This was due to both blood clotting in the dialyser, meaning it was unable to be returned; and due to dialysing methods, that did not emphasise the importance of returning blood to the patient. Blood was further lost by diagnostic blood sampling and through dialysis access.<sup>39</sup>

Over time, as haemodialysis techniques developed, efforts were made to reduce the blood loss of haemodialysis. More efficient washback techniques were adopted to maximise blood return at the end of haemodialysis sessions.<sup>40</sup> Kiil plate dialyser and Kolff double coil dialyser were replaced with modern hollow fibre dialysers. Current annual blood loss in haemodialysis is now estimated to be only 1.33 litres of whole blood, compared to the historical estimates of 5-7 litres.<sup>41</sup> This equates to annual iron loss of approximately two grams.<sup>42</sup>

Non-dialytic blood loss also affects haemodialysis patients more than the general population. Occult blood loss through the gastrointestinal tract is increased in patients with renal impairment, likely compounded by the use of anticoagulants in this group.<sup>43</sup> Furthermore, the impairment of oral iron absorption seen in haemodialysis patients adversely affects their ability to replenish this iron loss.<sup>44</sup>

### *Other factors*

The reduction of red cell lifespan associated with end stage renal failure which may be up to 70%.<sup>45</sup>

Two factors contribute to this. There is a disruption of the red cell membrane as a result of the uraemic conditions.<sup>46</sup> Mechanical haemolysis may occur due to the passage of the red cells through the extracorporeal circuit.<sup>47</sup>

## 1.4 The management of anaemia in haemodialysis patients prior to the 1980s

### 1.4.1 An overview

Anaemia management in haemodialysis patients has progressed significantly over the past 50 years. When haemodialysis first started, blood transfusion was the only way to maintain haemoglobin levels. This, however, was fraught with complications and alternatives were required. Concerted efforts were made to improve dialysis techniques and lessen blood loss.

Following the introduction of erythropoietin stimulating agents (ESA) in the 1980s, anaemia management was revolutionised. Iron treatment was introduced to work in conjunction with the ESA to enhance anaemia management. The iron and ESA combination, however, is not universally effective in the way that it is currently administered. Alongside the ongoing work on novel agents for the treatment of renal anaemia, there is an immediate need to improve the effectiveness of ESA and iron prescribing.

### 1.4.2 Blood transfusion

Blood transfusions were used liberally in haemodialysis patients up to the mid-1960s. The threshold for transfusion was variable and the local unit used the cut of a haematocrit less than 25%. This resulted in a transfusion rate of 4.1 units per patient month. With the realisation that repeated transfusions led to complications, improvement in dialysis techniques were made to try to reduce blood loss and therefore transfusion dependence. These were partially effective and did manage to reduce the transfusion rate to 2.6 units per patient month.<sup>48</sup> However this still exposed patients to complications of blood transfusions such as those discussed below.

#### *Complications of blood transfusion*

As the number of patients receiving maintenance haemodialysis increased, complications of blood transfusion became more apparent. These are detailed below.

### *Iron overload*

A single unit blood transfusion contains between 200 and 250 milligrams of iron.<sup>49</sup> Even with the reduction in transfusion rate following improvement of dialysis technique, patients were still receiving in excess of 0.5 grams of iron a month. With no natural excretion mechanism to regulate iron levels, this resulted in an accumulation of iron in these patients receiving the transfusions. Post mortem analysis of these individuals demonstrated iron overload, primarily in the liver and spleen.<sup>50</sup> Iron overload has also been shown to result in impaired glucose tolerance, left ventricular dysfunction and hypogonadism.<sup>51</sup>

### *Infection*

Historically, blood transfusions have had high rates of infective, primarily viral, complications. Hepatitis B was the first virus known to cause a prevalent problem in haemodialysis patients. This affected both those initially exposed to the virus at time of a blood transfusion and through subsequent cross contamination between patients and staff. Outbreaks were not infrequent and took a long time to come under control.<sup>52</sup> By the 1970s, a different blood borne hepatitis virus, initially known as non-hepatitis A non-hepatitis B but latterly hepatitis C, also began to affect the haemodialysis populations.<sup>53-55</sup> In the early 1980s, blood transfusions were contaminated by the human immunodeficiency virus (HIV), for which at the time was no treatment.<sup>56</sup> Rarely, bacterial contamination of transfusions led to septic complications in the recipient.<sup>57</sup> Modern day screening and handling of blood products have led to infective complications becoming less common.

### *Transfusion reactions and development of HLA antibodies*

Blood transfusion reactions used to be commonplace. These reactions have included acute lung injury, acute haemolytic reactions secondary to ABO-incompatibility, febrile non-haemolytic reactions as well as allergic reactions.<sup>58 59</sup>

Preparation of blood for transfusion has changed. Leucocyte depletion did not use to be standard practice which led to recipients developing what later became known as human leucocyte antigens

(HLA).<sup>60</sup> These antibodies were present in up to 50% of maintenance haemodialysis patients at the end of the 1970s.<sup>61</sup> For those awaiting future kidney transplantation, the development of HLA can both preclude transplantation or have an adverse effect on transplant kidney graft survival.<sup>62</sup>

#### *Change in blood transfusion practice*

Although blood transfusion rate had been reduced by improvement in haemodialysis technique, patients remained susceptible to the complications of blood transfusions. The threshold for transfusion changed from a haematocrit of less than 25% changed to whether a patient was symptomatic from anaemia. Blood transfusion rate dropped to 0.34 units per patient month with a concurrent reduction in the mean packed cell volume in the patients from 24.5 to 19.9 in the local haemodialysis unit<sup>48</sup> with similar findings reported in other haemodialysis units.<sup>63</sup>

#### 1.4.3 Erythropoietin stimulating agents (ESA)

The use of blood transfusions as the primary treatment of anaemia in haemodialysis patients began to decline following the discovery and subsequent widespread introduction of ESA into clinical practice. Erythropoietin insufficiency had long been thought of as the pathophysiological basis of renal anaemia (see section 1.3). Using therapeutics to increase erythropoietin levels became the logical approach to treat renal anaemia.

#### *Development of ESA therapy*

The development of ESA was the result of two key advances in scientific discovery: recombinant technology and the isolation of purified erythropoietin.

Recombinant technology allowed the mass production of a single protein. In 1982, Ely Lilly was the first person to use this technique to synthesise human pro-insulin, the precursor to insulin. This technique used restriction endonucleases to isolate the deoxyribonucleic acid (DNA) of interest. DNA ligases were then used to insert the isolated DNA into a plasmid. The plasmid was then re-inserted into the carrier vector *Escherichia Coli* from which it was derived, chosen for its ability to undergo rapid replication. This was placed into an amino acid rich medium to ferment, alongside promoters, ribosome binding

sites and terminator sequences which are needed in the replication process. Restriction enzymes then isolated the protein of interest which was then purified by liquid chromatography and crystallisation.<sup>64</sup>

Secondly, a purified form of human erythropoietin was first isolated from urine. Using the carrier vector Chinese hamster ovary cells, recombinant technology allowed the first mass synthesis of recombinant human erythropoietin in 1985.<sup>65 66</sup>

#### *Early trials using ESA therapy*

Winearls first reported ESA use in humans in 1986. Within a week of initiation of ESA treatment in 10 maintenance haemodialysis patients, an increase in both reticulocyte count and haemoglobin were seen. By week 12, the mean haemoglobin had increased from 61g/l prior to treatment to 103g/l. Despite the small numbers involved in the study, there were complications of the ESA treatment. Two of the participants had clotting of their fistula access and another patient developed hypertensive encephalopathy.<sup>67</sup> This was likely related to the high doses of ESA used which far exceed those of later studies.

A phase I and II study by Eschbach was reported in the New England Journal of Medicine (NEJM) the following year. Following the complications seen by Winearls in the previous study, this study used smaller, weight adjusted doses. Twenty-five patients were divided into six treatment groups that were treated with ESA for 3 weeks at differing doses. Once the dose reached 15 units of ESA per kilogram (kg) body weight, some recipients began to show an increase in reticulocyte count. A widespread, consistent increase in reticulocyte count was seen with ESA doses above 50 units per kg. The graduated response in reticulocyte count according to dose is echoed by the different mean haematocrit increases seen in the different treatment groups. Those treated at a dose of 15 units per kg had a haematocrit of 0.24 at the end of the study in contrast to a haematocrit of 0.41 in those treated with 500 units per kg. Following the initial 3-week treatment period, the dose of the ESA was titrated to maintain a haematocrit between 0.35 and 0.40 for the next seven months. This longitudinal study was used to try and identify side effects of treatment, and with the lower doses of ESA used, none were noted.<sup>68</sup>

The first multi-centre phase III study was reported in 1989. Involving 333 haemodialysis patients, researchers treated patients with an initial ESA dose of 300 units per kg, although this was later reduced to 150 units per kg as the study progressed. Following a patient achieving the target haematocrit of 0.35, ESA dose was reduced to maintain the haematocrit between 0.32 and 0.38. Three hundred and twenty-seven patients achieved the target haematocrit within 18 weeks of initiation of ESA treatment. Non-responders were individuals who were known to have myelofibrosis, chronic inflammatory conditions and blood loss. Aside from the increase in haematocrit levels, this was the first study to report on the positive effects that the medication had on patients' quality of life with improvement seen in exercise tolerance, appetite, energy levels and perception of health.<sup>69</sup>

#### *Early adverse effects of ESA*

##### *Hypertension and seizures*

Early studies reported that ESA was well tolerated with few side effects. The most prevalent reported side effect in early studies was hypertension. In this phase I and II study, Eschbach reported hypertension in 4 out of 25 patients with one resultant seizure.<sup>68</sup> His phase III study in 1989 reported worsening hypertension in 32% of patients with seizures in 5.4%.<sup>69</sup> This effect of ESA on hypertension has also been demonstrated experimentally by the use of ambulatory blood pressure monitoring. This demonstrated an increase in 24 hour ambulatory systolic blood pressure of greater than 10mmHg in 8 out of 13 participants and a loss of nocturnal diastolic blood pressure dip.<sup>70</sup> The degree of hypertension correlated with the dosage of ESA used.<sup>71</sup>

##### *Vascular access thrombosis*

Vascular access thrombosis as a complication of ESA use was also seen in early studies. A European study of 150 haemodialysis patients using ESA found 22 out of 150 (14.7%) developed a thrombotic problem with their vascular access requiring either thrombectomy or fibrinolysis over the course of a year. Sixteen out of 22 affected individuals had had a concomitant significant increase in haemoglobin.<sup>72</sup> Later, a study was conducted involving two matched ESA recipient cohorts, one with native dialysis

access and the other with vascular graft access. This demonstrated that the increased rate of access thrombosis was restricted to those with vascular access grafts rather than native access.<sup>73</sup>

#### *Pure red cell aplasia*

Some patients on ESA treatment started to develop an inexplicable fall in haemoglobin. This was due to a condition called pure red cell aplasia (PRCA), secondary to the development of anti-erythropoietin antibodies. These prevented the interaction of both endogenous erythropoietin and the ESA with the EpoR. Patients therefore developed a profound anaemia unresponsive to ESA.<sup>74</sup> PRCA was first identified in 1998 with a peak in incidence in 2002. Eprex, a subcutaneously administered ESA produced by Amgen, was initially thought to be responsible for most cases of PRCA. Later however registry data suggested that the development of PRCA was not restricted to Eprex use. Rates of PRCA were 35.8 per 100,000 patients years of Eprex use versus 14.0 per 100,000 patients years of the other ESA formulations, the difference between these rates did not reach statistical significance.<sup>75</sup>

#### *Introduction of ESA into clinical practice*

The early studies largely demonstrated both the efficacy of ESA, with the huge positive impact on quality of life, and safety. This led to both the Food and Drug Authority (FDA) in the United States of America (USA) and the Medicines and Healthcare products Regulatory Agency (MHRA) in the UK approving ESA for clinical use in 1989. Rapidly, the use of ESA became standard clinical care with 60% of in-centre haemodialysis patients in the USA receiving the medication by the following year.<sup>76</sup> Guidance as to how to prescribe ESA was initially lacking and the expense of ESA dictated the dose.<sup>72</sup>

<sup>77</sup> This led to a disparity between the dosing used in trials and that used in clinical practice<sup>78</sup> In the UK, it was not until 1997, that the Renal Association in conjunction with the Royal College of Physicians, first produced guidelines on ESA dose.<sup>79</sup>

#### 1.4.4 Iron therapy

Historically, iron levels in haemodialysis patients have varied reflecting the clinical practice of the time. Iron overload caused by blood transfusions was a significant problem for haemodialysis patients but as

transfusions became less common, iron deficiency prevalence increased, and, iron began to be used therapeutically. The balance between iron overload and deficiency has been difficult and remains a huge clinical conundrum.

#### *Iron levels prior to the introduction of ESA*

A single unit of blood transfusion contains between 200 and 250 milligrams of iron.<sup>49</sup> With haemodialysis patients receiving regular transfusions, iron overload was a common finding amongst patients.<sup>80</sup> Post mortem studies of haemodialysis patients demonstrated iron deposition, commonly affecting the liver and myocardium.<sup>50 81 82</sup>

As the threshold for transfusion (see section 1.4.2), the amount of concomitant iron received by patients declined. With the ongoing blood and therefore iron loss on haemodialysis, patients became progressively iron deficient. This resulted in the introduction of regular iron therapy to these patients in an attempt to maintain haemoglobin levels. Both oral and intravenous iron therapy were used to provide maintenance iron therapy. By 1977, 38 out of 41 surveyed haemodialysis units in the United Kingdom were giving maintenance iron therapy, 16 of which were giving exclusively parenteral iron, 14 only oral iron and the remainder a combination.<sup>82</sup>

By the early 1980s, oral iron became the mainstay of anaemia management in those not receiving blood transfusions. It was as efficacious as parenteral iron therapy with a lesser risk of allergic reactions and iron overload.<sup>83 84-88</sup>

#### *The impact of ESA use on iron levels*

Following the initial introduction of ESA, iron storage levels of haemodialysis patients declined significantly. One such example of this was seen in a haemodialysis patient, who was previously transfusion dependent, and maintained on 150 units per kg of ESA. The iron storage markers indicated a rapid reduction of available iron. Serum ferritin decreased from 5,738 nanograms per litre (ng/l) to 4,067ng/l and transferrin saturation from 100% to 49%. Erythrokinetic studies, confirmed that the rate

of erythropoiesis and therefore iron inclusion in red cells was 3.6 times that it had been prior to the introduction of ESA.<sup>68</sup>

Following the introduction of ESA, iron consumption increased. It became apparent that the previously common problem of iron overload secondary to repeated transfusion<sup>89</sup> was being ameliorated with an associated reduction in serum ferritin levels<sup>69</sup> and there was histological evidence of improvement in liver iron deposition.<sup>90</sup>

### *Maintenance iron therapy*

Blood, and therefore iron loss, on haemodialysis and increased iron utilisation with implementation of ESA led to patients becoming iron deficient.<sup>91</sup> Prior to the introduction of ESA, the mainstay of maintenance iron therapy was oral. With the increased iron consumption by ESA, maintenance iron therapy switched to the more efficacious parenteral iron dextran. This approach had the additional advantage of improved compliance in comparison to oral therapy. Parenteral iron, however, was not without risk. McCarthy et al reviewed 665 courses of parenteral iron dextran administration, of which 21 (3.2%) were associated with adverse effects. Of the most serious adverse reactions, two patients reported wheezing or dyspnoea with a further 7 reports of urticarial type reactions. The most common side effect was gastrointestinal disturbance, with 18 episodes of nausea, vomiting, diarrhoea and abdominal pain reported.<sup>92</sup> Parenteral iron therapy tolerance did however improve when the dextran formulation was superseded by iron sucrose. This had a preferential side effect profile with a severe adverse event rate reduction from 11.3 per million with high molecular weight iron dextran to 0.6 per million with iron sucrose.<sup>93</sup>

There remains no consensus as to how to give maintenance iron therapy. Up until the 2010 guidelines, iron status was deemed adequate if ferritin was maintained between 200 and 500ng/l with and transferrin saturation greater than 20% in patients.<sup>94</sup> The more recent 2017 Renal Association guidelines took the emphasis away from ferritin but used alternative biomarkers that functionally assessed iron inclusion in red cells.<sup>95</sup> How to achieve the targets of the guidelines is not prescribed. It

remains up to local units to decide whether to adopt a pro-active small regular dosing of iron or a reactive regime with higher dosing, as the optimal approach is unclear.<sup>96</sup>

#### *DRIVE (Dialysis patients' response to intravenous iron with elevated ferritin) study*

Published guidelines advise to dose iron according to ferritin and transferrin saturations. Despite this, no formal randomised control trials were used to determine to what levels were best to treat. The inadequacy of this approach was highlighted by the DRIVE trial. The study stratified participants into those with ferritin between 500 and 800µg/l and those with a ferritin between 800 and 1200µg/l. Those who were treated with regular intravenous iron had a greater increase in haemoglobin compared than the controls who did not receive any maintenance iron therapy. The magnitude of response to treatment was not predicted by the serum ferritin level.<sup>97</sup> The positive haemoglobin response to iron, despite seemingly adequate iron markers, indicated the need for more sensitive and specific markers of iron status to dictate iron therapy.

#### *Iron overload*

A pragmatic approach to the knowledge that some individuals were iron deficient despite biochemically believed to be iron replete was required. This is because of the concern of iron overload. Deposition of excess iron in organs had been seen previously when iron was received as part of blood transfusions, and is further discussed in section 1.4.2. Free iron may be utilised by invading bacterial pathogens resulting in an increased infection risk. Free iron can increase oxidative stress, with resultant endothelial dysfunction with its potential atherosclerotic risk.<sup>98</sup>

Amelioration of iron overload is difficult in the haemodialysis patient. Historically, the mainstay iron chelator has been desferrioxamine, which is renally excreted.<sup>99</sup> The chelated iron thereby has no pathway for excretion so the effects of the therapy are limited, with toxicity risks due to its accumulation.<sup>100</sup> The mechanism of excretion of the newer iron chelator, deferasirox, is via the biliary system and is therefore a potential therapeutic option for those suffering from iron overload.<sup>101</sup>

The difficulty of identifying iron overload, and its subsequent management, need underlies the rationale for an improved understanding of the relationship between biomarkers of renal anaemia and its required treatment, and the safety of an approach which may result in higher doses of iron therapy being administered.

## 1.5 Treatment targets in renal anaemia

### 1.5.1 Early studies following the introduction of ESA

Following the introduction of ESA, clinicians had the ability to adjust medication doses to try achieve different haemoglobin, or haematocrit, targets. Gradually, data emerged as to the positive benefits of correction of haemoglobin on mortality. What remained unclear was to what extent the haemoglobin needed to be corrected for an improvement in patient mortality to be achieved.

### 1.5.2 Early mortality studies

Following the introduction of ESA, registry based observational data suggested a higher haemoglobin conferred a positive benefit on mortality. The four largest studies are discussed in table 1. These studies provided the foundations for the continued use of ESA in haemodialysis. The variability of results highlights the need for randomised control trials to determine an optimal haemoglobin target for renal anaemia.

| Publication                          | Finding                                                                                                                                                                                                                                                                                                                                                              |
|--------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Foley et al<br>(1996) <sup>102</sup> | A prospective study of 432 end stage renal failure patients which show increased mortality for those with a haemoglobin below 95g/l (relative risk (RR)=2.08). A decrement in haemoglobin of 10g/l during the duration of dialysis therapy was associated with both cardiac failure (RR=1.28) and death (RR=1.14)                                                    |
| Madore<br>(1997) <sup>103</sup>      | Prevalent haemodialysis patients from the United States Renal Data System showed an increased odds ratio (OR) of death once haemoglobin was less than 100g/l with the OR 2.01 with a haemoglobin less than 80g/l compared with the reference range of 100-110g/l. There was no positive effect of a haemoglobin greater than 110g/l compared to the reference range. |
| Locatelli<br>(1998) <sup>104</sup>   | The Lombardy registry showed a decreased mean risk of all-cause mortality by 0.95 per unit increase of haematocrit measured between the range 0.27 and 0.32.                                                                                                                                                                                                         |

|                          |                                                                                                                                                                                                                                                                                                  |
|--------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Ma (1999) <sup>105</sup> | Medicare haemodialysis patients were stratified according to haematocrit. Using a haematocrit of 0.30 to 0.33 as a reference, there was a 1.51 relative risk of death in those with a haematocrit less than 0.27 and a 0.90 relative risk of death if the haematocrit was between 0.33 and 0.36. |
|--------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

*Table 1. A summary of key early studies that assess the impact on haemoglobin levels on mortality of haemodialysis patients. All four studies showed that a lower haemoglobin (or haematocrit) is associated with an increased risk of death, however the defined reference range differed between the studies. This evidence provided the basis of the rapid implementation of ESA into clinical practice to correct anaemia. The upper limit of haemoglobin in which mortality is lowest is not defined within these studies.*

### 1.5.3 Improved morbidity following introduction of ESA

The beneficial effect of improvement in haemoglobin was not restricted to mortality. There was a reduction in morbidity seen in those whose haemoglobin improved following introduction of ESA, with a reduction in hospital admission rates secondary to cardiovascular and cerebrovascular disease<sup>102</sup>  
<sup>106,104</sup> Patients reported a much improved quality of life. This success was the drive for their rapid widespread implementation into clinical practice without sound randomised control trials.<sup>107-109</sup>

### 1.5.4 Seminal randomised control trials

Observational studies demonstrated the predilection of clinicians to treat patients to a higher haemoglobin in the presence of co-morbidities such as diabetes and known ischaemic heart disease.<sup>102</sup> To fully assess the impact of corrected haemoglobin on outcome, formal randomised control trials were required where pre-conceived beliefs of the clinician did not influence target haemoglobin. The following randomised control trials, with a target haemoglobin defined by a protocol in a blinded manner, would attempt to overcome the limitations of previous studies. These studies, however, only include chronic kidney disease and not haemodialysis patients. The lessons learnt, however, were thought to be applicable to both chronic kidney disease and haemodialysis patients.

### *The Normal Haematocrit Trial*

This study, published in 1998, recruited 1,233 haemodialysis patients, all of whom had either evidence of heart failure or known ischaemic heart disease. Six hundred and eighteen participants were randomised to receive increasing doses of ESA to achieve a target 'normal' haematocrit of 42% and 615 were randomised to a 'near normal' target haematocrit of 30%. Analysed on an intention to treat basis, at the interim analysis at 29 months, there were 183 deaths and 19 non-fatal myocardial infarctions on the 'normal' haematocrit group. This compared with 150 deaths and 14 non-fatal myocardial infarction in the 'near normal' group. The increased risk of ratio of 1.3 for death with the higher target haemoglobin led to the early termination of the study. Patients in the 'normal' haematocrit group did have a reduced blood transfusion rate, improved physical function score and increased access thrombosis.<sup>110</sup>

### *Correction of Anaemia with Epoetin Alfa (CHOIR)*

This study, published in NEJM in 2006, randomised 1,432 chronic kidney disease patients, on a one to one basis, to achieve either a higher (130-135g/l) or lower (105-110g/l) target haemoglobin. The achieved mean haemoglobin in the 715 patients in the higher group was 135g/l and 113g/l amongst the 713 patients of the lower treatment group. The composite end point in the study was death, myocardial infarction, hospitalisation for heart failure or stroke. Within the 16 months following study initiation, 125 patients in the higher target haemoglobin group reached an endpoint in contrast to 97 in the lower target haemoglobin group. This equated to a 1.34 increased hazard ratio of reaching an end point in the higher versus normal target haemoglobin groups, leading to the early termination of the study. Contrary to the Normal Haematocrit Trial, those achieving a higher target haemoglobin did not report an improved quality of life score in comparison to those who had a lower haemoglobin.<sup>111</sup>

### *Cardiovascular Risk Reduction by Early Anemia Treatment (CREATE) trial*

In the same month as the CHOIR study, NEJM also published the CREATE study. This study was smaller with 603 participants, and the entry criteria restricted to those with more advanced chronic kidney

disease. Target haemoglobin in the higher group was 130 to 150g/l, with patients receiving ESA upon randomisation to achieve this; and 105 to 115g/l in the lower group with ESA initiated once haemoglobin had decreased to less than 105g/l. The primary end point of the study was time until a first cardiovascular event within a three year follow up period. There were 58 patients in the higher haemoglobin group and 47 lower haemoglobin group reaching a primary end point. This did not represent a significant difference in the relative risks of a cardiovascular event. Those in the higher haemoglobin group did however report a significantly higher quality of life.<sup>112</sup>

The conclusion from these two studies supported the partial correction of haemoglobin, aiming for a low-normal target, rather than complete correction of the haemoglobin.

#### *Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT)*

Three years later, the TREAT investigators published a larger study in the same journal. This study randomised 4,038 type 2 diabetic patients with chronic kidney disease to either the 'high Hb' group receiving ESA to achieve a target haemoglobin of 130g/l; or 'low Hb' group in which patients only received ESA if haemoglobin decreased to less than 90g/l. Mean haemoglobin achieved in the 'high Hb' group was 125g/l and 106g/l in the 'low Hb' group. There was no significant difference in death in cardiovascular events between the two groups although an increased risk of stroke and malignancy was seen in the higher target haemoglobin group. There was however a positive effect on quality of life for those with the earlier introduction of ESA.<sup>113</sup>

#### *Current limitations in clinical practice*

Despite the randomised control trials, target haemoglobin remained uncertain due to the contradictions between the trials, which supported a lower haemoglobin target than observational data.<sup>114 115</sup> A post-hoc analysis of the study of the Normal Haematocrit Trial helped delineate the issue. Following a review of those in the higher haematocrit group, ESA sensitivity was identified as a key factor in determining patient outcome. This study had achieved target haemoglobin by increasing ESA dose by 50% at the start of randomisation, then further 25% increases to achieve the target. Sensitivity

to ESA was assessed and patients were then divided into quartiles accordingly. A positive association was identified between a lack of ESA response and mortality, although not between ESA dose at baseline and mortality.<sup>116</sup>

The post-hoc analysis of the CHOIR trial provided further information subdividing patients into those that did and did not achieve their target haemoglobin and those on high and low ESA dose. This demonstrated that those that did not achieve their haemoglobin target had a higher mortality than those that did, and a greater proportion of those randomised to the higher target haemoglobin did not achieve their target. Equally, those in the higher target haemoglobin group treated with a high dose of ESA had higher mortality rates than those who achieved the target haemoglobin with a lower dose.<sup>117</sup> These post-hoc analyses, supported by similar findings by the TREAT investigators,<sup>118</sup> has led to a re-evaluation of approach to treating anaemia in the haemodialysis groups.

In summary, poor ESA responsiveness, independent of ESA dose, is a predictor of a poor outcome for a patient. This is demonstrated by a theoretical model shown in Figure 7 below. In this figure, the darker colour represents a poor outcome. The figure shows those that attain the highest haemoglobin with the smallest ESA dose have the best outcome; whereas those achieving a low haemoglobin despite a high ESA dose have the worst outcome. The two grey circles in the figure represent the potential ways in which outcome can be improved. This can either be achieving a higher haemoglobin with the same ESA dose, normally by appropriate iron supplementation; or reducing the ESA dose without any reduction in Hb. This is by trying to determine which individuals receive an increasing dose of ESA to which they are unresponsive.

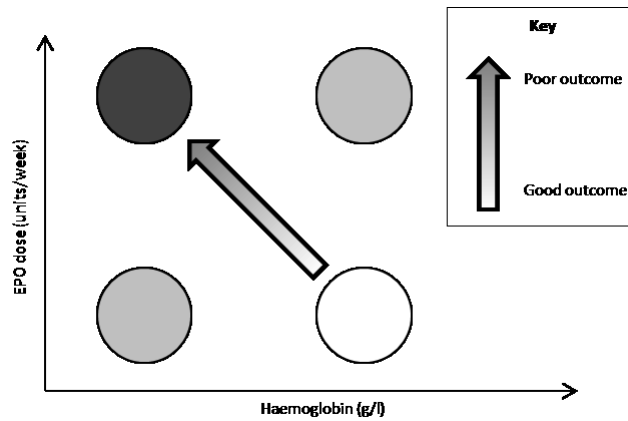


Figure 7. A theoretical model demonstrating expected outcome for a patient according to haemoglobin and ESA dose. Those who have the highest haemoglobin but the lowest ESA dose have the best outcome. Conversely, those achieving only a lower haemoglobin despite high ESA dose have a far worse outcome. Achieving a high haemoglobin with a high ESA dose or a lower haemoglobin with a lower ESA dose leads to an intermediate outcome. This model provides the theoretical basis for improving anaemia treatment. Those within the high ESA dose and lower haemoglobin are able to improve their outcome by either reduction in ESA dose to achieve that haemoglobin or achieving a higher haemoglobin with the same ESA dose. Dose have the potential to be achieved with the judicious use of iron.

## 1.6 Iron metabolism

Knowledge of iron physiology has facilitated development of biomarkers to assess iron status; with novel biomarkers reflecting an evolution of understanding. These biomarkers (such as ferritin, transferrin and hepcidin), with further study, will allow continued progress in developing individualised treatment strategies for renal anaemia. Furthering this goal is a key focus of this present study. The physiology of iron and the identification of biomarkers are reviewed in the following section.

### 1.6.1 An overview

Iron is able to exist in multiple oxidation states, from -2 to +6; although most of the time in physiology, it is in either the ferrous ( $\text{Fe}^{2+}$ ) or ferric ( $\text{Fe}^{3+}$ ) states. Iron is a key component of haemoglobin which is then able to transport oxygen around the body. The variable oxidation states of iron allow haemoglobin to reversibly bind, transport and release oxygen. These same properties of iron also facilitate its ability to act as a co-factor in multiple enzymatic reactions and as an electron donor for mitochondrial respiration and adenosine triphosphate (ATP) generation.<sup>119 120</sup>

Iron levels, however, need to be closely regulated with any excess iron safely stored. The properties which underlie its important role in physiology also result in its potential toxicity. Excess iron converts hydrogen peroxide and superoxides into free radicals. Further free radical generation is caused by invading bacteria using iron as a cofactor in its reactions. The free radicals to attack cellular membranes, proteins and DNA.<sup>121</sup>

### 1.6.2 Distribution of iron

Approximately 1,800mg of iron, representing the majority of the total in the body, is found within red blood cells. In the physiological state, erythrocytes have a lifespan of 100 days.<sup>122</sup> As erythrocytes reach the end of their lifespan, they are digested by macrophages within the reticuloendothelial system. The iron from the senescent erythrocytes is recycled and used in the generation of new red cells.<sup>123</sup> Approximately 3mg of iron at a time is in transport in the circulation bound to transferrin. The transferrin delivers iron to the bone marrow to become incorporated into the developing erythrocyte.

Erythrocyte development uses approximately 20 to 25mg per day of iron. The lack of physiological excretion mechanism and effective iron economy means iron loss is restricted to 1-2mg per day. This occurs through desquamation of the skin and loss of epithelial cells from the gastrointestinal tract and renal tubules and needs to be replenished through dietary intake.<sup>124</sup> Iron distribution throughout the body is summarised in Figure 8.

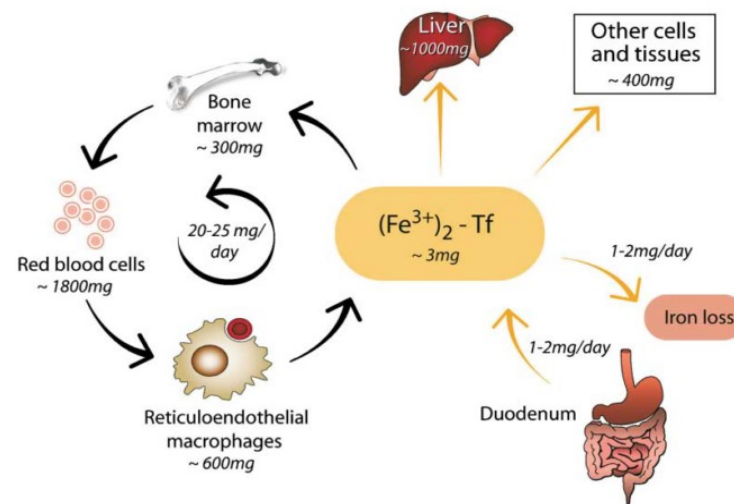


Figure from Hentze et al, 2010<sup>125</sup>

*Figure 8. The iron cycle. This figure demonstrates the distribution of iron throughout the body. The majority of the 1800mg of iron in the body is in the red blood cells. When red cells undergo senescence, they are digested by the macrophages. Macrophages contain approximately 600mg of iron at any one time. Three milligrams of iron is bound to transferrin (Tf) throughout the circulation. It is either transported back to the bone marrow for further erythrocytosis, taken to the liver for storage and a further 400mg is used for a multitude of functions by other cells throughout the body. The small amount of iron loss on a daily basis is replaced via the diet, with dietary iron absorbed in the duodenum.*

### 1.6.3 Haemoglobin

The 200 billion new erythrocytes produced on a daily basis consume 80% of the total body iron requirements.<sup>125</sup> Each circulating erythrocyte contains 640 million haemoglobin complexes. These

complexes are formed of four haem and four globin chains which are bound together, with each able to transport one oxygen molecule. Each haem is formed through an eight stage process, resulting in the complexing of an iron atom and a cyclic tetrapyrrole.<sup>126</sup>

#### 1.6.4 Iron cycle

#### 1.6.5 Dietary iron uptake

Dietary intake of iron, from either plant or animal sources, is absorbed in the duodenum and jejunum.<sup>127</sup>

Iron from animal sources undergoes vesicular uptake via the haem receptor, haem carrier protein 1 (HCP1).<sup>128</sup> Iron from plant sources is reduced by duodenal cytochrome b (Dcytb) and is then taken up by the divalent metal transporter 1 (DMT1).<sup>129 130</sup>

The rate at which iron is absorbed is influenced by both systemic and local factors. The local factors which affect dietary iron absorption include the luminal pH, intestinal motility, *Helicobacter pylori* infection and mucosal pathologies such as coeliac disease.<sup>131</sup> Systemic factors will be discussed in more detail later in section 1.6.10.

#### 1.6.6 Cellular iron export

Once iron has entered the enterocytes of the duodenum, it forms a complex with the transferrin (Tf) protein. Transferrin is the main carrier protein for iron in the body.<sup>132</sup> The iron-transferrin complex can then cross the basolateral membrane of the enterocytes, through the ferroportin (FPN) transporter, into the circulation. The frequency of FPN in the cell membrane reflects the quantity of cellular iron export. FPN is therefore most prevalent in enterocytes, macrophages and hepatocytes.<sup>133-135</sup>

#### 1.6.7 Transferrin, the iron transport protein

Transferrin is a glycoprotein synthesised by the hepatocytes of the liver. It is the protein that binds iron and then transports it around the body. Each transferrin is able to bind two  $\text{Fe}^{3+}$  ions, in association with an anion, such as bicarbonate. This safe method of iron movement, bound to transferrin, prevents toxic free radical formation.<sup>136</sup>

### 1.6.8 Cellular iron uptake

The circulating iron bound to transferrin can be taken up into cells via the transferrin receptor-1 (TfR-1).<sup>137</sup> TfR-1 is a transmembrane glycoprotein expressed on all cells throughout the body with the abundance reflective of the rate of iron utilisation or the iron storage capacity of a cell. TfR-1 binds the iron-carrying transferrin which is endocytosed via clathrin-coated pits into the cell.<sup>125</sup> Once in the cell, the complex breaks up with the iron remaining in the cytosol. The transferrin leaves the cell to bind further iron.<sup>138</sup> Caeruloplasmin or hephaestin are intracellular proteins which convert the iron in the cytosol into the ferric state. Ferric iron binds to ferritin, which is the protein which is able to allow the safe intracellular storage of iron.<sup>139 140</sup>

### 1.6.9 Ferritin, the iron storage protein

The ferritin protein, first described by Laugberger in 1937, constitutes of 24 subunits folded to form a 4-helical structure. This has a large internal cavity capable of binding up to 4,000 iron atoms. The 24 subunits of ferritin are made of a combination of heavy (H) and light (L) types. The H subunit oxidises iron, whereas the L subunit stabilises the iron-bound ferritin protein. The relative ratio of the two different subunits determines the iron storage capacity of different organs within the body. The liver and spleen have high levels of the L subunits whereas the heart, brain and kidneys have a higher ratio of H subunits. The presence of the H and L subunits allow safe storage of iron by ferritin preventing the formation of toxic free radicals. The ferritin assay only allows clinicians to measure serum ferritin rather than the functional tissue ferritin.<sup>141-144</sup>

### 1.6.10 Iron homeostasis

The process of iron uptake, transport and storage described above has to be carefully co-ordinated to maintain homeostasis. This is required to balance the need for iron for erythropoiesis versus the avoidance of excess iron toxicity.<sup>125</sup> The regulatory mechanisms required to achieve this outcome are described below.

### *Cellular regulation of iron*

Iron regulatory proteins, of which there are two: iron regulatory protein 1 and iron regulatory protein 2 (IRP 1 and IRP 2). These regulate the intracellular synthesis of both transferrin and ferritin. These contain an iron response element (IRE) which encodes the mRNA for ferritin and transferrin.<sup>145</sup>

In the setting of low intracellular free iron levels, the binding of the IRE by IRPs results in an increase in the synthesis of transferrin and decrease in ferritin. Conversely, with high intracellular free iron levels, there is a reduction in this binding between the IREs and IRPs. This results in ongoing ferritin formation whilst the transferrin mRNA is destabilised. The result of this is that there is safe intracellular iron storage with reduced intracellular iron uptake.<sup>146-153</sup>

### *Systemic control of iron*

The understanding of iron metabolism was completely revolutionised with the discovery of hepcidin. Known as the “master regulator” of iron metabolism, the circulating protein hepcidin controls dietary iron absorption and distribution throughout the body. Consequently, in combination with an understanding of how hepcidin levels vary, it is a key biomarker in determining how ESA and iron treatment can interact in ensuring the optimal haemoglobin target is reached. The correlation of hepcidin levels with clinical outcomes following treatment is a key strand in this study.

### *Hepcidin*

#### *Introduction*

Hepcidin is a 25 amino acid peptide encoded by the HAMP gene located on chromosome 19. It is synthesised as 84 amino acid preprohepcidin and undergoes successive cleavage to 60 amino acid prohepcidin and then to hepcidin.<sup>154</sup> The peptide is primarily produced in the liver, with lower levels detected in the heart, spinal cord, stomach, intestine, adipose tissue and lung.<sup>155</sup> Hepcidin was first identified in the blood by Krause et al in 2000 and by Park et al in the urine the following year. It was originally known as liver-expressed antimicrobial protein, reflecting the noted antimicrobial function when first discovered.<sup>156 157</sup>

## Function

The function of hepcidin is dependent on being able to act upon the sole intracellular iron exporter, FPN. Hepcidin is able to inhibit the binding of FPN to the cell surface, preventing the passage of iron into the circulation. The mechanism by which this occurs is shown in Figure 9.

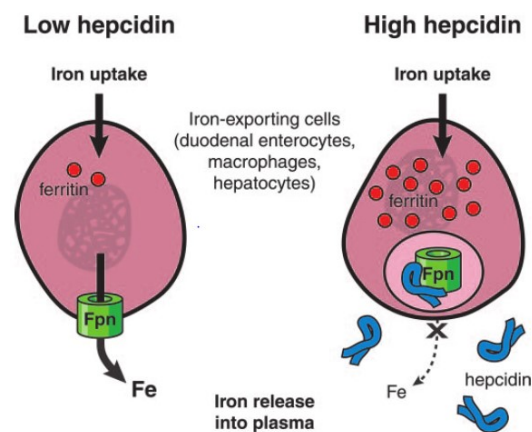


Figure taken from Ganz, 2007. <sup>158</sup>

*Figure 9. The impact of hepcidin levels of cellular iron uptake. In the setting of low hepcidin levels, the iron taken up into cells is immediately released into the plasma, exporting the cell by the ferroportin transporter. In contrast, when hepcidin levels are elevated, iron is unable to exit the cell. The hepcidin blocks the ferroportin binding at the cell surface so iron remains intracellular. Duodenal enterocytes therefore accumulate iron from the diet which remains intracellular so binds to ferritin to prevent it causing toxicity. Macrophages and hepatocytes retain the iron that they have previously taken up but further iron uptake is limited due to minimal circulating iron.*

## Hepcidin mouse models

Early studies of hepcidin used mice to further define its role in iron metabolism. The key studies are described below.

### *Low levels of hepcidin*

Researchers trying to produce an upstream transcription factor 2 (USF2) knock out model noted their mice had a phenotype consistent with haemochromatosis, and these mice were found to have reduced hepcidin levels. Following production of a more accurate USF2 knockout model, the phenotype of the mice normalised, and the serum hepcidin levels were normal.<sup>159</sup> Researchers were able to localise hepcidin production to the liver when the mice with a general knockout of the hepcidin encoding gene, HAMP, resulted in the same iron overload phenotype as knockout those with just knockout of HAMP in the liver.<sup>160</sup>

### *High levels of hepcidin*

The production of a transgenic mouse model (TTR-HEPC1) produced mice with elevated hepcidin levels who were small and died soon after birth. Post mortem analysis revealed these mice had decreased body iron stores with a severe microcytic hypochromic anaemia.<sup>161</sup> The impact of elevated hepcidin has been further shown using synthetic hepcidin. Injection of this into mice resulted in a dose-dependent rapid depletion of iron levels within the first 12 hours, which subsequently normalised within 96 hours.<sup>162</sup>

### *Hepcidin regulation*

The three key determinants of hepcidin control: iron concentration, iron requirements for erythropoiesis and inflammation are shown in Figure 10.

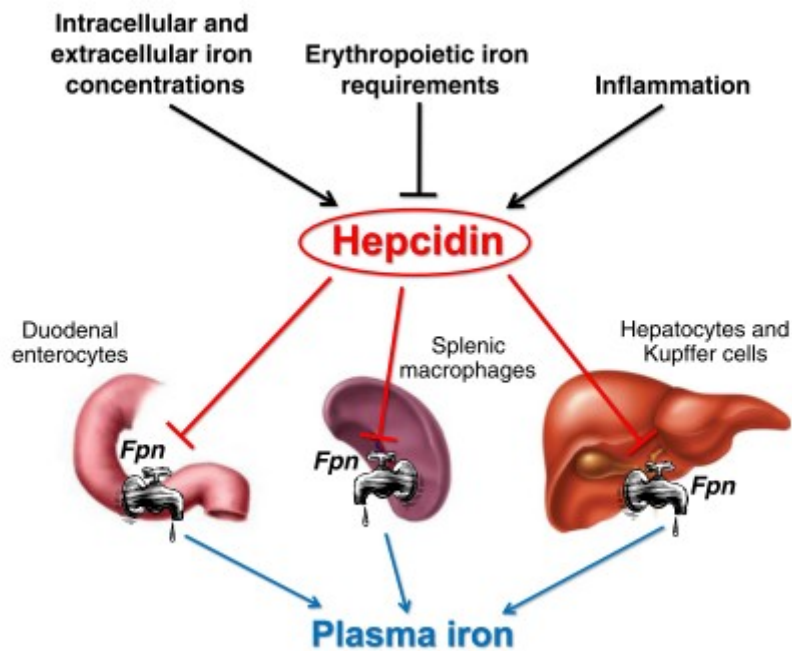


Figure taken from Ganz, 2012. <sup>163</sup>

Figure 10. Regulators of hepcidin levels. Elevated intracellular and extracellular iron concentrations and inflammation cause upregulation of hepcidin levels. In contrast, hepcidin is downregulated in response to increased erythropoietic iron demands. When hepcidin levels are elevated in the settings of inflammation and high iron concentrations, hepcidin blocks the ability of FPN to allow intracellular iron to be exported. When hepcidin levels are low in the setting of high erythropoietic iron requirements, stored intracellular iron is exported into the circulation and delivered to the bone marrow for erythropoiesis. The impact of hepcidin is on all the major cell types involved in the iron cycle; these includes the duodenal enterocytes, splenic macrophages and hepatocytes and Kupffer cells.

#### Iron stores

Iron stimulates the production of hepcidin. This facilitates intracellular iron storage in conjunction with ferritin in the setting of high extracellular iron concentrations. Conversely, in the absence of extracellular iron, the low hepcidin levels result in the iron stored in conjunction with ferritin, being exported into the circulation.

Increased intracellular hepatic iron levels increases intracellular bone morphogenetic peptide-6 (BMP6) synthesis. This gets excreted into the circulation which activates receptors which cause phosphorylation of a SMAD complex thereby stimulating hepcidin expression. Circulatory iron bound to transferrin causes its dissociation from the TfR-1 receptor, which is present on all cells, preventing intracellular iron uptake with preferential binding to transferrin receptor-2 (TfR-2), which is restricted to the liver. This further increases intracellular hepatic iron levels and therefore further enhancing BMP-6 synthesis.<sup>164-166</sup>

The influence of iron levels has been demonstrated experimentally in mice. Measurement of hepcidin mRNA in mice two months following parenteral iron administration demonstrated a fourfold increase in its expression in comparison to controls.<sup>167 168</sup> Mice from an iron replete to an iron deplete diet have also been shown to reduce hepcidin liver mRNA levels with a concomitant increase in expression of genes encoding proteins involved in the duodenal uptake of iron.<sup>169</sup>

Healthy volunteers, with normal baseline iron parameters, given oral iron on seven consecutive days, showed an increase in serum hepcidin levels. This increase was seen within three days of the start of iron supplementation, and persisted beyond the course of treatment.<sup>170</sup> Conversely, induction of iron deficiency in humans, by repeated phlebotomy, has been shown to suppress of hepcidin levels.<sup>171 172</sup>

### *Inflammation*

The sequence of events by which inflammation causes release of hepcidin is demonstrated in Figure 11.

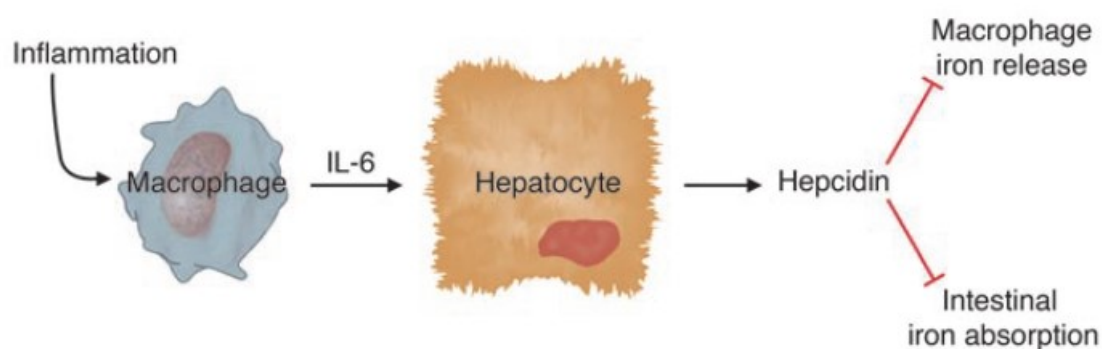


Figure from Andrews, 2004.<sup>173</sup>

*Figure 11. Impact of inflammation on hepcidin levels. Interleukin 6 is released from the macrophage in response to inflammation. This induces hepcidin mRNA expression within the hepatocyte stimulating the release of hepcidin. This in turn decreases iron bioavailability by preventing macrophage iron release and reducing intestinal iron absorption.*

Both acute and chronic inflammation cause hepcidin upregulation. Experimentally, acute inflammation can be precipitated by injection of heat-treated *Brucella abortus* into mice. This results in marked anaemia in these mice, which is not seen in the control mice. Performing the same experiment using HAMP knockout mice, did not result in the anaemia as in wildtype mice. HAMP knockout mice did have a shortened life expectancy however suggestive of a protective adaptive role of the upregulation of hepcidin.<sup>174</sup> Similar upregulation of hepcidin is seen in response to chronic inflammation. Mice repeatedly administered turpentine had a six-fold increase in hepcidin levels with a twofold decrease in serum iron levels compared to controls.<sup>175</sup>

A similar upregulation in hepcidin levels has been seen in humans in response to inflammation. Human hepatocytes incubated in a monocyte conditioned media upregulated IL-6 and hepcidin following exposure to lipopolysaccharide (LPS).<sup>176-178</sup> In healthy volunteers, LPS infusion resulted in an increase in interleukin 6 (IL-6) and CRP. This was then followed by an increase in urinary hepcidin levels, and a reduction in serum iron levels.<sup>179</sup> The increase in urinary hepcidin levels was likely to be secondary to an increase in serum hepcidin levels, rather than an increase in urinary hepcidin clearance, given the

concomitant reduction in serum iron as this would be unaffected by increased urinary hepcidin clearance.

The upregulation of hepcidin in response to inflammation is thought to be an adaptive immune response.<sup>157 180</sup> Iron provides nutritional support to invading pathogens; therefore, minimisation of available free iron prevents this support.<sup>181 182 183</sup> Dietary iron uptake is minimised as this iron becomes trapped within the duodenal enterocytes and unable to enter the circulation. This limits intracellular iron overload in hepatocyte and monocytes which is important as excess intracellular iron has a negative effect on cell-mediated immunity.<sup>184</sup>

### *Erythropoiesis*

Ongoing erythropoiesis causes a reduction in hepcidin levels. This allows stored intracellular iron to be exported into the circulation and thereby increases iron bioavailability which allows it to be incorporated into the developing erythrocyte. Conversely, hepcidin levels are increased in response to a reduction in erythropoiesis.<sup>163</sup>

Experimentally this has been demonstrated in mice. Phlebotomising mice induces erythropoiesis and decreases hepcidin production. Mice however pre-exposed to the chemotherapeutic agent, doxorubicin and carboplatin, or ESA-blocking antibody, showed no increased erythropoiesis and hepcidin levels remained static.<sup>185</sup> Direct induction of erythropoiesis by the use of ESA significantly reduces serum hepcidin in mice. This is an effect obviated in control mice pre-conditioned with total body irradiation or a control saline injection, that lacked the ability to increase erythropoiesis in response to ESA.<sup>186 187</sup>

Similar patterns of response have been shown in human studies where stimulation of erythropoiesis by ESA causes a marked, prolonged reduction in hepcidin levels.<sup>188</sup> Measurement was made of serial hepcidin levels of patients with beta thalassaemia prior to, and 4 to 8 days following, transfusion. Transfusion suppresses erythropoiesis. Following transfusion, the surrogate markers of erythropoiesis,

erythropoietin levels and growth differentiation factor 15 (GDF 15), were suppressed and hepcidin levels increased.<sup>189</sup>

### *Hepcidin abnormalities in humans*

#### *Primary abnormalities*

There are few known primary hepcidin abnormalities in humans. Haemochromatosis 2B, in which there is an abnormality of the encoding HAMP gene, results in a hepcidin deficiency with resultant iron overload.<sup>190</sup>

Hepcidin-secreting adenomas have been described in patients with glycogen-storage disease 1a. Long term follow up of 38 patients found 6 developed a severe microcytic anaemia. Two of these patients were found to have hepatic adenomas, one of which was resected. Staining of the specimen for hepcidin mRNA showed markedly increased levels in comparison to normal tissue. The patient subsequently had a resolution of his anaemia implicating the role of hepcidin in its pathophysiology.<sup>191</sup>

#### *Haemochromatosis*

The HFE gene, located on the short arm of chromosome 6, is responsible for the synthesis of the haemochromatosis protein, HFE. This protein binds the transferrin receptor and is a positive upstream regulator for hepcidin.<sup>192 193</sup> Haemochromatosis, in which there is a mutation in the encoding gene for HFE, results in phenotypical changes of iron overload. Hepcidin levels are low in most types of haemochromatosis, barring a few exceptions. This is contrary to what would be expected if the normal feedback mechanism of upregulation of hepcidin in response to iron was effective. This indicates that there is a dysregulation of the hepcidin feedback process that is responsible for haemochromatosis.<sup>194</sup>

#### *Thalassaemia*

Thalassaemia is characterised by a deficiency in one of the globin chains used in the synthesis of haemoglobin. This leads to ineffective erythropoiesis with high numbers of early stage erythrocyte progenitors but low numbers of the more developed reticulocytes. Hepcidin levels are low in patients with thalassaemia and iron overload is not an uncommon feature. The rationale for this has been

proposed that an erythroid factor, produced in the early stages of red cell synthesis, is excessively released during the ineffective erythropoiesis. This is a negative regulator to hepcidin production in an attempt to increase iron availability for incorporation into the developing red cells.<sup>195</sup>

## 1.7 The revised model of renal anaemia

The original model of renal anaemia, which centralised the role of erythropoietin deficiency, has been revised following the discovery of hepcidin.

### 1.7.1 Hepcidin levels in renal disease

Hepcidin levels are elevated in renal disease. The normal diurnal variation in hepcidin levels seen in the healthy population is able.<sup>196-198</sup> As renal disease progresses, hepcidin levels increase and this correlates with the degree of anaemia in patients.<sup>198 199</sup>

#### *Mechanisms of elevated hepcidin levels in renal disease*

There are three factors that underlie the increase in hepcidin levels with renal disease. Firstly, there is a reduction in urinary hepcidin clearance. Secondly, the pro-inflammatory nature of renal disease results in increased production of hepcidin, which is further exacerbated in end stage renal failure due to the use of extracorporeal circuits for renal replacement therapy. Thirdly, there is a failure to increase erythropoietin levels in response to anaemia in renal disease. This leads to a lack of suppression of hepcidin levels by the negative feedback of erythropoiesis.<sup>200-205</sup>

#### *A comprehensive overview of renal anaemia with focus on hepcidin*

In renal disease, just as within the healthy population, the purpose of the iron cycle is to ensure adequate delivery of iron to the bone marrow to be incorporated into developing erythrocytes. The efficiency of this system is maximised by effective recycling of the iron laden red cells with reuse of the iron. Once circulating erythrocytes become senescent, macrophages digest these to make the iron available again. Hepcidin influences how the macrophages subsequently release the iron procured from the digested red cells either into storage or back to the bone marrow for further incorporation into developing erythrocytes according to need at that moment in time.

Beyond the three influencers on hepcidin levels of iron status, inflammation and erythropoiesis, there are some additional factors that influence hepcidin and the developing red cells unique to renal disease. There is a reduction in urinary hepcidin excretion in renal disease leading to increased hepcidin levels.

The relative reduction in erythropoietin production, with a failure to appropriately increase erythropoietin in response to anaemia, and the impact of uraemic inhibitors in the developing red cells in the bone marrow also contribute to the development of renal anaemia.

Medication can be used in an attempt to treat anaemia caused by renal disease. Iron administration tries to ensure iron availability despite the hepcidin blockade on making iron available from the macrophage. ESA enhance bone marrow activity which has been negatively regulated by uraemic inhibitors. The resultant iron cycle is shown diagrammatically in the figure below.

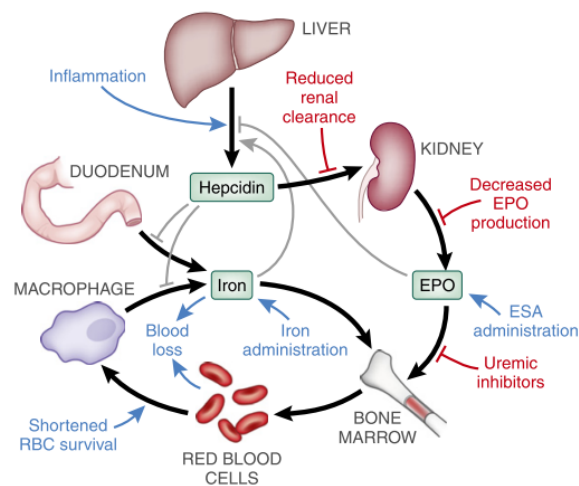


Figure from Babitt, 2012.<sup>206</sup>

Figure 12. Current understanding of renal anaemia. Iron travels in the circulation to the bone marrow where it is incorporated into developing erythrocytes. The mature erythrocytes enter the circulation. They however have a shortened lifespan due to the renal disease. Furthermore, patients with renal disease are more vulnerable to both idiopathic blood loss and due to iatrogenic causes. At the end of the erythrocyte lifespan, they are digested by macrophages and the iron is recycled by the macrophages. The rate of the subsequent release of iron from macrophages is limited by hepcidin. Levels of hepcidin are upregulated in response to inflammation, which is compounded by a reduction in renal hepcidin clearance. Iron levels can be supplemented by the use of parenteral iron. ESAs can be used to partially overcome the relative reduction in endogenous erythropoietin in

*renal disease. Even with the iron and ESA overcoming the lack of bioavailable iron and erythropoietin, synthesis of new red blood cells is limited by uraemic inhibitors on the bone marrow.*

## 1.8 Current management of anaemia in haemodialysis

In the United Kingdom, management guidelines for anaemia in haemodialysis patients are directed by the Renal Association (RA) in conjunction with the National Institute of Clinical Excellence (NICE). Historically, the biomarkers used to assess iron status were ferritin to assess iron storage; and either serum transferrin saturation (TSat) or percentage of hypochromic red cells (HRC) to assess iron availability. Target levels indicative of adequate iron status were ferritin level 200-500µg/l and TSat greater than 20% or HRC<6%. Clinicians were advised to use intravenous iron to reach and remain within these parameters whilst avoiding a consistent ferritin greater than 800µg/l which may indicate iron overload.<sup>207</sup> This approach was advocated by the international bodies Kidney Disease: Improving Global Outcomes (KDIGO) and Kidney Disease Outcomes Quality Initiative (KDOQI) who advise on the use of ferritin and TSat to evaluate iron status.<sup>208 209</sup>

The most recent collaborative publication between NICE and the RA in 2015 placed a greater emphasis on the use of red cell parameters of iron to assess iron status, and relegated the use of TSats and ferritin. These guidelines recommended the use of HRC as first line iron biomarker, reticulocyte haemoglobin content (CHr) or its equivalent (Ret-He). The thresholds of defining iron deficiency were HRC<6%, and CHr or Ret-He less than 29pg, with supplemental iron indicated if these were met.<sup>95</sup>

The lack of randomised control trial evidence that supports new guidelines means that clinicians are wary of changing their clinical practice. This project has the potential to provide some of that evidence. Meanwhile, longstanding anaemia protocols continued to be followed. The protocol of the local unit is shown in Figure 13. The protocol considers haemoglobin and ferritin completely independently of one another; ESA dose is adjusted in accordance to haemoglobin and iron administered in accordance with ferritin, regardless of haemoglobin.

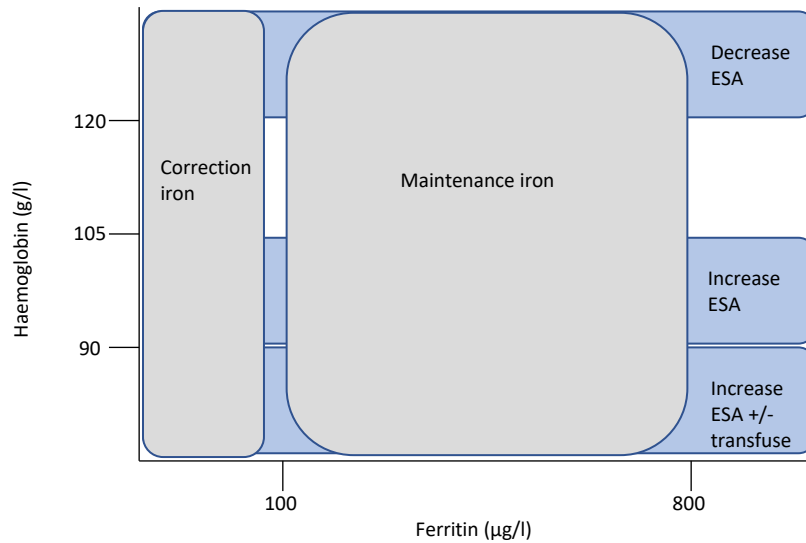


Figure 13. Standard clinical practice, including within the local renal unit, which considers haemoglobin and ferritin independent of one another. For those with a ferritin less than 100µg/l, a corrective dose of iron given as 5 doses of 200mg iron sucrose on successive dialysis session is administered. In those with whose ferritin is between 100 and 800µg/l, patients receive a low dose of maintenance iron, in this unit, 100mg iron sucrose. ESA is increased or decreased in response to haemoglobin regardless of ferritin level.

In the view of the present author, this approach is oversimplified and does not take into account the multiple factors needed to determine an optimal treatment plan. Specifically, studies such as TREAT and CHOIR discussed above have highlighted the need to assess treatment dose in relation to existing factors such as haemoglobin levels and ESA responsiveness.

## 1.9 Inadequacy of current approach to anaemia in haemodialysis patients

Ferritin is not a sensitive biomarker in determining an individual's iron status. It has been used to determine whether an individual is iron deficient, requiring parenteral iron, or replete, requiring ESA. Multiple studies have found that the degree of response to parenteral iron is independent of baseline ferritin.<sup>210 211</sup> The DRIVE study (section 1.4.4) showed a positive response to intravenous iron despite high baseline ferritins. FIND-CKD demonstrated improved haemoglobin in pre-dialysis patients treated with iron to target a higher ferritin level. Overall, more aggressive iron policies have been trialled which target higher ferritin levels and have been shown to increase mean haemoglobin and decrease ESA dose.<sup>212</sup>

### *PIVOTAL study*

With the knowledge that some patients positively responded to higher iron doses, the Proactive Intravenous Iron Therapy in Haemodialysis Patients (PIVOTAL) trial was undertaken to evaluate the safety of a higher iron dosing. This is the largest RCT of iron therapy to date which randomised to either a proactive iron regime, where individuals received maintenance iron up to 400mg iron sucrose per month, versus reactive, when iron was only administered if ferritin was less than 200µg in a month. The primary outcome of the study was a composite of death, non-fatal myocardial infarction, stroke or hospitalisation for heart failure. Those treated proactively received a median iron dose of 264mg per month versus 145mg in the reactive group. There was a primary end point in 333 out of 1093 individuals in the proactive group versus 343 out of 1048 in the reactive group, (hazard ratio 0.88,  $p < 0.0001$  for inferiority). This validates the safety of the higher iron administration in haemodialysis patients.<sup>213</sup>

From the aforementioned studies, one can see that the current clinical protocol (shown in Figure 13) undertreats some individuals with iron and overtreats others. Universally increasing the target ferritin in haemodialysis patients, with a more aggressive iron policy, puts patients however at risk of toxicity from iron. The dangers of this have been described in section 1.4.4. Furthermore, ESA therapy is not

without risk as described in section 1.4.3. The optimal approach would therefore be to adopt a more patient centred care with individualised treatment plans.

#### *Iron requirements*

The approach to determining iron or ESA requirements may benefit from evaluation of alternate biomarkers to ferritin. The improved understanding of renal anaemia, as described in section 1.1, has led to other proteins, such as hepcidin, being measured. These have the potential to be superior predictive biomarkers in determining response to iron and ESA compared to ferritin and thus require full evaluation. Combining this with the functional approach of measuring iron incorporation in red cells, the approach encouraged in the 2015 NICE renal anaemia guidelines, may help to optimise prescribing of iron and ESA medications.

### 1.10 Aim and hypothesis of study

Optimal patient management of anaemia would require the accurate determination of the individual need for either iron or incremental ESA dose increase in response to anaemia at a given point in time, based upon available biomarkers. The aim of a treatment model would to ensure judicious use of these medications to achieve maximal response, as demonstrated in Figure 7.

The enhanced understanding of haemoglobin synthesis and the iron cycle has led to identification of new measurable biomarkers. These include the red cell parameter, Ret-He, and proteins from the iron cycle, transferrin and hepcidin. The identification of these new biomarkers, however, has not yet translated into improved clinical practice. In large part, this is because there has been limited research into how they correlate with patient outcomes following treatment. This is a gap that the present study seeks to address.

This prospective study aims to measure the biomarkers in participants who are then randomised to receive either iron or ESA therapy. Response to these treatments is evaluated against the baseline biomarkers to determine which of these are effective at predicting the response to treatment.

This study hypothesises that iron and ESA are both efficacious treatments of anaemia; but new biomarkers, in combination with existent biomarkers, will prove to be more predictive of response than those employed in standard clinical practice today.

## 2 Methods

### 2.1 Introduction

The study design aimed to assess the effectiveness of biomarkers in predicting an incremental increase in haemoglobin response to treatment with either ESA or iron sucrose. This study was a prospective open label, randomised control trial. Ethical approval was provided by the Westminster Research Ethics Committee (14/LO/0805) and the study was logged with EudraCT (2014-001383-36) and clinicaltrials.gov (NCT03658876). The full study protocol is detailed in appendix 1.

### 2.2 Study monitoring

The study was a phase IV study therefore registered with the Medicines and Healthcare products Regulatory Agency. Monitoring of the study was performed by the Joint Research Compliance Office (JRCO) of Imperial College London. Data collection was recorded in the web based data entry system, INFORM, which is administered by the Clinical Trials Unit, Imperial College London.

### 2.3 Recruitment

Three satellite haemodialysis units of Imperial Renal and Transplant Centre participated in the study. These units were located at Ealing, Northwick Park and Watford General Hospitals.

### 2.4 Inclusion criteria

Patients of the three satellite units aged 18 years or older, who were able to give informed consent and were established on haemodialysis for more than 3 months considered for inclusion within the study.

### 2.5 Exclusion criteria

Patients excluded from the study were those who had a known active malignancy or bone marrow disorders. Recipients of regular blood transfusions or those transfused within the past three months or unable to tolerate the study drugs were also excluded.

Nursing staff of the haemodialysis units were consulted before recruitment started to identify those individuals who known to have an exclusion criteria. Potential participants to the study had their medical notes screened prior to being approached to participate.

After study commencement, the study exclusion criteria were refined as not all participants were able to adhere to the trial protocol. Those with frequent hospital admissions due to factors unrelated to the study or those in whom a clinical decision had been made by their lead consultant to target a different haemoglobin were unsuitable for continued participation in the study. Frequent re-assessment of a patient's frailty status was made, following discussion the with lead haemodialysis nurses, throughout the duration of the study.

## 2.6 Consent

Study recruitment took place during haemodialysis sessions at each of the three units. Potential participants were provided with both a verbal explanation of the study and given the patient information leaflet (appendix 2). Patients agreeable to participation were given the option to either sign the consent form either at the time or at a later date (appendix 3).

## 2.7 Trial design

This is a “real world” study of a prevalent haemodialysis population. Baseline demographic data was collected following a participant enrolling in the study. This data was limited to features thought to be of importance in the outcome of the study, using existing data systems, rather than specific proformas.

Participants in the study continued on the same maintenance ESA dose that they had been receiving prior to entry into the study but ceased to receive any low-dose maintenance iron therapy that had been within the standard clinical protocol (as shown in Figure 13).

All participants in the study continued to have bloods taken on a monthly basis as part of standard clinical care of a haemodialysis patient. These tests included haemoglobin and ferritin levels. The results

of things were reviewed in accordance with Figure 14. Each month, those who had a haemoglobin between 90 and 104g/l inclusive with a ferritin between 100 and 800 µg/l underwent randomisation.

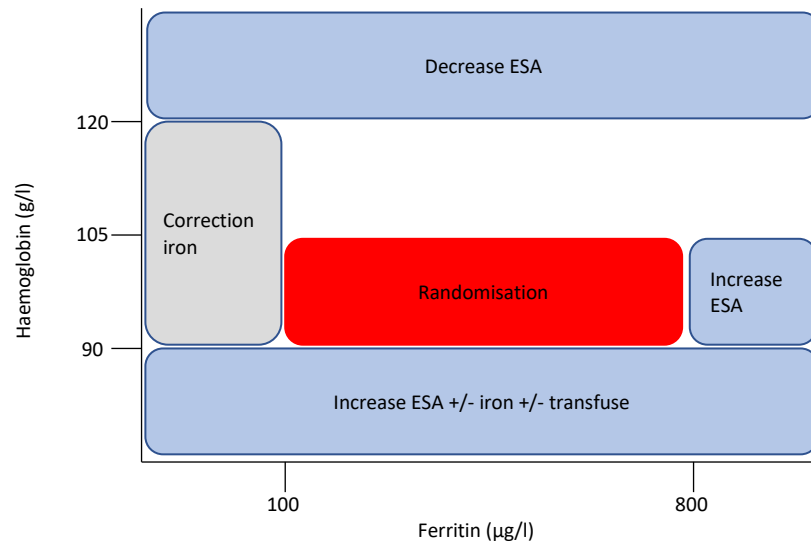


Figure 14. The trial protocol considers haemoglobin and ferritin, measured in the routine monthly haemodialysis bloods, in conjunction with each other rather than independently. Those with a haemoglobin between 90 and 104g/l inclusive, and a ferritin between 100 and 800µg/l, were randomised according to study protocol. Outside of the randomisation criteria, ESA was reduced if haemoglobin was greater than 120g/l and increased if less than 90g/l. The ESA increase could be in conjunction with intravenous iron or transfusion, if felt clinically indicated, with further investigations for the anaemia considered. Those with a ferritin less than 100µg/l, received intravenous iron. No changes to medication were given to those with a haemoglobin between 105 and 120g/l, with a ferritin greater than 100µg/l.

## 2.8 Randomisation

Participants were randomised to two treatment groups 'iron' and 'ESA' on a 1:1 ratio. Randomisation was performed using the online randomisation tool, randomization.org.

The 'iron' group received a correction dose of intravenous iron. This was a total of one gram of iron sucrose given as 5 doses of 200mg iron sucrose on successive haemodialysis sessions.

Those randomised to the 'ESA' group received an incremental increase in their regular dose of ESA. This stepwise increase is shown in Table 2. The ESA used in this study was epoietin beta (Neorecormon) and administered by nursing staff at the end of a haemodialysis session.

| Dose escalation of epoietin beta<br>(neorecormon) |             |
|---------------------------------------------------|-------------|
|                                                   | 0 units     |
|                                                   | 1000 units  |
|                                                   | 2000 units  |
|                                                   | 3000 units  |
|                                                   | 4000 units  |
|                                                   | 6000 units  |
|                                                   | 8000 units  |
|                                                   | 12000 units |

*Table 2. The ladder by which ESA doses were increased at randomisation to 'ESA' group. Although the aim prior to the study was to increase dosing by 50% at each interval; this was not possible due to only particular syringe dosages being manufactured. This stepwise increase reflects the graduated dosing intervals in common practice locally.*

## 2.9 Blinding

This was an open label study. It was not feasible to blind the medications as these would need to be administered by the regular dialysis unit nursing staff. The primary outcome of change in haemoglobin is unable to be influenced by knowledge of what medication is received so the open label study design has no negative repercussions on the integrity of the study.

## 2.10 Sample size calculation

The purpose of this study is to propose a treatment model that could then be validated within a future study. As such, this is considered a pilot study and therefore sample size estimates are based upon the number of proposed variables that may be important in a future treatment model.

For each randomly assigned treatment, iron and ESA, the aim was to define characteristics predictive of a response to treatment. There was an anticipated number of potential explanatory variables of eight, however as data inspection could identify further candidates, calculations were made so that up to ten variables may be explored within the statistical model.

It is generally agreed that the number of observations should be 10-fold greater than the number of explanatory variables for initial regression models, so 200 treatment observations should be observed (100 for each group). Audit data suggests that 10% of patients would reach a randomisation indication each month, so 4000 patient months should be observed. To allow for exclusion criteria, declining consent and loss to follow-up, the target should be closer to 5000. This equates to 420 patients over a year, or 280 patients over 18 months.

Although the focus of this study was not to calculate the effectiveness of the two drugs being used, the setup is such that it is able to calculate a difference in effectiveness of the two treatment groups. Previous audit work has shown that the effectiveness of ESA as a treatment at increasing haemoglobin is 80%. Aiming for 100 patients in each group, at a power of 80% with 95% confidence intervals, this study will be able to detect a difference of effectiveness of the treatment groups if iron is less than 62% effective. This will require 96 in each arm of the study.

## 2.11 Blood sampling

Study blood sampling was performed only on those who were randomised as part of the study protocol. Bloods were taken prior to receiving their allocated treatment, and for each of the subsequent two months. Blood was taken by nursing staff at the beginning of haemodialysis sessions prior to receiving either ESA or iron.

Ten millilitres (mls) of blood was taken; 3 mls in EDTA sample bottles and the remainder in heparinised sample bottles. The blood was immediately refrigerated. The heparinised sample underwent centrifuge at 3000 revolutions per minute for five minutes. The serum was then aliquoted into 0.5mls portions in

ependorfs. This was frozen and stored at -80°C. These samples are registered with the Imperial College Tissue Bank registration number Ren-DA-15-017.

## 2.12 Blood testing

### 2.12.1 Routine studies in haemodialysis patients

As part of routine clinical care, participants in the study had haemoglobin, C-reactive protein (CRP), ferritin, iron studies and albumin measured on a monthly basis. Parathyroid hormone (PTH) levels, folate and vitamin B12 were measured on a three-monthly basis.

### 2.12.2 Reticulocyte parameters

The EDTA sample was processed for a repeat haemoglobin and reticulocyte parameters by the author. These were measured using the Sysmex-XE 2100 analyser®. Haemoglobin reported in the study are the average between those in the monthly bloods and this second repeat samples.

The reticulocyte parameters of reticulocyte count and Ret-He are measured on the analyser by flow cytometry. Blood aspirated from the sample presented undergoes standardised dilution. A fluorescence marker, which binds to DNA present in reticulocytes, is then added to the diluted sample. This marked sample travels through a narrow flow cell into which a semiconductor laser beam is shone. By measurement of forward light scatter, side scattered light and side fluorescence light, the red cell parameters can be calculated.<sup>214</sup>

### 2.12.3 Hepcidin enzyme-linked immunosorbent assay (ELISA)

The ELISA assay was performed on stored serum samples in three batches along the duration of the study. The analysis was performed in conjunction with, and with the assistance of, the Department of Biochemistry, Imperial College. All samples were performed in duplicate, and analysis repeated if the calculated coefficient variant was greater than ten. The full methodology of the ELISA performed is found in appendix 4.

## 2.13 Treatment response

A positive treatment response was defined as an incremental increase in haemoglobin of greater than 5g/l within 2 months of treatment. Haemoglobins were taken in duplicate where possible: the first taken as part of routine monthly bloods and the second specifically for the study.

Using this as the outcome measure provided an important functional assessment of treatment efficacy. This approach, as well as effective in relating to the clinical question in the study, is a method adopted by other haemodialysis studies.<sup>215 216</sup>

Patients in whom the haemoglobin remained less than 105g/l following two months of treatment received the alternate medication with treatment response further measured two months later.

## 2.14 Summary

The individual patient pathway following a randomisation episode is demonstrated in Figure 15. The data collected following each randomisation is detailed within the appendix. Collated information is sourced from the combination of medical records, routine bloods taken as part of standard haemodialysis care and from additional bloods taken specifically for this research study.

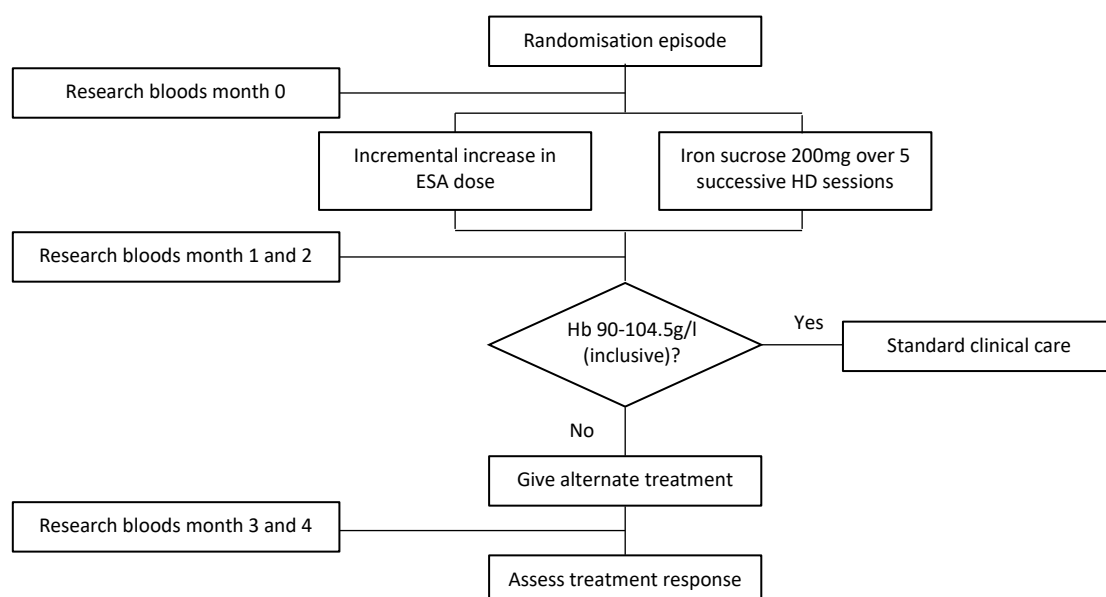


*Figure 15. Individual participant pathway following randomisation. At randomisation, 'month 0' bloods are taken prior to receiving either iron or ESA treatment. Additional research bloods are taken*

*the following two months in conjunction with the routine monthly bloods taken in all haemodialysis patients. Treatment outcome is recorded. For those whose haemoglobin remained within the randomisation criteria of between 90 and 104.5g/l and ferritin 100 to 800µg/l, the alternate treatment was given. Bloods were taken on the two successive months and treatment response was recorded. Those not within the randomisation criteria received standard clinical care as shown in Figure 14.*

## 2.15 Statistical analysis

Treatment episodes with iron and ESA were considered separately. Variables were assessed against the binary primary outcome of this study, treatment response.

For those continuous variables all data approximated to the normal distribution and therefore the means were reported. CRP and hepcidin levels underwent logarithmic transformation and were then able to approximate to the normal distribution. The independent samples t-test were used to compare the variable against the primary outcome. Chi squared testing was used to compare between groups of discrete variables.

Analysis of variance (ANOVA) testing was performed to assess the influence of individual biomarkers to treatment response. The biomarkers of value following ANOVA analysis underwent response and interaction modelling to determine its ability to differentiate between response to the two different treatment options.

Statistical analysis was performed using a combination of Microsoft Excel 2016 and SPSS version 23.0.

### 3 Results

The following section discusses the results of this study, starting with recruitment. The linear and site-specific recruitment are detailed with reference to the local population demographics. This is followed by the baseline demographics with reference to all randomisation episodes, the two different treatment groups, then subdivided into responders and non-responders of each of these treatments. Within the two different treatment groups, the trend in haemoglobin is reported prior to, and following, treatment.

The crucial aspect of the study is exploration of the baseline biomarkers in responders and non-responders to treatment in both treatment groups. These potentially important biomarkers are then further explored to identify as to whether they may have in differentiating between the response in the two different treatment groups.

#### 3.1 Recruitment

Following review of the study protocol by the lead consultants and head nurses of three satellite haemodialysis sites of the Imperial Renal and Transplant Centre, all centres agreed to participated in the study. These sites were chosen as they performed their routine haemodialysis bloods on different weeks of the month, making the logistics of a multi-site centre more feasible.

##### 3.1.1 Site specific recruitment

The sites from which participants were recruited were the haemodialysis units based at Watford General hospital (site 1), Ealing District General Hospital (site 2) and Northwick Park Hospital (site 3). These satellite centres are all run under the jurisdiction of Imperial Renal and Transplant Centre.

At sites 1 and 2, all established haemodialysis patients were considered for participation in the study. Site 3 is a large satellite site divided into five subsections, of which two were recruited from. Prior to recruitment, the current list of patients of the unit was reviewed by the head nurse who indicated which

patients were not suitable for participation based upon the study participation criteria (see sections 2.4 and 2.5).

The number of participants screened for this study excluded these individuals. Table 3 reports on numbers screened and subsequently enrolled in relation to the total size of the haemodialysis unit. The 'number screened' refers to those who were declared eligible by the lead nurse and were approached to participate. Recruitment for the study continued throughout its duration. This explains the relatively high number of patients screened.

|        | Total<br>number of<br>HD<br>patients | Number<br>screened | Number<br>recruited<br>(%) | Number<br>ineligible on<br>clinical<br>grounds (%) | Number<br>declined<br>(%) | Number<br>not able to<br>give<br>consent<br>(%) |
|--------|--------------------------------------|--------------------|----------------------------|----------------------------------------------------|---------------------------|-------------------------------------------------|
| Site 1 | 114                                  | 90                 | 84 (93.3)                  | 1 (1.1)                                            | 4 (4.4)                   | 1 (1.1)                                         |
| Site 2 | 122                                  | 95                 | 82 (86.3)                  | 0 (0)                                              | 9 (9.5)                   | 4 (4.2)                                         |
| Site 3 | 91                                   | 53                 | 30 (56.6)                  | 4 (7.5)                                            | 11 (20.8)                 | 8 (15.1)                                        |
| Total  | 327                                  | 238                | 196 (82.3)                 | 5 (2.1)                                            | 24 (10.1)                 | 13 (5.5)                                        |

*Table 3. Screening and recruitment data for each of the three study sites. Total number of patients stated is those within the unit at a given time, or for site 3 the number of patients in the subsections of the dialysis unit considered for the study. As per study protocol, new patients to each unit were eligible for study recruitment once established on haemodialysis for 3 months.*

Recruitment for the study started at site one in June 2015. Site two entered the study in August 2015 and site three in November 2015. The monthly numbers and cumulative total of the number recruited in the study is shown below in Figure 16.

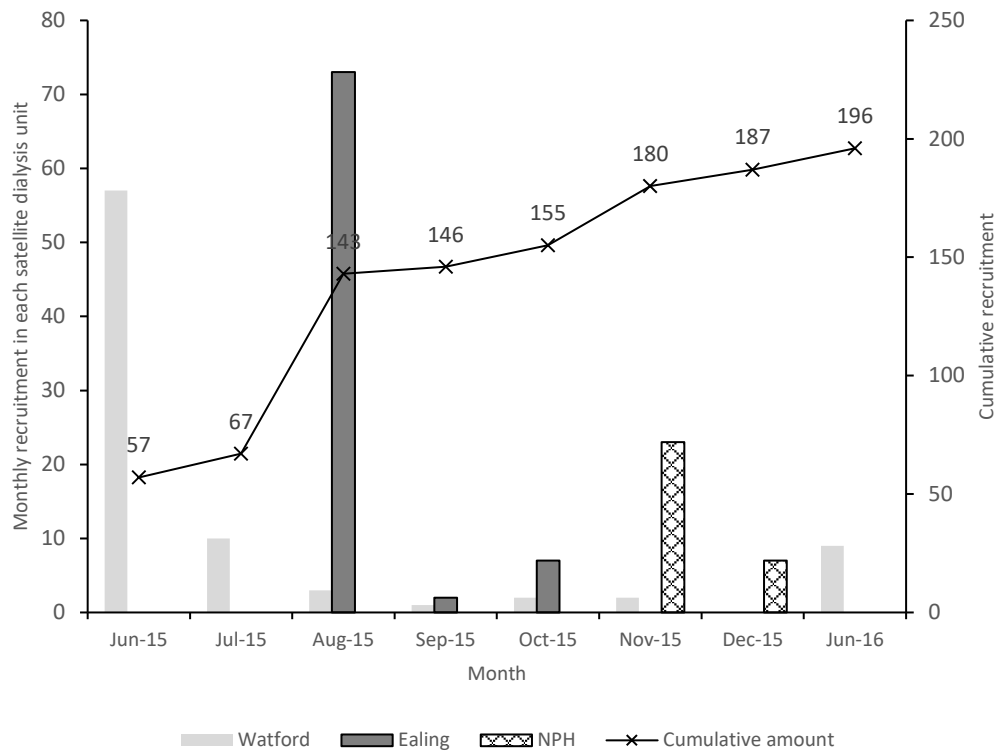


Figure 16. Site specific and cumulative monthly recruitment data across the duration of the study. Recruitment started at site 1 and with continual investigator presence on site, recruitment was rapid with 57 recruited in the first month. Once the study was established at the first site, focus switched to site 2 where 73 were recruited in the first month. Ongoing low-level recruitment continued throughout the duration of the study.

### 3.2 Baseline characteristics of recruited patients

In total, this study recruited 196 participants. Table 4 reports the baseline characteristics of the group both in its entirety and subdivided according to site.

|                           | Whole group      | Site 1 (Watford General Hospital) | Site 2 (Ealing District General Hospital) | Site 3 (Northwick Park Hospital) |
|---------------------------|------------------|-----------------------------------|-------------------------------------------|----------------------------------|
| Number                    | 196              | 86                                | 82                                        | 28                               |
| Age (years)               | 64.3 (25-91)     | 65.1 (32-91)                      | 62.5 (25-89)                              | 65.8 (34-83)                     |
| Male gender (%)           | 137 (69.9%)      | 58 (67.4%)                        | 60 (73.2%)                                | 19 (67.9%)                       |
| Ethnicity                 |                  |                                   |                                           |                                  |
| White                     | 93 (47.4%)       | 66 (76.7%)                        | 19 (23.2%)                                | 8 (28.6%)                        |
| Asian                     | 75 (45.2%)       | 12 (14.0%)                        | 49 (59.8%)                                | 14 (50.0%)                       |
| Black                     | 24 (12.2%)       | 5 (5.8%)                          | 14 (17.1%)                                | 5 (17.9%)                        |
| Other                     | 4 (2.0%)         | 3 (3.5%)                          | 0 (0%)                                    | 1 (3.6%)                         |
| Dialysis vintage (years)  | 3.77 (0-22)      | 2.73 (0-17)                       | 4.28 (0-22)                               | 5.39 (0-22)                      |
| Previous transplant       | 29 (14.8%)       | 13 (15.1%)                        | 10 (12.2%)                                | 6 (21.4%)                        |
| PTH (pmol/l) at enrolment | 63.8 (54.1-73.5) | 57.1 (40.7-73.4)                  | 73.0 (58.1-87.9)                          | 57.1 (41.1-73.1)                 |
| Co-morbidities            |                  |                                   |                                           |                                  |
| Diabetes                  | 87 (44.4%)       | 27 (31.4%)                        | 42 (51.2%)                                | 18 (64.3%)                       |
| Viral liver disease       | 5 (2.6%)         | 2 (2.3%)                          | 2 (2.4%)                                  | 1 (3.6%)                         |

|                         |            |            |            |            |
|-------------------------|------------|------------|------------|------------|
| Previous vascular event | 74 (37.8%) | 27 (31.4%) | 35 (42.7%) | 12 (42.9%) |
| Valve replacement       | 7 (3.6%)   | 2 (2.3%)   | 0 (0%)     | 5 (17.9%)  |

Table 4. The baseline demographics and co-morbidities of the participants in the study. These were largely the same between the different sites used within the study. Ethnicity however did differ between the sites, with site 1 having a predominantly white population but Asian population contributing a significantly higher proportion in the other sites. Sites 2 and 3 also had more diabetics within their population. Continuous data is reported as a mean with either the range, for age and dialysis, or standard deviation, bracketed. Discrete data is reported in conjunction with the percentages in brackets.

### 3.2.1 Demographics of the local areas that each dialysis unit serves

The following is to provide a local population comparator for the baseline characteristics of those recruited to the study. The following describes the difference in the age and ethnicity demographics of the local area for which each satellite dialysis unit serve. Site 1 serves a largely White population whereas sites 2 and 3 serve a largely Asian population.<sup>217</sup>

|          | Age demographics |                 |                 |                 |               | Ethnicity |           |           |           |
|----------|------------------|-----------------|-----------------|-----------------|---------------|-----------|-----------|-----------|-----------|
|          | <18 years (%)    | 18-44 years (%) | 45-59 years (%) | 60-74 years (%) | 75+ years (%) | White (%) | Asian (%) | Black (%) | Other (%) |
| Site 1   | 22.86            | 42.94           | 16.87           | 10.91           | 6.4           | 72.0%     | 18.0%     | 7.4%      | 2.6%      |
| Site 2   | 22.6             | 45.1            | 17.3            | 9.9             | 5             | 49.0%     | 29.8%     | 12.7%     | 8.5%      |
| Site 3   | 23.0             | 39.30           | 18.6            | 12.3            | 6.8           | 42.3%     | 43.0%     | 9.6%      | 5.2%      |
| National | 21.4             | 36.9            | 19.4            | 14.6            | 7.8           | 85.5%     | 7.6%      | 4.5%      | 2.2%      |

*Table 5. Demographic and ethnicity for the Local Authority within which each dialysis unit is located, with the data sourced from the Office of National Statistics. The higher proportion of white individuals at site 1 and Asian at sites 2 and 3 reflects the proportions seen within the general population. Total percentages within the table may not equal 100% due to rounding.*

### 3.3 Randomisation episodes

Over the 18 months of the study, there were 2423 observed patient months. This resulted in 185 randomisation episodes between 117 patients. Of these 185 episodes, 160 (86.5%) achieved an outcome and therefore analysable in the per protocol format of this study. The distribution of these episodes over the duration of the study is shown in Figure 17.

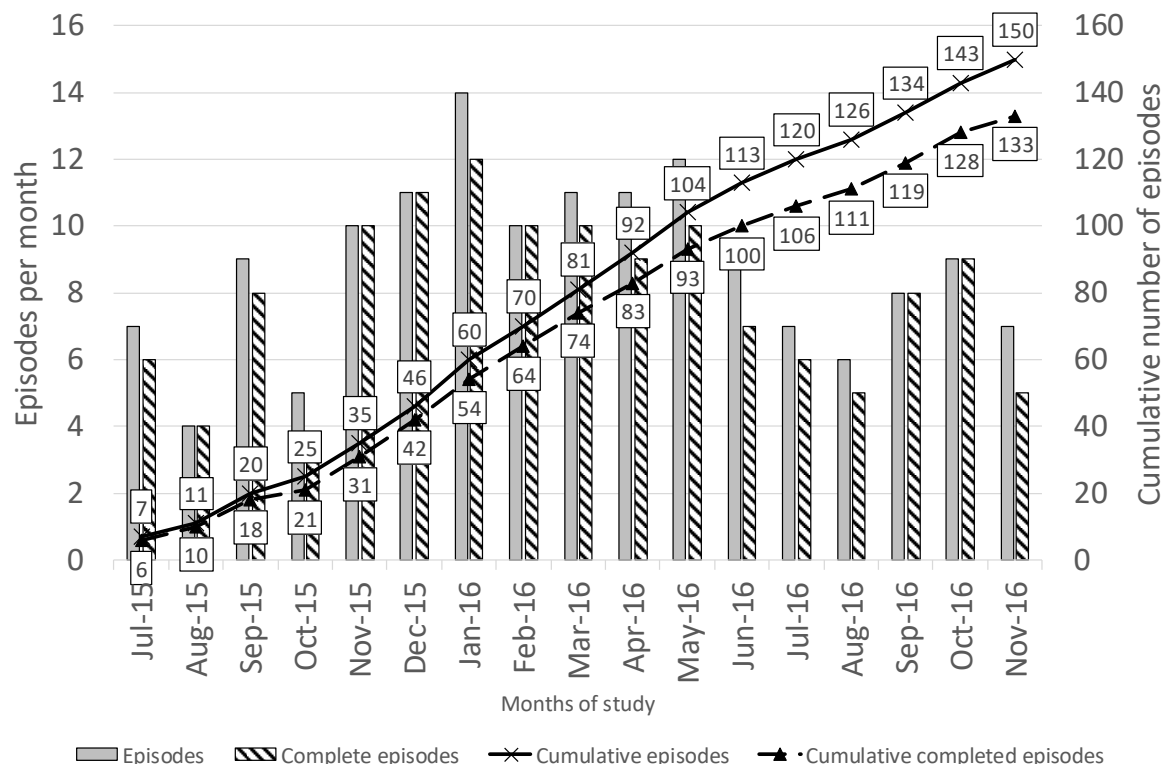


Figure 17. Monthly randomisation episodes, with a cumulative total, throughout the duration of the study. This only includes the randomisation, where individuals received the first part of the treatment, and not all treatment episodes thereby not including those that went on to receive the alternate treatment. Both completed and incomplete episodes are shown in the graph. Most incomplete treatment episodes are due to intercurrent illness or transplantation and this is described in more detail in figure 18.

A flow chart with each of the randomisation episodes and whether they achieved an outcome is shown in Figure 18. Following monthly bloods and subsequent screening for intercurrent illness, the haemoglobin randomisation threshold was met 179 times. Of these, 150 (83.8%) were randomised,

with the remaining 29 outside the ferritin range for randomisation. Seventy-four were randomised to receiving ESA and 76 to iron. One hundred and thirty-three people (88.7%) reached the two month follow up period with intercurrent illness and transplantation the two main reasons for non-completion.

Those who went on to receive the alternate treatment had a haemoglobin within the randomisation threshold of 90 to 104g/l which included both responders and non-responders to the first treatment. Of the 34 that received the alternate treatment, 25 had been non-responders to treatment and a further 9 who met the haemoglobin threshold.

In total, 160 out of 184 (87.0%) randomisation episodes met the two month follow up period required in the study for the per-protocol basis. Ten episodes of intercurrent illness (41.7% of total), four transplants (16.7%) and five protocol deviations (20.8%) were the chief contributors to non-completion of a randomisation episode. The rest are detailed as part of Figure 18, with the one death due to an out of hospital cardiac arrest presumed secondary to ischaemic heart disease.

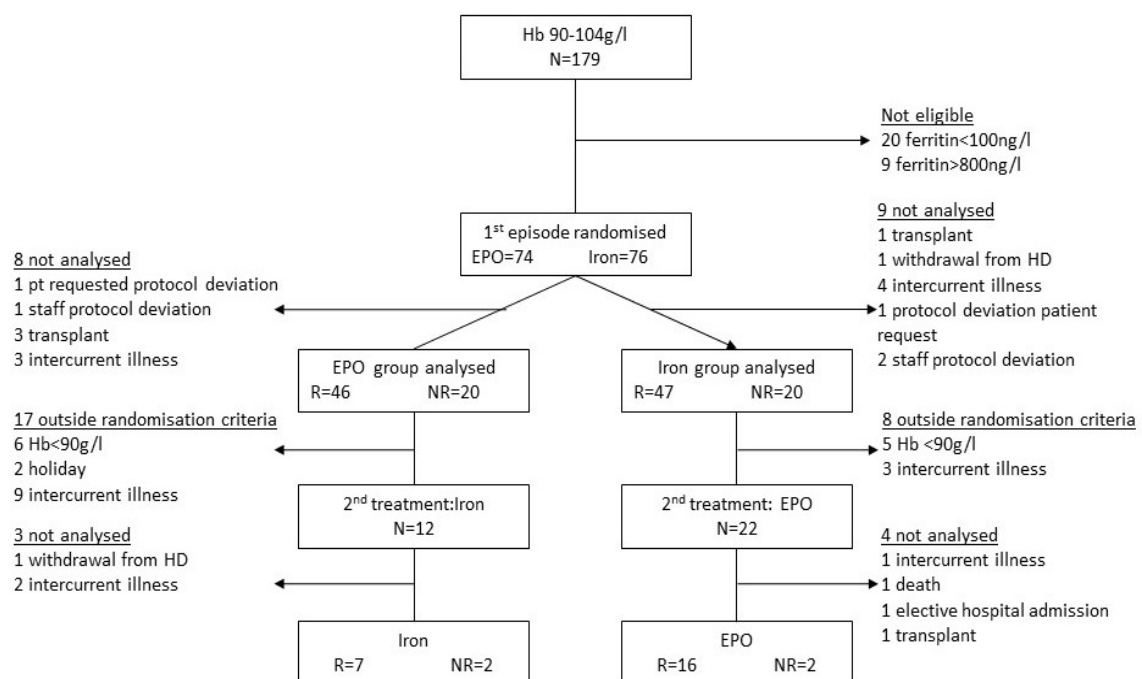


Figure 18. Flowchart of number of randomisation episodes throughout the duration of the study, and the follow up thereof. Following monthly bloods, there were 179 occasions of potential

randomisation in which haemoglobin was between 90 and 104g/l. On 29 of these, ferritin was outside the randomisation criteria. This led to randomisation, on a 1:1 basis, in 150 cases, with 74 receiving EPO and 76 iron. Sixty-six cases were analysed, with 46 (60.5%) having a positive treatment outcome. From the EPO group, 12 subsequently received iron. This group consisted of both responders to EPO whose haemoglobin persisted beneath 105g/l and non-responders. Intercurrent illness which had not been apparent earlier in the course of the randomisation and treatment episode was the main reason for individuals not receiving the alternate iron treatment as per protocol. Response rate following treatment with iron, as the second line agent, was positive with 2/9 (77.8%) responding. In the 76 individuals initially treated with iron, there was a positive treatment response in 47/67 (70.1%). Twenty-two individuals initially treated with iron then went on to receive EPO, with a positive response to treatment in 16/18 (88.9%) individuals.

### 3.3.1 ESA versus iron therapy

Table 6 shows the trend in haemoglobin over time in the different time points of the study within the two different treatment groups. The mean haemoglobin between the two different treatment groups does not significantly differ throughout the duration of the study. The lack of difference prior to, and at the point of, randomisation validates the integrity of the randomisation process. Following treatment, the lack of difference in the haemoglobin between the two groups supports the equality of the two different treatment options.

|      | Hb (g/l) 2 months prior to randomisation | Hb (g/l) 1 month prior to randomisation | Hb (g/l) at randomisation | Hb (g/l) 1 month post randomisation | Hb (g/l) 2 months post randomisation |
|------|------------------------------------------|-----------------------------------------|---------------------------|-------------------------------------|--------------------------------------|
| ESA  | 110.07<br>(108.67-113.47)                | 107.93<br>(106.05-109.80)               | 97.33<br>(96.35-98.31)    | 102.47<br>(100.55-104.39)           | 105.79<br>(101.85-109.74)            |
| Iron | 110.77<br>(108.47-113.08)                | 107.88<br>(106.07-109.68)               | 98.40<br>(97.52-99.28)    | 103.99<br>(102.18-105.81)           | 106.16<br>(103.60-108.71)            |

|          |       |       |       |       |       |
|----------|-------|-------|-------|-------|-------|
| <i>p</i> |       |       |       |       |       |
| values   | 0.859 | 0.969 | 0.109 | 0.252 | 0.877 |

Table 6. Monthly haemoglobins prior to and following randomisation. This shows that the mean haemoglobins of individuals randomised to ESA and iron does not differ, both in the lead up to and following treatment. This table charts the mean haemoglobin alongside the 95% confidence interval prior to and following treatment with both iron and ESA, with unpaired t-test used to calculate difference between groups at each respective time point.

Figure 19 further reinforces the findings of Table 6, that ESA and iron are equally efficacious treatments and provides a clearer demonstration of trend in haemoglobin over time. This separates out the haemoglobin at all time points within the study and differentiates between those that were treated as the first part of the treatment episode and those who went on to receive the subsequent treatment. Both following the initial treatment and the alternate treatment in the two different treatment groups, haemoglobin did not significantly differ regardless of the order in which treatment was received.

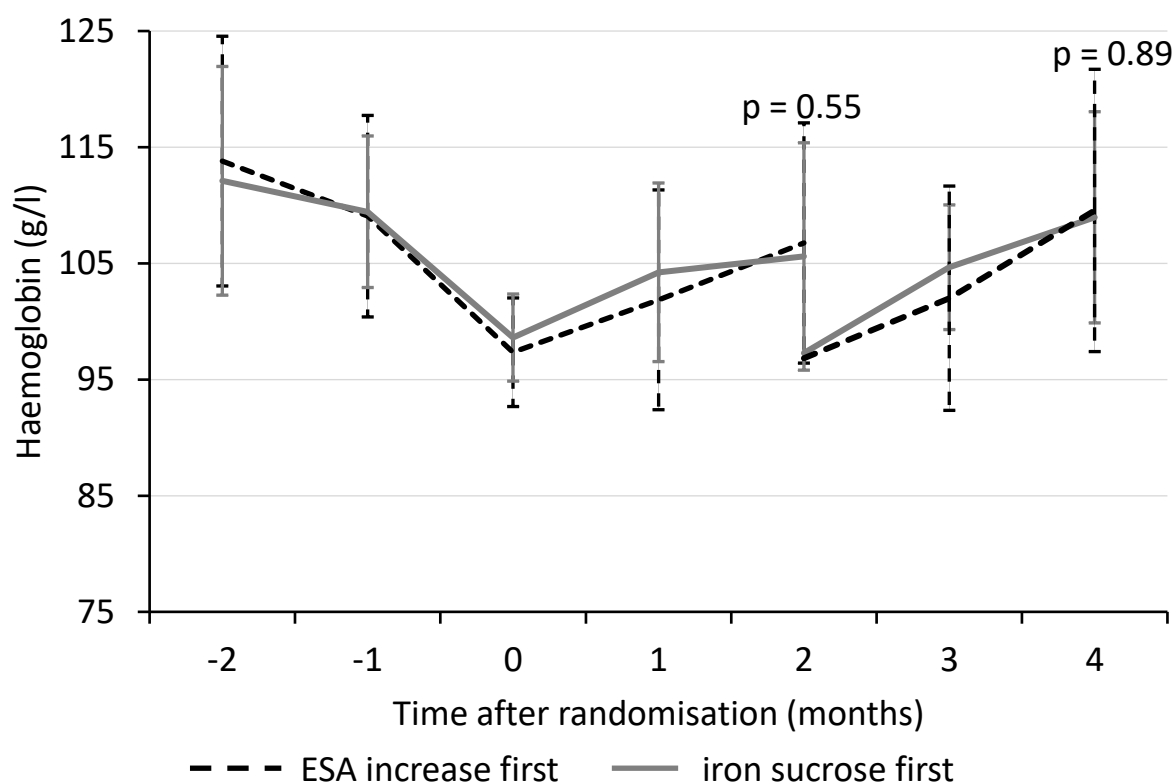


*Figure 19. Haemoglobin change prior to and following randomisation in the two different treatment groups. Mean of each group is plotted with standard error bars. There is no significant difference in haemoglobin between the two different groups both prior to randomisation or following randomisation.*

### 3.3.2 Response pattern in each treatment group for responders and non-responders

Following the 160 randomisation episodes, 114 (71.3%) had a positive response to treatment. As shown in Table 7, there was no difference in success between the different treatments. Out of the 84 recipients of ESA, 61 (72.6%) responded to treatment compared to 53 (69.7%) out of 76 responding to iron ( $p=0.687$ ).

|      | Positive response to treatment | No response to treatment |
|------|--------------------------------|--------------------------|
| ESA  | 61 (72.6%)                     | 23 (27.4%)               |
| Iron | 53 (69.7%)                     | 23 (30.3%)               |

*Table 7. Response rate in all individuals treated with either iron or ESA. The numbers of responders and non-responders are charted with their respective percentages in brackets. A positive response to treatment was defined as a haemoglobin increase of greater than 5g/l within two months of treatment. This table does not differentiate between those that had received the treatment as the first or second part of a treatment episode. It does however clearly demonstrate that the response rates to both treatments are largely equivalent.*

Table 8 reviews the response rate following first treatment and those who went on to subsequently receive the alternate treatment. This highlights the similar response rates to both ESA and iron, when used as first line treatments. Although a higher proportion seem to respond to ESA as a second line

treatment, numbers involved are small. The use of sequential treatments is discussed further in section 4.5 .

|                     |                              | Randomised |           | Opposite treatment in NR |          |
|---------------------|------------------------------|------------|-----------|--------------------------|----------|
|                     |                              | ESA        | Iron      | ESA                      | Iron     |
| N analysed          |                              | 66         | 67        | 18                       | 9        |
| Responders          | With Hb $\geq 105\text{g/l}$ | 37 (56.1)  | 37 (55.2) | 13 (72.2)                | 6 (66.7) |
|                     | With Hb $< 105\text{g/l}$    | 9 (13.6)   | 10 (14.9) | 3 (16.7)                 | 1 (11.1) |
| Non-responders (NR) | With Hb $\geq 90\text{g/l}$  | 14 (21.2)  | 15 (22.4) | 1 (5.6)                  | 2 (22.2) |
|                     | With Hb $< 90\text{g/l}$     | 6 (9.1)    | 5 (8.3)   | 1 (5.6)                  | 0 (0)    |

*Table 8. Response rate to the two studied medications, ESA and iron, subdivided into those who were receiving their first treatment and those who were receiving the alternate treatment following failure to respond to the first treatment. Response and non-response further divided according to haemoglobin following treatment. Absolute numbers of individuals are charted, with percentages in brackets. The proportion who respond to treatment but still have a suboptimal haemoglobin is equivalent amongst the different treatment groups; as is the proportion for whom the haemoglobin falls below 90g/l despite treatment with either iron or ESA. The most striking difference demonstrated in the table is that a greater proportion of individuals responded to their second treatment, be it either ESA or iron.*

### 3.4 Factors predictive of treatment response

#### 3.4.1 Baseline characteristics of those randomised to iron or ESA

The demographics and baseline characteristics of the individuals who completed randomisation episodes, grouped according to treatment received, are shown in Table 9 below. The lack of difference between the two groups reinforces the validity of the randomisation method used within the study.

|                           | ESA                    | Iron                   | <i>p value</i> |
|---------------------------|------------------------|------------------------|----------------|
| Randomisation episodes    | 84                     | 76                     |                |
| Age (years)               | 63.12<br>(25.1-91.3)   | 63.22<br>(60.11-66.34) | 0.963          |
| Gender                    | 60 (71.4%) male        | 54 (71.1%) male        | 1.000          |
| Ethnicity                 |                        |                        | 0.792          |
| White (%)                 | 41 (48.8%)             | 34 (44.7%)             |                |
| Asian (%)                 | 28 (33.3%)             | 31 (40.8%)             |                |
| Black (%)                 | 12 (14.2%)             | 9 (11.8%)              |                |
| Other (%)                 | 3 (3.6%)               | 2 (2.6%)               |                |
| Dialysis vintage (years)  | 3.56<br>(0-22)         | 3.97<br>(2.80-5.15)    | 0.607          |
| Previous transplant (%)   | 14 (16.7%)             | 18 (23.4%)             | 0.324          |
| PTH (pmol/l) at enrolment | 63.67<br>(46.47-80.86) | 62.52<br>(48.70-76.34) | 0.918          |
| Co-morbidities            |                        |                        |                |
| Diabetes                  | 35 (41.7%)             | 33 (43.4%)             | 0.873          |

|                         |            |            |       |
|-------------------------|------------|------------|-------|
| Viral liver disease     | 3 (3.6%)   | 1 (1.3%)   | 0.622 |
| Previous vascular event | 30 (35.7%) | 29 (38.2%) | 0.870 |
| Valve replacement       | 2 (2.4%)   | 2 (2.6%)   | 1.000 |

*Table 9. Baseline demographics and characteristics of those that received either ESA or iron treatment. This demonstrated equivalence between the two different groups within all baseline demographic data. Continuous data is reported along with standard deviation, or range, and discrete with percentages bracketed. P values are from t-testing and chi-square testing respectively.*

### 3.4.2 Baseline biochemical characteristics

#### *Baseline biochemical characteristics of all treatment episodes*

Table 10 charts the baseline biomarkers and ESA dose in the two different treatment groups at the time of randomisation. The biomarkers at baseline do not differ significantly between the two different treatment groups aside from in baseline ESA dose, ferritin and hepcidin. This may be explained due to the study design in which the two treatments were administered sequentially. The first treatment may have affected the baseline biomarker for the second episode. Table 11 will further test this hypothesis by restricting the included baseline biomarkers to the first episode only. Overall, the similarity in the baseline characteristics of the two groups in Table 10 gives justification in analysing the first and second treatment episodes together. The increase in analysable treatment episodes by doing this is offset against the impact on the first treatment in the baseline biomarkers of the second episode but deemed acceptable by the author.

|                          | ESA group (84)  | Iron group (76) | <i>p value</i> |
|--------------------------|-----------------|-----------------|----------------|
| ESA dose (units/session) | 2438.5          | 3276.3          | 0.045          |
|                          | (1973.0-2904.0) | (2596.5-3956.1) |                |

|                                          |                         |                          |        |
|------------------------------------------|-------------------------|--------------------------|--------|
| Hb (g/l) at time of randomisation        | 97.33<br>(96.35-98.31)  | 98.40<br>(97.52-99.28)   | 0.109  |
| MCV (fl)                                 | 93.39<br>(91.90-94.89)  | 91.72<br>(90.06-93.38)   | 0.137  |
| Reticulocyte count (x10 <sup>9</sup> /l) | 52.19<br>(46.79-57.60)  | 49.4<br>(43.94-54.95)    | 0.479  |
| Ret-He (pg)                              | 35.33<br>(34.58-36.08)  | 34.30<br>(33.49-34.10)   | 0.064  |
| Ferritin (µg/l)                          | 338.32<br>(298.0-378.7) | 249.3<br>(222.1-276.5)   | 0.000  |
| Hepcidin (µg/ml)                         | 79.03<br>(68.33-89.72)  | 102.09<br>(87.11-117.08) | 0.014  |
| Albumin (g/l)                            | 34.99<br>(33.92-36.05)  | 34.15<br>(33.05-35.24)   | 0.275  |
| CRP (mg/l)                               | 17.42<br>(11.03-23.82)  | 24.61<br>(15.73-33.50)   | 0.193  |
| Transferrin (g/l)                        | 1.82<br>(1.59-2.06)     | 1.69<br>(1.61-1.77)      | 0.316  |
| Transferrin saturations (%)              | 27.95<br>(25.10-27.95)  | 26.31<br>(23.78-28.84)   | 0.407  |
| Serum iron (µmol/l)                      | 13.30                   | 11.18                    | 0.0812 |

|                               |                        |                        |       |
|-------------------------------|------------------------|------------------------|-------|
|                               | (11.32-15.28)          | (10.01-12.34)          |       |
| B12 (pg/ml)                   | 494.0<br>(428.0-560.0) | 449.4<br>(380.5-518.3) | 0.931 |
| Folate (ng/ml)                | 8.11<br>(6.71-9.50)    | 12.43<br>(3.82-21.04)  | 0.327 |
| Warfarin                      | 6 (6.1%)               | 7 (9.2%)               | 0.774 |
| Number of antiplatelet agents |                        |                        |       |
|                               | 43 (51.2%)             | 37 (48.6%)             | 0.882 |
| 0 antiplatelet agents         | 31 (36.9%)             | 28 (36.8%)             |       |
| 1 antiplatelet agent          | 10 (11.9%)             | 11 (14.5%)             |       |
| 2 antiplatelet agents         |                        |                        |       |

*Table 10. Mean baseline characteristics of the two different treatment groups. This includes both first treatment given following randomisation and the alternate treatment given to those in whom the haemoglobin was still within the randomisation criteria. Those treated with ESA had a lower baseline ESA dose and hepcidin level at randomisation. Those treated with iron had lower ferritin and Ret-He. These differences may be accountable for by the sequential nature of treatments given to those that did not respond to the first treatment. Therefore, the following table will evaluate the baseline characteristics in just the first treatment episode. All continuous data in the table above is shown as a mean with 95% confidence intervals with p value calculated by t-testing. For discrete data, absolute values are tabulated with percentages in brackets with chi-square testing used to calculate p-values.*

#### *Baseline biochemical characteristics of first treatment episodes*

Restricting analysis to the first treatment episode would negate any effect that treatment may have beyond the two-month period. This has to be offset against the larger number of episodes available if

the episodes following alternate treatment are included in analysis. Table 10 and Table 11 report the baseline characteristics for all randomisation episodes, and just the first treatment episode, respectively. The similarity in these baseline characteristics provided reassurance that including all episodes should not affect the overall validity of the results of this study.

|                                        | ESA group (66)            | Iron group (67)           | <i>p</i> value |
|----------------------------------------|---------------------------|---------------------------|----------------|
| ESA dose (units/session)               | 2447.0<br>(1915.7-2978.3) | 3089.5<br>(2398.3-3780.8) | 0.144          |
| Hb (g/l) at time of randomisation      | 97.35<br>(96.20-98.50)    | 98.61<br>(97.70-99.53)    | 0.088          |
| MCV (fl)                               | 92.90<br>(91.33-94.48)    | 92.09<br>(90.30-93.88)    | 0.493          |
| Reticulocyte count ( $\times 10^9/l$ ) | 51.80<br>(45.28-58.33)    | 47.35<br>(41.56-53.13)    | 0.309          |
| Ret-He (pg)                            | 35.20<br>(34.37-36.02)    | 34.50<br>(33.61-35.39)    | 0.251          |
| Ferritin ( $\mu g/l$ )                 | 301.2<br>(261.4-341.0)    | 260.0<br>(231.4-288.6)    | 0.096          |
| Hepcidin ( $\mu g/ml$ )                | 79.31<br>(66.96-91.66)    | 101.92<br>(87.89-115.95)  | 0.02           |
| Albumin (g/l)                          | 34.79<br>(33.60-35.97)    | 34.38<br>(33.31-35.45)    | 0.610          |

|                               |                        |                         |       |
|-------------------------------|------------------------|-------------------------|-------|
| CRP (mg/l)                    | 19.41<br>(11.36-27.47) | 22.35<br>(13.51-31.20)  | 0.624 |
| Transferrin (g/l)             | 1.82<br>(1.60-2.04)    | 1.66<br>(1.55-1.76)     | 0.175 |
| Transferrin saturations (%)   | 22.93<br>(19.77-26.08) | 23.63<br>(20.07-27.18)  | 0.764 |
| Serum iron (μmol/l)           | 12.82<br>(7.72-17.93)  | 9.84<br>(8.33-11.35)    | 0.260 |
| B12 (pg/ml)                   | 478.2<br>(410.6-545.9) | 448.2<br>(372.9-523.4)  | 0.554 |
| Folate (ng/ml)                | 8.40<br>(6.76-10.03)   | 13.07<br>(372.9-523.44) | 0.350 |
| Warfarin                      | 5 (7.6%)               | 6 (9.0%)                | 1.000 |
| Number of antiplatelet agents |                        |                         |       |
|                               | 32 (48.5%)             | 36 (53.4%)              | 0.766 |
| 0 antiplatelet agents         | 26 (39.4%)             | 25 (37.3%)              |       |
| 1 antiplatelet agent          | 8 (12.1%)              | 6 (9.0%)                |       |
| 2 antiplatelet agents         |                        |                         |       |

Table 11. Baseline characteristics of the 133 treatment episodes in which patients were receiving their first treatment, within the six-month time frame determined by the study protocol. Most of the differences found in table 10 between the two different treatment groups have been eliminated by exclusion of any sequential treatments. There does however remain a significantly lower hepcidin level in those who went on to receive ESA treatment. The table reports continuous data as means

*with accompanying 95% confidence intervals; discrete data is presented as absolute numbers with percentages bracketed. P values are from t-testing and chi-square testing respectively.*

Despite restriction of the baseline biomarkers to those that were receiving their first treatment only, there were still significantly lower hepcidin levels in the group treated with ESA therapy. One possible explanation for this is the lower baseline ESA dose in the ESA group. The need to be on higher doses of ESA at baseline is associated with the pro-inflammatory state, and inflammation is an important upregulator of hepcidin levels, as described previously in section 1.7.1.

### 3.4.3 Analysis of completed episodes

The principle findings of this study are reported in the section below. First, the raw data is presented for the ESA and iron groups separately, comparing biomarker values in responders and non-responders to treatment. Section 3.7.2 works through the translation of the raw data into answering the clinical question of identification of a suitable biomarker predictive of response to ESA and iron treatment.

### 3.5 ESA group

#### 3.5.1 Haemoglobin response following treatment with ESA therapy

Figure 20 follows the mean trend over time in haemoglobin between those who went on to respond to a stepwise increase in ESA therapy versus those who failed to respond. Those who failed to respond to treatment had a steeper decline in haemoglobin prior to treatment although there was not a significant difference in haemoglobin between responders and non-responders.

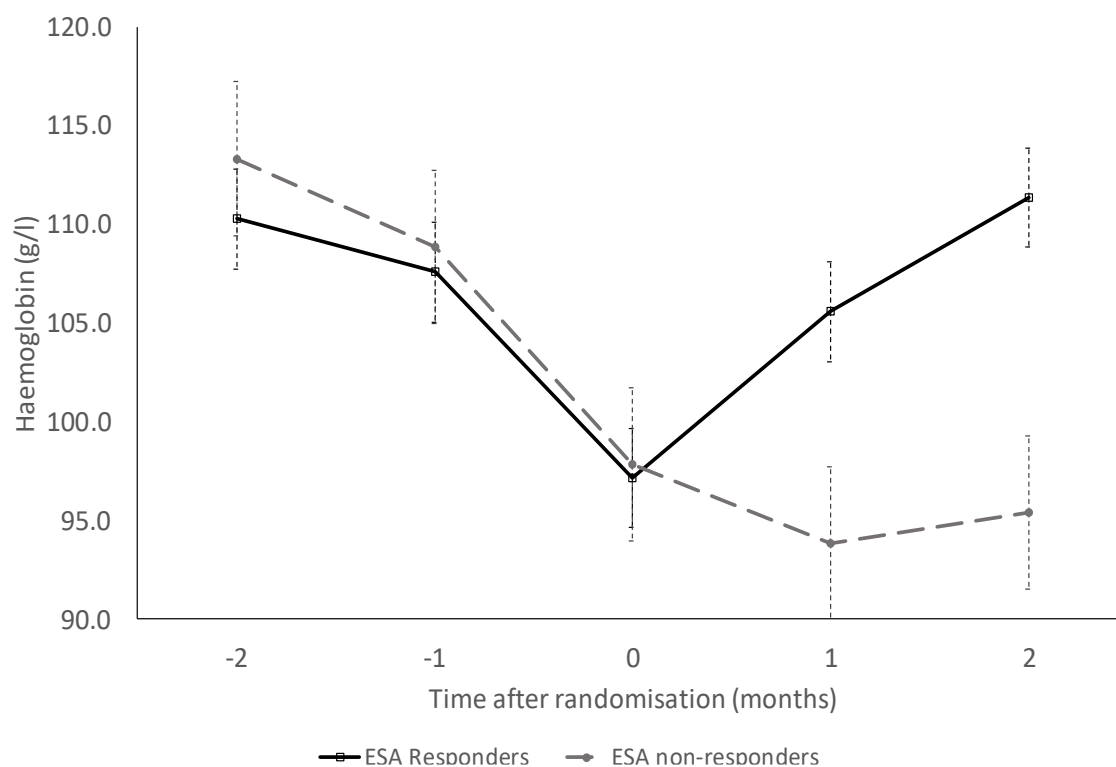


Figure 20. Haemoglobin change in responders and non-responders to ESA therapy prior to and following treatment. A marked haemoglobin increase in responders occurs within the first month of treatment, and the continued increase in haemoglobin continues for a further month. Those who fail to respond continue to have a decline in haemoglobin following treatment which plateaus in the following month.

#### 3.5.2 Baseline demographics and co-morbidities of responders versus non-responders to ESA

Differences between the baseline demographics and characteristics were compared between responders and non-responders to ESA treatment. An interesting observation was that those who had

received a previous renal transplant were more responsive to ESA as shown in Table 12. Previous studies have shown that a failed renal transplant does result in erythropoietin resistance and higher ESA requirements<sup>218</sup>, but the impact of a dose change has not been described before. Given that there were only 14 individuals with a failed renal transplant randomised to ESA included within the study, the significance of this is unknown but merits further evaluation. Aside from this, there were no differences in the baseline characteristics between the two different treatment groups.

|                                           | Responders       | Non-responders    | P value |
|-------------------------------------------|------------------|-------------------|---------|
| Numbers                                   | 62 (73.8%)       | 22 (26.1%)        |         |
| Age (years)                               | 64.2 (31-91)     | 60.1 (25-89)      | 0.278   |
| Male gender (%)                           | 47 (75.8%)       | 13 (5.1%)         | 0.136   |
| Ethnicity                                 |                  |                   |         |
| White (%)                                 | 29 (46.8%)       | 12 (54.5%)        | 0.978   |
| Asian (%)                                 | 21 (33.9%)       | 7 (31.8%)         |         |
| Black (%)                                 | 9 (14.5%)        | 3 (13.6%)         |         |
| Other (%)                                 | 3 (4.8%)         | 0 (0%)            |         |
| Dialysis vintage (years)                  | 3.92 (0-22)      | 2.54 (0-12)       | 0.270   |
| Previous transplant (%)                   | 14 (22.6%)       | 0 (0%)            | 0.050   |
| PTH (pmol/l) at enrolment<br>in the study | 55.2 (41.1-69.4) | 87.4 (33.1-141.8) | 0.102   |
| Comorbidities                             |                  |                   |         |
| Diabetes (%)                              | 27 (43.5%)       | 8 (36.4%)         | 0.563   |

|                                         |            |           |       |
|-----------------------------------------|------------|-----------|-------|
| Vascular disease (IHD, PVD, stroke) (%) | 22 (35.5%) | 8 (36.4%) | 0.941 |
| Viral liver disease (%)                 | 3 (4.8%)   | 0 (0%)    | 0.299 |
| Valve replacement (%)                   | 1 (1.6%)   | 1(4.5%)   | 0.438 |

*Table 12. Baseline demographics and characteristics in those treated with ESA divided according to subsequent response to ESA. The only significant difference between the two groups is that those with a previous transplant were significantly more likely to respond to ESA treatment. The table reports continuous data as means with accompanying 95% confidence intervals; discrete data is presented as absolute numbers with percentages bracketed. Statistical significance is assessed by t-test for continuous variables or chi-squared testing for discrete.*

### 3.5.3 Biomarkers and medications predicting treatment response

There is no single biomarker that is able to definitively predict response to ESA. A lower CRP has the strongest association with a positive response to treatment. The mean CRP in responders to ESA treatment is 13.46mg/l versus 28.59mg/l in non-responders ( $p=0.038$ ). A lower baseline ESA dose prior to receiving treatment also favoured a positive treatment response (2529.5 versus 2913.0 units/session,  $p=0.243$ ). Although a higher serum B12 cannot technically be considered to be a predictor of treatment response as it is modifiable by treatment, it was notable that responders to ESA therapy had a higher serum B12 level than those that did not respond (531.3 vs 389.0 pg/ml,  $p=0.059$ ). The importance of this finding is discussed in section 4.4.1.

| Predictor                         | Responders (62) | Non-responders (22) | p value |
|-----------------------------------|-----------------|---------------------|---------|
| Baseline ESA dose (units/session) | 2259.5 (62)     | 2913.0 (22)         | 0.243   |
| MCV (fl)                          | 92.99 (62)      | 94.53 (22)          | 0.371   |

|                                           |                             |                              |       |
|-------------------------------------------|-----------------------------|------------------------------|-------|
|                                           | (91.24-94.73)               | (92.84-96.22)                |       |
| Reticulocyte count<br>( $\times 10^9/l$ ) | 50.83 (62)<br>(44.82-56.67) | 56.02 (22)<br>(50.19-61.86)  | 0.404 |
| Ret-He (pg)                               | 35.35 (62)<br>(34.64-36.05) | 35.28 (22)<br>(34.57-35.99)  | 0.942 |
| Ferritin ( $\mu g/l$ )                    | 354.5 (62)<br>(306.3-402.7) | 292.68 (22)<br>(244.5-340.9) | 0.182 |
| Hepcidin ( $\mu g/ml$ )                   | 75.80 (61)<br>(64.96-86.64) | 87.98 (22)<br>(77.14-98.82)  | 0.330 |
| Albumin (g/l)                             | 35.26 (62)<br>(34.13-36.38) | 34.23 (22)<br>(33.10-35.35)  | 0.400 |
| CRP (mg/l)                                | 13.46 (62)<br>(8.01-18.91)  | 28.59 (22)<br>(23.14-34.04)  | 0.038 |
| Transferrin (g/l)                         | 1.90 (61)<br>(1.59-2.21)    | 1.60 (22)<br>(1.28-1.91)     | 0.293 |
| Transferrin<br>saturations (%)            | 26.90 (60)<br>(24.26-29.54) | 30.82 (22)<br>(28.18-33.46)  | 0.174 |
| Iron ( $\mu mol/l$ )                      | 12.11 (60)<br>(10.98-13.24) | 13.68 (21)<br>(12.55-14.80)  | 0.226 |
| B12 (pg/ml)                               | 531.3 (62)<br>(448.6-614.0) | 389.0 (22)<br>(306.3-471.7)  | 0.059 |

|                |                           |                          |       |
|----------------|---------------------------|--------------------------|-------|
| Folate (ng/ml) | 8.71 (62)<br>(7.07-10.30) | 6.50 (22)<br>(4.86-8.09) | 0.167 |
| Warfarin       | 5 (8.0%)                  | 1 (4.5%)                 | 0.582 |
| Anti-platelets |                           |                          | 0.547 |
| 0              | 32 (51.6%)                | 11 (50.0%)               |       |
| 1              | 24 (38.7%)                | 7 (31.8%)                |       |
| 2              | 6 (9.7%)                  | 4 (18.2%)                |       |

*Table 13. Biomarkers and medications in those treated with ESA, divided according to treatment response. Those who had a lower CRP at baseline was the strongest factor differentiating between those who responded and did not respond to treatment. A higher B12 level, higher folate level, higher ferritin and lower baseline ESA dose also increased the tendency to treatment response. The table reports continuous data as means with accompanying 95% confidence intervals; discrete data is presented as absolute numbers with percentages bracketed. Statistical significance is assessed by t-test for continuous variables or chi-squared testing for discrete.*

### 3.6 Iron group

#### 3.6.1 Haemoglobin response following treatment with iron therapy

Those treated with iron therapy received 200mg on each of the first five sessions following randomisation. Those who successfully responded to treatment had a gentler decline in haemoglobin prior to treatment compared to non-responders. Following treatment, responders showed the biggest increase in haemoglobin in the first month following treatment, but still continued to increase in the second month. Non-responders continued to decrease their haemoglobin in both of the first two months following treatment but at a less marked rate than prior to treatment.

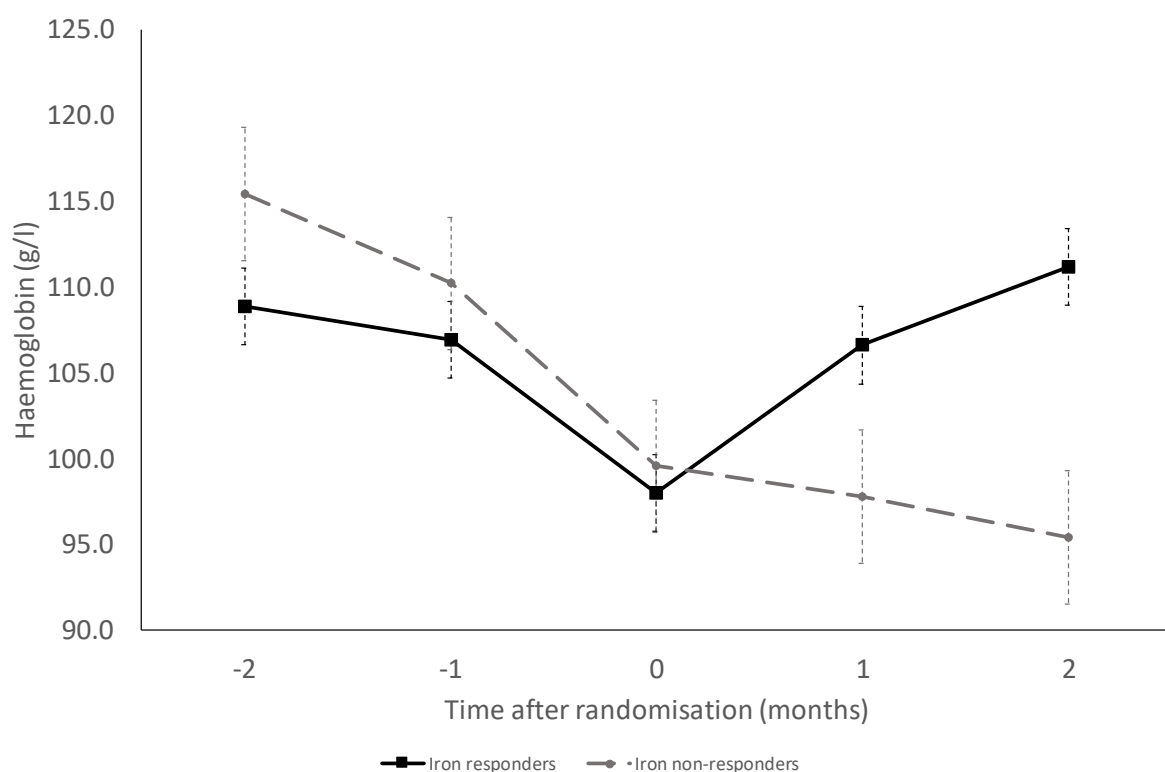


Figure 21. Mean haemoglobin prior to and following treatment with 5 doses of 200mg iron therapy.

The difference in haemoglobin prior to treatment between responders and non-responders is visually more marked than that between that seen within the ESA treatment group. Following treatment, the haemoglobin continues on a downward trend in non-responders in the next two months. In those effectively treated with iron therapy, haemoglobin increases in both of the two months following treatment.

### 3.6.2 Baseline demographics and co-morbidities of responders versus non-responders

Females showed a stronger preponderance than men to respond to iron with 20 out of 22 (90.9%) females responding to treatment but only 34 out of 54 (63.0%) males,  $p=0.024$ . Those on warfarin in this study all responded to the administered iron however antiplatelet agents did not seem to have the same impact on iron response.

|                                        | Responders             | Non-responders          | P value |
|----------------------------------------|------------------------|-------------------------|---------|
| Numbers                                | 54 (71.1%)             | 22 (28.9%)              |         |
| Age (years)                            | 62.68 (31-86)          | 64.48 (33-86)           | 0.605   |
| Gender                                 | 34 (63.0%) male        | 20 (90.1%) male         | 0.056   |
| Ethnicity                              |                        |                         |         |
| White (%)                              | 23 (42.6%)             | 11 (50.0%)              | 0.856   |
| Asian (%)                              | 23 (42.6%)             | 8 (36.4%)               |         |
| Black (%)                              | 7 (13.0%)              | 2 (9.1%)                |         |
| Other (%)                              | 1 (1.9%)               | 1 (4.5%)                |         |
| Dialysis vintage (years)               | 4.23 (0-22)            | 3.39 (0-22)             | 0.531   |
| Previous transplant (%)                | 14 (25.9%)             | 4 (18.2%)               | 0.559   |
| PTH (pmol/l) at enrolment in the study | 59.59<br>(46.24-72.73) | 69.28<br>(32.77-107.18) | 0.602   |
| Co-morbidities                         |                        |                         |         |

|                                               |            |            |       |
|-----------------------------------------------|------------|------------|-------|
| Diabetes (%)                                  | 22 (40.7%) | 11 (50.0%) | 0.624 |
| Vascular disease<br>(IHD, PVD, stroke)<br>(%) | 20 (37.0%) | 9 (40.9%)  | 1.000 |
| Viral liver disease<br>(%)                    | 1 (1.9%)   | 0 (0%)     | 1.000 |
| Valve<br>replacement (%)                      | 2 (3.7%)   | 0(0%)      | 1.000 |

*Table 14. Baseline characteristics in responders and non-responders of those treated with iron. This shows that female gender is associated with a positive response to iron therapy, however aside from this, baseline characteristics do not significantly differ between responders and non-responders. The table reports continuous data as means with accompanying 95% confidence intervals; discrete data is presented as absolute numbers with percentages bracketed. Statistical significance is assessed by t-test for continuous variables or chi-squared testing for discrete.*

### 3.6.3 Biomarkers and medications predicting treatment response

Overall, more biomarkers were predictive of a response to iron in comparison to ESA. Ret-He, MCV, hepcidin, transferrin and TSats were lower in responders to treatment, whereas CRP was higher. This is further discussed in section 4.4.2. Of the baseline medications, more recipients of warfarin responded to iron treatment than non-responders however there was no apparent impact of use of anti-platelet agents. Although mechanistically, a positive response to iron would counteract the increased obscure blood loss which would be expected within this group, one would also expect that anti-platelet use would have an impact. A caveat to the potential impact of either of warfarin or anti-platelet agents affecting, or not affecting, iron response rates is that the numbers of individuals taking these medications in the study are small.

| Predictor | Responders | Non-responders | P value |
|-----------|------------|----------------|---------|
|-----------|------------|----------------|---------|

|                                           |                                |                                |       |
|-------------------------------------------|--------------------------------|--------------------------------|-------|
| Baseline ESA dose<br>(units/session)      | 3550.30                        | 2644.91                        | 0.157 |
| MCV (fl)                                  | 90.60 (54)<br>(88.97-92.23)    | 94.47 (22)<br>(92.48-96.45)    | 0.034 |
| Reticulocyte count<br>( $\times 10^9/l$ ) | 50.53 (54)<br>(44.03-57.02)    | 46.79 (22)<br>(42.49-51.09)    | 0.543 |
| Ret-He (pg)                               | 33.79 (54)<br>(33.06-34.52)    | 35.55 (22)<br>(34.46-36.63)    | 0.047 |
| Ferritin ( $\mu g/l$ )                    | 250.61 (54)<br>(220.95-280.27) | 246.00 (22)<br>(217.08-274.92) | 0.880 |
| Hepcidin ( $\mu g/ml$ )                   | 91.39 (54)<br>(77.22-105.56)   | 130.99 (20)<br>(111.52-150.46) | 0.021 |
| Albumin (g/l)                             | 34.07 (54)<br>(32.84-35.31)    | 34.33 (21)<br>(33.32-35.34)    | 0.834 |
| CRP (mg/l)                                | 29.51 (54)<br>(18.51-40.52)    | 12.59 (22)<br>(9.48-15.69)     | 0.085 |
| Transferrin (g/l)                         | 1.90 (53)<br>(1.57-1.74)       | 1.77 (22)<br>(1.69-1.85)       | 0.182 |
| Transferrin<br>saturations (%)            | 24.38 (53)<br>(21.86-26.89)    | 31.19 (21)<br>(28.20-34.18)    | 0.017 |
| Iron ( $\mu mol/l$ )                      | 10.28 (53)                     | 13.42 (21)                     | 0.017 |

|                   | (9.04-11.53)                          | (12.26-14.59)                       |       |
|-------------------|---------------------------------------|-------------------------------------|-------|
| B12 (pg/ml)       | 460.2 (54)<br>(378.0-542.4)           | 437.68 (22)<br>(392.3-438.1)        | 0.767 |
| Folate (ng/ml)    | 8.85 (54)<br>(7.14-10.57)             | 6.28 (22)<br>(5.27-7.30)            | 0.111 |
| Warfarin (%)      | 7 (13.0%)                             | 0 (0%)                              | 0.094 |
| Antiplatelets (%) | 28 (51.6%)<br>19 (35.2%)<br>6 (11.1%) | 9 (40.9%)<br>9 (40.9%)<br>5 (22.7%) | 0.393 |

*Table 15. Baseline biomarkers in responders versus non-responders to iron. Those with a lower MCV, Ret-He, hepcidin, transferrin saturation and iron level were significantly more likely to respond to iron treatment. The use of warfarin, a higher CRP, a higher baseline ESA dose and a higher transferrin level also increased the tendency to treatment response. Significantly, ferritin levels did not differ between responders and non-responders to treatment. The table reports continuous data as means with accompanying 95% confidence intervals; discrete data is presented as absolute numbers with percentages bracketed. Statistical significance is assessed by t-test for continuous variables or chi-squared testing for discrete.*

### 3.7 ESA versus iron selection

The previous section described the difference in baseline biomarkers for each of the treatment groups separately. This however is only the first stage in trying to determine a useful predictor. To be able to differentiate between the treatments requires that the direction of treatment response is different between the two biomarkers. Thus, an ideal biomarker should be lower in responders than non-responders to one of the treatments but higher in responders than non-responders in the alternate treatment.

The following section adopts a step by step approach to try answer the research question. This starts with box-plots which give graphical representation of how to answer the question and the findings are later validated using statistical testing.

#### 3.7.1 Box-plot and ANOVA analysis

The box-plots are able to provide a visual representation of the difference in means of the four different treatment response groups: ESA responders, ESA non-responders, iron responders and iron non-responders. The practical application of any statistical difference is the incorporation of the findings into a treatment model. The box-plot demonstrates the direction of treatment response in the two different groups, which allows thresholds by which a treatment model can be developed. The ANOVA calculation is the strength of that parameter in determining the binary outcome of treatment response or non-response and is included for the parameters that were considered for inclusion within a future treatment model.

(i) MCV

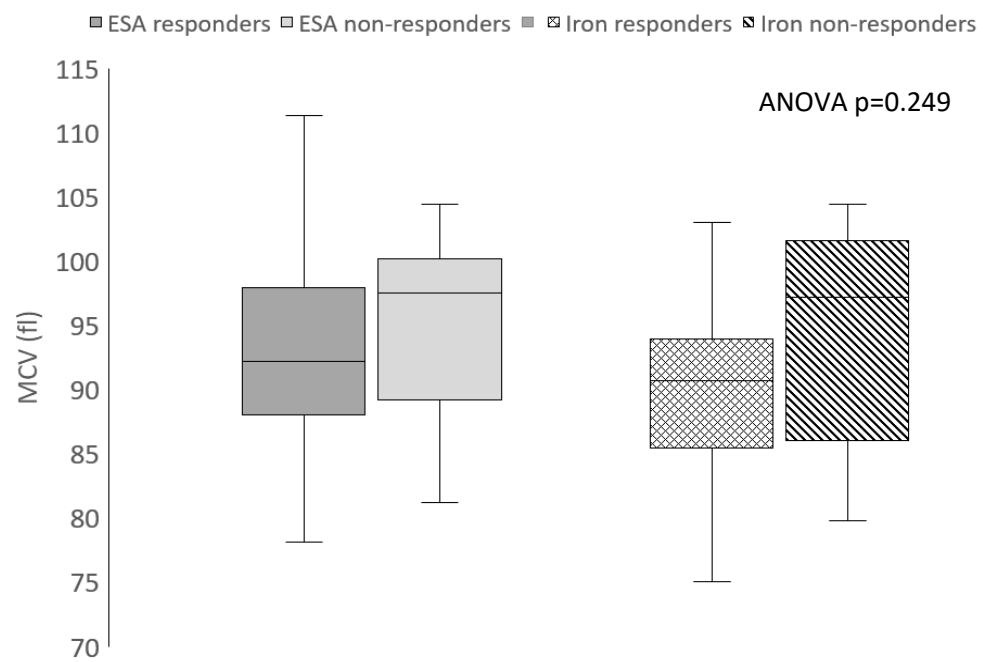


Figure 22 (i). The median MCV in non-responders is higher than in responders to both treatments, indicating that a lower MCV is supportive of overall treatment response. Despite this visual indicator, statistically the ANOVA does not support this.

(ii) Reticulocyte count

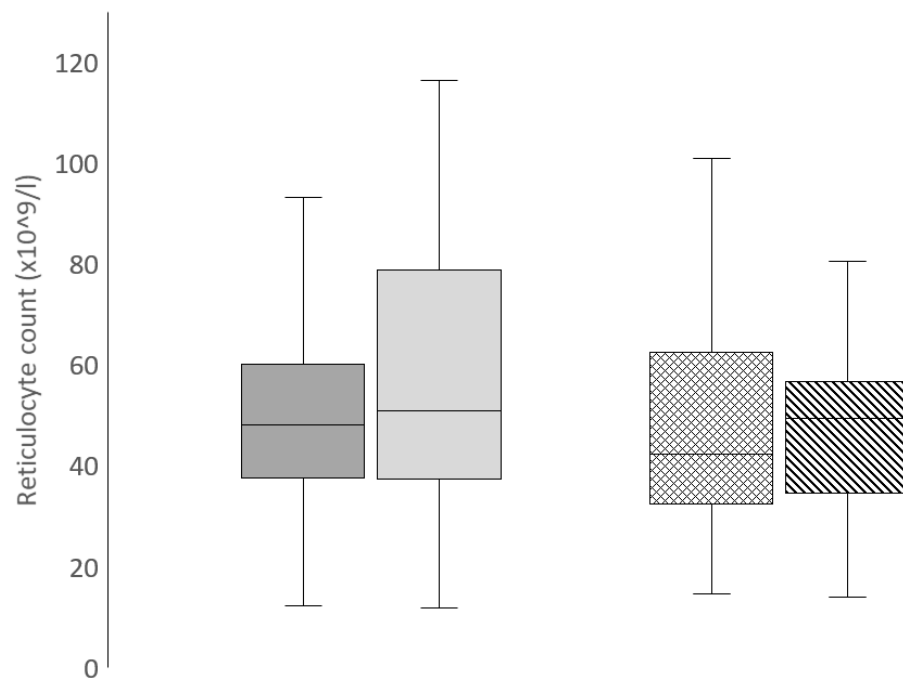


Figure 22(ii). The median reticulocyte counts in the four groups are very similar. As suggested by previous data, reticulocyte is not thought to be an important predictor of treatment response and this was therefore not included in ANOVA analysis.

(iii) Ret-He

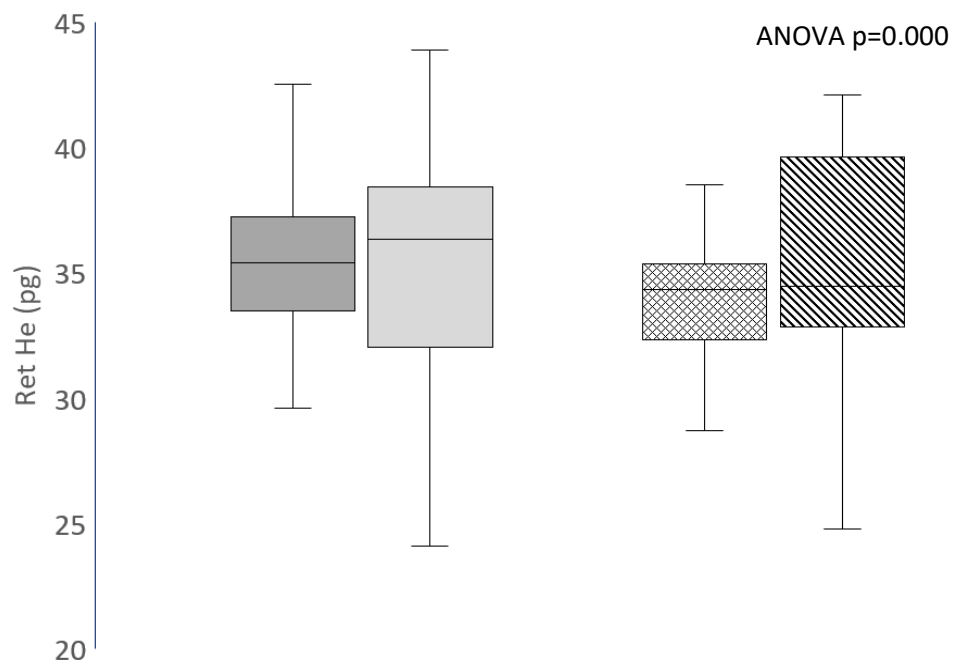


Figure 22(iii). Visually, median Ret-He values are similar across all group groups. The range of Ret-He in non-responders includes significantly lower values in non-responders to both treatments. This parameter was included within the ANOVA analysis as red cell parameters, such as CHr or equivalent tests, which would be Ret-He form part of the most recent NICE guidelines.

(iv) Ferritin

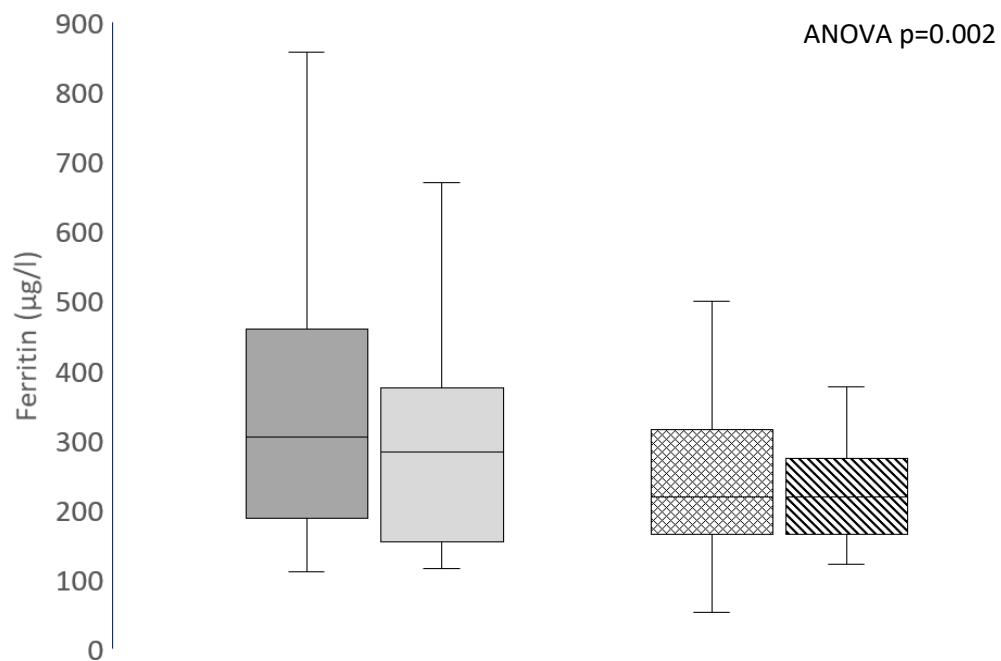


Figure 22 (iv). Ferritin has been the mainstay parameter of anaemia treatment since the introduction of ESA therapy. It was therefore deemed important to include this within the ANOVA analysis. Visually, there seems to be a difference in the treatment groups with higher ferritin levels in those treated with ESA. This is likely a by-product of the study format, discussed previously in section 3.3.

(v) Hepcidin

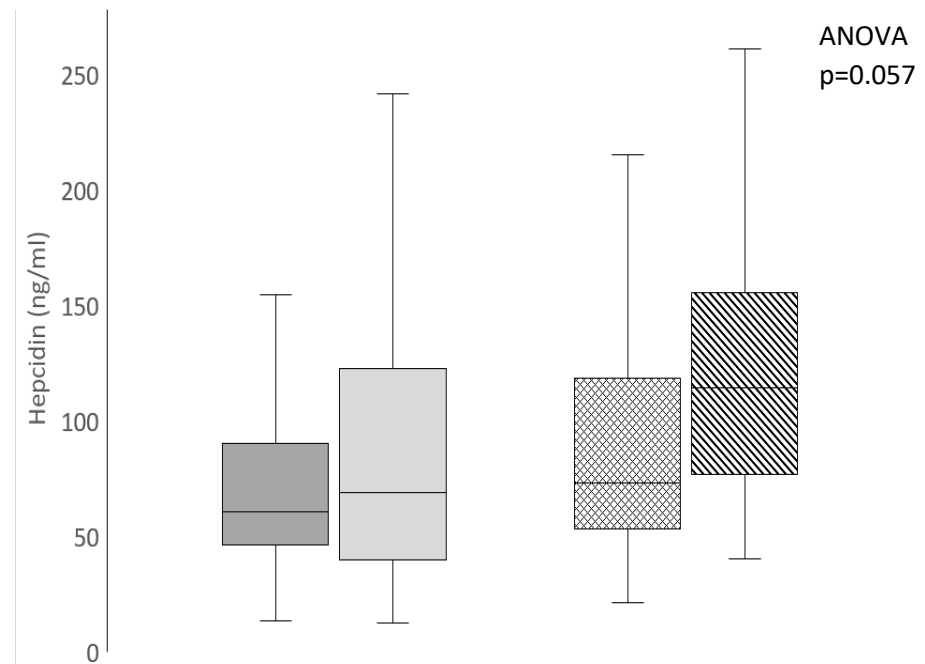


Figure 22 (v). Hepcidin differs significantly amongst the four groups as shown by the ANOVA analysis.

The importance of this within a future treatment model is unclear given that this difference is potentially attributable to the higher hepcidin levels in those treated with iron therapy. This is explored further in section 4.3.

(vi) Albumin

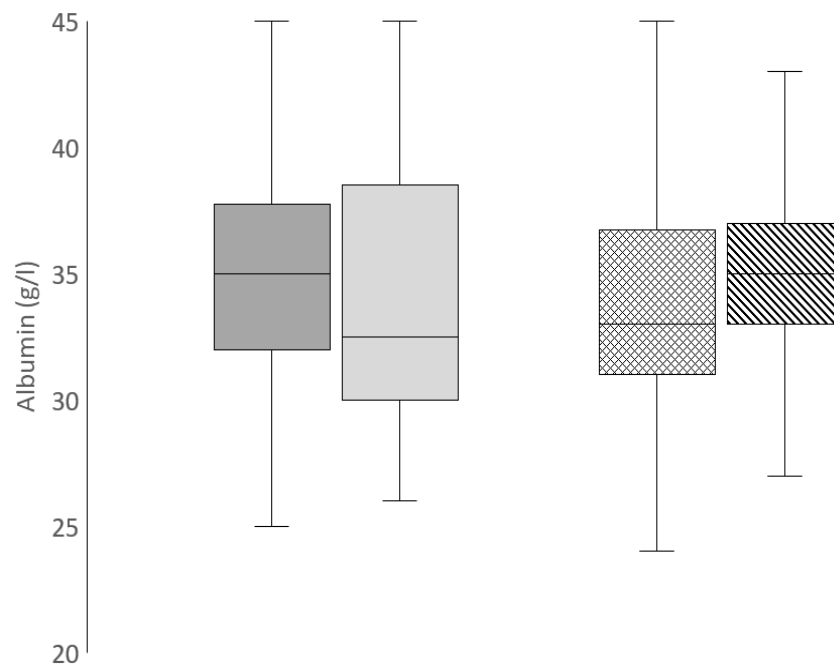


Figure 22 (vi). The four boxes of treatment outcomes overlap between the four treatment groups.

The lack of precedence of using this parameter in treatment, without supportive data following box-plot analysis, resulted from its exclusion from the ANOVA analysis.

(vii) Serum iron

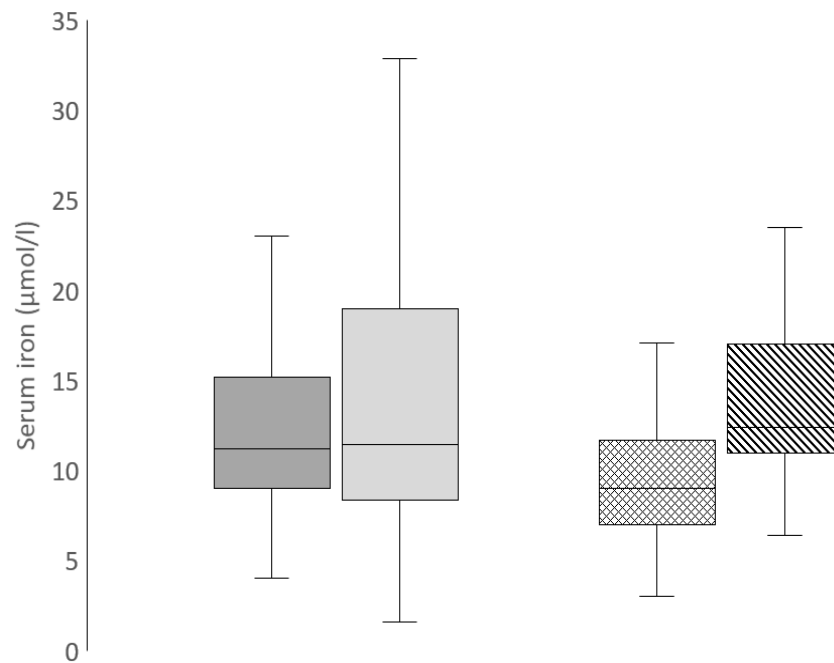


Figure 22 (vii). Serum iron levels largely overlapped in responders and non-responders to ESA treatment, although visually this biomarker seems a good differentiator between responders and non-responders to iron. It however was not used in the ANOVA analysis due to lack of precedence and the use of other iron biomarkers, that form part of the same pathway, within the ANOVA analysis.

(viii) Transferrin saturations

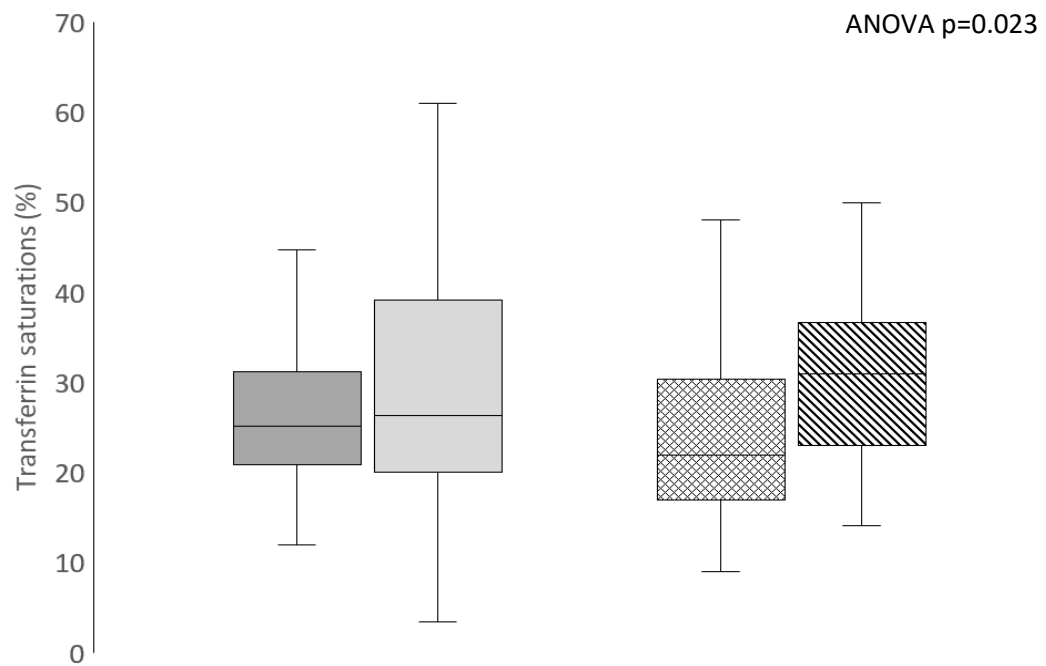


Figure 22 (viii). Transferrin saturation was recommended as part of the Renal Association guidelines for determining iron treatment in individuals in whom ferritin was elevated for other reasons. The box-plot shows that TSats levels differ between responders and non-responders to iron, with less of a difference seen in those treated with ESA. The importance of this visual difference is emphasised by the significant ANOVA result.

(ix) CRP

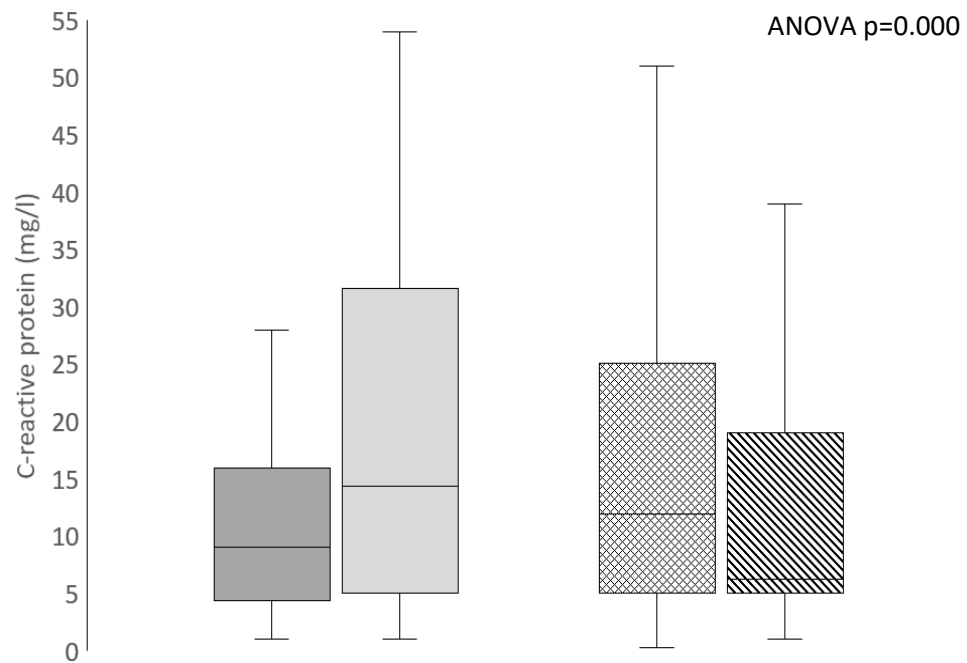


Figure 22(ix). Visually, CRP is the ideal biomarker with higher levels seen in responders to iron but lower levels in responders to ESA. The different direction of treatment response is key to helping differentiate between the two different treatment options at a single point in time. The strength of CRP is emphasised by the ANOVA result.

(x) Transferrin

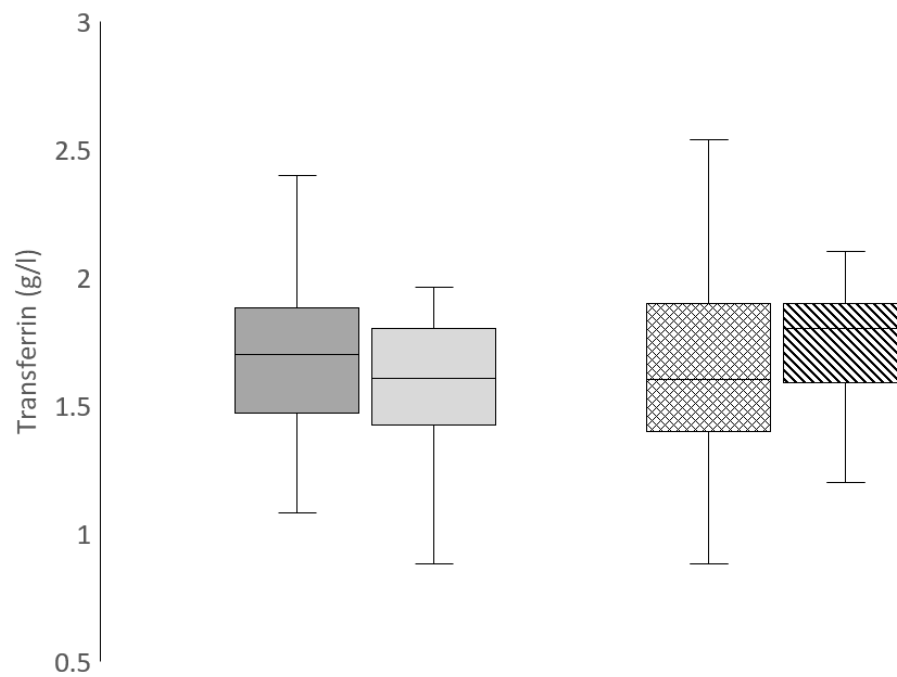


Figure 22 (x). Transferrin levels visually appear to be similar amongst all treatment groups. This parameter was excluded in deference to TSats, a parameter that relies on transferrin levels in its calculation.

(xi) ESA dose

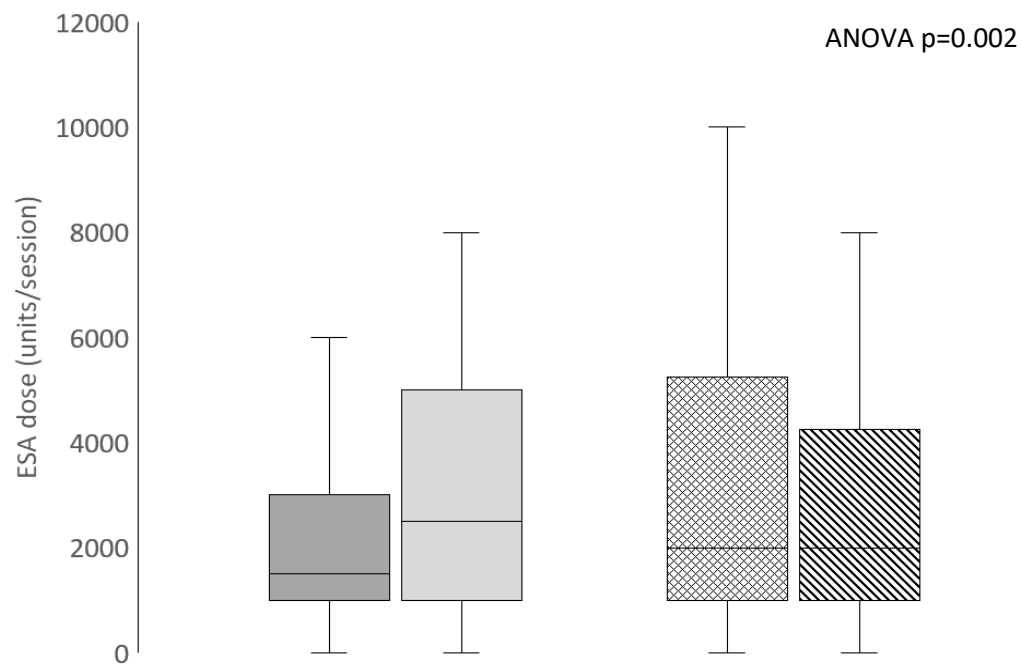


Figure 22 (xi). Visually, baseline ESA dose seems to be another strong differentiator of treatment response to ESA or iron. The clear difference in responders and non-responders to ESA is useful as traditionally, there have been few biomarkers predictive of ESA treatment response. This difference supported inclusion of this parameter in the ANOVA analysis, and the resultant significant finding.

### 3.7.2 Main and interaction effects

Table 16 below shows the response and interaction effect described above. It highlights CRP as the biggest differentiator of whether a patient is likely to respond to one treatment or the other as those with a higher CRP are far more likely to respond to iron and a lower CRP more likely to respond to ESA. ESA dose prior to treatment is also a strong predictor of treatment response as a lower ESA dose means an individual is more likely to respond to treatment, whereas those on higher ESA dose respond to iron. Ret-He and hepcidin are also both able to differentiate between the response to each of the treatments but with less strength than that of ESA dose and CRP. It is these findings that are important in the development of a treatment model.

| Parameter                         | Main response difference<br>comparing responder and non-<br>responders | Interaction with treatment<br>model |
|-----------------------------------|------------------------------------------------------------------------|-------------------------------------|
| MCV (fl)                          | 0.030                                                                  | 0.349                               |
| Ret-He (pg)                       | 0.170                                                                  | 0.140                               |
| Ferritin (µg/l)                   | 0.236                                                                  | 0.307                               |
| Hepcidin (µg/ml)                  | 0.013                                                                  | 0.186                               |
| CRP (mg/L)                        | 0.881                                                                  | 0.008                               |
| TSat (%)                          | 0.015                                                                  | 0.507                               |
| Baseline ESA dose (Units/session) | 0.968                                                                  | 0.058                               |

*Table 16. This shows the p values calculated of both the response to treatment and interaction.*

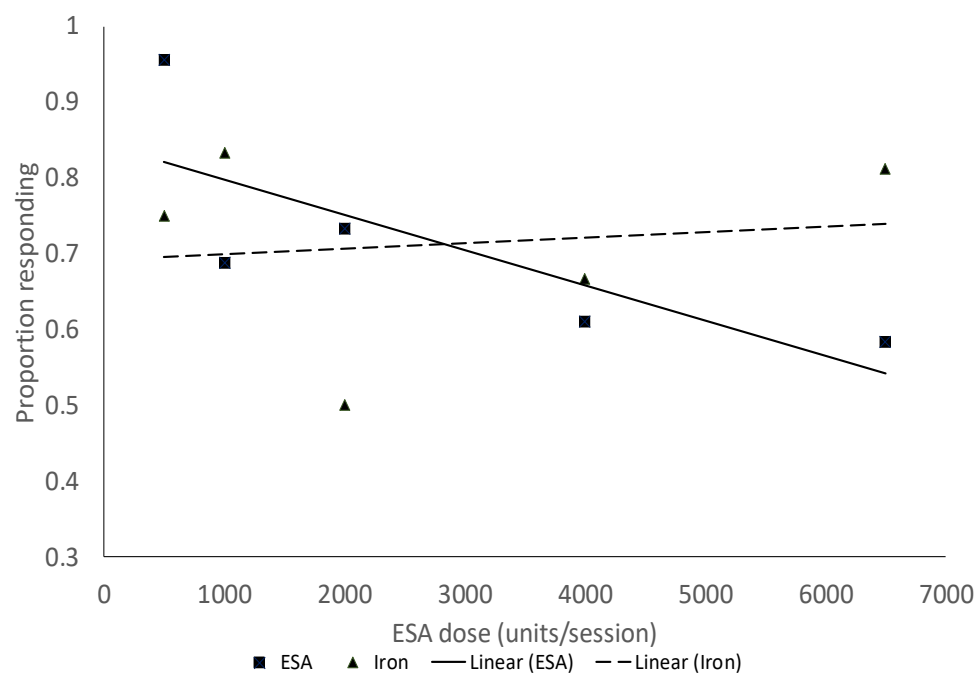
*Although MCV, hepcidin and TSats differ between responders and non-responders to each of the treatments, it is CRP and baseline ESA dose in which the direction of response is different between the treatment groups. This table can be used, in conjunction with the graphs above, to evaluate the importance of each individual parameter in both predicting response to each treatment, and the direction thereof.*

### 3.8 New model for treatment

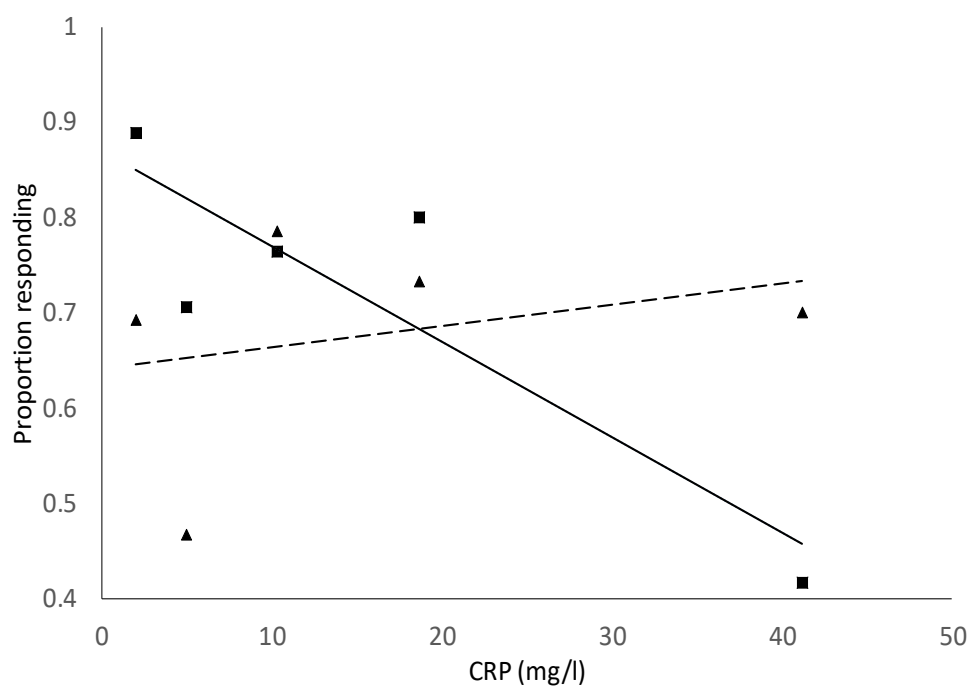
For this study to have a clinical impact, the findings have to be translated into a new treatment algorithm. Features of an effective algorithm are that it has to be run largely autonomously, with delivery at nurse led satellite haemodialysis units. Computer application development can facilitate what would have previously considered a more complex model, containing more variables. Entry of data into this application to generate a treatment output is a feasible way for treatment modelling to be delivered.

The model generated from this study uses the four strongest variables at predicting treatment response derived from the interaction modelling in Table 16. These parameters are: baseline ESA dose, CRP, Ret-He and hepcidin. The model works on a points allocation system. This allocates a point if an individual's biomarkers meet a threshold. These thresholds are derived from calculating the quintiles of the biomarkers against the proportional response rate to treatment. A linear line of best fit was plotted as felt this provided an easier visual representation of how the two different treatment options compared to each other, although a quadratic line would have perhaps been more accurate. This is shown below in Figure 23.

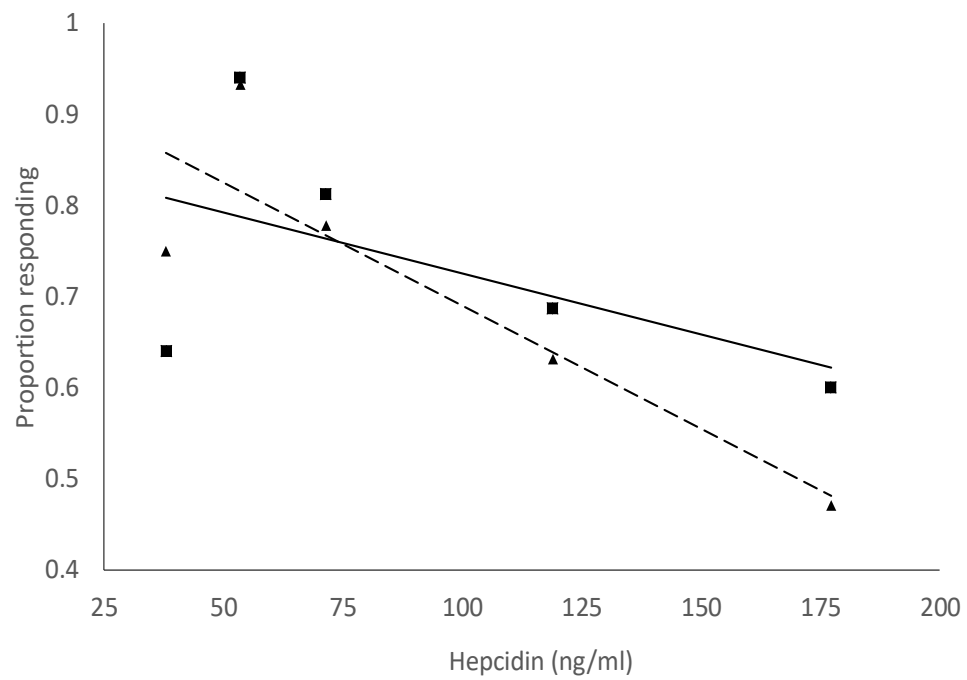
(i) ESA dose



(ii) CRP



(iii) Hepcidin



(iv) Ret-He

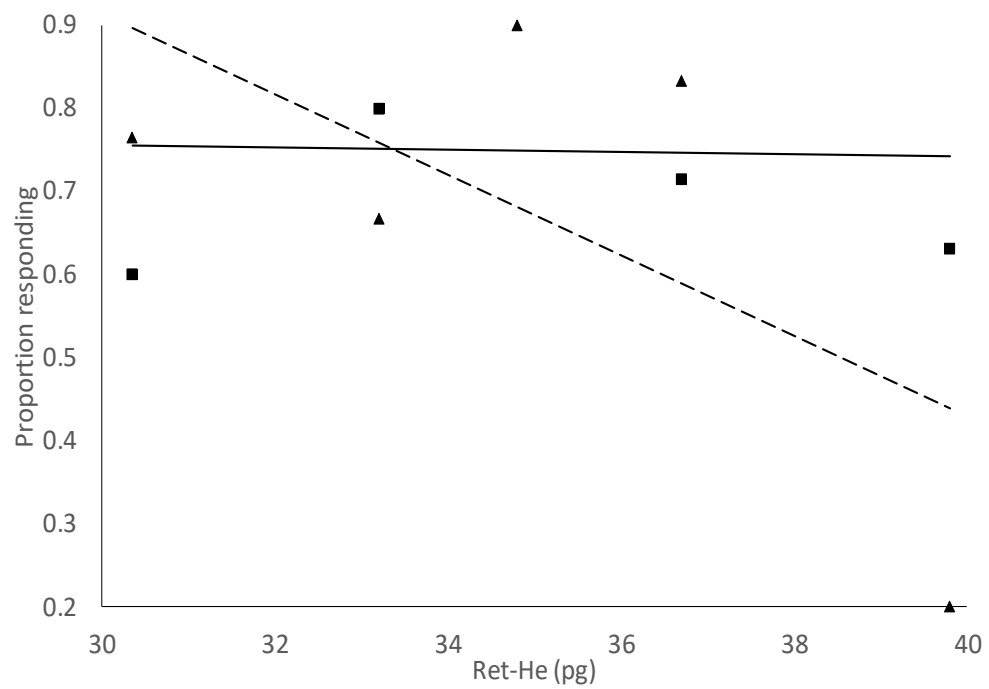


Figure 23. Linear lines of best fit demonstrating the proportional treatment response according to biomarkers prior to treatment. The parameters reported are (i) ESA dose (ii) CRP (iii) hepcidin (iv)

*Ret-He. The legend that is present as part of figure 23 (i) is applicable to all graphs in this figure. The inclusion of these parameters only was based upon the information following ANOVA analysis and the main and interaction modelling. The strength of ESA dose and CRP in differentiating between treatment response for the two different treatments is emphasised by the positive gradient for the line of best fit for iron treatment in that iron response rates increase with increasing ESA dose and CRP; and a negative gradient for the line of best fit for ESA with decreasing response rates as baseline ESA dose and CRP increase. The difference in the gradients between the two different treatments in the Ret-He graph is less marked and is even the same direction, although with differing gradients, in the hepcidin graph.*

The treatment model works on the basis of monthly bloods being reviewed and being allocated a total point score. The total number of points allocated determines whether iron or ESA should be given to the anaemic individual. This treatment model advocates the use of the variables CRP, baseline ESA dose, Ret-He, and hepcidin as the most important differentiators between treatment response to iron or ESA. In this treatment model, the more points allocated following review of the monthly bloods, the more likely the individual is to respond to iron rather than ESA treatment. The thresholds by which points are allocated are charted on Table 17 below and these were derived from Figure 23. Below the thresholds, no points would be allocated for that variable i.e. an individual who on monthly bloods had a CRP of less than 3, a baseline ESA dose less than 999 units per week, a Ret-He greater than 38pg and a hepcidin greater than 128µg/ml would receive zero points and would therefore be allocated ESA treatment. Some variables are more predictive of treatment response than others and this is demonstrated by the ANOVA and interaction modelling. These variables therefore have more points attached to them.

| Threshold               | Proportion meeting threshold (%) |
|-------------------------|----------------------------------|
| CRP >3 mg/l (1 point)   | 83.8                             |
| CRP >20 mg/l (2 points) | 25.0                             |

|                                       |      |
|---------------------------------------|------|
| ESA dose >999 units/week (1 point)    | 86.3 |
| ESA dose > 3999 units/week (2 points) | 28.8 |
| Ret-He <32pg (1 point)                | 17.5 |
| Ret-He <38pg (1 point)                | 82.5 |
| Hepcidin <51µg/ml (1 point)           | 24.8 |
| Hepcidin <128µg/ml (1 point)          | 77.1 |

*Table 17. Point allocation according to baseline parameters of the most important determinants of treatment response. The recurrence of a parameter in the point allocation system reflects the weight of influence of the parameter in predicting treatment response. The respective proportion of the studied population meeting that parameter is also shown in the table.*

Using the treatment thresholds in Table 17, the points for each randomisation episode within this study can be calculated. By comparing this to whether the individual subsequently responds to treatment, a proportional response rate according to the points can be plotted and is shown below in Figure 24. Using the points allocation system, it is possible to increase the proportional response to ESA therapy from 73.8% to 93.3%, for those that receive less than two points, and iron therapy from 71.1% to 100%, in those that have 6 points.

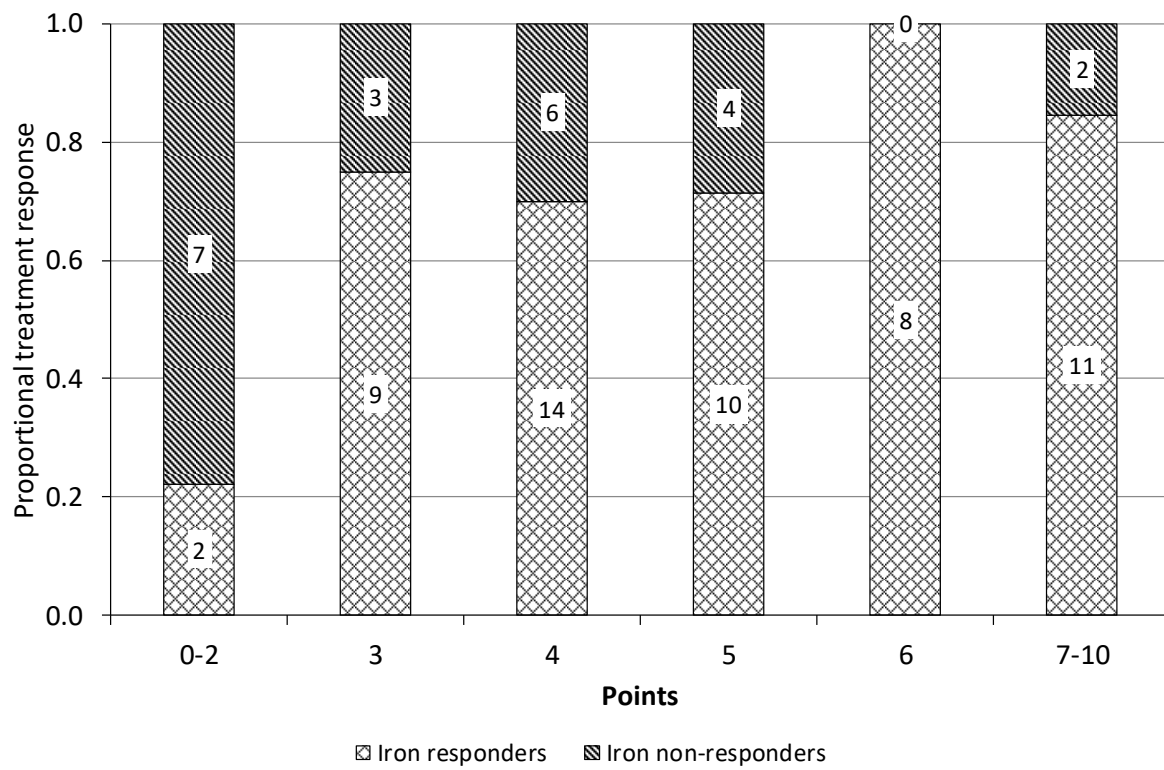
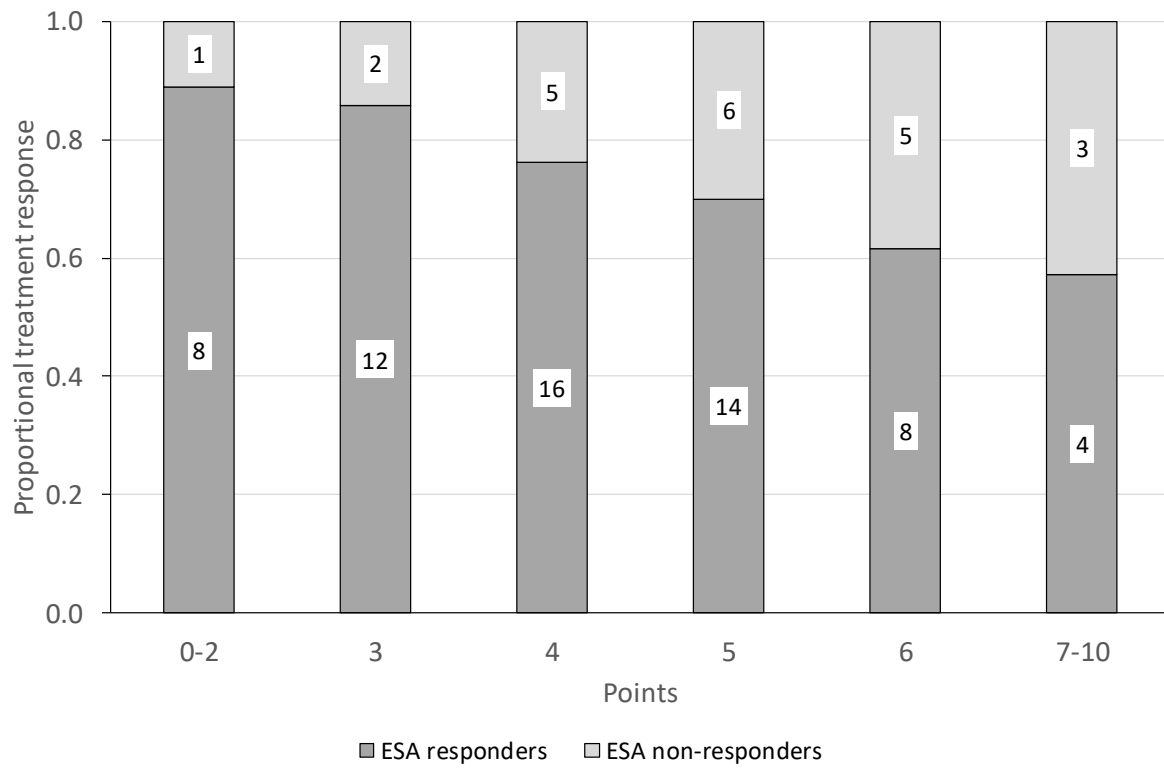


*Figure 24. Proportion of individuals responding to (i) ESA and (ii) iron at each points allocation. The first graph shows that treatment response rate to ESA steadily decreases with more points. Response to iron is slightly more unpredictable but increases with more points.*

Figure 24 demonstrates the success of each treatment individually with each point total. In individuals with mild anaemia, the pragmatic approach would be to give one treatment at a time, with the one most likely to be successful. Thus, the combination of the two graphs in Figure 24 into a single graph underlies the proposed treatment model.

Figure 25 is a graph generated from a combination of the two parts of figure 24. This uses a bar to show treatment response with each points allocation cut off. Individuals in whom the monthly bloods gave them a total score below the cut off would be treated with ESA and above, iron. Each bar is divided into four sections: those with a positive response to ESA, those with a negative response to ESA, those with a positive response to iron and those with a negative response to iron. These four sections are shown as a proportion of the total number of individuals from the study population that had that number of points.

The most effective treatment cut off is the one with the smallest proportion of non-responders. In the graph, this would be the bar with the smallest total of light grey and diagonal areas. Reviewing Figure 25, the most effective way of delivering treatment would be to give ESA to those who have four or less points and iron to those with five or more. This would result in an overall treatment rate of 82.3%. This is a decrease in non-response to treatment in the study from 28.8% to 17.7%, a 38.5% reduction. Adopting these cut offs would result in 53.2% of individuals receiving ESA with 81.7% responding to treatment and the remainder receiving iron to which 82.9% respond.

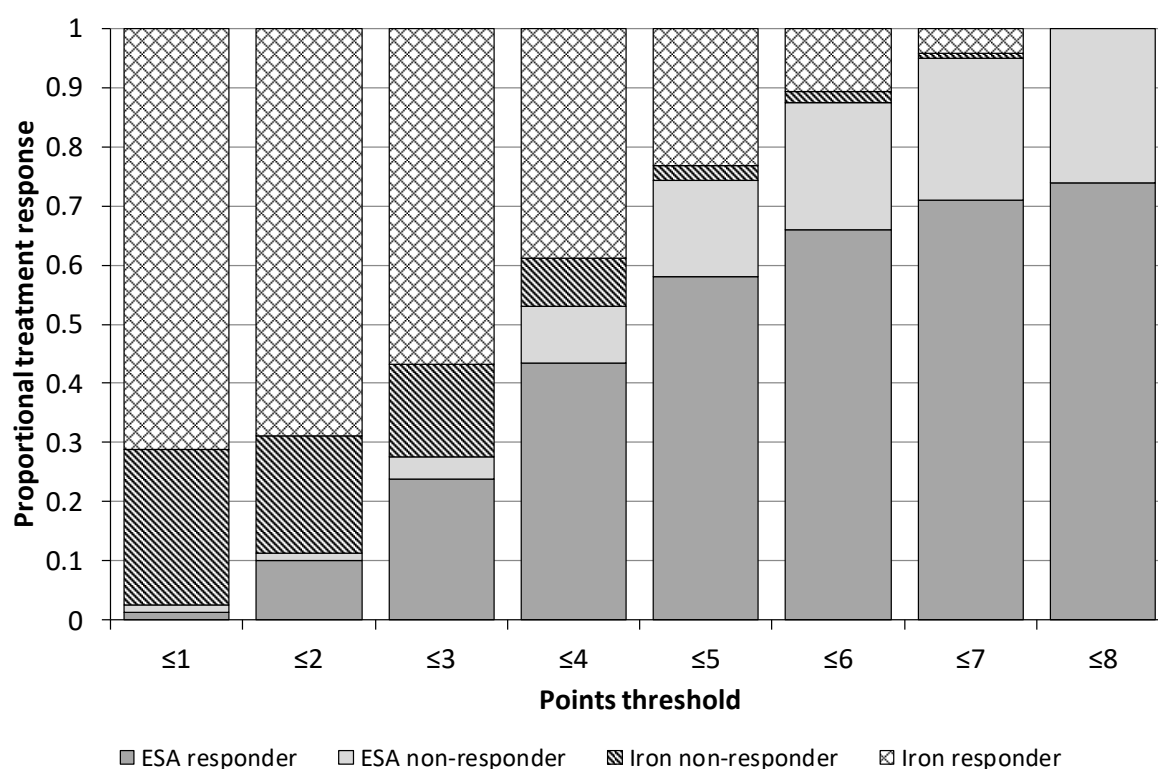


Figure 25. The proportion of individuals being allocated to each treatment group, and the likelihood of successful treatment thereof, at the different points cut off. This is an amalgamation of the two graphs in figure 24. This provides a visual representation of which point score would lead to maximal overall response rate. This is the bar for which the light blue and green section make up the smallest proportion of the whole vertical bar. From this graph, administering iron to those with a score of less than or equal to four resulted in 83% of individuals having a positive response to treatment.

Referring back to Table 17, examples of the practical application of Figure 25 follow using the points threshold of less than or equal to four receiving iron. Monthly bloods for an individual with an Hb 98g/l, CRP 25 mg/l (3 points), Ret-He 34 pg (1 point), hepcidin 154 µg/ml (0 points) and a baseline ESA dose of 4000 units/week (3 points) would total 7 points. This individual would receive intravenous iron with 82.8% of individuals expected to increment haemoglobin as a result. If, however on monthly bloods, the CRP was 2mg/l (0 points), Ret-He 34 pg (1 point), hepcidin 101 µg/ml (1 point) and ESA dose 3000 units/ week (1 point), the total points score would be 3 points and thus treatment would be incrementation of ESA dose. In this situation, 81.8% of individuals would be expected to respond.

So far, this proposed treatment model has only been evaluated against the data from which it is derived. Testing the model in a new haemodialysis population, and comparing it against standard clinical practice, is the next stage of the protocol development process.

### 3.8.1 Summary and potential impact on clinical practice

This is the largest study that prospectively addresses the real life, clinical conundrum of whether an anaemic patient requires either ESA or iron at a given point in time. This allows combination treatment to be restricted to those with more severe anaemia likely to respond to both medications. Overall, this reduces treatment exposure with its potential side effects and financial cost and requires proper economic assessment in the future.

There is a clearly defined trajectory that would introduce the findings of this study into clinical practice. The treatment algorithm needs to be assessed, within a clustered randomised trial format into multiple satellite haemodialysis units. This study would be conducted with the primary outcome of the study as proportion of population achieving a therapeutic haemoglobin, and the secondary outcomes of total dose of ESA and iron used. If the results of this study favour the use of the new treatment model, this could then become standard clinical care.

### 3.8.2 Technical aspects of study design

It was the aim of the study to reflect the actuality of a haemodialysis cohort; a heterogeneous, co-morbid group of individuals. The broad inclusion criteria of this study and limited exclusion criteria maximised numbers of participants and randomisation episodes within the study. The result of electing for this method of study, is that there is an expected withdrawal rate of individuals from the study and incomplete randomisation episodes is discussed below.

#### *Withdrawals from study*

Haemodialysis patients are a co-morbid group of individuals; and intercurrent illness was expected to affect a significant proportion of individuals in the duration of the study. A number of participants were active on the renal transplant waiting list with the expectation that some of them would receive a

transplant and therefore leave the study. Thus, as described in section 2.3, recruitment for the study pre-emptively accounted for these expected withdrawals to achieve the required number of randomisation events.

Seventy-four individuals withdrew throughout the duration of the study. The reasons for withdrawal are shown in Table 18 below. Of the withdrawals, 23 (31.1%) were due to participants receiving a renal transplant with a further 8 (10.8%) either changing haemodialysis unit or renal replacement modality. These numbers are similar to that found in other studies.

Through the course of the study, it was clear that the frailty of an individual would preclude a patient from being able to adhere to the protocol, with them either having a revised haemoglobin target or being unable to wait the two months to enact further change as required by the study protocol. The protocol of the study was therefore adapted to exclude these individuals with 28 (37.8%) withdrawn based upon this. Only having four participants withdrew due to not wishing to comply with the protocol underlies the acceptability of this study to haemodialysis patients. The 31 withdrawn due to changing dialysis unit or modality is at a similar rate to that seen in other large longitudinal haemodialysis studies.

| Cause of withdrawal from study      | Details                                                                                                                  |
|-------------------------------------|--------------------------------------------------------------------------------------------------------------------------|
| Change of dialysis unit or modality | 23 received a transplant<br><br>7 changed to a non-participating dialysis unit<br><br>1 converted to peritoneal dialysis |
| Medical reasons                     | 5 acquired a protocol specified ineligibility<br><br>28 withdrawn due to frailty<br><br>3 deaths                         |
| Protocol reasons                    | 3 used alternate ESA or iron agents<br><br>4 declined to follow treatment protocol                                       |

*Table 18. Reasons for withdrawal for study. This has been subdivided into change of modality, medical reasons and either inability or participant refusal to continue in the study. The majority of study withdrawals were due to increasing frailty of an individual throughout the duration of the study, with a slightly lesser number withdrawing due to receiving a renal transplant. Neither of these were thought to be disproportionately high compared to what would otherwise be expected within a haemodialysis cohort. To account for this, the study aimed to maximise its recruitment.*

### *Serious adverse events*

Prior to the commencement of this study, in conjunction with the Imperial Dialysis Research Group, the decision was made to define as serious adverse event (SAE) as either death or an emergency hospital admission in a study participant. Blood transfusions were considered adverse events (AE). All 55 SAEs and the 7 AEs were immediately reported to the local JRCO.

SAEs were analysed in relation to randomisation episodes. This was to distinguish those SAEs attributable to the study design. Twenty-five SAEs occurred in participants who were in the observational phase of the study; these individuals had never been randomised and the SAE was not related to anaemia. There were 30 SAEs that occurred post randomisation which have been subject to further analysis, which are summarised in Table 19.

Prior to the start of the study, one of the potential concerns was whether the change in anaemia management, with the withdrawal of maintenance iron, would precipitate worsening of anaemia in the whole cohort. To try determine this, the episodes were classified according their timing in relation to a randomisation episode and haemoglobin at this time. Thus, the SAEs in which the haemoglobin is less than the Renal Association minimum target of 100g/l were analysed to determine whether the lower haemoglobin is the cause of, or secondary to, the SAE. Anaemia was considered non-contributory to an SAE within 14 days of randomisation as this would be prior to when a potential increase in haemoglobin in response to treatment would be expected.

Infection was the cause of the majority of SAEs in those with a haemoglobin less than 100g/l. It accounted for 3 SAEs between 15- and 30-days post randomisation and a further episode between days 61 and 120 days. In these situations, the anaemia was considered secondary to the infection and was not the cause of hospital admission. The other 2 episodes in which the affected individual had a haemoglobin of less than 100g/l was a recurrence of vasculitis and a symptomatic pleural effusion. The pleural effusion occurred over 4 months following a randomisation episode. This episode, although not caused by, was potentially exacerbated by the nature of the protocol with the reduction in maintenance iron therapy. Importantly, there were no SAEs directly and definitely precipitated by the study. The medications used in this study were previously used, and tolerated, in all participants of this study. There were no direct SAEs related to administration of the medication.

The disparity seen between the numbers in Figure 18 and Table 19 is due to the broader definition of intercurrent illness, which included those treated in the outpatient setting, rather than the SAEs which were restricted to inpatient.

| Time from randomisation | Proportion with Hb less than 100g/l<br>at time of SAE | Cause                                                         |
|-------------------------|-------------------------------------------------------|---------------------------------------------------------------|
| 0-14 days               | 3/3 (100%)                                            | 3 infection                                                   |
| 15-30 days              | 3/9 (33.3%)                                           | 6 infection<br><br>2 cardiac arrhythmia<br><br>1 hip fracture |
| 31-60 days              | 1/6 (16.7%)                                           | 1 vasculitis<br><br>2 cardiac events<br><br>3 infection       |
| 61-120 days             | 1/5 (20%)                                             | 1 stroke                                                      |

|                       |             |                    |
|-----------------------|-------------|--------------------|
|                       |             | 1 liver failure    |
|                       |             | 3 infection        |
| Greater than 120 days | 1/7 (14.3%) | 1 hip fracture     |
|                       |             | 1 pleural effusion |
|                       |             | 3 infection        |
|                       |             | 1 cardiac event    |
|                       |             | 1 other            |

*Table 19. SAEs with relation to time since randomisation episode. This subdivision help enables identification of the contribution of the randomisation episode to the SAE and focuses upon those SAEs for which anaemia may contribute and therefore potentially precipitated by the study protocol. Those who had a SAEs within the first 2 weeks of treatment all had a low haemoglobin. It is possible that subclinical infection was present at time of randomisation, which precipitated anaemia, but only became apparent later. Infection as a cause of adverse event was prevalent throughout the duration of the study and the main contributor to SAEs, as would have been predicted prior to the study.*

#### *Adverse events*

Adverse events (AE) were defined as blood transfusion for anaemia, restricted to the outpatient haemodialysis unit. Those admitted to hospital, and transfused, were accounted for in the SAEs in the study. Seven patients received a blood transfusion during the study. Three of these had been previously randomised within the study. Two of these were following a clinical decision made by the lead consultant in conjunction with the patient when the haemoglobin was greater than 90g/l due to patient symptoms. One of these individuals was subsequently withdrawn from the study due to overall frailty. The third individual who was transfused had a haemoglobin less than 90g/l when it was re-checked within two weeks of being randomised. The indication for the repeat haemoglobin check was not clear, but transfusion decision was based upon a history of heart failure.

Five patients received a blood transfusion in line with the study protocol when the haemoglobin was less than 90g/l. Three had a recent acute infective illness, treated with outpatient antibiotics, resulting in a temporary acquired ESA resistance. One had a recent elective hospital admission and was recovering from surgery. The final patient was transfused according to protocol, however was also considered frail, and deemed not suitable for continuing participation in the study. The randomisation element of the study did not contribute to the blood transfusion requirement of these patients. There is a potential for the lack of maintenance iron to have contributed to the participant's anaemic status. The transfusion frequency seen within this study did not exceed that seen in previous audit data of transfusion rates at site 3.

#### *Protocol deviations*

There were two inadvertent protocol deviations during the duration of the study. These were the failure of timely administration of the prescribed course of iron. One of these was delayed and the other one was omitted. The delayed medication was administered 3 weeks late with an insufficient follow up time to analyse the episode. The participant in whom the iron medication was omitted had a haemoglobin within target range on the next month's bloods so the prescription was cancelled.

Clinician decision led to four protocol deviations. One individual had simultaneous up titration of ESA and received iron in anticipation of forthcoming surgery. Two received blood transfusion outside the specifications of the study. The final deviation was following an early, off protocol, repeat of haemoglobin measurement which prompted a further change in treatment. There were three participant requests for protocol deviations asking for early up titration of ESA dose as participants felt they were unduly symptomatic of anaemia.

### 3.9 Review of the study format and limitations

As with all research, the results of this study should be considered with respect to the study format. Since the study was designed, SONG-HD has highlighted deficiencies in haemodialysis research, some of which are applicable to this study. The lack of patient involvement in this study's design, with the importance of identifying outcomes measures that are important to them, would form an important part of the next stage of implementation of the findings of the study.<sup>219</sup> These outcome measures would also have to be generalisable beyond this specific study, and to enable the findings of this study to be used in conjunction with other studies to determine clinical practice.<sup>220</sup>

The majority of this study limitations were considered to be unavoidable within the constraints of study funding and limited personnel available to carry out this study. The ongoing review of the format of the study throughout its duration allowed the modification of the exclusion criteria, as discussed in section 2.5 to try and limit the impact of identified issues. Nonetheless, despite the limitations described below, this study provides a good foundation to positively influence the treatment of anaemia in haemodialysis patients.

#### 3.9.1 Recruitment

This was a single centre study, located within three haemodialysis satellite units. It benefited from being within the largest renal centre in the UK and was therefore able to recruit large numbers despite being single centre. Different satellite centres, despite being within a single unit, do run slightly differently and this can affect delivery of the study.

Recruitment started at site 1 from which the highest recruitment ratio was attained reflecting the increased time invested in recruitment at this site. Familiarity has been shown to increase tendency to participate in studies and this is a likely unintentional by-product of the format of the study.<sup>221</sup>

One limitation of this as a single centre study is that the demographics of the units are not representative of that of the UK population, yet even within the study the demographics of the satellite units differed significantly. National data supplied by the Renal Registry from 2014 showed only 21.5% of the prevalent renal replacement therapy population were of an ethnic minority compared with the higher proportion within this study as shown in Table 4. Within an ethnically diverse population, a proportion of individuals are non-English speakers. Although the patient information leaflet was only available in English, translators were used to try overcome some of the language difficulties. Despite this, language difficulties did decrease the enrolment rate of the study.

Only limited baseline demographic data was collected as part of the study. This was a practical consideration made prior to study initiation as the study was to be delivered by one individual. This therefore reflected the pre-conceived ideas of the researchers as to the important factors that may affect outcome of the study. A more encompassing data collection with additional co-morbidities and demographics including smoking status would have been preferential.

### 3.9.2 Randomisation rates

Previous audit data from site 3 had indicated that the rate of patients meeting randomisation criteria on the monthly bloods was approximately 10%. This figure however would have included patients that are were not eligible for the trial on the account of being transfusion dependent, having a haematological disorder or an active infection. The occurrence of 185 randomisation episodes over 2423 observed patient months equates to a rate of 7.6%. This is appropriate compared with the audit data. As well as the aforementioned medical reasons, those who were recently randomised would not be eligible for further randomisation further decreasing randomisation rates.

### 3.9.3 Incomplete randomisation episodes

The reasons for the 25 incomplete randomisation episodes were shown in Figure 18. Incomplete randomisation episodes had been forecast in the planning of the project. Inevitably, in a cohort of haemodialysis patients, there is significant co-morbidity and high infection rates.<sup>222</sup> Temporary

exclusion of these individuals was an important aspect to the study as it results in a temporary elevated ESA resistance.<sup>223</sup> This would confound the ability to correlate between the biomarkers taken at randomisation and subsequent treatment response which was assessed at two months. Analysis of results on a per-protocol rather than in an intention to treat basis is an attempt to obviate the influence of a newly acquired temporary ESA resistance.

#### 3.9.4 Differing baseline characteristics of completed randomisation episodes

The lack of difference in the baseline demographics between the two treatment groups demonstrates the effectiveness of the online randomisation tool. There are two significant differences identified in the baseline biochemical parameters in the two different treatment groups which are accounted for by the study design, with the use of sequential treatments in non-responders to the first treatment. To try identify the true difference in baseline characteristics, not attributable by the study design, *Table 11* reports the baseline biomarkers in those in just the first treatment episode only.

The finding of an increased hepcidin level in the ESA group is unexplained. Samples for hepcidin were batched together and processed at a later date. The impact of this on the overall findings of the study is uncertain. Whether the role of hepcidin has either been diluted or overemphasised on treatment outcome will only be ascertained by conducting further studies.

#### *Variability of blood results*

Although standard operating procedures were developed for study blood sampling, the use of three different satellite units would result in varying levels of adherence. In particular, this would likely affect the timing of the blood sampling during the dialysis session. In a study conducted by a single individual, this is particularly pertinent as it is not possible for a single individual to provide onsite governance of this and very much reliant on nursing staff assistance in blood sampling.

Intra-assay variability is a problem encountered throughout medical practice. Aside from the hepcidin assay, all assays used within this study were the standard, validated ones used in clinical practice. The

monthly bloods taken as part of routine clinical practice were couriered and processed centrally for sites 1 and 2, at the NHS trust responsible for the clinical service. Monthly bloods for site 3 are processed at the local hosting trust of satellite site 3.

The hepcidin ELISA assay was conducted with the assistance of the local biochemistry department, who perform ELISA assays within their regular clinical practice. Duplicate samples were used and processed simultaneously for each specimen. The coefficient variant was calculated and if greater than 10, the result was not used and the assay repeated. Given the importance of haemoglobin in defining the primary outcome of the study, duplicate samples were taken and the average calculated by which treatment outcome was measured.

## 4 Discussion

### 4.1 Introduction

Until the most recent guideline publication in 2015, ESA and intravenous iron were dosed according to haemoglobin and ferritin. Using these two markers to guide treatment led to an unpredictable treatment response; some individuals ended up receiving medication to which they were ultimately unresponsive and other individuals were suboptimally treated for anaemia. More accurate biomarkers that can improve an anaemia treatment algorithm and result in more judiciously dosed medication. This would reduce unnecessary exposure to both ESA and iron, and their potential adverse effects, with its resultant benefit.

This study provided a framework by which an analysis of individual biomarkers and their relative strengths at predicting response to both ESA and intravenous iron. It is the first large-scale study to do so, for both medications within the same study, in a prospective fashion. The results presented in section 3 are discussed in this chapter.

## 4.2 Ferritin as a predictor

This study adds to the previous evidence of the inadequacy of the biomarker ferritin as a predictor for determining response to iron, and ESA. There was no difference identified in ferritin levels between responders and non-responders to iron therapy (250.61µg/l vs 246.00µg/l,  $p=0.880$ ). Figure 22 demonstrated that higher ferritin levels predict an increased likelihood of response to either treatment option, and this finding is reinforced by the statistical analysis in Table 16.

The positive response to iron therapy, which is undetermined by ferritin levels, echoes findings in the recently published PIVOTAL trial. The study found that those on a proactive iron administration regime, administered regardless of ferritin, overall required less ESA to maintain haemoglobin compared to those treated with iron reactively, only when ferritin levels fell.<sup>224</sup> This study further reinforced the previous findings of DRIVE (described in section 1.4.4) that individuals respond to iron treatment regardless of ferritin.<sup>225</sup>

The fundamental role that ferritin had in dosing iron and ESA was not borne from large, validated studies, and thus its lack of strength of determining iron status is unsurprising. It was the first and most well understood protein that had a fundamental role in the iron cycle.<sup>226</sup> Measuring serum ferritin was an affordable, rapid test that became readily and widely available. In a cohort of otherwise well individuals, the correlation between ferritin levels and clinical iron status was evident<sup>227</sup> and also appeared present in haemodialysis patients.<sup>228 229</sup>

Since these original studies in the mid-1970s, the characteristics of the haemodialysis cohort has changed significantly. Initially, haemodialysis was rationed to a limited number of young patients, without other co-morbidities. The evolution of haemodialysis has led to an increasingly elderly, frail and co-morbid population being dialysed.<sup>230</sup> With this comes an increased burden of inflammation and there is associated upregulation of ferritin, regardless of iron status.<sup>231 232</sup> It is for this reason that ferritin is a less reliable indicator of iron status in the modern day haemodialysis cohort than was suggested by early studies.<sup>233</sup>

### 4.3 Hepcidin in clinical practice

Despite the pivotal role of hepcidin in renal anaemia and iron metabolism, its utility as a biomarker in clinical practice has yet to be fully defined. Previous work on hepcidin as a biomarker focused upon its ability to predict response to iron, but this is the first study that treats anaemic patients with both iron and ESA alongside one another so the role of hepcidin in renal anaemia can be more fully considered. As discussed in the results in sections 3.5.3 and 3.6.3, hepcidin was analysed as a predictor of iron and EPO response independently. In those treated with iron, higher hepcidin levels were seen in non-responders to iron (mean 130.9ng/l versus 91.39ng/ml,  $p=0.021$ ).

The reason for this can be explained by an understanding of hepcidin physiology. The three main regulators of hepcidin levels are iron, inflammation and erythropoiesis (see section 1.6.10). Iron deficiency reduces hepcidin levels to increase the bioavailability of iron for ongoing erythropoiesis. Hepcidin blockades ferroportin, the only channel by which stored intracellular iron can be exported from a cell. With reduced hepcidin levels, more ferroportin is active and able to export iron into the systemic circulation. This iron can therefore be delivered to the bone marrow and become incorporated into erythropoiesis.<sup>163</sup> Low hepcidin levels are therefore a marker of an iron deficient state and individuals therefore respond to the administration of iron as given in the study.

Inflammation induces hepcidin production. This is thought to confer protection to the host in the pro-inflammatory state of infection. This role is due to both the bactericidal nature of hepcidin and the prevention of iron availability for invading bacteria.<sup>234</sup> This potentially explains why some individuals with a high hepcidin level respond to iron as the high hepcidin level is a reflection of the pro-inflammatory state rather than sufficient iron stores. Administration of exogenous iron directly into the circulation can bypass the ferroportin blockade which prevents endogenous iron being bioavailable.

The finding of an increased haemoglobin response to iron in those with lower hepcidin levels is not unique to this study. Takasawa treated dialysis patients with oral iron and measured a significantly lower mean hepcidin-25 level of 10.8ng/ml in responders to oral iron treatment compared with

32.8ng/ml in non-responders ( $p<0.05$ ).<sup>235</sup> Similarly, in a pre-dialysis cohort, Drakou found hepcidin-25 levels lower in responders (mean 1.4ng/ml) versus non-responders (mean 3.4ng/ml) to iron therapy ( $p=0.025$ ).<sup>236</sup> This finding is echoed in work by Chand who found that hepcidin-25 was a significant predictor of degree of haemoglobin increment in the six weeks following intravenous iron administration.<sup>237</sup>

Not all studies however support the use of hepcidin to predict response to iron treatment. In a small subset of the FIND-CKD study, Gaillard did not find a significant association between hepcidin prior to receiving oral iron therapy, and response following either 8 and 24 weeks of treatment.<sup>238</sup> In haemodialysis patients, Tessitore found no association between hepcidin levels and those in whom haemoglobin incremented following 1 gram of iron.<sup>239</sup> Like this study, Tessitore included individuals with a raised CRP in contrast to Gaillard who eliminated those in whom the CRP was elevated.

Hepcidin levels showed a trend to being lower in responders to ESA compared to non-responders. The mean hepcidin level was 75.80ng/ml in that incremented haemoglobin in response to treatment versus 87.98ng/ml in non-responders ( $p=0.330$ ). The influence of iron levels and inflammation on hepcidin levels was described above. The third factor that influences hepcidin levels is erythropoiesis. The blunting of erythropoiesis by elevated hepcidin levels may explain the failure to respond to ESA, the mechanism of action of which is to accelerate erythropoiesis. This theory is supported by the finding that more effective reduction of hepcidin levels by haemodialysis, reduces ESA requirements.<sup>240</sup>

The three influencing factors on hepcidin levels of inflammation, erythropoiesis and iron status provide a rationale for the findings of this study in that elevated hepcidin levels are a general indicator of a lower tendency to respond to either of the treatments. The two different treatments however affect hepcidin levels in differing ways. Iron therapy increases hepcidin levels whereas ESA therapy decreases hepcidin.<sup>196</sup> Knowledge of this may influence how iron and ESA therapy can be given sequentially in certain situations to maximise treatment response. The alternate approach, alluded to in the introduction, is changing the tact of treatment and targeting the hepcidin pathway directly may be of

benefit in the treatment of renal anaemia.<sup>241</sup> The potential importance of this has been shown with the development of both hepcidin agonists and antagonists. Hepcidin agonists have a role in the iron overloaded individual. Although focus has so far been in thalassaemia and siderophilic disease, where iron overload is more prevalent, it may be possible to expand its use beyond this to the haemodialysis patients.<sup>242</sup> This would help mitigate iron overload in individuals who have been rendered iron overloaded by transfusion dependence or as a result of clinical protocols that have given excess iron due to limitations of biomarkers able to determine true iron status. Hepcidin antagonists would theoretically be of benefit in CKD patients, in whom hepcidin levels are elevated and would reduce the reliance on iron and ESA medications, both of which are thought to have adverse effects (see sections 1.4.3 and 1.5.4). Hepcidin antagonists are currently undergoing development.<sup>243</sup>

Ascertaining the evidence for the hepcidin pathway being the key target for renal anaemia treatment is undermined by the inconsistent association between hepcidin levels and haemoglobin response to treatment described in the studies earlier in this section. The eventual introduction of hepcidin antagonists into clinical practice could take two approaches. Universal introduction of the medication, without any knowledge of which individuals would benefit, is similar to the approach taken when ESA medication was first introduced. The alternative approach would be targeting individuals. This may assist by trying to identify which individuals are most likely to benefit by targeting those with high hepcidin levels. This difficulty of this is that , individual intra-variability of hepcidin levels is marked.<sup>244</sup> Our study attempted to mitigate this by consistent method and time of blood sampling. Secondly, technically, measurement of hepcidin is notoriously difficult. This study benefited from the local expertise which produced the seminal radioimmunoassay that was able to measure hepcidin.<sup>245</sup> This assay has been used in a number of research studies that showed concordance between theoretical scientific knowledge and clinical results, an indicator of an effective assay.<sup>188 196</sup> The same group has gone on to develop a synthetic version of the antibody, used within the radioimmunoassay, to develop an antibody that is able to be used in ELISA assays. This ELISA has been validated against the mass spectroscopy measurements used previously by this research group (*personal communication*). ELISA

has the advantage over radioimmunoassay in that it is able to be performed within any biochemistry laboratory, without the specialist equipment that a radioimmunoassay requires. This increases accessibility to the assay as specialist laboratory equipment is not required to perform ELISA in contrast to radioimmunoassay. Improvement, with standardisation, of the hepcidin assay is key to improving its role within research and making a viable biomarker of value within clinical practice. Such as the strength of belief of the importance of hepcidin in renal anaemia, improvement of the hepcidin assay has become the focus of an international steering committee. Once a reliable, reproducible assay has been produced, with minimal inter-assay variability, focus can then be made on reducing the costings which is essential to make this a viable clinical biomarker. <sup>246 247</sup>

## 4.4 Best predictors

The ideal biomarker is able to determine whether there would be a preferential response to either of the two treatments, ESA or iron, at a given point in time. When a single biomarker is unable to do so, a combination of biomarkers incorporated into a treatment algorithm provides an alternative approach to direct towards a particular treatment. Although both treatments can be used simultaneously, taking a more pragmatic step wise approach in those with only a mild anaemia, would reduce overall treatment exposure with its inherent complications.

The first stage required for identification of an ideal biomarker to define anaemic status is to consider each biomarker independently, as was done in sections 3.5.3 and 3.6.3. The key findings of this comparison discussed in the section below. Although this analysis did not identify a single predictive biomarker, the findings facilitated the development of the new treatment model discussed in section 1.1.

### 4.4.1 ESA

Dosing of ESA has historically been titrated according to haemoglobin, as demonstrated in the local clinical protocol seen in Figure 13. There has been no dedicated biomarker that has specifically informed clinicians how to dose the ESA. This study has identified the importance of a lower ESA dose and lower CRP in predicting a positive treatment response. Although these factors are not strong enough biomarkers to be used in isolation in determining treatment, they have a crucial role in the development of a multifactorial treatment algorithm.

#### *C-reactive protein*

A lower CRP prior to treatment was the strongest single predictor of a subsequent positive treatment response; the mean CRP was 13.46mg/l in responders compared to 28.59mg/l in non-responders ( $p=0.038$ ). The association of a lower CRP with subsequent positive treatment response echoes findings of previous studies.<sup>248</sup> For a likely similar aetiology, there is a positive correlation seen between CRP and severity of anaemia, and CRP and required ESA dose to maintain a steady haemoglobin.<sup>249</sup>

A raised CRP is a common finding within the haemodialysis population with prevalence reported between 30 and 50%.<sup>250</sup> This study excluded individuals with overt infection from randomisation, the purpose of which was to focus on the cohort whom have elevated CRP without apparent cause in attempt to tease out the role of inflammation, but not infection. This approach differs from previous papers that have neither sorted or excluded those with overt infection, cohorting all reasons for raised CRP together.<sup>249</sup> By restricting this study to those without an obvious cause of elevated CRP, this study further emphasises the impact of CRP in isolation on ESA treatment response. The ready availability of CRP, as a good marker of inflammation, means that it is practical for this biomarker to form part of a future treatment algorithm.

The impact of inflammation, and therefore CRP on ESA treatment response, is mediated by IL-6 production. This suppresses erythropoiesis, with maximal effect at late stage of erythroid precursor maturation, which is also the target of ESA action (see section 1.2.3). The pro-inflammatory state therefore blunts the therapeutic effect of increasing the ESA dose. The inflammation also upregulates hepcidin limiting bioavailable iron. This prevents iron incorporation into the developing red cell so successful erythropoiesis and haemoglobin incrementation.<sup>251</sup>

#### *ESA dose*

The finding that a lower baseline ESA dose positively predicts a tendency to respond to ESA treatment echoes findings of the Normal Haematocrit Cardiac Trial. In this study, a subset of participants had a 50% increase in their ESA dose and those with a lower baseline dose did, overall have a greater haematocrit response to the change in treatment.<sup>116</sup>

More common than reporting the response to a change in ESA dose, most studies report retrospectively on ESA response in relation to total dose used. Longitudinal studies have long reported the association between high ESA doses, elevated erythropoietin resistance index and increased mortality.<sup>252 253</sup> Registry studies have shown a dose response curve to ESA that plateaus once larger ESA doses are used.<sup>254</sup> The findings of this study are therefore consistent with previous work.

The need to include baseline ESA dose in any future treatment model is supported by the response and interaction modelling following ANOVA analysis. Uptitration of ESA dose in those with a low baseline ESA dose is more likely to result in a positive treatment response.

### *Vitamin B12*

B12 levels can be supplemented so cannot be classified as a predictor of ESA response. Nevertheless, it is apparent that higher levels of B12, even within the normal range, are associated with an increased response to ESA treatment (responders 531.3pg/ml versus non-responders 389.0pg/ml,  $p=0.059$ ).

B12 deficiency limiting the effectiveness of ESAs is particularly pertinent within the local ethnically diverse haemodialysis population. Religious beliefs within the local population have made vegetarianism common, and this diet is associated with decreased vitamin B12 intake.<sup>255</sup> Even though dietary uptake of vitamin B12 is normal in renal failure, loss of B12 through the dialysis membrane, particularly with high flux dialysis, means a higher than normal dietary intake is required to maintain serum levels.<sup>256</sup>

There are two different approaches to reduce the impact of inadequate vitamin B12 levels on blunting treatment response to ESA. Firstly, the current B12 assay measures both active and inactive vitamin B12 levels. One could use an assay which provides functional measurement of B12 status. This would selectively identify the individuals that would benefit from B12 supplementation to optimise ESA use. There are two commercially available assays that provide a functional assessment of B12 levels; the holotranscobolamin (holoTC) assay, which measures the active form of vitamin B12, and the methylmalonic acid (MMA) assay. Intracellular MMA is converted to succinyl coenzyme A by vitamin B12. Levels of MMA rise when there is insufficient B12 present to undertake this conversion. Measurement of MMA levels therefore provide an indirect, functional test of the need for further B12. Both the holoTC and MMA assays, however, have limited availability and yet to be validated within the renal haemodialysis population.<sup>257 258</sup>

The second approach would be to target higher B12 levels using the current assay. This would involve the widespread administration of supplemental vitamin B12, treating those who are both functionally deplete and replete of vitamin B12. This approach is a legitimate way to proceed given that the medication is well tolerated. Vitamin B12 can be given intramuscularly, in the form of hydroxycobalamin, or orally as cyanocobalamin. The issue with this approach is that intramuscular injections are painful and have an associated bleeding risk, particularly given the bleeding diathesis of renal failure. An oral medication would add to the already large pill burden. Until such a time that supplementation can be given either intravenously via the dialysis machine, or within the dialysate, widespread vitamin B12 supplementation is unlikely to become commonplace.

### *Summary*

Predicting response to ESA remains difficult with no one effective biomarker which determines likelihood to respond. A lower CRP and lower baseline ESA dose point towards a tendency to respond to treatment and therefore would be important factors to be included in any treatment model.

Identification of non-responders in advance of a treatment plan is important. For the individual, there is reduced unnecessary exposure to ESA. High ESA dosing is associated with adverse outcome, as shown in section 1.5.4. If the biomarkers predict a low likelihood of ESA response, an alternative treatment plan can be formulated which may involve either supplemental iron or blood transfusion. In addition, more targeted treatment is of financial benefit.

### 4.4.2 Intravenous Iron

Table 15, in section 3.6.3, showed prediction of response to iron to be influenced by the biomarkers: hepcidin, Ret-He, serum iron and transferrin saturation with a lesser effect of CRP. This section discusses these biomarkers, their potential roles and the implications of the findings in clinical practice.

### *Reticulocyte haemoglobin equivalent*

An alternate approach to measuring proteins of the erythropoiesis and the iron cycle is to directly measure the ability of the red cell to incorporate iron. This approach provides a functional assessment

of iron status and tries to negate the impact that inflammation has on biomarkers.<sup>259</sup> The Ret-He measurement quantifies iron incorporation into the developing reticulocyte, and thereby reflects recent iron deficiency within the past 3 to 4 days.<sup>260 261</sup> The publication of the 2015 NICE renal anaemia guidelines, whilst this study was in progress, recommended the use of a functional iron status assay as a first line investigation in anaemia management.

The 2015 NICE guidelines advocated the use of %HRC as the first line measurement of iron status within the haemodialysis population. Its use however is limited by the need to have processed the sample within 6 hours, which is not always feasible within the satellite haemodialysis units setup. If %HRC is unavailable, the alternatives suggested are the more stable reticulated haemoglobin content (CHr) or Ret-He.<sup>95</sup> CHr measurements are performed using the ADVIA® manufactured analyser and Sysmex® analysers measure the Ret-He. CHr and Ret-He are able to be used interchangeably however most evidence generated thus far has been using the ADVIA® measured CHr. As Sysmex® supply most UK analysers, specific evidence to support its use will reassure physicians about the effectiveness of this parameter. Ascertaining the threshold below which treatment of iron deficiency is of benefit is difficult, similar to that encountered when ferritin was first introduced (see section 1.4.4). Guidelines currently suggest treatment for iron deficiency with a Ret-He below 29pg<sup>262 263</sup> however a positive response to treatment was seen in individuals with a Ret-He above this number.

This study found a mean Ret-He of 33.79pg in those who went on to respond to iron treatment. Fishbane randomised 32 haemodialysis patients to receive 1 gram of intravenous iron dextran, using the functional test used in this study of change to determine iron deficiency. Of the participants, 21.8% were deemed to be iron deficient with a mean CHr of 23.7pg in responders versus 27.9pg in non-responders,<sup>264</sup> much lower values than seen in our study. Buttarello's study advocated a higher Ret-He threshold for iron deficiency. Of his 59 participants who completed the study, the mean Ret-He of responders was 30.6pg versus 32.9pg in non-responders. He reported a low sensitivity of 45% with a higher specificity of 83% using the threshold of 30.6pg for iron deficiency.<sup>265</sup> The variability of Ret-He

values in responders to treatment underlies the limitations of this parameter as an individual biomarker for defining iron status.

#### *Transferrin saturations*

Like ferritin, iron studies including TSat, have been in widespread clinical use and formed part of national guidelines. This study however found the utility of iron studies was superior to that of ferritin with a significantly lower mean of 24.38% versus 31.19% seen in responders versus non-responders to iron ( $p=0.017$ ).

Like other proteins, iron studies fluctuate significantly with inflammation. The reduction in transferrin synthesis with the pro-inflammatory nature of advanced renal disease means less transferrin is available to bind and transport iron. The proportion of transferrin bound to iron, and therefore TSATs, increases due to the reduction in transferrin production despite no change in iron levels. This can therefore give a false reassurance that iron supplies are replete.

#### *MCV*

Consistent with previous studies, this study did demonstrate that a lower MCV was predictive of response to iron (90.6fl vs 94.5fl,  $p=0.034$ ).<sup>235</sup> In an otherwise healthy individual, MCV is an effective marker of iron status, with a lower level meaning response to iron is more likely.<sup>266</sup> Within the co-morbid haemodialysis cohort, MCV has been shown to be a marker of overall health status with higher morbidity scoring and mortality seen in those with a higher MCV.<sup>267</sup> This is likely to be related to increased endothelial dysfunction seen in those with a higher MCV.<sup>268</sup> In this study, even those who responded to ESA had a trend to a lower MCV, although this did not reach significance (see Table 13). A lower MCV therefore predicts a positive treatment response to either anaemia treatment undermines MCV as a good marker for differentiating between the two different treatment options.

## 4.5 Sequential treatments

The protocol of this study utilised single treatments when haemoglobin is greater than 90g/l. The effectiveness of those who received the alternate treatment following a failure to respond to the initial treatment is shown in section 3.3.2, Table 8. Below this level, the use of the combination of both iron and ESA may have a role to expedite treatment response but appropriately timing these is crucial to maximise efficacy. The approach of this study provides an ideal opportunity to evaluate how iron and ESA can be used in combination with each other and the sequencing thereof which is discussed below.

### 4.5.1 Iron and ESA therapy in combination

Hepcidin is known to be elevated in renal disease. Although the primary mechanism by which this exacerbates anaemia is through the modulation of the iron cycle, hepcidin also has a direct impact on erythropoiesis (see section 1.6.10). This has been shown in mice models that overexpress the hepcidin encoding gene *Hepc1*; phenotypically, these show reduced sensitivity to endogenous erythropoietin and a failure to upregulate reticulocyte counts in response to anaemia.<sup>269</sup>

Hepcidin levels are downregulated in response to ESAs. In healthy volunteers, this occurred within 24 hours of receiving the medication, maximal at day 3 but persisted beyond 2 weeks.<sup>188</sup> A similar, but less well defined, pattern of response has been seen in those with chronic kidney disease with hepcidin downregulated during the course of a haemodialysis session.<sup>270</sup> The lower hepcidin levels allow stored intracellular iron to be exported into the circulation to be used in erythropoiesis.<sup>196</sup> By the same rationale, administering intravenous iron prior to ESA upregulates hepcidin further suppressing erythropoiesis.

From this understanding of how hepcidin is regulated by ESA, it may be possible to maximise the effectiveness of anaemia treatment; by administering ESA prior to intravenous iron and giving the medications towards the end of the haemodialysis session when hepcidin levels are at their lowest. Both these hypotheses merit further exploration.

## 4.6 Conclusion

This study is a large, prospective exploratory study that randomised patients to receive either ESA and iron, to try determine which biomarkers predictive of response to both ESA and iron. Although both of these two treatments achieved the same response rate when used randomly to treat anaemia within a cohort of haemodialysis patients, it is clear that some individuals would preferentially respond to one treatment over the other. The failure to respond to treatment however in nearly 30% of individuals means that an improvement in treatment strategy is required.

This study promotes the idea that treatment should be titrated according to optimal haemoglobin only, using multiple biomarkers at the time of anaemia to determine how best to achieve this. This study developed a four variable treatment model using the variable baseline ESA dose, CRP, hepcidin and Ret-He. In a retrospective analysis, this resulted in a reduction of non-response to treatment from 29.1% to 17.7%. More judicious use of medication within a validated treatment model will limit non-efficacious ESA treatment being administered, which has been shown to be associated with increased adverse effects in the seminal TREAT, CHOIR and CREATE trials, and allow more liberal, but safe use of iron, supported by the findings of the PIVOTAL study.

Validation of this model, within a clustered randomised control format which has the primary outcome of number of individuals achieving target haemoglobin, is the next step required to bring this type of model into clinical practice.

## 5 References

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## 6 Appendix

Appendix 1. Full study protocol as approved by the JRCO

Study acronym:

**PRIME** (Predictors of Response to Iron and Erythropoietin)

Study title: Predictors of response to treatment with iron and erythropoietin in dialysis anaemia

Version number and date: Version 5, 23/09/16

Main sponsor: Imperial College Healthcare NHS Trust

Funders: Dialysis Research Group, Imperial Renal and Transplant Centre, Hammersmith Hospital, Imperial College Healthcare NHS Trust

REC reference: 14/LO/0805

EudraCT reference: 2014-001383-36

Protocol Authorised by: Joint Research Compliance Office, Imperial College

**Study Management Group**

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**Study Coordination Centre**

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**Clinical Queries**

Clinical queries should be directed to Dr Sarah Hildebrand who will direct the query to the appropriate person.

**Sponsor**

Imperial College Healthcare NHS Trust is the main research Sponsor for this study. For further information regarding the sponsorship conditions, please contact the Head of Regulatory Compliance at:

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**Funder**

The study is funded by the Dialysis Research Group, Imperial Renal and Transplant Centre, Hammersmith Hospital, Imperial College Healthcare NHS Trust, London W12 0HS

This protocol describes the PRIME study and provides information about procedures for entering participants. The protocol should not be used as a guide for the treatment of other participants; every care was taken in its drafting, but corrections or amendments may be necessary. These will be circulated to investigators in the study, but centres entering participants for the first time are advised to contact the trials centre to confirm they have the most recent version.

Problems relating to this trial should be referred, in the first instance, to the study coordination centre. This trial will adhere to the principles outlined in the Medicines for Human Use (Clinical Trials) Regulations 2004 (SI 2004/1031), amended regulations (SI 2006/1928) and the International Conference on Harmonisation Good Clinical Practice (ICH GCP) guidelines. It will be conducted in compliance with the protocol, the Data Protection Act and other regulatory requirements as appropriate.

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**APPENDIX 1. EXAMPLE LIST OF EXPECTED TOXICITIES**

**APPENDIX 2. SUMMARY OF INVESTIGATIONS, TREATMENT AND ASSESSMENTS**

**Glossary of abbreviations**

**EPO:** erythropoietin

**ESA:** erythropoietin stimulating agent

**IMP:** investigational medical product

**GCP:** Good Clinical Practice

**Keywords:** Anaemia, Haemodialysis, Iron, Erythropoietin

**STUDY SUMMARY TITLE** Predictors of response to treatment with iron and erythropoietin in dialysis anaemia

**DESIGN:** An open label, randomised trial

**AIMS:** To define the clinical and laboratory characteristics of functional iron deficiency in haemodialysis patients.

**OUTCOME MEASURES:**

The outcome of this study is the set of diagnostic parameters associated with functional iron deficiency. The gold standard for this diagnosis is a haematological response to iron or non-response to erythropoietin.

**POPULATION** Haemodialysis patients of Imperial Renal and Transplant Centre

**ELIGIBILITY** All haemodialysis patients are eligible. Patients are excluded if they evidence of non-renal anaemia such as gastrointestinal bleeding, malignancy or a bone marrow disorder.

**TREATMENT:** Intravenous iron sucrose 1g given over five consecutive dialysis sessions along with continuation of maintenance erythropoietin at the same dose, or an increase in the dose of maintenance erythropoietin without additional iron treatment.

**DURATION:** 18 months (to complete 31<sup>st</sup> December 2016)

## 1. INTRODUCTION

### 1.1 BACKGROUND

In the UK, about 30 000 patients with complete kidney failure are alive because they receive dialysis treatment.

The majority of dialysis patients are affected by renal anaemia, which increases cardiovascular mortality and is associated with poor quality of life. The principle cause of anaemia in this setting is erythropoietin (EPO) deficiency, since the kidneys are the main source of EPO production. Renal anaemia can be treated effectively in the majority of patients by erythropoietin injections which replace this deficient hormone, and over 95% of dialysis patients have their anaemia corrected with maintenance EPO, usually given intravenously via the dialysis circuit on a weekly basis.

Erythropoietin is ineffective in the context of iron deficiency, since iron is needed to form new blood cells, and dialysis patients are frequently iron deficient, due to the regular loss of blood which is stuck in the circuit after each dialysis session. Renal failure also causes malabsorption of oral iron, so intermittent intravenous iron injections, given via the dialysis circuit, are also a standard part of the treatment of renal anaemia. Iron transport to the bone marrow is slow (another feature of renal failure) and injections often improve the anaemia even when iron stores are adequate – this is termed functional iron deficiency, and is the main cause of a poor response to erythropoietin.

Resolving the anaemia is usually straightforward: although patients vary in their response<sup>271</sup>, the dose of erythropoietin can simply be escalated until the desired haemoglobin is achieved. This strategy is supported by many large observational studies demonstrating better survival in those with corrected haemoglobin. However, those who are more resistant to erythropoietin have worse outcomes, and interventional studies revealed that significant harm results from the high erythropoietin doses that result from haemoglobin-targeted treatment. This leaves a paradox: anaemia should be treated and is easy to treat, but poorly targeted treatment leads to high erythropoietin doses and worse outcomes. It is clearly therefore desirable to target treatment more accurately and better define functional iron deficiency.

Functional iron deficiency can only be defined after treatment: those whose anaemia responds well to iron +/- poorly to erythropoietin are functionally iron deficient. There is currently no reliable test for functional iron deficiency before treatment, and the clinical features are not known. In current practice iron is usually guided by ferritin, which is a mediocre marker of iron storage<sup>272</sup> and in the diagnosis of functional iron deficiency is probably no better than tossing a coin<sup>273</sup>. However, the incorporation of ferritin into current protocols prevents further analysis of functional iron deficiency, since the majority of the group do not receive iron and are therefore never identified<sup>274</sup>. Functional iron deficiency can therefore only be studied by allowing patients to have iron treatment or not, independent of the level of ferritin, ie randomly. Those not receiving iron should receive the only alternative treatment: an increase in maintenance erythropoietin dose.

Potential biomarkers of functional iron deficiency have been proposed, such as hepcidin, which has emerged over the last decade as the master hormone of iron regulation. Our group was the first to develop a reliable immunoassay for this peptide<sup>245</sup>, to measure circulating hepcidin in dialysis patients, and to characterise the influence of bone marrow physiology on hepcidin. Amongst a handful of potential biomarkers, hepcidin is the most promising, but the potential of routine clinical and laboratory parameters is also unknown.

This study therefore aims to fully characterise functional iron deficiency, in terms of routine clinical assessment as well as novel biomarkers, by assessing haemoglobin responses to iron, given by random assignment, without reference to ferritin.

## **1.2 RATIONALE FOR CURRENT STUDY**

### *Research question:*

What is the clinical profile of functional iron deficiency in haemodialysis patients?

The hypothesis for the study is that functional iron deficiency is clinically identifiable prior to treatment: ie. clinical / laboratory parameters exist which reliably predict whether a dialysis patient's anaemia will respond to iron.

### *Benefit:*

Functional iron deficiency can only be identified retrospectively and is usually missed. Reliable diagnostic criteria would enable targeted therapy and avoid the harms of inappropriately high erythropoietin dosing.

### *Risks:*

Blood sampling: up to 7 additional 10ml blood samples may be taken during the study all via the dialysis circuit. This constitutes negligible risk.

Treatment side-effects: iron and erythropoietin are routine aspects of clinical care for all dialysis patients with excellent safety profiles. The main concern is the potential for haemoglobin overshoot or iron overload. The laboratory criteria for randomisation, and maximum of 3 randomisation episodes per participant, with at least a 6 month gap between episodes, make both of these no more likely than with standard clinical care.

Treatment non-response: failure to correct the anaemia over 2 months occurs in about 20% of patients with standard clinical care. It is not known whether the study intervention will result in more or less non-response, but the laboratory criteria for randomisation minimises the impact of any increase in non-response. A slightly increased risk of mild anaemia persisting over 2 months, and a slightly increased risk of transfusion are the potential risks of randomisation. The use of transfusion during this study will be monitored to detect a greater than expected risk.

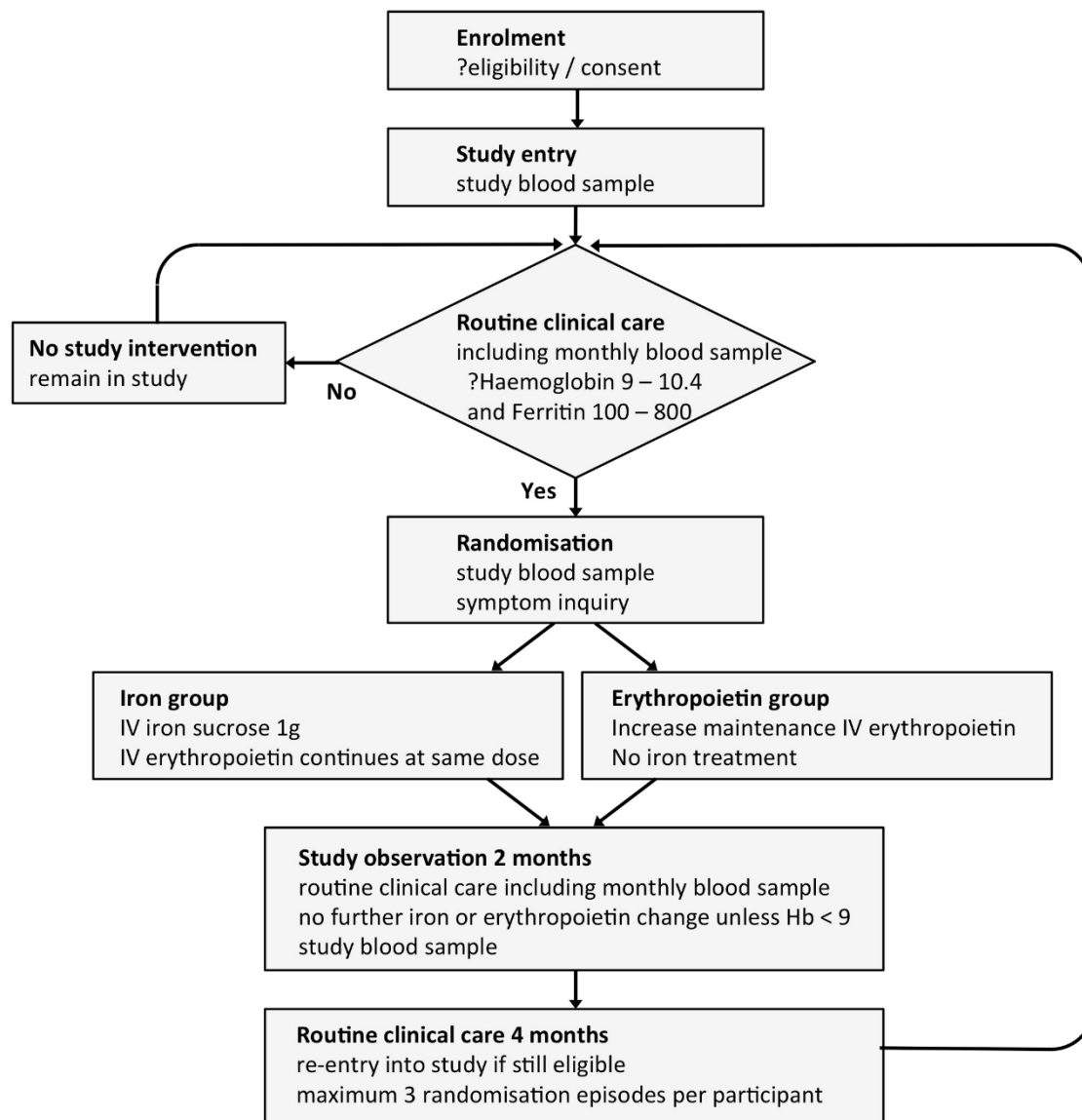
## **2. STUDY OBJECTIVES**

The study objectives are:

- 1) To treat a cohort of mildly anaemic dialysis patients with iron, and another cohort with an increase in their erythropoietin dose, with treatments allocated randomly, so that the cohorts are otherwise the same.
- 2) Identify the independent predictors of treatment response within each group to define the characteristics of functional iron deficiency.
- 3) Use these results to develop an evidence based protocol for renal anaemia treatment, in which those with functional iron deficiency are identified and treated, and are therefore able to avoid the high doses of erythropoietin which are harmful in this setting.

### 3. STUDY DESIGN

This is an open label randomised study. This study aims to collect 200 ‘randomisation’ episodes over and 18 month duration. Initial estimates of recruitment of 420 participants have been revised with the aim now to recruit 200 patients. All participants in the study are haemodialysis patients. Withdrawals from the study are inevitable due a change in clinical status of the patient, change of dialysis base unit and transplantation. This study therefore aims to continually recruit throughout the duration of the study to maintain the number of active participants.



#### 3.1 STUDY OUTCOME MEASURES

The outcome of this study is the set of diagnostic parameters associated with functional iron deficiency. The gold standard for this diagnosis is a haematological response to iron, usually associated with non-response to erythropoietin. Within each group “responders” will be defined as those with a rise in haemoglobin over this period of at least 0.5g/dl. The remainder will be defined as “non-responders”.

## **4. PARTICIPANT ENTRY**

### **4.1 PRE-RANDOMISATION EVALUATIONS**

Health records review and brief symptom inquiry will be used to establish eligibility. Randomisation occurs only when participants develop randomisation criteria.

### **4.2 INCLUSION CRITERIA**

Patients eligible for inclusion in the study have to meet the following criteria:

- Age greater than 18
- Able to consent
- Subject is not pregnant or planning a pregnancy

### **4.3 EXCLUSION CRITERIA**

Patients are excluded from participation in the study through the following:

- Overt blood loss in the past 6 months
- Received transfusion in the past 3 months
- Blood transfusion dependency
- Bone marrow disorder
- Active malignancy or haematological malignancy
- Less than 3 months of haemodialysis
- Unable to adhere to protocol secondary to known allergy or intolerance to medications in protocol

### **4.4 WITHDRAWAL CRITERIA**

Participants will be withdrawn from the study if they develop one of the conditions defined in the exclusion criteria, a clinical decision is made to treat patients outside of standard clinical criteria, or participant choice. They may do so via contact information provided on the patient information leaflet, or through communication with clinical staff who will inform the research team. With their permission, data from their earlier study involvement will still be included.

## **5. RANDOMISATION AND ENROLMENT PROCEDURE**

### **5.1 RANDOMISATION OR REGISTRATION PRACTICALITIES**

Participants will be recruited from the prevalent haemodialysis population from Imperial College Healthcare NHS Trust satellite haemodialysis sites located at Watford General, Ealing and Northwick Park Hospitals. The patient information leaflet will be provided to all potential participants. The researcher will be available during that haemodialysis session and subsequent sessions where patients will have the opportunity to consent.

Once a participant has been randomised to the study, the response is assessed over the subsequent 2 months. If the haemoglobin measured in the standard monthly bloods taken as part of a patient's routine remains less low (between 9.0g/dl and 10.4g/dl inclusive), the patient gets the alternative treatment; similarly they will receive the alternative treatment if they meet the same criteria the following month.

After 6 months from the initial date of randomisation, upon meeting the original randomisation criteria, the patient can be re-randomised in the study. Prior to this point i.e. between 4 and 6 months after initial randomisation episode, they will be treated as per standard clinical protocol.

Given the duration of study of 18 months, participants can be randomised a maximum of three times.

### **5.2 UNBLINDING**

This is an open label study.

## **6. TREATMENTS**

### **6.1 TREATMENT ARMS**

All drugs will be given intravenously via the haemodialysis circuit, within the terms of their licence, in the same manner in which they are administered as part of routine clinical care.

The iron group will receive:

Iron sucrose 1g divided over 5 consecutive haemodialysis sessions

Maintenance erythropoietin will continue at the same dose

The erythropoietin group will receive:

Maintenance erythropoietin at an increased dose – one point on the scale below. For patients who were receiving erythropoietin outside the scale below, but within the range, the dose will be increased by 25%.

No intravenous iron

Erythropoietin scale (units per dialysis session):

1000

2000

3000

4000

6000

8000

12000

There is no specific labelling or packaging required as this is an open label trial.

### **6.2 DOSE MODIFICATIONS FOR TOXICITY**

There is no dose modification for toxicity required. All treatments are to be used within normal dosing ranges.

### **6.3 PREMEDICATION**

No premedication is required.

### **6.4 INTERACTION WITH OTHER DRUGS**

There are no medications that interact with these drugs.

### **6.5 DISPENSING AND ACCOUNTABILITY**

The drugs will be supplied by usual NHS routes as these drugs are currently being used for anaemia in haemodialysis patients. We anticipate a small increase in the use of iron and reduction in the total use of erythropoietin.

There will be no accountability logs for this study as all of these medications are, and will be continue to be, prescribed on a patient's drug chart.

## 7. PHARMACOVIGILANCE

### 7.1 DEFINITIONS

**Adverse Event (AE):** any untoward medical occurrence in a patient or clinical trial subject administered a medicinal product and which does not necessarily have a causal relationship with this treatment. *An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of an investigational medicinal product (IMP), whether or not considered related to the IMP.*

**Adverse Reaction (AR):** all untoward and unintended responses to an IMP related to any dose administered. *All AEs judged by either the reporting investigator or the sponsor as having reasonable causal relationship to a medicinal product qualify as adverse reactions. The expression reasonable causal relationship means to convey in general that there is evidence or argument to suggest a causal relationship.*

**Unexpected Adverse Reaction:** an AR, the nature or severity of which is not consistent with the applicable product information (eg investigator's brochure for an unapproved investigational product or summary of product characteristics (SmPC) for an authorised product). *When the outcome of the adverse reaction is not consistent with the applicable product information this adverse reaction should be considered as unexpected. Side effects documented in the SmPC which occur in a more severe form than anticipated are also considered to be unexpected.*

**Serious Adverse Event (SAE) or Serious Adverse Reaction:** any untoward medical occurrence or effect that at any dose

☐ **Results in death**

☐ **Is life-threatening** – *refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe*

☐ **Requires hospitalisation, or prolongation of existing inpatients' hospitalisation**

☐ **Results in persistent or significant disability or incapacity**

☐ **Is a congenital anomaly or birth defect**

Medical judgement should be exercised in deciding whether an AE/AR is serious in other situations. Important AE/ARs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, should also be considered serious.

**Suspected Unexpected Serious Adverse Reaction (SUSAR):** any suspected adverse reaction related to an IMP that is both unexpected and serious.

### 7.2 CAUSALITY

Most adverse events and adverse drug reactions that occur in this study, whether they are serious or not, will be expected treatment-related toxicities due to the drugs used in this study. The assignment of the causality should be made by the investigator responsible for the care of the participant using the definitions in the table below.

If any doubt about the causality exists the local investigator should inform the study coordination centre who will notify the Chief Investigators. The pharmaceutical companies and/or other clinicians may be asked to advise in some cases.

In the case of discrepant views on causality between the investigator and others, all parties will discuss the case. In the event that no agreement is made, the MHRA will be informed of both points of view.

| <b>Relationship</b>   | <b>Description</b>                                                                                                                                                                                                                                                                                                  |
|-----------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Unrelated</b>      | There is no evidence of any causal relationship                                                                                                                                                                                                                                                                     |
| <b>Unlikely</b>       | There is little evidence to suggest there is a causal relationship (e.g. the event did not occur within a reasonable time after administration of the trial medication). There is another reasonable explanation for the event (e.g. the participant's clinical condition, other concomitant treatment).            |
| <b>Possible</b>       | There is some evidence to suggest a causal relationship (e.g. because the event occurs within a reasonable time after administration of the trial medication). However, the influence of other factors may have contributed to the event (e.g. the participant's clinical condition, other concomitant treatments). |
| <b>Probable</b>       | There is evidence to suggest a causal relationship and the influence of other factors is unlikely.                                                                                                                                                                                                                  |
| <b>Definitely</b>     | There is clear evidence to suggest a causal relationship and other possible                                                                                                                                                                                                                                         |
| <b>Not assessable</b> | There is insufficient or incomplete evidence to make a clinical judgement of the causal relationship.                                                                                                                                                                                                               |

### 7.3 REPORTING PROCEDURES

All adverse events should be reported. Adverse events are 2 months following a treatment episode in the study.

Depending on the nature of the event the reporting procedures below should be followed. Any questions concerning adverse event reporting should be directed to the study coordination centre in the first instance. A flowchart is given below to aid in the reporting procedures.

#### 7.3.1 Non serious AR/AEs

All such toxicities, whether expected or not, should be recorded in the toxicity section of the relevant case report form and sent to the study coordination centre within one month of the form being due.

#### 7.3.2 Serious AR/AEs

Fatal or life threatening SAEs and SUSARs should be reported on the day that the local site is aware of the event. The SAE form asks for nature of event, date of onset, severity, corrective therapies given, outcome and causality (i.e. unrelated, unlikely, possible, probably, definitely). The responsible investigator should sign the causality of the event. Additional information should be sent within 5 days if the reaction has not resolved at the time of reporting.

#### SAEs

An SAE form should be completed and faxed to the study coordination centre for all SAEs within 24 hours. However, relapse and death due to <condition>, and hospitalisations for elective treatment of a pre-existing condition do not need reporting as SAEs.

## SUSARs

In the case of suspected unexpected serious adverse reactions, the staff at the site should:

Complete the SAE case report form & send it immediately (within 24 hours, preferably by fax), signed and dated to the study coordination centre together with relevant treatment forms and anonymised copies of all relevant investigations.

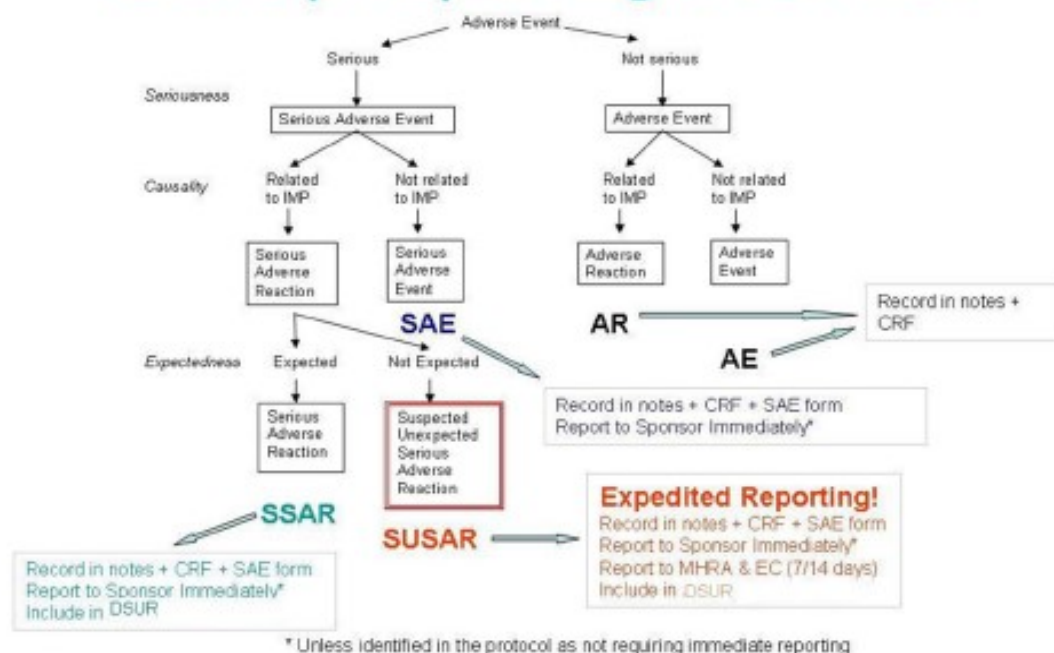
**Or**

Contact the study coordination centre by phone and then send the completed SAE form to the study coordination centre within the following 24 hours as above.

The study coordination centre will notify the MHRA, REC and the Sponsor of all SUSARs occurring during the study according to the following timelines; fatal and life-threatening within 7 days of notification and non-life threatening within 15 days. All investigators will be informed of all SUSARs occurring throughout the study.

Local investigators should report any SUSARs and /or SAEs as required by their Local Research Ethics Committee and/or Research & Development Office.

## Safety Reporting Overview



\* Unless identified in the protocol as not requiring immediate reporting

**Contact details for reporting SAEs and SUSARs**

**Fax: 020 3313 5169, attention Dr Damien Ashby**

**Please send SAE forms to: Dr Sarah Hildebrand at address above**

**Tel: 020 8383 5256 (Mon to Fri 09.00 – 17.00)**

## **8. ASSESSMENT AND FOLLOW-UP**

The study will continue for 2 years and will complete on December 31<sup>st</sup> 2016. All patients on dialysis for over 3 months have permanent kidney failure, and receive lifelong follow up. This will either be within the dialysis units, where monthly blood tests and quarterly clinics are part of standard care, or following transplantation in transplant clinics, where quarterly visits are standard.

### **8.1 LOSS TO FOLLOW-UP**

Due to the permanent nature of renal failure, and the incorporation of this study into the times of their normal clinical care, minimal participants will be lost to follow up. It is expected that patients will exit the study before 2 years due to death, change in geographic location, or transplantation. Participants that withdraw from the study or are lost to study follow up, will continue to receive the standard of care appropriate to their clinical condition.

### **8.2 TRIAL CLOSURE**

Closure of the trial will occur if there are unacceptable risks identified by the monitoring team.

## 9. STATISTICS AND DATA ANALYSIS

Data and all appropriate documentation will be stored for a minimum of 10 years after the completion of the study, including the follow-up period.

The 2 groups of patients (those treated with iron and those treated with increased erythropoietin) will be analysed separately – there is no comparison between the groups. Epidemiologic methods will be used within each group as follows.

Within each group “responders” will be defined as those with a rise in haemoglobin over two months of at least 0.5g/dl. The remainder will be defined as “non-responders”. Clinical characteristics available at baseline (prior to randomisation) will be examined as potential predictors of response.

Baseline clinical characteristics would include novel biomarkers measured at randomisation (such as hepcidin), routine blood tests (eg. ferritin, CRP), data from additional clinical assessment (eg. symptom inquiry, current erythropoietin dose) and fixed clinical characteristics (age, gender, comorbidities).

Baseline characteristics will be compared in responders and non-responders by t-test, Mann-Whitney or Fisher’s test as appropriate. Factors found to distinguish the groups will then be entered into a logistic regression model to determine the independent predictors of response for each treatment.

Clinical criteria defining likely responders for each treatment will then be defined, using thresholds designed to satisfy: (1) that groups are mutually exclusive; (2) that overall likelihood of response is the same for each group; and (3) that at least 90% of patients would fall into one of the groups. These criteria would be used to develop a treatment protocol which should lead to more accurately targeted treatment, and which could be tested against current practice in an interventional study.

Sample size has been estimated as follows. A two group t-test with a 0.05 two-sided significance level, will have 90% power to detect a 20ng/ml (1 standard-deviation) difference in plasma hepcidin between responders and non-responders (divided 60:40), when there are 52 treatment episodes. Up to 4 additional variables may be significantly associated with response and entered into a logistic regression model with hepcidin, which should contain 20 additional treatment episodes for each variable. Therefore 132 episodes should be observed in each of the iron and erythropoietin groups, making 264 randomisation events. Assuming the monthly rate at which patients reach criteria for randomisation is 5%, this will require 440 patients to be observed for 12 months.

## **10. MONITORING**

### **10.1 RISK ASSESSMENT**

This is a low risk study as reviewed by the local Dialysis Research Group.

Treatment side-effects: the study involves licensed medicinal products within the licensed dose range, indication and form. There is no blinding within the trial which means that administration of the medication will be easier for clinical staff as it will be given in the regular form and the medications will be handled as per standard NHS care. Independent of the IMP, the safety parameters that are used in regular clinical practice for management of anaemia are adopted within the trial design. The risk of the medications themselves is therefore no higher than that of standard clinical care.

Treatment over-response: the potential for haemoglobin overshoot or iron overload is no great than with standard clinical care, due to the laboratory criteria for randomisation, and maximum of 3 randomisation episodes per participant, with at least a 6 month gap between episodes.

Treatment non-response: failure to correct the anaemia over 2 months occurs in about 20% of patients with standard clinical care. It is not known whether the study intervention will result in more or less non-response, but the laboratory criteria for randomisation minimises the impact of any increase in non-response. A slightly increased risk of mild anaemia persisting over 2 months, and a slightly increased risk of transfusion are the potential risks of randomisation. The use of transfusion during this study will be monitored to detect a greater than expected risk. This data will be reviewed regularly by the Dialysis Research Group, and via six-monthly formal reports to the sponsor.

Blood sampling: up to 7 additional 10ml blood samples may be taken during the study all via the dialysis circuit. This constitutes negligible risk.

Haemodialysis patients do potentially include a vulnerable group, mainly the elderly and those with limited language skills. All potential participants will be well known to the clinical staff looking after them and advice will be taken with regards to the vulnerable patients and those unlikely to be able to give informed consent. Translators will be used for people with limited English. Potential participants will be able to give consent either at the time or in any subsequent dialysis sessions therefore there will be no time pressure placed upon them. The investigator obtaining consent is Dr Sarah Hildebrand who is GCP certified.

No sensitive data will be collected. Any personalised data will be kept within NHS computers with standard procedures adopted with regards to access. Data will be fully anonymised prior to transfer to any statistical program. Consent for data collection will be obtained at time of inclusion in the trial.

Rational, quality of design and safety have been reviewed by the Dialysis Research Group. Statistical advice has been provided by the Imperial College Statistical Advisory Service. Randomisation will be performed by a computer program with no influence of any clinical data.

### **10.2 MONITORING AT STUDY COORDINATION CENTRE**

The trial master file and consent forms will be kept at Hammersmith House, Hammersmith Hospital. Copies of the consent form will be placed within the participant's clinical notes, verified by the nursing

staff responsible for the participant within the dialysis unit. Their presence will be verified at any randomisation episode.

Data entry will be conducted by Dr Sarah Hildebrand with any missing or unusual data identified. These will be regularly reviewed by the Chief Investigator, Dr Damien Ashby. The sponsor will receive regular reports and may carry out checks of study conduct.

### **10.3 MONITORING AT LOCAL SITE**

All dialysis units are satellites of the Imperial Renal and Transplant Centre, sharing the same policies and governance structure. There is therefore no requirement for local site monitoring.

## **11. REGULATORY ISSUES**

### **11.1 CTA**

This study has Clinical Trials Authorisation from the UK Competent Authority; MHRA. Reference: 32611/0015/001-0001

### **11.2 ETHICS APPROVAL**

The Study Coordination Centre has obtained approval from the Westminster Research Ethics Committee. The study must be submitted for Site Specific Assessment (SSA) at each participating NHS Trust. The Study Coordination Centre will require a copy of the Trust R&D approval letter before accepting participants into the study. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

### **11.3 CONSENT**

Consent to enter the study must be sought from each participant only after a full explanation has been given, an information leaflet offered and time allowed for consideration. Signed participant consent should be obtained. The right of the participant to refuse to participate without giving reasons must be respected. After the participant has entered the trial the clinician remains free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant's best interest, but the reasons for doing so should be recorded. In these cases the participants remain within the study for the purposes of follow-up and data analysis. All participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

### **11.4 CONFIDENTIALITY**

Participants' identification data will be required for the registration process. The Study Coordination Centre will preserve the confidentiality of participants taking part in the study and is registered under the Data Protection Act.

### **11.5 INDEMNITY**

Imperial College Healthcare NHS Trust holds standard NHS Hospital Indemnity and insurance cover with NHS Litigation Authority for NHS Trusts in England, which apply to this study.

### **11.6 SPONSOR**

Imperial College Healthcare NHS Trust will act as the sponsor for this study.

### **11.7 FUNDING**

The Dialysis Research Group, who are supported by the Imperial College Healthcare Charity are funding this study. There is no specific payment intended for staff or participants.

### **11.8 AUDITS AND INSPECTIONS**

The study may be subject to inspection and audit by Imperial College Healthcare NHS Trust under their remit as Sponsor, the Study Coordination Centre and other regulatory bodies to ensure adherence to GCP.

## **12. TRIAL MANAGEMENT**

The Chief Investigator, Co-Investigators and Study Manager will form the trial management group.

Day-to-day management of the study will be co-ordinated by the Study Manager.

The Dialysis Research Group will act as data monitoring committee on a quarterly basis.

### **13. PUBLICATION POLICY**

All publications and presentations relating to the study will be authorised by the Trial Management Group. The first publication of the study results will be in the name of the Trial Management Group, if this does not conflict with the journal's policy. If there are named authors, these will include at least the trial's Chief Investigator, Statistician and Study Manager. Members of the TMG and the Data Monitoring Committee will be listed and contributors will be cited by name if published in a journal where this does not conflict with the journal's policy. Authorship of parallel studies initiated outside of the Trial Management Group will be according to the individuals involved in the project but must acknowledge the contribution of the Trial Management Group and the Study Coordination Centre.

#### 14. REFERENCES [List of useful and relevant references for the study]

1. Locatelli F, Barany P, Covic A, et al. Kidney Disease Improving Global Outcomes (Kdigo) Guidelines on Anaemia Management in Chronic Kidney Disease: A European Renal Best Practice (Erpb) Position Statement. *Nephrology Dialysis Transplantation*. Jun 2013;28(6):1346-1359.
2. Rocha LA, Barreto DV, Barreto FC, et al. Serum Ferritin Level Remains a Reliable Marker of Bone Marrow Iron Stores Evaluated by Histomorphometry in Hemodialysis Patients. *Clinical Journal of the American Society of Nephrology*. Jan 2009;4(1):105-109.
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4. Macdougall IC, Horl WH, Jacobs C, et al. European Best Practice Guidelines 6-8 - Assessing and optimizing iron stores. *Nephrology Dialysis Transplantation*. 2000 2000;15:20-32.
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6. Busbridge M, Griffiths C, Ashby D, et al. Development of a novel immunoassay for the iron regulatory peptide hepcidin. *British Journal of Biomedical Science*. 2009 2009;66(3):150-157.

**Imperial College  
London**

**Imperial College Healthcare** NHS Trust

**ANAEMIA IN  
HAEMODIALYSIS  
PATIENTS**



**INFORMATION FOR PATIENTS**

You are being asked to participate in research. This leaflet explains what this involves and why it is being done.

**No, of course not, it is completely up to you.**  
Participation, or choosing not to, will not affect your medical care.

**Who will benefit?**  
There is no direct benefit to participants by taking part. However, it is hoped that this research will lead to an improved individualised treatment plan for anaemia for each patient.

**Who would be able to see my records?**  
Any information that is collected about you during the study will be kept strictly confidential. Any results which are published or shared outside the hospital will have names and personal details removed first.

The study will continue for 2 years. We would however like to keep all data and blood samples taken, in an anonymised format, for 10 years. This will enable us to re-analyse data in light of any future developments

**Who is carrying out the study?**  
This study is being done by staff of the Imperial College Kidney and Transplant Centre, at

Hammersmith Hospital. The doctor who will be collecting the data and who will be available to talk to about the study is Dr Sarah Hildebrand, clinical fellow and doctoral student. The consultant in charge of this research is Dr Damien Ashby. If you have any further questions about the research, please speak to Dr Sarah Hildebrand, who can be contacted via the dialysis nurses or email [PRIME.trial@imperial.nhs.uk](mailto:PRIME.trial@imperial.nhs.uk). It has been independently approved by the Westminster Research Ethics Committee.

**What to do if something goes wrong?**  
The design of this study has been carefully considered and we envisage that it is unlikely something goes wrong. If, however, something goes wrong, please inform the dialysis nursing staff who can inform the consultant in charge of your care.

In an emergency, please contact the renal registrar on call at the Hammersmith Hospital on 020 8383 1000 and ask for bleep 9977 or via De Warden Ward on 020 8383 6690. The Patient Advisory Liaison Service at Hammersmith is contactable on 020 3313 0088 or email [pals@imperial.nhs.uk](mailto:pals@imperial.nhs.uk)

*D. Ashby*  
4/8/16

### What is this research all about?

The outlook for patients with kidney disease has improved substantially over the last 20 years. Diagnosis is more accurate, and treatment becoming more effective. This progress depends on ongoing research, most of which is conducted in large specialist centres, such as Hammersmith Hospital.

The vast majority of patients on haemodialysis suffer from anaemia. People receive both intravenous iron and erythropoietin (epo) to help manage this. Although both iron and epo are safe medications, the correct doses are hard to predict, with under and over dosing being quite common. Long term use of excessive doses is suspected to be harmful.

By determining why patients' responses to these medications vary, we hope that we can produce a new anaemia protocol which individualises treatment and gives patients the correct treatment at the correct dose.

### What is being done?

This study is part of a PhD project by Dr Søren Hildebrand.

Currently, dialysis patients have routine blood tests on a monthly basis – if you become anaemic, your EPO is increased. Many people would have responded better with an iron injection instead, but there is no reliable test.

This study aims to work out who should have iron instead of EPO, by assigning treatment at random and observing who responds best, in terms of their blood results, including existing tests as well as some new tests which might turn out to be better

### What would I have to do?

Every part of this study is incorporated into your routine dialysis sessions, so you don't have to do anything extra. Monthly blood samples will be taken by nursing staff from the dialysis machine as usual.

If you become mildly anaemic, a small extra blood test will be taken (10ml) and treatment will be with either extra EPO or iron injection. The process of

determining which of these treatments is known as randomization and is performed by a computer programme and can not be influenced by any researcher or member of staff.

If the anaemia hasn't improved by 2 months, both treatments would be given. A small extra blood sample would be taken each month and your tests would be closely monitored.

### Why am I being asked?

All haemodialysis patients are being asked to participate.

### Could there be any side effects?

EPO and iron are both safe treatments which most patients receive regularly. This study will slightly reduce EPO dose and increase iron use. Reduced EPO could prolong a period of mild anaemia, which might be noticed as tiredness. There is no known side effect of increased iron use except that allergic reactions can rarely occur.

### Do I have to take part?

Patient initials \_\_\_\_\_ Study ID \_\_\_\_\_

**RESEARCH CONSENT FORM**

**PREDICTORS OF RESPONSE TO TREATMENT WITH IRON AND ERYTHROPOEITIN IN DIALYSIS  
ANAEMIA (PRIME)**

This form documents your agreement to take part in this research study. You should initial each statement if true and sign at the bottom of the page

I have received a detailed explanation of this study, have read the information sheet (version 5, dated 2<sup>nd</sup> November 2014) and have been able to ask any questions I wanted

☐

I am aware that I am under no obligation to take part and can withdraw at any stage without having to give a reason

☐

I agree to take part in the study

☐

I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

☐

I agree for all data and specimens to be stored anonymously for 10 years to allow for future analysis of newer blood tests of anaemia

☐

**Participant** Name \_\_\_\_\_

Signature \_\_\_\_\_ Date \_\_\_\_\_

**Researcher** Name \_\_\_\_\_

Signature \_\_\_\_\_ Date \_\_\_\_\_

#### Appendix 4. Hepcidin enzyme-linked immunosorbent assay (ELISA) methodology

The heparinised serum samples that had been stored at -80°C were defrosted to room temperature. All samples were analysed in duplicate to try maximise the integrity of the results. A coefficient variant was calculated between the two duplicate samples. If this was greater than 10, the result was not used and the assay was repeated.

Fifty microlitres of Assay Diluent RD1-21 was pipetted to each well on a micro plate.

Fifty microlitres of either the sample, standard or control was added to each well. Standards were made following reconstitution of human hepcidin standard concentration 10000pg/ml with distilled water and subsequent dilution with calibrator diluent RD5-26. The hepcidin standard solutions are listed in below. This was incubated at room temperature for 2 hours.

Each well was then aspirated and washed 4 times using the wash buffer. The wash buffer was made of 20mls wash buffer concentrate and 480mls of distilled water. Excess fluid was removed at each time that it was washed. Following the fourth wash, the micro plate was inverted and blotted against clean paper towels.

Two hundred microlitres of human hepcidin conjugate was added to each well. This was covered with a new adhesive strip and incubate for a further 2 hours at room temperature.

The aspiration and wash process described in step 3 was then repeated.

Two hundred microlitres of substrate solution was then added to each well. The substrate solution was made of equal quantities colour reagent A and B. The result was incubated for 30 min, protected from the light.

After 30 minutes, 50µl of stop solution was added to each well. This precipitated a change in colour from blue to yellow.

A microplate reader was used to determine the optical density of each well. This was set to 450nm, with wavelength correction set at 540 nm.

The optical density was converted to a hepcidin measurement.

| Hepcidin standard solutions concentration |
|-------------------------------------------|
| 1000 pg/ml                                |
| 500 pg/ml                                 |
| 250 pg/ml                                 |
| 125 pg/ml                                 |
| 62.5 pg/ml                                |
| 31.3 pg/ml                                |
| 15.6 pg/ml                                |

0 pg/ml

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*Table showing the different concentrations of hepcidin standard solutions prepared by reconstitution of hepcidin concentrate*