CONTRIBUTING FACTORS AFFECTING ERYTHROPOIESIS

AND ANALYSIS OF ERYTHROPOIESIS

BIOASSAY IN RENAL PATIENTS

IN KWAZULU NATAL

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AUTHORS DECLARATION

This study represents original work by the author. It has not been submitted to any other Tertiary Institution. Where use of the work of others was made, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Department of Clinical Technology, Faculty of Health Sciences, Durban University of Technology, under the supervision of Prof J K Adam and the Dorris Duke Medical Research Institute, Nelson Mandela School of Medicine, Durban, South Africa under the supervision of Professor A.G. Assounga (Department of Medicine, Nelson Mandela School of Medicine).

SIGNED:

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I hereby certify that the above statement is correct.

SIGNED: ______________________________

PROFESSOR J. K. ADAM

SIGNED: ______________________________

PROFESSOR A.G.ASSOUNGA (PhD)
DEDICATION

I dedicate this work to:

My Lord Jesus Christ, who has loved and blessed me in abundance and has been my strong tower throughout my journey. Lord Jesus, you are the light of my life and I honour you for your word never fails. In everything I do, I give you all the glory, honour and praise. You are my everlasting eternal Father in heaven who has never left me with want.

My dearest husband, Rev Winston Benjamin who has been my mentor and encouraged me throughout my studies even through the very challenging times. Your motivation and sacrifice has brought us here. You have been a blessing in my life. Thank you my dearest, I love you dearly. My three bundles of joy who has been, my strength and prayed daily for me to persevere to completion, Jordan, Trinity and Jubilant. You were my driving motivation. I love you all three immensely.

My mother, Mrs Monica Naidoo who, trained me in the ways of the Lord Jesus. My appreciation and love extends much for you for your desire of progress and prayer. My late father, Mr Logan Naidoo for pioneering the medical field. Thank you and I love you mum and dad. My three brothers, Mervin, Lesley and Vernon for all those memories passed, I cheer them. I love you guys immensely.
ABSTRACT

Erythropoietin (EPO) is widely used in patients with chronic renal failure and is a necessity. However, due to the cost implications and the medical complications in our population it is imperative to review the factors affecting the process of erythropoiesis and the analysis of cell proliferation and cell viability in the bioassay. Complications such as hypertension and risk of worsening a malignancy cannot be ignored. We had previously analysed variations of erythropoietin levels in haemodialysis patients over a six month period. This study aims to evaluate erythropoiesis in conjunction with various laboratory, demographic, clinical parameters and inflammatory markers, in the population of haemodialysis patients. EPO, antibody level and antibody activity were analysed in the population groups as EPO responsive and EPO sensitive patients.

This is a prospective, experimental and controlled study. Fifty nine patients were randomly selected from haemodialysis units of Addington and King Edward VIII Hospitals following an informed consent and 15 healthy individuals were also selected as controls. Demographic parameters (age, sex), clinical parameters (weight, height, skin folding, EPO doses and blood pressures (BP) were recorded. Pre-dialysis serum was used to measure laboratory markers (haemoglobin, transferrin, ferritin, albumin, ESR, C reactive protein, creatinine and urea). EPO levels and antibody levels were measured by ELISA, the optical density of each well was determined within fifteen minutes using the microplate reader set at 450 nm. All results were statistically analysed using SPSS statistical package version 21 (IBM®).
Patients requiring very high doses of EPO to reach Hb of 11g/dL, and they remained anaemic after at least three months of adequate EPO doses were considered to be EPO resistant. Those who responded to the usual EPO doses were labelled EPO sensitive. The bioassay was used to quantify cell proliferation and cell viability in the presence of EPO. The UT 7 cells were cultured in medium, in the presence of serum from the EPO resistant, EPO sensitive patients and the healthy, control subjects. Luminescence was read with the Glorunner Microplate Luminometer and was recorded in relative light units (RLU).

The analysis revealed: a non-significant positive correlation between haemoglobin and erythropoietin levels. However, a strong negative correlation was found between CRP and albumin level (R= -0.591; (p=0.001), which was not significant. No correlation was found between haemoglobin or erythropoietin levels and CRP or albumin. There was a positive correlation with systolic and diastolic blood pressures and mean arterial pressures which was statistically significant (p <0.05). EPO dosages and Hb levels were correlated significantly (p < 0.05). No correlation of EPO levels and Hb; age and Hb was found to be significant (p = 0.08). The UT 7 cells cultured in serum in medium alone with RHuEPO containing cells were statistically significant (p <0.01)). Reduction of ATP stimulation between medium and serum was observed. However, mean arterial pressures had a significant association with EPO resistance (p = 0.041) odd ratio- 1.066.

In conclusion, EPO level is not a useful tool for the monitoring of its use as it does not correlate with EPO goal of red blood production in our patients. The neutralizing antibodies
did not correlate with any of our variables contributing to erythropoiesis, and are therefore not confirmed as playing a major role in erythropoiesis.

From the analysis of our results the key contributing factors of EPO doses, malnutrition and age were more significant in erythropoiesis. However the higher doses of EPO significantly increased the blood pressures and the mean arterial pressures (MAP). The analysis of the bioassay showed lack of difference between EPO responsive and EPO sensitive patients. This observation warrants further studies to clarify the role of serum of haemodialysis patients in erythropoiesis.
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My supervisor, Professor AG Assounga, for his expert advice, motivation, dedication, constructive criticism, constant encouragement, guidance and patience during the research and preparation involved in this dissertation. I sincerely thank him for being my mentor and for the opportunity of working under his expert guidance.

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The Superintendent (Addington and King Edward VIII Hospitals), for granting me permission to access patients at the haemodialysis units. Both unit managers, Sr Green, Sr Toboko and their staff of the haemodialysis units, Addington Hospital and King Edward VIII Hospitals for all their assistance in the collection of patient samples and data.
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## Abbreviations

<p>| Ab | - | Antibody |
| APR | - | Acute phase response |
| ALB | - | Albumin |
| ACD | - | Anaemia of chronic disease |
| ABS | - | Antibodies |
| AIF | - | Apoptosis inducing factor |
| AMiC | - | Arterial micro calcification |
| AIDS | - | Auto immune deficiency syndrome |
| BP | - | Blood pressure |
| BMI | - | Body mass index |
| BM | - | Bone marrow |
| BSA | - | Bovine serum |
| BFU-E | - | Burst-forming unit-erythroid |
| CRP | - | C reactive protein |
| ChIP | - | Chromatin immunoprecipitation |
| CGN | - | Chronic glomerulonephritis |
| CKD | - | Chronic kidney disease |</p>
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<td>chronic renal failure</td>
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<td>CFU-E</td>
<td>colony forming units- erythroid</td>
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<td>CSF</td>
<td>colony stimulating factors</td>
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<td>CHF</td>
<td>congestive heart failure</td>
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<td>CERA</td>
<td>continuous EPO receptor activator</td>
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<td>CAD</td>
<td>coronary artery disease</td>
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<td>CHOIR</td>
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<td>DOQI</td>
<td>Disease Outcomes Quality Initiative</td>
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<td>DMT1</td>
<td>divalent metal transporter 1</td>
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<td>ELISA</td>
<td>enzyme-linked immunoassay</td>
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<td>ESRD</td>
<td>end-stage renal disease</td>
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<td>EPOR</td>
<td>EPO receptor</td>
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Fas Ligand (FasL) - Fas signalling

FERR - ferritin

FDA - food and drug administration

FACT-F - functional Assessment of Cancer Therapy-Fatigue

GI - gastrointestinal

GFR - glomerular filtration rate

HD - haemodialysis

Hb - haemoglobin

HV group - healthy volunteers

HR - heart rate

HSP70 - heat shock protein 70

HF-HD - High flux HD

HIV - human immune deficiency virus

HuIFN - human interferon

HIF - hypoxia-inducible factor

HIF-1 - hypoxia-inducible transcription factor.

HREs - hypoxia-response elements

IBD - inflammatory bowel disease
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>IVD</td>
<td>in vitro diagnostic</td>
</tr>
<tr>
<td>ICH</td>
<td>International Conference on Harmonization</td>
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<tr>
<td>CI</td>
<td>interval</td>
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<tr>
<td>IWL</td>
<td>involuntary weight loss</td>
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<tr>
<td>FE</td>
<td>iron</td>
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<td>IRI</td>
<td>ischemia-reperfusion injury</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>LBM</td>
<td>lean body mass</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LF-HD</td>
<td>low flux HD</td>
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<tr>
<td>MHD</td>
<td>maintenance haemodialysis</td>
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<tr>
<td>MS</td>
<td>malnutrition score</td>
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<tr>
<td>MIA</td>
<td>malnutrition, inflammation, and atherosclerosis</td>
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<tr>
<td>MICS</td>
<td>malnutrition-inflammation complex syndrome</td>
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<tr>
<td>MIS</td>
<td>Malnutrition-Inflammation Score</td>
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<tr>
<td>MICERA</td>
<td>methoxy polyethylene glycol-epoietin beta</td>
</tr>
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<td>MAC</td>
<td>mid-arm circumference</td>
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<tr>
<td>MAMC</td>
<td>midarm muscle circumference</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>DOH</td>
<td>National Department of Health</td>
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<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>Nab</td>
<td>neutralizing antibodies</td>
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<tr>
<td>N/D</td>
<td>night/day ratio</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>NCD</td>
<td>noncommunicable diseases</td>
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<tr>
<td>NTBI</td>
<td>non–transferrin-bound iron</td>
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<tr>
<td>NF</td>
<td>nuclear factor</td>
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<tr>
<td>NF-kB</td>
<td>Nuclear factor-kappNucaB</td>
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<tr>
<td>OR</td>
<td>odds ratio</td>
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<tr>
<td>OL-HDF</td>
<td>online hemodialfiltration</td>
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<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
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<tr>
<td>HbSS</td>
<td>Patients with sickle-cell disease</td>
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<tr>
<td>HbAS</td>
<td>patients with single heterozygous sickle haemoglobin</td>
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<tr>
<td>PEW</td>
<td>protein energy wasting</td>
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<tr>
<td>PMA</td>
<td>phorbo l myristate acetate</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
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<tr>
<td>Acronym</td>
<td>Abbreviation</td>
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<tr>
<td>POP</td>
<td>populations</td>
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<tr>
<td>Hgb</td>
<td>predialysis hemoglobin</td>
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<td>PHDs</td>
<td>prolyl hydroxylases</td>
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<tr>
<td>nPCR</td>
<td>protein catabolic rate</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PEM</td>
<td>Protein-energy malnutrition</td>
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<tr>
<td>PAH</td>
<td>pulmonary arterial hypertension</td>
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<tr>
<td>PRCA</td>
<td>pure red cell aplasia</td>
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<tr>
<td>QoL</td>
<td>quality of life</td>
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<tr>
<td>RIP</td>
<td>radioimmunoprecipitation</td>
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<tr>
<td>RHuEPO</td>
<td>Recombinant Erythropoietin</td>
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<tr>
<td>RBCs</td>
<td>red blood cells</td>
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<tr>
<td>RLU</td>
<td>Relative light unit</td>
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<tr>
<td>REPs</td>
<td>renal Epo-producing cells</td>
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<tr>
<td>RRT</td>
<td>replacement therapy (RRT)</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>SGK1</td>
<td>serum and glucocorticoid-regulated kinase-1</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>STAT-5</td>
<td>Signal transducer and activator of transcription 5</td>
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<tr>
<td>SFT</td>
<td>skin fold thickness</td>
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<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
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<tr>
<td>TAMCIS</td>
<td>Tel Aviv Medical Center Inflammation Survey</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
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<tr>
<td>Tf</td>
<td>transferrin</td>
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<tr>
<td>TFR1</td>
<td>transferrin receptors</td>
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<td>T Sats</td>
<td>Transferrin Saturation</td>
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<tr>
<td>TREAT</td>
<td>Trial to Reduce Cardiovascular Events with Aranesp Therapy</td>
</tr>
<tr>
<td>TSF</td>
<td>triceps skin-fold thickness</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>vHL</td>
<td>von Hippel Lindau</td>
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<tr>
<td>pvHL</td>
<td>von Hippel-Lindau tumor suppressor protein</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER ONE: INTRODUCTION

Erythropoiesis is the production of red blood cells. This process takes place when there is a decrease in oxygen in blood. This decrease oxygen in blood is sensed by the kidneys. Erythropoietin, a hormone that is secreted by the kidneys is responsible for the production of red blood cells (erythrocytes). Erythropoietin stimulates the process of erythropoiesis in the haemopoietic tissues. The process begins with the production and differentiation of red cell precursors, which eventually produces red blood cells (erythrocytes). In adults this process takes place in the bone marrow (Palis and Segel, 1998; Sherwood et al., 2005). Greater amount of physical activity can increase the production of erythrocytes (Tao et al., 2010). The production of erythrocytes in specific diseases is known as extramedullary erythropoiesis. Extramedullary erythropoiesis in specific diseases occurs in the spleen and liver and not in the bone marrow.

Erythropoiesis and Renal Failure

The onset of normochromic normocytic anaemia becomes more progressive due to the limited production of erythropoietin. The decrease in the glomerular filtration rate in patients with end stage renal disease has an effect on the progression of normochromic normocytic anaemia. Due to the poor activation of reticulocytes and the poor survival of erythrocytes there is an increase in the ureamia-induced platelet dysfunction. This is usually followed with the risk of bleeding (Macdougall et al., 2012). The limited production of erythrocytes in the bone marrow in end stage renal disease progresses to
the normochromic normocytic anaemia. The haematocrit readings of these patients are usually between 20%-35%. However the normal readings for normal males are between 42%-54% and for females between 20%-35%. Nutritional deficiencies, iron metabolism abnormalities, and circulating uremic toxins contribute to anaemia by the decrease in production of erythrocytes. The stimulation of the bone marrow to produce erythrocytes by the hormone, erythropoietin, is decreased in end stage renal disease and with other contributing factors to anaemia. Hypertension, ureamia, acid base imbalance and electrolyte composition contributes to accelerated breakdown of erythrocytes or red blood cells contributing to anaemia (Volker, 2010). Aluminum, ion overload or deficiencies in iron stores are common in the development of microcytic hypochromic anaemia. The phosphate-binding agent to manage hyperphosphataemia contains aluminum in their medications. Municipal water which is supplied to domestic households contains aluminum. Aluminum is also present in water that is not treated or in nondealuminized dialysis water. Chelation with desferoxamine is used for the treatment of microcytic hypochromic anaemia (Von Bonodorff et al., 1990). Malaria and malnutrition are major limiting factors affecting the process of erythropoiesis in haemodialysis patients in KZN. Malaria and malnutrition are not common in other countries as it is in KZN, South Africa.

The Kidney Disease Outcomes Quality Initiative (DOQI) general guidelines (2006) are not specific for South African haemodialysis population. In this population group, malnutrition, genetics and other medical complications contribute to the process of erythropoiesis. Anaemia contributes to the morbidity and mortality in End stage renal
disease (ESRD) patients. The treatment of anaemia with the use of recombinant erythropoietin (RHuEPO) in ESRD has been successful. The availability of iron, good protein nutrition and absence of inflammation plays an important role in erythropoiesis. Therefore patients that are on RHuEPO can be affected by any of the above factors listed (Volker, 2010). Recombinant erythropoietin in ESRD has been used worldwide for many years and it improved the lifestyles of many patients. The increase use of RHuEPO developed significant side effects such as pure red cell aplasia and complications to existing cancer. Furthermore, RHuEPO is very expensive for patients in South Africa, knowing that they have to also maintain a basic lifestyle. The cost of dialysis therapy is increasing and with other treatments that are required, patients find it unaffordable (Nissenson et al., 2002).

The bioassay screening method used demonstrates the antibody (Ab) activity. Bioassays are one of the exclusive assays that can quantitatively measure neutralizing activity against therapeutic proteins (Thorpe and Swanson, 2005). Neutralizing antibodies can bind to the portions of drug molecules involved in receptor binding or cell activation, thereby blocking the therapeutic effect of the drug (Martinez et al., 2014). However, the bioassay will be set up to demonstrate the activity of antibodies on recombinant erythropoietin in HD patients. This type of study is the first to be done on renal patients in South Africa.

The bioassay has been the most accurate Ab assay in terms of speed, specificity, sensitivity, and the ability to characterize binding of antibodies (Ab isotopes and relative
binding affinities), and the ability to regenerate the sensor. Therefore, the bioassay is recommended to be used for the further investigation for the measurement of neutralizing capacity of antibodies. They require days of cultivation for the cells to proliferate and microscopic quantification of the erythroid colonies growing in culture (Thorpe and Swanson, 2005). With the use of the bioassay one can determine whether the antibodies are blocking the process of erythropoiesis.

In this prospective quantitative study, the nutrition of the patients will be assessed by monitoring transferrin and albumin levels as well as anthropometric analysis. Inflammation will be monitored by the measurement of C reactive protein (CRP) and erythrocyte sediment rate (ESR) of blood. A convenience and purposive sample of forty four haemodialysis patients will be selected. Forty four patients will be receiving recombinant erythropoietin (RHuEPO) and fifteen will not be receiving RHuEPO as per the dialysis prescription.

The study will assess the various factors which play a role in erythropoiesis, i.e., those that promote and those that inhibit the process. The use of the bioassay is to quantitatively identify and measure neutralizing antibodies against recombinant erythropoietin (Thorpe and Swanson, 2005). The bioassay will be used to analyze the significance of the antibodies present in the serum of the haemodialysis patients in KZN.
CHAPTER TWO: LITERATURE REVIEW

2.1. End Stage Renal Disease in South Africa

The challenges of chronic renal disease has increased nationwide and is the greatest in developing countries such as South Africa (SA). This is related to the rise of diabetics and hypertensives of non-communicable diseases (NCD) to 130%. The HIV virus which is a further complication in ESRD contributes 30% to mortality and 19.9% of adults to morbidity (De Francisco et al, 2009). Non Communicable Diseases (NCD) remains the major contributing factor to the mortality rate (Katz, 2005). The primary cause of end-stage renal disease (ESRD) is hypertension. Statistics reveal that hypertension is common in 34.6% of Blacks, 4.3% Whites, 20.9% of mixed race people, and 13.9% of Indians. Diabetes is believed to occur in 10% to 16% of South Africans. These risk factors, together with a high HIV/ESRD concern (8%), result in an increased concern of CKD. Another researcher stated that other nontraditional risk factors, such as low birth weight, must also be considered (Lysaght, 2002). Despite rates of ESRD suspected to be about 400 per million populations (pop), only 99 per million receive renal replacement therapy (RRT) (Lysaght, 2002). Novel methods have to be established in the developing world to tackle the NCD and communicable disease burden. Katz (2005) investigated the option of an integrated approach to chronic diseases as an answer to some of this burden. Both an urban-based and a rural-based NCD prevention and treatment program was reviewed (Katz, 2005).
Glomerular filtration rates below 25-30mL/min contribute to anaemia in 90% of patients with end stage renal disease. Anaemia which contributes to morbidity and mortality becomes a concern in ESRD (Pisoni et al., 2004). Although the etiology is multifactorial the suboptimal production of erythropoietin caused by renal mass loss appears to be of major importance. Since the commencement of Erythropoiesis Stimulating Agents (ESA´s) in the treatment of anaemia in CKD, new advancements have been researched. Erythropoietin stimulating agents (ESA´s) contributes to the process of erythropoiesis and hence increase in the haemoglobin levels (Barros et al., 2011).

### 2.2 History of Anaemia in End Stage Renal Disease

Morbidity, mortality and other medical complications are closely related to ESRD. Anaemia is associated with ESRD and develops early in the diagnosis of the disease. Anaemia contributes to the poor quality of life (Fung et al., 2007; Bross et al., 2010). There had been many adverse effects of anaemia with these patients-many complications and increase in mortality from cardiovascular complications. Prior to the commencement of RHuEPO, severe anaemia in chronic dialysis patients was treated with regular blood transfusions (Buttarello et al., 2010).

Regular blood transfusions to the patients caused complications such as, iron overload, viral hepatitis and HIV. This also increased production of antibodies to human antigens and further complicated transplantation options (Torti and Torti et al., 2002). The
introduction to the treatment of recombinant human erythropoietin in the late 1980s brought a promising change to the treatment of anaemia in ESRD patients (Weiner et al., 2005; Hasegawa et al., 2011).

Severe anaemia was frequent and blood transfusions were often necessary to assist the patient’s wellbeing, malaise, loss of appetite, weakness, etc. Further observational studies performed during the late 80’s suggested that high haemoglobin (Hb) values may have been beneficial in CKD patients and the use of ESA’s became universal (Barros et al., 2011). Randomized trials tried to show the positive impact of rising Hb levels with ESA’s in patient's outcome (Barros et al., 2011).

Anaemia in CKD patients had predominantly been due to the deficiency of the erythropoietin hormone. This may be contributed due to iron deficiency, inhibition of erythropoiesis caused by nutritional deficiencies, inflammation, secondary hyperparathyroidism, or accumulation of inhibitory uraemic toxins, decreased erythrocytes and severe anaemia (Besarab and Levin, 2000; Somvanshi et al., 2012). Anaemia in CKD results in tiredness, decreased ability to exercise. The cognitive and immune function does not function at optimum levels. Patients have a poor quality of life (Quigley et al., 2004). These authors further described that anaemia of CKD significantly contributed to the disease by causing or exacerbating existing co morbidities. Early treatment of anaemia in ESRD could improve the patient’s wellbeing. This could also contribute in slowing the progression of the renal disease (Somvanshi et al., 2012).
Variety of new strategies for stimulating erythropoiesis was demonstrated (Somvanshi et al., 2012). The authors stated that their results were not only of interest scientifically but that they also contribute to new therapeutic agents in the future. As with all treatments for anaemia, there will be both efficacy and safety considerations. Recombinant human erythropoietin therapy proved that Hb levels could increase. It had taken nearly 20 years to realize the limitations of this therapy and the potential for harm if used too aggressively. There have been no newer agents with outcomes of data showing superiority to existing ESAs. There are insufficient information and data on any new agents. The authors had suggested that the newer agents will need to be subjected to the same degree of scientific investigation as the existing ESAs. It might be many years before the true efficacy. Safety balance of these novel scientific strategies would be realized (Somvanshi et al., 2012).

2.3 Anaemia in renal failure

Anaemia is present in patients with chronic kidney disease (CKD). The continuous loss of blood, decreased erythrocyte life span and deficiency in vitamins contribute to the progression of anaemia in ESRD patients. Decreased production of erythropoietin (EPO), decreased iron, and inflammation also contribute to the anaemia (Formanowicz et al., 2007). The authors described that unexplained disturbances is that of iron homeostasis in CKD. The process of maintaining the balance of iron within the body is very crucial (Sackmann et al., 2007).
Chronic kidney disease patients are compromised already and further complicated by them having a decreased production of erythropoietin followed by anaemia. Anaemia treatment of majority patients that are on conventional haemodialysis (HD) are known to be in stage 5 of CKD (Popat, 2011). End stage renal disease patients are treated by other modalities which are the renal replacement therapies (RRT). These include peritoneal dialysis and kidney transplantation. Each of this treatment also has other factors that contribute to the patients existing anaemia and is managed accordingly (Saudi, 2002).

2.3.1 Factors responsible for causing Anaemia

There are several factors other than the minimal renal erythropoiesis. These factors include anaemia, elevated destruction of erythrocytes which are enhanced by the chemical effects of uraemic toxins. The platelet not functioning at optimal standards increases the risk of blood loss. Blood loss is due to clotting inside haemodialysers and sets during HD sessions and common bleeding. More factors include the haemolysis related with contamination of dialysate water; and water-soluble losses of folate and vitamin B_{12} through haemodialyser membranes, affecting erythropoiesis (Bowry and Gatti, 2011). Many factors affect the patients that are on dialysis therapy. These patients have a reduced amount of erythropoietin. Long term therapy of dialysis patients allows these patients to reduce their production and survival of erythrocytes. Other co-morbidities accompanied with ESRD also contribute to anaemia. These are the bone
disease (secondary to hyperparathyroidism or aluminum intoxication) and increased inflammatory activity (Ada et al., 2006).

2.4. Anaemia and Cardiovascular Outcome

Anaemia in ESRD, with cardiovascular complications contributes to the patient’s quality of life. The decrease in physical activity, vitality and the general co morbidities of anaemia requires more attention. All of these co-morbidities have a direct effect on cardiovascular outcomes (Foley et al., 1996). Increased mortality among HD patients is due to the cardiovascular complications. Anaemia allows overload on cardiac function, ultimately leading to left ventricular hypertrophy which is a marker of morbidity and mortality (Besarab et al., 1998). There are increased side effects with the higher levels of haemoglobin and this is of concern to the wellbeing of the patient. There is however a minimal clinical improvement with higher haemoglobins levels which is not significant (Clement et al., 2009; Makoto et al., 2013; Ohashi et al., 2013). Prior to the clinical use of erythropoietin stimulating agents (ESAs) in the early nineties, anaemia had been the major concern of CKD. Anaemia was treated with regular blood transfusions (Barros et al., 2011).
2.5 End Stage Renal Replacement therapies

Pre-dialysis patients on anaemia management in a specific population had been investigated. There were greater than 30% of the patients who had follow up treatments with their nephrologists when they commenced dialysis therapy. These were done for a time period of less than six months. The follow up treatment of less than six months contributed to the decreased effectiveness in the progression of ESRD and the co-morbidities (Valderrábano et al., 2003). The authors described that cardiovascular comorbidities were extremely common, particularly in diabetic patients and, in their investigation. Diabetes was the most common cause of end stage renal disease. Patients that had follow up treatments to a Nephrologist for greater than a year, and was associated with less frequent (predominantly heart-related) complications. There were a minimum number of patients who received epoietin treatment before starting dialysis. Majority of the patients started dialysis with severe anaemia. The epoietin treatment was started with very low Hb concentrations. These were lower than the Hb concentrations recommended by the European Best Practice Guidelines (EBPG) (Valderrábano et al., 2003).

End stage renal disease (ESRD) has increased in the South African population (Hogan, 2009). Dialysis is the primary modality to patients with chronic kidney disease. Patients wait in anticipation for donated kidneys because this will allow them to not have daily dialysis therapy. There is a strain on national resources when patients remain on the
transplant list for a very long period of time continuing dialysis therapy. Nephrologists also find it difficult in the protocol of accepting patients onto the transplant program (Hogan, 2009).

Hoagan (2009) further explained that health system in South Africa, like in other countries, was structured by the presence of both a private sector as well as the public sector. These were with different financial and human resources. The existence of these two sectors extensively contributed to the unfair access to chronic renal dialysis for the population. The author explained that the objective of the National Health Services of South Africa was to provide all South African citizens and permanent residents equitable access to chronic dialysis therapy (Hogan, 2009).

2.6 Erythropoiesis

2.6.1 Process of Erythropoiesis

The process of erythropoiesis in adult had been distinguished by the rapid growth development of haematopoietic stem cells to lineage-specific progenitors. Their shape and further characteristics had become identifiable precursors which enucleate to form developed erythrocytes (Palis et al., 2010). Early stages of erythropoiesis are distinguished by the appearance within the yolk sac of a transient, lineage-restricted progenitor group. This group activates a series of erythroid precursors. In blood
circulation the enhanced development of precursors occur. The process is distinguished by nuclear condensation and embryonic haemoglobin accumulation (Brunelli et al., 2008). The development stages are reliant on erythropoietin signaling through its cognate receptor. Many erythroid-specific transcription factors include GATA1 and EKLF (Bieber, 2001; Palis et al., 2010).

**Figure 1:** The process of Erythropoiesis (Guyton and Hill, 2000)

The hormone erythropoietin in the bone marrow stimulates differentiation of progenitor cells into erythroid precursors. The process of erythropoiesis is maintained by the EPO
hormone (Haase, 2010; Jelkmann, 2011). During this process, many intermediate cell stages can be recognized beginning with the nucleated proerythroblast (pronormoblast). The cell nucleus is then removed at the orthochromatic erythroblast (normoblast) stage. This is prior to an enucleated polychromatic erythrocyte often known as the reticulocyte (Figure1). These are sent into the circulation where they develop into functional erythrocytes (Haase, 2010; Jelkmann, 2011).

### 2.6.2 Regulator for Erythropoiesis

The maintenance of erythropoiesis and its activation in the presence of hypoxia is the erythropoietin response to ischaemic stress. The cloning and manufacturing of recombinant human EPO has resulted to safe therapy in HD patients. This had been present with anaemia for average of twenty five years and has promoted clinical investigations of EPO action (Buttarello et al., 2004).

The oxygen delivery to brain, heart and other non-haematopoietic tissues is facilitated by nitric oxide which is increased by erythropoietin. Erythropoietin is also functional in the vascular endothelial response where it increases nitric oxide. These have been reported *in vitro* from the animal and cell models of ischaemic stress, and injury (Zhang et al., 2014). The major component for life is oxygen, and the body has developed an elite mechanism to contain oxygen in the lungs and transport it to the tissues. Haemoglobin (Hb) is sustained within red blood cells (RBCs). The erythrocytes or RBCs
are the key components for carrying the oxygen in blood. The erythrocytes are firmly maintained and deliver oxygen as when the body requires it (Elliot et al., 2008).

The foundational criteria for oxygen supply to the tissues to maintain the red cell volume. The comparisons of the very energetic processes of erythropoiesis and depletion of the red blood cells are not secure. As stated by the authors the haematocrit has been consistent in the normal individuals (Weiss and Goodnough, 2005). This is achieved by a very diligent and tactful homeostatic mechanism which relates to the delivery of oxygen and the process of erythropoiesis. The process is powered by the primary glycoprotein hormone erythropoietin (EPO). The clarity of the entire process is that when there is a minimum decrease in the production of the hormone there will be a direct response of decreased erythrocytes in circulation. Anaemia will thereafter follow with the decrease of erythrocytes in circulation. Anaemia in Chronic renal failure is the most common predominant example. He further stated that the erythropoietin therapy is extensively used in other clinical conditions and the advent of novel therapeutic approaches. He discussed that this is the ideal time to review the physiology and pathophysiology of this remarkable and essential hormone (Kendall, 2001).

The attributes of a variety of physiological and pathophysiological conditions are the contingency of the hypoxic regions. A presentation of lack of nutrients, oxygen, and ischaemia due to the deprivation of the blood supply in tissues manifests in Hypoxia (Weidemann and Johnson, 2009). When the blood flow is deprived to the middle of the
tumours at the junctions of ischaemia and carcinogenesis this allows the expansion of
tumours to establish hypoxic sites (Paul et al., 2003; Ikeda, 2005). On more than one
level the hypoxic stimulus aggravates the adaptive processes. Individual cells
reorganize their metabolic enzyme composition proceeding anaerobic metabolism. This
follows an increase in the levels of glycolytic enzymes and glucose distributors in
hypoxic cells and tissues (Beleslin-Cokic et al., 2004).

During the mechanism of erythropoiesis the erythroid precursor cells grow rapidly and
individualize into RBCs (Singh et al., 2014). At the beginning stages the haematopoietic
progenitor cells located primarily in the bone marrow individualize into burst-forming unit
erthyroid cells. They are called burst-forming unit erythroid cells because of the
characteristic ‘burst’ colonies. These were developed in cell culture in a semisolid
medium (Fibach, 2011; Singh et al., 2014). The cells will continue to characterize into
colony-forming unit erythroid cells. They will then further access the responsiveness
erthropoietin. The coalescence of Hb and individualization into proerythroblasts is due
to the stimulation of colony-forming unit erythroid with EPO. The erythroblasts will then
be formed. The red blood cells will a nucleate cumulating in reticulocytes. They are
called reticulocytes because of the 'reticulin' accompanied with the existence of RNA.
After several days, reticulin decreases and the cells become completely developed
RBCs. With normal circumstances in adults, erythrocytes have a longer lifespan (3–4
months) (Bieber, 2001; Singh et al., 2014).
Recent observations, by Frank (2003), may have widespread implications for the understanding of various inflammatory complications. It implicates that the released heme may be a physiological response that is necessary to recruiting inflammatory cells to initiate inflammatory processes.

The process of erythrocyte formation is dependent on the sensitivity of vital organs. The kidney responds to the hypoxia and plays a vital role in intervening in the process of erythropoiesis. The identification of erythropoietin (EPO) has resulted in many research understanding of the molecular basis of oxygen-regulated erythropoiesis. Erythropoietin is fundamental for the normal process of erythropoiesis. This finding has also resulted in the understanding of hypoxia-inducible factor (HIF), the transcription factor. They are all involved in the regulation of EPO synthesis and mediate the cellular adaptation to hypoxia (Weidemann, 2009; Haase, 2010).

2.7 Cytokines of the haematopoietic system - Haematopoietic Receptors

Cytokines of the haematopoietic system collaborate with their receptors to activate formation of productive blood cells. These are the pluripotential, multipotent, and lineage-committed progenitors/precursors (Miller, 2013). Haematopoietin or cytokine receptor group is individualized by single-span membrane proteins. These exist without enzymatic activity in cytoplasmic domains. He stated that although a few receptor subgroups are dimers of a single unique receptor subunit, as is the erythropoietin
receptor. Many subgroups consist of a multisubunit complex with unusual and specific ligand-binding subunit and a common shared signaling subunit (Miller, 2013). The binding of specific ligands to extracellular domains results in conformational changes and activation of members of the Janus kinase (JAK) family. They bind to receptor cytoplasmic domains. Miller (2013) further stated that this receptor tyrosine phosphorylation and intervenes a downstream signaling cascade. This however regulates the activation of signal transducers including the signal transducer and activator of transcription (STAT), Ras, mitogen-activated protein kinases, PI 3-kinase, and Akt. He described that the receptors signal a number of functions necessary in haematopoiesis. These include the stimulation of proliferation, commitment to specific lineage, differentiation/maturation protection from apoptosis, and functional activation of mature cells (Miller et al., 2013).

2.8 Erythropoietin

The process of developing erythrocytes or red blood cells (RBCs) is maintained by the erythropoietin (EPO) hormone. Erythropoietin hormone regulates the blood haemoglobin (Hb) levels consistently in normal circumstances. Red blood cells (RBCs) circulate for 100–120 days. Red blood cells are taken up by macrophages in the bone marrow. It is also taken up in the spleen and the liver. To ensure that the reduction of erythrocytes does not have further implication, every second the bone marrow continues to produce about 2.5 million reticulocytes (Jelkmann, 2013). The primary fundamental hormone for the development of erythrocytes is the erythropoietin
molecule. During the deprivation of oxygen supply, the kidney will be stimulated to produce and secrete erythropoietin. Thereafter there will be an increase in the production of erythrocytes. The colony forming units- erythroid (CFU-E), proerythroblast and basophilic erythroblast subsets in the individualization will all be targeted (Haase, 2010). Erythrocyte progenitors and precursors are affected foundationally by the hormone EPO, located in the bone marrow in adults by enhancing their survival through protecting these cells from apoptosis (Maxwell et al, 1997).

The fundamental erythropoietin factor is erythropoietin which cooperates with all different growth factors (e.g., IL-3, IL-6, glucocorticoids, and SCF). They all contribute in the mechanism of erythroid lineage from multipotent progenitors (Kerenyi and Orkin, 2010). The start Erythropoietin receptor expression easily activated by erythropoietin which enhanced by burst-forming unit-erythroid (BFU-E) cells. Colony-forming unit-erythroid (CFU-E), responds with maximal erythropoietin receptor density. It is totally reliant on erythropoietin for further differentiation. The precursors, proerythroblasts and basophilic erythroblasts, all are significant of the erythropoietin receptor (Kerenyi and Orkin, 2010).

Erythropoietin (EPO) is a member of the cytokine family of molecules and humoral regulator of erythropoiesis (erythrocyte development). Erythropoietin (EPO) effects on proliferation and survival of erythroid progenitor cells are mediated via the erythropoietin receptor (EPOR) and downstream intracellular signaling events (Papayannopoulou et al., 2009; Shaheen and Broxmeyer, 2009). Erythropoietin has been the primary cytokine
to be functional in clinical practice. The treatment with erythropoietin progressed to EPO insufficiency. Erythropoietin therapy had proven excellent results of the ESRD patients that had presented with severe anaemia. These patients had previously had to receive blood transfusions and had resulted in many complications such as an increase in the iron levels (Nairz et al., 2011). Another investigator had explained that it may be that not all cancer cells have EPOR or respond to EPO, and that the immune dampening effects of EPO noted are as important, or are more important, than direct EPO-stimulating effects on proliferation and survival of tumour cells (Broxmeyer, 2011).

2.8.1 Erythropoietin produced by the peritubular fibroblasts

The EPO gene have been expressed by several tissues but the ability to produce substantial amounts of EPO during hypoxia/anaemia is restricted to the faetal liver and the adult kidney (Maxwell et al., 1997; Suzuki et al., 2007; Obara et al., 2008; Haase, 2010). One of the vital functions of the kidney is to determine the onset of hypoxia. The kidney is known to contribute 90% of plasma EPO in adult animals (Koury et al, 1988). There have been many difficulties in determining and purification of the renal EPO-producing cells. These have limited the understanding of the mechanisms that are responsible for the development of erythropoietin in the kidney. Renal EPO-producing cells are frequently known to be peritubular fibroblast-like cells in the kidney (Obara et al., 2008, Maxwell, 1993). The hypoxia-dependent EPO-producing cell line derived from human renal cancer was also described recently to exhibit fibroblast-like phenotype (Frede et al., 2011).
2.8.2 Erythropoietic Response

The erythropoietin result in haemodialysis patients is contributed by several physiological factors. The effect of these factors being represented as confounders was included in many epidemiologic studies (Gaweda et al., 2010). This study tested the hypothesis that iron stores, inflammation, dialysis adequacy, nutritional status, and hyperparathyroidism are as nonlinear effect modifiers of the erythropoietic response. The researchers quantified the magnitude of those effects over clinically relevant ranges.

Predialysis haemoglobin (Hb), transferrin saturation, serum albumin, dialysis adequacy (Kt/V) quarterly, predialysis serum ferritin and intact parathyroid hormone were collected monthly from a group of dialysis patients over a period of 13 to 69 months. This group consisted of 209 haemodialysis patients receiving epoietin alfa and the retrospective data was analysed accordingly. The study analyzed the dynamic relationship between haemoglobin and epoietin alfa, considering nonlinear effect modification by ferritin, transferrin saturation, Kt/V, albumin, and parathyroid hormone individually (Gaweda et al., 2010).

The maximum Hb response to epoietin alfa was achieved for serum ferritin between 350 and 500 ng/ml, transferrin saturation greater than 30%, Kt/V >1.4, and albumin >3.8 g/dl. Haemoglobin(Hb) sensitivity to EPO decreased by about 30% as parathyroid
hormone increases from 0 through 1000 pg/ml. Serum ferritin, transferrin saturation, Kt/V, serum albumin, and intact parathyroid hormone are markers of nonlinear effect modification of the erythropoietic response in haemodialysis patients (Gaweda et al., 2010).

Erythropoiesis, physiologically involves a significant reaction of EPO and iron. Serum Ferritin and Transferrin Saturation (T Sats) have been used as the two foundations variables of iron available for erythropoiesis (Gaweda et al., 2008). However, several clinical investigations have not successfully demonstrated the significance that these markers have on the erythropoietic response. Their analysis had explained that ferritin and transferrin saturation variables are related with the moderation of the erythropoietic response. The findings are consistent that iron deficiency indicated by low levels of T Sats or ferritin is associated with an impaired erythropoietic response (Kalantar-Zadeh et al., 2009). The results indicated that the impaired erythropoietic response occurs when ferritin levels are increased (>500 ng/ml). Increased ferritin in plasma compromises the process of the functional process of erythropoiesis. The process of the development of erythrocytes that is discontinued could be explained by the malnutrition-inflammation complex (Rattanasampattikul et al., 2013).

2.8.3 The structure of the Erythropoietin (EPO) molecule

The 30.4 kilo Daltons glycoprotein and class I cytokine made up of 165 amino acids is the description of the erythropoietin (EPO) molecule (Figure 2) (Mocini et al., 2007).
The authors further explained that EPO is made up of four acidic oligosaccharide side chains (3 N-linked and 1 O-linked). Forty percent of their molecular weight is supported by its carbohydrate portion. The N-linked polysaccharide side chains maintain to be vital for the biosynthesis and secretion of EPO. They assist the systemic transit of EPO from kidney to the site of erythropoiesis by maintaining stability in blood, and limit hepatic clearance (Boissel et al., 1993).

The variable nature of the sialic acid content gives rise to EPO isoforms with differences in their charge (Catlin et al., 2002). The investigators explained that as the number of sialic acid groups on the carbohydrate portion of EPO increases, so does its serum half-
life, whereas the receptor-binding capacity decreases. The clearance however, appeared to have a stronger influence and the receptor-binding affinity does not have a higher significance *in vivo* activity as compared to the clearance. There are two EPO receptor (EPOR) binding sites on every EPO molecule. There are two affinities of the EPOR for EPO in solution. There is one of high affinity and one of low affinity (needs 1,000 times the concentration of EPO for activation (Weidemann and Johnson, 2009).

### 2.8.4 Molecular biology of erythropoietin (EPO) synthesis

The human EPO gene (2.9 kb; 7q11-q22) contains 5 exons and 4 introns. The hypoxia-response elements (HREs) are functional in the activation of EPO. They are binding sites for Hypoxia-inducible transcription factors (HIF-1 and HIF-2) binds to the binding sites which are hypoxia-response elements. The α- and β-subunits make up the heterodimeric proteins which are the HIFs. The C-termini of the HIF-α subunits are a group of domains. Proline residues are hydroxylated in the presence of O₂ in this group of domains. This reaction requires HIF-α-specific prolyl hydroxylases (PHDs). Prolyl hydroxylated HIF-α goes through a series of steps of immediate proteasomal degradation. Prolyl hydroxylated HIF-α is bound by the *von Hippel-Lindau tumor suppressor protein* (pVHL) in complex with an ubiquitin-protein E3-ligase. The significant transcription factor activating erythropoietin is the HIF-2. During oxygen deprivation, the HIF-2α subunits dimerize with HIF-1β in the nucleus when there is the presence of hypoxia noted. However the EPO expression is stimulated by the responding complex allows (Jelkmann, 2012).
The prolyl hydroxylases (PHDs) are Fe$^{2+}$ containing enzymes requiring HIF stabilizers which does not allow the processes of HIF-α degradation and stimulation of erythropoiesis (Bernhardt et al., 2010). A Phase I clinical trial has presented that single injections of the HIF stabilizers (FibroGen Inc., San Francisco, CA, USA) produced a 13-fold inflation in the plasma EPO concentration in healthy volunteers (Brügge et al., 2007).

2.8.5 Physiological stimuli for erythropoietin (EPO) synthesis and regulation

The hormone (EPO) levels in blood circulation are at decreased values when anaemia is not present. The EPO levels are at an average 10 mmol/l. Erythropoietin (EPO) concentration could be increased a 1000-fold. This can be accumulated to 10,000 mU/ml of blood, during deprivation of oxygen (Jacobson et al., 1957; Fisher et al., 1996). The researchers explained that EPO is formed mainly by peritubular capillary lining cells of the renal cortex. They are extremely advanced, epithelial-like cells. Jelkmann et al., (2007) explained that it is synthesized by renal peritubular cells in adults with a small amount being produced in the liver. Regulation is believed to rely on a feedback mechanism measuring blood oxygenation. The authors explained that synthesized transcription factors for EPO, known as hypoxia-inducible factors, are hydroxylated and proteosomally digested in the presence of oxygen (Jelkmann, 2007).
The peritubular interstitial fibroblasts in the renal cortex and outer medulla of the kidney are functional in the development of an average of 90% of native EPO in adults (Jelkmann, 2007). An advanced process incorporating oxygen supply to the tissues appears to maintain the development process of erythropoietin. Hypoxia-inducible factor (HIF) moderates transcription of the EPO gene in the kidney. This process induces the EPO synthesis. This mechanism depends on the availability of oxygen supply to the tissues. Hypoxia-inducible factor (HIF) is speedily disintegrated in well-oxygenated cells through tagging for degradation in the proteasome by the von Hippel-Landau tumour suppressor protein (pVHL). During the reduction of oxygen supply, pVHL stops its proteolysis of HIF, causing the levels of HIF to rise. This concurrently inflates EPO production (Diskin et al., 2008; Bahlmann and Fliser, 2009).

2.8.6 Structure of erythropoietin receptors (EPOR)

The erythropoietin receptor (EPOR) is characterized as a 66 kilo Daltons membrane glycoprotein consisting of 484 amino acids and 2 peptide chains. They are part of a big cytokine and growth factor receptor family (Catlin et al., 2002). Research has shown that the EPOR has different compatibility for EPO. The erythropoietin receptor (EPOR) isoforms with greater compatibility for EPO may be accountable for the erythropoietin effects of EPO. Isoforms with a decreased compatibility for EPO binding may have nonerythropoietin effects. These are like tissue protection (Johnson et al., 2006).
Cytoplasmic domains of the EPOR consist of many of phosphotyrosines that are phosphorylated by the activation of a representative of the Janus-type protein tyrosine kinase family (JAK2) (Percy et al., 2008). This is combined to the regular beta subunit of the EPOR. Together with the activation of the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and protein kinase B (Akt) pathway (Figure3), the phosphotyrosines also contribute as docking sites for signal transducer and activators of transcription (STATs) such as STAT5.

Figure 3: The main pathways of the effects of Erythropoietin (EPO)

(Moore and Bellomo, 2011)
Dephosphorylation of JAK can be activated by phosphatase with the subsequent internalization and degradation of the EPO/EPOR complex. They finalize the process of the EPO activity. This does not allow extreme stimulation, which may follow to excessive erythrocytosis (Li et al., 2004). The intracellular domain of the EPOR contains phosphotyrosines, which are phosphorylated by activation of a member of the Janus-type protein tyrosine kinase family (JAK2) bound to the EPOR.

2.8.7 Post-receptor (intracellular) effects of erythropoietin (EPO)

There are many of familiar directions through which EPO stimulates its erythropoietin outcomes. In this they also present to confer tissue protection. Erythropoietin (EPO) "classically" binds to two EPORs, which become joined as a homodimer and change (Percy et al., 2008). The reaction stimulates JAK2, which is bound to the familiar beta subunit of the EPOR and leads to phosphorylation of tyrosine residues of the EPOR. This finally stimulates many of the signaling pathways (Figure 3).

Erythropoietin (EPO) uniquely signals through the "signal transducer and stimulator of transcription 5" (STAT-5) pathway. The STAT proteins are direct substrates of Janus kinases (JAKs). This is the outcome in tyrosine phosphorylation of the STATs including phosphorylation of the phosphatidylinositol 3-kinase (PI3K) they are coherent in phosphorylation of Akt (Figure 3).
The fundamental component of advancements that promote anti-apoptotic effects is Akt (Figure 4). They do not stimulate the caspases, the major mediators of apoptosis, mitochondrial dysfunction, and subsequent release of cytochrome C (Rusai et al., 2010). The potential of erythropoietin ability to conserve cellular integrity and not allow inflammatory apoptosis is closely linked to conservation. This includes the conservation of mitochondrial membrane potential, modulation of Apaf-1, inhibition of cytochrome C release, and inhibition of caspases. Research also explained that serum and glucocorticoid-regulated kinase-1 (SGK1) may add to the mediation of EPO's renoprotective outcomes (Myklebust et al., 2000).

Figure 4: Apoptotic pathways influenced by EPO (Moore and Bellomore, 2011)
2.8.8 Apoptotic pathways influenced by erythropoietin (EPO)

Stimulated STAT5 activates transcription of promitogenic and antiapoptotic genes. These are related with the apoptotic maintenance and cytoprotection. Serine threonine kinase (PKB/Akt) activates cell growth, development and anti apoptotic outcomes. The phosphorylation of mitogen-activated protein kinases (MAPKs) presents to enhance to the cell protection of EPO (Figure 4). Proteinkinase C (PKC) is also functional in the prevention of apoptosis, cell development and growth. It maintains the EPO-induced erythroid proliferation and differentiation. Proteinkinase C (PKC) interacts with phosphorylation of the EPOR. The interaction results in a significant modulation of the EPOR (Miller et al., 1999; Yasui et al., 2012).

Erythropoietin (EPO) may be functional in the regulation of cellular calcium homeostasis by influencing the rise in calcium influx (Figureueroa et al., 2002). Nuclear factor-kappNucaB (NF-kB), a mediator of inflammatory and cytokine response, is enhanced in EPO signaling. The cytoprotection of EPO partly depends on Akt and subsequent NF-kB activation(Figure 1 and Figure 2) NF-kB participates in the release of EPO during HIF-1 induction. Protein kinase B (Akt) can increase NF-kB and HIF-1 stimulation with resultant increase in EPO expression (Yang et al., 2003). The induction of heat shock protein 70 (HSP70) by EPO is similar to renal protection in ischaemic kidneys (Lui et al., 2007). The HSP70 does not allow apoptosis by preventing movement of apoptosis inducing factor (AIF) to the nucleus. This inhibits Apaf-1/cytochrome C binding in the cytosol(Figure 4) (Beere et al., 2000; Elliott et al., 2008).
Activation of proteins and peptides by erythropoietin can increase the process of Erythropoiesis. The expression of the erythropoietin gene (EPO) is prevented by GATA-2 and NFκB. It is activated by the hypoxia-inducible factor (HIF) – 1 which induces EPO expression during oxygen deprivation but disintegrates under normoxic environments.

**Figure 5:** The activation of proteins and peptides by erythropoietin (Foley, 2010)
Erythropoietin joins to the erythropoietin receptors on the cells of the bone marrow, activating the process of erythropoiesis. The increased formation of erythrocytes or red blood cells can be elevated by the following interventions: inhibition of PH, GATA-2 or NFκB, alteration of EPO–EPO receptor interactions, activation of the EPO receptor, or by making the EPO gene autonomous. Abbreviations: EPO, erythropoietin; HIF-1, hypoxia-inducible transcription factor-1; NFκB, nuclear factor κB; PH, prolyl hydroxylase (Figure 5) (Foley, 2010). The fundamental priority of the EPO hormone is to stimulate the process of erythropoiesis. This will follow the progression from a tightly controlled proliferation and development pathway (Salahudeen et al., 2008).

### 2.8.9 Red blood cell effects of Erythropoietin

Primary haematopoietic progenitor cells develop into burst-forming unit-erythroid cells (BFU-Es). Continuous activation with EPO causes the development of CFU-Es into erythroblasts. They form reticulocytes after the nuclei have disintegrated. When a few days have passed reticulocytes lose reticulin and progress to erythrocytes (red blood cells). Reticulocytes and erythrocytes cease expressing EPOR and they no longer become responsive to EPO (Silva et al., 1999).

Erythropoietin (EPO)-binding to EPORs on erythroid progenitor cells leads to activation of the JAK2-STAT5 signaling pathway and phosphorylation of PI3K and Akt1 (Figure 3) (Kralovicset et al., 2005; Chatterjee, 2007). Protein kinase B (Akt) mediated
phosphorylation of Bad in the Bad-Bcl-xL complex releases the anti-apoptotic protein Bcl-xL, which suppresses erythroid progenitor cell apoptosis (Joyeux-Faure, 2005). Several pathways that promote cell survival and anti-apoptotic effects is enhanced by the Protein kinase B (Akt). This is achieved by inhibition of FOXO3a, inactivation of GSK3β, induction of XIAP, inactivation of caspases which is activated by the Protein kinase B (Akt). The prevention of cytochrome C release contributes further to this process (Figure 4). Erythropoietic properties of EPO are enhanced by these effects. Protection is vital for other cell types and may contribute to the reported neuronal and renal protective effects (Chatterjee, 2007).

2.9 Potential risks of Erythropoietin

No reports of sudden toxic effects of the recombinant erythropoietin, although they were not scripted for patients that were hypersensitive to non-human cell-derived products. The dialysis individuals who are susceptible for hypersensitive reactions to human albumin are to be restricted of therapy from formulations stabilized with this protein. Recombinant erythropoietin (RhuEPO) has not been widely researched in pregnancy. Therefore therapy is to be used with caution (Jelkmann, 2013). The strengths and limitations of the network meta-analysis had included 40 studies, had similarities of the trial in terms of the patient populations. These were adults with CKD and anaemia, patients mostly on dialysis, and with similar prevalence of co-morbid conditions (Mills et al., 2013). The RhuEPO treatments used were epoietin alfa, darbepoietin alfa, epoietin beta, methoxy polyethylene glycol-epoietin beta, and biosimilar epoietin. They were all
injectable recombinant proteins and the outcomes of the treatments were assessed, these included mortality, cardiovascular events, benefits, blood transfusions requirements, hypertension and vascular access thrombosis. The significant differences in some of the studies e.g. non-dialysis versus dialysis patient populations, were the trial contexts and methodology have been similar. Network meta-analysis allows estimates of comparative efficacy between the different ESAs even if they were not compared directly against each other in the included trials (Mills et al., 2013).

2.9.1 Pure red cell aplasia

Although the many added contributions of EPO risks remain to be part of a concern. Pure red cell aplasia is an unknown adverse event, which is differentiated by anaemia, low reticulocyte count, absence of erythroblasts, resistance to EPO, and neutralizing antibodies against EPO. This is a complication that does not occur often (McKoy et al., 2008).

2.9.2 Cancer

A Cochrane Review analysed 53 (erythropoietin stimulating agents) ESAs in a trial with a total of 13,933 patients with cancer. There were 1530 deaths on-study, and 4993 overall deaths. Increased mortality and enhanced tumour growth has been reported in cancer patients being administered erythropoietin. Due to the unknown advancement
many patients with a few malignancies may be in a hypercoagulable state, causing EPO therapy unadvisable (Bohlius et al., 2009). Erythropoietin stimulating agent (ESA) treatment is effective in alleviating anaemia in anaemic cancer patients. When used in patients with a target Hb at no more than 13 g/dL, ESA treatment is not associated with increased frequency of severe adverse events. Erythropoietin stimulating agents (ESAs) significantly increased the Hb concentration [OR 7.85, 95% confidence interval (CI): 5.85 to 10.53, P<0.001] and reduced the red blood cell (RBC) transfusion rate (OR 0.52, 95% CI: 0.42 to 0.65, P<0.001). Erythropoietin stimulating agents (ESAs) did not increase the accumulated adverse events (OR 0.95, P=0.82), or the on-study mortality (OR 1.09, P=0.47) (Li et al., 2014). Erythropoiesis-stimulating agents (ESAs) are widely used in the management of anaemia in cancer patients. Despite their apparent effectiveness, recent studies have suggested that ESAs could result in serious adverse events and even higher mortality(Li et al., 2014). However, there has been a great deal of work both scientifically and in clinical trials in oncology that have revealed certain concerns and risks of ESA use in patients with cancer (Hazzan et al., 2014).

Thromboembolic events in cancer patients are considered a critical risk factor associated with the use of ESAs (Bernhardt et al., 2008). The Cochrane Database review in 2012, included 91 randomized controlled trials with 20,102 participants on managing anaemia in cancer patients receiving or not receiving anticancer therapy. The use of ESAs reduces the risk of RBC transfusions but also there has been evidence that ESAs increase the risk for thromboembolic complications and deaths (Tonia et al., 2012). Increase in platelet numbers is associated with ESA therapy. Thrombopoiesis
could be stimulated in an indirect way, as iron depletion due to increased erythropoiesis can result in thrombocytosis (Henry et al., 2012). A recent report proposed the use of anti-thrombotic therapy in order to reduce the ESA-associated risk of thrombosis (Aapro et al., 2009).

Meta-analyses on fatigue- and anaemia-related symptoms were measured with the Functional Assessment of Cancer Therapy-Fatigue (FACT-F) and FACT-Anaemia (FACT-An) subscales (primary outcomes) or other validated instruments. In cancer patients, particularly those receiving chemotherapy, ESAs provide a small but clinically important improvement in anaemia-related symptoms. Erythropoiesis-stimulating agents (ESAs) reduce the need for red blood cell transfusions; however, they increase the risk of thromboembolic events and mortality. The impact of ESAs on quality of life (QOL) is controversial and led to different recommendations of medical societies and authorities in the USA and Europe (Bohlius et al., 2014).

Erythropoietin binds to erythropoietin receptor (EPOR), stimulates proliferation, prevents apoptosis, and induces differentiation of RBC precursors (Osterborg et al., 2007). Erythropoietin receptor (EPOR) mRNA or protein expression in a range of cancer cell lines indicated that ESA could affect cancer cell biology (Sinclair et al., 2008; Paragh et al., 2009). Randomized clinical trials and systematic reviews demonstrate an increased risk of thromboembolism in those treated with either epoietin or darbepeoietin. Epoietin is recommended as a treatment option for individuals with chemotherapy associated
anaemia with haemoglobin that is decreased to less than 10 g/dL in order to decrease transfusion (Rizzo et al., 2010).

Presumably, EPO-R expression may vary among cancer cells of different tissue origins or types. It is conceivable that the benefits and risks of ESAs vary among patients with different types of cancers (Li et al., 2014).

2.9.3 Thrombosis

Observation in patient groups with elevated levels of haemoglobin (> 120 g/L) presented with a progression of thrombosis. Patients with haemoglobin >120 g/L not being accounted in clinical trials for EPO decreases the risk for thrombosis. However, systematic assessment for thrombosis should be routinely practiced in any erythropoietin trials of critically ill patients. This is due to them having an increased risk for thrombosis (Moore and Bellomo, 2011).

2.9.4 Hypertension

The clinical overview of hypertension is one of the primary interventions in patients receiving HD therapy. The blood pressure (BP) during therapy in Chinese dialysis patients was reviewed. The 44-h ambulatory BP was done in 90 patients on those that were consistently on chronic haemodialysis. Patients were distinguished as 'dipping',
‘non-dipping’ or ‘reverse-dipping’ based on night/day ratio (N/D) of systolic BP on days they did not receive dialysis therapy. The presence of blunted circadian BP pattern was significantly increased (92.2%). More than fifty percent of the patients (55.6%) was classified as reverse-dipping. There was a significant relation with increased levels of erythropoietin (EPO) doses used and deteriorated circadian rhythm. Patients in the dipping group also presented with a dipping state for heart rate (HR) compared with the other two groups (N/D of HR: 81.5±6.6 vs. 92.1±6.0 and 91.3±10.7, \( P=0.02 \)). There had been 26.7% patients whom had a monitored nocturnal BP. Patients receiving nocturnal treatment of EPO, had lower N/D of systolic BP as with patients without (100.1±7.0 vs. 105.2±7.1, \( P=0.01 \)). Non-dipping and reverse-dipping are increasingly significant in Chinese patients. Erythropoietin (EPO) therapy and autonomic dysfunction may enhance the blunted circadian rhythm. An urgent management of nocturnal blood pressure is an urgent need and nocturnal dosing may be of positive contribution (Liu et al., 2014).

Hypertension presents in an average of 30% of patients whom have been receiving EPO therapy for a long period. This had presented with an elevated endothelin release. The up regulation of tissue renin and angiotensin levels had also been elevated. The differences in the balance of vasoactive substances (prostaglandin/prostacyclin/thromboxane) and an increase of calcium by EPO have other effects. They (at least in chronic kidney disease) impair the vasodilating mechanism of nitric oxide. For further clinical studies, it is with much concern that patients with uncontrolled hypertension
were not volunteers with studies of EPO in acute kidney injury (Moore and Bellomo, 2011).

Nitric oxide (NO) is the signaling pathways of many others that are related with EPO (Bahlmann et al., 2004; Sautina et al., 2010). It has been stated that recombinant erythropoietin (RhuEPO) activates vascular NO production directly (Banerjee et al., 2000; Kanagy et al., 2003; Beleslin-Cokic et al., 2004). Recombinant erythropoietin also stimulates NO production indirectly through increased shear stress in endothelial cells (Walker et al., 2000). Recombinant EPO (RhuEPO) provides cardio protection to NO activation as stated in recent studies (Bullard and Yellon, 2005; Teng et al., 2011). The advancement for EPO-induced cardio protection is related to the stimulation of the contribution of nitric oxide signaling. An initial dose of RhuEPO progressed to an optimal NO-mediated decrease of systemic blood pressure. This had been related to the decrease of the cardiac systolic function.

The EPO-induced cardio protection of the myocardium from ischaemic damages were not associated with nitric oxide activation or NO-mediated haemodynamic responses (Ahmet et al., 2012). The stimulation of nitric oxide in patients who were on recombinant EPO therapy was a contributing factor for the increase of blood pressure activation through the effect of vasodilatory mechanisms (Kanagy et al., 2003). Carbamylated EPO, a cytoprotective, nonerythropoietic derivative of EPO is known not to enhance further risks. The advancements of EPO promote the interest in the further progression of tissue-protective therapy. This progression requires further research prior to the evaluation in safe clinical trials (Moore and Bellomo, 2011).
The German Multicenter EPO Stroke Trial, a Phase II/III trial were planned to develop contributing positive outcomes from the “Gottingen” EPO Stroke Study. However, the clinical outcomes were negative on patients with ischaemic stroke. All the patients were on EPO treatment. An increased mortality rate was observed in the patients on RHuEPO than those on the placebo. The increase on mortality rate was observed to be greater in those pretreated with thrombolysis (Ehrenreich et al., 2002; Ehrenreich, 2009; Paragh et al., 2009). Many clinical studies and pilot reviews proposed that recombinant human erythropoietin (EPO) offered neuroprotection. This advancement of RHuEPO therapy could contribute as being valuable for patients with ischaemic stroke. As of current research and clinical trials, there has been an elevation of thromboembolic complications. The mortality risk has been increased in ESRD patients with cancer that are on RHuEPO therapy. However EPO had been known in the past more than twenty years to have been a moderately safe drug which had been favourable to patients (Ehrenreich et al., 2002; Ehrenreich et al., 2009).

Erythropoietin stimulating agents (ESA) was found to be increasingly related to the rate of mortality among patients. Colony stimulating factors (CSF) for stroke included erythropoietin in addition to other CSFs. The physiological results followed by an acute or sub-acute ischaemic or haemorrhagic stroke treated with CSF was the fundamental result. The population group for the randomized trial was 1275. There were 11 randomized controlled trials which was a part of the analysis. There had been seven
Erythropoietin (EPO) therapy was associated with a significant rise in mortality. By the end (odds ratio (OR) 1.98, 95% confidence interval (CI), 1.19 to 3.3, p=0.009) and there had been no effective elevation in major adverse events. There had been an effective rise in the process of erythropoiesis with no adverse effects on platelet and white cell count, or infarct volume (Bath et al., 2013).

2.9.6 Cardiac risks

Additionally, the complication of the larger EPO dosages was seen in the analysis of the "CHOIR predialysis CKD trial". This revealed that at the four months and nine months of the study there has been an observation that the larger EPO dose was effectively associated with the composite endpoint of death, coronary heart failure, stroke, or myocardial infarction (Szczech et al., 2008; Ismayil et al., 2012).

Correction of Haemoglobin and Outcomes in Renal insufficiency (CHOIR) trial published the results of an open label, randomized trial of 1432 individuals with chronic kidney disease. There were two groups of patients that were allocated to acquire the desired haemoglobin levels. The first group of patients was allocated to acquire Hb-13.5 g/dL and the second group was to acquire Hb-11.3 g/dL. However of the two hundred and twenty-two of these patients, there were sixty five (29.3%) of these patients that have demised. A total of hundred and one patients (45.5%), were hospitalized for cardiac
complication such as congestive heart failure (CHF). The balance of hospitalized patients presented with cardiac complications, (11.3%) which were twenty five myocardial infarctions (MI) and 23 strokes (10.4%) which were twenty three patients. This trial was discontinued in the beginning of May 2005. The discontinuation of this trial was due to the repeat of the results, statistics and the contributing factors. The result of this investigation was the haemoglobin goals of 13.5 g/dL as related to the haemoglobin goal of 11.3 g/dL, has concluded with an elevated contingency to life. This resulted in a further effect on no contribution to patients well being (Singh et al., 2007).

The final modification of anaemia does not decrease the dangers of cardiovascular presentations. Hypertensive episodes are associated with the presentation of headaches and vascular disorders. Chronic kidney disease patients that were in stage 3 or 4 were selected for different clinical trials. This accrued to six hundred and three patients. They were further divided into different cohorts and were followed up for a period of 36 months. The patients in the first cohort were immediately treated with epoietin beta. The treatment was stopped as soon as the desired haemoglobin level of 13-15.0 g/dL was acquired. Patients in cohort two trial initiated epoietin beta when the haemoglobin result was less than 10.5 g/dL. They had to then to maintain a haemoglobin level of 10.5-11.5 g/dL (Drüeke et al., 2006).

Results were reported from an international randomized, double-blinded trial. This trial was to decrease the cardiac events with patients on Aranesp therapy (TREAT) and observing those on darbepoietin and placebo. Patients with type 2 diabetes, chronic
kidney disease and a haemoglobin level lower than or 11 g/dL were selected for this clinical trial. There had been a very large experimental group of 4038 individuals who were selected. A selection of 2012 patients was selected to be on darbepoietin alfa treatment group. The second selection was 2026 patients who were on the placebo group. The trial was finalized with a median follow-up of 29.1 months. The entire median baseline for the patient’s haemoglobin was 10.4 g/dL. The median acquired haemoglobin was valid at 12.5 g/dL in the treatment group. However, the results was 10.6 g/dL haemoglobin in the control group (p<0.001) vs the treatment group. The complex result of mortality or a nonfatal cardiovascular presentation was not statistically of value between the groups. However, "Fatal or nonfatal stroke was more common to present in the patients assigned to darbepoietin alfa (101 patients [5.0%] vs. 53 patients [2.6%]; hazard ratio, 1.92; 95% CI, 1.38 to 2.68; p<0.001)” (Pfeffer et al., 2011).

Chronic kidney disease patients that are receiving higher doses of RHuEPO are at an increased risk to complications. The administration of RHuEPO to achieve the desired levels of Hb of higher than 11 g/dL has a higher danger for mortality and serious adverse cardiac implications. There has been no current report from research studies that has located an ideal Hb level, ESA dose, or a desired dose that has no increased risks.
2.10 Networking erythropoiesis

Figure 6 represents a model of the multiprotein complexes which orchestrates gene expression or repression in erythroid cells. It shows the comparison of GATA-1, SCL/TAL1, and LDB1 whole-genome occupancy maps with gene expression profiling data.

Figure 6: Model of multiprotein complexes (Kerenyi and Orkin, 2010)
A relatively small cadre of lineage-restricted transcription factors largely orchestrates erythropoiesis, but how these nuclear factors interact to regulate this complex biology is still largely unknown. However, recent technological advances, such as chromatin immunoprecipitation (ChIP) paired with massively parallel sequencing (ChIP-seq), gene expression profiling, and comprehensive bioinformatic analyses, offer new insights into the intricacies of red cell molecular circuits (Kerenyi and Orkin, 2010).

Red blood cells (RBCs) fulfill the essential functions of transporting oxygen to tissues and facilitating gas exchange in the lungs. Red blood cells (RBCs) are continuously produced throughout life in a tightly controlled growth process termed erythropoiesis (Kendall, 2001). Erythroid differentiation is accompanied by temporally regulated changes in cell surface protein expression, a reduction in cell size, progressive haemoglobinisation, and nuclear condensation, which culminates in extrusion of the nucleus, RNA, and mitochondria (Richmond et al., 2005).

Erythropoiesis is largely mediated by a relatively small number of lineage restricted transcription factors, including GATA-1, SCL/TAL1, LMO2, LDB1, and KLF1 (Cantor and Orkin, 2002). Kerenyi and Orkin (2010) further explained the importance of these transcription factors in erythropoiesis which has been demonstrated by cell-based ex vivo assays, as well as in knockout mouse models and rare patients with anaemias. The critical transcription factors are present in diverse multiprotein complexes. However, how distinct multiprotein complexes activate or repress transcription, and thereby regulate the erythroid maturation program, remains incompletely understood. New
techniques, including ChIP coupled with massively parallel sequencing (ChIP-seq), gene expression profiling, and bioinformatic analyses, provide new information about the regulatory networks that coordinate erythroid cell maturation and function. The authors summarized their recent findings relevant to the understanding of gene expression regulation in red blood cells (Kerenyi and Orkin, 2010).

GATA-1 activates gene expression specifically in concert with SCL/TAL1 (Figure 4). However, partners for GATA-1 in gene repression are less clear. GATA-1 is thought to facilitate gene repression via interaction with the NuRD complex; this may be mediated through a direct interaction between GATA-1 and FOG-1 (Hong et al., 2005; Rodriguez et al., 2005), as well as via the transcriptional repressor Gfi-1b in concert with the LSD1–CoREST corepressor complex (Figure 4); (Rodriguez et al., 2005; Saleque et al., 2007).

2.10.1 Other new interventions

A study has shown that a novel synthetic peptide-based EPO receptor agonist (peginesatide) can correct anaemia in chronic kidney disease patients with antibody-mediated PRCA. This small clinical trial in 14 patients indicated a high success rate for peginesatide in rescuing patients with anaemia in chronic kidney disease. The biological rationale for the use of peginesatide in this setting was based on the fact that the amino-
acid sequence is different from that of recombinant EPO, and it does not cross-react with anti-EPO antibodies (Macdougall et al., 1999; Macdougall et al., 2009).

2.11 Factors affecting Erythropoiesis

2.11.1 Introduction

Erythropoietic response in haemodialysis patients depends on several physiologic factors (Gaweda et al., 2010). Most epidemiologic studies included the effect of these factors by representing them as confounders. Gaweda et al., (2010) in their study tested the hypothesis that iron stores, inflammation, dialysis adequacy, nutritional status, and hyperparathyroidism acted as a nonlinear effect modifiers of the erythropoietic response. They quantified the magnitude of those effects over clinically relevant ranges.

2.11.2 General Factors affecting Erythropoiesis

The most common physiologic marker which is routinely measured as a part of monthly or quarterly laboratory collections in haemodialysis patients was sampled for investigation. They had made a physiological assumption that iron stores, nutritional-inflammatory status, dialysis adequacy, and bone metabolism disorders may have a moderating effect on erythropoiesis (Gaweda et al., 2010). The physiologic mechanism through which the erythropoietic response is compromised at high ferritin levels could
be explained by the malnutrition-inflammation complex syndrome inhibiting erythropoiesis (Kalantar-Zadeh et al., 2004). Albumin has been positively correlated with the erythropoietic response (Agarwal et al., 2008 and Locatelli et al., 2006). The authors further explained that severe hypoalbuminemia was associated with a significant impairment of the erythropoietic response. Similarly to high ferritin, the mechanism behind this effect modification can be attributed to the malnutrition-inflammation complex syndrome (Gaweda et al., 2010).

The decrease in oxygen supply in blood is of significance in the developmental process of erythropoiesis. The altitude contributes to the required dosage of EPO and desired Hb a patient may require. The patient’s location of residence is of concern due to the differences of altitudes. In a retrospective cohort study, clinical data from 341,737 incident haemodialysis patients registered in the U.S. Renal Data System were combined with elevation data from the U.S. Geological Survey. Patients that resided on greater altitudes were related with lower EPO doses and haematocrit levels were greater. Those that resided at ocean level compared to patients living above 6000 feet received 19% less EPO (12.9 versus 15.9, thousand units/wk). They had haematocrit levels 1.1 points higher (35.7% versus 34.6%). These results were presented within subgroups defined by sex, race, age, calendar time, cause of chronic renal disease. Other factors that affected these outcomes were the profit margins of the dialysis clinics. The persisted after adjustment for various potential confounding factors were also common. The decrease in erythropoiesis to EPO decreased with elevation. The authors explained that there is an increase in the formation of erythrocytes of those chronic
renal failure patients that are not residing at sea level and are residing at higher heights. These patients are also known to have activated greater response with endogenous and exogenous erythropoietin (Jauréguy and Choukran et al., 2006; Brookhart et al., 2008).

2.12 The effect of anti-oxidant on erythropoietin stimulating agent (ESA) therapy

There are certain products that can contribute to consistent inflammation in uraemia. The administration vitamin E has antioxidant properties. This contributes to the regulation of cytokine biology. Children with ESRD who are on erythropoietin stimulating agent therapy are known to improve with Vitamin E supplementation. A group of ten children receiving haemodialysis dialysis therapy was investigated for the treatment of anaemia. They children were administered epoietin and vitamin E versus epoietin alone. They were further examined during dialysis therapy (Nemeth et al., 2000).

After two weeks of monitoring the onset of the symptoms of acute oxidative stress had presented in the children who had received only the epoietin. The vitamin E was then commenced in these children. The treatment with vitamin E after two weeks presented with significant lower indices of oxidative stress compared with epoietin therapy by itself. An effective elevated level in Hb and haematocrit (P < 0.01) had been acquired. This had resulted in a two week period of commencing the epoietin and vitamin E therapy together. Familiar occurrences presented at the fifth and eighth weeks in the absence of
vitamin E administration. It had been reported that dialysis membranes that were vitamin E-modified had a significant effect on anaemia. This occurrence had been clearly demonstrated that the concentration effect of plasma vitamin E had a significant effect on the development of erythrocytes (Usberti et al., 2002).

These findings are from a minimum patient number group. For patients with end stage renal disease, the bigger prospective randomized investigations are required to decide the favorable effects of vitamin E (Nemeth et al., 2000).

**2.13 Immunogenicity of erythropoietin stimulating agents (ESA)**

Biopharmaceuticals such as epoietin are recombinant transcriptions of human proteins. However, this has resulted to autoimmunity and the tolerance of disintegration of the B-cells. The process of the disintegration of the B cell has not been discovered as of present. The immunogenicity is activated by many other variables (Hal, 2011). The existence of aggregates is the fundamental variable contributing to auto reactive B cells. The above has also been reported from other recent investigations. The assumption of the B cells being directly stimulated is due to the antigens and the structure of the repetition of self-antigens existing in protein aggregates appear like the repeated self-epitope structure of viral capsids (Schellekens and Jiskoot, 2006; Van Beers et al., 2010).
2.14 The pleiotropic effects of erythropoietin in infection and inflammation

Erythropoietin (EPO) effects are a result of the many functions that are predisposed by the erythropoietin as a cytokine. The anti-apoptotic and immune-modulatory functions are incorporated by the erythropoietin molecule. The evidence of this function is validated in the binding to two distinct receptors. They are expressed on erythroid, parenchymal and immune cells, respectively (Van Beers et al., 2010; Nairz et al., 2012). Diseases such as autoimmune encephalomyelitis and inflammatory bowel disease is alleviated during the course of improvement in haemolytic anaemia. Erythropoietin (EPO) has been found to inhibit macrophage functions. The macrophages have resulted to be repressed by the action of the erythropoietin molecule. This however has an impact on the *Salmonella* infection. Therefore the target modulation of extra-erythropoietic functions causes an attractive environment for infection and inflammation (Nairz et al., 2012).

Erythropoietin (EPO) is the main erythropoietic controller as, the renal type I cytokine erythropoietin displaying as a vital controller for the process of red blood cells (De Maria et al., 1999; Pevny et al., 1999). The apoptosis of erythroid progenitor is placed under constrain by the erythropoietin molecule. This is activated due to the erythropoietin interfering with Fas Ligand (FasL)-Fas signaling and following differentiation of the erythroid. The fully formed erythroid cells maintain apoptosis-inducing ligands such as FasL and tumour necrosis factor (TNF). Apoptosis-inducing ligand (TRAIL) is related to decrease in the development of immature progenitors by contact-dependent negative
feedback mechanism. They all add up in the cleavage of GATA1. The vital transcription factor in erythroid advancement, by caspases, results in maintained apoptotic removal of erythroid precursors that are not completely developed (De Maria et al., 1999; Pevny, et al., 1999). Liu et al., (2006) explained that by down-modulating both, FasL and Fas expression (Figure 5), EPO causes a higher formation of erythrocytes (RBCs). This enhances the increase of oxygen in blood the circulation (Liu et al., 2006).

2.14.1 Immune-modulatory effects of EPO

![Image: Immune-modulatory effects of EPO (Hass, 2010)]

**Figure 7:** Immune-modulatory effects of EPO (Hass, 2010)
The prolyl hydroxylases (PHD) within the peritubular fibroblasts are sensitive to hypoxia. The production of erythrocytes is then activated by the prolyl hydroxylases (Figure 7). They then in turn transactivate the key transcription protein hypoxia inducible factor (HIF) (Haase, 2010). The haemoglobin (Hb) level thereafter decreases, following a greater oxygen upload in peripheral tissues. The renal erythropoietin formation can be stimulated followed by decreased blood flow in the kidney (Ekkardt and Kurtz, 2005).

Erythropoietin (EPO) is secreted by peritubular fibroblasts in response to activation of hypoxia inducible factor (HIF)-1α, while nitric oxide (NO) and other pro-inflammatory mediators are produced (Figure 7).

The kidney is not the only organ which is able to adapt EPO production in response to low oxygen tension. Chronic hypoxia is also sensed in the epidermis by a HIF-dependent mechanism; nitric oxide (NO) (Boutin et al., 2008). The mediated redistribution of blood flow from inner organs to the skin results in a secondary increase in renal EPO production. Although the kidney is the pivotal production site for EPO, extra-renal EPO formation, for instance in the liver, may also insignificantly contribute to circulating EPO concentrations in adults (Haase, 2010).

The heterodimeric transcription factor (HIF) is essential for the control of EPO expression. Whereas the amount of HIF-1α is regulated post-translationally by three PHDs, HIF-1β is constitutively expressed and assumed not to be subject to regulations...
(Jelkmann, 2007). Under normoxic conditions, oxygen levels are sufficient for PHDs to continuously hydroxylate HIF-1α at two specific proline residues. Proline hydroxylation, which is modified by concentrations of ferrous iron and 2-oxoglutarate, tags HIF-1α for proteosomal degradation following binding by the von Hippel Lindau (vHL) E3 ubiquitin ligase complex. During cellular hypoxia, in contrast, HIF-1α is stabilized enabling transcription of HIF target genes such as EPO and vascular endothelial growth factor (VEGF). Apart from HIF-1α, two additional isoforms exist. Hypoxia-inducible factor (HIF)-2α is expressed in renal fibroblasts in response to hypoxia. The Hypoxia-inducible factor (HIF)-2α can dimerise with HIF-1β to drive the expression of target genes including EPOR contrasts (Warnecke and Eckardt, 2009; Eckardt, 2005 and Haase, 2010). Due to the lack of a transcriptional activation domain in HIF-3α, this isoform suppresses the expression of hypoxia-responsive genes (Eckardt, 2005; Haase, 2010).

Hypoxia inducible factor (HIF) 1α and hypoxia inducible factor (HIF) 2α promote EPO expression and nuclear factor NF-κB inhibits EPO expression (La Ferla et al., 2002). As NF-κB is one of the central transcription factors to initiate and perpetuate inflammation, its negative effect on EPO expression in inflammatory conditions may contribute to the pathogenesis of the anaemia of chronic disease (ACD), which frequently occurs in patients suffering from inflammatory diseases thereby negatively impacting on organ function and quality of life (Weiss and Goodnough, 2005). Jelkmann(1998), described in his study that, via the stimulation of NF-κB, interleukin (IL)-1 and TNF- alpha EPO expression can be inhibited. Accordingly, the injection of lipopolysaccharide (LPS) into
mice results in reduced expression of EPO mRNA in kidneys and decreased levels of circulating EPO (Jelkmann, 1998).

The hepatic formation of EPO is stimulated by IL-6 in an NF-κB and HIF-1α-dependent manner, which provides evidence for cross-regulatory interactions between inflammatory mediators and EPO formation (Ramadori et al., 2010). The balancing of HIF-dependent gene transcription is of particular importance in compartments with low oxygen tension such as sites of infection and inflammation (Peyssonnaux et al., 2005; Jantsch et al., 2008). Most interestingly, NF-κB directly affects HIF-1α expression under hypoxic conditions and induces the accumulation of HIF-1α in macrophages challenged with bacteria, thus linking HIF-1 expression to innate immune response (Lui et al., 2007; Rius et al., 2008).

Pharmacological inhibition of PHD initiate HIF-dependent gene expression efficiently attenuates ischemia-reperfusion injury (IRI) and experimental colitis in animal models. The EPO production response to pharmacological PHD inhibition or to which extent EPO may contribute to the promising tissue-protective and anti-inflammatory effects of these compounds remains to be investigated in further detail (Cummins et al., 2008; Bernhardt et al., 2009). Hypoxia inducible factor (HIF) regulated renal EPO production provides the essential endocrine signal for the expansion of bone marrow RBC progenitors to compensate for anaemia and hypoxia (Figure. 7) (Haase, 2010).
2.15 Types of Recombinant Erythropoietin (EPO)

In clinical practice there are several forms of EPO available for the treatment of anaemia (Table 1). In the first generation there were two forms of recombinant human EPO (RhuEPO): epoietin α (alpha) (Epogen, Amgen and Procrit/Eprex; Johnson & Johnson/Janssen-Cilag) and EPO beta (β) (Neo Recormon; Roche), which were administered three times a week (Halstenson et al., 2001). However, the development during the last decade of ESAs with a higher degree of glycosylation and prolonged half-life allowed for a less frequent administration (Macdougall et al., 1999).

A preparation called long-acting darbepoietin α (Aranesp; Amgen) produces a similar physiological response compared with RHuEPO (Lopez et al., 2009). It has more chains of carbohydrates and sialic acidic residues, which gives it a different pharmacokinetic profile from that of RHuEPO, with a half-life approximately three times higher (25.3 h vs. 8.5 h by iv administration), and a plasma clearance four times slower (Allon et al., 2002; Nissenson et al., 2002; Pigeolet et al., 2009).
Table 1: Erythropoietic agents available (Lopez et al., 2009)

<table>
<thead>
<tr>
<th></th>
<th>Half-life (h)</th>
<th>Periodicity</th>
<th>Initial dose</th>
<th>Target levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epoetin β</td>
<td></td>
<td>9</td>
<td>1-3 times per week</td>
<td>100-150 IU/kg per week, (max 300 IU/kg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td></td>
<td>Hemoglobin: 11-12 g/dL, Hematocrit: 33%-36%, ↑ hemoglobin every 2 wk: 0.5-1 g/dL</td>
</tr>
<tr>
<td>Epoetin α</td>
<td>7</td>
<td>7</td>
<td>1-3 times per week</td>
<td>100-150 IU/kg per week, (max 300 IU/kg)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Darbepoetin α</td>
<td>25</td>
<td>48</td>
<td>Every 1-2 wk</td>
<td>0.45 µg/kg every 2 wk</td>
</tr>
<tr>
<td>CERA</td>
<td>133</td>
<td>137</td>
<td>Every 2-4 wk</td>
<td>0.8 µg/kg every 2 wk</td>
</tr>
<tr>
<td></td>
<td></td>
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</tbody>
</table>

The frequency of administration of once weekly or even every 2-4 week was allowed (Vanrenterghem et al., 2002). The equivalence relationship between RHuEPO and darbepoetin α from the molecular weight of both proteins is 1:200. In clinical practice, the multiplication factor is not so simple, as the required dose of RHuEPO can be higher, which probably is related to an increase in resistance to EPO (Scott, 2002; Krapf et al., 2009).

Continuous erythropoietin receptor activator (CERA) (Epoietin β-methoxy polyethylene glycol, CERA; Roche) is another erythropoietic agent that activates repeatedly the EPO receptor (Locatelli and Reigner, 2007; Levin et al., 2007; Provenzano et al., 2009). It has an elimination half-life in humans of about 130 h, and so can be administered every
Several studies have shown that it has an effect similar to EPO in maintaining haemoglobin levels (Sulowicz et al., 2007). Continuous erythropoietin receptor activator (CERA) is not available in United States, but is currently used in Europe. An additional advantage is that CERA can be kept out of the fridge and used for up to 1 month (at < 25°C).

New generation of erythropoietic analogues develop as synthetic erythropoiesis of protein and peptides become mimetics of EPO (Sytkowski et al., 1999; Kochendoerfer et al., 2003; Vadas et al., 2008). These new agents stimulate erythropoiesis through activation of EPO receptors. Hematide (developed by Affymax) is a synthetic peptide agonist of the EPO receptor, and although it has no structural homology with EPO, it is able to activate the EPO receptor and stimulate erythropoiesis over a 1-month period, with good tolerance and stability at room temperature (Johnson et al., 2000).

Darbepoietin alfa is another Food and Drug Administration (FDA)-approved ESA for similar indications. Because darbepoietin alfa is long acting, less frequent dosing is required (weekly or biweekly for individuals on dialysis). Randomized trials were performed to confirm the data from dose-finding studies, which suggest that darbepoietin alfa can be administered effectively as infrequently as once per chemotherapy cycle (weekly or once every three weeks) (Glaspy et al., 2001). Comparative studies were performed to evaluate darbepoietin alfa in individuals with cancer (Glaspy, 2001). The safety and efficacy of both darbepoietin and epoietin alfa
are similar in anaemia of chronic kidney disease or chemotherapy-induced anaemia (Allon et al., 2002; Herrington et al., 2005).

Food and Drug Administration (FDA) approved methoxy polyethylene glycol-epoietin beta (Mircera), an erythropoietin receptor activator to treat anaemia associated with chronic renal failure for individuals in the U.S., including those on dialysis and individuals not on dialysis. The label also specifies that epoietin beta is not indicated to treat anaemia due to cancer chemotherapy (Product Information, 2007). Methoxy polyethylene glycol-epoietin beta may be administered intravenously or subcutaneously once every two weeks or once a month to attain the haemoglobin target.

Individuals experienced greater risks for death, serious adverse cardiovascular reactions and stroke when administered ESAs to target haemoglobin level of greater than 11 g/dL for individuals with CKD on dialysis. No trial has identified a haemoglobin target level, ESA dose, or dosing strategy that does not increase these risks (product information labels, 2013).

### 2.16 Pure Red Cell Aplasia (PRCA)

Pure red cell aplasia (PRCA) is a primary haematologic disease, but can also occur as a result of various infections, haematologic malignancies, autoimmune diseases, severe malnutrition, and exposure to certain drugs and toxins. The first clinical sign of ESA-
induced PRCA is severe resistance to treatment, which manifests as a rapid decline in haemoglobin levels to 5–6 g/dl or transfusion dependence (Cameron, 1999). On exclusion of other causes of ESA hypo responsiveness, measurement of blood cell counts should be performed; a reticulocyte count of <10 × 10⁹/l in the presence of normal white cell and platelet counts justifies bone marrow examination and measurement of anti-EPO antibodies. Although bone marrow examination in a patient with ESA-induced PRCA will usually demonstrate an absence of erythroblasts, the diagnosis is confirmed by the presence of neutralizing anti-EPO antibodies. The presence of circulating anti-EPO antibodies is usually determined with an immunoassay (radioimmunoprecipitation or enzyme-linked immunosorbent assay) or surface plasmon resonance methods. Samples that are positive for the presence of anti-EPO antibodies are then tested for the presence of EPO-Nabs (neutralizing antibodies) using a cell-based bioassay (Pollock et al., 2008).

Antibody-mediated pure red cell aplasia is a very rare but devastating condition affecting patients receiving treatment with erythropoietin-stimulating agents. New cases continue to emerge, generally in clusters, consistent with an ‘environmental’ trigger to its pathogenesis. Defining the causes of antibody-mediated pure red cell aplasia is clearly of importance for patients with chronic kidney disease, but any developments in this area may also have relevance to other disease areas as therapeutic delivery of endogenous proteins rapidly increases (Macdougall et al., 2012).
Pure red-cell aplasia (PRCA) in patients with chronic renal failure (CRF) and renal anaemia treated with recombinant human erythropoietin (RHuEPO) has significantly increased (Cesk, 2003). Due to the positive effects of RHuEPO on quality of life, lowering of morbidity and mortality of patients with CRF, the increased incidence had attained a widespread interest, though PRCA remains only a rare complication. The responsibility for the development of PRCA lies with the neutralizing anti-erythropoietin antibodies. The rise of antibodies and development of PRCA was related to the subcutaneous administration of erythropoietin and in the vast majority of patients to the treatment with one of the epoietin alpha (Cassadevall et al., 2002; Eckardt and Cassadevall, 2003).

Neutralizing antibodies (Nabs) to recombinant human erythropoietin (RHuEPO) increased in European dialysis patients which raised concerns that US dialysis patients may be at similar risk. The frequency of diagnosis codes that include pure red cell aplasia (PRCA) were clinically investigated in the Medicare end-stage renal disease data (Eckardt and Cassadevall, 2003). The dialysis patient's data were used to identify incidents from 1995 through 1999. The patients were aged 67 years or older and did not have a diagnosis code for aplastic anaemia (AA) which included PRCA or they were not administered RHuEPO before dialysis therapy initiation. Patients were assessed for complicating conditions, RHuEPO dose, haematocrit level, blood transfusion, bone marrow testing, and AA diagnosis (maximal follow-up, 13 months) (Collins et al., 2004).
The investigators explained that of 101,782 patients who were eligible for the study, 9,896 patients had diagnosis codes for AA after dialysis therapy initiation. The 3,894 patients who underwent bone marrow tests (required for PRCA diagnosis) 19 patients had diagnosis codes for AA based on bone marrow examination and no other complicating conditions. They further explained that 5 patients were administered blood transfusion in 1 or more months during follow-up, of whom only 1 patient had persistent low haematocrit levels while being administered RHuEPO and blood transfusion. This latter patient was identified from a total of 101,782 patients, or 70,706.75 person-years of RHuEPO exposure (Collins et al., 2004; Cournoyer et al., 2004).

The investigators had further explained that the diagnostic codes in the Medicare data were inadequate for the conclusive study of PRCA in US dialysis patients. Despite limitations of their study, it had appeared that few cases of RHuEPO-associated PRCA had occurred in US Medicare dialysis patients. Additional investigation was needed to determine whether apparent differences between US complication rates and those elsewhere result from differences in the manufacturing and/or use of erythropoietin products (Collins et al., 2004).

2.17 Erythropoietin Resistance

The evidence of EPO resistance stems from studies with an inadequate response to EPO in 5%-10% of patients or in patients who develop EPO resistance after a good
initial response (López-Gómez and Valderrabano, 1999; Locatelli et al., 2010). There is a resistance to EPO when a sufficient dose of it, equal to or greater than 300 U/kg per week, does not reach the desired concentration of haemoglobin (European best practice guidelines, 2006). It has been shown that the most frequent and important cause of resistance to EPO was iron deficiency, but there are also other less frequent factors (Valderrabano, 2003).

Blood loss was the most frequent cause of absolute iron deficiency. This was defined by ferritin levels < 100 ng/mL, transferrin saturation < 20%, and/or hypochromic red cells increased by 10% (Lacombe, 1996). Relative or functional iron deficiency results from difficulty in transferring stored iron to red blood cells and is defined by the existence of transferrin saturation < 20% whilst maintaining high levels of ferritin > 100 ng/mL. The main causes of relative iron deficiency were acute and chronic inflammatory processes and chronic liver diseases (Lacombe, 1996). The elevated level of parathyroid hormone in CKD patients is another cause of non-negligible resistance to EPO (Rao et al., 1993). The presence of an inflammatory disorder increases the resistance to treatment with erythropoietic agents. These include the production of inflammatory cytokines that interfere with iron metabolism, reducing their availability in the bone marrow and causing functional iron deficiency. The process of dialysis may be associated with an increase in the induction of cytokines and the appearance of an inflammatory response syndrome (Sitter et al., 2000).
Haemodialysis patient’s carnitine metabolism is altered and carnitine deficiency is more likely in patients with a protein-deficient diet and a high dialysis dose (Lago et al., 1995). Carnitine may improve anaemia in haemodialysis patients with EPO resistance by mechanisms not yet known or stabilizing the membrane as an antioxidant agent (Bommer, 1999). Some of the haemodialysis patients treated with L-carnitine demonstrated an increase in the number of reticulocytes. This was due to the stimulation of erythropoiesis (De los Reyes et al., 1998). The dose used in patients with EPO resistance is 1 gram intravenously (I.V.) post-haemodialysis (Eknoyan et al., 2003).

Folic acid is involved in the process of regeneration and maturation of haematopoietic precursors (Pronai et al., 1995). The authors discussed that folic acid deficiency was associated with an ineffective erythropoiesis, and a megaloblastic anaemia. Malnutrition, malabsorption, alcoholism, and various drugs that lower intestinal absorption, such as diphenylhydantoin, contraceptives and barbiturates, could cause folic acid deficiency. In dialysis patients with resistance to EPO, with normal ferritin levels, adjuvant treatment with folic acid may assist. The authors had discovered that the use of folic acid (10 mg/d) in haemodialysis patients improved the response to EPO, especially when presented with high mean corpuscular volume, even with normal levels of folic acid (Pronai et al., 1995).

The effectiveness of erythropoietic response to stimulation was assessed by resistance index of ESAs. The resistance index of ESAs expressed the relationship between the
dose of erythropoietic agents (IU/kg per week) divided by the haemoglobin levels (g/dL).

Resistance index of ESAs varied from one patient to another and also in the same patient over time. The values ranged from 0 in patients able to maintain adequate haemoglobin level through the endogenous production of EPO, to > 50 IU/kg per week and per g/dL of haemoglobin in patients who could not maintain adequate haemoglobin, even after high-dose EPO therapy (> 300 IU/kg per week)(Pérez-García et al., 2001; Kaysen et al., 2006; Lopez-Gomez et al., 2008).

An alternative mechanism for relative ESA resistance may be chronic inflammation. Chronic inflammation in patients with sickle-cell disease showed elevations in C-reactive protein and interleukin-6A similar. This could occur in patients with single heterozygous sickle haemoglobin. Inflammation has been suggested to impair both iron utilization and erythropoiesis (Hedo et al., 1993; Bourantas et al., 1998). Adamson et al., (2009) suggested that numerous studies had shown in the ESRD population that indices of active inflammation, including C-reactive protein, have been associated with ESA resistance. The presence of variant haemoglobin had to induce inflammatory responses beyond those already experienced in haemodialysis. Finally, antibodies to erythropoietin may contribute to an impaired ESA response. Such antibodies have been reported in patients with β-thalassemia and sickle/β thalassemia, but not common among ESRD patients (Voulgari et al., 2004).

2.17.1 Haemoglobin Variability
Haemoglobin (Hb) variability presents a comparison with mortality in retrospective analyses of 34,963 HD patients while other studies suggest that low Hb levels may be the critical factor associated with poor outcomes (Yang et al., 2007). Brunellie et al., (2008) found that a greater Hb variability was associated with diminished survival. However, the association was not evident in the subgroup of patients with $\geq$10.5 g/dL, though this might have been due to the smaller size of this subgroup. In a much larger retrospective study of 152,846 patients, the investigators characterized six different types of Hb variability patterns and then evaluated hospitalizations and co-morbidities in these categories. They observed that the group of patients with consistently low Hb levels had the highest percentage of hospitalizations and the highest number of co-morbid conditions (Ebben et al., 2006; Chan et al., 2014).

Haemodialysis (HD) patients that had longer therapy time than other patients within their population groups had Hb levels $<11$ g/dL. They had a greater risk of mortality than the others who were on a shorter time period on HD therapy. This was similar to another study with a sample of 159,720 HD patients receiving epoietin ($P < 0.001$) (Gilbertson et al., 2008). In another large retrospective study of HD patients, the proportion of time spent on HD with Hb levels below a target of 11 g/dL was compared against the risk of death (Ishani et al., 2008). The results showed a clear trend of increased mortality with increasing time below this target level. For patients with Hb levels $<11$ g/dL for 80–100% of the time, the risk was $\sim 1.8$ times as high as for patients with no time below this level. Pisoni et al., (2004) explained that this trend was also observed in the DOPPS study, where higher Hb values at the start of the study among
patients on dialysis therapy for longer than 180 days were associated with a lower risk for mortality and hospitalization (Pisoni et al., 2004)(Figure 2). These data suggest that maintaining patients within the target Hb range is an important goal in the treatment of renal anaemia (Locatelli et al., 2010).

2.18 Malaria

2.18.1 Malaria in South Africa

In South Africa, malaria is endemic in three provinces – Limpopo, Mpumalanga and KwaZulu-Natal (Figure 8) and transmission occurs predominantly between September and May (DOH, 2011). Over the past decade, the National Department of Health (DOH) has focused intense efforts on preventing the local transmission of malaria, and on ensuring the prompt and effective management of cases, especially in the endemic provinces. The DOH has recorded significant success in reducing the burden of malaria through the implementation of its key interventions: Vector Control, Case Management, Surveillance, Health Promotion, and Epidemic Preparedness and Response
Notified malaria cases in the aforementioned provinces have decreased by 88% over the past decade, from 64 622 cases in 2000 to 7 626 in 2010. In the same period, malaria-related deaths have been reduced by 81%, from 458 to 87 deaths (DOH, 2011).

Malaria has been a notifiable disease in South Africa since 1956 (Government notice No. 2 081 of 1956). One of the key challenges that the DOH has historically faced is the irregularity of routine notifications, particularly from the private sector and public health
facilities in non-endemic areas. In recent years, reporting appears to be gaining in efficiency, with an increase in malaria notifications from the non-endemic areas, especially Gauteng (Figure 8). In this province, trends for malaria cases and related deaths show an increase over the past 2 years, with a peak in January. This seasonality is related to travellers returning from malaria-endemic areas, particularly Mozambique (Figure. 2.8), (Weber et al., 2010).

Malaria prevention and control strategies are being implemented robustly in the endemic provinces (Moonasar et al., 2011). Similar strategies in the non-endemic provinces have lagged behind. Measures for malaria prevention and management were successful. Malaria treatment is dependent on a high suspicion for malaria in patients with acute febrile illness. Urgent treatment with effective medication must be implemented (Moonasar et al., 2011).

2.18.2 Malaria in Chronic Kidney Disease in relation to erythropoiesis

A number of infections with parasitic agents such as Plasmodium, Schistosoma, Leishmania or hookworms result in anaemia (King et al., 2005; Lafuse et al., 2013). In the case of intestinal infections, this anaemia is believed to be caused primarily by intestinal haemorrhage reduced iron absorption or decreased bioavailability of iron (Periago and Bethony, 2012). Inflammatory responses to the infections including the
secretion of proinflammatory cytokines and the resultant upregulation of hepcidin additionally appear to inhibit erythropoiesis, as in the anaemia of chronic disease (Morceau et al., 2009; Burte et al., 2013). In the case of malaria, evidence exists that parasitic products may also directly impede erythroid proliferation and/or differentiation (Haldar and Mohandas, 2009; Thawani et al., 2013).

Erythroid inhibition by *P. falciparum* was used to detect erythropoiesis inhibiting activity in a medium conditioned by blood-stage cultures of the malaria parasite *P. falciparum*. The assay was able to distinguish between erythropoiesis-inhibiting and -promoting conditions to the same extent as manual cell counting using trypan blue exclusion and was able to detect differential responses to different concentrations of the inhibitory medium (Boehm and Bell, 2014).

2.18.3 The role of Erythropoiesis in relation to anaemia during blood stage

**Malaria**

Severe anaemia is a severe occurrence of malaria. Inappropriately low reticulocytosis in malaria patients with anaemia suggests insufficient erythropoiesis of which the mechanisms and implications are not clear. The principle growth factor that promotes erythropoiesis is erythropoietin (EPO). Studies determining the serum level of EPO in malaria infected patients have been inconclusive. Furthermore, the role of EPO and the
erythropoietic response to EPO stimulation during malaria have never been examined. The purpose of the experiments performed was to determine the role of EPO and erythropoiesis in relation to anaemia during blood-stage malaria using the murine model of *Plasmodium chabaudi*. A murine erythropoietic specific ELISA, which was determined to be less biased by the presence of other cytokines in the samples as compared to the conventional EPO bioassay, was first developed to facilitate the research. The kinetics of erythropoiesis and the levels in the serum were characterized (Chang and Stevenson, 2004).

Erythropoiesis during blood-stage malaria is mainly regulated by the degree of anaemia and that renal cytokines may have only a minor effect on this response. The significance of EPO and erythropoiesis in blood-stage malaria was studied. This was accomplished by the neutralization of endogenous EPO or by administration of exogenous EPO. The EPO-induced reticulocytosis was shown to be important for the alleviation of malarial anaemia and survival (Chang et al., 2002). However, the reticulocytosis in response to EPO stimulation was severely suppressed by infection with malaria. Dissection of the upstream events of erythropoiesis demonstrated that blood-stage malaria compromises the generation of reticulocytes by suppressing the proliferation, differentiation, and maturation of erythroid-lineage cells at various stages of erythroid development (Chang and Stevenson, 2004).

Anaemia is a common life-threatening complication of *Plasmodium falciparum* malaria in African infants and young children. The anaemia develops when accelerated removal of
erythrocytes is not compensated by the bone marrow. Bone marrow suppression is generally present in all malaria patients (Nkumah and Hvid, 1997; Kurtzhals et al., 1999). The fact that some malaria patients develop severe anaemia, whereas others retain normal or near normal haemoglobin (Hb) must thus be explained by the amount of erythrocyte destruction during the period until return of normal bone marrow function. The anaemia may either develop rapidly with severe, acute haemolysis, or take a slow smouldering course, with a relatively slow rate of erythrocyte destruction in the presence of persistent bone marrow suppression (Phillips and Pasvol, 1992).

Data on the duration of bone marrow suppression after a malaria attack are conflicting. In some studies there has been evidence of hypo proliferative erythropoiesis and dyserythropoiesis for weeks following treatment (Philips et al., 1986; Camacho et al., 1998). In areas where malaria is endemic, a high number of children with severe anaemia but without detectable parasites are hospitalized each year coinciding with the peak of malaria transmission. The history of recent febrile illness and the presence of detectable circulating antigens strongly suggest that these children suffer from the late effects of a recent malaria attack (Kurtzhals et al., 2003).

2.19 Global haemodialysis growth

The global population of end-stage renal disease (ESRD) patients undergoing haemodialysis in mid 2001 was estimated to exceed 1.1 million patients, at a cost of
US$70 - 75 billion. Moreover, the size of this population has been expanding at a rate of 7% per year and is likely to exceed 2 million patients by 2010 (Lysaght, 2002). In 2003, an estimated 430,000 South Africans were living with ESRD. Even though ESRD may be more prevalent and severe in developing countries than in developed countries, it has remained under-researched in Africa in comparison with major infectious diseases such as auto immune deficiency syndrome (AIDS), tuberculosis and malaria (Naicker, 2003). Although kidney transplantation is considered the treatment of choice, the limited number of organ donors means that the majority of patients need a compensatory method of treatment. Haemodialysis has been developed as available, safe and efficient method for the maintenance of patients with ESRD. The procedure requires patients to be dialysed three times per week for approximately four hours per session. Patients are also required to adhere to strict dietary restrictions (which include limiting potassium and phosphate intake) and fluid restrictions (Fincham et al., 2008).

2.20 Protein energy malnutrition

Malnutrition is not a common effect in first world countries when there is a prevalence of insufficient food intake. Decreased absorption an abnormal metabolic rates allows the onset of protein-energy malnutrition. Thus, in developed countries, diseases, such as cystic fibrosis, chronic renal failure, childhood malignancies, congenital heart disease, and neuromuscular diseases, contribute to malnutrition. Fad diets, inappropriate management of food allergies, and psychiatric diseases, such as anorexia nervosa, can also lead to severe protein-energy malnutrition (Scheinfeld and Mokashi, 2010).
Both groups of patients, on acute and chronic therapy are at a greater risk of unknown and progressive weight loss. This can progressively enhance protein-energy malnutrition. Involuntary weight loss (IWL) is defined as a loss of 4.5 kg or greater than 5% of the usual body weight over a period of 6-12 months. Protein-energy malnutrition results in weight decreasing greater than 10% of normal body weight (Scheinfeld and Mokashi, 2010). Older patients are more prone to malnutrition because of known factors that contribute to the poor nutrition. These regular factors appetite, dependency on help for eating, impaired cognition, higher nutrient density requirements, and other demands of age, illness, and disease on the body (Scheinfeld and Mokashi, 2010).

There is an escalation in the less advantageous countries of 42% and 77% of the common presentation of malnutrition in chronic dialysis. Naicker (2003) reported that 77% of patients were hypoalbuminemic. The author further explained that patients were consuming 1014kCal with a protein consumption of 0.34g/kg body weight. The ESRD low level protein and energy intakes resulted in reduction in cell-mediated immunity. This also progressed to the higher complication of infection. The prevention of satisfactory antibody results to vaccines was noted (Naicker, 2002).

Target organ failures associated with uraemia are most often considered to be caused by processes other than uraemia. The author explained that heart disease is considered the product of hypertension, lipid abnormalities, rather than the uraemic state (Lowrie, 1998). Erythropoietin deficiency, blood loss, and iron deficiency are believed to cause
anaemia, rather than the uraemic state. Malnutrition is believed to be the product of poor nutrient intake and perhaps nutrient losses, rather than uraemia. The author reviewed the evidence, suggesting that anaemia and malnutrition shared a common cause; the acute-phase inflammatory process which is a normal host-defense mechanism. With the high prevalence of heart disease among patients with end-stage renal disease (ESRD), there is data indicating activation of the acute-phase process in patients with kidney failure. This process has a significant role in the risk for cardiovascular disease among patients without kidney failure. There is a strong likelihood that heart disease will share with anaemia and malnutrition in the acute-phase state as a contributing cause. Thus, instead of disconnected target organ failures, each with different antecedent causes, we see emerging the likelihood of a unifying pathobiology for uraemia. The antecedents of morbidity and mortality appear as a web of organ failures connected by a common pathobiology. Whereas each failure most likely has contributing causes other than the acute-phase state, they probably share the state as a causative, contributing, or exacerbating factor (Lowrie, 1998).

Protein-energy wasting, inflammation and refractory anaemia are common in individuals receiving chronic dialysis over a period of many years. A decreased responsiveness to is often the result of refractory anaemia is due to resistance to erythropoiesis-stimulating agents (ESA). Protein-energy malnutrition (PEM) and inflammation are often presented. It is often related in maintenance dialysis patients (Rattanasompattikul et al., 2013). There are multiple variables that contribute to PEM and inflammation causing them to be similar. Investigations that are done with specific criteria result in hypoalbuminemia.
The inflammation and PEM are associated with a significant decreased dialysis prognosis. Low appetite and a hyper catabolic state are among common features. Protein-energy malnutrition (PEM) in dialysis patients has been suggested to be secondary to inflammation. This evidence is not the final result and an opposite causal direction could be present. Malnutrition-inflammation complex syndrome (MICS) is a relative term. There are multiple consequences of MICS which are the co morbid illnesses, oxidative and carbonyl stress and nutrient loss through dialysis. Anorexia, low nutrient intake, uraemic toxins, decreased clearance of inflammatory cytokines, volume overload, and dialysis-related factors are the balance of the consequences of MICs (Rattanasompattikul et al., 2013).

Malnutrition-inflammation complex syndrome (MICS) is commonly known to be the central consequence of many related complications. They include erythropoietin resistance, increased cardiovascular atherosclerotic disease and decreased quality of life. A high mortality and hospitalization rate had been reported in dialysis individuals. Because MICS follows a decreased body mass index, occurrences of other complications in dialysis patients can result. These include hypercholesterolemia, hypocreatininemia, and hypohomocysteinemia, a “reverse epidemiology” of cardiovascular risks. However obesity, hypercholesterolemia, and increased blood levels of creatinine and homocysteine presented to be protective. They are consequently related to better results. There is no definite knowledge to investigate the degree of severity of MICS or how to control it. Many other diagnostic tools and treatment methods are described. Productive methods to MICS may ameliorate the
cardiovascular epidemic and poor outcome in dialysis patients. Clinical trials focusing on MICS and its possible causes and consequences are urgently required to improve poor clinical outcome in dialysis patients (Kalantar et al., 2003).

Protein-energy wasting has been a predominant effect and vital factor for the increase in deaths in ESRD patients on therapy. Many different ways are often required to evaluate the nutritional status in ESRD. All contributing factors to malnutrition, for example, are to be further investigated (Segall et al., 2009).

2.20.1 Malnutrition and patients on dialysis therapy

The decrease in body mass index (BMI) and the decrease of cholesterol levels are related to poor outcomes and the increase in complications such as death (Yen et al., 2010). This presentation known as “reverse epidemiology” is based on the malnutrition-inflammatory complex. Inflammation in patients with a lower nutritional reserve will take a longer therapy time to heal. A reduced protein-calorie intake, chronic acidosis and failure of vascular access are also known to occur (Locatelli et al., 2006).

The need for increased EPO doses is necessary for a reduced nutritional status and the progression of inflammation (Locatelli et al., 2006; De Lurdes et al., 2011). The increase consequence of patients with chronic kidney disease is the Protein-energy malnutrition
and nutritional reduction within the body (Qureshi et al., 1998; Stenvinkel et al., 1999; Kopple et al., 2000).

There have been increased reports culminated over many years that malnutrition has resulted with the occurrence of cardiac co morbidity, inflammation and poor survival in chronic renal failure. In co morbid diseases such as chronic renal failure, protracted infections, cancer and malnutrition of visceral proteins often occurs in many (Avram et al., 1994, Jansen et al., 2001, Stenvinkel et al., 2002).

The predominant component during a clinical assessment of malnutrition is the biochemical indicator (Caimi et al., 2005). Serum albumin is a resourceful variable of nutrition in ESRD patients. A linear increase in the mortality with deteriorating serum albumin in patients on dialysis therapy is maintained (Kaysen, 1998). Malnutrition may be the result of decreased serum albumin. The occurrence of hypoalbuminemia could be due to an inflammatory reaction, older people and the severity of hydration (Heimburger et al., 1994; Jones et al., 1997; Heimbürger et al., 2000). Serum albumin is the most commonly used nutritional marker used to assess nutrition in ESRD patients (Stenvinkel et al., 2002). Inflammation is another cause of problems attributed to malnutrition (Stenvinkel and Alvestrand, 2002).

A recent study compared several simplified nutritional screening tools in haemodialysis patients with the Malnutrition-Inflammation Score (MIS), a fully quantitative version of
the SGA. The BMI, serum albumin, and serum total-iron-binding capacity within the Geriatric Nutritional Risk Index, combining weight loss and serum albumin loss, was the simplest and most accurate risk index for identifying haemodialysis patients at risk of protein energy wasting (PEW) according to the MIS (Yamada, 2008). However, by including serum albumin, a marker of inflammation, these were significant in the risk due to inflammation. Indeed, MIS was found to be comparable with serum C-reactive protein and serum interleukin-6 concentrations in predicting hospitalization and mortality (Kalantar-Zadeh et al., 2004). De Mutsert et al., (2009) explained from their study that all 4 subscales of the SGA were strongly associated with mortality, which suggested that they were equally important in the assessment of the nutritional status of dialysis patients.

Wasting or cachexia is prevalent among patients with chronic kidney disease (CKD). It is to be distinguished from malnutrition, which is defined as the consequence of insufficient food intake or an improper diet (Mak et al., 2011). Malnutrition is characterized by hunger, which is an adaptive response, whereas anorexia is prevalent in patients with wasting/cachexia. Energy expenditure decreases as a protective mechanism in malnutrition whereas it remains inappropriately high in cachexia/wasting (Fouque et al., 2008). In malnutrition, fat mass is preferentially lost and lean body mass and muscle mass is preserved. In cachexia/wasting, muscle is wasted and fat is relatively underutilized. Restoring adequate food intake or altering the composition of the diet reverses malnutrition. Nutrition supplementation does not totally reverse
cachexia/wasting. The diagnostic criteria of cachexia/protein–energy wasting in CKD are considered (Mak et al., 2011).

Reduced muscle mass appeared to be the most valid criterion for the presence of PEW in CKD and criteria for cachexia (Fouque, 2008). Mid-arm circumference has been shown to correlate with quality of life and survival in adult patients on maintenance haemodialysis (Evans et al., 2008; Noori et al., 2010). Accurate assessment of the adequacy of muscle mass is even more challenging. Dual X-ray absorptiometry, near-infrared interactance, and bioelectrical impedance have been used in investigations of ESRD patients but these techniques have limitations in ESRD and are not currently accepted as clinically useful tools (Bross et al., 2010).

Indirect measures, such as creatinine appearance (estimated by quantification of creatinine in a 24-h urine collection and in the collected spent dialysate) have been proposed as an index of muscle mass in patients with CKD and ESRD (Fouque et al., 2008). In adults with CKD Body fat mass lower than 10% of body weight is considered an additional criterion for PEW (Fouque et al., 2008). This is due to the known association between total body fat below 10% and increased mortality risk in adult maintenance dialysis patients (Kalantar-Zadeh et al., 2006). A more recent study showed that higher fat mass in dialysis patients might actually be protective in survival predictability (Noori, 2010). Abdominal fat deposition is linked to inflammation and PEW, resulting in an increased mortality risk in maintenance HD patients (Cordeiro et al., 2010).
The wasting or cachexia syndrome should be distinguished from malnutrition, which is defined as the consequence of insufficient food or an improper diet. Responses in malnutrition are adaptive whereas those in wasting/cachexia are maladaptive. Hunger, which is an adaptive response, characterizes malnutrition whereas anorexia is prevalent in patients with wasting/cachexia (Kalantar-Zadeh, 2004). Energy expenditure decreases as a protective mechanism in malnutrition whereas it remains inappropriately high in cachexia or wasting (Sea et al., 2004; Wang et al., 2004). In malnutrition, such as in simple starvation, fat tissues are preferentially lost and lean body mass (LBM) and muscle mass is preserved until the advanced stages, whereas in cachexia/wasting, muscle is wasted and fat is relatively underutilized (Mak and Cheng, 2006; Mitch, 2006). Restoring adequate food intake or altering the composition of the diet reverses malnutrition. Nutrition supplementation does not totally reverse the cachexia/wasting syndrome (Mak and Cheng, 2007; Cano et al., 2007).

Factors responsible for survival advantages of African American and Hispanic dialysis patients have been investigated and may have major clinical and public health implications (Kalantar-Zadeh et al, 2006). This may not only apply for CKD patients but also for other populations with chronic disease states and poor survival. Understanding the factors led to methods for improving clinical outcomes in other groups of dialysis patients as well as in populations with other chronic disease states associated with poor survival (Kalantar-Zadeh et al., 2006). These issues were time-sensitive for ESRD patients, since the imminent payment for provision of medical care to dialysis patients in
the US currently does not include an adjustor for race (Coutts, 2008; Sullivan, 2008; Zigmond, 2009). Some nutritional surrogates such as BMI were associated with both race and survival in maintenance haemodialysis patients, in which higher BMI was associated with greater survival.

Nutritional and inflammatory status was the main cause of the survival advantages of minorities who undergo haemodialysis treatment. The authors hypothesized that the apparent survival advantage is due to a more favorable nutritional/inflammatory profile in minority HD patients. Trials of nutritional and anti-inflammatory interventions were required to be examined in order to determine whether longevity could be improved in dialysis patients and other populations with chronic diseases (Streja et al., 2011). The existence of elements of malnutrition-inflammation complex syndrome (MICS) as indicated by a high malnutrition-inflammation score (MIS) and increased levels of proinflammatory cytokines, such as IL-6, as well as decreased nutritional values, such as low serum concentrations of total cholesterol, prealbumin, and TIBC correlates with EPO hypo responsiveness in maintenance haemodialysis (MHD) patients (Kalantar-Zadar et al., 2003).

2.20.2 The effects of inflammatory bowel disease on erythropoiesis

Blood loss, and to a lesser extent, malabsorption of iron are the main causes of iron deficiency in inflammatory bowel disease (IBD). There is also a variable component of
anaemia related to chronic inflammation. The anaemia of chronic renal failure has been treated for many years with recombinant human erythropoietin (RHuEPO), which significantly improves quality of life and survival. Subsequently, RHuEPO has been used progressively in other conditions that occur with anaemia of chronic processes such as cancer, rheumatoid arthritis or IBD, and anaemia associated with the treatment of hepatitis C virus. Erythropoietic agents complete the range of available therapeutic options for treatment of anaemia associated with IBD, which begins by treating the basis of the inflammatory disease, along with intravenous iron therapy as first choice. In cases of resistance to treatment with iron, combined therapy with erythropoietic agents aims to achieve near-normal levels of haemoglobin (11-12 g/dL) (Lopez et al., 2009).

2.21 Role of Nutrition in haemodialysis patients

Co morbidities may be different in the statistics and severity of the progression in chronic renal failure patients. Co morbid conditions and clinical outcomes are related in developing the significant effect of co – morbidities. This will result in both being analysed to decrease the risk in survival studies. Many variables are used to clearly analyse the co morbidities (Khan et al., 1998; Hemmelgam et al., 2003).

The existence of co morbidities include the following, coronary artery disease (CAD), cerebrovascular disease, peripheral vascular disease, congestive heart failure, malignancy and cardiac arrhythmias. These are also followed by chronic lung disease,
and chronic liver disease. Known malnutrition is enhanced by many co morbid attributes. The result of decreased energy surplus, loss of somatic and protein visceral protein is due to the high level of catabolic activity (Stenvinkel et al., 2000). A decrease in nutritional intake contributes largely to the mortality of patients (Bergstrom and Lindholm, 1998). Hypoalbuminemia and mortality are enhanced at the beginning of therapy of the patients on HD and PD (Foley et al., 1996).

2.22 Iron in relationship to erythropoiesis

Functional or absolute iron reduction is a complication for the effects of epoietin therapy in chronic renal failure patients. The I.V iron therapy could cause immediate and long-term effects. Therefore, it becomes important to select patients who need iron supplementation and possibly have the parameters useful in predicting an effective response (Fishbane et al., 2001). There are many guidelines that recommend T Sats% and serum ferritin for being sensitive to when there is a decrease in the iron blood levels. However, T Sats% is enhanced by the regular changes in iron levels. The authors explained that during elevated chronic inflammatory diseases, i.e. uraemia, ferritin is to be increased due to it being an acute phase protein. The guidelines for treating chronic dialysis individuals on RHuEPO therapy and I.V. iron agree on the lower values of T Sats% (<20%) at which therapy has to be started but disagree on the upper value of ferritin, which should not be exceeded to avoid the risk of acute and chronic toxicity (Fishbane et al., 2001).
Dialysis patients on RHuEPO progress to a decrease in plasma iron levels. The decrease in iron levels being absolute, can lead to malnutrition, gastrointestinal bleeding, chronic blood retention in the dialysis circuit, and frequent transfusions. Functional iron deficiency is the prevention of the bone marrow to erythropoiesis. This results in the decrease of iron transport from storage. In this situation the body’s total iron stores may be normal. The deficiency in iron has a negative effect on EPO therapy. For effective treatment of EPO therapy, patients are to be administered intravenous (I.V.) iron (Coyne, 2006).

Parenteral iron administration has potential risks that are immediate (e.g., toxic effects and anaphylactic reactions) and long-term (e.g., decreased polymorph nuclear leukocyte function, increased risk of infections and organ damage). It is essential to group individuals who require iron supplementation. Iron removal from the body has no known maintained mechanism pathways. The total amount of iron being absorbed by blood entering the body each day represents less than 0.1% of the total body iron endowment. All iron in blood comes from the blood system (Figure 9) (De Domenico et al., 2008). The most important pathway of iron metabolism is the unidirectional recycling of iron from the erythrocyte to the erythroid bone marrow. This mechanism is done through the macrophages (Andrews and Schmidt, 2007). Chasis and Mohandas discussed the role of macrophages within the erythroblast islands, the specialized niches in which erythroid precursors proliferate, differentiate, and enucleate in the bone marrow (Chasis and Mohandas, 2008).
Around the macrophage are the erythroblasts which make up the haematopoietic sub compartments. The macrophage is a principle cell in providing iron to the maturing erythroblasts for heme synthesis (Bessis and Breton-Gorius, 1962).

Ferritin synthesized by macrophages is secreted by exocytosis. However it is received by the erythroblasts being a vital source of iron for heme synthesis (Leimberg et al., 2008). However there is no confirmation to macrophages being functional in releasing iron by the ferroportin-dependent exporting pathway. This understanding requires further investigation. The alternative for the body’s requirement is the cycling of iron from the hepatocytes. This process is to be reversible. The final option is the absorption of iron. This mechanism is to be through the duodenal and upper jejunum that balances the 1-2 mg daily iron loss occurring through cellular exfoliation (Mariani et al., 2009).

![Figure 9: Iron metabolism (Mariani et al., 2009)](image-url)
2.22.1 Iron transport and utilization

The systemic transporter of iron in the body is transferrin, an abundant, high affinity iron-binding protein. Under normal circumstances, transferrin (TF) carries nearly all serum iron collected from duodenal absorptive epithelium, macrophages and hepatocytes, and dampens its reactivity (Andrews, 1999). Very small amounts of iron may be loosely associated with albumin or small molecules. In normal human subjects, iron occupies approximately 30% of the iron-binding sites on plasma TF1. The saturation of TF by iron shows diurnal fluctuation exhibiting a morning peak and an evening nadir. It is likely to be higher in the portal circulation, where recently absorbed iron from the intestine enters the circulation and passes through the liver. This first-pass effect may explain the periportal iron accumulation in the hepatic lobule observed in iron overload disorders associated with inappropriately increased intestinal iron absorption (Figure10)(Deugnier et al., 1992; Piperno, 1998).
**Figure 10:** Iron Cycle in Haemodialysis

(Mariani et al., 2009)

**Figure 11:** Erythropoiesis (Schott and Fitzgerald, 1997)
Hentze et al., (2004) stated that TF saturation is most likely lower than average in plasma leaving the erythroid bone marrow, where most of the iron present is extracted for use by erythroid precursor cells. The erythroid bone marrow is the largest consumer of iron. Normally, two-thirds of the body iron endowment is found in developing erythroid precursors and mature red blood cells (Figure11). Erythroid precursors express cell surface transferrin receptors (TfR1) that take up iron from holo-TF by receptor-mediated endocytosis.

Levy et al., (1999) explained that targeted disruption of the murine TfR1 gene causes embryonic lethality, attributable to severe anaemia. Goldwurm et al., (2000) further explained that spontaneous mutations disrupting the TF gene demonstrate the importance of TF in animals and humans. The lack of TF results in severe iron deficient microcytic anaemia. The iron deficiency occurs only in haematopoietic cells whereas other tissues develop massive iron overload. This underscores the importance of the TF-TfR1 endocytic cycle in erythropoiesis, but further demonstrates that non-haematopoietic cells have alternative mechanisms to assimilate iron (Hentze et al., 2004).

The overall iron homeostasis highlights changes that result from iron deficient erythropoiesis. Hepcidin synthesis is markedly depressed and intestinal iron absorption is increased in congenital hypo-transferrinaemia, apparently to try to compensate for the lack of iron available to erythroid precursors. This is a compelling evidence for a signaling mechanism that allows the erythroid bone marrow to communicate its iron
needs to the intestine’s absorptive epithelium (Trombiniet al., 2007; Nemeth et al., 2008).

2.22.2 Iron absorption

Iron in food is described as being present as ferric iron or as heme (Mariani et al., 2009). Heme is a biologically important iron containing compound and a key source of dietary iron but the mechanism by which the enterocyte takes up heme and catabolises it to utilize the iron is still poorly understood (Mariani et al., 2009). There are two prevailing hypotheses explaining the mechanisms of this process; first, heme is taken up by receptor mediated endocytosis; secondly, the recent discovery of a heme transporter (PCFT/HCP1) that may have the capability of transferring heme from the small intestinal lumen directly into the cytoplasm (West and Oates, 2008).

One important criticism of the receptor mediated endocytosis hypothesis is that it assumes iron released from heme is transported out of the internalized vesicles in order to join the labile iron pool. Currently, no such transport process has been identified. However, it is possible that divalent metal transporter 1 (DMT1) may fulfill this role in a manner analogous to its role in the transferrin receptor cycle. In recent years, two mammalian heme transporters have been discovered, namely PCFT/HCP1 (Shayeghi et al., 2005; Inoue et al., 2008) and FLVCR20 possibly acting at the apical and basolateral site of the small intestinal enterocyte, respectively. At this early stage, however, the physiological relevance of these transporters to intestinal heme iron
absorption is unclear. PCFT/HCP1 has been independently characterized as a folate/proton symporter and appears to play a key role in intestinal folate absorption. Interestingly, the folate transport capabilities of PCFT/HCP1 are, at least in order of magnitude higher than that observed for heme, suggesting that folate may be the more physiologically relevant target of this transport protein. FLVCR is a heme exporter relevant for erythropoietic activity, which acts as an overflow valve for excess manufactured heme that would otherwise result in cellular toxicity. Intestinal cell line CaCo-2 does express the protein and it may be hypothesised that it can exert similar function in intestinal cells (Uc et al., 2004).

Non-haem iron requires to be converted to ferrous iron by the apical ferric reductase duodenal cytochrome B. However, the physiological significance of this pathway is the subject of continued debate. Following the reduction, iron crosses into the cytoplasm via an apical iron transporter, DMT1 (Andrews, 1999). The physiological relevance of DMT1 in iron absorption is confirmed in the Belgrade (b) rat and mk mouse which exhibited a microcytic, hypochromic iron deficiency anaemia due to a G185R mutation to DMT1, resulting in a dramatic decrease in DMT1 function (Fleming et al., 1998).

2.22.3 Cells regulating body iron homeostasis

Besides enterocytes other cell lines, namely macrophages, hepatocytes in adults and placental cells during faetal life, have core functions within iron metabolism. They
acquire iron from different sources (senescent erythrocytes and holotransferrin) and deliver it to the rest of the body according to its needs (Figure12). To do this important function these cells have a specialized mechanism for exporting iron to plasma, that is the iron exporter ferroportin (Ganz, 2013). Ferroportin needs copper-ferroxidases to release iron to plasma transferrin, namely hephaestin in duodenal cells, ceruloplasmin in hepatocytes, and macrophages (Frazer et al., 2002). When defective, these proteins reduce cellular iron retention in specific cell types as shown in hephaestin deficient mice and in humans with aceruloplasminaemia (Frazer et al., 2002).

Ferroportin acts under the control of hepcidin and this interaction explains the systemic regulation of iron metabolism (Nemeth et al., 2004 b). It has been shown that hepcidin-induced internalization of ferroportin requires binding and cooperative interaction with Janus kinase (Jak2) (De Domenico et al, 2011). Hepcidin binding to ferroportin results in the phosphorylation of ferroportin. This step is necessary for its internalization by clathrin-coated pits and the kinase responsible for the phosphorylation is Jak2. This finding provides a mechanistic explanation for the dominant inheritance of hepcidin resistant ferroportin disease and suggests that Jak2 might represent an important link at the interface between erythropoiesis and iron metabolism.

Enterocytes, macrophages and hepatocytes acquire iron from different sources and deliver it to the rest of the body through the iron exporter ferroportin, which needs copper ferroxidases to release iron to plasma transferrin. Ferroportin acts under the
control of hepcidin and this interaction can explain the systemic regulation of iron metabolism (Mariani et al., 2009).

**Figure 12: Iron Storage (Mariani et al., 2009)**

Following phagocytosis the damaged erythrocytes, tissue macrophages, particularly in the spleen and lysescells, cataboliize haemoglobin to heme oxygenase to liberate iron. Some iron remains stored in macrophages, although some is exported to plasma transferrin. Ferroportin is critical for macrophage iron export and can be regulated to change the ratio between stored and released iron (Ganz, 2013). Hepatocytes
represent the main depot for iron storage in normal conditions and in non-transfusional iron overload.

Although the transferrin cycle may be involved in hepatocyte iron acquisition to some extent, non–transferrin-bound iron (NTBI) uptake pathways become particularly important when serum iron levels exceed transferrin binding capacity (Anderson, 1999; Barisani et al., 2008). The identity of the hepatocyte NTBI uptake system is not known, but DMT1 is unlikely to be involved, because hepatocytes can accumulate iron in the absence of DMT1 (Gunshini, 2005). Non–transferrin-bound iron (NTBI) transporter includes a zinc transporter family which mediates the uptake of zinc and NTBI in hepatocytes (Goldwurm et al., 2000; Nicolas et al., 2002).

Hepatocytes have a large capacity to store excess iron (Liuzzi et al., 2006). Most storage of iron is probably in the form of ferritin, which can be mobilized when needed elsewhere in the body. However, massive iron overload results in hepatotoxicity; hepatitis leads to fibrosis and cirrhosis (Liuzzi et al., 2006). The liver is probably exposed to more NTBI than are other tissues because of the first-pass effect of the portal circulation. Other tissues have NTBI uptake activities and load iron when NTBI is present in the plasma. The heart and endocrine tissues are particularly susceptible to cardiomyopathy and endocrinopathies and are predominant from hepatic complications of iron overload. L-type calcium channels mediate the uptake of NTBI in the myocardium (Oudit et al., 2003; Mwanjewe and Grover, 2004).
2.22.4 Hepcidin

The vital regulator for iron is Hepcidin which is a peptide (25 amino acids). The process of the formation of hepcidin is in the hepatocytes. The elevated hepcidin levels leads to the decrease in iron absorption and an inhibition of iron release from its storages (macrophages and hepatocytes) as hepcidin binds to ferroportin promoting its internalization and degradation in lysomes (De Domenico et al., 2007; De Domenico et al., 2011). Hepcidin is increased in HD patients, regulated by inflammation and is linked to EPO resistance. Hepcidin correlates with IL-6, the cytokine that stimulate its production (Nemeth et al., 2004 a; Moroi et al., 2008; Song et al., 2010).

2.22.5 Effect of iron levels in Erythropoiesis

The glycoprotein hormone erythropoietin (EPO) is the key component in the process of formation of erythrocyte. It activates the differentiation of erythroid cell precursors. This results increase erythropoiesis and haemoglobin levels (Jelkmann et al., 2008). Recombinant human erythropoietin (RHuEPO) mimics the action of endogenous EPO. It had been introduced in clinical practice about twenty 20 years ago. It is now extremely beneficial in the treatment of anaemia. However, it is improving as research continues. The presence of erythropoietin receptors (EPOR) in various nonerythropoietic tissues indicates that EPO also has nonhaematopoietic activity. However its effects outside the erythron have not been completely described (Uchida et al., 1983).
The erythroid cell proliferation activated by recombinant erythropoietin is related with a change in the iron metabolism. This is to deliver an increased need for iron to synthesize haemoglobin (Andrews, 2008). Iron homeostasis is primarily modulated by hepcidin, a liver peptide hormone. This hormone inhibits cellular iron release by triggering the internalization and degradation of the cellular iron exporter ferroportin. Thereafter the iron absorption by the intestine is prevented and iron is released by reticulocyte endothelial cells (Nemeth et al., 2004c; De Domenico et al., 2008; Muckenthaler et al., 2008).

Hepatic hepcidin, described as an expression is down-regulated in response to iron deficiency, anaemia, or hypoxia. However, iron release and availability are processed at greater levels. The authors further explained that the hepcidin expression is up-regulated in response to iron overload or inflammation (Nicolas et al., 2002; Burns et al., 2011).

Iron deficiency is a common cause of anaemia in chronic renal failure. The causes include decreased intake or absorption of iron, iron sequestration as a result of inflammation, blood loss. Therefore there is an increased iron use for erythropoiesis in response to erythropoiesis stimulating agents (ESA). A decrease in erythropoiesis and response to RHuEPO due to inflammation, progresses to anaemia. This is usually in the more advanced stages of ESRD (Bruce et al., 2008). Ideal therapy of anaemia in ESRD is the involvement of iron and ESA (KDOQI, 2006). Due to impaired absorption
and gastrointestinal there is restriction to oral iron therapy. Other adverse effects contribute to the individual's compliance (Nielsen et al., 2005).

Hypo responsiveness to erythropoietin therapy is a common presentation in ESRD haemodialysis patients, due to a variety of comorbid conditions, particularly aluminum toxicity and iron deficiency (Tarng et al., 2009). Ferritin is regularly used as a marker of iron storage and a pattern exists between serum ferritin and liver iron (Goodnough et al., 2000). Growth and development of erythroid precursor cells are the limiting factors in the erythropoietic response. Limiting factors to this response is also due to the sudden blood loss and in anaemia therapy that is administering increased dosages. Clinical trials have demonstrated a dose-response relationship between erythropoietin and red blood cell production. Erythropoietin dosage is not related to patient gender or age. It is suggested that patient-specific factors such as accompanying chronic disease, restricted erythropoiesis, or other factors that normally cause the wide distribution of the haemoglobin level account for the variability in erythropoietic response to erythropoietin (Goodnough et al., 2000).

Hepcidin is a peptide hormone that has been implicated in controlling the release of iron from cells. Infection, malignancy, and chronic inflammation may result in inefficient macrophage iron release and subnormal intestinal iron absorption, contributing to the anaemia of chronic disease (Weinstein et al., 2002).
2.22.6 Iron deficiency

The effect of erythropoiesis is enhanced by a few factors. These are the Iron stores, nutritional-inflammatory status, dialysis adequacy, and bone metabolism disorders. The haemoglobin response depends on the factors affecting the production of EPO (Baron and Kenny, 1986; Santiago, 2012).

The haemoglobin level of the ESA therapy is restricted by physiological representations (Coyne et al., 2007). The authors hypothesized that this limiting activity might be nonlinear. Erythropoiesis is closely related with the activity of EPO and iron. Serum Ferritin and T Sats have been practical as vital components available for erythropoiesis. However, several studies did not report on the outcome of these components of the erythropoietic response. The analysis showed that Ferritin and T Sats was related with the erythropoietic response. Iron deficiency stated the decreased T Sats or ferritin was related with an impaired erythropoietic response. Impaired erythropoietic response was also predominant when ferritin increased (>500 ng/ml). The physiologic process through which the erythropoietic response was compromised was the increased ferritin levels. This can be described as the inhibition of erythropoiesis by the malnutrition-inflammation complex syndrome (Kalantar-Zadeh et al., 2004).

Dialysis adequacy and the erythropoietic response have been established to be linked in previous studies (Locatelli et al., 2006). Movilli et al., (2003) established a negative
correlation between the total EPO dose and Kt/V when Kt/V is below 1.33 and no correlation when it is above 1.33. The physiology behind this is not yet fully understood. It could be explained by a number of phenomena, including inflammation or vascular access complications. Albumin has been positively correlated with the erythropoietic response (Locatelli et al., 2006; Agarwal et al., 2008).

Severe hypoalbuminaemia is associated with a significant impairment of the erythropoietic response (Gaweda et al., 2010). Similarly to high ferritin the mechanism behind this effect modification could be attributed to the malnutrition-inflammation complex syndrome. A detrimental effect of hyperparathyroidism on the Hb response to EPO has also been reported (Gaweda et al., 2010). Increased PTH levels were associated with a decreased erythropoietic response. The potential physiologic mechanisms that may affect the erythropoiesis and could be represented by this marker include bone marrow fibrosis and the hormonal effects on erythroid precursors (Gaweda et al., 2010).

The process of the development of erythrocytes is activated in physiologic and clinical presentations of hypoxia and other presentations of depletion of oxygen. The distribution of tissue oxygen is regulated by the erythropoietin and the availability of iron transport is required. The duodenal iron absorption and increased iron availability from macrophages are together with the mobilization from storage organs such as the liver. All the known processes are regulated to allow the supply of iron is provided in erythropoiesis (Andrews, 2008; De Domenico et al., 2008).
Inflammation is regulated by lower levels of iron and iron-binding capacity (transferrin) in anaemia. The high ferritin iron in bone marrow macrophages, results in poor development of transport of iron from storage (De Francisco et al., 2009). Iron metabolism is deactivated by the cytokines by multiple processes. This however results in functional iron deficiency. Increased doses of epoietin may elevate the activation for red blood cell production. The increase in activation of red blood cells therefore exceeds the maximum capacity of liver iron stores. The elevated ferritin and lowered transferrin formation stops iron transport to the reticulo-endothelial storage pool. However there is no availability of iron to the delivery to erythroid precursors. The mucosal uptake and mucosal transfer of iron thereafter presents a reduction in dialysis patients with elevated CRP levels (>8 mg/L versus <8 mg/L, P<0.01) (Kooistra et al., 1998). Individuals who had increased ferritin levels (P<0.02) and decreased transferrin levels (P<0.01) presented with elevated CRP. These individuals had to receive increased epoietin doses to regulate the haematocrit between 30% and 35% (P<0.05). Anaemia of inflammation therefore maintains the functional iron deficiency (De Francisco et al; 2009).

2.22.7 Predictors of iron deficiency

Sixty nine patients on haemodialysis 3 times weekly verified the clinical usefulness of the biochemical and cellular parameters as the predictors of iron deficiency in patients undergoing long-term haemodialysis (Buttarello et al., 2010). The baseline values of serum ferritin and percentage of transferrin saturation were poor predictors of iron
responsiveness. Better ability was demonstrated by reticulocyte indices. The authors further explained that even though percentage of hypochromic RBCs (HYPO %) has been demonstrated as one of the best predictors of iron deficiency, it was not included in the KDOQI guidelines because the measured value depended on the time elapsed between collection and analysis (Buttarello et al., 2010).

2.22.8 Iron therapy

Dialysis patient’s response to intravenous iron with elevated ferritin (DRIVE) study demonstrated the efficacy of intravenous ferric gluconate to improve haemoglobin levels in anaemic haemodialysis patients (Kapoian et al., 2008). The authors described that these patients were receiving adequate epoietin doses maintained ferritin levels between 500 and 1200 ng/ml and transferrin saturation (T Sats) ≤25%. Those patients with T Sats and serum ferritin levels less than or equal to 1200 ng/ml while maintaining optimal Hb levels were given one gram loading course of intravenous ferric gluconate. The haemodialysis patients that received the intravenous ferric gluconate had effectively and safely reduced their epoietin doses. The magnitude of this epoietin dose reduction was similar to that seen in many reports of patients having classic iron deficiency. Accordingly, this study provided additional evidence to support the conclusion of the DRIVE study that intravenous iron is beneficial in maintaining Hb concentration while decreasing epoietin doses in anaemic haemodialysis patients with a low T Sats and ferritin levels up to 1200 ng/ml (Kapoian et al., 2008).
The randomized controlled trial showed that intravenous ferric gluconate was effective in improving anaemia in haemodialysis patients with ferritin of 500 to 1200 ng/ml, transferrin saturation (TSAT) ≤ 25%, and adequate epoietin doses (Coyne et al., 2007). Thus, disproving the widely held belief that patients with ferritin more than 500 ng/ml are unlikely to benefit from intravenous iron administration (Van Wyck et al., 2002; Aronoff, 2004; National Kidney Foundation Kidney Disease Outcomes Quality Initiative, 2006). In the context of an increased epoietin dose, intravenous ferric gluconate patients were more likely to mount a haematologic response than controls, regardless of baseline levels of ferritin, TSats, C-reactive protein, Hb, soluble transferrin receptor, epoietin dose, or reticulocyte Hb content (Dreuke et al., 2006; Kapoian et al., 2008).

Erythropoiesis stimulating agents (ESA) and iron must be present in sufficient quantities to effectively produce red blood cells in patients with chronic kidney disease (Kapoian et al., 2008). Studies in haemodialysis patients have repeatedly shown that greater use of intravenous iron invariably results in lower epoietin doses while maintaining or increasing haemoglobin (Hb)/haematocrit levels (Kaneko et al., 2003).

Sirken et al. (2006), has expressed concern that administration of intravenous iron to patients with an elevated ferritin may increase infection rate or infectious complications. The authors explained that the risk of infections from intravenous iron should coincide with intravenous iron administration or follow shortly thereafter. They further stated that during the 12 week of the DRIVE and DRIVE-II studies there had been less risk of hospitalizations from infections among patients who were given 1 g of intravenous ferric...
gluconate compared with the control group. The control groups had no ferric gluconate. The lack of difference in rates of infections between intravenous ferric gluconate and control may not be generalized to all intravenous iron preparations. Different rates of bacteremia have been reported with different intravenous iron preparations in patients on maintenance haemodialysis (Sirken et al., 2006; Lawler et al., 2010).

Chronic kidney disease (CKD) is characterized by a high mortality rate derived largely from CVD. In patients with CKD, high levels of pro-inflammatory cytokines and increased oxidative stress are common features that may contribute to malnutrition, anaemia, ESA resistance and atherosclerosis by different pathogenetic mechanisms. Inflammation is multifactorial in cause, and while it may reflect underlying CVD, the acute-phase response may also contribute to both oxidative stress and progressive vascular injury. Recent findings suggest that anaemia is associated with increased oxidative stress and various anti-oxidant treatment strategies have been associated both with a reduction in oxidative stress and in the required dose of ESA in patients with CKD. Conversely, there may be pro-oxidant effects from treatment of anaemia with ESA and iron. Controlled trials are needed before evidence-based recommendations for the management of inflammation-induced anaemia and ESA resistance can be defined. In particular, the risks and benefits of intravenous (I.V.) iron and the effects of various iron dosage schedules warrant further careful evaluation in prospective studies (Barany, 2009).
2.22.9 Inflammation Markers

The ferritin molecule, with an average molecular weight of 450kDa, is the main storage molecule of iron because it stores iron in a safe and soluble manner (Torti, 2002). This allows for regulated release of iron and mitigates the risk for oxidation via free iron atoms (Worwood, 1990; Harrison and Arosio, 1996). During the acute-phase response, proinflammatory cytokines increased the synthesis of various subunits of ferritin molecule through an increased translation of preformed ferritin mRNA (Torti and Torti, 2002). Alterations are usually parallel to increased hepcidin activity (Nemeth and Ganz, 2006; Barisani et al., 2008).

Inflammation is closely related to protein-energy wasting in dialysis patients (Fouque et al., 2008). Simultaneous combination of these two conditions, also referred to as malnutrition-inflammation cachexia syndrome (MICS), is observed frequently in CKD patients (Kalantar-Zadeh et al., 2003). Inflammation-induced hyperferritinaemia may result in a so-called “functional iron deficiency, “which can block iron mobility and, hence, be useful in “acute” inflammation by iron containment in the reticulo endothelial system but harmful under “chronic” inflammation by leading to refractory anaemia, such as in CKD or other chronic disease states (Kalantar-Zadeh et al., 2006). The results of this study are consistent with the role of inflammation in confounding such iron markers, especially ferritin. Serum iron, T Sats, and ferritin are the most commonly used laboratory indicators in the diagnosis and management of iron deficiency anaemia in CKD patients (Kalantar-Zadeh et al., 2006). Iron stores, such as non–iron-related
factors, like inflammation and nutritional status may have a bearing on variability of iron markers, especially serum ferritin variability (Kalantar-Zadeh et al., 2004).

Maintenance haemodialysis patients (MHD) with ferritin values above 500ng/ml and with low T Sats are associated with inflammation. Strategies to dissociate inflammation from iron metabolism to mitigate the confounding impact of inflammation on iron and to improve iron treatment responsiveness may improve anaemia management in chronic kidney disease (Rambod et al., 2008). The investigators found that the probability of having a moderately high serum ferritin 500 ng/ml was explained by both the iron stores and inflammation. The seemingly paradoxical combination of serum ferritin 500 ng/ml and T Sats<25% in MHD patients was associated with increased level of inflammatory markers (Rambod et al., 2008).

This finding implied that inflammatory states should be taken into account when interpreting a moderately high serum ferritin, especially in the setting of low T Sats. Hence, in dealing with MHD patients with ferritin levels >500 ng/ml, the possibility of high levels of circulating inflammatory cytokines should be considered as an alternative explanation to iron overload (Rambod et al., 2008; Majoni et al., 2014).
2.22.10 Transferrin

Transferrin is a vital regulator in distributing Fe in the human body via blood circulation. In physiologically status transferrin occurs in four variants. Studies were carried out on a group of 55 ESRD patients treated by HD (ESRD group). The reference values were obtained from a group of 20 healthy volunteers group. The concentration of selected haematological variables, and selected liver metabolism variables were assessed by routine laboratory tests. The concentration of cytokines was measured by ELISA method, and selected early stages of proteins. This is the C-reactive protein, by the immunonephelometry method. The concentration of transferrin and its variants is changing in several conditions, especially during acute phase response, which may take place in chronic renal failure (CRF). The purpose of this investigation was the assessment of the associations between changes of glycosylation of transferrin and selected blood cell count and iron metabolism parameters in ESRD patients. These patients were treated by maintenance haemodialysis “(MHD)” with long lasting anaemia despite treatment by RHuEPO (Formanowicz et al., 2007a).

End stage renal disease associates many characteristics of the inflammatory state. Anaemia had occurred in the presence of elevated levels of pro-inflammatory cytokines. Decreased resultant dialysis therapy contributes to the occurrences. Suppression of bone marrow erythropoiesis, suppression of EPO production, increased intestinal bleeding and modulation of iron metabolism is associated with inflammatory cytokines.
These cytokines are effective in contributing to the progression of anaemia (Nemeth et al., 2004c).

Inflammation in their trial was an important factor that influenced transferrin (TF) variants (Formanowicz et al., 2007b). The authors established that during 1 year of the haemodialysis treatment the serum concentration of the total transferrin was constant, but lower if compared to controls, whereas the percentage of the individual transferrin variants changed. They had further explained that alterations in the glycosylation profile of serum proteins may occur faster than in its general concentration, which suggested peripheral binding of selected variants and synthesis and glycosylation are regulated distinctly. Formanowicz et al., (2007b) had investigated in his study the iron deficiency in dialysis patients. There are three known main mechanisms that contribute to the high frequency of iron deficiency in these patients, in addition to the increased demand for iron and consequent storage in RBCs when erythropoiesis is stimulated with RHuEPO (Bernstein, 1987; Fishbane and Maesaka, 1997). These include abnormal iron absorption, external blood loss and functional iron deficiency. The authors in the previous study observed that the usual tests for iron deficiency in dialysis patients did not indicate absolute iron deficiency (ferritin of more than 100 ng/ml; T Sats of more than 20%), but patients responded to additional iron administration with a rise in haematocrit at a stable RHuEPO dose or maintain a stable haematocrit with a lower RHuEPO dose (Formanowicz et al., 2007b; Formanowicz et al., 2012c). They further explained that patients with functional iron deficiency, therefore, have insufficient available iron to keep up with the demands of the stimulated erythropoiesis that occur
when RHuEPO is administered. In some patients, the inability to mobilize iron rapidly depends on the presence of reticulo-endothelial blockade, which may be the effect of the increased levels of cytokines. Circulating cytokines may impair iron metabolism via several different mechanisms (Francisco et al., 2009).

2.22.11 C-reactive protein (CRP)

2.22.11.1 C-reactive protein (CRP) as a marker for inflammation and malnutrition

Serum albumin is a well-known marker of nutrition in ESRD patients. Serum albumin is still the most commonly used nutritional marker in ESRD patients. C-reactive protein (CRP), the major acute phase response (APR) protein is elevated in these patients. High CRP levels are linked to the degree of atherosclerosis in coronary, peripheral, and extra cranial brain arteries (Bradbury et al., 2009; Dashti et al., 2012). The aim of the study was to investigate nutritional factor (albumin) and CRP levels in ESRD patients. The cross-sectional study had a sample number of a total of 300 patients who had ESRD and had been on haemodialysis treatment for at least 6 months. The laboratory tests consisted of measurement of CRP and albumin using high sensitive ELISA kits. The study patients included 157 males (52.3%) and 143 females (47.7%) with average age of 41.5 ± 14.3 years. Mean CRP level was 7.96 mg/ dl (±1.52), mean serum albumin was 4.07 g/dl (±0.19). Of 300 patients, 21 died (7%). These were patients with serum albumin <4 g/dl and CRP>9.5 mg/dl. This study showed that low albumin and
high CRP levels are the main predictors for death. There was a significant difference between CRP and albumin levels in ESRD patients (P<0.0001). Measuring CRP as a marker of inflammation can be helpful in managing these patients (Dashti et al., 2012).

The authors had described that measuring CRP as a marker of inflammation could be of benefit in managing their patients. Their findings had important implications for clinical practice. End stage renal disease (ESRD) patients with low albumin and/or high CRP levels should receive close follow up and all sources of malnutrition and inflammation should be controlled (Dashti et al., 2012). Inflammation as measured by an elevated C reactive protein (CRP) level appears to be an independent predictor of greater ESA dose requirements. Patients with the highest CRP levels required significantly higher ESA doses to achieve comparable Hb levels even after controlling for potential confounding variables (Bradbury et al., 2009).

**CRP on Glomerular Filtration rates**

Increased CRP level with decreasing estimated GFR have been investigated. Inflammation is an important cause of uraemic cachexia, and baseline serum C-reactive protein (CRP) level independently predicts a decrease in fat mass over time in patients on maintenance dialysis (Eustace et al., 2004).
2.22.11.2 CRP and Cardiovascular Disease

Cardiovascular disease is the leading cause of death in patients with end-stage renal disease. Besides traditional risk factors, disturbances in mineral and bone metabolism and inflammation are thought to be responsible for the increased risk of death. In the last years, C-reactive protein (CRP) has gained a lot of attention in the general population, especially with regard to its link with atherosclerosis (Van der Sande et al., 2006). Although several studies suggest that CRP may be useful as a parameter in predicting future cardiovascular events in both the general population and in patients with end-stage renal disease, there is doubt about the clinical evidence of this assumption. A statistical association between CRP and cardiovascular disease was observed in various studies, but the predictive power of this association is markedly diminished when adjusted for other risk factors.

The relative contributions of CRP as a marker, as a causative agent, or as a consequence of atherosclerotic vascular disease are unclear, both in the general population and in the dialysis population. The CRP levels are highly variable and influenced by intercurrent events in dialysis patients. In dialysis patients, it is possible to reduce the CRP levels by statins, although these agents do not reduce the cardiovascular mortality in diabetic dialysis patients (Van der Sande et al., 2006). Chronic inflammation and the impact of different HD modalities on morbidity and mortality rates in a large population (1.235 million people) of ESRD patients were followed up for a period of 30 months. The authors explained that CRP and pro-
inflammatory cytokines are independently associated with all-cause and CV mortality in dialysis patients. Multivariate analysis adjusted for co morbidity and demographic showed CRP as the most powerful mortality predictor (P< 0.001) followed by IL-6 . The combined determination of CRP and IL-6 seemed to be the best option in the context of clinical studies (Panichi et al., 2008).

High plasma levels of CRP (>5 mg/dL) and of pro-inflammatory cytokines (IL-6 > 3.2 pg/mL, IL-8 > 1 pg/mL) showed an increased risk for CV (RR 1.9; P< 0.001) and all-cause mortality (RR 2.5 P< 0.001). The highest levels of risk for CV (RR 2.1; P< 0.001) and all-cause mortality (RR 2.7; P< 0.001) were reached in patients with low albumin plasma values (<3.5 g/dL). By the stepwise regression analysis and the 2 log L test, CRP was the strongest predictor of all-cause and CV mortality even after adjustment for age, dialytic age, diabetes, co morbidities and BMI followed by IL-6 (Panichi et al., 2008).

Arbel et al., (2012) stated that the main finding of their study was that among the commonly used inflammatory biomarkers that were available in daily practice (CRP, quantitative fibrinogen, and WBC) the CRP correlated best with the different anthropometric measurements.
CRP protein stimulates tissue factor production and neutrophil aggregation. The tendency to coagulation could indicate a direct contribution of CRP to mortality. Greater CRP levels indicate patients at risk of progression of cardiovascular disease (Harris et al., 1999). In the above stated study, the authors observed that a large proportion of patients had elevated serum CRP levels (= 10mg/L). The presence of elevated CRP in a significant number of ESRD patients confirms the existence of chronically activated APR. Recent data from ESRD patients also showed that elevated CRP levels had significant association with hypoalbuminaemia, malnutrition, increased morbidity and mortality in ESRD patients (Arici and Walls, 2001; Kop and Weinstein., 2007). Bergstrom et al., (1998) were first to show that elevated CRP was a strong predictor of mortality. The association between CRP and nutritional measures were seen (Arici and Walls, 2001). Recent studies showed CRP was a significant and independent predictor of death in chronic haemodialysis patients (Zimmermann et al., 1999).

Two-year patient survival was significantly lower in the elevated CRP group than in the normal CRP grouping (Arici and Walls, 2001). Several studies in patients with ESRD have shown that CRP levels are linked with cardiovascular disease. C reactive protein (CRP) levels were found to be associated with various classic markers of cardiovascular disease in ESRD population (Helal et al., 2010; Kato et al., 2010).
Strong association exist among laboratory findings for malnutrition and acute phase processes and pathobiology implied by these laboratory abnormalities influences mortal risk in patients primarily through depletion of vital body proteins. Deplete stores of vital proteins may result in part from down regulation of protein synthetic processes and up-regulation of catabolic processes. The finding that hypoalbuminaemia was the main prognostic factor for death was largely reported. This had suggested that chronic inflammation may be the factor that relates hypoalbuminaemia to morbidity and mortality. However, data consistent with the possibility that malnutrition may affect survival independently do exist (Fellah et al., 2009). Markedly elevated other circulating cytokine levels are found in ESRD patients, which may be due to impaired removal of cytokines, and increased synthesis due to various infectious processes, co morbid conditions such as coronary heart disease and chronic heart failure (Pecoits-Filho et al., 2002).

Inflammation can be assessed by means of inflammatory biomarkers; such as interleukins and CRP, and associations between levels of these biomarkers and CVD and mortality have been well documented in the renal literature. Interleukin (IL) 6 and CRP also are associated with protein energy malnutrition because inflammation affects nutritional status through inhibition of protein synthesis and induction of catabolism (Honda et al., 2006).
2.23. Anthropometric measurements associated with inflammatory status

Malnutrition being a relatively common problem in chronic dialysis patients affects approximately one-third of both haemodialysis (HD) and peritoneal dialysis patients. Malnutrition may occur secondary to poor nutritional intake, increased losses, or to an increase in protein catabolism (Ohkawa et al., 2000). Anthropometry could provide a reasonably accurate method for assessing body fat and protein stores. The measurements of skin fold thickness (SFT) at the biceps or triceps provided an estimate of body fat, whereas mid-arm circumference (MAC) and midarm muscle circumference (MAMC) could be used to estimate muscle mass (Ohkawa et al., 2000; Steiber et al., 2003; Daugirdas et al., 2007). Inflammation was an important factor associated with Hb variability and that high C-reactive protein (CRP) levels (a widely used surrogate marker of inflammatory activity) are a predictor for less stable Hb control in CKD patients (Dellanna et al., 2006).

A cross-sectional study design was conducted in which the data was collected and analyzed during a five-year period in the Tel Aviv Medical Center Inflammation Survey (TAMCIS). Included in the study were 13,033 apparently healthy individuals at a mean age of 43. Of these, 8,292 were male and 4,741 female. A significant age-adjusted and multiple-adjusted partial correlation was noted between all anthropometric measurements and all inflammatory biomarkers (Wolach et al., 2008).
Anthropometric measurements, waist circumference and BMI are strongly associated with inflammation (Warnberg et al., 2006; Apovian et al., 2008). The high correlations and the different inflammatory variables were significant after controlling many possible parameters. The anthropometric measurements were used to evaluate the metabolic status of patients. The authors explained that visceral fat of the abdomen could be infiltrated by macrophages. This could lead to insulin resistance and endothelial dysfunction (Warnberg et al., 2006; Apovian et al., 2008). Metabolic syndrome is closely related to the inflammatory status of the patient (Frohlich et al., 2000). Anthropometric measurements are used in order to evaluate the metabolic status of patients. These measurements, particularly waist circumference and body mass index (BMI), are strongly associated with inflammation. The high correlations between these variables and the different inflammatory variables were significant in the study done, after controlling for many known and possible confounding parameters (Arbel et al., 2012).

Inflammatory variables are similarly correlated with anthropometric variables and are used often. Afshar et al., 2007 had discussed that patients on HD required regular periodic assessment of nutritional status. This was based on the malnutrition score (MS) which allowed implementation of preventative interventions, such as nutritional counselling or psychosocial interventions, as well as the use of dietary supplements, in order to decrease patients' mortality and morbidity (Afshar et al., 2007). The authors further explained that there had been a significant correlation between the protein catabolic rate (nPCR) and the mortality rate. The protein catabolic rate (nPCR) was used in this study as a predictor of increased mortality. They further suggested that the
MS was a reliable, precise, and rapid method for estimating the nutritional status in patients on HD. The nPCR could be used as a predictor of increased mortality (Afshar et al., 2007).

Nutritional status of patients on HD at the Mostafa Khomeini Hospital, Tehran, Iran displayed that the malnutrition score (MS) is compatible with the anthropometric measurement results (Afshar et al., 2007). Based on the malnutrition score (MS) which consisted of seven components weight change, dietary intake, gastrointestinal (GI) symptoms, functional capacity, co morbidity, subcutaneous fat, and muscle wasting. The investigators conducted a cross-sectional descriptive-analytic study on 54 HD patients (35 males, 19 females) with age range of 18 to 82 years (mean 44.2 ± 19.8 years). Each component of the MS had a score from one (normal) to five (very severe). Anthropometric measurements including triceps skin-fold thickness (TSF), mid-arm circumference (MAC) and mid-arm muscle circumference (MAMC) were taken on all patients. The investigators concluded that the MS is compatible with the anthropometric measurement results and can be used as a reliable, rapid, and precise method for nutritional assessment in office, hospital and HD centres. It was the preferred method in comparison with other time-consuming methods for nutritional assessment (Afshar et al., 2007).
2.23.1 Nutrition and inflammation

In maintaining chronic patients on dialysis reports stated that the analysis of nutrition was one of the main predictors of the increase in deaths and hospitalizations. These conditions did not accommodate patients to be assessed by the global assessment or malnutrition–inflammation score (MIS). The Objective Score of Nutrition (OSND) and the hospitalization days and frequency of hospitalization were significant. The lean body mass and fat mass were also significant with the OSND. Beberashvili et al., 2010 have further explained that it contributed in offering comprehensive characteristics in chronic haemodialysis patients. This further contributed to the hospitalizations, mortality, analysis of nutrition and inflammation (Beberashvili et al., 2010).

Despite the complications of the decrease in inflammation, the albumin levels were significant in analyzing the nutritional status. The authors continued to explain that multiple other medical complications are often a characteristic in chronic haemodialysis patients are frequently present in these patients. The investigators further analysed albumin as a nutrition parameter. This is vital when it is with clearer parameters. They discussed that methods that are calculated percentage of body fat and muscle mass. These results are to be used significantly for analyzing nutrition of chronic renal failure patients on therapy (Stosovic et al., 2011).
End stage renal disease is related with many physiological and metabolic complications. The complications are associated with poor outcomes. These are hypertension, dyslipidemia and the anorexia-cachexia syndrome (Slee, 2012). Certain hormonal variables function as vital components in the ESRD progress and in pathogenesis. Specific hormonal, inflammatory, and nutritional-metabolic factors play key roles in CKD development and pathogenesis. They are the inflammatory and nutritional metabolic factors. The increased levels of proinflammatory cytokines such as interleukin-1 and -6, tumour necrosis factor and altered hepatic acute phase proteins, including reduced albumin, increased C-reactive protein, and perturbations, in normal anabolic hormone responses with reduced growth hormone-insulin-like growth factor-1 axis activity. Proinflammatory cytokines displays a poor outcome in ESRD and is partially a vital component during a presentation. Moreover the recruse in the formation of red blood cells with the decreasing glomerular filtration rate of the patient progresses to anaemia in ESRD (Kazory and Ross, 2009; Slee, 2012).

2.24 ESR

Al-Homrany (2002) analysed the resultant presence of the increased ESR in ESRD patients on therapy. The investigation further analysed other serum factors. The ESR was calculated with the westergren method at Predialysis session. Thereafter ESR was calculated by post dialysis in 200 stable (i.e. with no other known medical complications). A further fifty patients were added to the sample group and these were presenting with other acute medical complications. Erythrocyte sedimentation rate
(ESR) and anaemia did not correlate. There had been no identified reason for this result. There had been no significant correlation of the increased ESR, age or gender of the patients (Al-Homrany, 2002).

2.25 Parathyroid (PTH) effect

Secondary hyperparathyroidism resulted in an increased progression to bone disease. Decreased PTH is usually not the effect of hypo dynamic bone. A recent 5-year cohort study in 748 stable haemodialysis outpatients presented with increase in the survival of MICs. This is the result of malnutrition-inflammation cachexia-syndrome (MICS). This resulted in the absence of MIC and the average decreased PTH is 100 to 150 p/ml range. The Japanese ESRD patients presented higher rates of survival with decreased levels of PTH. The relation however can have a complicating effect between serum PTH and alkaline phosphatase (Feroze, 2011).

2.25.1 Secondary hyperparathyroidism

The parathyroid hormone (PTH) is considered by Eutox Work Group, (2012) as a middle molecule uraemic toxin with biological effects. Secondary hyperparathyroidism is a condition resulting from the deregulation of calcium and phosphorus homeostasis in the kidney. It seems that PTH could be a marker of hypo responsiveness to ESAs in dialysis patients (Al-hilali et al., 2007; Kalantar-zadeh et al., 2009). Several mechanisms
have been proposed, such as interference with RBC production, as PTH causes bone marrow fibrosis. Parathyroid hormone (PTH) has an inhibitory effect on BFU-E and interferes with EPO endogenous production (Al-hilali et al., 2007), interference with RBC survival as PTH increases osmotic fragility of erythrocytes (Drueke and Eckardt, 2002; Brancaccio et al., 2011).

### 2.25.2 Vascular Calcification

Vascular calcification in patients with CKD is a significant problem and is associated with cardiovascular mortality. Investigation of factors related to vascular calcification is important. The risk factors of abnormal mineral metabolism and diabetes, resistance to ESAs may be associated with vascular calcification in patients with CKD (Won et al., 2014). Eighty-nine patients who received vascular access surgery between September 2010 and November 2012 in Uijeongbu St. Mary’s Hospital were evaluated, and thus they had just commenced HD or prepared to start HD. Finally, 82 patients were included in this study. The study was approved by the ethics committee of the Institutional Review Board of The Catholic University of Korea, Uijeongbu St. Mary’s Hospital (Won et al., 2014).

The ESAs darbepoietin or epoietin was administered subcutaneously at the end of dialysis. The target haemoglobin level was 1g/dL. The initial epoietin dose was 60 – 120 IU/kg per week in two to three doses per week, and the darbepoietin dose was
0.45 μg/kg per week which was given once a week. A ratio 200 was used to convert darbepoietin to the equivalent epoietin dose. Erythropoietin stimulating agents (ESA) doses were recorded over 3 months from 1 month before vascular access surgery, and the mean values were used in this study. The ESA hypo responsiveness index (EHRI), defined as the weekly ESA dose per kilogram of body weight divided by the haemoglobin level (g/dL), was calculated. Thus, higher EHRI values meant a reduced response to ESAs. Patients with iron deficiency received parenteral iron at a dose of 100 mg/week until the target ferritin and T Sats levels were achieved, and then they took 512 mg of oral ferrous sulfate (160 mg of elemental iron) per day. If the serum ferritin level was >800 ng/mL and/or T Sats was >50%, iron therapy was stopped (Won et al., 2014).

Won et al., (2014) stated that their investigation had been derived from an improved understanding of the mechanisms common to ESA hypo responsiveness and vascular calcification. The uraemic state is characterized by increased oxidative stress and it is also related to the inflammatory conditions. Inflammation plays a key role in the ESA hypo responsiveness of HD patients who have sufficient iron (Kwack et al., 2006; Won et al., 2012). Inflammatory iron contributes to ESA hypo responsiveness via proinflammatory cytokines such as IL-6, which antagonize the action of endogenous and exogenous erythropoietin by directly inhibiting erythroid progenitor cells and by the disruption of iron metabolism (Van der Putten et al., 2008).
The mechanism of vascular calcification has been studied for decades, and growing evidence now suggests that vascular calcification is not the simple precipitation of calcium and phosphate but is considered to be a highly regulated pathological process that resembles osteogenesis (Neven et al., 2011).

### 2.26 Albumin effects in relation to malnutrition and inflammation

Patients with arterial microcalcification (AMiC) had lower levels of serum albumin and total cholesterol compared with patients without AMiC. Serum albumin has been the most common nutritional marker in CRF patients (Won et al., 2014). The results from their investigation suggested that there was an association between malnutrition and AMiC. This relationship can be explained on the basis of inflammation. Several reports have suggested the existence of a syndrome consisting of malnutrition, inflammation, and atherosclerosis (MIA syndrome) in some patients with CRF (Stenvinkel et al., 2000; Zeki et al., 2006). Inflammation plays a significant role in causing hypoalbuminaemia in CRF patients. Proinflammatory cytokines cause malnutrition by stimulating protein catabolism and by reducing albumin synthesis (Stenvinkel et al., 2000; Schneider et al., 2009).

In a cohort of patients ESA resistance was not only associated with cardiovascular mortality but also with non-cardiovascular mortality. A remarkable finding since the hypothesized mechanism through which high ESA dose would increase mortality,
namely elevated arterial blood pressure, altered endothelial function and prothrombotic effects (Singh, 2010), would mainly result in cardiovascular causes of mortality. Erythropoietin stimulating agents (ESA) resistance comprises more than just high ESA dose and is linked to malnutrition, inflammation, low iron stores, hyperparathyroidism, and other co morbid conditions with increased mortality risk (Kalantar-Zadeh et al., 2009). It may, therefore, be regarded as an indicator for disease severity, but even after adjustment for a wide range of these confounders ESA resistance is still associated with mortality. Thus the inability to achieve a proper haematopoietic response seems to be the best marker of worse prognosis. Secondary analyses of anaemia correction trials also confirm this association between ESA resistance and mortality (Szczech et al., 2008; Solomon et al., 2010).

2.27 Inadequate dialysis- KT/V

Intensity or adequacy of dialysis (measured by Kt/V) is a factor that can modulate the response to ESA therapy. Inadequate dialysis is associated with the need for higher ESA doses. Some studies showed that convective treatments present benefits in ESA response, as compared with other treatments (Bowry and Gatti, 2011). High flux HD (HF-HD) and online hemodialfiltration (OL-HDF)improve the response to ESAs, as compared to low flux HD (LF-HD), probably due to a better removal of middle and large molecules that impair erythropoiesis (Johnson et al., 2007; Stefansson et al., 2012).
2.28 Significance of age of patients

The epidemiology of the HD population has changed worldwide. The elderly HD population is growing (Collins et al., 2012). Older patients experience a higher incidence of cardiovascular complications and reduced level of activity in daily life (Rostand et al., 1982; Foley et al., 1998; Nakai et al., 2012). Increased Hb does not necessarily improve prognosis in patients with cardiovascular disease (Maekawa et al., 2008). Many elderly patients have low Hb levels despite the high doses of erythropoiesis-stimulating agent (ESA) received. The elderly population might tolerate low haemoglobin levels (Nakai et al., 2012).

Three thousand three hundred and forty one patients were selected from Japan’s Dialysis Outcomes and Practice Patterns study and analysed. The primary outcome occurred in 567 patients during the median follow-up of 2.64 years. Haemoglobin levels for the entire population were 10.3 ± 1.3 g/dL. The median of epoietin dose was 3000 IU/week. Interaction was found between ages stratified by the age of 75 years and haemoglobin values (P = 0.045) with use of Cox’s proportional hazard model. The nonelderly population had poorer prognosis with haemoglobin <10 g/dL, while elderly population only with haemoglobin <9 g/dL (Hanafusa et al., 2014). The haemoglobin target for optimal anaemia control in the elderly population differs from that in the nonelderly HD population (Hanafusa et al., 2014).
Oxygen consumption in the elderly population is reported to be lower than that in the younger people (Hsieh et al., 2010), as well as the exercise capacity measured by peak exercise oxygen uptake (Sietsema et al., 2002). The sedentary characteristics of this population might be permissive of the reduced oxygen delivery due to low Hb levels (Avesani et al., 2012). Decreased physical activity itself can be related to poorer prognosis (Tentori et al., 2010; Matsuzawa et al., 2012).

2.29 Decreased erythropoiesis in the advancement of chronic kidney disease (CKD)

Anaemia was estimated to be present in 15.4% of people with any stage of CKD in a study done in the United States. Data from the National Health and Nutrition Examination Survey (NHANES) in 2007–2008 and 2009–2010 were used to determine the prevalence of anaemia in subjects with CKD. The prevalence of anaemia increased with stage of CKD, from 8.4% at stage 1 to 53.4% at stage 5 (Stauffer, 2014). Erythropoietin production decreases as kidney function worsens (Clase et al., 2007; Robinson et al., 2007; Moossavi and Freedman, 2009, Lasch et al., 2009) consistent with the known pathogenesis of CKD. Fishbane et al., (2009), found high rates of iron deficiency in adult men (57.8 to 58.8%) and women (69.9 to 72.8%) with CKD stages 3–5 in the NHANES III and 1999–2004 surveys, indicating that anaemia in higher stage CKD may have multiple causes.
The prevalence of anaemia in CKD in other studies focused on older adults (>64 years of age). A 29.9% of KEEP 2000–2008 participants and 19.9% of NHANES 1999–2006 patients in CKD stages 3–5 and over age 64 had co morbid anaemia, this therefore defined haemoglobin levels <13.5 g/dL for men and <12 g/dL for women (Stevens et al., 2010). The prevalence of anaemia in stage 3–5 CKD patients aged >64 in the 2007–2010 NHANES surveys was 24.4%. A higher prevalence of anaemia (64.9%; defined as haemoglobin<13 g/dL for men, <12 g/dL for women) was observed in US nursing home residents aged >64 with CKD stages 3–5 (Robinson et al., 2007).

2.30 Blood pressures and erythropoietin

Erythropoietin (EPO) functional activity in vitro, pulmonary artery endothelial cell networking and smooth muscle cell proliferation presented upregulation of EPO in the circulation and in the lungs of patients with pulmonary arterial hypertension (PAH). Upregulation of EPO in plasma, in addition to in vitro lung effects, suggests that EPO may be an important factor in the development or progression of PAH (Karamanian et al., 2014; Kim et al., 2014).

2.31 Effect of the production of neutralizing anti erythropoietin antibodies

2.31.1 Erythropoiesis in serum-end stage renal disease
The impact of erythropoiesis in the serum of ESRD patients had been investigated by Pollock et al., 2008). The authors explained that PRCA generally occurs after the production of neutralizing anti-erythropoietin antibodies. The development of pure red cell aplasia cases required the simple measurements of blood cell counts. Erythropoiesis-stimulating agents hypo responsiveness was attributable to other causes. The investigators further explained that these criteria indicated that the patient’s response to erythropoietin-stimulating agent therapy was very poor. They suggested bone marrow examination and measurement of anti-erythropoietin antibodies. If pure red cell aplasia was confirmed, then cessation of erythropoiesis-stimulating agent therapy and initiation of immunosuppressive therapy were recommended (Pollock et al., 2008).

2.3.1.2 The absence of erythroblasts from an otherwise normal bone marrow

In classic cases, the bone marrow aspirate and/or trephine biopsy showed a virtual absence of red cell precursors (in many cases 5% erythroblasts), whereas the cellularity of the bone marrow was normal, with normal myeloid cells and megakaryocytes. The investigators stated that because iron use is largely abolished in PRCA as a result of the absence of marrow erythropoietic activity, serum ferritin increases to very high levels, as does the transferrin saturation.
Pure red cell aplasia (PRCA) is the absence of erythroblasts from an otherwise normal bone marrow (Figure 13).

**Figure 13:** Haematologic stains of bone marrow (BM) from a normal patient (left) and a patient with antibody-mediated pure red cell aplasia (PRCA; right)  
(Pollock et al., 2008)

Thus, serum ferritin levels of greater than 1000 g/L and transferrin saturation levels of greater than 70% are characteristic of this condition (Pollock et al., 2008).
2.31.3 Anti-Erythropoietin Antibodies

A recent study investigated that antibodies that are induced by therapeutic proteins, such as the ESA, may affect the safety, efficacy, and pharmacokinetic and pharmacodynamic characteristics of the proteins. It is, therefore, essential that procedures be identified or developed for detecting such antibodies and characterizing their important properties. The investigators explained that some type of immunoassay is first used to screen samples (serum or plasma) for the presence of antibodies, and then positive screening results are confirmed. Preferably by using an assay based on an alternative technology. Confirmed positive samples were assessed for neutralizing characteristics using a bioassay. A variety of assays have been used to detect ant-erythropoietin antibodies, including radioimmunoprecipitation (RIP) assays, ELISA, and surface plasmon resonance procedures (Swanson et al., 2004; Hoesel et al., 2004; Thorpe and Swanson, 2005).

Antibody-mediated pure red cell aplasia (PRCA) in patients in response to ESA treatment are unlikely to respond to treatment with other erythropoietic agents, because there is substantial cross reactivity among erythropoietic agents, including endogenous erythropoietin (Weber et al., 2002). Asari and Gokal (2004) explained that an exception to this rule was seen in a patient who had Eprex-associated PRCA and responded to darbepoietin, without any complications, in the presence of persisting epoietin antibodies. A clinical study analysed serum samples for the presence of anti-ESA
antibodies, using a validated surface plasmon resonance (SPR)-based immunoassay or SPRIA.

The pre-existing non-neutralizing anti-ESA antibodies were found in 6% of the patients from clinical studies in nephrology, oncology and congestive heart failure (CHF). After ESA treatment, 2.3% of the subjects developed binding, non-neutralizing antibodies with 0.1% confirmed as having an IgG isotype and were specific to the ESA protein. Antibodies, IgM were detected at baseline and post-ESA treatment and reported to be specific to the glycosylation of the ESA. No clinical study participants progressed to PRCA. In contrast, anti-ESA antibody-positive patients from the post-market setting with a confirmed IgG subclass were specific to the ESA protein. Patients that had progressed to PRCA were noted to have high antibody concentrations with neutralizing activity and a diverse IgG subtype (Pollock et al., 2008).

A low prevalence of non-neutralizing anti-ESA IgM specific to glycosylation on the ESA and IgG1 antibodies specific to the ESA protein was detected across all clinical patient populations. Patients with PRCA were noted to have high IgG antibody concentrations, neutralizing antibodies and the presence of anti-ESA IgG4 antibodies (Barger et al., 2012).

The detection and evaluation of the presence of serum anti-EPO antibodies in patients with ESRD on regular haemodialysis (Al-Shifaa Hospital in Gaza), receiving
recombinant EPO therapy were detected by the ELISA technique. The results revealed that 18 patients (22.5%) had the anti-EPO antibodies in their blood, while 62 patients (77.5%) did not. Significantly high occurrences, 40%, 67% and 69% respectively of anti-EPO antibodies among patients receiving recombinant EPO for various renal complications were revealed in studies done (Castelli et al., 2000; Puri, 2004; El-Din et al., 2010). The low percentage of anti-EPO antibodies in positive patients compared to others may be explained by the relatively lower doses of EPO (2000 IU/week) used to treat patients (Alqahwaji et al., 2014). Commercial preparations of EPO could affect the immune system differently as different populations and ethnicities have different immune responses (Watson et al., 2013).

2.32 The Bioassay

The bioassay is a screening method to demonstrate the antibody activity. Bioassays are one of the exclusive assays that can quantitatively measure neutralizing activity against therapeutic proteins (Thorpe and Swanson, 2005). Neutralizing antibodies can bind to the portions of drug molecules involved in receptor binding or cell activation, thereby blocking the therapeutic effect of the drug (Zong et al., 2001; Zoller-Pazner et al., 2004). Bioassay demonstrating the effect of antibodies on recombinant erythropoietin in HD patients has not been undertaken. The present study was the first study undertaken on renal patients in South Africa.
The bioassay has been the most versatile Ab assay in terms of speed, specificity, sensitivity, and the ability to characterize binding of antibodies (Ab isotopes and relative binding affinities), and the ability to regenerate the sensor. Therefore, the bioassay is recommended to be used for further investigation for the measurement of neutralizing capacity of antibodies. They require days of cultivation for the cells to proliferate and microscopic quantification of the erythroid colonies growing in culture. With the use of the bioassay one can determine whether the antibodies are blocking the process of erythropoiesis or not (Thorpe and Swanson, 2005).

This study is of great importance as it investigates the significance of the antibodies present as well as limiting factors and enhancing factors that affect erythropoiesis in chronic renal failure patients. The Erythropoiesis bioassay will test the effect of patient’s plasma on Erythropoiesis and assist in the understanding the role of plasma factors on Erythropoiesis, and this will contribute to open other avenues of research in this field.

Bioassays are usually relatively time-consuming (because of long incubation times) and are prone to non-specific matrix effects and interference by inhibitory molecules. Their sensitivity is moderate, and they can be relatively difficult to validate. Erythropoietin bioassays that are used to measure neutralizing antibodies can be based on primary cultures of bone marrow–derived erythroid cells or on continuously growing, factor-dependent cell lines that proliferate in response to erythropoietin. Neutralizing antibodies present in serum from patients with ESA-induced PRCA block this erythropoietin-dependent cellular proliferation. Cell lines offer some advantages over
primary bone marrow cells for erythropoietin bioassay. These include greater reproducibility and consistency of cell growth characteristics and erythropoietin responsiveness. Bone marrow samples may be available only in limited quantities, and the erythroid cultures need 7 days of incubation. Cell lines that have been used to demonstrate epoietin-neutralizing activity are TF-1 and UT-7, derived from patients with erythroleukaemia, and 32D-EPO, a murine haematopoietic cell line transfected with the human erythropoietin receptor (Hammerling et al., 1996; Wei et al., 2004).

Different erythropoietin-sensitive cell lines have different inherent sensitivities to the neutralizing effects of anti-erythropoietin antibodies in the bioassay; however, cell lines are sensitive to growth factors other than erythropoietin, and tight controls must be included in the bioassays. It is important to elaborate a clear, prospective strategy for detecting and measuring erythropoietin antibodies and correlating results with clinical data relevant for diagnosis of PRCA. Purpose-specific validation, qualification, and standardization of assays for measuring and characterizing anti-erythropoietin antibodies are essential if meaningful results are to be produced. In general, immunoassays for the detection of antibodies have target sensitivity at least 500 ng/ml (so that if a patient has at least 500 ng/ml of anti-drug antibody in his or her circulation, then the assay would detect the patient as “positive”). Bioassays, because of their cell-based nature and higher inherent variability, have a target sensitivity of 1 g/ml for neutralizing antibodies. It is important to note that assay sensitivity is based on the performance of a high-affinity positive control antibody and as such may not mimic the type of immune response generated in any given patient.
For this reason, bioassays are useful for measuring the immunogenic potential of proteins expressed in different cells (Wadhwa et al., 2003). The bioassays have been used to quantify EPO-neutralizing Abs, including EPO-stimulated growth of erythroid precursors in plasma-clot cultures of mononuclear blood cells or bone marrow cells and proliferation of the EPO-dependent human erythroleukemia cell line, UT-7 (Casadevall et al., 1996).

The usefulness of immortalized cell lines lies in the ability to culture these cells over extended periods of time before cell death occurs. Proliferation of such cells can be quantified by using tritiated thymidine to assess nucleic acid synthesis and tetrazolium dyes to assess metabolic activity (Hammerling et al., 1996; Wu et al., 2003). In addition, genetic variants of cell lines can be produced and cultured for the purposes of biochemical or immunochemical studies. For example, genetic variant of the UT-7 cell line transfected with the wild-type c-mpl thrombopoietin receptor gene or gene mutants was used to map the functional domains on the thrombopoietin receptor protein responsible for cellular proliferation and megakaryocytic differentiation (Takatoku et al., 1997). The UT-7 cell line and a murine erythroleukemia cell line, HCD57, were used to map the receptor binding domains of EPO (Boissel et al., 1993, Woo et al., 2014).

Casadevall and colleagues (2002), tested serum obtained from patients with confirmed Ab-mediated PRCA for the presence of EPO-neutralizing Abs (Casadevall et al., 2002). Pure red-cell aplasia and anti-erythropoietin antibodies in patients treated with recombinant erythropoietin were investigated. Using bone marrow cells from healthy
donors, they showed that all PRCA serum samples effectively inhibited proliferation of bone marrow cultures \textit{in vitro}, thus demonstrating 100% concordance in this bioassay. In this study, the EPO-dependent UT-7 cell line was used to further characterize neutralizing Abs obtained from one of the patients. Similar bioassays with autologous bone marrow cells and the UT-7 cell line were used earlier by Casadevall and colleagues to demonstrate the presence of EPO-neutralizing Abs in a patient with confirmed Ab-mediated PRCA, and the RIP assay was used to confirm the bioassay results (Casadevall et al., 2002). Krantz and colleagues (1967), characterized a serum factor that inhibited erythropoiesis in patients with EPO-resistant PRCA by using an \textit{in vitro} culture system of autologous bone marrow cells (Krantz and Kao., 1967). Studies on red cell aplasia and the demonstration of a plasma inhibitor to heme synthesis and an antibody to erythroblast nuclei have been investigated (Krantz and Moore., 1973).

\textbf{2.32.1 \textit{In vitro} bioassays}

Polyamines were investigated as an Inhibitor on erythropoiesis of haemodialysis patients by \textit{in vitro} bioassay analysis of EPO activity using faetal mouse liver cells. The bioassay could detect EPO activity levels as low as 0.4 mU/mL; i.e., almost lower than the normal threshold. Their subjects included the patients of the HD unit. These consisted of 20 dialysis patients, 14 men and 6 women, who underwent haemodialysis for 35–151 months (mean: 81.9 ± 34.9 months). The patients did not receive any blood transfusion for a period of 6 months prior to the study, and all had normal levels of iron and ferritin. Their age ranged from 11 to 69 years (mean: 46.2 ± 14.2 years). Patients
with a primary renal disease other than chronic glomerulonephritis (CGN) were excluded from this study. With regards to anaemia, the RBC level was from 265 × 10⁴ to 326 × 10⁴ (mean: 298 ± 19 × 10⁴), and the dose of RHuEPO was 3000–6000 units. The control patients in this study were 20 nonanaemic volunteers, 10 men and 10 women, whose ages ranged from 20 to 58 years (mean: 32.8 ±10.3 years) (Katsunori et al., 2006).

In a few haemodialysis patients treated with RhuEPO, the initial response to RHuEPO was excellent, but gradually decreased. This explains the potential presence of inhibitory substances in the uraemic sera influence on erythropoiesis (Katsunori et al., 2006). Serum EPO levels had been measured with various methods (Marsden et al., 1999; Swanson et al., 2004). The investigation carried out by Katsunori and team showed that the EPO levels assessed by RIA did not correspond to the physiological activity of EPO, which might be due to growth of colonies in the CFU-E milieu that was observed in the *in vitro* bioassay. The authors explained that this could be due to some substances in the uraemic sera of haemodialysis patients inhibiting EPO activity and consequently impairing erythropoiesis (Katsunori et al., 2006; Grossberg et al., 2009).

Existence of many uraemic toxins have been shown, and their role in the development of anaemia in the haemodialysis patients. Polyamines mainly consist of putrescine, spermidine, spermine and their molecular weights range from 88 to 202 Daltons (Allen, 1999; Espinoza and Aguilera, 1999). It is well-known that they are uraemic toxins, and their level increases with the onset of chronic renal failure and decreases immediately
by haemodialysis. However, in spite of their small molecular weight, they cannot be removed sufficiently by haemodialysis, so their levels in the uraemic sera and erythrocytes are higher than those of healthy subjects (Fiocchi, 1987).

Some substances in the serum of the haemodialysis patients could influence erythropoiesis, and they would confirm that polyamines were the inhibitors, which play a role in the development and progression of anaemia in some patients. Their results suggested that the polyamines have an inhibitory effect on the proliferation or maturation of erythroid precursor cells and are intimately involved in the pathogenesis of renal anaemia in the chronic haemodialysis patients (Katsunori et al., 2006).

![An in vitro bioassay](image)

**Figure 14:** An *in vitro* bioassay
Bioassays are functional assays (Figure 14) that distinguish antibodies with neutralizing potential from those that simply bind to a protein such as erythropoietin but do not directly affect its biologic activity. Bioassay data may correlate with clinical response, whereas *in vitro* binding assay results may not correlate (Pollock et al., 2008).

This distinction was important as a general concept but is particularly significant for erythropoietin and PRCA because patients who have developed ESA-induced, antibody-dependent PRCA have been found in the majority of the cases to produce erythropoietin-neutralizing antibodies. Bioassays are usually relatively time-consuming (because of long incubation times) and are prone to nonspecific matrix effects and interference by inhibitory molecules. Their sensitivity is moderate, and they can be relatively difficult to validate (Pollock et al., 2008).

Erythropoietin bioassays that are used to measure neutralizing antibodies can be based on primary cultures of bone marrow–derived erythroid cells or on continuously growing, factor-dependent cell lines that proliferate in response to erythropoietin. Neutralizing antibodies present in serum from patients with ESA-induced PRCA block this erythropoietin-dependent cellular proliferation (Grossberg et al., 2009). Cell lines offer some advantages over primary bone marrow cells for erythropoietin bioassay. These include greater reproducibility and consistency of cell growth characteristics and erythropoietin responsiveness. Bone marrow samples may be available only in limited quantities, and the erythroid cultures need 7 days of incubation (Pollock et al., 2008).
2.32.3 The Elisa and its sensitivity to detect antibodies to RhuEPO

Sensitive and efficient methods for detecting anti-erythropoietin (anti-EPO) antibodies were needed for analysis for large scale screening of human serum samples (Gross et al., 2006). The authors explained that the ELISA was an alternative to labor-intensive radioimmuno precipitation assays. Apparently conflicting reports have questioned its sensitivity. The investigators tried to challenge this issue by directly comparing different reported ELISA approaches to determine whether RHuEPO-coating methods affect detection of anti-EPO antibodies. Investigators reporting low sensitivity had used ELISAs in which RhuEPO was directly coated to micro titer plates while the high sensitivity ELISA used plate-bound streptavidin to bind biotinylated RHuEPO. Using anti-EPO positive human sera, results confirmed a large (100- to 300-fold) difference in sensitivity between the ELISAs and suggested that the inferiority of the low sensitivity (Gross et al., 2006). ELISA was caused by the direct coating of RHuEPO which may disrupt epitopes by masking recognition sites or introducing conformational changes. Therefore, the bridging ELISA could be an appropriate and effective system for antibody analysis and screening of human sera with high sensitivity and specificity. This could be performed with streptavidin binding of biotinylated antigen. Their finding may also be more generally applicable to the detection of antibodies against other protein antigens (Gross et al., 2006).
2.32.4 Cell-based bioassay for the detection of neutralizing antibodies

*In vitro* cell-based bioassay capable of detecting neutralizing antibodies (NAb) to recombinant human erythropoietin (RHuEPO) in clinical samples was developed and validated. The bioassay used the IL-3-dependent murine 32D cell line transfected with human EPO receptors (EPOR) (Wei et al., 2004). This cell line responded to RHuEPO with proliferation measurable by [methyl-³H] thymidine incorporation into the cellular DNA. The reduction of RHuEPO-induced cell proliferation response indicates the possible presence of anti-RHuEPO NAb. In addition, a specificity assay using murine IL-3 (mIL-3) induced proliferation of the same cell line was developed and validated. The specificity assay allowed the testing of samples that inhibited the biologic activity of RHuEPO to evaluate whether the inhibition was specific and not attributable to cytotoxicity of the serum sample. Both assays were conducted in a 5% human serum matrix in 96-well micro titer plates (Wei et al., 2004).

The Guidelines of the International Conference on Harmonization (ICH) were followed for the validation of different assay parameters including analytical recovery, precision, sensitivity, specificity, selectivity, and robustness. The anti-RHuEPO NAb assay is capable of detecting concentrations of NAb equivalent to 500 ng/ml of the positive control antibody in undiluted human serum. The anti-RHuEPO NAb assay yielded consistent results with cells cultured for up to 30 days. The positive control antibody maintained its ability to inhibit the biologic activity of RHuEPO upon freezing and thawing. The presence of free RHuEPO in serum samples interfered with the detection
of the antibody. The validated assay was sensitive, specific and robust and was successfully used to monitor NAb development in patients (Wei et al., 2004).

The expiry of the originator patients for RHuEPO will inevitably result in a surge in the marketing of non-innovator products, some of which may not have undergone the rigorous comparability testing of an approved biosimilar, and therefore may exhibit altered immunogenicity (Ferguson et al., 2013). There is a need for widely available bioassays that allow for rapid and routine monitoring of patients receiving RHuEPO for the development of NAb. Recommendations have been made for the development of validated cell-based bioassays for NAb (Gupta et al., 2011). These studies addressed a number of the considerations for assay design, by including selection of the cell line and assay endpoint. The initial assessment of matrix interference and demonstration of inhibition of the endpoint by a positive control serum and by a patient sample, thereby suggesting that the measurement of inducible endogenous gene expression by qRT-PCR in a responsive cell line provided a suitable assay for quantifying RHuEPO bioactivity and for detecting anti-RHuEPO NAb in patient sera (Gupta et al., 2011).

2.32.5 The Contributions of Bioassays to other Studies

The importance of establishing a common method of reporting neutralizing antibody levels was emphasized by the fact that patients injected repeatedly with a human interferon (HuIFN) may develop such antibodies that can abrogate the beneficial effects
of the treatment. Recommendations have been discussed by the World Health Organization (WHO) concerning the methodology of neutralization tests and how the resultant data was to be reported. A WHO international collaborative study on two human sera with antibodies against HuIFN-α and HuIFN-β provided the opportunity not only to test the theoretical concepts concerning the neutralization reaction with data obtained in different bioassay systems in different laboratories but also to obtain enough data points for statistical evaluation with bioassays having a great range of sensitivity to IFN. The analyses substantiated and extended the original conclusions of other studies done that the neutralization followed the reaction mode of low-affinity antibody. The constant proportion of the hypothesis by which the antibody reduces IFN activity in a set ratio of added/residual biologically active IFN, is a consequence of the low molar concentration of free IFN at the neutralization end point. This helped make the results from different laboratories employing different bioassay systems more readily comparable and interpretable, provided that the bioassays are sufficiently sensitive to IFN (Grossberg et al., 2001). A recent research proved that anti-ESA antibody concentrations above 1000 ng/mL are likely to show neutralizing antibodies (Gross et al., 2008).

2.32.6 Detection of antibodies

Detection of neutralizing antibodies (NAb) in patient serum relies on measuring the inhibition of a RHuEPO-stimulated response in a cell-based bioassay and the sensitivity of such an assay will be improved if a low stimulatory concentration of RHuEPO can be
used. This allows the detection of lower titres of NAb in patient serum and enables higher dilutions of patient serum to be assessed. Despite the detection of reproducible, dose-responsive expression of both EGR1 and PIM1 in UT-7 cells, the UT-7/EPO cell line was selected for the detection of NAb in patient serum as this allowed a lower stimulatory concentration of RHuEPO to be used. A RHuEPO concentration of 0.02 U/ml routinely stimulated an increase in EGR1 expression of >30 fold over unstimulated UT-7 EPO cells (Kelley et al., 2005).

The presence of Nab was detected in a pool of human sera known to contain Nab, by inhibition of cell proliferation in UT-7/EPO cells stimulated with 0.01 U/ml RHuEPO. The bioassay was based on the stimulation of EGR1 gene expression, 0.02 U/ml RHuEPO was fully neutralized by pre-incubation with a 1/200 dilution of serum (Kelley et al., 2005).

Patients who had received epoetin alfa or darbepoetin alfa treatment were divided into two groups on the basis of antibody type, anti-epoetin alfa or anti-darbepoetin alfa. The patients were then further subdivided on the basis of the presence of neutralizing antibodies in a bioassay. Binding antibodies were detected in 43 of 243 patients. They were then analyzed for anti-epoietin alfa antibodies; 14 of the 43 patients with binding antibodies were found to have neutralizing antibody activity. Binding anti-ESA antibodies were detected in 40 of 400 patients when analyzed for anti-darbepoietin alfa antibodies; 13 of the 40 patients with binding antibodies were found to have neutralizing antibodies. Compiled results from all subjects at all time-points were observed to have
anti-ESA antibodies which indicated that patient’s positive for anti-ESA IgG neutralizing antibodies are likely to have higher antibody concentrations (Figure 16) and anti-EPO IgG4 subtype. In addition, samples from three patients with PRCA demonstrated specificity to the ESA protein (Figure 15 B). Post-market study samples with detected anti-ESA antibodies were analyzed for antibody isotype, neutralizing antibodies and relative antibody concentration (Barger et al., 2012).

Positive control antibody with neutralizing activity and IgG anti-ESA antibodies bound to all immobilized ESA demonstrated signal inhibition in the presence of excess E.coli EPO. Representative IgM anti-ESA antibodies bound only to glycosylated ESAs did not demonstrate signal inhibition in the presence of E.coli EPO. Positive control antibody binding to aglycosylated darbepoetin alfa confirmed that the aglycosylation did not alter the protein folding or binding epitopes (Figure 15 A) (Barger et al., 2012).
Serum samples confirmed to have anti-ESA IgG antibodies, selected from clinical studies \((n = 5)\) and from post-market setting \((n = 3)\), were re-analyzed after pre-incubating the serum samples with excess soluble \(E.\text{coli}\) EPO. In all eight samples, antibody binding was inhibited by the soluble \(E.\text{coli}\) EPO (Figure 15 B).

**Figure 15:** Detection of antibodies in serum samples (Barger et al., 2012)

Results shown for all post-market anti-ESA antibodies for non-neutralizing antibody (non-NAb) and neutralizing antibody (NAb) groups with median values are illustrated in Figure 16. The median anti-darbepoietin alfa (darbepo) antibody concentrations were higher for NAb \((n = 21)\) than for non-Nab \((n = 51)\); P-value <0.0001. Similarly, the median
anti-epoietin alfa (EPO) antibody concentrations were higher for Nab \((n=29)\) than for non-Nab \((n=59)\); \(P\)-value <0.0001. Specimens with indeterminate concentrations were not included in the calculated median. Antibody concentrations were relative to a rabbit polyclonal anti-epoietin alfa antibody (Barger et al., 2012).

**Figure 16:** Post market anti-ESA antibodies for non-neutralizing antibody (non-Nab) and neutralizing antibody (Nab) (Barger et al., 2012).

Three randomized studies estimated the rate of decline in kidney function using surrogate measures. Serum creatinine doubled in 26/31 (84%) anaemic pre-dialysis patients not treated with erythropoietin versus 21/35 (60%) of non-anaemic pre-dialysis patients not treated with erythropoietin versus 22/42 (52%) anaemic pre-dialysis patients treated with erythropoietin for 36 weeks and followed for a median duration of
28 months (Kuriyama et al., 1997). The differences between groups 2 and 3 were not statistically significant. Limited data suggested that the presence of diabetes might reduce the effect of erythropoietin on progression. A study by Teplan et al., (2003) (n = 186) using inulin clearance changes suggested that supplementary dietary ketoacids and erythropoietin might independently contribute to decreased progression in patients on a low protein diet. The composite endpoint of serum creatinine doubling, initiation of dialysis, or death was met in 23/43 (54%) of those in whom erythropoietin treatment was delayed until haemoglobin levels decreased to less than 9 g/dl as compared 13/45 (29%) of those in whom treatment was initiated for milder anaemia (haemoglobin concentration of 9 to 11.6 g/dl) (Teplan et al., 2003; Gouva et al., 2004).

2.32.7 UT 7 Cell Lines

The majority of cell-based bioassays measure the antibody-mediated inhibition of a proliferative response to RHuEPO by determining $^3$H-thymidine incorporation into newly synthesized DNA (Wei et al., 2004; Kelley et al., 2005; Thorpe and Swanson, 2005). The requirement for 2–4 days of incubation and the use of radioactive reagents renders this assay both time consuming and expensive and it is also accompanied by the hazards associated with the use of radioactive reagents. Furthermore, non-specific factors in patient serum may inhibit proliferation.
The measurement of endogenous gene expression in a responsive cell line offers an alternative measure of RHuEPO biopotency. A microarray analysis of the human erythroleukaemic cell line, UT-7 (Komatsu et al., 1991), identified that the expression of the PIM1 gene, which encodes a serine/threonine protein kinase, increased in response to RHuEPO (Yu et al., 2006).

RHuEPO-stimulated PIM1 expression, measured by branched DNA technology, was inhibited by human sera spiked with a rabbit anti-RHuEPO polyclonal antibody. Bioassays based on the measurement of endogenous gene expression, quantified by qRT-PCR or branched DNA technology, was used to assess the bioactivity of therapeutic proteins such as vascular endothelial growth factor (Burns et al., 2008) and recombinant interferon (IFN) α-2a and IFN α-2b (Silva et al., 2008; Moore et al., 2009). Endogenous gene expression, quantified by qRT-PCR, has also been used successfully to detect NAb in patients who have received recombinant IFN products for the treatment of relapsing remitting multiple sclerosis (Bertolotto et al., 2007). Bioassays can be used to detect anti-RHuEPO NAb using the human erythroleukaemic cell line, UT-7 and a RHuEPO-sensitized sub-line, UT-7/EPO (Komatsu et al., 1993).

UT-7 is a human leukemic cell line capable of growing in interleukin-3 (IL-3), Granulocyte/ macrophage colony-stimulating factor (GM-CSF), or erythropoietin (EPO) (Komatsu et al., 1991). Erythropoietin (EPO) also stimulates in vitro megakaryopoiesis; EPO initially binds to a specific cell-surface erythropoietin receptor EPOR in the process of its signal transduction. However, intracellular events provoked by signals from the
EPO/EPOR system have not yet been elucidated. Previously established human leukemic cell line, UT-7, was based on its ability to be maintained in granulocyte/macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) (Komatsu et al., 1991). Using a proliferation assay, the investigators found that UT-7 cells could also respond to EPO. The UT-7 cell has a large number (7,200 to 13,000/cell) of EPO binding sites (Miura et al., 1990; Komatsu et al., 1993) and is one of the best available human cell lines to study the expression and function of EPO-R. This cell line could differentiate to megakaryocytes in response to phorbolester acetate (PMA) (Komatsu et al., 1991).

A cell line in which growth is totally dependent on the presence of EPO would provide great help in further analysis of the molecular mechanisms by which EPO promotes the growth and differentiation of haematopoietic cells. The abundant expression of the EPO binding sites expressed on UT-7 cells encouraged a long-term culture of the cells in the presence of EPO. A subline, UT-7/EPO, was finally established that could proliferate continuously in medium containing only EPO as the growth factor. Neither GM-CSF nor IL-3 could support the growth of these cells. The expression of erythroid lineage markers in UT-7/EPO cells was then analyzed to determine whether the culture conditions promoted erythroid development of the cells. The expression of the c-myh oncogene and GM-CSF receptor (GM-CSF-R) in UT-7/EPO by RNA blot hybridization analysis was examined. Down regulation of the expression of these genes was reported to be an essential event for EPO-induced erythroid differentiation (Todokoro et al., 1988; Liboi et al., 1992). Transcription factor GATA-1 has been reported to be essential
for the transcriptional activation of erythroid, mast cell, and megakaryocytic-specific
genes (Martin et al., 1990; Romeo P-H, 1990; Yamamoto et al., 1990).

Therefore, the expression of GATA-1 mRNA in UT-7 and UT-7/EPO cells was
examined. The analyses clearly demonstrated that UT-7/EPO cells were committed to
the erythroid lineage. The availability of EPO-R on the surface of UT-7/EPO cells was
significantly decreased, suggesting that changes in post receptor signaling events were
responsible for the establishment and maintenance of the UT-7/EPO phenotype
(Komatsu et al., 1993). In studying the effect of EPO on the proliferation and
differentiation of UT-7 in long-term culture, UT-7 cells were maintained in the presence
of EPO and, after 6 months, a subline designated UT-7/EPO was established (Komatsu
et al., 1991). The UT-7/EPO phenotype differed from that of the parent cells (UT-7) in
the following respects: (1) UT-7/EPO lost the response to IL-3 and GM-CSF, resulting in
complete dependence on EPO for maintenance and growth; (2) it lost the ability to
express the megakaryocytic program in response to PMA; and (3) it acquired the ability
to respond to BA, resulting in increased haemoglobin synthesis.

Among the several human and murine EPO-dependent cells, UT-7/EPO cells are
unique in that growth can be maintained solely by EPO and in that the cells have a large
number of EPO binding sites on the surface. Therefore, taking advantage of this fact,
the cell line may serve as a useful system for the bioassay of EPO and modified EPO
molecules because MTT reduction paralleled the concentration of EPO in the range of
0.01 to 1 U/mL of EPO (Figure16).
The proliferative responses of UT-7 cell lines to recombinant human EPO (mU/mL), GM-CSF (0;ng/mL), IL-3 (A; U/mL), or IL-6 (m; ng/mL) were analyzed by MTT assay (Figure 16). The cells had been maintained for 1 year in the presence of (A) 1 ng GM-CSF/mL, (B) 10 U IL-3/mL or (C) 1 U EPO/mL at the time of this assay. Cells were plated at 1 O*/well in IMDM in the presence of increasing concentrations of growth factors. MTT incorporation was measured after 3 days of culture.
CHAPTER THREE: METHODS

AIM

The aim of this prospective study was to assess the contributing factors affecting erythropoiesis in chronic renal failure (CRF) patients in KwaZulu Natal. The study design was prospective, experimental and quantitative. The aim of the study was to assess the contributing factors adversely affecting erythropoiesis in haemodialysis (HD) patients in KwaZulu Natal (KZN). The impact of erythropoiesis in serum of CRF patients with the use of a bioassay was monitored.

OBJECTIVES

- Nutritional status of the haemodialysis patients by monitoring the transferrin and albumin blood levels and anthropometric analysis was assessed.
- The role of inflammation in erythropoiesis by measuring the C reactive protein (CRP), erythrocyte sedimentation rate (ESR) levels and ferritin levels was assessed.
- The effect of iron and ferritin levels on erythropoiesis was demonstrated.
- The measurement of native erythropoietin and antibodies against recombinant erythropoietin (RHuEPO) in serum of the haemodialysis (HD) patients was achieved. Various factors which played a role in erythropoiesis was analysed i.e., those that promoted and those that inhibited the process.
- The use of the bioassay was to quantitatively identify and measure the neutralizing antibodies against recombinant erythropoietin.
A convenience and purposive sample of fifty nine patients was selected from Addington and King Edward Hospitals. A further 15 healthy individuals who were staff had volunteered to participate in the study. The total sample size of participants was 74. These included 59 patients who were the chronic renal failure (CRF) patients on haemodialysis and 15 healthy individuals. Among the 59 CRF patients on haemodialysis, forty four patients received recombinant EPO (RHuEPO) treatment and fifteen patients did not receive RHuEPO. They all were analysed for demographics. The forty four patients receiving RHuEPO were analysed for all laboratory investigation. The control group (healthy individuals) consisted of fifteen (15) healthy individuals who had volunteered to participate in the study. They were not in CRF, not for haemodialysis treatment and therefore were not on RhuEPO treatment. The fifteen healthy individuals had their Hb levels done. The smaller number of patients in the group that did not receive recombinant EPO is due to the standard operating procedures of CRF and EPO administration. Due to ethical regulations of EPO in Chronic renal failure patients on haemodialysis, patients cannot stay more than three months on HD therapy without being on recombinant EPO. The sample numbers selected was verified by a biostatistician.

The patients were randomised into experimental and control groups. The haemodialysis patients were bled on a monthly basis for six months. Haemoglobin (Hb), transferrin (Tf), albumin (Alb), iron (Fe), ferritin (Ferr), CRP and ESR levels were obtained from the
patients routine monthly blood tests. Blood was drawn from the arterial blood lines of
the haemodialysis circuit during the duration of the study. The nutritional status of the
patients was assessed by monitoring the transferrin and albumin blood levels. The
inflammation of the patients was monitored by the CRP and ESR levels. The role of
inflammation in erythropoiesis was monitored using the CRP levels. The utilisation of
the patient’s iron and ferritin levels for erythropoiesis were assessed. An extra 15mls of
blood (with the patients consent) was drawn from the arterial blood lines of the
haemodialysis circuit with the routine monthly blood samples. This blood was used for
ELISA and bioassay (these were done in duplicate) which were carried out during the
period of the trial.

The ELISA was set up to measure EPO levels and to assess antibodies to RhuEPO.
The bioassay was carried out using cell lines that depend on erythropoietin for growth.
This procedure quantitatively measured any neutralizing activity (if any) against the
therapeutic proteins. An anthropometric analysis was performed on patients, i.e. height,
weight, arm circumference and the skin fold thickness was measured on a monthly
basis by the principal investigator. The measurements were taken post dialysis together
with the routine post dialysis assessments (Nelson et al., 1990; Woodruff, 2002).
Predialysis blood pressures were recorded and mean arterial pressures calculated.
Patient’s dry weights were recorded. All patients selected for the trial were given
detailed verbal description for all measurements required. All record of recombinant
erthropoietin doses and injections given to the patients in the last hour of the
haemodialysis procedure were documented by the principal investigator. The three
blood specimens (15mls) drawn for the assay procedures were couriered by the principal investigator to the Nelson Mandela School of Medicine. The blood tubes were taken and marked according to a number (protecting patient identity).

3.1 SELECTION CRITERIA

Patients enrolled into the study were required to meet the following inclusion and exclusion criteria.

**Inclusion criteria**

a) Haemodialysis Patients in Chronic Renal Failure  
b) All patients irrespective of sex, culture, race.  
c) Patients receiving treatment at either Addington or King Edward VIII Hospitals  
d) Patients who are 18 - 50 years old.

**Exclusion criteria**

a) Acute HD patients  
b) Patients who are pregnant  
c) Patients who are aware of undergoing a live related transplant in the next few
months to a year

d) Patients who have severe cases of immunological disorders.

Before commencement of the actual investigation, ethical approval was obtained from the Durban University of Technology Ethical Committee and permission was also obtained from the Higher Degree’s Committee and the Department of Nephrology, Haemodialysis Units at Addington and King Edward VIII Hospitals (Appendix C and D). In order to facilitate the study, the research plan was presented to the Departments of Nephrology, Haemodialysis units and the nursing staff at Addington and King Edward VIII Hospitals.

A letter requesting permission to conduct the study at Addington and King Edward VIII Hospital was forwarded to KZN Health Ethekwini District (Appendix B). This letter was forwarded with an ethical clearance certificate and an approved proposal. Thereafter KZN Health Ethekwini District forwarded the request for permission from the Health Department in Pietermaritzburg. After the Department of Health granted permission, the study could commence. The approval letter attached in appendices.

Patients who met the inclusion criteria were recruited in the Haemodialysis units. A letter of information and consent form, drawn up by the researcher in both English and IsiZulu was presented to all patients participating in the study. Patients were notified as to the purpose and the requirements of the study. Patients were informed that their right
to participate in the trial was entirely voluntary and that they were entitled to withdraw at any point without affecting the medical treatment rendered to them. They were also informed that all information used in the research would remain confidential and that any data reported in scientific journals or published would not include information identifying them as a patient in the study (refer to appendix E and F for consent in English and IsiZulu). All patients recruited into the study were under the consultant care of Professor A.G. Assounga, Head of Department of Nephrology, Addington and King Edward VIII Hospitals.

Patients, who were willing to participate signed the consent form and were randomized into the control or experimental groups and continued their treatments as usual. The HD patients were bled for a period of six months. Anthropometric analysis were performed post HD for six months (Figure 18). Recombinant Erythropoietin (RHuEPO) doses were monitored for the study period.

The haemoglobin, transferrin, albumin, iron, ferritin, CRP and ESR levels were obtained from patient’s routine monthly blood tests. The blood samples were drawn from the arterial blood lines of the haemodialysis circuit at the commencement of the procedure (Figure 17).
3.2 Anthropometric measurements

Anthropometric measurements were performed on the patients selected for the study as per the subjective global assessment criteria for chronic renal failure patients on HD. (Nelson et al., 1990; Woodruff, 2002). The height, weights, mid arm circumference and skin fold thickness was measured on a monthly basis for six months post dialysis (Figure 18 a, b). Skin fold measurements were taken from the supra-iliac areas. These were all done in duplicates.
3.3 Blood samples

An extra 15 mls of blood was taken together with the routine monthly blood tests and was used for the Elisa tests and the bioassay. The duplicate blood tubes were couriered by the principal investigator to the Nelson School of Medicine. The blood tubes were taken and marked accordingly to numbers, protecting the patient’s identity (Figure 19 a). The samples were centrifuged to separate the serum from blood components (Figure 19 b). Blood was spun down at 1750x g for 10 min. Serum was then collected and labelled accordingly and stored at -70 degree celsius (Figure 20).
a) Blood tubes from Addington and King Edward HD unit’s  
b) Blood samples spun to separate serum from the blood components

**Figure 19:** Preparation of blood samples (Benjamin, 2013)
3.4 Enzyme Linked Immunosorbent Assay

Positive control sera were chemically coupled to enzymes eg. horseradish peroxidase or alkaline phosphatase. Antibodies (Abs) that bind to antigen coated wells, are detected with the addition of an enzyme coupled to horseradish peroxidase or alkaline phosphatase.

The direct method calls for linking the enzyme directly to the antibody of interest. Depending on the availability of the Abs and its activity, direct conjugation of the enzyme to the Abs may interfere with specificity or success of binding with the target. The preferred method is the indirect ELISA; the antigen or the cell of interest is
immobilised onto the well. The primary Abs from a known host animal is allowed to bind to the immobilised target. A secondary antibody directed against the primary host animal’s immunoglobulin (usually IgG) is conjugated to a reporter enzyme and allowed to bind to the primary Abs. The complex is visualised by addition of a substrate and formation of a coloured reaction product. The two most common enzyme labels for secondary Abs are alkaline phosphatase used in conjunction with the substrate ABTS and H$_2$O$_2$, which results in blue green reaction product (Javious, 1998).

**Preparation of all Samples**

All samples were brought to room temperature. Samples in the eppendorf tubes were vortexed (DDMRI, Natal). All samples used were labelled according to the plan set out for the ELISA plate layout in duplicate. 100µL of each specimen was pipetted for the appropriate step.

**Preparation of the Enzyme Linked Immunosorbent Assay Kit (ELISA)**

The quantikine *in vitro* diagnostic (IVD) enzyme linked immunosorbent assay kit (R&D Systems, Oxon, UK) was brought to room temperature. The reagents (Table 2) used from the kit were followed by the directions provided by the manufacturer. All reagents used in the experiment were vortexed before use. All assays carried out consisted of controls.
### Table 2: Reagents provided in the Elisa Kit

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythropoietin microplate</td>
<td>96 wells – mouse monoclonal antibody</td>
</tr>
<tr>
<td>EPO conjugate (200µL)</td>
<td>Conjugated to horseradish peroxidase</td>
</tr>
<tr>
<td>EPO Standards (0.0-200mIU/mL)</td>
<td>Recombinant human EPO</td>
</tr>
<tr>
<td>EPO Assay diluent (100µL)</td>
<td>Buffered protein base</td>
</tr>
<tr>
<td>Specimen diluent (100µL)</td>
<td>Protein stabiliser buffer</td>
</tr>
<tr>
<td>EPO wash buffer (1X)</td>
<td>100ml</td>
</tr>
<tr>
<td>Colour reagent A (100µL)</td>
<td>0.01N buffered H₂O₂</td>
</tr>
<tr>
<td>Colour reagent B (100µL)</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>Stop solution (100µL)</td>
<td>2N sulphuric acid</td>
</tr>
<tr>
<td>Plate covers</td>
<td>Adhesive strips</td>
</tr>
</tbody>
</table>

**Preparation of substrate solution**

Colour reagent A and colour reagent B were mixed together in equal volumes and labelled. The substrate solution was used within 15 minutes and protected from
exposure to light. Two hundred microliters (200µL) of the resultant mixture was required per well. All unused substrate was discarded.

**Protocol: For the erythropoietin concentration levels in blood**

1. All reagents and samples stored at -70°C were brought to room temperature. Each sample was assayed in triplicate and the experiment was carried out in duplicate in a 96-well polystyrene microplate coated with a mouse monoclonal antibody against recombinant human erythropoietin.

2. Eighty wells were used and the excess microplate strips were removed from the plate frame and returned and sealed in the foil pouch containing the desiccant seal.

3. A pipette was used to pipette 100 µL of erythropoietin assay diluent into each well using separate tips for each well.

4. A sample diagram as Appendix E for the standards, controls, and specimens showing the plate layout was followed accordingly. The standard, control, and specimen were added into each of the polystyrene microplate wells respectively of which each measured at 100µL. The controls were labelled accordingly. All samples used were documented accordingly. The standards were used according to the manufacture’s instruction manual.
5. The erythropoietin microplate frame was gently tapped for one minute to mix the contents in the well. The microplate was covered with the adhesive strip provided to seal the plate.

6. The wells were incubated for one hour and five minutes at room temperature on a horizontal orbital microplate shaker (Whitehead Scientific, Labnet, Brekenfell, South Africa), 50 RPM (Figure 22).

7. After incubation on the horizontal orbital microplate shaker, the plate sealer was gently removed and the contents from each well had been thoroughly aspirated. The microplate was blotted dry on clean paper towelling.

8. Two hundred micro litres (200µL) of erythropoietin conjugate was added into each well. The plates were covered with a plate sealer and incubated for one hour and five minutes at room temperature. This was left to incubate on the horizontal orbital microplate shaker at 50 RPM.

9. The plate sealer was gently removed and each well was aspirated of its contents and washed four times. A thorough washing of the plate was extremely important to reduce the background. A multi-channel pipette was used to fill each well with 400 µL of diluted wash buffer. Complete removal of liquid at each step was essential for good performance. After the last wash the remaining wash buffer in the wells were best
accomplished by inverting the plate over the sink and blotting it against clean paper towels. Using the multichannel pipette 400µL of wash buffer was added to each well; the plate was inverted and blotted. This procedure was repeated for a total of four times.

10. Colour reagents A and colour reagent B were allowed to come to room temperature (refer to Appendix D) for substrate preparation. Two hundred microlitres of substrate solution was dispensed into each well (Figure 23). The plate was resealed with the adhesive strip provided and incubated on the bench top for 25 minutes at room temperature.

11. The colour generation was monitored very closely appearing blue at various wells. The wells were incubated at room temperature for 20-25 minutes.

12. The stop solution of 100µL was dispensed into each well (Figure 24). The plate was gently tapped to ensure thorough mixing. The colour changed to yellow.

13. The optical density of each well was determined within fifteen minutes, using the microplate reader (EL X 800, Universal Microplate Reader, and BioTek Instruments) set at 450 nm (Figure 21).
**3.5 ANTIBODY ELISA**

**3.5.1 DETECTION OF ANTIBODIES USING THE ELISA WITH SERUM**

DILUTIONS OF 1:50

**PREPARATION OF REAGENTS**

Recombinant Erythropoietin – Beta
The reagent was brought to room temperature. It was vortexed and added to the wells accordingly.

**Bovine serum albumin**

The solution was vortexed and added to the wells accordingly.

**Horseradish peroxidase – conjugated goat antihuman IgG**

The reagent was brought to room temperature and vortexed before adding to each well.

**Substrate solution**

The reagents were mixed together in equal volumes and were used within 15 minutes. The substrate solution was protected from light. All unused prepared substrate solution was discarded.

Colour reagent A and colour reagent B were allowed to come to room temperature (refer to appendix for reagent preparation). They were mixed together in equal volumes and used within fifteen minutes of preparation.
Stop solution

Reagent was brought to room temperature and vortexed before use.

Protocol

1. All reagents were prepared accordingly. Ninety-six wells polystyrene microfiber plates were coated with RHuEPO-beta at 10mg/L in PBS Ph 7.4 and then incubated overnight at 4°C.

2. The plates were emptied and washed five times with PBS using an electronic washer (EL X 50, Autostrip Washer, and Biotek Instruments). The plates were then post coated till the top of each well with PBS containing 30g/L bovine serum albumin (BSA) and incubated for 4 hours at room temperature.

3. The contents of the wells were flicked out and 200µL of serum dilutions of 1:50 was added to the wells. Conjugate was added to the positive control wells and PBS added to the negative control wells, no EPO wells and in the non-specific binding wells. The wells were incubated at one hour at room temperature.
4. Plates were then washed five times as described previously by an electronic washer (Figure 22). After washing, 100µl of freshly prepared substrate solution was added to each well (Figure 23).

5. The plates were then washed five times as described previously.

6. Subsequently, 100µL of horseradish peroxidase conjugate goat antihuman (IgG), (Sigma, Missouri USA) were added to the wells including the wells that were labelled as non specific binding wells. PBS was added in the positive control wells, negative control wells, no EPO wells, and non-specific binding wells.

7. The wells were then incubated for one hour at room temperature.

8. After thirty minutes the reactions were stopped by adding 100µL stop solution (sulphuric acid). The absorbance was measured with a microplate reader (EL X 800, Universal Microplate Reader, BioTek Instruments) at 450 nm.
**Figure 22:** Plates washed with an electronic washer EL X 50

Autostrip Washer Biotek Instruments (Benjamin, 2013)

**Figure 23:** One hundred microlitres (100uL) of freshly prepared peroxidase substrate solution was added to each well (Benjamin, 2013)
Figure 24: Colour reaction observed after peroxidase was added into each well (Benjamin, 2013).
### Table 3: Protocol for the detection of antibody using the ELISA

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Incubation: 80 µl recormon added into each well</td>
<td>Overnight at 4°C</td>
</tr>
<tr>
<td>2. Buffer rinse: Washed in PBS(ph 7.4) five times</td>
<td>30 min</td>
</tr>
<tr>
<td>3. Bovine serum albumin-post coated: Wells filled to top with 30g/l BSA</td>
<td>4 hrs at 37°C</td>
</tr>
<tr>
<td>4. Adding of serum dilution: contents flicked out 1:50 serum dilution added to wells</td>
<td>5 min</td>
</tr>
<tr>
<td>5. Incubation: Conjugate and PBS added to respective wells</td>
<td>20 min</td>
</tr>
<tr>
<td>Incubated</td>
<td>1 hr at 37°C</td>
</tr>
<tr>
<td>6. Buffer rinse: Plates were washed five times</td>
<td>30 min</td>
</tr>
<tr>
<td>7. 2nd Antibody added to wells: in all patient wells in EPO and 2nd antibody well PBS in other three sets</td>
<td>30 min</td>
</tr>
<tr>
<td>8. Incubation</td>
<td>1 hr at 37°C</td>
</tr>
<tr>
<td>9. Washing</td>
<td>30 min</td>
</tr>
<tr>
<td>10. Substrate Added:</td>
<td>20 min</td>
</tr>
<tr>
<td>Step</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>10</td>
<td>freshly prepared substrate into each well</td>
</tr>
</tbody>
</table>
| 11 | Reaction stopped after 30 min  
100ul of sulphuric acid added to each well | 15 min |
| 12 | **Absorbance measured:** with a microplate reader | 15 min |
3.6 Bioassays

Principle of the technique:

The cell-based bioassay for EPO determines the effect of proliferation of human leukaemia cell line in response to the presence of EPO. A linear increase of cell metabolism as well as cell proliferation is observed by adding EPO into the culture medium. This proliferation assay determines the relative potency of EPO contained in the test material. A colorimetric assay is used to quantify cell proliferation and cell viability. Patients requiring very high doses of EPO to reach Hb of 11g/dL, and if they continued to remain anaemic after at least three months of adequate EPO doses were grouped to be EPO resistant. Those who responded to usual EPO doses were grouped as EPO sensitive.

This assay is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase from living cells. The potency of EPO in the test item is quantified by comparing the responses of test item to that of the reference materialising the standard statistical methods for a parallel line assay (Thorpe and Swanson, 2005).
3.6.1 Cell Culture

The UT-7 cell line was established from marrow cells obtained from a patient with acute myeloid leukaemia (Creative Bioarray Laboratories, Lang Island, New York). The frozen UT 7 cells were thawed and suspended in RPMI culture medium with 10% bovine faetal serum FCS, (Hyclone Laboratories, Logan, UT) and 10 units/ul EPO (epoietin beta 4000iu/0,3 ml). The UT 7 cell lines were incubated for 12 hours in the carbon dioxide (CO₂) incubator. After incubation, UT 7 cells were washed and resuspended in the culture medium RPMI with in 10 units/ul EPO (epoietin beta 4000iu /0,3ml). The UT 7 cells were cultured alone in a medium with three different serums; three resistant individual patients, three EPO sensitive individuals, three healthy individuals and controls. All serum samples (the resistant; Hb<11g/dL, sensitive; Hb>10g/dL, normal healthy individuals and controls) were added in the 96 well plate. They were cultured in duplicate. The 96 well plates were left to incubate in CO₂ incubator for 24 hours.

3.6.2 ATP Standard

The ATP (Sigma Capital Laboratory, New Germany, Dbn, RSA) standard was prepared by serial dilutions of 1mg ATP according to manufacturer’s instructions to concentrations of 10000ng/ml, 1000ng/ml, 100ng/ml, 10ng/ml and 1ng/ml with distilled water. 100ul of each level of the calibrator panel were dispensed into the designated wells. The amount of ATP produced was calculated from the ATP standard curve thus
an ATP standard was run with each assay plate and a standard curve generated for each test.

### 3.6.3 CellTiter Glo Luminescent cell viability assay (Promega Whithead Scientific (PTY) Ltd, Brakenfell, Capetown, RSA)

This test was a uniform single step method of determining the number of metabolically active cells in culture based endogenous on the amount of ATP detected. Intracellular ATP was measured using the firefly luciferin/luciferase reaction. This assay uses a stable form of luciferase based on the gene from the firefly *Photurispennsylvanica*.

A mutant form of the gene was created in which the characteristics that improve performance were selected, eliminating the problems caused by endogenous ATPase, Ph, detergents and hence creating a reagent that has a sensitive and stable luminescence. The luminescence generated is proportional to the number of viable cells. The CellTiter Glo assay reagent was prepared according to the manufacture’s instruction (Promega, 2005).

An equal volume of 100ul of the CellTiter Glo assay reagent equal to that of the cell culture medium was dispensed into each well. The plate was then manually shaken for average of 2 minutes to induce cell lysis and release the intracellular ATP to react. The
cells were then incubated for 10 min at room temperature to stabilise the luminescent signal.

The cells were read with the GloRunner Microplate Luminometer (Turner Biosystem, Sunnyvale, Canada, USA) at a wavelength of 550nm for 1 second per well. Luminescence was recorded in relative light units (RLU). Turner Biosystem GloRunner microplate luminometer software was used to access and analyse the results.

3.6.4 Construction of an ATP standard curve

Microsoft Excel program was used to draw up the standard curve. The average of the triplicate RLU readings for each known ATP dilution concentration was calculated. The standard curve was drawn with the known ATP concentration value on the X axis and calculated RLU value on the Y axis. The straight line equation $y= 0.000x + 0.383$ was used to determine the ATP values of the test samples.

3.6.5 Data Analysis

All data were recorded and analysed accordingly.
Challenges and Limitations

In our study we had a long delay in the delivery of the UT 7 cell lines. It was firstly ordered from China and thereafter found out that the company was unable to deliver it in maintaining the storage requirements. Thereafter we had to re order it from Creative Bioarray, 45-16 Ramsey Road Shirley, New York 11967, USA. There had been a time delay in the plan of the research and this held the study behind by 18 months.

The study had limitations in regards to the number of patients in the experimental groups that were not receiving RHuEPO. The control groups of healthy individuals were also a small number in this study. Larger number of patients in the above groups would have been more significant however the study sample numbers were approved by the biostatistician.

3.7 Statistical Analysis

The data collected was captured and subsequently analysed using the Statistical Package for Social Sciences (SPSS version 18). Descriptive statistics such as mean, median, mode and standard deviation was used to summarize blood results. Two samples independent test or Mann Whitney test was used to examine the difference in blood results between the control and cases. Pearson correlation or Spearman rank correlation was used to estimate the strength of linear relationship between presence of antibodies and process of erythropoiesis. Multiple linear regressions were used to
estimate the effect of iron and ferritin levels on erythropoiesis. Multiple linear regressions were also used to measure the effect of CPR, ESR and ferritin.
CHAPTER FOUR: RESULTS

4.1 Erythropoietin levels and haemoglobin blood levels of patients during trial period

A dot plot graph of erythropoietin (EPO) levels and haemoglobin levels (Hb) was constructed (Figure 25) with EPO levels on the x axis and the haemoglobin (Hb) levels on the y axis. There was a positive correlation between erythropoietin and haemoglobin levels (R=0.24) which was not significant (p = 0.895).

Figure 25: Erythropoietin levels (x axis) and haemoglobin levels (y axis)
4.2 Erythropoietin dosages and haemoglobin levels during trial period

A positive correlation ($R= 0.646$) was noted between EPO dosage and Hb levels, which was also significant ($p= 0.01$) (Figure 26).

**Figure 26:** Erythropoietin doses (x axis) and haemoglobin levels (y axis)
4.3 C Reactive Protein (CRP) and Haemoglobin levels

A dot plot graph was constructed (Figure 27) with haemoglobin levels indicated on the x axis and CRP on indicated on the y axis. There was a negative correlation between haemoglobin levels and CRP levels ($R = -0.112$) but it was not significant ($p = 0.571$).

![C Reactive Protein (CRP) and Haemoglobin levels](image)

**Figure 27:** C Reactive Protein (CRP) (x axis) and Haemoglobin levels (Hb) (y axis)
4.4 Erythropoietin weekly dosages and blood pressure (systolic and diastolic blood pressure)

As represented in Figure 28, the increase in systolic and diastolic blood pressure increases with the erythropoietin weekly dosages. A positive correlation was noted with the weekly EPO dosages and the blood pressures (systolic and diastolic blood pressures), which was significant (p <0.05).

**Figure 28:** EPO doses (x axis) and blood pressure (systolic and diastolic blood pressures) (y axis)
4.5 Erythropoietin dosage and mean arterial pressure

There was a positive correlation (R = 0.398) between EPO doses and the mean arterial blood pressure (MAP) which was also significant (p = 0.008) (Figure 29).

Figure 29: Erythropoietin dosage (x axis) and Mean arterial pressure (y axis)
4.6 Erythrocyte Sedimentation Rate (ESR) and Haemoglobin (Hb)

A negative correlation ($R = -0.26$) was noted between the erythrocyte sedimentation rate (ESR) and the haemoglobin levels which was not significant ($p = 0.898$) (Figure 30).

**Figure 30**: Erythrocyte Sedimentation Rate (ESR) (x axis) and Haemoglobin (Hb) (y axis)
4.7 Skin fold thickness and Haemoglobin (Hb) levels during the trial period

A dot plot graph was constructed of the skin fold thickness on the x axis and haemoglobin levels on the y axis (Figure 31). There was a positive correlation (R=0.55) between the skin fold thickness and the haemoglobin levels but it was not significant (p = 0.745).

Figure 31: Skin fold thickness(x axis) and Haemoglobin (Hb) (y axis)
4.8 Percentage Transferrin saturation (T Sats) and Haemoglobin levels during the trial period

A positive correlation (R=0.55) was noted between % transferrin saturation (T Sats) (x axis) and haemoglobin levels (y axis) which was also not significant (p = 0.745) (Figure 32).

Figure 32: Percentage % Transferrin saturation (T Sats) and Haemoglobin levels (Hb) (y axis)
4.9 Ferritin and Haemoglobin levels during the trial period

A dot plot graph of ferritin levels and haemoglobin levels was constructed (Figure 33) with ferritin levels on the x axis and haemoglobin levels on the y axis. There was a positive correlation between ferritin and Hb levels ($R=0.73$) but it was not significant ($p=0.662$).

![Ferritin and Haemoglobin levels](image)

**Figure 33:** Ferritin levels (x axis) and Haemoglobin levels (y axis)
4.10 Albumin and haemoglobin levels during the trial period

A dot plot graph of ferritin levels and haemoglobin levels was constructed (Figure 34) with ferritin albumin on the x axis and haemoglobin levels on the y axis. There was a positive correlation between albumin and Hb levels ($R = 2.88$) and it was not significant ($p=0.802$).

Figure 34: Albumin levels (x axis) and haemoglobin levels (y axis)
4.11 Age of patients and haemoglobin levels during the trial period

A dot plot graph of age and haemoglobin levels was constructed (Figure 35) with age of patients on the axis and Hb levels on the y axis. The age and Hb levels were statistically analysed with each other (R=0.427) and (p= 0.08), the relationship is positive but not significant.

**Figure 35**: Age of patients (x axis) and Haemoglobin levels (y axis)
4.12 Creatinine levels and haemoglobin levels of patients during the trial period

The creatinine levels are presented on the x axis and haemoglobin levels are represented on the y axis (Figure 36). The creatinine levels and haemoglobin levels of patients were compared with each other, a positive relationship noted but not significant (p>0.05). There was also no correlation of the creatinine and haemoglobin levels.

Figure 36: Creatinine levels(x axis) and Haemoglobin levels(y axis)
4.13 Correlations of Erythropoietin (EPO) Antibody (Ab)

The antibody levels were correlated with all the variables as indicated in Table 4. There were correlations as shown in Table 4 for the variables indicated and all the variables however had p>0.05, this was not significant with the antibody.

**Table 4: Erythropoietin (EPO) Antibody Correlations**

<table>
<thead>
<tr>
<th>Correlations of Epo antibody(AB)</th>
<th>P value</th>
<th>Pearsons coefficient</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab and haemoglobin</td>
<td>0.535</td>
<td>-0.112</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and Epo level</td>
<td>0.971</td>
<td>0.007</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and Epo weekly dose</td>
<td>0.505</td>
<td>-0.117</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and ferritin</td>
<td>0.477</td>
<td>-0.128</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and transferrin</td>
<td>0.887</td>
<td>0.26</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and albumin</td>
<td>0.259</td>
<td>0.202</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and % T Sats</td>
<td>0.173</td>
<td>0.243</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and urea</td>
<td>0.396</td>
<td>-1.53</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and creatinine</td>
<td>0.322</td>
<td>0.178</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and Epo resistance</td>
<td>0.820</td>
<td>0.41</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and frequency</td>
<td>0.962</td>
<td>0.09</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and ESR</td>
<td>0.395</td>
<td>0.178</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and diastolic BP</td>
<td>0.159</td>
<td>0.356</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and systolic BP</td>
<td>0.347</td>
<td>0.153</td>
<td>Nil</td>
</tr>
<tr>
<td>Ab and mean arterial pressure</td>
<td>0.311</td>
<td>0.174</td>
<td>Nil</td>
</tr>
</tbody>
</table>
4.14 Clinical parameters of patients and controls

The means of all the clinical parameters of patients and controls are indicated in Table 5. Age within the patient group and healthy controls was significant \( (p = 0.035) \).

Table 5: Clinical Parameters

<table>
<thead>
<tr>
<th>Means of variables</th>
<th>Patients n=59</th>
<th>Healthy Controls n=15</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Means ±</td>
<td>Means ±</td>
<td></td>
</tr>
<tr>
<td>Skin fold thickness</td>
<td>22.86 ± 2.06</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arm circumference</td>
<td>38.74 ± 0.65</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td>1.63 ± 0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(M)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>45.37 ± 1.74</td>
<td>34.27 ± 2.49</td>
<td>( P = 0.0035 )</td>
</tr>
<tr>
<td>(yrs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic Bp</td>
<td>153.0 ± 2.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Diastolic Bp (mmHg) 89.4 ± 1.9 - -
Mean arterial pressures (mmHg) 110 ± 2.0 - -

4.15 Laboratory parameters of patients and controls

The means of all the laboratory findings of patients, controls and p values are represented in (Table 6). Erythropoietin levels within the patient group and healthy controls was not significant (p = 0.442). Haemoglobin levels within the patient group and healthy controls was also not significant (p <0.0001).
Table 6: Laboratory parameters

<table>
<thead>
<tr>
<th>Means of variables</th>
<th>Patients n=59</th>
<th>Patients n=59</th>
<th>Healthy Controls n=15</th>
<th>Healthy Controls n=15</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Means ±(SEM)</td>
<td>Means ± (SEM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>10.03 ± 0.28</td>
<td>14.03 ± 0.51</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td>941.30 ± 68.45</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Transferritin (g/dL)</td>
<td>1.45 ± 0.05</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>% Saturation</td>
<td>30.96 ± 2.34</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>32.49 ± 0.92</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>EPO levels (mU/mL)</td>
<td>22.67 ± 5.41</td>
<td>10.28 ± 2.34</td>
<td>0.4429</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPO wkly dose (iu)</td>
<td>8007 ± 840.2</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
</tr>
</tbody>
</table>
4.16 Means for Inflammatory markers of patients

The means ± SEM of all the inflammatory markers of patient’s, controls and p values are represented in Table 7.

**Table 7: Inflammatory Markers**

<table>
<thead>
<tr>
<th>Means of variables</th>
<th>Patients n=59</th>
<th>Patients n=59</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Means</td>
<td>± SEM</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>37.03</td>
<td>± 5.41</td>
</tr>
<tr>
<td>CRP (g/dl)</td>
<td>31.87</td>
<td>± 9.78</td>
</tr>
</tbody>
</table>
4.17 Means of Clinical, Laboratory and Inflammation parameters for Months

(February, April and July)

The means of all the clinical parameters, laboratory findings and inflammatory markers for three months (February, April and July) is represented in Tables 8, 9, 10. The means for the laboratory findings, inflammatory markers and clinical parameters did not change drastically from their values over the three months. No significance was noted in any of the parameters.

**Table 8: Means of Clinical parameters for Months (February, April, and July)**

<table>
<thead>
<tr>
<th>Means of Clinical parameters</th>
<th>February</th>
<th>April</th>
<th>July</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin fold thickness (mm)</td>
<td>18.75 ± 27</td>
<td>15.21 ± 10.7</td>
<td>19.83 ± 2</td>
</tr>
<tr>
<td>Arm circumference (cm)</td>
<td>23.27 ± 23.3</td>
<td>22.58 ± 33</td>
<td>28.35 ± 7</td>
</tr>
</tbody>
</table>
Table 9: Means of Laboratory parameters for Months (February, April and July)

<table>
<thead>
<tr>
<th>Means of lab findings</th>
<th>February</th>
<th>April</th>
<th>July</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>10.59 ± 9.4</td>
<td>10.82 ± 8.4</td>
<td>10.65 ± 11.6</td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td>1141.35 ± 763</td>
<td>1146.56 ± 585</td>
<td>1070.71 ± 585</td>
</tr>
<tr>
<td>Transferritin (g/dL)</td>
<td>1.61 ± 0.64</td>
<td>1.41 ± 0.39</td>
<td>1.46 ± 0.45</td>
</tr>
<tr>
<td>% Saturation</td>
<td>37 ± 10</td>
<td>31.4 ± 11</td>
<td>33.1 ± 8</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>33.28 ± 7</td>
<td>34.09 ± 7</td>
<td>35.13 ± 4</td>
</tr>
<tr>
<td>EPO levels (mIU/mL)</td>
<td>37.40 ± 0.6</td>
<td>35.46 ± 7.0</td>
<td>22.66 ± 8.6</td>
</tr>
<tr>
<td>Antibody levels (OD)</td>
<td>3.645 ± 3</td>
<td>3.604 ± 2</td>
<td>3.754 ± 0.8</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>24.49 ± 16.2</td>
<td>24.42 ± 18.3</td>
<td>24.74 ± 2.9</td>
</tr>
</tbody>
</table>
Table 10: Means of Inflammatory markers of for Months (February, April, and July)

<table>
<thead>
<tr>
<th>Means Inflammatory Markers</th>
<th>February</th>
<th>April</th>
<th>July</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR (mm/h)</td>
<td>38.73 ± 22</td>
<td>29.82 ± 27</td>
<td>34.07 ± 34</td>
</tr>
<tr>
<td>CRP (g/dl)</td>
<td>34.26 ± 27</td>
<td>19.3 ± 6</td>
<td>22 ± 2</td>
</tr>
</tbody>
</table>

4.18 Correlations of Nutritional and Inflammatory markers

C Reactive protein (CRP) and albumin levels were compared with each other. C reactive protein (CRP) and albumin levels showed a negative correlation (R= -5.91) and (p < 0.05) this was significant (Table 11). However the other variables did not correlate with each other and was not significant. There was a negative correlation of EPO level and albumin levels (R=-1.40) (p > 0.05), not significant. EPO levels and transferrin levels did not correlate with each other negatively (R= -2.76), (p=1.20) which is not significant. There was negative between transferrin and %T saturation (R=-1.24) (p >
0.05) which is also not significant. Transferrin and albumin levels correlated with each other positively but not significant R=0.377 (p > 0.05).

**Table 11: Correlations between nutritional and inflammatory markers**

<table>
<thead>
<tr>
<th>Correlations</th>
<th>P value</th>
<th>Pearsons correlation R</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epo level and Albumin</td>
<td>0.437</td>
<td>-1.40</td>
<td>nil</td>
</tr>
<tr>
<td>Epo level and Transferritin level</td>
<td>1.20</td>
<td>-2.76</td>
<td>nil</td>
</tr>
<tr>
<td>Transferrin and %T Sats</td>
<td>0.459</td>
<td>-1.24</td>
<td>nil</td>
</tr>
<tr>
<td>CRP and Hb</td>
<td>0.571</td>
<td>-0.112</td>
<td>nil</td>
</tr>
</tbody>
</table>
4.19 Bioassay: Cell Culture- Titration curve

The titration curve (Figure 37) with, equation allows the conversion of relative light unit (RLU). The average of the triplicate RLU readings from the luminometer for each known ATP dilution concentration was calculated ng/ml. The standard curve was drawn with the known ATP concentration value on the Y axis and calculated RLU value on the X axis. The straight line equation \( y = 0.0004x + 0.383 \) was used to determine the ATP values of the test samples.

Figure 37: ATP Titration curve
4.20 Effect of serum of haemodialysis patients and healthy controls on UT 7

Cell proliferation

The culture of UT 7 cells in the presence of serum of haemodialysis patients and healthy controls were compared with each other using ANOVA (analysis of variance), p <0.0097, this was statistically significant. The ATP production in UT 7 cell lines without serum was statistically significantly higher (p< 0.0097) than with serum from any of the three subject groups (EPO resistant serum, EPO responsive serum and the healthy controls serum) (p<0.0097), this was statistically significant (Figure 38). There was no statistically significant difference in the UT 7 cell line cultured in the presence of EPO resistant serum, EPO responsive serum and the healthy controls serum.

Figure 38: Effect of serum of haemodialysis patients and healthy controls on UT 7 cell proliferation, p>0.05
4.21 Logistic regression Analysis for Erythropoietin Resistance with different variable

Erythropoietin resistance was analysed using different variables (Table 12). All the variables were statistically analysed using p value, Odd ratio and Confidence intervals. Majority of the variables in Table 12 as indicated were statistically not significant (p > 0.05). The variables that had been statistically significant and close to significance (p < 0.05) were the MAP and the diastolic and systolic blood pressures. The mean arterial pressure was statistically positively associated with EPO resistance (p=0.041), odd ratio -1.066 and confidence interval [1.003 – 1.133]. The association of the Systolic BP with EPO resistance was not significant (p = 0.068), odd ratio 1.040 and confidence interval [0.997- 1.084]. As similarly the association of diastolic Bp with EPO resistance was also close to significance (p = 0.066), odd ratio -1.055 and confidence interval [0.996 – 1.117].
Table 12: Logistic regression Analysis for Erythropoietin Resistance

<table>
<thead>
<tr>
<th></th>
<th>P value</th>
<th>Odd Ratio</th>
<th>Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>0.074</td>
<td>3.8333</td>
<td>[0.879 -16.711]</td>
</tr>
<tr>
<td>Age</td>
<td>0.073</td>
<td>0.953</td>
<td>[0.903 - 1.004]</td>
</tr>
<tr>
<td>Height</td>
<td>0.864</td>
<td>1.742</td>
<td>[0.003 - 981.237]</td>
</tr>
<tr>
<td>Weight</td>
<td>0.852</td>
<td>0.995</td>
<td>[0.944 – 1.049]</td>
</tr>
<tr>
<td>Arm Circumference</td>
<td>0.428</td>
<td>0.942</td>
<td>[0.814 – 1.091]</td>
</tr>
<tr>
<td>Skin fold thickness</td>
<td>0.991</td>
<td>1.000</td>
<td>[0.954 – 1.047]</td>
</tr>
<tr>
<td>Haemoglobin (Hb)</td>
<td>0.097</td>
<td>0.734</td>
<td>[0.510 – 1.057]</td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.484</td>
<td>1.001</td>
<td>[ 0.999 – 1.002]</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.763</td>
<td>1.017</td>
<td>[0.911 – 1.135]</td>
</tr>
<tr>
<td>Transferrin</td>
<td>0.506</td>
<td>0.406</td>
<td>[0.047 – 4.533]</td>
</tr>
<tr>
<td>% T Sats</td>
<td>0.278</td>
<td>0.971</td>
<td>[0.922 – 1.024]</td>
</tr>
<tr>
<td>ESR</td>
<td>0.431</td>
<td>0.974</td>
<td>[0.911- 1.041]</td>
</tr>
<tr>
<td>CRP</td>
<td>0.537</td>
<td>1.004</td>
<td>[0.989 – 1.020]</td>
</tr>
<tr>
<td>Mean arterial pressure(MAP) *</td>
<td>0.041</td>
<td>1.066</td>
<td>[1.003 – 1.133]</td>
</tr>
<tr>
<td>Weekly Epo dose *</td>
<td>0.001</td>
<td>1.000</td>
<td>[1.000 – 1.001]</td>
</tr>
<tr>
<td>Test</td>
<td>Mean</td>
<td>SD</td>
<td>95% CI</td>
</tr>
<tr>
<td>--------------</td>
<td>----------</td>
<td>---------</td>
<td>----------</td>
</tr>
<tr>
<td>Epo Level</td>
<td>0.917</td>
<td>1.001</td>
<td>[0.976 – 1.027]</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.161</td>
<td>0.998</td>
<td>[0.996 – 1.001]</td>
</tr>
<tr>
<td>Urea</td>
<td>0.570</td>
<td>0.969</td>
<td>[0.868 – 1.081]</td>
</tr>
<tr>
<td>Systolic Bp</td>
<td>0.068</td>
<td>1.040</td>
<td>[0.997 – 1.081]</td>
</tr>
<tr>
<td>Diastolic Bp</td>
<td>0.066</td>
<td>1.055</td>
<td>[0.996 – 1.084]</td>
</tr>
</tbody>
</table>

P value > 0.05 – not significant

P value , 0.05- significant *
CHAPTER FIVE: DISCUSSION

We have analysed all demographic data (age and sex); clinical variables (height, age); erythropoiesis (ferritin, transferrin, T Sats, EPO dosage, EPO levels); nutritional variables (arm circumference, skin fold thickness, weight, transferrin, T Sats, and inflammatory variables (ESR and CRP). The ELISA was set up to measure EPO levels and to assess antibodies to RhuEPO. The bioassay was carried out using cell lines that depend on erythropoietin for growth. This procedure quantitatively measured any neutralizing activity (if any) against the therapeutic proteins.

The contributing factors affecting erythropoiesis mainly demographic, nutritional, clinical and inflammatory markers have been analysed. Decreased erythropoiesis is due to the deficient production of EPO, iron deficiency, and chronic disease with endogenous EPO resistance (Van der Putten et al., 2008). The prevalence of CKD stages greater than 3 is the highest reported in Africa (Matsha et al., 2013). The anemia of these patients is, mainly, due to decreased kidney’s secretion of EPO. In CKD patients there is a failure in increasing the EPO levels in response to hypoxia, as occurs in other types of anemia. These patients present an EPO deficiency, rather than it being totally unavailable, as EPO remains detectable even in the most advanced stages of CKD (Artunc and Risler, 2007). Erythropoiesis production decreases as end stage renal failure progresses with the known pathogenesis of chronic kidney disease (Stauffer, 2014). Due to the decrease in erythropoiesis, patients in end stage renal disease develop anaemia (Swinkels et al., 2008). Insufficient production of erythropoietin by kidneys contributes to
the complications in haemodialysis patients. Most patients with CKD and anaemia can be effectively treated with ESAs (Swinkels et al., 2008). A systematic review of 14 randomised controlled and uncontrolled trials in pre-dialysis CKD patients demonstrated that treatment of anaemia with ESAs improved energy levels and physical function (Gandra et al., 2010; Chan et al., 2014).

Other studies have also observed that there are several known causes of suboptimal response to ESA. These include: deficiencies in iron, vitamin B12, and folate; infection, chronic inflammatory state, neoplasia, severe hyperparathyroidism, aluminium intoxication, inadequate dialysis, myelosuppressive agents, haemoglobinopathies, myelodysplasia and antibody-mediated pure red cell aplasia (Macdougall and Cooper., 2002). There is however a marked variability in the response to EPO therapy and 5-10% of patients developed resistance to RHuEPO (KDOQI et al., 2006; KDOQI et al., 2007). However, other factors contribute to the anemia in these patients, such as reduced red blood cell (RBC) life span, iron deficiency, uremic toxins, HD procedure, blood loss and inflammation (Ribeiro et al., 2013). Higher targets of Hb levels have been achieved due to the use of higher ESA doses. The increased risk for adverse cardiovascular outcomes could also result from the higher ESAs doses and not only from the normalization of Hb (Santos et al, 2011). The Clinical Evaluation of the dose of Erythropoietin (C.E. DOSE) trial enrolled HD patients that were randomized 1:1 to 4000IU/week versus 18000 IU/week of I.V. epoietin alfa or beta, or of any other ESA in equivalent doses. The primary outcome was death, non fatal stroke, non fatal myocardial infarction and hospitalization for cardiovascular causes (Strippoli, 2010).
The age of our population group did not positively correlate to the haemoglobin levels (R=0.427) and (p=0.08); this is statistically not significant. However, our older patients presented with lower Hb levels as that to the younger patients as was found in our patient population group. A similar finding as been reported there could be some specific differences in the Hb target for such HD patients. Many of the existing guidelines recommend the same target Hb across all ages. High Hb might be beneficial for young patients with high physical activity. The elderly population might tolerate low hemoglobin levels. Further investigation of individualized anemia management is required (Hanafusa et al., 2014).

The elderly haemodialysis patient population is growing at a fast rate and achieving the desired Hb levels for this age group is of importance. However, minimal information is known about the relationship between hemoglobin level and survival according to age. Similarly studies stated that the differences in the effect of Hb on survival between the elderly and nonelderly populations, showed that nonelderly patients had increased risk if they had Hb<10 g/dL, whereas only for the <9 g/dL for the Hb category among the elderly population (Hanafusa et al., 2014).

The epidemiology of the HD population has been changing worldwide. The elderly HD population is growing (Collins et al., 2012). Old people experience a high incidence of
cardiovascular complications and reduced level of activity in daily life (Foley et al., 1998; Nakai et al., 2012). It is known that increased Hb does not necessarily improve prognosis in patients with cardiovascular disease (Maekawa et al., 2008). Moreover, many elderly patients have low Hb levels despite the high doses of erythropoiesis stimulating agent (ESA) received (Nakai et al., 2008).

This was also evident in a prospective randomized controlled trial recruiting 1255 haemodialysis patients with type 2 diabetes mellitus, aged 18–80 years, from 178 German dialysis centres presented older age and male sex were predictors of ESA resistance (Schneider et al., 2013). Previous findings by Panichi and colleagues showed that patients who belonged to the highest ESA resistant group were older and predominantly male (Panichi et al., 2011). Similarly, in an observational study of 1710 patients, ESA resistance was associated with older age female sex was a risk factor for ESA resistance in this study (Lopez-Gomez et al., 2008). Age and sex however are unmodifiable risk factors. Therefore the identification of potentially modifiable factors is of particular interest (Schneider et al., 2013).
Erythropoietin (EPO) and Haemoglobin (Hb) levels

Our data displayed no significant correlation with Epo levels and Hb. We summarized the patients and the controls. Erythropoietin (EPO) and Hb levels did not correlate with each other $R=0.24$ and $(p=0.895)$, this was not significant. Another study demonstrated clearly that there is a relative EPO deficiency in renal anaemia rather than an absolute lack even in patients with severe renal failure (CKD stage 5). EPO concentrations were neither lower than the values from renal patients without anaemia different from the normal range. With increasing stages of CKD the correlation between haemoglobin and EPO concentrations was gradually attenuated and was completely lost in CKD stage four and five. After the analysis of the regression lines of patients with CKD, a continuous decline in both slope and y-intercept with increasing stages of CKD was found. This indicated both reduced responsiveness and reduced capacity of EPO secretion (Artunc and Risler, 2007).

In a similar study, Fehr et al., (2004) investigated EPO concentrations with various degrees of renal insufficiency and anaemia. In patients with advanced renal failure (calculated creatinine clearance $<40$ ml/min), they also could not find a significant correlation between EPO and Hb concentrations whereas for patients with a clearance $>40$ ml/min a negative correlation of -0.35 was obtained. From linear regression analysis they derived a formula to calculate EPO from Hb levels (EPO in U/l¼2.5_ $(140\_Hb$ g/l$)$), which mathematically does not account for the exponential rise of the EPO levels with decreasing Hb levels. This equation is only valid for Hb values $>140$ g/l;
otherwise negative EPO values are calculated (Fehr et al., 2004). In anemic patients with Hb< 11 g/dl, EPO levels under the cutoff was considered as relative EPO deficiency, and it is suggested that many of the anemic patients in CKD stage 3 may have renal anemia. Measurement of serum EPO may be useful in diagnosis and treatment of renal anemia (Uehata et al., 2009).

A study done presented was to test the hypothesis that pentoxifylline inhibits proinflammatory cytokine production in vivo, giving rise to enhanced erythropoiesis (Cooper et al., 2004).

**Erythropoietin Dosage and Haemoglobin**

Our overall results showed a positive correlation with the Hb levels and the Epo doses, as seen in the graph plot (Fig 4.2). (The p value =0.01) this is statistically significant. R = 0.646. Our population group attained higher haemoglobin levels with a relative higher dose of EPO.

We thereafter compared the erythropoietin (EPO) dosage requirements to the haemoglobin levels for our patients. The average weekly doses that are administered are 4000iu, 8000iu, 12000iu, 18000iu respectively. There is a population of our patients that has increased EPO dosages without the similar outcome of increased haemoglobin.
This has been seen in other studies as well as reported in patients with lower haemoglobin values received larger doses of ESA (Kainz, 2010). This was also shown by other studies Lopez-Gomez, (2008) who reported greater co-morbidity in patients with severe anaemia. Recent studies revealed evidence that indicates more harm than benefit from targeting higher haemoglobin levels with ESA therapy. Patients who needed higher doses of ESA experienced increased mortality at any haemoglobin level, and patients who achieved target haemoglobin levels had better outcomes than those who did not (Badve et al., 2013). It is also known from other studies that some co-morbidities like antecedents of malignant neoplasm are associated with EPO responsiveness. Cytokines like IL6 are induced by malignant tumours and may impair erythropoiesis. Also, TNF-α is known to inhibit this pathway (Miller, 1990).

The haemoglobin levels from our data did not change much with the increased dosages of epo. This is however significant and further investigation regarding this will be beneficial. Other studies reported that MHD patients who required higher doses of ESA to maintain their hemoglobin levels had a higher risk for death. Haemoglobin over time was associated with a better survival independent of the baseline hemoglobin level, whereas a fall in hemoglobin conferred increased death risk. Both of these associations displayed a dose-response phenomenon. Their reports showed that a higher magnitude of rise or fall in hemoglobin was associated with a greater or lower chance of survival, respectively (Regidor et al., 2006).
Other studies analysed the role of clinical parameters for ESA resistance. This was done as information on patient’s demographic characteristics is easily accessible in the daily clinic routine and free of additional costs. In the study population (n = 1015), univariate analyses revealed that older, male patients with previous or current smoking habits had and a shorter dialysis vintage were associated with ESA resistance (Schneider, 2013).

Our creatinine and urea levels were analysed using Logistic regression analysis for EPO resistance. For creatinine (p value = 0.372), odd ratio - 0.998 and confidence interval [0.996 – 1.001] and urea (p value=0.570), odd ratio – 0.969 and confidence level (Table 4.7). Both of the levels of creatinine and urea are not significant to the EPO resistance. There is currently no conclusive evidence in the literature reporting significant differences in erythropoietin doses between dialysis patients receiving high dose HD and conventional HD (Rocco et al., 2011).

**The effects of erythropoietin weekly dosage on blood pressure**

The weekly dosages of recombinant erythropoietin beta were compared with results of the blood pressure (systolic and diastolic blood pressures). This is a positive correlation of the EPO weekly doses and the blood pressure (systolic and diastolic blood pressure) as represented in the graph (Figure 28); the systolic and diastolic blood pressures increased with the erythropoietin dosages.
This was also observed in another study where the patients who had received higher dosages of RHuEPO had higher mean arterial pressures (MAP) as that of the patients whom received lower dosages had presented with the lower range of MAP (Figure 25). An increase in arterial blood pressure and possibly hypertension which affects 1–10 users in 100 in ESA-treated CKD patients is most undesirable (Jelkmann, 2013).

Erythropoiesis stimulating agents (ESAs) are contraindicated in patients with uncontrolled hypertension. The increase in blood pressure can be partly explained by the elevated blood viscosity and the reversal of hypoxia-induced vasodilatation in association with the increase in Hb concentration. Patients with non-renal anemia do not usually develop hypertension on ESA therapy. The use of ESAs may increase the incidence of thromboembolism and the risk of cardiovascular events, including death. It seems likely that the occurrence of cardiovascular events is partly related to the elevation in Hb concentration and haematocrit (Jelkmann, 2013).

The mean arterial pressures in our study showed significant increases with the increase in erythropoietin (EPO) therapy dosages (Figure 29). The \( p = 0.008 \) this is statistically significant. There is a positive correlation \( R = 0.398 \) of the mean arterial blood pressures and the EPO doses.

There is overwhelming evidence from clinical studies that EPO treatment induces significant and sustained increases in mean arterial Bp in both normal patients and
patients with CKD. Among CKD patients, the incidence of hypertension is EPO dose-dependent. Erythropoietin apparently exerts its strongest hypertension effect in the HD patient population (Krapf and Hulter, 2009).

Another author had reported similar effects of EPO on blood pressure, the up regulation of EPO in plasma, in addition to in vitro lung effects, suggested that EPO may be an important factor in the development or progression of PAH. Future studies of the antigens characterized in our study and the role of EPO in PAH are warranted (Karamanian et al. 2014)

**Anthropometric measurements**

Skin fold thickness and haemoglobin levels were compared with each other. There were no significance of the skin fold thickness and haemoglobin levels, \( p = 0.745 \) and indicated no correlation, \( R=0.55 \) (Figure 31). Poor nutritional status has been associated with worse patient survival in maintenance haemodialysis patients. Anthropometric values are important nutritional measures, incorporating muscle and fat mass. However, the association of changes in anthropometry, including mid arm circumference (MAC) and skin fold measurements, with mortality in haemodialysis patients remains unknown (Su et al., 2013). Another study revealed that the magnitude of correlation was great between different anthropometric variables and the different inflammatory variables with strong association for BMI and waist circumference (Arbel et al., 2012). The Hemo
study of 1,846 haemodialysis patients decrease measurements in skin fold thickness were not associated significantly with outcomes except for participants with BMI ≤25 kg/m². Declines in MAC are associated significantly with all-cause mortality and cardiac outcomes in haemodialysis patients, most notably in those with BMI ≤25 kg/m² (Su et al., 2013).

The methods that measure percentage body fat and muscle mass assessment of the nutrition status of haemodialysis patients should be used together with the serum albumin levels. This cohort study had been done with anthropometric measurements of 271 patients included skin folds, mid-arm circumference, mid-arm muscle circumference, percentage of body fat, body mass index, body height, and dry weight (Stosovic et al., 2011). However, other data of a cohort of 331 maintenance haemodialysis (MHD) patient’s poor appetite correlated with an increased erythropoietin dose requirement and hence a higher risk of refractory anemia. However, markers of body composition such as BMI, percentage body fat and upper arm skin fold thickness and muscle circumference did not correlate with appetite intensity (Kalantar-Zadeh et al., 2004).

The high prevalence of malnutrition, the impact of this complication on morbidity and mortality, and the characteristics of the patients, are reasons to highlight the importance of nutritional therapy in the HD population. Early indications that nutritional markers improve after androgens (potential to be valuable as adjuvants or substitutes for RHuEPO in the treatment of renal anaemia in select patients) therapy in dialysis
patients have been clearly confirmed by recent studies. Patients receiving nandrolone decanoate (NAND) showed a significant improvement of anthropometric and biochemical nutritional variables when compared with subjects treated with RHuEPO. Androgens seem to offer partial solution for the treatment of renal anaemia in the RHuEPO era (Navarro, 2002).

**Albumin**

Our results showed that haemoglobin levels and albumin levels did not correlate with each other, $R = -0.288$ and ($p=0.80$) this is not significant (Figure 34). We further analysed the erythropoietin levels and albumin levels (Table 11). The erythropoietin levels in our study did not correlate with albumin levels $R = -1.40$ and ($p =0.437$) this was not significant. Another study reported that serum albumin concentration is an important predictor of both baseline Hb and EPO sensitivity in chronic haemodialysis patients. Factors that improve serum albumin may also improve Hb in haemodialysis patients (Agarwal et al., 2008). A large German multicentre retrospectively analysed albumin in relation to Hb target and ESA doses supported the findings that there is a strong relationship with ESA therapy (Costa et al., 2009).
C Reactive Protein and haemoglobin levels

C Reactive Protein (CRP) and haemoglobin levels did not correlate with each other, \( R = -0.112 \) (\( p = 0.571 \)) this was statistically not significant (Figure 27). The correlations of the both variables are also indicated in Table 11. Other studies reported on biological monitoring of case studies revealed episodic elevations of C-reactive protein (CRP) ranging from 1 mg/l to 60 mg/l associated with a decrease of hemoglobin (Hb) levels without any other obvious cause of resistance to RHuEPO. Serum ferritin, intact parathyroid hormone and aluminum levels were within the normal ranges. The mean \( Kt/V \) was 1.15. The patient had no malnutrition and patients native fistula showed no malfunction. Therefore authors suggested that chronic inflammatory syndrome (CIS) highlights the importance of screening for vascular calcification in the early stages of kidney disease and during the haemodialysis follow-up (Amrani et al., 2014).

Erythrocyte Sedimentation Rate (ESR) and Haemoglobin (Hb)

The erythrocyte sedimentation rates (ESR) and haemoglobin levels were statistically analysed during the trial period (Figure 30). There were no correlations between the erythrocyte sedimentation rates (ESR) and haemoglobin levels (\( R = -0.26 \); \( p = 0.898 \)), which is not significant.
% Transferrin Saturation

Our results represented no correlation between the % transferrin saturation (T Sats) and the haemoglobin levels. The Person’s coefficient, $R=0.55$, ($p = 0.745$) and it is statistically not significant (Figure 32). However, a larger use of iron therapy caused a substantial increase in ferritin levels. Indeed, in these patients the median ferritin level increased from 556 to 650ng/mL with 34% of the patients exceeding the value of 800ng/mL. Conversely the percentage of patients with $TSA\geq 50\%$ remained around 10%. Interestingly, every 100 mg of IV iron raised TSAT by 0.43% in those subjects having T Sats values $<30\%$ but only by 0.10% in those having higher T Sats levels; Hb values remained unchanged (Robinson et al., 2012). This may suggest that targeting to T Sats levels $\geq 30\%$ with IV iron therapy does not improve erythropoiesis and exposes patients to the risk of iron overload.

Iron

The means of the ferritin and haemoglobin levels over three months (Table 9) displayed ferritin 1141.35µg/ml; 1146.56µg/ml; 1070.71µg/ml and haemoglobin 10.59g/dL; 10.89g/dL; 10.65g/ dL respectively. The levels for three months did not change greatly. These observations suggest that Hb levels $<11g/dL$ are achieved with mean ferritin in the thousand to thousand one hundred µg/ml. Haemoglobin levels and ferritin levels were compared with each other (Figure 33). The haemoglobin and ferritin levels did not
correlate with each other, R=0.73 and (p = 0.662) this is statistically not significant. This indicates that the haemoglobin levels are not totally dependent on the level of ferritin.

Another study reported that Iron deficiency, whether functional or absolute, is the most common factor that limits the supplementation among maintenance haemodialysis patients in Japan is far lower compared with Western countries (Yamamoto and Tsubakihara, 2011). In the 2007 Japan-DOPPS (J-DOPPS) study, the mean ferritin level for the maintenance haemodialysis patients studied was 244ng/ml, and the percentage of patients with a ferritin level of100ng/ml was 41.3%. Notably, 47.2% of the patients with hemoglobin of 11 g/dL had a ferritin level of 100 ng/ml and only 40.6% received intravenous iron. These observations suggest that a substantial percentage of patients could achieve hemoglobin of 11.0 g/dL without iron supplementation (Hasegawa et al., 2011).

Consistently, high ferritin level, large fluctuations in ferritin level and hypo responsiveness to ESA are associated with high risk for CCVD, infection and death. Patients with a stable target hemoglobin level have less risk for adverse events compared with other maintenance haemodialysis patients whose ferritin level is in the lower range of the KDIGO guidelines. This was reported from a study done in Japan with sixty dialysis centers over a three year period (Kuragano et al., 2014). Iron stores and inflammation, anemia and hypoxia also affect iron metabolism. These stimuli would be expected to decrease hepcidin production and remove the inhibitory effect on iron absorption and iron release from macrophages so that more iron is available for
compensatory erythropoiesis. It was confirmed that these effects indeed take place (Weinstein et al., 2002 and Nicolas et al., 2002). Hepcidin may be the principal iron-regulatory hormone, the key mediator of anemia of inflammation, and a bridge between innate immunity and iron metabolism (Ganz, 2003).

Iron stores and inflammation, anemia and hypoxia also affect iron metabolism. These stimuli would be expected to decrease hepcidin production and remove the inhibitory effect on iron absorption and iron release from macrophages so that more iron is available for compensatory erythropoiesis confirmed that these effects indeed take place (Weinstein et al., 2002 and Nicolas et al., 2002).

The wide range of ferritin iron content in a pilot study underlined the problems using serum ferritin as a marker of iron stores. Other chemokines other than pro-inflammatory cytokines such as IL-6 may be associated with secretion of ferritin with low iron content. Hepcidin levels correlated with CRP levels, but not with ferritin iron content or serum ferritin. Larger studies are needed to clarify which factors determine ferritin iron content in CKD patients (Barany et al., 2009).

Lower albumin levels as well as CRP levels independently predicted ESAs resistance. Strategies to improve nutritional status and to lower the burden of inflammation are likely to play an important role in order to avoid ESA resistance as well as to prevent cardiac events in these patients (Schneider, 2013). In Kwa Zulu Natal we have to place
emphasis on the haemodialysis population needs and prioritize malnutrition and inflammation. Hyperthyroidism contributes to the impaired response to recombinant erythropoietin in patients with renal disease. Pathologic links between anaemia and the parathyroid hormone (PTH) include reduced erythropoiesis (Drueke and Eckardt, 2002).

**Antibodies**

Our results indicated that the antibodies have no contribution to erythropoiesis. There has been no correlation with all of the variables that were monitored during the trial period of the study (Table 4). Antibodies are found to have a negative effect on erythropoiesis with extreme cases of PRCA being reported and antibodies being responsible (Casadevall et al., 1996). The antibody mean levels as per the optical density (OD) were between ±3.602 and ±3.754 OD during the months February, April and July months (Table 9). The incidence of anti-EPO antibodies is high among haemodialysed patients treated with recombinant human-erythropoietin and is associated with increased incidence of anemia possibly due to immune-mediated inhibition of erythropoiesis (Alqahwaji et al., 2014). Anti-EPO antibodies developing after the use of one EPO products may cross-react with one of the other commercially available recombinant erythropoietin products (Casadevall et al., 2002). It has been found that up to 67% of patients treated with RHuEPO to correct anemia of chronic renal failure developed anti-EPO antibodies (El-Din et al., 2010). Another study found that Hb tended to be inversely proportional to the level of anti-EPO antibodies (Howman and Kulkarni, 2007).
Erythropoietin Resistance

Patients requiring very high doses of EPO to reach Hb of 11g/dL, and they are remaining anaemic after at least three months of adequate EPO doses, this is considered to be EPO resistant. Those who responded to usual EPO doses were labelled EPO responsive. The bioassay was used to quantify cell proliferation and cell viability in the presence of EPO. The UT 7 cells cultured in medium, in the presence of serum from the EPO resistant, EPO responsive patients, healthy and control. The standard curve, drawn with the known ATP concentration value on the X axis and calculated RLU value on the Y axis is shown in Figure 37. The straight line equation $y=0.0004x + 0.383$ was used to determine the ATP values of the test samples.

The experiment showed a reduction of ATP stimulation between the medium and the serum (Figure 38). The UT 7 cells alone with the sera, the serum containing wells statistically significant. The difference is found, $p < 0.0097$. There is a significant reduction of ATP proliferation in UT 7 alone wells compared to those exposed to any of the sera. The ATP production in UT 7 cell lines without serum was statistically higher with serum from any of the three from the subject group (EPO resistant serum, EPO responsive serum and the healthy controls serum. This was statistically significant ($p<0.0097$). There was no statistically significant difference in the UT 7 cell line cultured in the presence of EPO resistant serum, EPO responsive serum and the healthy controls serum. This could be due either to the fact that the serum contains less EPO or
to inhibition in the sera. Recently as proposed certain compounds such as cytokines or hepcidin contribute to the poor response of erythropoiesis. However, no significant difference was found between the serum from the resistant, not resistant, healthy individuals and control.

Erythropoietin resistance was analysed using Logistic Regression for different variables (gender, age, height, arm circumference, skin fold thickness, Hb, ferritin, albumin, transferrin, % T Sats, ESR, CRP, MAP, weekly dose, EPO level, creatinine, urea, GFR, systolic, and diastolic BP) as shown in Table 12. The variables were statistically analysed using p value, odd ratio and confidence level. All variables analysed were statistically not significant (p > 0.05). The majority of the variables were not associated with EPO resistance. We analysed the mean arterial blood pressures and there was a significant association with the positive odd ratio- 1.066, (p=0.041) and confidence interval [1.003 – 1.133]. The systolic and diastolic blood pressures were closely significant and associated with EPO resistance but did not reach the statistical values of significance. For systolic BP (p =0.068), odd ratio- 1.040 and confidence interval-1.040), this was not significant. For diastolic Bp (p=0.068), odd ratio- 1.040 and confidence interval-1.040), this was also not significant.

The patients age has been closely near to being significant (p =0.073). This is closely and positively associated with EPO resistance. The lack of difference between EPO responsive and EPO resistant needs further investigation as these results are based on the selection of a limited number of patients and controls (Table 12). Haemoglobin as
well is not significant but the \( p = 0.097 \) was close to significance when we analysed the logistic regression analysis very closely with EPO resistance. The variables that were very close to the value of significance could be due to us requiring a larger number for our population group. A larger population group is required to analyze this data to benefit the further understanding of EPO resistance.

At the moment no conclusive evidence on the neutralizing antibodies, the additional serum from healthy individuals, EPO resistant and EPO responsive did not show response. Therefore the neutralizing antibodies may not play a major role in this process. More studies are required to investigate the role of neutralizing antibodies. All sera in general have behaved the same.

The means ± SEM of all the clinical parameters of patient's, controls and \( p \) values are indicated as in Table 5. Age within the patient group and healthy controls \( (p = 0.035) \) is significant, other variables within the healthy group was not measured. The means ± SEM of all the laboratory findings of patients, controls and \( p \) values are indicated as in (Table 6). Erythropoietin levels within the patient group 22.67 ± 5.41 and healthy controls10.28 ± 2.34 \( (p= 0.4429) \). Haemoglobin levels means 10.03 ± 0.28 in patient group and in healthy individuals SEM 14.03 ± 0.51 \( (p <0.0001) \).The means of ± SEM for the inflammatory markers, ESR 37.03 ± 5.41, CRP 31.87 ± 9.78 (Table 7).
Anaemia is a common complication in haemodialysis (HD) patients. This anaemia is associated with a decreased bone marrow production of erythrocytes, due to the inability of the failing kidneys to secrete erythropoietin (EPO). The introduction of recombinant human EPO (RHuEPO) therapy led to a significant reduction in anaemia and improved patients' quality of life. However, there is a marked variability in the sensitivity to RHuEPO (Costa et al., 2009). We adopted the European Best Practice Guidelines (EBPG) for the management of anaemia which is failure to achieve target of Hb of 11-12g/dL with dose of EPO of 300iu/kg/wk. Six patients out of 47 patients receiving EPO were resistant. They are considered to be resistant which represents 12.76%. We have an average of 12.7.6% resistance (resistance patients- all patients receiving > 15000 IU EPO) (KDIGO, 2012; Kliger et al., 2013). Authors from another study explained that caution should be used in the specific patient populations with some particular risk factors. These include patient populations especially among the diabetic patients (symptomatic limb arteriopathy, stroke or asymptomatic ischaemic heart disease, cancer) or in those who are hypo responsive to ESA treatment. The patients that are in the control groups of trials testing partial versus complete anaemia correction Hb values ranged between 9 and 12 g/dL. In the opinion of the group it was reasonable to use ESA therapy to generally maintain CKD patients with Hb values ranging between 10 and 12 g/dL (Robinson et al., 2012).

Short term chronic kidney disease patients (CKD) produce more erythropoietin (epo) as compared to the long term CKD patients. A similar trend has been reported consistent with the known pathogenesis of CKD that erythropoietin production decreases as kidney
function worsens (Stauffer, 2014). ESA treatment used to target high haemoglobin levels in people with CKD is associated with neutral impacts on survival and increased risks of stroke, vascular access thrombosis and hypertension without any reduction in cardiovascular events (Phrommintikul et al., 2007; Palmer et al., 2010; Chan et al., 2014).

Other studies also showed that there is usually small but important minority of patients who show an inadequate response to RHuEPO, and no obvious cause (such as iron deficiency) can be found (Drueke et al., 2001). Failure to respond to RHuEPO may be due to enhanced immune activation, which is known to occur in renal failure patients (Stenvinkel and Barany, 2002; Macdougall and Cooper, 2002). Some proinflammatory cytokines (IFN-γ, TNF-α, and IL-1) suppress erythropoiesis in vitro (Means et al., 1992; Allen et al., 2002). We have recently shown that T cells from renal failure patients responding poorly to RHuEPO generate more IFN-γ and TNF-α compared with both good responders to RHuEPO and healthy controls (Cooper et al., 2003).

The Dutch multi-centre prospective cohort study analysed dialysis patients who started dialysis between January 1997 and January 2007. They found that the effect of ESA resistance, ESA dose and haemoglobin were closely related. ESA resistance was defined as haemoglobin level < 11 g/dL with an above median ESA dose (i.e. 8000 units/week in HD and 4000 units/week in PD patients (Suttorp et al., 2013). ESA resistant patients had lower GFR, lower albumin levels and higher ferritin levels. PD patients with higher ESA doses had more co morbidities and higher CRP in general,
while for HD the co morbidities and higher CRP were more pronounced in ESA resistant patients (Suttorp et al., 2013). A large study in prevalent HD patients from Japan also defined ESA resistance based on categories of ESA and Hb. They showed that patients with an ESA dose from 6,000 units/week and Hb below 10 g/dL had a HR of 1.94 for all-cause mortality (Fukuma et al., 2012). A study done in Italy found a 62% increase in mortality rate and 43% increase in cardiovascular events for those who presented with ESA resistance (Panichi et al., 2011). Higher interleukin-6 (IL-6) levels and lower albumin and transferrin saturation (TSAT) values are independent risk factors for presenting a higher ESA resistance index (ERI) (Panichi et al., 2011).

Although the etiology of resistance to RHuEPO therapy is still being researched on studies reported that inflammation seems to have an important role in resistance to RHuEPO therapy and is associated with “functional” iron deficiency (Costa et al., 2009; Locatelli et al., 2013).
CHAPTER SIX: CONCLUSION

The aim of this prospective study was to assess the contributing factors affecting erythropoiesis in chronic renal failure (CRF) patients in KwaZulu Natal. The study assessed the nutritional status of the patients by monitoring the transferrin and albumin blood levels and anthropometric analysis. The role of inflammation in erythropoiesis was analysed by measuring the CRP, ESR levels and ferritin levels. The effect of iron and ferritin levels on erythropoiesis was demonstrated. Native erythropoietin and antibodies against RHuEPO in serum of the HD was measured. The study monitored the impact of erythropoiesis in serum of CRF patients with the use of a bioassay.

This is a prospective, experimental and controlled study. Fifty nine patients were randomly selected from haemodialysis units of Addington and King Edward VIII Hospitals following an informed consent and fifteen healthy members were also selected as controls. We assessed the contributing factors affecting erythropoiesis and analysis of erythropoiesis bioassay in renal patients in KwaZulu. We measured the different variables the demographics, clinical, laboratory, the Elisa, Antibody Elisa, and the analysis of the bioassay.

Demographic parameters (age, sex), clinical parameters (weight, height, skin folding, EPO doses, Bp) were recorded. Pre-dialysis serum was used to measure laboratory markers (haemoglobin, transferrin, ferritin, albumin, ESR, C reactive protein, creatinine and urea). EPO levels and antibody levels were measured by ELISA, the optical density
of each well was determined within fifteen minutes using the microplate reader set at 450 nm. All results were statistically analysed using SPSS statistical package version 21 (IBM®). The bioassay was used to quantify cell proliferation and cell viability in the presence of EPO. The UT 7 cells cultured alone in medium, present with serum from the resistant, non resistant, healthy individuals and control. Luminescence of cells read with the Glorunner Microplate Luminometer and was recorded in relative light units (RLU).

The analysis revealed a lack of correlation between haemoglobin and erythropoietin levels. However a strong negative correlation was found between CRP and albumin level (Pearson coefficient = -0.591, p=0.001). No correlation was found between haemoglobin or erythropoietin levels and CRP or Albumin. There is a positive correlation between an association with blood pressure (p value <0.05) and mean arterial pressures. EPO dosages and Hb levels were significant (p=0.01). No correlations of EPO levels and Hb. Age and Hb were not significant (p= 0.08). The UT 7 cells with the sera, serum containing cells were statistically significant (p value=0.0097). Reduction of ATP stimulation between medium and serum was observed. No difference existed between the effect of serum from the resistant, non resistant, healthy individual and control group. Logistic Regression analysis for erythropoietin resistance, of majority different variables was statistically not significant from our data. However mean arterial pressures were significant (p =0.041), odd ratio 1066.
The highlighted finding from the study was that there was no correlation of EPO level and Hb concentration. Other factors can contribute to erythropoiesis. The EPO levels in chronic renal failure (CRF) and healthy individuals is almost the same. The second finding was the resistance to erythropoiesis and the factors such as anaemia, malnutrition and inflammation plays a contributing role in the process. The patients who responded to erythropoiesis were on very high doses of erythropoietin. Erythropoietin doses correlated positively with the systolic and diastolic blood pressures together with the mean arterial pressures. Long term cardiovascular complications to patients are a concern.

Erythropoietin (EPO) levels in patients are not a useful tool for the monitoring of its use as it does not correlate with EPO goal of red blood cells production. The antibodies are found to have a negative effect on erythropoiesis. The neutralizing antibodies are not confirmed playing a major role in erythropoiesis. From the analysis of our results the key contributing factors were EPO doses, malnutrition and age were more significant in erythropoiesis. However, the higher doses of EPO significantly increased the blood pressures. The analysis of the bioassay showed lack of difference between EPO responsive and EPO resistant patients could be due either to the fact that serum contains either less EPO or an inhibition in erythropoiesis.

As with all biologics, immunogenicity concerns may persist because of the fragility of the manufacturing process and the worldwide experience with pure red blood cell aplasia as a result of epoietin therapy. The uptake of biosimilar epoietin after approval in the
United States will depend on the balance of cost advantage against safety concerns. Competition in the marketplace will likely decrease the cost of the reference agent as well (Wish, 2014).

From our analysis of our results obtained we recommend that erythropoietin therapy should be reviewed for the desired haemoglobin levels required. Treatments for anemia include iron supplementation and erythropoietin stimulating agents. The lowest possible EPO doses for erythropoiesis are recommended to avoid other effects such as increase in blood pressure.


alternative for the malnutrition-inflammation score in assessment of nutritional risk of

Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to
the Apaf-1 apoptosome. Nature Cell Biology, 2: 469–75.

(2004). Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide


Letter of Information and Consent

Title of the Research Study:
Contributing factors affecting erythropoiesis and analysis of erythropoiesis bioassay in renal patients in KwaZulu Natal.

Principal Investigator:
Mrs Sherilene Cheryl Benjamin, student enrolled for the Doctorate Degree: Clinical Technology (Nephrology) at Durban University of Technology.

Brief Introduction and Purpose of the Study:
You are invited to be a volunteer for a research study. The information in this letter will help you understand what the research is about and how it will benefit your quality of...
dialysis. If there are any questions, which are not clearly explained in this letter, do not hesitate to ask the renal staff or investigator.

Renal patients have a decreased production of red blood cells and have to be supported by either blood transfusions or an injection that helps you to produce the red blood cells that are required by your body on a daily basis.

**Outline of the Procedures:**

This study will assess the factors affecting the production of red blood in haemodialysis patients in KZN. Patients will be divided into an experimental group consisting of thirty (30) patients who will be on RhuEPO treatment and a control group of fifteen (15) patients who will not be receiving RhuEPO treatment as prescribed by your doctor.

This study will monitor your monthly bloods taken in the haemodialysis unit.

Haemoglobin, albumin, transferrin levels and other factors affecting blood formation will be measured on monthly basis in order to determine the factors affecting erythropoiesis. Anthropometric measurements, ie. (armcircumference, skinfold thickness, weight and height) will be monitored post dialysis on a six month basis.

You are required to attend every dialysis. An extra 15 mls of blood will be drawn from your dialysis lines on a monthly basis for a period of six months together with your routine blood tests. There will be no additional risks to patients. Any remaining blood will be stored in a freezer for future investigations.

**Benefits:**

The new information gained from the study may help to improve patients quality of life undergoing dialysis therapy.
Reason/s why the Subject May Be Withdrawn from the Study:

Your participation in this trial is entirely voluntarily. Your withdrawal at any time will not affect your medical treatment. There are no risks involved.

Remuneration:

There will be no remuneration for the participant.

Costs of the Study:

The patient will be liable for the normal costs for the routine medical procedures needed; no extra costs will be added.

Confidentiality:

All information obtained in this trial will be strictly confidential.

Data that may be reported in scientific journals or published will not include information that will identify you as a patient in this study.

Persons to Contact in the Event of Any Problems or Queries:

Mrs S.C. Benjamin  Prof J. K. Adam  Prof A.G. Assounga
Principal Investigator  Promoter  Co/Promoter
0735080362  031-373 5291  031-2401325

Ethics of IRB Committee

Durban University of Technology
Faculty of Health Sciences
Informed Consent Form

Statement of Agreement to Participate in the Research Study:
I,…………………………………………….subject’s full name,

ID number…………………………………………….., have read this document in its entirety and understand its contents. Where I have had any questions or queries, these have been explained to me by ……………………………………………….to my satisfaction. Furthermore, I fully understand that I may withdraw from this study at any stage without any adverse consequences and my future health care will not be compromised. I, therefore, voluntarily agree to participate in this study.

Subject’s name .......................... Subject’s signature..........................

Date: .....................

Researcher’s name ..........................Researcher’s signature..........................

Date: .....................
Witness name .......................... Witness signature..........................

Date:..............................

Promoters name ....................... Promoters signature ......................

Date: ..............................
Re: Request to conduct research at King Edward VIII Hospital and Addington Hospital

I, Mrs Sherilene Cheryl Benjamin, a student enrolled for the doctorate degree: Clinical Technology (Nephrology) at Durban University of Technology request permission to perform a research study at the haemodialysis unit at the King Edward VIII and Addington Hospitals. The title of my research study is: “Contributing factors affecting erythropoiesis and analysis of erythropoiesis bioassay in renal patients in Kwazulu Natal.” The study will include 30 haemodialysis patients receiving recombinant
erythropoietin (RHuEPO) (Hb<9 g/dL) and 15 haemodialysis patients not receiving RHuEPO(Hb>9 g/dL)as per their dialysis prescription.

My promoters are:

1) Professor J.K. Adam, Associate Professor & Programme Co-ordinator Department of Biomedical and Technology, DUT. (tel: 031-3735291)

2) Professor A.G. Assounga, Chief Specialist, Head of Department of Nephrology, Department of Health, KwaZulu Natal. (tel: 031-2604441)

The study requires the involvement of haemodialysis patients and the collection of data and analysis of blood results. This clinical study will include the following:

a) The study will be explained to the patients and they will be given a choice to participate.
b) Informed consent forms will be given to patients willing to participate.
c) The staff of the renal unit involved will be informed of the study.
d) There will be strict confidentiality maintained at all times.
e) The study will monitor the routine monthly bloods that are done in the haemodialysis unit, ie., haemoglobin, albumin, transferring, iron, ferritin, C reactive protein (CRP) and erythrocyte sediment rate (ESR) in blood.
f) The post dialysis weight and height that are daily recorded from the haemodialysis patients will be monitored over the period of six months.
g) An extra 15 mls of blood will be drawn from the arterial blood line on a monthly basis for a period of six months together with their routine monthly bloods. The blood samples will be used to carry out the bioassays and Elisa tests at the laboratory of the Nelson R School of Medicine. There would be no expenses incurred to the hospitals.
h) Anthropometric measurements, ie., arm circumference and skin fold thickness, will be measured post dialysis together with the other predialysis assessments during the study period. Blood pressures pre dialysis was recorded and mean arterial pressures calculated. Patient’s dry weights were recorded.
i) The bloods and other information obtained will be analysed by the principle investigator and information will be available to those concerned.

This study will assess the factors affecting the production of red blood in haemodialysis patients in KZN, SA. Monitoring of these factors affecting erythropoiesis will be
conducted on a monthly basis for six months. Patients will be required to attend every dialysis session and receive their treatment regularly.

I will be grateful if this study could receive your approval as soon as possible, to enable us to commence.

Yours Sincerely

S.C. Benjamin (Mrs)

(Principle investigator)
Dear Dr. C Rangiah,

Medical Manager
Addington Hospital
P O Box 9775
Beach
Durban
4001

Dear Sir/Madam

Re: Request to conduct research at King Edward VIII Hospital and Addington Hospital

I, Mrs Sherilene Cheryl Benjamin a student enrolled for the doctorate degree: Clinical Technology (Nephrology) at Durban University of Technology.
I request permission to perform a research study at the haemodialysis unit at the King Edward VIII and Addington Hospitals. The title of my research study is: Contributing factors adversely affecting erythropoiesis in haemodialysis patients in Kwazulu Natal. My promoters are:

1) Professor J.K. Adams, Associate Professor of Department of Biomedical and Clinical Technology, DUT. (tele: 0313735291)

2) Professor A.G. Assounga, Head of Department of Nephrology, Addington and KingEdward VIII Hospital. (tele: 0312604441)

The study requires the involvement of haemodialysis patients and the collection of data and analysis of blood results. This clinical study will include the following:

a) The study will be explained to the patients and they will be given a choice to participate.
b) Informed consent forms will be given to patients willing to participate.
c) The staff of the renal unit involved will be informed of the study.
d) There will be strict confidentiality maintained at all times.
e) The study will monitor the routine monthly bloods that are done in the haemodialysis unit, ie. (haemoglobin, albumin, transferring, iron, ferritin, C reactive protein (CRP) and erythrocyte sediment rate (ESR) in blood.
f) The post dialysis weight and height that are daily recorded from the haemodialysis patients will be monitored over the period of six months.
g) An extra 15mls of blood will be drawn from the arterial blood line only on a monthly basis for a period of six months together with their routine monthly blood tests bloods and couriered to the Nelson Mandela School of Medicine by the principle investigator. The blood samples will be used to carry out the bioassays and Elisa tests.
h) Anthropometric measurements ie. (arm circumference and skin fold thickness) will be measured post dialysis together with the other predialysis assessments during the study period. Blood pressures pre dialysis was recorded and mean arterial pressures calculated. Patient’s dry weights were recorded.
i) The bloods and other information obtained will be analysed by the principle investigator and information will be available to those concerned.

This study will assess the factors affecting the production of red blood in haemodialysis patients in KZN, SA. Monitoring of these factors affecting erythropoiesis will be
conducted on a monthly basis for six months. Patients will be required to attend every dialysis session and receive their treatment regularly.

Thank you for allowing me to use the haemodialysis units to undertake this study. I will be grateful if this study could receive your approval as soon as possible, to enable us to commence.

Yours Sincerely

S.C. Benjamin (Mrs)

(Principle investigator)
Dear Sir

Re: Request to conduct research at King Edward VIII Hospital and Addington Hospital

I, Mrs Sherilene Cheryl Benjamin a student enrolled for the doctorate degree: Clinical Technology (Nephrology) at Durban University of Technology.

I request permission to perform a research study at the haemodialysis unit at the King Edward VIII and Addington Hospitals. The title of my research study is: Contributing
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2) Professor A.G. Assounga, Head of Department of Nephrology, Addington and KingEdward VIII Hospital. (tele: 0312604441)

The study requires the involvement of haemodialysis patients and the collection of data and analysis of blood results. This clinical study will include the following:

a) The study will be explained to the patients and they will be given a choice to participate.

b) Informed consent forms will be given to patients willing to participate.

c) The staff of the renal unit involved will be informed of the study.

d) There will be strict confidentiality maintained at all times.

e) The study will monitor the routine monthly bloods that are done in the haemodialysis unit, ie. (haemoglobin, albumin, transferring, iron, ferritin, C reactive protein (CRP) and erythrocyte sediment rate (ESR) in blood.

f) The post dialysis weight and height that are daily recorded from the haemodialysis patients will be monitored over the period of six months.

g) An extra 15mls of blood will be drawn from the arterial blood line only on a monthly basis for a period of six months together with their routine monthly blood tests bloods and couriered to the Nelson Mandela School of Medicine by the principle investigator. The blood samples will be used to carry out the bioassays and Elisa tests.

h) Anthrometric measurements ie. (arm circumference and skin fold thickness) will be measured post dialysis together with the other predialysis assessments during the study period. Blood pressures pre dialysis was recorded and mean arterial pressures calculated. Patient’s dry weights were recorded.

i) The bloods and other information obtained will be analysed by the principle investigator and information will be available to those concerned.

This study will assess the factors affecting the production of red blood in haemodialysis patients in KZN, SA. Monitoring of these factors affecting erythropoiesis will be
conducted on a monthly basis for six months. Patients will be required to attend every dialysis session and receive their treatment regularly.

Thank you for allowing me to use the haemodialysis units to undertake this study. I will be grateful if this study could receive your approval as soon as possible, to enable us to commence.

Yours Sincerely

S.C. Benjamin (Mrs)

(Principle investigator)
FEBRUARY – AUGUST 2010 (7 MONTHS)

Introduction to proposal
Literature review and identification of methodology
Background
Submission of proposal

AUGUST – NOVEMBER 2010 (4 MONTHS)

Permission application from the Department of Health
Consent to notify patients of study
Consent from staff in units and collection
Selection of patients (study population)

NOVEMBER 2010 – APRIL 2011 (6 MONTHS)

Lab tests
Collection of samples
Checking parameters
Checking blood levels
APRIL 2011 – JUNE 2011 (3 MONTHS)

Analysing
Presentation of results

JUNE 2011 – JULY 2011 (1 MONTHS)

Bringing all information together

JULY 2011 – SEPTEMBER 2011 (3 MONTHS)

Final write up
Correction of all information

Literature review and project write up is ongoing.
FLOW CHART- SUMMARISING THE RESEARCH PROCESS

Patient in chronic renal failure

↓

Patient attends haemodialysis treatments in Durban

↓

Selection of patients are done using the inclusion and exclusion criteria

↓

Consent forms and information letters issued by student/staff

↓

Patients not selected if they fell into the exclusion criteria

↓

excluded from the study

↓

Explanation of study can be given on request /if necessary

→→→

↓

Patient interested Patient unwilling to participate

↓

→ Consent forms signed and handed

In for collection

↓

Patient has accepted and agreed Patient disagreed to be part of the Study

↓

Research student plans out monthly bloods to be done

↓

Patient continues with their treatment
Treatment of erythropoietin continued

↓

An average of 15mls of blood taken from blood lines on a monthly basis for 6 months

↓

Sent to research laboratory → tests are done → routine monthly blood tests, ELISA, Bioassays, Anthrometric Blood pressure measurements

↓

Continuous recording of blood results

↓

Data collected and to be analysed

↓

Statistical analysis and interpretation of data

↓

Final write up, completion of thesis.
APPENDIX E

PREPARATION OF REAGENTS FOR ANTIBODY ELISA ASSAY

10 mg/L rHuEPO Beta in PBS ph 7.4

rHuEPO beta 4000iu in 3ml

1000iu = 8.3ug / 8.3ul

Total volume = 8.3 x 4 / 10 = 3.32ml

10 mg/L rHuEPO Beta in PBS ph 7.4

rHuEPO beta (4000iu) 0.3ml

PBS 3ml

30g/L bovine serum albumin (BSA)

Bovine serum albumin 35g per 100ml (35%)

5mls BSA prepared as follows

bovine serum albumin 500ul

PBS 4500ul

Horseradish peroxidase – conjugated goat anti-human IgG

Horseradish peroxidase – conjugated goat anti-human IgG 5ul
**Preparation of substrate**

The substrate was prepared with two reagents, colour reagent A (0.01 N buffered hydrogen peroxide) and colour reagent B (.35 g/L tetramethylbenzidine) in equal volumes. Both of the reagents were supplied in the ELISA kit.

**Substrate solution**

- Hydrogen peroxide in citrate buffer 11ml
- Tetramethylbenzidine 11ml

The solution was vortexed.

The substrate solution was freshly prepared and used within 15 minutes.

**Stop Solution (2N Sulphuric acid)**

Supplied from the ELISA kit
Appendix F

RESEARCH QUESTIONS

HOSPITAL

PATIENT NAME .................................................................

DATE OF BIRTH .................................................................

AGE .................................................. GENDER .........................

COMMENCEMENT OF DIALYSIS ................... DIAGNOSIS....................

DAYS OF HD SESSIONS .................. FREQUENCY ..................

EPO WEEKLY DOSAGE ...........................

BLOOD PRESSURE RECORDINGS .........................

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