THE ASSESSMENT OF NATIVE ERYTHROPOIETIN AND ANTIBODIES TO RECOMBINANT ERYTHROPOIETIN IN HAEMODIALYSIS PATIENTS

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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 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:_____I

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DR J K ADAM (D TECH: CLIN TECH)

DEDICATION

I dedicate this work to:

My heavenly father, The Lord Jesus Christ who has never forsaken me, and makes all things possible;

My late father, Mr Logan Venketas Naidoo for his motivation and inspiration; and My husband, Rev Winston Benjamin, my son, Jordan; my daughter, Trinity and to my new arrival for their continuous support and encouragement.

ABSTRACT

Anaemia being one of the most severe complications of end stage renal disease is presently being managed with treatment by recombinant erythropoietin (RHuEPO). Recombinant erythropoietin (RHuEPO) produced by recombinant human DNA technology (RHuEPO) is becoming a standard part of therapy in chronic renal failure. In such patients anaemia is a leading cause of morbidity and mortality. Haemodialysis patients have grown dependent on RHuEPO therapy due their anaemia. The assessment of native erythropoietin levels and the detection of antibody levels in blood in the study were carried out to assist in the clear target of the treatment of anaemia. Haemoglobin levels were monitored over a trial period of six months during the RHuEPO therapy. Ferritin, transferrin saturation (T Sats %) and transferrin levels were monitored accordingly. Monitoring of erythropoietin levels in haemodialysis patients has been one of the first done in our population group in South Africa.

Forty haemodialysis patients on RHuEPO therapy and ten haemodialysis patients not on RHuEPO therapy and ten healthy individuals from the Haemodialysis unit at Addington Hospital, Durban, South Africa were recruited to participate in the trial. Blood samples were collected then were centrifuged at 5 degrees celsius. Plasma was isolated, stored and subsequently used in enzyme linked immunosorbent assay (ELISA). Two ELISA were set up, one to measure EPO level and another for anti EPO antibodies. The dilutions of 1:50 were selected to detect the presence of antibodies. These have all been done in duplicates. Optical density of each sample was measured using a microplate reader at 450nm.

The haemodialysis patients receiving RHuEPO presented with higher EPO levels as compared to the haemodialysis patients not receiving RHuEPO and the healthy individuals. However, in the study the HB levels were not increased over the trial period with higher RHuEPO doses. Higher doses of RHuEPO therapy showed no clear increase in haemoglobin levels. From a total of forty patients, twelve patients (35%) in the months of August and eight (25%) in the month of November were tested positive for antibodies to RHuEPO. Using statistical analysis, no correlation was observed between the antibody levels and the erythropoietin levels. However, we did not test whether the antibodies found were neutralizing or not. Bioassays for EPO may be used for that purpose.

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LIST OF ABBREVIATIONS

^{0}C - degrees celcius

AB - antibody

AB - antibodies

ARF - acute renal failure

BI - biocore immunoassay

CFU-E - colony forming unit

CFU-G/M - Colony forming unit Granulocytes/Monocytes

CFU-M - Colony forming unit Megakarycytes

CH 4 - methane

CKD - chronic kidney disease

CRF - chronic renal failure

DOQI - Dialysis outcomes quality initiative

ELISA - enzyme-linked immunoassay

EPBG - European Best Practice Guidelines

EPO - erythropoietin

ESA - erythropoietin stimulating agents

ESRD - end stage renal disease

Fe+++ - ferric

H2O2 - hydrogen peroxide

HB - haemoglobin

HD - haemodialysis

HIF - hypoxia induced transcription factor

IgG - immunoglobulin G

IgM - immunoglobulin M

IL-3 - interleukin – 3

IV - intravenously

KT/V - dialysis adequacy

LSC - lymphoid stem cells

mIU/mL - milli units per milliliter

NESP - novel erythropoiesis - stimulating protein

OD - optical density

OPTA - Optimal treatment of renal anaemia

PBS - phosphate buffered saline

PD - peritoneal dialysis

PRCA - pure red cell aplasia

RBC - red blood cell

RHuEPO - recombinant erythropoietin

RIA - radioimmunoassay

RPM - rates per minute

RT - room temperature

SUBCUT - subcutaneous

TSAT - transferrin saturation

uL - microlitres

WBC - white blood cell

CHAPTER ONE: INTRODUCTION

The primary regulator of the growth and survival of erythroid progenitors, which mature into red blood cells, is the native glycoprotein hormone erythropoietin (Krantz., 1991). Over many years, the availability of recombinant erythropoietin (RHuEPO) has led to its widespread use in stimulating red cell synthesis for the treatment of severe anaemia associated with acute and chronic disease (Fisher., 1997). The results of RHuEPO demonstrated that the packed cell volume was restored. This decreased the necessity of regular blood transfusion in patients requiring dialysis. Recombinant human erythropoietin has been the most successful application of molecular genetic technology to the development of therapeutic agents. Two different RHuEPO products have been developed, alpha and beta, with differences in carbohydrate structure (Winnearls et al., 1988; Eschbach et al., 1987).

Recombinant human erythropoietin (RHuEPO), the glycoprotein that appeared to be immunologically and biologically equivalent to the endogenous compound. It is effective in the process of erythropoiesis and is proportional to the respective doses (Casadevall et al., 1996). The recombinant erythropoietin produced in mammalian cell lines had a widespread use. However, little information had been available regarding their immunogenic character. However, few reports have been published on the development of anti-erythropoietin antibodies (Bergrem et al., 1993; Peces et al., 1996).

Although RHuEPO had been seen to be weakly immunogenic and the production of antibodies seemed to be rare, this is a potential risk (Urra et al., 1997). Immune responses to many therapeutically used exogenous proteins have been described. The

human derived and recombinant human proteins have been less immunogenic than animal proteins (Chamberlain., 2002; Koren et al., 2002).

Pure red cell aplasia, is an autoimmune disorder in which the immunoglobulin gamma (IgG) antibodies or cytotoxic T- lymphocytes are directed against the erythroid precursors (Casadevall., 2002; Eckardt and Casadevall., 2003). Erythropoietin stimulating agents-induced PRCA had been caused by the development of neutralizing anti-erythropoietin antibodies in patients (Eckardt and Casadevall., 2003). The antibody titre decreased in all patients after RHuEPO therapy was stopped and the immunosuppressant caused a decrease in antibody levels. Erythropoiesis recovered to the level maintained prior to the treatment of erythropoietin stimulating agents (ESA) (Casadevall et al., 2002). Chronic renal failure patients developed unexplained anaemia following an initial good response to RHuEPO therapy. In all the patients anaemia was secondary to PRCA, and neutralizing anti-erythropoietin antibodies were present (Casadevall., 2002).

The optimal treatment of renal anaemia (OPTA) had been the initiative in aiming to improve anaemia to achieve target haemoglobin levels of greater than 11g/l with highest efficiency European Best Practice Guidelines (EBPG) (Thomas et al., 2003). Erythropoiesis was influenced directly via reduced erythrocyte stem cell proliferation, suppressed erythropoiesis and the endogenous or native erythropoietin (EPO) production. The accelerated destruction of erythrocytes, and the decrease of the reactive increase in EPO levels in response to haemoglobin levels was observed (Locatelli et al., 2004).

Haemodialysis patients have become dependent on the treatment of anaemia with recombinant RHuEPO which improves patient's quality of life and decreases morbidity. The aim of this study was to assess native erythropoietin and antibodies and recombinant erythropoietin in haemodialysis patients. The objectives were to observe the antibody response to erythropoietin and the effects it has on the rate of production of red blood cells. The comparison of haemoglobin levels, formation of antibodies and the levels of erythropoietin was measured.

CHAPTER TWO: STUDY BACKGROUND AND LITERATURE REVIEW

2.1 STUDY BACKGROUND

2.1.1 INTRODUCTION

Renal diseases are responsible for a great deal of morbidity and not equally major causes of mortality. To place the problem in some perspective, approximately 70,000 deaths are attributed to renal disease in the USA in contrast to about 700,000 to heart disease, 550,000 to cancer, and 170,000 to stroke (Llach, 1993). Morbidity, however, is by no means insignificant. Millions of people are affected annually by nonfatal kidney diseases. Dialysis and transplantation keep many patients alive who would formerly have died of renal failure, adding to the pool of renal morbidity (Llach, 1993). The cost of such programmes now exceeds several billion dollars annually. The anatomic and functional independence of the components of the kidney implies that damage to one almost always secondarily affects the others. There is a tendency for all forms of chronic renal disease ultimately to destroy all components of the kidney, culminating in chronic renal failure and what has been called end-stage kidneys (Sodeman, 1967). The functional reserve of the kidney is large, and much damage may occur before there is evidence of functional impairment. Chronic renal failure characterized by prolonged symptoms and signs of ureamia, is the end result of all chronic renal parenchymal diseases (Sodeman, 1967). Anaemia in chronic kidney disease is due to the decreased production of the glycoprotein hormone erythropoietin (EPO). Although EPO can be produced in many of the body tissues, erythropoietin required for erythropoietin is generally produced by endothelial cells in proximity to renal tubules (Daugirdas et al., 2001).

The decrease in production of erythropoietin due to the excretory function being lost correlates with the declining glomerular filtration rate. The severity of anaemia varies in chronic kidney disease.

2.1.2 BONE MARROW FAILURE

2.1.2.1 Pure red cell aplasia

Pure Red Cell Aplasia is a rare form of marrow failure characterized by a marked hypoplasia of marrow erythroid elements in the setting of normal granulopoeisis and thrombopoeisis. Pure red cell aplasia can be primary, without any associated disease, or arise secondarily to neoplasms, particularly thymic tumours (thymomas) and large granular lymphocytic leukaemia, drug exposures or autoimmune disorders (Eckardt and Smith, 2003). This disease, being unusual but dramatic is characterized by severe anaemia due to the isolated depletion of erythroid tissue and is believed to be an immunological dysfunction. The production and turnover of erythropoietin appear to be normal, but the bone marrow response to this hormone is inadequate (Erslev and Gabuzda, 1985).

Chronic renal failure is invariably associated with anaemia with the reduction in red blood cell count, haemoglobin content, packed cell volume (haemoglobin content is below normal level), and is proportional to uremia. The reduced red cell production, related to advanced destruction of the kidneys and inadequate synthesis of erythropoietin appears to be the dominant cause of anaemia. The administration of recombinant erythropoietin results in significant improvement of the anaemia associated with renal failure (Kumar et al, 2004).

2.1.2.2 Symptoms of Anaemia

The decreased oxygen delivery to tissues and to the heart causes fatigue and dyspnoea. The symptoms develop slowly and the patients overall sense of well-being is diminished. Patients may experience difficulty in concentrating, dizziness, sleep disorders, cold intolerance and headaches. The heart maintains the systemic oxygen delivery with increased cardiac output and left ventricular hypertrophy (Daugirdas et al., 2001). Haemostatic dysfunction, impaired immune function, diminished cognitive and sexual function may be experienced. Angina and transient ischemic attacks may be observed (Kleophas et al., 2003; Guyton and Hall, 2000).

2.1.2.3 Treatment for anaemia

The forms of recombinant erythropoietin available are epoetin alpha and darbepoetin alpha. Epoetin alpha is a glycoprotein manufactured by deoxyribonucleic acid (DNA) technology and has a weight of 30,400 daltons and a circulating half life after intravenous administration of approximately 8.3 hours. Darbepoietin alpha is a synthetic analog of erythropoietin with increased carbohydrate content which increases the molecular weight by approximately 20% as compared to native erythropoietin (Kumar et al, 2004).

2.1.3 ERYTHROCYTES

2.1.3.1 Morphology

Erythrocytes or red blood cells are the non-nucleated formed element in blood. The cytosol of the red blood cell contains haemoglobin molecules synthesised before loss of nucleus during red blood cell production. The red of these cells is due to the presence of the colouring matter haemoglobin in these cells. Each red blood cell contains about 280 million haemoglobin molecules, each carrying up to four oxygen molecules. The kidney has a hemopoietic function to stimulate the production of erythrocytes by secreting the hormone erythropoietin. Erythropoietin is the important general factor necessary for erythropoiesis. Thyroxin increases the production of red blood cells. It is one of the important factors necessary for erythropoiesis. Thyroxin accelerates the process of erythropoiesis and increases blood volume (Ganong, 1995; Guyton and Hill, 2000).

Red blood cells are disc shaped and biconcave. The biconcave contour of red blood cells helps in rapid diffusion of oxygen and other substances into the interior of the cell. The large surface area enables for absorbtion and removal of different substances. Red blood cells can squeeze through capillaries very easily. It is with haemoglobin and without nucleus (Guyton and Hill, 2000; Mckerry and Salerno, 2001).

2.1.3.2 Haemoglobin

Haemoglobin is the colouring matter of red blood cells. It is a conjugated protein. Haemoglobin consists of a protein combined with an iron containing pigment. The protein part is globin and the iron containing pigment is heme. Iron is present in ferrous form (Fe++) form. It is in an unstable form. In certain conditions the iron may be present in a stable form i.e., ferric (Fe+++).

Globin contains four polypeptide chains of which two are alpha chains and two are beta chains. The pigment is called porphyrin formed by four pyrole rings, i, ii, iii and iv. The pyrole rings are attached to one another by methane bridges (CH4). The iron is attached to the N- of globin molecule. The synthesis of haemoglobin starts in the proerythroblastic stage of erythropoiesis (Blaney, 2000; Ganong, 1995; Thibodeau, 1999).

2.1.3.3 Functions of red blood cells

Erythrocytes transport oxygen from the lungs to the tissues. The haemoglobin in red blood cells combines with oxygen and 97% of oxygen is transported as oxyhaemoglobin. Red blood cells transport carbon dioxide from tissues to the lungs in the form of carbhaemoglobin. Approximately 30% of carbon dioxide is transported in this form. Haemoglobin functions as a good buffer in red blood cells. It regulates the hydrogen ion concentration thereby maintaining acid base balance.

Red blood cells carry the blood group antigens like A agglutinogen, B agglutinogen and Rh factor. This helps in determination of blood group and blood transfusion (Ganong, 1995; Guyton and Hall, 2006).

2.1.4 ERYTHROPOIESIS

The human body generates an average of 2.5 million new red blood cells per second from the bone marrow to replenish the continuous removal of RBCs. Erythropoiesis is controlled by an intricate interaction between various humoral factors and cytokines. A specific cytokine, a sialoglycoprotein known as erythropoietin, which directly acts on certain progenitors and precursors in the bone marrow, controls the proliferation, differentiation, and maturation of RBCs. The expression of EPO is increased in the kidneys during a hypoxic state. The ultimate effect is to increase erythropoiesis in an attempt to maintain oxygen delivery to vital organs (Ng et al, 2003; Hole, 1992).

2.1.4.1 Production of red blood cells

During embryonic life, the erythropoiesis occurs in three stages: mesoblastic stage, hepatic stage and myeloid stage.

The first two months of intrauterine life, primitive red blood cells are produced from mesenchyme of yolk sac. From the third month of intrauterine life, the liver is the main organ that produces red blood cells. Some are also produced in the spleen and lymphoid organs. During the last three months of intrauterine life, the red blood cells are produced in the red bone marrow and liver. In new born babies, in growing children and in adults, the red blood cells are produced only from the red bone marrow. During disorders of the bone, the red blood cells are produced in the spleen (Sodeman, 1967; Sembulingum, 2005).

The stem cells are the primitive cells in the bone marrow, which gives rise to the blood cells. These stem cells are called pluripotent stem cells. In early stages, the

stem cells are not designed to form a particular blood cell. At this stage the cells are called uncommitted pluripotent haemopoietic stem cells. When the cells are designed to form a particular type of blood cell, the stem cells are called committed pluripotent haemopoietic stem cells. The committed stem cells are of two types namely the lymphoid stem cells (LSC) which gives rise to lymphocytes and the colony forming blastocytes, which gives rise to blood cells (Sodemane, 1967; Guyton and Hall, 2006). There are different units of colony forming cells as follows:

i) Colony forming unit – Erythrocytes (CFU).The stem cells of this unit develop into erythrocytes.
ii) Colony forming unit – Granulocytes / Monocytes (CFU-GM) These cells give rise to granulocytes (neutrophils, basophils and eosinophils) or monocytes.
iii) Colony forming unit – Megakaroycytes (CFU-M). From these cells, platelets develop (Guyton and Hall, 2006; Thibodeau, 1999)

2.1.4.2. Changes during erythropoiesis

The stem cells of the colony forming unit -E (CFU-E) pass through different stages and finally become the matured red blood cells. During erythropoiesis four important changes are noticed i.e., reduction of cell size, disappearance of nucleoli and nucleus, appearance of haemoglobin and change in staining properties of the cytoplasm (Guyton and Hill, 2000; Ganong, 1995).

2.1.4.3 Stages of erythropoiesis

The following stages are between the stem cell to the formation of the matured red blood cell as seen in (Figure 2.1).

- Proerythroblast which is the first cell derived from the stem cell (CFU-E) also called megaloblast. It has a very large nucleus and does not contain haemoglobin.

- Early normoblast presents itself where the cell is slightly smaller, and the nucleoli disappear. The cytoplasm is basophilic in nature and stains with basic dyes, and is called basophilic erythroblast which develops into intermediate normoblast.

- Intermediate normoblast is a smaller cell as compared to the early normoblast. The nucleus is still present, the chromatin network shows further condensation and the haemoglobin starts appearing. The diameter of the cell is reduced, the nucleus becomes very small. The quantity of haemoglobin increases. In the late normoblast the nucleus disintegrates and disappears.

- Late normoblast, the reticulocyte is known as the immature red blood cell and is slightly larger than the matured red blood cell. The number may increase whenever there is increased production and release of red blood cells into the circulation.

- At the matured erythrocyte stage, the reticular network disappears and the cell becomes the matured red blood cell. It is with haemoglobin and without nucleus (Guyton and Hill, 2000; Ganong, 1995; Hole, 1992).

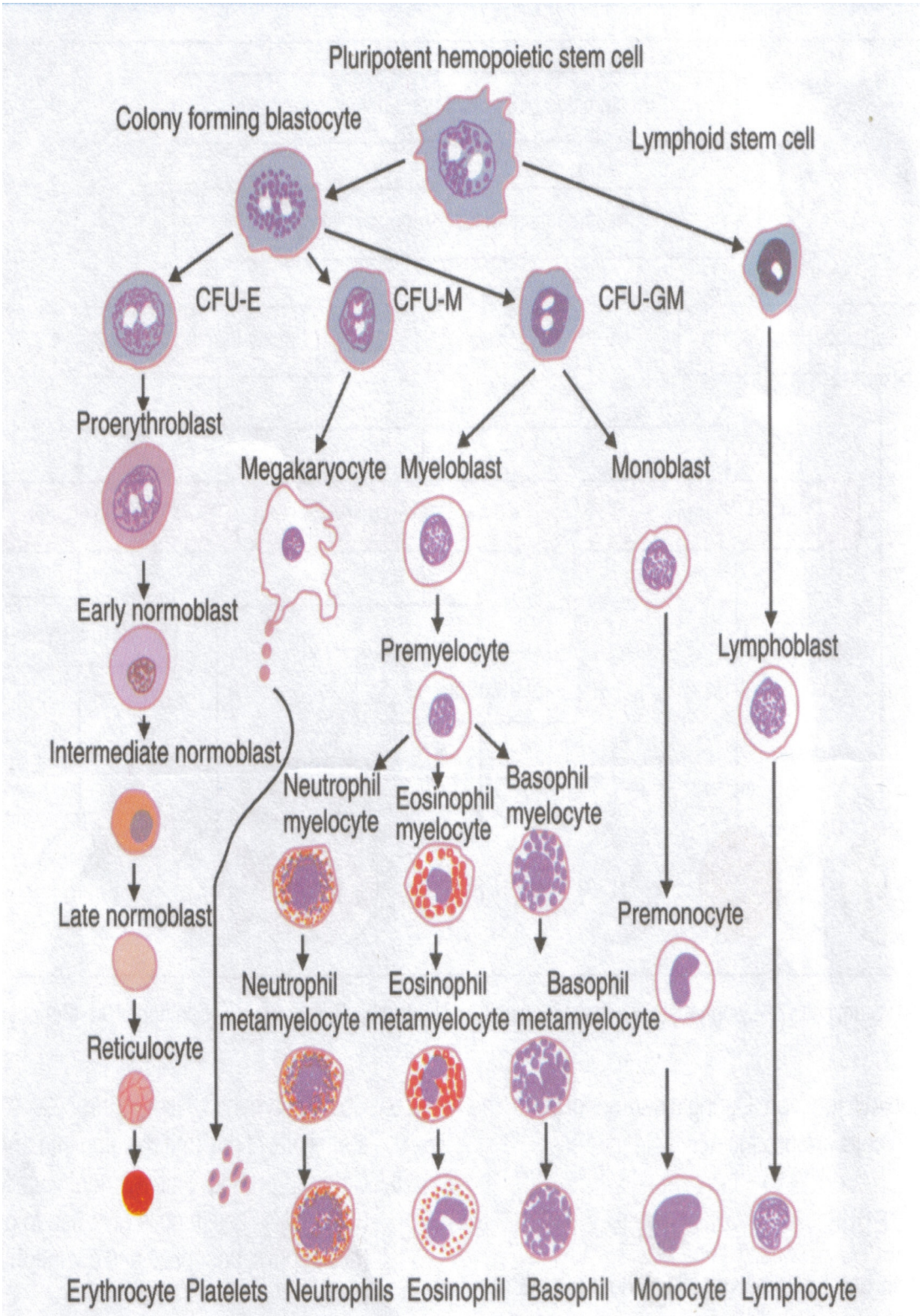


Fig. 2.1 Schematic representation of blood cell formation (Guyton and Hill., 2000)

2.1.5 ERYTHROPOIETIN

2.1.5.1 Biochemistry and Physiology

Erythropoietin in its physiologically active form is a 165-amino acid monomeric protein with a molecular weight of 30.4 kiloDalton, of which approximately 40% is carbohydrate (Tortora and Anagnostakos, 1990). The carbohydrate residues of erythropoietin are not required for its biological activity or target cell specificity but to prevent its rapid removal from circulation which is common to all other plasma glycoproteins. Erythropoietin acts by binding specifically to erythroid progenitor cells in the bone marrow, thereby inducing their differentiation and development into mature red blood cells, which are then released into circulation (Tortora and Anagnostakos, 1990). Erythropoietin is extremely hydrophobic, which are the current formulations of recombinant erythropoietin in solution contain c carrier protein (human serum albumen or artificially synthesized version) or amino acids to reduced absorbtive losses. The recombinant product has been found to be identical to native erythropoietin, possessing the same physicochemical, immunological and physiological properties, (Sembulingham, 2005; Ganong, 1995).

Erythropoietin is the most important general factor for erythropoiesis. It is also called haemopoietin or erythrocyte stimulating factor. Erythropoietin is a glycoprotein, produced primarily by the kidneys in fibroblastoid interstitial cells in the inner renal cortex, and, is secreted by peritubular capillaries. In the liver, erythropoietin is produced by both hepatocytes and interstitial fibroblastoid cells. Erythropoietin causes the formation and release of new red blood cells into circulation. The hormone promotes the production of proerythroblasts from the stem cells in the CFU

– E of the bone marrow. It also promotes development of proerythroblasts into matured red blood cells through the normoblastic stages and reticulocyte, and the release of matured erythrocytes into blood through the capillary membrane of the bone marrow (Hole, 1992; Thibodeau, 1999).

2.1.5.2 Stimulation

By the rapid increase in red cell production is a way maintained for the normal oxygen carrying capacity. This is despite the continuation of the mechanism which threatens hypoxia to the tissues. These may include a lack of oxygen, loss of red blood cells through bleeding/haemolysis and failure of synthesis of haemoglobin. The major system that controls erythropoiesis is erythropoietin. In the adult a steady state of erythropoiesis occurs within the haemopoietic marrow. Therefore, the stimulus that activates EPO production is low oxygen availability in the kidney (Guyton and Hill, 2000)

2.1.6 ERYTHROPOIETIN AND DIALYSIS

Patients in end stage renal failure and severe uraemia are supported with intermittent chronic haemodialysis. Haemodialysis is not a substitute for kidney function. The patients blood is allowed to flow through a cellophane tube permeable to nonprotein, nitrogenous material, fluid and electrolytes (Llach, 1993). In end stage renal disease erythropoietin remains deficient and anaemia generally persists. By maintaining hematocrit levels (20-25 %) anaemia remains more stable (Llach, 1993). Due to the frequency of haemodialysis required, patients lose blood in the haemodialysis circuit. Chronic blood loss in haemodialysis patients is caused through blood retention by the

dialysis lines and the filter used. Patients lose blood when sampling is done for laboratory testing, when any accidents related to vascular access occur, during surgical blood loss, and occult gastrointestinal bleeding (Roitt, 1988; Llach, 1993).

2.1.7 IMMUNE REPONSES

The immune system can prevent a disease or can aggravate a disease. The immune system recognises many different “foreign” substances (antigens) and can distinguish them from those substances that are native to the body. This system has the ability to recognise a foreign antigen and distinguish itself by means of immune response (Roitt, 1998).

2.1.7.1 Antibodies

Antibodies or immunoglobulins are produced by plasma cells in response to the presence of antigens. The immunoglobulins form 20% of the total plasma proteins. These antibodies produced by B lymphocytes, are found in almost all tissues of the body. Five types of antibodies known i.e., immunoglobulin alpha (IgA), immunoglobulin delta (IgD), immunoglobulin epsilon (IgE), immunoglobulin gamma (IgG) and immunoglobulin mμ (IgM). They are different in their sequences and in their antigenic structure (Roitt, 1988; Benjamini et al, 2000). Among these antibodies IgG forms 75% of the antibodies in the body (Guyton and Hall, 2006). Immunoglobulin G antibodies protect the body against bacteria and viruses by enhancing phagocytosis, neutralizing toxins, and triggering the complement system. Immunoglobulin M antibodies are the first antibodies to appear after an initial exposure to any antigen. Immunoglobulin delta antibodies are involved in

stimulating antibody producing cells to manufacture antibodies. Immunoglobulin epsilon antibodies are involved in allergic reactions and are located on mast and basophil cells. Immunoglobulin alpha antibodies are elevated during stress levels (Tortora and Anagnostakos, 1990; Elgert, 1996; Benjamini et al, 2000).

2.1.7.1.1 THE GENERAL STRUCTURE OF THE PORTIONS OF AN ANTIBODY

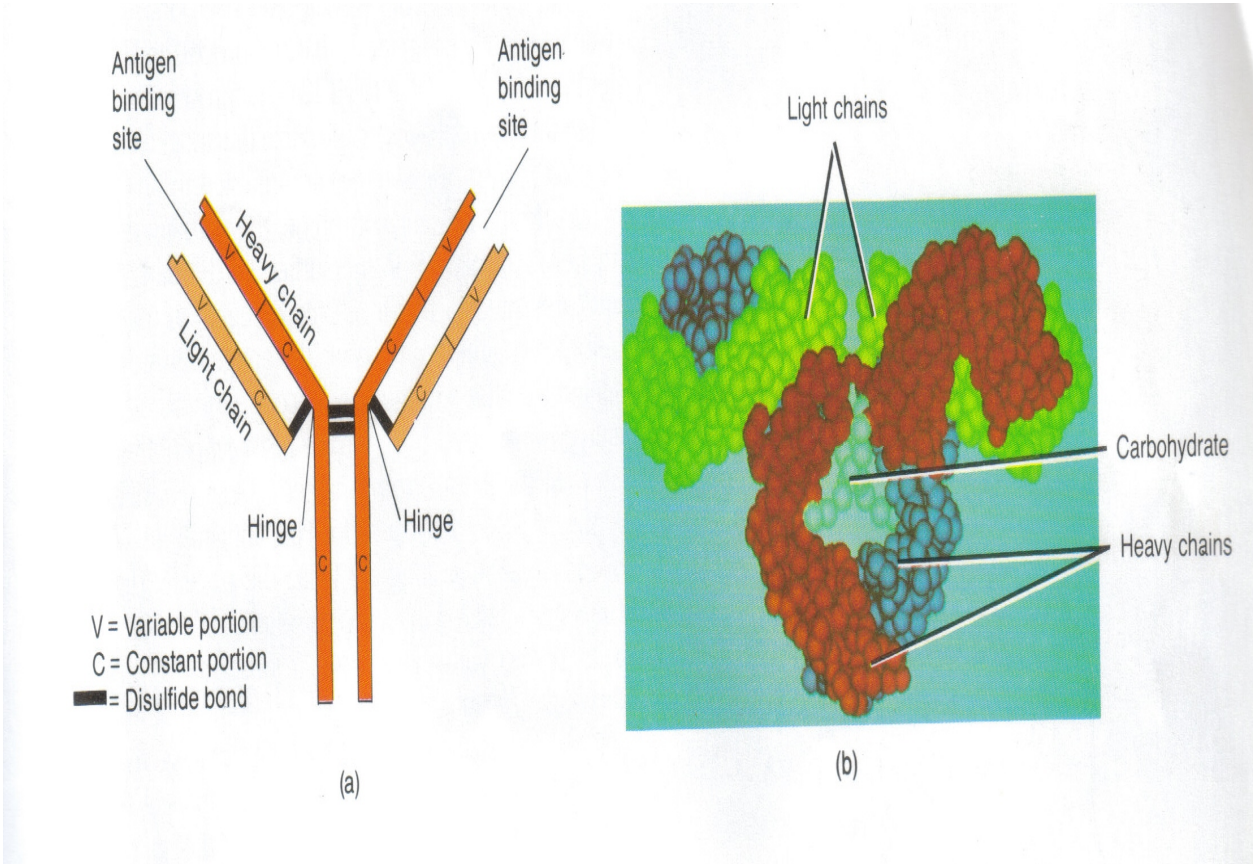


Fig 2.2 a) general structure of antibody b) computer generated model of an IgG antibody (Tortora and Anagnostakos., 1990)

Antibodies are protein and are formed by two pairs of polypeptide chains. One of the chain is called the heavy chain and consists an average of 400 amino acids. The other pair called the light chain consists of an average of 200 amino acids (Figure 2.2).

Each antibody consists of two halves which are identical to each other (Tortora and Anagnostakos, 1990). The two halves are held together by disulphide bonds (S – S). Each half of the antibody consists of one heavy chain (H) and one light chain (L). The two chains are also joined by disulphide bonds (S-S) (Figure 2.2). The disulphide bond allows the movement of the amino acid chains. In each of the antibody, the light chain is parallel to one end of the heavy chain. The light chain and the part of the heavy chain parallel to it form one arm. The remaining part of the heavy chain forms another arm. A hinge joins both the arms; (Tortora and Anagnostakos, 1990; Elgert, 1996).

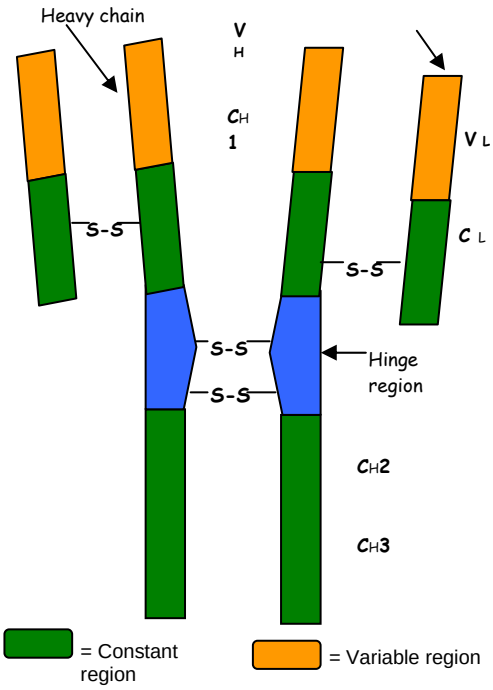


Fig. 2.3 The structure of an antibody (IgG) molecule

VL= variable region of light chain. VH= variable region of heavy chain. CL= constant region of light chain. CH1, CH2 and CH3= constant regions of heavy chains.

Within each heavy and light chain, there are two distinct regions called the constant region and the variable region (Figure 2.3). Within the constant region the amino acids are similar in number and placement (sequence) in all the antibodies of each type. The identification and the functions of different types of immunoglobulins depend upon the constant region. This region binds the antibody receptor situated on the surface of the cell membrane. The constant region causes complement fixation and is also called the complement binding region (Semibulingam, 2005; Elgert, 1996). The variable region is smaller and the amino acids occupying this region are different in numbers and placement (sequence) in each antibody. This region allows the antibody to recognize the specific antigen and to bind itself with the antigen and called antigen binding region (Elgert, 1996; Hole, 1992).

2.1.7.2 Structure of the classes of antibodies

2.1.7.2.1 Immunoglobulin Gamma (IgG)

Immunoglobulin gamma is the major antibody in blood and is able to enter tissue spaces and coat antigens together. Thus (IgG) antigen uptake is primarily induced by protein antigens, which constitutes about 80% of the antibody in serum (Elgert, 1996). The IgG has four polypeptide chains which are covalently held together by disulphide bonds. They are composed of two light chains and two heavy chains (Elgert, 1996). Human IgG consists of four subclasses (isotypes) which are numbered in order of their serum concentrations (IgG1, IgG2, IgG3, and IgG4). The four subclasses have 90 to 95% identity with each other in the C-region domains as seen in (Figure 2.4). The y chain is made up of four domains, one in the v portion and there in the C portion of the chain. The y1 chain is the shortest heavy chain, with

446 amino acid residues. On the CH2 domain of all y chains is attached one carbohydrate group that controls the structure of this domain. The main distinguishing characteristic among the four IgG subclasses is the pattern of the interchain linkages in the hinge region (Elgert, 1996; Benjamini et al, 2000).

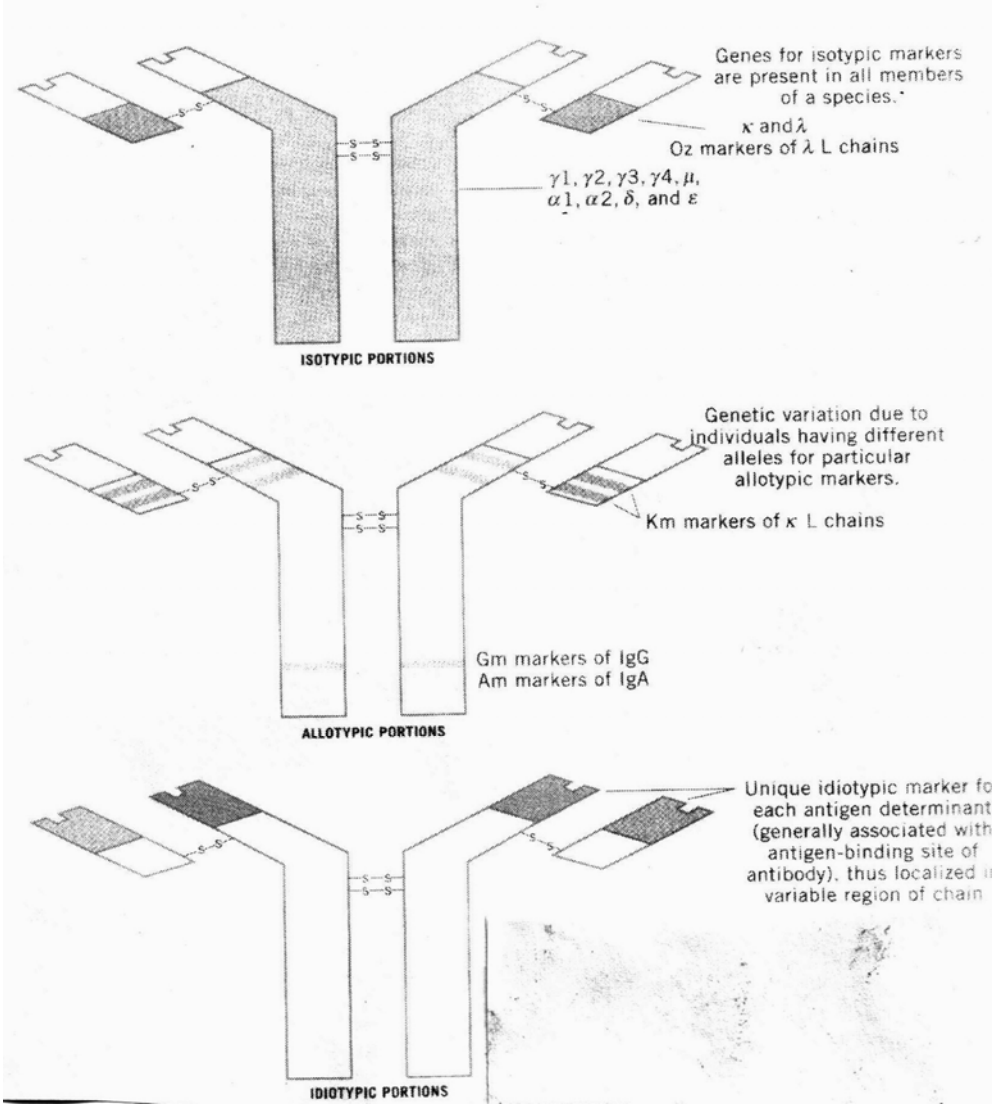


Fig 2.4 Nomenclature of antibody variability (Elgert, 1996)

2.1.7.1.2 Immunoglobulin Mμ (IgM)

Immunoglobulin M is the largest antibody and tends to remain in blood, thus successfully killing bacteria. Immunoglobulin mμ primarily induced by polysaccharide antigens, is a 950-kD pentamer and makes about 8% of the antibody

in serum. The five monomeric IgM molecules are arranged radially, the Fab fragments pointing outward and the Fc fragments pointing to the centre of the circle (Figure 2.5). Immunoglobulin mμ is the first antibody to appear during an immune response and the first to be formed by developing fetus. Immunoglobulin mu can quickly clump an antigen and efficiently activate complement due to its many antigen-binding sites. On the surface of mature B cells, IgM acts as one of the main receptors together with IgD. When IgM is a surface receptor, it is in its monomeric form (Benjamini et al, 1996; Elgert, 1996; Roitt, 1988).

The IgM μ chain consists of 576 amino acid residues, with 452 making up the C region. Unlike y and alpha chains, which have three C-region domains, the u chain has four. The five carbohydrate groups are in the CH 1 and CH3 domains and in the part of the μ chain where the j chain binds. The CH2 domain of the μ chain is equivalent to the hinge regions the alpha chains. The μ chain has two interchain disulphide bonds (Figure 2.5). The membrane form of IgM is made up of 41 additional amino acid residues, of which 25 form a transmembrane segment of hydrophobic (nonpolar) amino acids followed by hydrophilic (polar) amino acids (Elgert, 1996; Roitt, 1988; Blaney et al, 2000).

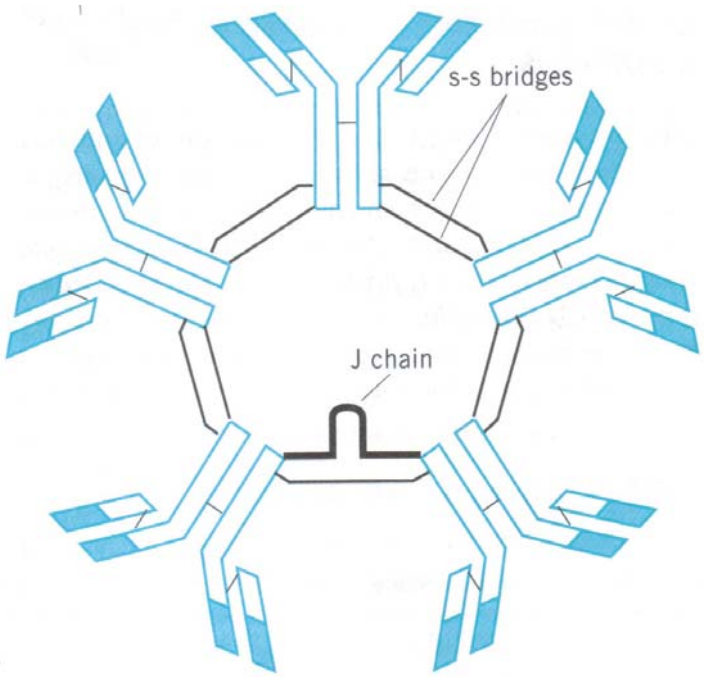


Fig 2.5 Structure of the human immunoglobulin mμ (Elgert, 1996)

This is a pentameric (five monomeric IgM molecules) polypeptide chain that has four domains in each of the heavy chains. Disulphide bonds cross-link adjacent C3 and C4 domains of individual monomeric molecules. The possible location of the J chain is given in (Figure 2.5).

2.1.7.1.4 Mechanisms of Actions of immunoglobulins

The antibodies protect the body from invading organisms in either directly or indirectly through the complement system (Figure 2.6).

Direct actions of antibodies

Antibodies directly inactivate the invading organism by one of the following methods:

-Agglutination – foreign bodies like red blood cells or bacteria with antigens on their surfaces are bound together into a clump by the antibodies

- Precipitation – the soluble antigens like tetanus toxin are converted into insoluble forms and then precipitated.

-Neutralization - The antibodies cover the toxic sites of antigenic products.

-Lysis – the most potent antibodies are active in this method. They rupture the cell membrane of the organisms and destroy them. The indirect actions of antibodies are greater and play a more important role in the defence mechanism than the direct actions (Roitt, 1988).

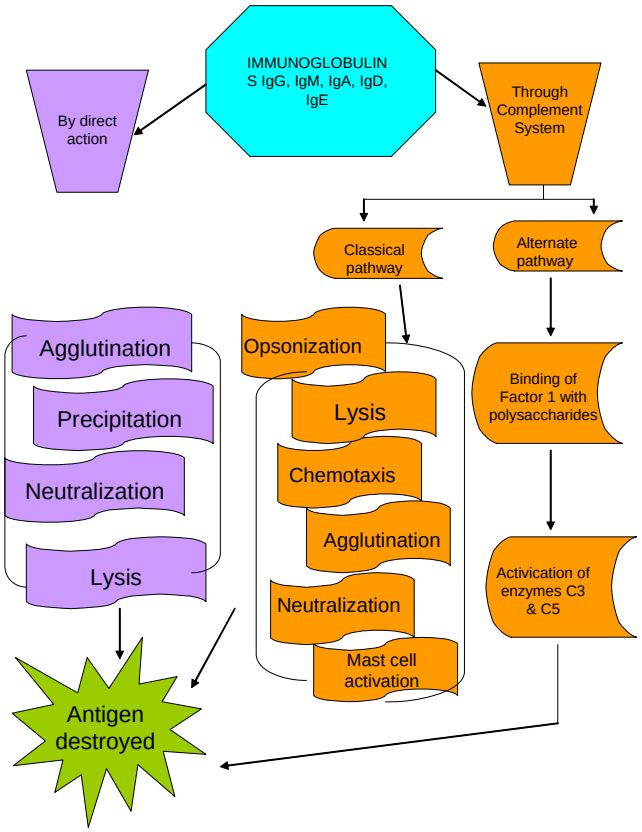


Fig 2.6 The mechanism of action of the immunoglobulins

The Complement system

The complement accelerates various activities during the fight against the invading organisms. It is a system of plasma enzymes which are in an inactive form, and are activated in two ways, the classical pathway and the alternate pathway.

A. The classical pathway

The plasma enzyme C1 binds with the antibodies and triggers a series of events in sequence. These enzymes that are formed during these events cause the following activities.

- Opsonization i.e., The activation of neutrophils and macrophages to engulf the bacteria, which are bound with a protein in plasma called opsonin
- Lysis
- Chemotaxis: i.e. attraction of leukocytes to the site of antigen antibody reaction
- Agglutination
- Neutralization
- Mast cell activation: The activation of mast cells and basophils liberates histamine. Histamine dilates the blood vessels and increases capillary permeability. Plasma proteins from blood can enter the tissues and inactivate the antigenic product (Sembulingam, 2005; Roit and Delves, 2001).

B. The Alternate pathway

This is another way in which the complement system can be activated. The protein in circulation called factor 1 binds with polysaccharides present in the cell membrane of the invading organisms. This binding activates C3 and C5, which attacks the antigenic products of invading organisms (Sembulingum, 2005; Roitt and Delves, 2001).

2.1.7.3 ANTIGENS

Antigens are substances which induce specific immune reactions in the body. They are self antigens or autoantigens present on the body"s own cells and foreign or nonself antigens which enter the body from outside. The nonself antigens are classified into two types depending upon the response developed against them in the body. The first type of antigen induces the development of immunity or production of antibodies (immunogenicity) and the second type of antigens reacts with specific antibodies, allergic activity (Sodeman, 1967; Sembulingum, 2005; Roitt, 1988).

Antigens are mostly conjugated proteins like lipoproteins, glycoproteins and nucleoproteins. The immune system can recognise a foreign substance and distinguish it from substances that are native to the body. The B and T cells of the immune system play an important role in recognizing the antigens (Roitt, 1988; Elgert, 1996; Roitt and Delves, 2001).

2.1.8 IMMUNOLOGIC TECHNIQUES

2.1.8.1 The Enzyme Linked Immunosorbent Assay

This assay uses the properties of plastics such as polyvinyl and polystyrene to absorb monomolecular layers of proteins onto their surface (Benjamini et al., 1996). This assay can be direct or indirect. Positive control serums are chemically coupled to enzymes eg, horseradish perioxidase or alkaline phosphatase. Antibodies (Abs) that bind to antigen coated wells are detected with the addition of an enzyme –coupled to horseradish perioxidase or alkaline phosphatase (Roit and Delves, 2001; Javious, 2000).

The direct method links 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, where the antigen or the cell of interest is immobilised onto the well (Figure 2.7). The primary Abs from a known host animal is allowed to bind to the immobilised target (Cruse and Lewis, 2004; Javious, 2000). A secondary antibody directed against the primary host'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 hydrogen peroxide (H2O2), which results in blue green reaction product (Javious, 2000; Cruse and Lewis, 2004).

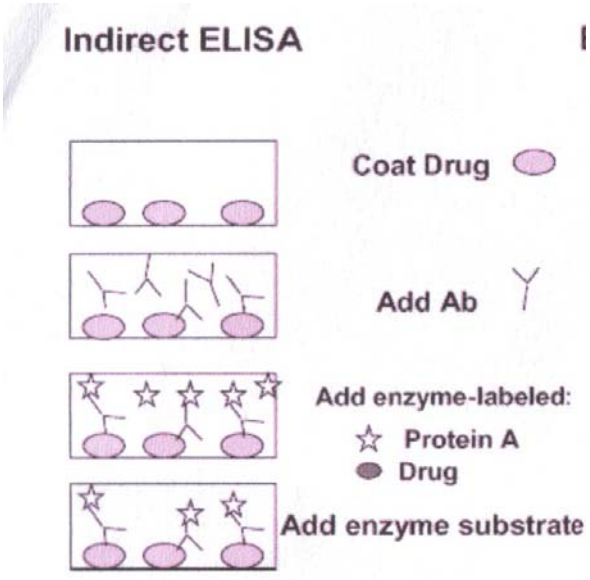


Fig 2.7 The indirect Elisa (Cruse and Lewis, 2004)

The Positive Aspects of ELISA are as follows:

- They are highly sensitive
- They are commonly used
- They are automated and nonradiometric
- Used for Ab isotype analysis
- They are inexpensive, versatile and adaptable

The Negative aspects of ELISA are as follows:

- They have high background readings, toxic enzyme reagents
- They do not accurately measure low affinity Abs
- They are susceptible to interference from rheumatoid factor (false positives) or other naturally occurring Abs
- The competition among Abs isotypes therefore, can hamper isotype analysis (Pollock, 2004; Williams and Williams, 1997; Javious, 2000).

2.1.8.2 Radioimmunoprecipitation Assays

Radioimmunoprecipitation Assays measures Abs bound to antigens in a fluid phase compared to the ELISA which detect Abs that are bound to antigens coated onto solid substrate of plastic wells. They are highly sensitive, commonly used, measure multiple parameters radiometric, nonautomated procedure requiring centrifugation or microfiltration.

2.1.8.3 BIAcore 3000 Immunoassays

They use surface plasma resonance to optically measure Abs that bind to target antigens immobilised on a special dextran coated glass surface. This procedure takes much time and is a competitive analysis. The costs involved are high. Curently there is poor access to instrument, and a lack of expertise among researchers (Javious, 2000; Pollock, 2004).

2.1.8.4 Bioassays

These can only accurately measure neutralising Abs activity against biologically active proteins. It is the only test used to measure EPO neutralising antibodies. They are moderately sensitive (Pollock, 2004, Williams and Williams, 1997; Javious, 2000).

2.2 LITERATURE REVIEW

2.2.1 THE IMPORTANCE OF ERYTHROPOIETIN IN CIRCULATION

Oxygen is required for all processes of life at a cellular level (Bun and Poyton, 1996). Red blood cells act as the oxygen transporters in blood via the high affinity of the haemoglobin molecule. Erythropoiesis regulates to maintain homeostasis and to meet changes in oxygen supply and demand. The principle factor in the regulation of erythropoiesis is the glycoprotein hormone named erythropoietin (Moritz et al., 1997).

Erythropoietin (EPO), the glycoprotein and specific hormone that stimulates red cell production was first shown to exist in circulation by Reissmann in 1950 and subsequently, Jacobson et al., (1957) found that it was produced primarily by the kidney.

Although other factors may synergise with EPO, mice that are null mutants for either the EPO gene or its receptor die about day 13 of development, demonstrating the critical importance of this hormone. The hormone is very sensitive to changes in oxygen availability, and its levels are finely tuned by changes in levels of oxygenation, by a classical negative feedback loop. Erythropoiesis is the process whereby a fraction of primitive multipotent haemopoietic stem cells becomes committed to the red cell lineage.

The oxygen carrying pigment of mammalian erythrocytes, or red blood cells, is haemoglobin, which is synthesized in BFUes. Stem cells require one major growth

factor for their replication and to express their receptor. In adults erythropoiesis requires the actions of interleukin-3 (IL-3) and EPO acting on their appropriate receptors (Moritz et al., 1997).

Renal anaemia has been one of the main factors of death and discomfort in haemodialysis patients by the associated dysfunction of many physiological functions. It is often more severe in chronic renal failure (CRF), which is due to greater suppression of erythropoiesis (Eschbach, 1994). The following functions however, improve in chronic renal failure patients but are not resolved, i.e. the decreased utilisation of oxygen, increased cardiac output and dilation, decreased cognition, concentration, and skin perfusion. Therefore, the immune responsiveness, pulmonary diffusion and menstrual cycling are also altered.

The anaemia is controlled with the treatment of RHuEPO (Paseual et al., 1991) The treatment of renal anaemia with recombinant human erythropoietin (RHuEPO) has shown to steadily improve the quality of life and alleviate the symptoms that originate in the pathophysiology of anaemia (Hampl, 2000). Eschbach (1994) documented marked improvement in quality of life, morbidity and mortality in uremic patients with increasing correction of renal anaemia.

Several clinical trials had been performed on both dialysis and pre-dialysis patients. They all showed significant improvement in their haemoglobin levels (Eschbach, 1994). The results obtained from this study apply no more, due to the recent findings. Reports have suggested that the progression of kidney disease can be slowed down by the treatment of anaemia by erythropoietin (Kuriyama et al., 1997). It is now

evident that that erythropoietin therapy has no potential adverse effect in the progression of kidney disease (Ritz, 2000).

The correction of anaemia should be considered earlier in the course of chronic kidney disease, thereby reducing cardiac damage and increasing the quality of life, before the onset of irreversible organ damage. The benefits from an early treatment of anaemia, and its long term impact on morbidity and mortality have been shown to have a great effect on dialysis patients (Eknoyan, 2001).

2.2.2 EARLY TREATMENT OF ANAEMIA IN CHRONIC KIDNEY DISEASE

Studies carried out had shown that there has been a disparity in the clinical adoption of two effective interventional measures. The treatment of hypertension early in the course of kidney disease has been widely accepted in clinical practice (Peterson et al., 1995). There has been a severe negligence in the treatment of anaemia with Epoetin which, apart from the cost issue, there had been the concern of kidney function worsening with the use of epoetin (Gracia et al., 1988).

The beneficial effects of treating anaemia in dialysis dependent patients with erythropoietin on the improvement of cardiac status, exercise capacity, cognitive function and quality of life are well established. Garabed (2001) discussed a reduction in morbidity and mortality after the treatment of anaemia with epoetin.

In the course of progressive kidney disease, hypertension has been regarded as an independent risk factor for cardiovascular disease, and secondary

hyperparathyroidism. Eknoyan (2001), concluded in his study that, one will have to derive the full benefit of epoetin therapy. He had explained the negative effects of hypertension on anaemia and the contribution to the aggravation of cardiovascular disease (Eknoyan, 2001).

He further described that the evidence accrued in the course of the past decade indicated that the correction of the anaemia of kidney disease improved but did not normalise cardiac function, exercise capacity and quality of life in dialysis patients. His findings further indicated that in order to limit cardiac damage and maximise exercise capacity and quality of life, correction of anaemia should be considered earlier in the course of kidney disease, well before the onset of irreversible organ damage. The extent of the benefits that can be derived from the early treatment of anaemia, and its long term impact on morbidity and mortality of those who started on dialysis, awaits confirmation from the results of the on going clinical trials (Eknoyan, 2001).

2.2.2.1 Management of anaemia

A study carried out by Frankenfield et al (2001), significantly showed that an inadequate delivery of a haemodialysis dose (Kt/V <1.2) and poor nutritional status (based on low serum albumin concentrations were identified in the End stage renal disease (ESRD) Core Indicators Project. This had a negative impact on the correction of anaemia with Epoetin. The iron deficiency, whether absolute or functional, was clearly shown to be the most important factor responsible for the lack of response to epoetin therapy (Frankenfield et al., 2000).

Another study showed that the lowest dose of Epoetin was received by patients with adequate iron status, while those with functional iron deficiency received the highest dose. This was due to the fact that the haemodialysis patients with adequate iron status had significantly higher mean haemoglobin levels compared to all patients with absolute iron deficiency, which had higher haemoglobin levels than those with functional iron deficiency (Jacobs et al., 2000).

A survey on anaemia management had been designed to assess the care given to pre dialysis patients prior to commencement of haemodialysis or peritoneal dialysis treatment. This pre dialysis survey showed that more than 30% of the patients had been followed by nephrologists for less than six months when they started dialysis. This invalidated or reduced the effectiveness of the measures taken to delay the progression of chronic renal insufficiency and control corporeity. Cardiovascular commodities are extremely common, particularly in diabetic patients and have been shown in to be the most common cause of chronic renal insufficiency. The authors stated that being under the care of nephrologists for greater than one year has been associated with less frequent heart related complications. They stated that only a small proportion of patients received treatment with Epoetin before starting dialysis treatment. The authors further described that the patients usually start dialysis treatment with severe anaemia. The treatment with Epoetin is usually commenced at haemoglobin levels far below than those recommended by the European best practice guidelines (Valderrabano et al., 2003).

Valderrabano et al (2003), reported that their findings may have drawn attention to the risks of late treatment of anaemia and may, therefore, have contributed to the

improvement of the management of anaemia during the initial stages of chronic renal insufficiency.

2.2.3 EPO DEFICIENCY

The impression that had been created in the early days was to improve dialysis clearance. This was the removal of small and middle molecules which resulted in an increased hematocrit or haemoglobin. Haemodialysis was changed from twice weekly to thrice weekly, which resulted in improved erythropoiesis. However, routine use of red cell transfusions was found to suppress erythropoietin, and was, therefore, discontinued at the same time (Escbach et al., 1970). However, better middle molecule clearance had not improved Hb levels in dialysis patients. Neither had better small molecule clearance improved the effectiveness of erythropoietin (Locatelli et al., 2000).

The resistance to human recombinant erythropoietin (RHuEPO) therapy challenged the concept that erythropoietin deficiency was the main cause of anaemia of chronic kidney disease (CKD). In healthy individuals, serum erythropoietin levels increased logarithmically as RBC mass decreased. Patients with renal failure did not experience an increase in erythropoietin levels as anaemia progressed (Erslev et al., 1980).

This suggested that the damaged kidney was unable to increase erythropoietin secretion in response to anaemic stress. Bilateral nephrectomy was the procedure to improve blood pressure in dialysis patients. However, anaemia worsened and RBC transfusions increased. Therefore, this had revealed that the endocrine function of the

kidney persisted in the absence of any exocrine function (Kominami et al., 1971).The immediate response to RHuEPO in iron repleted dialysis patients resulted in the correction of anaemia (Eschbach et al., 1987; Winnearls et al., 1986), which suggested that erythropoietin deficiency is a major cause of CKD.

Erythropoietin deficiency remained the central focus as replacement therapy corrected anaemia in almost all iron repleted patients with CKD, provided that enough of the hormone is given. Suggestions were made in conclusion that, functional iron deficiency should be avoided aluminium levels and parathyroid toxicities to be controlled. The study further concluded that no haematological condition that affected erythropoiesis or RBC survival be developed. Inflammatory conditions were the most prevalent cause of temporary refractoriness to RHuEPO. The relationship of nutrition, serum albumin, gender and weight to individual responsiveness to RHuEPO remained to be better understood (Eschbach et al., 2002).

2.2.4 DEVELOPMENTAL REGULATION OF ERYTHROPOIESIS

Erythropoiesis occurs first in the yolk sac, then in the liver, moving to the bone marrow and in rodents, the spleen during development. The origin of the erythropoeitic precursors and some factors were important in the changing location of erythropoiesis. The major site of EPO production was the liver but recent studies have shown that the EPO gene is expressed strongly in the fetal kidney. The metanephric EPO, mRNA is increased by anaemia and decreased by glucocorticoids and contributed substantially to circulating hormone levels in haemorrhaged ovine fetuses. The placenta and the brain showed the sites of EPO and EPO receptor production which had important actions in development (Moritz et al., 1997).

In mice and rats the sites of erythropoiesis occur as in humans, but the embryonic haemoglobin is replaced by adult haemoglobin without the formation of fetal haemoglobin. The yolk sac is seeded by hemapoietic precursors by the eighth day and the liver by the tenth day (Moritz et al., 1997).

2.2.5 RECOMBINANT ERYTHROPOIETIN

2.2.5.1 Structural and Biological Characteristics

The human erythropoietin gene is encoded in chromosome 7q11-22. The N-glycosylation confers the biological activity of erythropoietin. Before the release of erythropoietin into circulation, the primary structure of a mature erythropoietin (RHuEPO) contains 165 amino acids (Figure 2.8). The molecular mass of the polypeptide backbone and the glycosylated form of erythropoietin is estimated to be 18 kilodaltons (kDa) and 30 (kDa) respectively. The secondary structure contains 50% of alpha helix movement, with the arrangement of two alpha-helical pairs running antiparallel similar to that of growth hormone. The glycosylated (or sugar) moiety of erythropoietin has an important role in terms of biosynthesis, tertiary structure of the molecule, and in vivo biological activity (Ng et al., 2003).

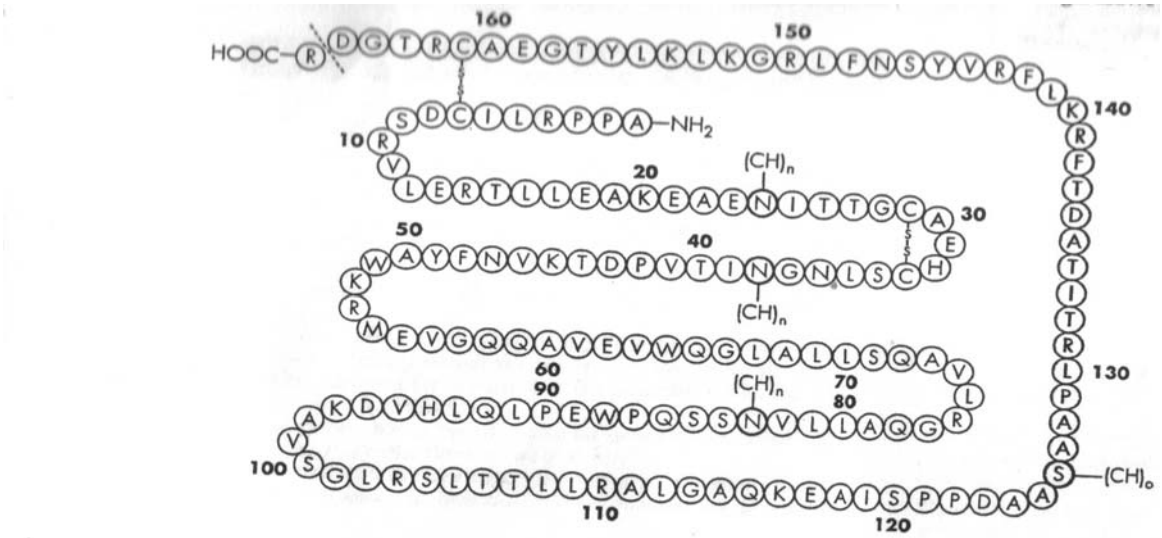


Fig 2.8 The primary structure of erythropoietin (RHuEPO) (Ng et al., 2003)

In a recent study conducted, it was demonstrated that RHuEPO had restored the packed cell volume and improved the overall wellbeing of the dialysis patient. Thereafter, RHuEPO was granted licence to be used as therapeutic agent for patients with chronic kidney disease (Winearls et al., 1986; Eschbach, 1989). The N-glycosylated moiety of RHuEPO has three main functional units: the main core, the branched portions and the terminal component, with each having a specific role (Table 2.1). The function of the O- glycosylated unit, a component constituting about 3% of the total mass of RHuEPO remained to be defined. There are four different RHuEPOs: alpha, beta, delta, and omega. The commercially available RHuEPO are the EPO-alpha and EPO-beta. Although they act on the same erythropoietin receptor, there are some differences in the pharmacokinetics and pharmacodynamics among the RHuEPOs. The recombinant erythropoietin has the same polypeptide backbone and equal number of glycosylation sites as the endogenous/native form (Ng et al., 2003).

Table 2.1 The functional units of the N – glycosylation moiety of erythropoietin

Main core sugar	Branched chain sugar	Terminal sugar
Mannose "rich"/GicNAc structure	GicNAc branches	Containing sialic acids, repeating unit of poly-N-acetyllactosamine, and galactose
Maintaining conformation of polypepfide chain	Supportive function to terminal sugars	Correlating to EPO receptor binding and interaction with other molecules
	Conferring stability of EPO in circulation	Directly correlating with in vivo biological activity of EPO
	Degree of branching positively correlating with in vivo biological activity of EPO	

2.2.5.2 Modifications of RHuEPO

An increase in the number of glycosylation sites was observed to enhance the biological activity of RHuEPO. The hyperglycosylated RHuEPO, known as the NESP (erythropoiesis stimulating protein, darbepoetin alpha) was therefore, introduced (Macdoughall, 2001).

The polypeptide backbone of the RHuEPO was modified by a process called “site mutagenesis,” which leads to the creation of five N- glycosylation sites as compared with three in RHuEPO.Novel erythropoiesis stimulating protein (NESP) had a higher negative charge and a three fold longer half life. It required less frequent dosing

schedule and produced a similar clinical outcome and safety profile to RHuEPOs in treating anaemia of chronic kidney disease and of malignancy (Smith et al., 2001). To decrease the rate of elimination and enhance the biological activity use is made of a “protective vehicle”, thereby increasing the half life of RHuEPO. Methods such as microencapsulation and pegylation to RHuEPO were currently being assessed (Ng et al., 2003).

2.2.5.3 Functions of erythropoietin

Erythropoietin is essential for the proliferation, differentiation, and maturation of RBCs in bone marrow. Erythropoietin was observed to be critical for the survival of RBC progenitors in bone marrow and to have immunomodulatory activity (Silva et al., 1996; Huraib et al., 1997).

2.2.6 ADMINISTRATION OF RHuEPO

2.2.6.1 Route of administration

2.2.6.1.1 Mechanisms for increased efficiency of subcutaneous administration

The efficiency of subcutaneous administration has been ascribed to the prolonged half life of RHuEPO. Kaufman (1988) described the influence of erythropoietin on erythrokinetics. It had been discussed that under the influence of several growth factors, including erythropoietin, interleukin-3, granulocyte-macrophage stimulating factor, stem- cell factor, and insulin-like growth factor 1, the burst-forming units, erythroids are stimulated to divide and differentiate into colony forming units

erythroid. These cells had the highest density of erythropoietin receptors. Erythropoietin stimulation caused these cells to proliferate and become erythroblasts. Erythroblasts eventually lost their erythropoietin receptors and matured into circulating red blood cells (Kaufman, 1998).

Proinflammatory cytokines, such as tumour necrosis factor, interleukin-1, interleukin-6, transforming growth factor-beta, and interferon gamma were shown to interfere with erythroid maturation, by the inhibition of erythropoiesis and of production of erythropoietin (Kaufman,. 1998). Taniguchi et al (1997) stated that the inhibition of erythropoiesis could occur through the down regulation of the erythropoietin receptor. However, a further study showed the cytokines that were produced during inflammation could account for the anaemia in chronic kidney disease. These cytokines were also produced during haemodialysis, due to the contact of blood mononuclear cells with the haemodialysis membrane (Dinarello et al., 1990).

Kaufman (1988) explained further that the biological mechanism could contribute to the benefits of subcutaneous administration. However, he stated that there had been no direct evidence for this mechanism and it could just be hypotheses. His final explanation was that this could be due to the prolonged half life of subcutaneously administered erythropoietin. This resulted in sustained stimulation of the progenitor cells. The kinetics of erythropoietin binding to its receptor and subsequent downstream events were not well characterized. It is also not known whether sustained receptor occupancy, resulted in greater proliferation of red blood cell precursors (Kaufman, 1998).

Kaufman (1988) added that the suppressor cytokines produced during haemodialysis lead to a relative inefficiency of intravenous administration. The intravenous erythropoietin administered was present when the cytokines were at their highest levels. Subcutaneous erythropoietin has a prolonged half life, therefore EPO is available for binding to its receptor when the levels of the dialysis stimulated proinflammatory cytokines had decreased.

2.2.6.1.2 Neocytolysis

Alfrey et al (1992), proposed another mechanism for the benefits of subcutaneous administration. They suggested that when circulating erythropoietin levels are rapidly reduced, red blood cells released from the bone marrow were destroyed, hence the name neocytolysis. They have presented evidence that neocytolysis was the cause of the reduction in red cell mass. This mechanism could account for the reduced efficiency of intravenous erythropoietin administration in haemodialysis patients. Therefore, the haemodialysis patients had wide fluctuations in erythropoietin levels, with immeasurable levels on nondialysis days (Alfrey et al., 1997).

2.2.7 ADVANTAGES OF SUBCUTANEOUS ADMINISTRATION OF RHuEPO

2.2.7.1 Dosage Requirements

The reduced dosage requirement has been both clinically and economically desired and had been particularly important when long term management was necessary.

Several parallel group (De Schoenmakere et al., 1998; Eidemak et al., 1992; Jensen et al., 1996; Kaufman et al., 1998; Muirhead et al., 1992; Schaller et al., 1994; Virot et al., 1996) and crossover studies (Albitar et al., 1995; Besrab et al., 1992; Paganini et al., 1995; Parker et al., 1997; Steffensen et al., 1992; Taylor et al., 1994; Zehnder and Blumberg., 1992) were investigated. The goal of these investigators was to prove whether, the route of administration had an effect on the dosage requirements. Majority of these studies showed that compared with the intravenous route, a lower dose of EPO along the subcutaneous route was required to maintain the haemoglobin levels in the patients with chronic kidney disease. These are seen in Tables 2.2.

In one of the largest studies carried out by Kaufman et al (2003), 208 long term haemodialysis patients were randomised and given RHuEPO. One hundred and one patients received RHuEPO intravenously, and 107 patients received RHuEPO subcutaneous administration of over 26 weeks. The outcome of this study showed that the mean weekly dose that was required to maintain the target haematocrit of 30-33% was 27-32% lower in the subcutaneous group than in the intravenous group, which was consistent in the parallel group studies.

Table 2.2 Subcutaneous RHuEPO administration in parallel and crossover group studies

Table i. S.c. RHuEPO administration with dosage reduction in patients with renal anaemia (parallel-group studies)

	Duration (weeks)	**Patients** (n)	**Dosage reduction** (%)
De Schoenmakere et al.	52	30	no change
Eidemak et al.	6 - 14	29	25
Jensen et al.	16	50	no change
Kaufman et al.	26	208	27 - 32
Muirhead et al.	24	128	25
Schaller et al.	35	90	33
Virot et al.	16	49	25

Table ii. S.c. RHuEPO administration with dosage reduction in patients with renal anaemia (crossover studies)

	Duration (weeks)	**Patients** (n)	**Dosage reduction** (%)
Albitar et al.	26	16	53
Besarab et al.	15	29	34 - 43
Paganini et al.	24	72	26
Parker et al.	40	44	60
Steffensen et al.	12	12	47
Taylor et al.	8	16	4
Zehnder & Blumberg	36	6	43 - 59

Greater dosage reductions had been observed in crossover studies. A further study investigated patients who received intravenously RHuEPO for six months before they switched to subcutaneous administration of RHuEPO for ten months, followed by a further six months of intravenous therapy. These investigators reported a 60%

reduction in the RHuEPO dosage, and maintained the haemoglobin levels, with the subcutaneous administration. There were no differences between the groups in their blood pressure, serum chemistry or dialysis adequacy (Kaufman et al., 2003).

A comprehensive randomised controlled trial had shown that once weekly s.c. Epoetin beta effectively managed anaemia in haemodialysis patients, even in patients who required a higher dose. Once weekly dosing of s.c Epoetin beta added greater flexibility and an improved capacity to tailor dosing frequency to patients needs. This had reduced the clinic time for the patients who had not self administered. It had improved the convenience of new formulations and delivery systems. The study had proved that the patient compliance would improve and self administration would be encouraged (Weiss, 2001).

2.2.7.2 The efficacy of once weekly subcutaneous Epoetin beta

A randomised controlled multicentre trial studied the efficacy of once weekly compared with two or three times weekly of subcutaneous epoetin administration. Eighty eight patients in the once weekly group and thirty patients in the control group were treated for at least 16 weeks. Stable haemoglobin levels were maintained without epoetin dose increase in 73% of the patients in the both groups. This multicentre trial concluded that once weekly subcutaneous administration of epoetin beta was safe and effective in maintaining the haemoglobin levels in stable haemodialysis patients. The two or three times weekly administration was of the same total dose. This weekly regimen decreased the number of injections per year which calculated to be an average of 104 injections (Weiss et al., 2000). Another randomised study stated that s.c epoetin beta administered once weekly to

haemodialysis patients was statistically equivalent to thrice weekly treatment in maintaining an adequate and stable hematocrit. The additional option of once weekly dosing provided greater opportunities for the individualization of treatment regimens according to specific needs (Locatelli et al., 2002).

2.2.7.3 Efficacy of Multidose Epoetin Beta

Epoetin had initially been available as a set single dose formulation, which often resulted in wastage because the unused solution was discarded. Furthermore, several vials had to be combined to provide the exact dose. It was further acknowledged that a more convenient, flexible and less wasteful method for self administration of subcutaneous epoetin would encourage this method of administration and reduce costs. It would also have an effect on the overall compliance and treatment outcomes (Raftery et al., 2000).

Thereafter, three studies had been conducted using the multidose formulation which had been recently developed in conjunction with the pen delivery device. The investigators discovered that the formulation offered a greater dosage flexibility and had been well tolerated in the healthy volunteers and patients that were selected for the respective studies with end stage renal disease (Raftery et al., 2000; Gallar et al., 2000; Franke et al., 1997). The favourable tolerability profile with the pen delivery system was consistent in 420 patients with end stage kidney disease treated (Gallar et al., 2000).

2.2.7.4 The Epoetin Beta Multidose Formulation

An open single group study was conducted at 139 German centres, to assess the tolerability and safety of the epoetin beta multidose formulation which was administered with a pen device in patients with anaemia due to renal failure and stabilised on a maintenance dose of epoetin. The study also assessed the efficacy, local tolerability and effect on laboratory variables. The outcome of the study showed that the haemoglobin levels remained stable despite the significant reduction in the epoetin beta dose. The subcutaneous administration of the multidose epoetin beta formulation with the Reco-Pen device had been well tolerated and effective (Lafferty et al., 1999).

This open group study in the 139 German centres, proved that the treatment of the new formulation of the pen device had enabled greater dosing flexibility, improving the capacity to individualize dosing to patient needs. The improved capacity to individualize the dose had contributed to the considerable reduction in subcutaneous epoetin beta dosage requirement (Kleophas et al., 2003).

Further studies revealed that the cartridge formulation of epoetin was well tolerated both systemically and locally (Franke et al., 1997). Hyperkalaemia had been reported in the early studies of epoeitin (Eschbach et al., 1987). Later studies did not support the significantly increased incidence of this effect (Buccianti et al., 1993). Ruiz et al (2000) presented no cases of hyperkalaemia in their study. Moreover, the general biochemical and iron assessments remained unchanged throughout the study, indicating the tolerability of the new formulation of epoetin beta. It was established,

however, that switching dialysis patients from the intravenous injection of epoetin to the subcutaneous route permitted the haematocrit levels to be maintained at a reduced dosage (Kaufman et al., 1998). The use of subcutaneous epoetin allowed the maintenance of haematocrit and haemoglobin levels at a lower dosage compared with the intravenous route, had the obvious cost implications for the management of patients with renal anaemia (Ruiz et al., 2000).

2.2.7.5 Subcutaneous versus intravenous Epoetin in maintenance treatment of anaemia

Recombinant human erythropoietin represented a significant advance in the treatment of anaemia associated with chronic kidney disease. Patients undergoing haemodialysis, the intravenous route of administration was chosen in the initial clinical studies because of the convenient access to the venous return catheter. In 1997, the Dialysis Outcomes Quality Initiative (DOQI) recommended the s.c route for epoetin administration (NKF, 1997). Thereafter, revision of the guidelines was undertaken and the National Kidney Foundation (NKF) recommended the s.c route of administration for haemodialysis patients (NKF, 2001).Despite these recommendations, approximately 90% of patients with end stage kidney disease in the United States were administered epoetin intravenously (McCellan et al., 2001).

However, numerous trials had shown that the subcutaneous route of administration was more efficacious than the intravenous route, allowing the same target haemoglobin or hematocrit level to be maintained at a reduced epoetin dose (Kaufman et al., 1998). Darbepoietin alfa was introduced as an alternative to epoetin

alpha. It had a longer half life than epoetin alpha and could be administered once weekly (Aljama et al., 2001). According to the darbepoetin alpha usage guidelines, there was no difference in the mean weekly s.c and i.v. doses administered to the 809 patients in their study (Aljama et al., 2001). This contrasted sharply with the epoetin doses reported in another study in which the analysis of the s.c rout was associated with as much as 32% lower weekly dose, which resulted in cost savings relative to the i.v. administration (Kaufman et al., 1998).

Beesrab et al (2002) demonstrated in their study that epoetin was an effective treatment for anaemia in patients with chronic kidney disease. The cost analysis presented in the trial showed substantial cost savings with s.c.administration compared with i.v administration. The European guidelines had recommended the use of s.c administration, which had not only a sound rationale in terms of efficacy and safety, but also had a sound economic basis.

2.2.7.6 The activity and isoform composition

The bioactivities of epoetin alfa and epoetin beta were compared in an animal model study. They were both produced by recombinant methods in Chinese-hamster-ovary cells. A significantly higher in vivo to in vitro bioactivity was seen with epoetin beta versus epoetin alfa, indicating that that epoetin beta was more biologically potent. The results of the study showed that that epoetin alfa and epoetin beta differed in their isoform composition, characterised by divergences in glycosylation pattern, providing the most likely reason for differences in potency (Storring et al., 1998). This was confirmed by a publication which reported differences in glycosylation patterns among various recombinant human erythropoietin, including epoetin alfa

and epoetin beta. The slight difference in the glycosylation was due to epoetin alfa having more sialic acid residues than epoetin beta. Additionally, these researchers found that the epoetin beta batches obtained from two different sources, were consistent and had identical charge patterns (Skibeli et al., 2001).

2.2.8 PURE RED CELL APLASIA (PRCA)

2.2.8.1 OCCURENCE

Erythropoietin deficiency being the most common cause of anaemia in patients with chronic kidney disease (CKD) has been the most common cause of morbidity and mortality and contributed significantly to the development of left ventricular hypertrophy. Since the 1990's erythropoietin (EPO) produced by recombinant human technology (RHuEPO) had become a standard part of therapy for anaemic CKD patients requiring dialysis, and the use has been increased in patients prior to dialysis treatment. Effective treatment of renal anaemia with RHuEPO, improved patient survival, decreased morbidity, and increased the quality of life. Importantly, however, there have been reports of antibodies against RHuEPO causing pure red cell aplasia (PRCA) in kidney patients treated with RHuEPO via the subcutaneous route (Bergrem et al., 1993). Pure red cell aplasia is a severe isolated anaemia, with sudden onset and almost complete absence of red cell precursors. Acquired PRCA, a haematological disorder, generally arises spontaneously, sometimes in association with a thymoma, a lymphoid cancer, or rheumatoid arthritis. Autoimmunity is often implicated in PRCA, and immunosuppressive therapy has been found to be successful in many patients (Casadevall, 2000). Anti-erythropoietin antibodies were described with red cell aplasia. These antibodies inhibited the binding of

erythropoietin to its receptor and blocked the differentiation of erythroid progenitors in vitro. Recently, studies have reported that antibodies against RHuEPO cause pure red cell aplasia (PRCA) in chronic renal failure patients treated with RHuEPO (Casadevall et al., 2002).

Early introduction of recombinant erythropoietin (RHuEPO) in the treatment of renal anaemia with RHuEPO had been weakly immunogenic (Urra et al., 1997). The production of RHuEPO antibodies had been rare, but considered a potential risk. However, Paulitschke et al., (2000) observed that RHuEPO resulted in improvement in the quality of life of patients treated for renal anaemia and no significant increase in treatment risk occurred.

Jacobs et al., (2000), compared different routes of administration of recombinant erythropoietin and its effectiveness in the improvement of haemoglobin levels. Intravenous route of administration had been the least immunogenic compared to the subcutaneous route of administration of epoetin alpha in patients with a high incidence of PRCA (Jacobs et al., 2000; Cassidy et al., 2002).

Pure red cell aplasia (PRCA) is an autoimmune disorder in which (IgG) antibodies or cytotoxic T-lymphocytes are directed against erythroid progenitors. Anti-erythropoietin antibody production is clearly related to the treatment of recombinant erythropoietin to correct anaemia in chronic renal failure haemodialysis patients (Casadevall et al., 2002).

Erythropoietin induced antibodies remained a very rare complication for many years. However, this situation has now been changed. A significant increase in the

frequency of this complication has led to severe occurence of PRCA (Eckardt and Casadevall, 2003). The immunogenicity of erythropoietin has been recognised. The severity of PRCA with erythropoietin treatment has been reported. Therefore, patients diagnosed with PRCA and treated with erythropoietin alpha had to be discontinued. Sharma (2004) conducted a study on patients using the subcutaneous route of therapy and discovered that the cause of the immunogenic response, i.e., increased antibody mediated PRCA, resulted from the polysorbate 80 on uncoated rubber syringe stoppers (Sharma, 2004).

The presence of anti-erythropoietin antibodies had been demonstrated directly by immunoprecipitation of radio labelled erythropoietin using native, deglycosylated and denatured erythropoietin (Casadevall et al, 2002). When antibodies neutralize and cross-react with available erythropoietin stimulating agents and endogenous erythropoietin, EPO therapy has to be discontinued immediately in patients known to be immunogenic (Locatelli et al, 2004). The production of antibodies against recombinant therapeutic proteins (RHuEPO) can limit the clinical efficacy of these agents (Pollock, 2004). The degree and nature of the antibody responses to the recombinant proteins (RHuEPO) reflect their overall immunogenicity. ELISA – based assays have been developed for detection of anti-erythropoietin antibodies (Eckardt and Casadevall, 2003).

This project investigated the antibody production in haemodialysis patients treated with recombinant erythropoietin (RHuEPO). An ELISA-based assay was set up to detect the antibodies and simultaneously measured the levels of native erythropoietin. The study examined the relationship of the RHuEPO and the native EPO. It also assessed whether the antibody was the real cause of the decrease of

haemoglobin levels with time. Although PRCA had become life threatening, the treatment of anaemia with recombinant erythropoietin remained critically important. The response of the immune system to RHuEPO was assessed in this project. The haemoglobin levels of the patients were monitored over a period of time and the effects of the EPO therapy were closely monitored in relation to antibodies.

2.2.9 CHARACTERIZATION OF ANT-ERYTHROPOIETIN ANTIBODIES

In 2000, Casadevall in her study described the antibodies against EPO, native and recombinant. She further demonstrated that the anti-erythropoietin antibodies recognized the carbohydrate or the protein moiety of the molecule. In her study, the native and deglycosylated RHuEPO was immunoprecipitated using the patient's serum. The immunoprecipitated radioactivity was measured and analysed by polyacrylamide gel electropheresis. The antierythroid protein antibodies immunoprecipitated glycosylated and deglycosylated forms of RHuEPO with the same efficiency (Casadevall, 2002).

One analysis revealed the presence of a single class of binding sites with a dissociation affinity constant and a maximum binding ability of RHuEPO (60iu/ml) were consistent with rough estimations made previously (Scatchard, 1949). Another investigation showed that the recombinant molecules are heavily glycosylated and the glycosylation of recombinant EPO was slightly different from that of the endogenous molecule (Sasaki, 1987). It had recently been shown that the anticarbohydrate antibodies could be produced in rabbits immunized with recombinant EPO (Pazur, 2000).

2.2.10 MECHANISM OF IMMUNOGENICITY

The expression of DNA in cell lines has a great potential for the treatment of diseases. In a study conducted by Eckardt et al., (2003), the endogenous production of a specific protein was inadequately low and the administration had beneficial modulating effects on the disease processes. Although these drugs are designed as copies of endogenous molecules, immunogenicity is a recognised risk. The authors described that the factors were associated with an immunogenic potential. These included subtle differences in protein structure and in glycosylation. The contaminants of the production process, the formulation, storage conditions, patient associated variables and the mode of application, with the intravenous route carried the lowest risk (Eckardt et al., 2003).

Epoetin alfa has more sialic acid residues than Epoetin beta (Casadevall et al., 2002). The antibodies investigated by Casadevall et al., (2002) were found to be directed against the protein itself rather than the carbohydrate moiety. This had been demonstrated when the removal of sugar by enzymatic digestion was seen to have no effect on the affinity of the antibody for erythropoeitic protein. This argues against the differences in glycosylation as the cause of immunogenicity (Bunn, 2002). Host cell contaminants and protein modification have been implicated in the immunogenicity of biopharmaceuticals (Schellekens, 2002).

All formulations of Epoetin alfa, beta, and darbepoetin alfa have been consistently manufactured in the same host cells, i.e., Chinese hamster ovary cells, though not necessarily in cells derived from the same cell line or from the same cell bank.

However, there is no evidence of protein modification. The investigator described that other factors could influence the immunogenicity of a therapeutic protein. This excluded the degree of divergence between the endogenous and recombinant molecule (Schellekens, 2002).

It has been observed that the processes and formulations that allow protein oxidation or aggregation, such as freeze drying, can also enhance immunogenicity (Cleland, 1993). It has been hypothesised by Casadevall (2002), that a change in the dialysis membranes might have produced changes in the RHuEPO molecule making it more immunogenic. He also stated, that the explanation may lie in a subtle modification of the molecule during manufacture or handling of the pharmaceutical product (Casadevall, 2002).

2.2.10.1 Unsatisfactory Response of Recombinant Erythropoietin Treatment

Ng et al., (2003) stated that when the haemoglobin levels increase < 10g/l after a four week standard dosage treatment, this describes the failure of the recombinant EPO to respond immediately. The authors further explained that the definition of resistance to RHuEPO therapy varies among different settings. In renal anaemia the resistance to recombinant EPO was defined by a failure to attain the target haemoglobin while receiving >300iu kilogram weekly or a continued need for such a dosage to maintain the target haemoglobin. In haematology or oncology setting, resistance to RHuEPO therapy is regarded as unsatisfactory haemoglobin increase of > 10g/l despite a four week high dose RHuEPO (900 iu/kg) therapy, in patients who previously failed on a four week standard dosage (450 iu/kg/week). The authors concluded that it is

important that other therapeutic and pathological factors can be a contribution (Ng et al., 2003).

2.2.11 ANTIBODY MEDIATED PURE RED CELL APLASIA

The incidence of antibody mediated pure red cell aplasia in patients with chronic kidney disease had increased in 1998 and reached a peak in 2002. Most of these cases were associated with Epoetin alfa and all of those cases included the subcutaneous administration of RHuEPO (Sharma et al., 2004).

The reported incidences of PRCA associated with epoetin use in patients with chronic kidney disease had increased, from only sporadic reports over a few years to a peak of seventy one cases in a study conducted by Casadevall in 2002. Of the 262 cases of suspected Epoetin associated PRCA that had been reported, 217 had anti-erythropoietin antibodies, 210 of those cases were associated with the subcutaneous use of epoetin alfa (Casadevall., 2002).

One of the authors had described the immune responses in the therapeutically used exogenous proteins. However, he further explained that the human derived and recombinant human proteins are less immunogenic than animal proteins. It had been described that the immunogenic response to erythropoietin in the patients treated with epoetin alfa had been unclear. The increased incidence of PRCA led to an intensified investigation of the manufacturing process and of the purified bulk and finished product (Koren., 2002).

A rigorous technical investigation had identified leachates extracted by polysorbate 80 from uncoated rubber syringe stoppers which had been the most likely cause of

increased immunogenicity associated with epoetin alfa and the increased frequency of PRCA. Thorough investigation of the epoetin molecule had been performed. The investigators had detected no changes that were outside of the normal ranges in the epoetin alfa molecule. The product had been no difference from other epoetin product. This was all demonstrated from a baseline frequency of PRCA (Sharma et al., 2004).

In an investigation, the stabilizer polysorbate 80 failed to demonstrate any evidence of an adjuvant effect, confirming the known safety profile of the polysorbate 80 as an excipient for protein formulations. The silicone oil also failed to show any adjuvant effect in mice and therefore, could be excluded, based on the lack of any temporal association between silicone oil and PRCA. Several other hypothesis were investigated over the course of the investigation. Leachates were shown to have a dose dependent adjuvant effect in a mouse model. The evidence from the investigation, between 1998 and 2003 showed the presence of leachates in the polysorbate prefilled syringes with the uncoated rubber stoppers. These had met all the criteria as the causative factor for the increased incidence of antibody –mediated PRCA as described by the investigators. They concluded that the increased immunogenicity associated with epoetin alfa was based on some of the criteria that had been described. These were the temporal correlation between the occurrence of leachates in the product and the increased incidence of PRCA. The exclusive presence of leachates in polysorbate 80 in syringes with uncoated rubber stoppers, and the absence of these leachates in other epoetin products were with lower rates of PRCA. The presence of leachates in microgram quantities was sufficient to initiate rare immune responses. The requirement of an adjuvant in the immune mechanism for generating a T cell mediated B cell response lead to IgG antibodies of the type

detected in PRCA patients. The leachates acted as adjuvants that are capable of stimulating the production of IgG antibodies in animal models (Sharma., 2004).

2.2.12 DRUG INDUCED AUTOIMMUNE PRCA

The serious adverse effect of epoetin had been observed to occur even when it was administered under proper medical supervision. Casadevall (2002), described thirteen patients with renal failure in whom aplasia of erythroid cells in the marrow and severe anaemia were caused by an immune response to epoetin. The authors provided convincing evidence that these patients had neutralizing antibodies with a high affinity and specificity for erythropoietin. In one case these antibodies recognized only a conformational epitope that required proper folding of the protein (Casadevall, 2002). The thirteen patients had a severe form of pure red cell aplasia, readily diagnosed by a progressive drop in haemoglobin levels in the presence of a low reticulocyte count, indicating the failure of new red cell production. The patients in this study became transfusion dependent. The diagnosis in one case was confirmed by examination of the bone marrow, which showed virtual absence of red cell precursors (Casadevall et al., 2002).

Krantz (1974), in his investigation stated that acquired pure red cell aplasia was an uncommon haematological disorder, which generally arises spontaneously. It could sometimes be in association with a thymoma, a lymphoid cancer, or rheumatoid arthritis. He also described that in adults, pure red cell aplasia is usually an autoimmune disorder in which IgG antibodies or cytotoxic T lymphocytes attack erythroid progenitors and precursors.

In patients with epoetin induced pure red cell aplasia, the anaemia is much more severe than the anaemia of renal failure that prompted the epoetin therapy. The antibody reacts not only with epoetin but also with the small amount of endogenous erythropoietin that patients with renal failure produce (Casadevall et al, 2002).

The fact that the serum erythropoietin levels were undetectable in the patients studied by Casadevall et al (2002), is further evidence that the antibody cross reacted with endogenous erythropoietin. The mechanism of the formation of antierythropoietin antibodies in these patients is of considerable interest. Prior to the introduction of epoetin therapy, fifteen years ago, patients with red cell aplasia were rarely observed to have had antibodies against endogenous erythropoietin (Steinberg et al, 1990).

Despite the differences in the carbohydrate structure, the antibodies characterized by Casadevall et al (2002), were not directed against the carbohydrate moiety on erythropoietin. However, the removal of sugar by enzymatic digestion had no effect on the antibody's affinity for the erythropoietin protein. It is therefore possible that a subtle difference in the carbohydrate structure of epoetin and endogenous erythropoietin created an epitope on the epoetin polypeptide to which an antibody binds, thereby inactivating not only epoetin but the endogenous hormone as well (Casadevall et al, 2002).

2.2.13 ANIT-EPO ANTIBODIES

The EPO produced by recombinant DNA technology is probably the most successful biopharmaceutical to date. It was introduced for the treatment of renal anaemia in the late

1980”s as an iv drug. Few years later when the first attempts were made to administer it subcutaneously other investigators were concerned about an increased risk of antibody formation. The subcutaneous application proved to be safe and feasible. Due to a lower dose requirement it became the preferred route of administration in many countries. Epoetin induced antibodies remained a very rare complication for many years. The significant increases in the frequency of such complication lead to severe inhibition of red cell production (Eckardt et al, 2003).

The understandable concern about the increased number of cases, PRCA due to anti EPO antibodies remained a very rare complication which is not fatal and principally reversible. Although it had been difficult, there does not seem to be an exorbitant increase in incidence. Bunn (2002) stated that it still appeared that the ratio of risk to benefit of epoetin is matched by few other pharmacological agents.

The antibody mediated pure red cell aplasia associated with the administration of erythropoietin stimulating agents (ESA) had been identified as a serious problem. When human recombinant erythropoietin was first introduced into the market, a number of cases of PRCA had been reported (Locatelli, 2004). Erythropoietin stimulating agent induced PRCA. This was caused by the development of neutralizing anti erythropoietin antibodies in patients with ESA therapy. Locatelli et al (2004), proved that the antibodies cross react with the patient’s endogenous erythropoietin and lead to an anaemia that is more severe, than, before the onset of erythropoeitic therapy (Locatelli, 2004).

As with all therapeutic proteins, the potential for immunogenicity with the ESA was recognized and tested for during development. There had been little evidence of any immunogenic effect in clinical trials over the first decade of clinical use. Studies showed that there were only four reports of antibody-mediated PRCA after the use of ESA"s in chronic kidney disease patients from their initial introduction in 1988 to 1997.The reported cases occurred between 1992 and 1997 (Bergem et al, 1993; Peces et al, 1996; Prabhakar et al, 1997).

2.2.13.1 Hyporesponse and PRCA in dialysis patients

An increase of antierythropoietin antibody induced PRCA has been reported. The data consisted mostly of spontaneous cases. The question arose about the frequency of the increase in EPO induced PRCA. A multicentre cohort study was conducted and the incidence and causes of recombinant EPO hyporesponse, of antibodies to EPO in patients on dialysis had been determined. The authors had also related the detection of anti EPO antibodies to the presence of PRCA (Kharagitsingh et al, 2005).

In two studies the resistance to recombinant EPO therapy was observed in haemodialysis patients. Patients who did not respond to high doses of EPO were referred to as hyporesponsive or EPO resistant (Dreuke, 1990; Stivelman, 1989).
The causes for EPO resistance have been identified, and the most important has been iron deficiency, infection and inflammation (Tarng, 1999). One of the rare causes of EPO resistance has been the antibody formation against EPO. A few cases of antibody production against EPO have been reported and published (Kharagitsingh et al, 2005). However, there have been a number of cases in which the development of

neutralizing anti EPO antibodies causing PRCA has been described during EPO exposure (Casadevall et al, 2002).

The occurrence of EPO induced PRCA has been estimated only from voluntary reports to the regulatory agencies or manufacturers and has been hampered by the problem of estimating the number of patients at risk (Statement, 2003).Therefore, a multicentre cohort study was conducted. The study consisted of a total of 1677 patients with a median follow up time of two years since the start of dialysis. The authors have observed that the EPO hyporesponsiveness in their patients without PRCA have has a 96% identifiable cause (Kharagjitsingh et al, 2005). Other investigators found other possible associations of inadequate EPO responses but their exact roles have not been clear (Eckhardt, 2002).

Kharagitsingh et al (2005) estimated the incidence of anti EPO antibodies based on the assumption that once antibodies are produced and the antigenic stimulus persisted, the antibody titers may fluctuate and may not be expected to disappear spontaneously. Therefore, the authors concluded that the incidence of inadequate EPO response in their population of dialysis patients has been in concordance with the literature. They have found that there had been a vast majority of patients with an inadequate EPO response. Based on their findings of the low incidence of EPO induced PRCA, they concluded that PRCA is a rare result of EPO resistance in dialysis patients. Furthermore, they had found the incidence of anti EPO antibodies to be low i.e., one per 1000 a year (Kharagjitsingh et al, 2005).

2.2.13.2 Treatment with RHuEPO: PRCA and Antierythropoietin

Benette et al (2005) observed that from 1998 to 2004, two hundred patients with chronic kidney disease treated for anaemia with epoetin, had resulted in pure red cell aplasia. The majority of the patients received an epoetin alfa product marketed exclusively outside the United States. They therefore, reported on the long term outcome of these patients. For the 170 chronic patients with kidney disease, who had developed epoetin associated PRCA, had three months or more follow up information available. Case reports were reviewed from the Food and Drug Administration and epoetin manufacturers for the information or clinical characteristics of the patients. These also included the immunosuppressive treatments, epoetin responsiveness, and hematologic recovery of these patients (Bennett et al., 2005).

Overall, 64% of the PRCA patients received immunosuppressive therapy, 19 of which underwent renal transplantation. Thirty seven percent experienced a hematologic recovery, with higher hematologic recovery rates among the PRCA patients who had received immunosuppressive therapy. Among the thirty four patients who received epoetin after the onset of PRCA, 56% had regained their epoetin responsiveness. The highest rates of epoetin responsiveness were observed among patients whose antierythropoietin antibodies were undetectable when the epoetin was administered. The chronic kidney disease patients with epoetin associated PRCA, epoetin discontinuation and immunosuppressive therapy or renal transplantation had been necessary for hematologic recovery. The reinitiation of epoetin therapy among these individuals could be considered if the antierythropoietin antibodies were undetectable (Bennett et al, 2005).

The European PRCA study group reported that the majority of the PRCA patients who achieved a hematologic recovery, had antierythropoietin antibody levels below the lower limit of detection in the referral laboratory of one of the co authors (Verhelst et al, 2004).

For the PRCA patients who had not cleared the antierythropoietin antibodies with immunosuppression alone, renal transplantation appeared to be a viable treatment option. Several Epoetin associated PRCA patients, who failed to recover the epoetin responsiveness after administration of multiple immunosuppressive agents, developed a reticulocytosis one day after renal transplantation and a complete hematologic recovery shortly thereafter (Gagnon et al, 2003; Praditpornsilpa et al, 2005).

Hematologic recovery occurred in all the patients with the exception of one of the renal transplant recipient with epoeitin associated PRCA. Small antigenic differences between endogenous erythropoietin and recombinant erythropoietin may exist in vivo which accounted for the high rates of recovery following renal transplantation and the resumption of natural erythropoietin production. Small peptide molecules without sequence homology with epoetin and non peptidic erythropoietin mimetics that bind to the erythropoietin receptor have been developed (Livnah et al, 1996).

Bennett et al (2005) stated that antibodies to these agents had unlikely inhibited the pharmacologic activity of epoetin and that conversely the antierythropoietin antibodies were unlikely to inhibit their erythropoeitic effects. They further explained that it was possible that erythropoietin mimetics would be successful in treating those

patients who have persistent epoetin associated PRCA despite multiple immunologic therapies.

2.2.14 THE CURRENT STATUS OF ERYTHROPOIETIN

The primary regulator of the growth and survival of erythroid progenitors, which mature into red blood cells, is the glycoprotein hormone EPO (Krantz, 1991). Erythropoietin exerts this effect by specifically interacting with a receptor present on the surface of progenitor cells which leads to receptor activation and initiation of an intracellular signal cascade. Over many years the availability of recombinant erythropoietin had lead to its widespread use in stimulating red cell synthesis for the treatment of severe anaemia associated with acute and chronic disease (Fisher, 1997).

It has been shown that chronic renal failure and cancer patients with severe anaemia, receiving EPO administration demonstrated a significant improvement in quality of life and functional capacity (Jollife et al., 1995).

The cost and inconvenience associated with chronic parenteral administration of EPO and other protein therapeutics, have led a number of investigators to seek ways to deliver proteins orally, transdermally and by inhalation. However, the size and intrinsic liability of proteins has hindered the progress. Other investigators described that a more difficult strategy was to obtain orally administered agents with small molecules that retained the full agonist activity of the larger protein molecules (Barbone et al., 1999).

Johnson and Jolliffe (2000) discussed that their motivation for attempting such a high risk research strategy for EPO had been enhanced by several potential benefits. They demonstrated that, in addition to the contemporary uses of EPO, an oral agent would be preferable. The oral agent would potentially extend to therapeutic applications in less severe anaemia conditions associated with rheumatoid arthritis and other chronic inflammatory disorders. They have discussed that in the past ten years since EPO was first approved for the use in man, the researchers have not been successful in delivering an orally active EPO mimetic (Johnson and Jolliffe, 2000). Investigators have described that a number of key steps have been reached in the search for small molecule agonists of the EPO receptor and a great deal of knowledge concerning EPO receptor structural biology has emerged from these efforts (Wilson and Jolliffe, 1999). The ultimate goal is to identify a small molecule that will act as an orally available agonist of the EPO receptor (Johnson and Jolliffe, 2000).

A number of key milestones have been achieved including the discovery of both the peptide and non peptide mimics of EPO. This, however, has been extended to create EPO mimetic fusion proteins (Kuai et al., 1999). It remains to be seen if small defined linkers can be designed to create molecules which not only bind the EPO receptor, but which will also be capable of promoting an active receptor assembly. This remains a significant technical hurdle and is complicated by recent findings which suggest that the overall geometric constraints can influence the magnitude of the EPO receptor activation (Livnah et al., 1998). The structural difference between the agonist and the antagonist structures is modest and is an additional complication that must be faced when striving to discover small molecule agonists of the EPO receptor with clinical potential (Wilson and Jolliffe, 1999). Some evidence has emerged that some fraction of the EPO receptor on the cell surface exists as a of the

two EPO receptor molecules in the resting state and it introduces an additional hurdle to be crossed by the theoretical small molecule agonist (Livnah et al., 1999).

The small molecule mimetic would then also be required to promote rearrangement of the inactive to the active state. A greater understanding of the structural and cellular biology of EPO receptor will likely provide the basis for additional advances. To date no molecules with the desired EPO mimetic potential and oral bioavailability have been discovered. Such discoveries are encouraged (Johnson and Jolliffe, 2000).

An investigation carried out by Bunn (2007), at the haematology division of the Harvard Medical School, described that there could be new agents that could stimulate erythropoiesis other than the recombinant erythropoietin. He stated that despite the enormous success of RHuEPO there has also been a concern over the cost factor. In addition, he stated that patients have objected to parenteral administration of at least three times a week (Bunn, 2007). A considerable anxiety was engendered from a spate of reports, from 1998 to 2004, of severe pure red cell aplasia in European patients treated with recombinant erythropoietin (Casadevall, 2002).

Severe pure red cell aplasia was encountered only rarely and was probably caused by defective formulation other than inherent antigenicity of recombinant erythropoietin. There has been considerable impetus in the pharmaceutical industry to develop less costly agents that match with the recombinant erythropoietin in safety and efficacy but is more easily administered and is non antigenic (Bunn, 2007).

2.2.15 CONCLUSION

Three strategies have appeared to be particularly promising in the improved stimulating agents for erythropoiesis. The first is the half life of EPO in circulation which can be prolonged by the addition of N- linked carbohydrate groups, by forming adducts with polyethylene glycol, and by preparation of EPO multimers. The second is the mimetic peptides which effectively can trigger signal transduction at the EPO receptor, thereby boosting red cell production. The third is the hypoxia inducible transcription factor (HIF) which could be pharmacologically induced by oral agents, and thereby result in enhanced expression of not only endogenous EPO but also of other genes important in the regulation of erythropoiesis (Bunn, 2007)

CHAPTER THREE: MATERIALS AND METHODOLOGY

The aim of this study was to assess native erythropoietin and antibodies to recombinant erythropoietin in haemodialysis patients. In order to achieve this, the erythropoietin levels in blood were measured in forty haemodialysis patients and ten controls (healthy individuals). Forty individuals participated in this study. The plan of the entire research process was set as in Appendix A and Appendix F. Blood samples were collected from patients and centrifuged at 3000 rates per minute (RPM). Serum was stored at -70^{0}C.

Optical density of erythropoietin was measured in the serum using the ELISA. The levels of erythropoietin were calculated for further investigation. The ELISA was set up to measure the native erythropoietin levels to assess the antibodies response to recombinant erythropoietin. Thereafter, the formation of antibodies was measured. The antibody response to erythropoietin and the effects it had on the rate of production of red blood cells was observed. Haemoglobin levels were monitored after the commencement of recombinant erythropoietin.

3.1 SAMPLING

Haemodialysis patients were recruited from the haemodialysis unit at Addington Hospital (South African State Hospital), after obtaining permission from the Health Department of Kwa-Zulu Natal and the Hospital Manager of Addington Hospital. Forty haemodialysis patients agreed to participate to the study and were included. The test groups consisting of three groups of patients were selected to participate in the study. Chronic renal failure patients on haemodialysis receiving recombinant

erythropoietin; one control group consisting of chronic renal failure haemodialysis patients not receiving recombinant erythropoietin and the other control group consisted of healthy individuals. The inclusion criteria were: Chronic renal failure patients on haemodialysis. The exclusion criteria were: patients who were aware of undergoing a live related transplant within the year, patients who were pregnant, acute patients receiving haemodialysis and patients who were already diagnosed with Pure Red Cell Aplasia were excluded from the study. All patients recruited into the study were under the consultant care of Professor A.G. Assounga (Head of Department of Nephrology who confirmed the diagnosis of chronic renal failure). A total of forty patients participated in this study and the population consisted of both males and females of different cultures and race. An informed consent form (Appendix B or Appendix C) was completed and signed by all participants. Ethical approval for this study was obtained from the Durban University of Technology Ethics Committee.

The sample size was distributed as follows:

Group A	- Haemodialysis patients recieving recombinant erythropoietin	30
Group B	- Haemodialysis patients not receiving recombinant erythropoietin	10
Group C	- Volunteers (healthy individuals)	10

Healthy Individuals not on haemodialysis comprised of, five females and five males. Erythropoietin levels and antibody levels in the bloods of all participants were measured using the appropriate protocols. The haemoglobin levels were also measured.

3.2 SPECIMEN COLLECTION

Blood samples were collected on a monthly basis for a total of six months. Venous whole blood (15 ml) was obtained from the haemodialysis patients receiving recombinant erythropoietin and haemodialysis patients not receiving erythropoietin, with a 20ml sterile syringe and transferred into three purple top tubes. All blood samples labelled accordingly were taken from the arterial line of the haemodialysis blood circuit on commencement of the haemodialysis procedure. The haemoglobin levels were obtained from patients routine monthly bloods which were tested at the Addington Laboratory. The two tubes were couriered to the Immunology research laboratory of the Nelson Mandela Medical School at Dorris Duke Medical Research Institute (DDMRI).

3.2.1 Centrifugation

- The blood samples were centrifugated at 3000 RPM at 5^0C.
- Three Eppendorf tubes were precisely labelled per patient with their initials and surnames, date of centrifugation and description of sample in Eppendorf tubes.
- After centrifugation sterile pipettes were used to collect the plasma and buffy coat from the blood tubes into the three labelled Eppendorf tubes per patient respectively, plasma 1 and plasma 2 and buffy coat.
- All samples were carefully stored in an Eppendorf box labelled and stored in the freezer at -70^0C.

3.3 ENZYME LINKED IMMUNOSORBENT ASSAY

Principle of Test

This assay can be direct or indirect. Positive control serums were chemically coupled to enzymes examples, horseradish perioxidase or alkaline phosphatase. Antibodies (Abs) that bind to antigen coated wells, were detected with the addition of an enzyme –coupled to horseradish perioxidase or alkaline phosphatase. The direct method called 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 method we used was the indirect ELISA as described by (Urra et al., 1997), the antigen or the cell of interest was immobilised onto the well. The primary Abs from a known host animal was allowed to bind to the immobilised target. A secondary antibody directed against the primary host animal's immunoglobulin (usually IgG) was conjugated to a reporter enzyme and allowed to bind to the primary Abs. The complex was visualised by addition of a substrate and formation of a coloured reaction product. The two most common enzyme labels for secondary Abs were alkaline phosphatase used in conjunction with the substrate ABTS and (hydrogen peroxide) H2O2 (R&D Systems, Oxon, UK), which resulted in blue green reaction product (Javious, 2000).

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. IOOµ 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 3.1) 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 3.1 Reagents provided in the Elisa Kit

Erythropoietin microplate	96 wells – mouse monoclonal antibody
EPO conjugate	Conjugated to horseradish perioxidase
EPO Standards	Recombinant human EPO
EPO Assay diluent	Buffered protein base
Specimen diluent	Protein stabiliser buffer
EPO wash buffer	100ml
Colour reagent A	0.01N buffered H2O2
Colour reagent B	tetramethylbenzidine
Stop solution	2N sulphuric acid
Plate covers	Adhesive strips

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 the exposure of light. Two hundred (200µL) of the resultant mixture was required per well. All unused substrate was discarded.

Protocol: the ELISA for detection of erythropoietin(EPO) levels in blood

1. All reagents and samples stored at -70^{o}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 manufactures instruction manual. The standard curve was plotted (Figure 3.1) and the formular calculated accordingly.

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.

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 (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. The plate was resealed with the adhesive strip provided and incubated on the benchtop 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. 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 (Anthos Labtech Instruments, Anthos, UK) set at 450 nm.

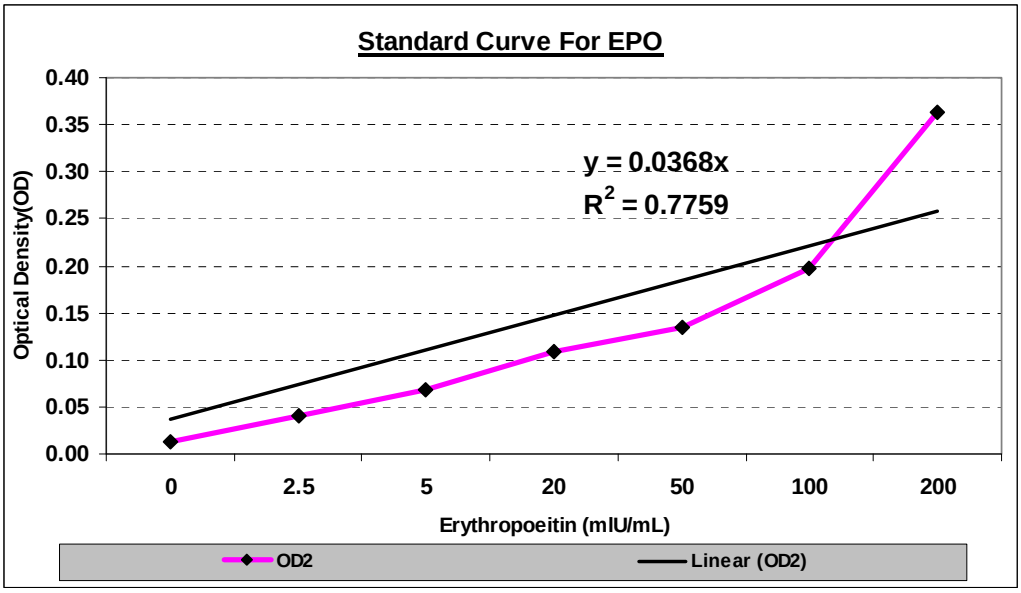


Fig 3.1 Standard curve for erythropoietin

ASSAY PROCEDURE SUMMARY

1. All reagents prepared as described

2. 100µL of EPO assay diluent added to each well

3. 100ul µL of standard, control, and specimen added to each well

4. Plates were sealed and incubated at room temperature (20-25^{0}C)

For 1 hour on the orbital microplate shaker at 500 ± 50 rpm.

5. Solution was aspirated thoroughly

6. 200ul of conjugate was added to each well

7. Plate was sealed and incubated at room temp (20-25^{0}C) using the orbital shaker

8. Wells were aspirated and washed four times with 1X Wash solution

200 µL of substrate solution added to each well

incubated at room temperature for 20-25min

9. 100 µL stop solution added to each well

within 15min reading was at 450nm

Fig. 3.2 Assay procedure Summary

3.4 AN ELISA FOR THE DETECTION OF ANTIBODIES

3.4.1 INTERPTRETATION OF ELISA FOR EPO - DIFFERENT SERUM DILUTIONS OF ANTIBODY DETERMINATION

Three different groups of patients were selected to assess the antibody levels using different dilutions. The patients were separated according to those receiving erythropoietin, those not receiving erythropoietin and the healthy individuals not on erythropoietin and not haemodialysis patients (Table 3.2)

Protocol

1. All reagents were prepared accordingly. Ninety-six wells polystyrene microtiter plates were coated with recombinant erythropoietin beta (Roche, SA) at 10mg/l in PBS Ph 7.4 and then incubated overnight at 4^0C.

2. The plates were emptied and washed five times with phosphate buffered saline (PBS). The plates were then postcoated till the top of each well with PBS containing 30g/L bovine serum albumin (BSA), (Sigma, Missouri, USA) and incubated for 4 hours at room temperature.

3. The contents of the wells were flicked out and 200µL of different serum dilutions of 1:50, 1:100, 1:200, 1:400, 1:800 was added to the wells (Figure 3.4).

4. 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.

Table 3.2 Protocol for the selection of different serum dilutions

Different serum dilutions	Patients on EPO	Patients not on EPO	Healthy individuals	Control Wells
1:50	200 µL	200µL	200µL	positive control
1:100	200 µL	200µL	200µL	Negative control
1:200	200 µL	200µL	200µL	nonspecific binding
1:400	200µL	200µL	200µL	Epo and 2nd antibody

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

6. Subsequently, 100µL of horseradish perioxidase 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. Plates were then washed five times as described previously.

9. After washing, 100µL of freshly prepared substrate solution was added to each well.

10. After thirty minutes the reactions were stopped by adding 100 µL of stop solution (sulphuric acid). The absorbance was measured with a microplate reader at 450 nm.

After reviewing the results with different dilutions, the 1:50 dilution presented with the most optimal result. This dilution was then selected. The antibody sample was measured in duplicate (Urra et al., 1997).

3.4.2 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 perioxidase – 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 (Table 3.3)

1. All reagents were prepared accordingly. Ninety-six wells polystyrene microtiter plates were coated with RHuEPO-beta at 10mg/l in PBS Ph 7.4 and then incubated overnight at 4^0C.

2. The plates were emptied and washed five times with PBS. 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. After washing, 100ul of freshly prepared substrate solution was added to each well.

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

6. Subsequently, 100µL of horseradish perioxidase 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 at 450 nm (Urra et al., 1997).

Table 3.3 Protocol for the detection of antibody using the ELISA

Procedure	**Time**
1. Incubation: 80 µL recormon added into each well	Overnight at 4^0C
2. Buffer rinse: Washed in PBS(ph 7.4) five times	30 min
3. Bovine serum albumin-postcoated: Wells filled to top with 30g/l BSA	4 hrs at 37^0C
4. Adding of serum dilution: contents flicked out 1:50 serum dilution added to each well	40 min
5. Incubation • Conjugate and PBS added to respective wells • incubated	20 min 1 hr at 37^0C
6. Buffer rinse Plates were washed five times	30 min
7. 2nd Antibody added to wells: in all patient wells in epo and 2nd antibody well PBS in other three sets	30 min
8. Incubation	1 hr at 37^0C
9. Washing	30 min
10. Substrate Added: freshly prepared substrate into each well	20 min
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

3.5 STATISTICAL ANALYSIS

Statistical analysis has carried out by the Instat 3 computer programme and reviewed by a statistician. Results are presented as the mean and standard error of the mean (SEM). Significance was calculated by a one – tailed test. A p-value < 0.05 was taken to be statistically significant.

CHAPTER FOUR: RESULTS

4.1 THE MEAN RESULTS OF ALL HAEMODIALYSIS PATIENTS OVER THE TRIAL PERIOD (SIX MONTHS)

4.1.1 Erythropoietin concentration in all the haemodialysis patients

The mean erythropoietin (EPO) concentration in the haemodialysis patients in the two groups of males and females varied over the trial period. The females presented with higher mean values of EPO concentration as compared to the male haemodialysis (HD) patients during the trial period (Figure 4.1). From August 2006 to November 2006 the male EPO concentration had increased. From December 2006 to January 2007, the EPO concentration had dropped slightly as compared to the increase in the months of August to November. The mean concentration of EPO between the two groups of male and females were almost the same in the last two months of the trial. The overall mean total of EPO concentration and ± of the female HD patients were higher than the male HD patients (Table 4.1). The differences in the means between the two groups were statistically significant ($p < 0.05$) as seen in Table 4.1.

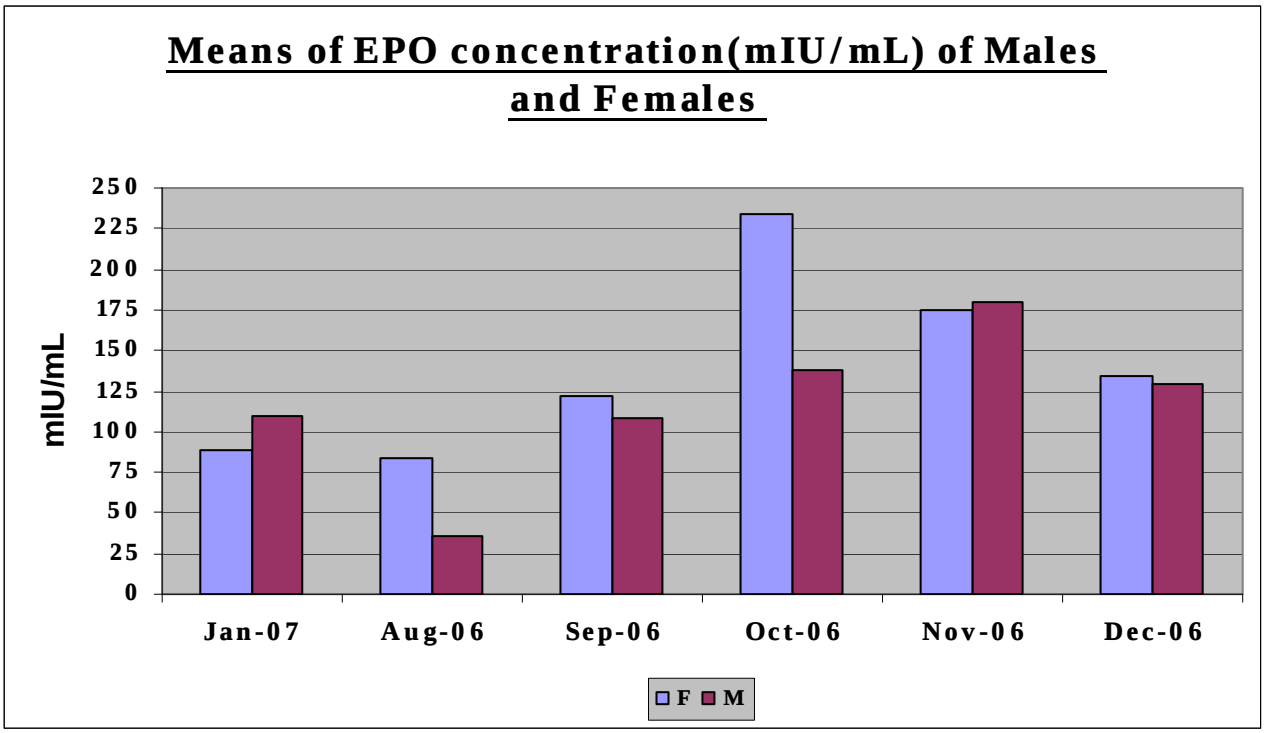


Fig 4.1 Erythropoietin concentrations of male and female haemodialysis patients

Table 4.1 Means of EPO concentration of males and females with statistical analysis

MEAN OF EPOCONC (mMI/uL)	**GENDER**	
MONTHS	**F**	**M**
Aug 06	84.15	35.45
Sep 06	121.63	107.91
Oct 06	234.51	137.32
Nov 06	174.52	179.30
Dec 06	133.71	129.57
Jan 07	88.64	109.57
MEAN TOTAL	139.42	119.49
SEM ±	**15.24**	**7.54**
P(T<=t) one tail	**0.02**	

4.1.2 Antibody levels in all haemodialysis patients

The mean antibody levels presented in optical density (OD) in both the groups of males and females were almost the same during the trial period (Figure 4.2). The females had a higher mean OD in August and January. The males had higher mean OD levels in September, October and November. However there had not been a very large difference in the antibody levels between the male and female groups. The overall mean total of the antibody levels (OD) in the female HD patients were 0.44, ± 0.02 and in the male HD patients were 0.43, ±0.01 (Table 4.2). The differences between the means in the two groups of patients were statistically insignificant ($p>0.05$) as seen in Table 4.2.

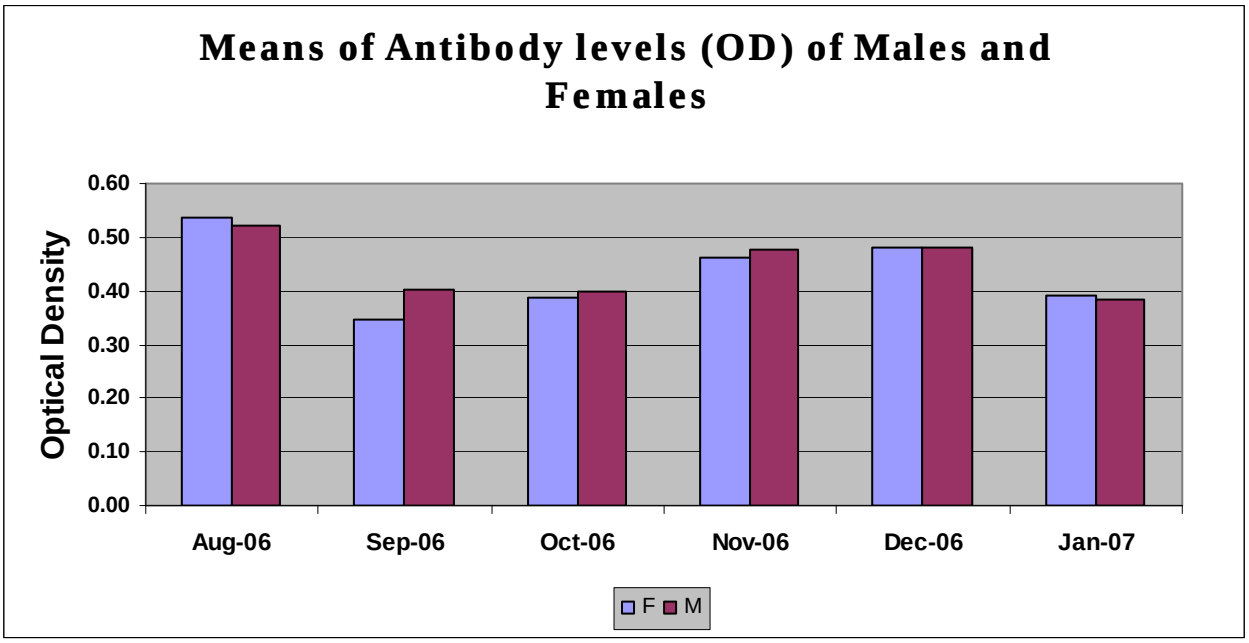


Fig 4.2 Antibody levels of male and female haemodialysis patients

Table 4.2 Mean Antibody levels of males and females with statistical analysis

MEANS OF ANIBODY LEVELS (OD)	**GENDER**	
MONTHS	**F**	**M**
Aug-06	0.54	0.52
Sep-06	0.35	0.40
Oct-06	0.39	0.40
Nov-06	0.46	0.48
Dec-06	0.48	0.48
Jan-07	0.39	0.39
MEAN TOTAL	0.43	0.44
SEM ±	**0.02**	**0.01**
P(T<=t) one tail	**0.34**	

4.1.3 Haemoglobin levels in all haemodialysis patients

The mean haemoglobin levels of the haemodialysis patients of the male and female groups varied over the trial period (Figure 4.3).The female patients presented with higher mean haemoglobin levels (HB) as compared to the male patients. However in the month of December the mean HB levels of the males were higher to that of the female patients. The overall mean total of the HB levels in the female HD patients were 9.97, ± 0.27 g/dL. In the male HD patients the overall mean total of the HB levels were 9.71, ± 0.26 g/dL (Table 4.3). The mean differences between the two means were statistically insignificant ($p>0.05$) as seen in Table 4.3.

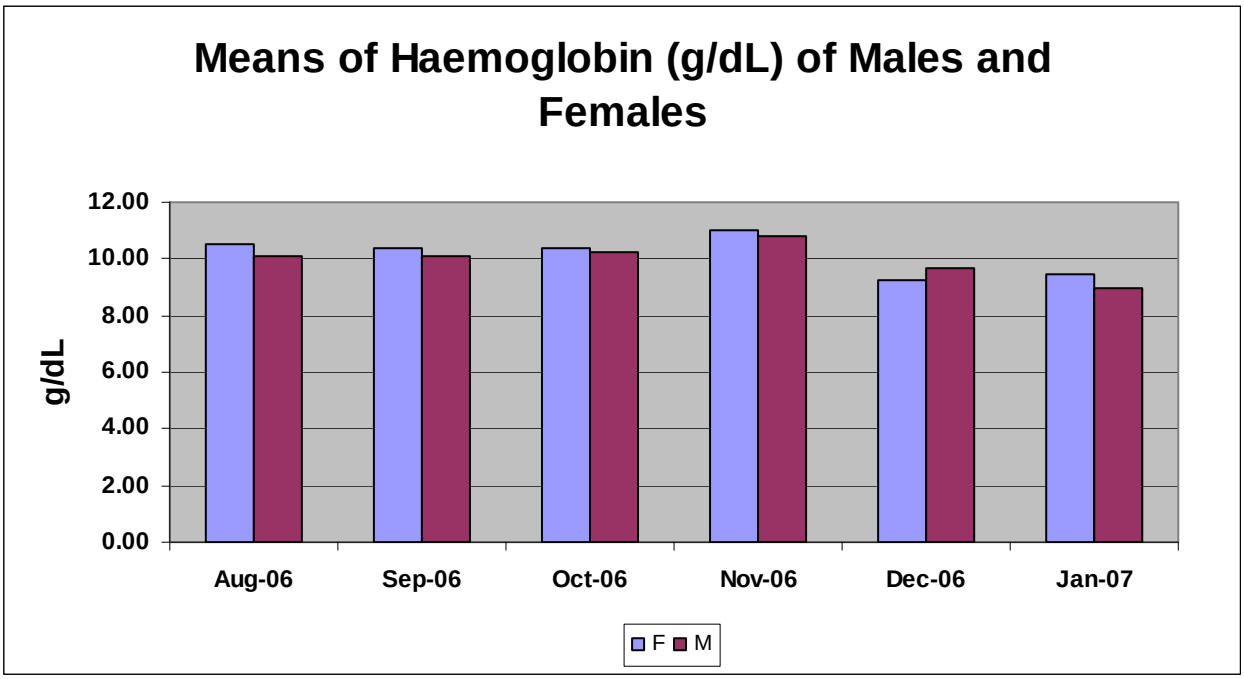


Fig 4.3 Haemoglobin levels of male and female haemodialysis patients

Table 4.3 Mean haemoglobin levels of males and females with statistical analysis

MEANS OF HB LEVELS (g/dL)	**GENDER**	
MONTHS	**F**	**M**
Aug-06	10.50	10.11
Sep-06	10.36	10.09
Oct-06	10.39	10.23
Nov-06	11.00	10.80
Dec-06	9.27	9.68
Jan-07	9.43	8.95
Grand Total	10.07	9.83
SEM ±	**0.26**	**0.27**
P(T<=t) one tail	**0.19**	

4.1.4 Transferrin saturation (%) of haemodialysis patients

The means of the transferrin saturation (%) levels in the both male and female haemodialysis patients were above 30 % during the trial period (Figure 4.4). These levels were above 30% during the six month trial period.

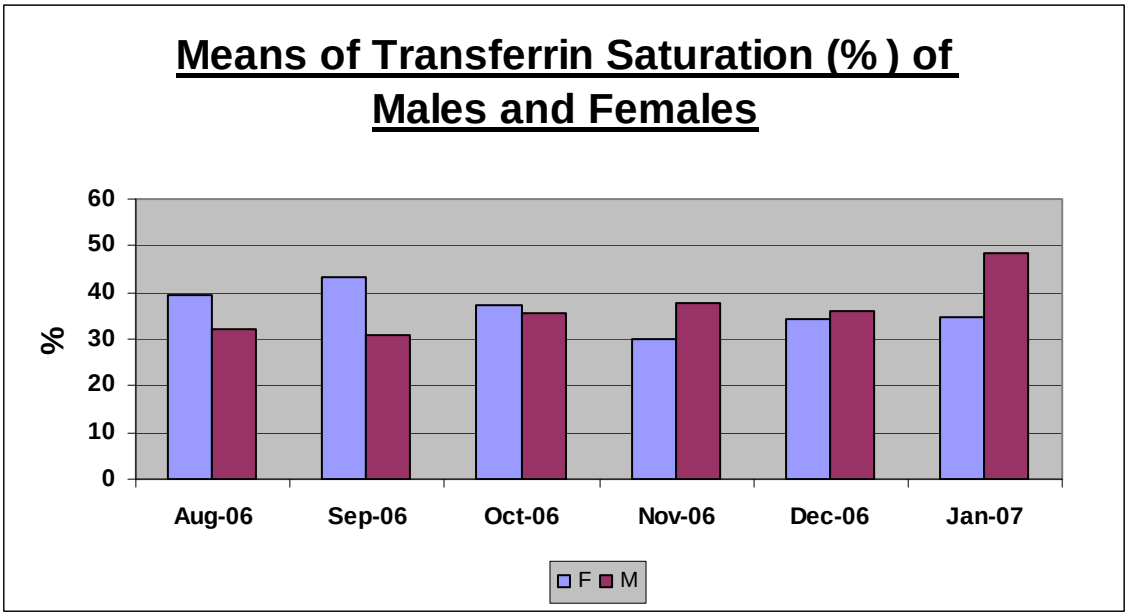


Fig 4.4 Transferrin saturation (%) of male and female haemodialysis patients

4.1.5. Transferrin levels of haemodialysis patients

The mean transferrin levels of the haemodialysis patients of the both groups were 1 and 2. The male transferrin levels were higher than the female in almost all the months of the trial period. However in the month of December the female transferrin levels were higher than the male HD patients.

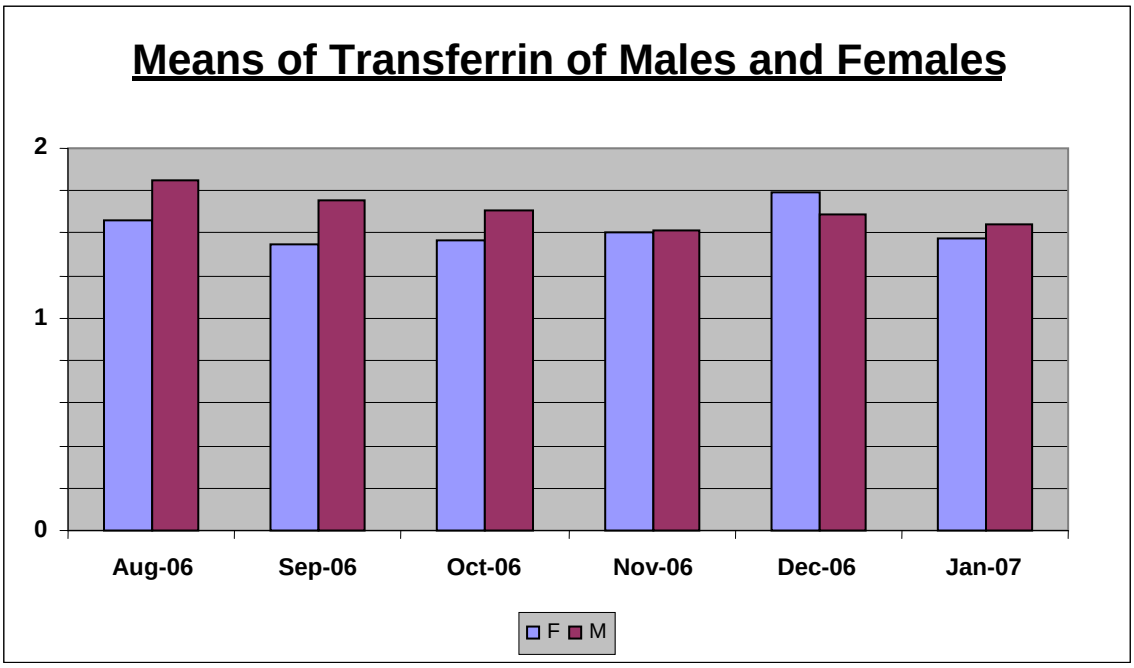


Fig 4.5 Transferrin levels of male and female haemodialysis patients

4.2 THE MEAN VALUES OF ALL MALE AND FEMALE HAEMODIALYSIS PATIENTS RECEIVING RHuEPO ACCORDING TO THEIRWEEKLY DOSES

4.2.1 Erythropoietin concentration of haemodialysis patients according to weekly RHuEPO doses

The erythropoietin concentration in the both groups did not increase largely with the increased doses of RHuEPO. However with the male and female HD patients on zero doses RHuEPO had high amounts of EPO as compared to the both groups with increased doses of RHuEPO. There were no females receiving 4000iu weekly RHuEPO.

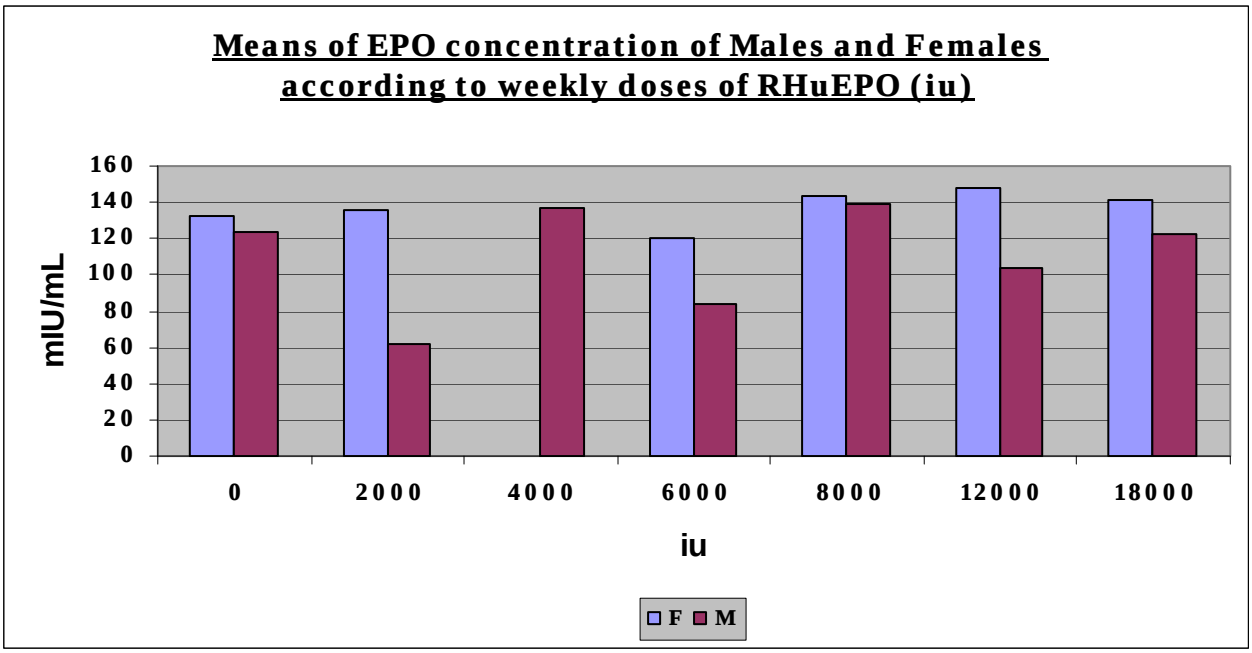


Fig 4.6 Erythropoietin concentration of male and female HD patients according to weekly RHuEPO doses

4.2.2 Antibody levels of haemodialysis patients according to weekly RHuEPO doses

The antibody levels presented in optical density (OD) were averagely consistent in their levels in both the male and female groups (Figure 4.8). The levels of antibodies were averagely similar in the male and female groups of those on zero doses of RHuEPO as compared to the other weekly doses of RHuEPO. All patients represented here had antibodies.

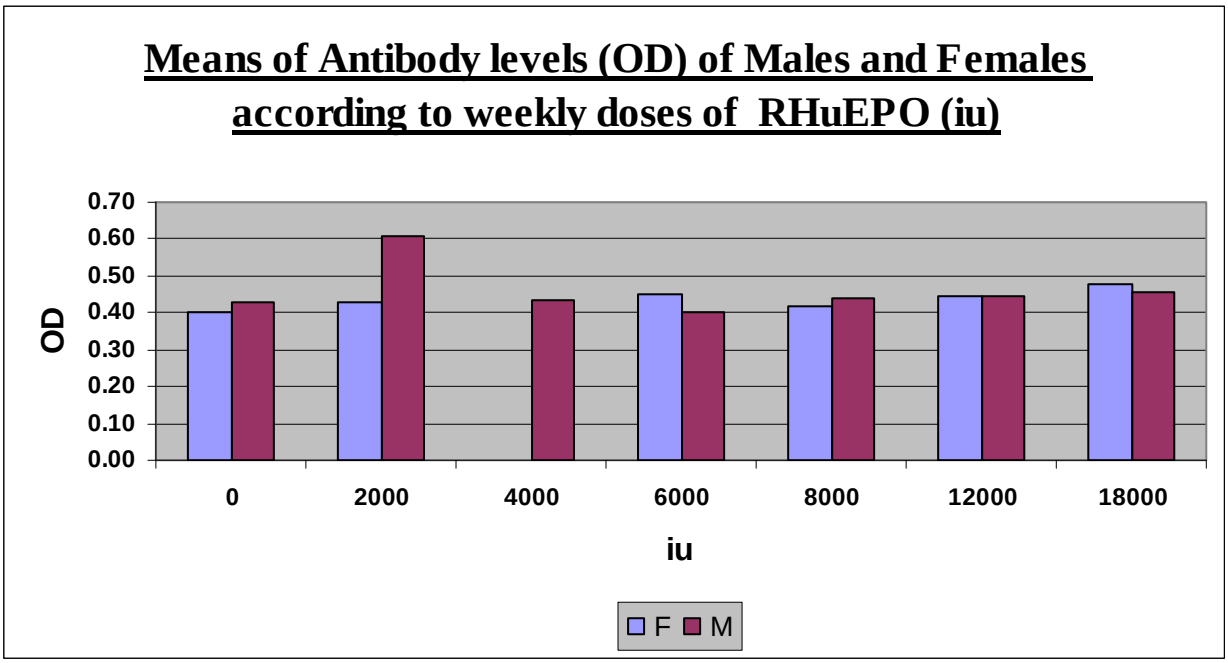
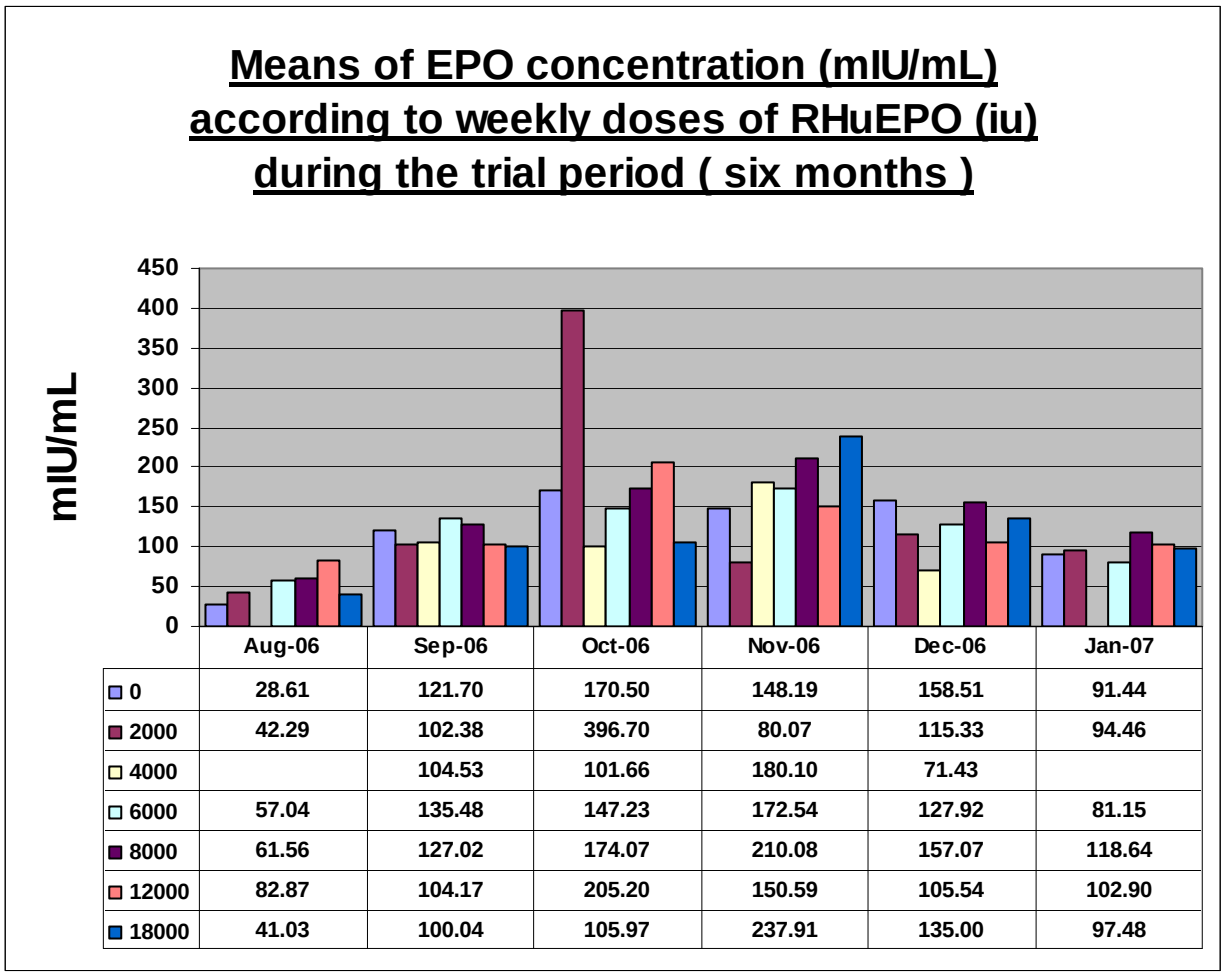


Fig 4.7 Antibody levels of male and female haemodialysis patients according to weekly RHuEPO doses

4.2.3 Erythropoietin concentration of haemodialysis patients according to weekly RHuEPO doses during the trial period

The mean EPO concentration of all haemodialysis patients represented (Figure 4.9) were grouped according to their weekly RHuEPO doses. This group included the males and females together. The EPO concentration fluctuated over the six month trial period. In all groups of weekly RHuEPO doses, including the group not receiving RHuEPO presented with varying levels of EPO concentration.

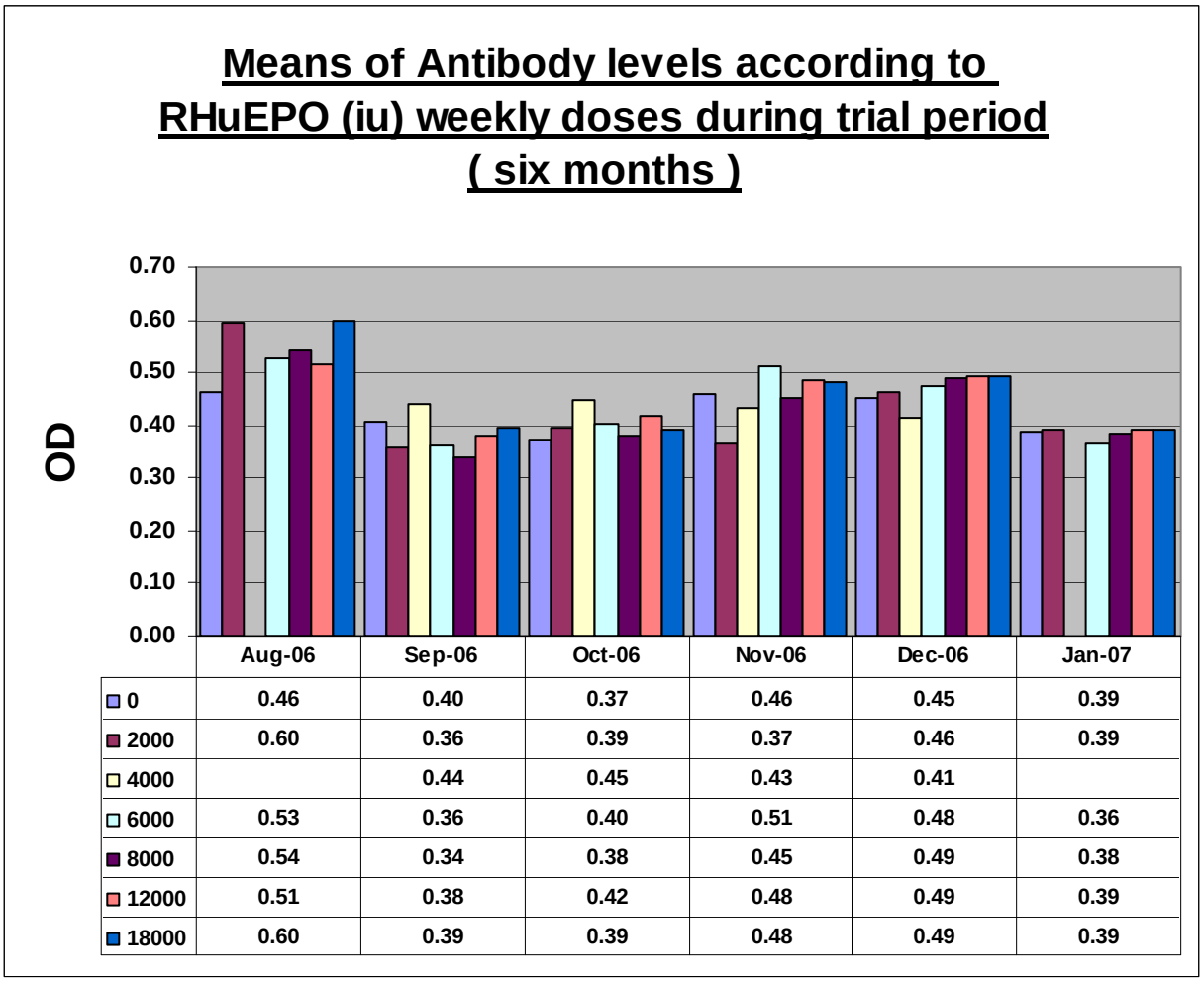


	Aug-06	Sep-06	Oct-06	Nov-06	Dec-06	Jan-07
0	28.61	121.70	170.50	148.19	158.51	91.44
2000	42.29	102.38	396.70	80.07	115.33	94.46
4000		104.53	101.66	180.10	71.43	
6000	57.04	135.48	147.23	172.54	127.92	81.15
8000	61.56	127.02	174.07	210.08	157.07	118.64
12000	82.87	104.17	205.20	150.59	105.54	102.90
18000	41.03	100.04	105.97	237.91	135.00	97.48

Fig 4.8 Erythropoietin concentration of all HD patients according to weekly RHuEPO doses

4.2.4 Antibody levels of haemodialysis patients according to weekly RHuEPO doses during the trial period

The antibody (AB) levels were ranging from 0.379 to 0.595 OD in all groups of RHuEPO over the six month period (Figure 4.10). The trend of the AB levels also fluctuated in the group that were on zero doses RHuEPO. The AB levels in the month of August were between 0.462 and 0.595 OD, and in the last month of the trial in January the AB levels were between 0.386 and 0.393 OD.

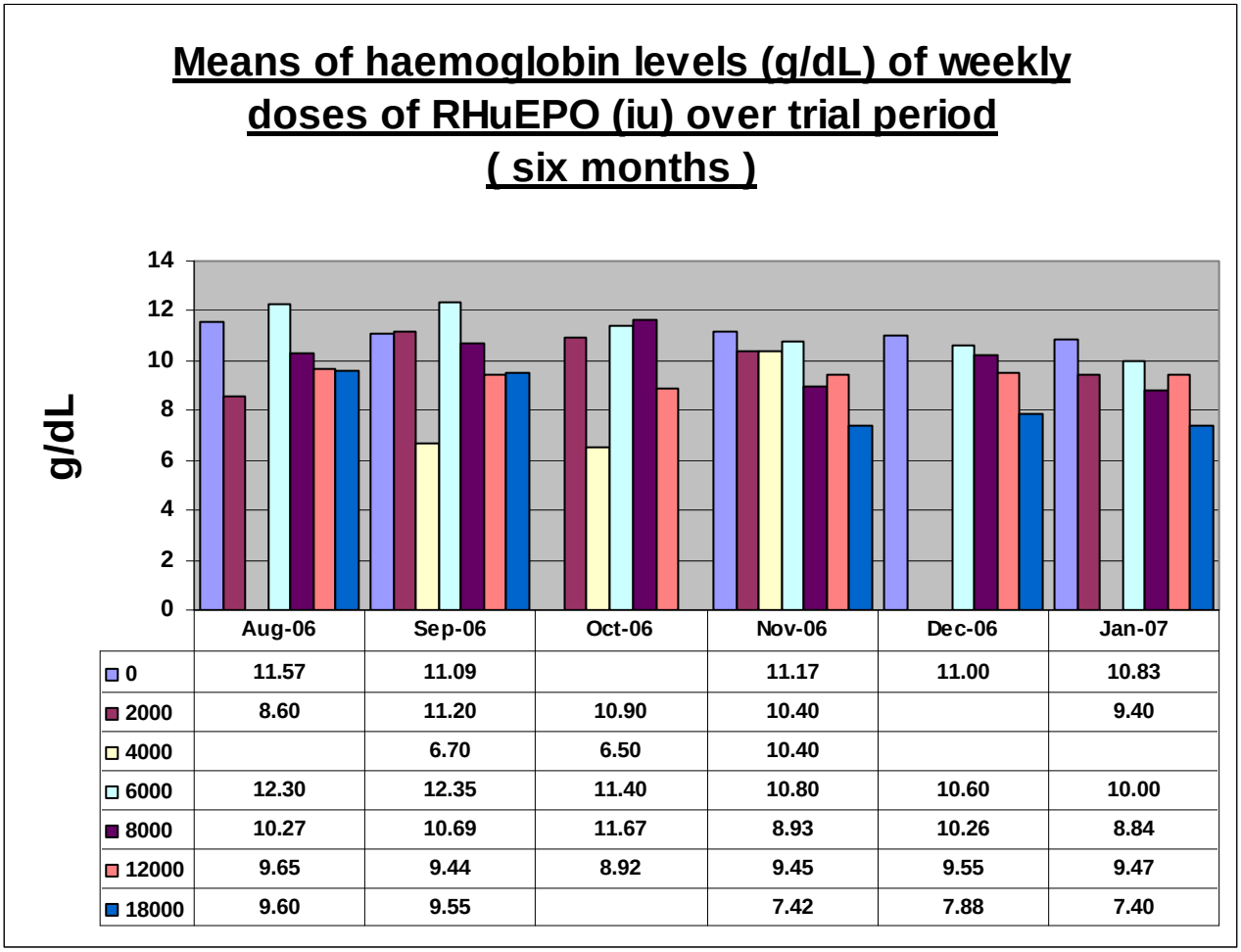


	Aug-06	Sep-06	Oct-06	Nov-06	Dec-06	Jan-07
0	0.46	0.40	0.37	0.46	0.45	0.39
2000	0.60	0.36	0.39	0.37	0.46	0.39
4000		0.44	0.45	0.43	0.41	
6000	0.53	0.36	0.40	0.51	0.48	0.36
8000	0.54	0.34	0.38	0.45	0.49	0.38
12000	0.51	0.38	0.42	0.48	0.49	0.39
18000	0.60	0.39	0.39	0.48	0.49	0.39

Fig 4.9 Antibody levels of all HD patients according to weekly RHuEPO doses

4.2.5 Haemoglobin levels of haemodialysis patients according to weekly RHuEPO doses during the trial period

The haemoglobin levels in all groups of weekly RHuEPO doses from August 2006 to January 2007 presented with a slight decrease at the end of the trial as compared to the beginning of the trial (Figure 4.11). During the course of the trial period there had been a slight increase and at the last month of the trial a decrease in HB had been presented.

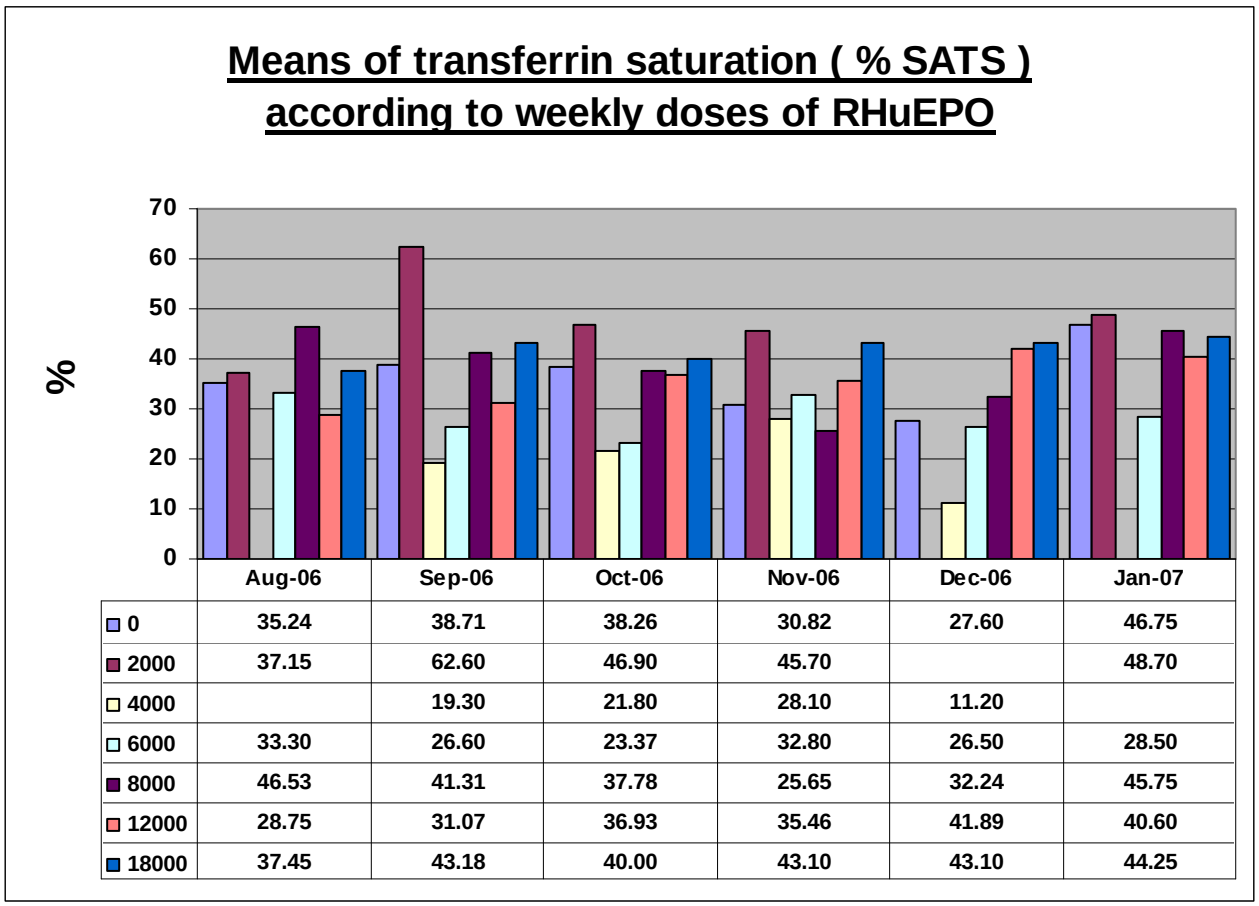


	Aug-06	Sep-06	Oct-06	Nov-06	Dec-06	Jan-07
0	11.57	11.09		11.17	11.00	10.83
2000	8.60	11.20	10.90	10.40		9.40
4000		6.70	6.50	10.40		
6000	12.30	12.35	11.40	10.80	10.60	10.00
8000	10.27	10.69	11.67	8.93	10.26	8.84
12000	9.65	9.44	8.92	9.45	9.55	9.47
18000	9.60	9.55		7.42	7.88	7.40

Fig 4.10 Haemoglobin levels of all HD patients according to weekly RHuEPO doses

4.2.6 Transferrin saturation (% SATS) of haemodialysis patients according to weekly RHuEPO doses during the trial period

The transferrin saturation in all groups of weekly doses of RHuEPO fluctuated during the six months (Figure 4.12). The HD patients on zero doses RHuEPO maintained % Sats from 30% and higher, whereas in December the %Sats had dropped to 27.6%. In the other groups the similar trend was observed.



	Aug-06	Sep-06	Oct-06	Nov-06	Dec-06	Jan-07
0	35.24	38.71	38.26	30.82	27.60	46.75
2000	37.15	62.60	46.90	45.70		48.70
4000		19.30	21.80	28.10	11.20	
6000	33.30	26.60	23.37	32.80	26.50	28.50
8000	46.53	41.31	37.78	25.65	32.24	45.75
12000	28.75	31.07	36.93	35.46	41.89	40.60
18000	37.45	43.18	40.00	43.10	43.10	44.25

Fig 4.11 Transferrin saturation of all HD patients according to weekly RHuEPO doses

4.3 Comparisons of EPO and HB

4.3.1 Erythropoietin concentration versus haemoglobin levels over six months

The EPO concentration of all haemodialysis patients with the haemoglobin levels for four months selected (Figure 4.12 to Figure 4.14) presented scattered on the graph. In all six months of the trial, August 2006 to January 2007 the EPO concentration versus the HB levels were scattered.

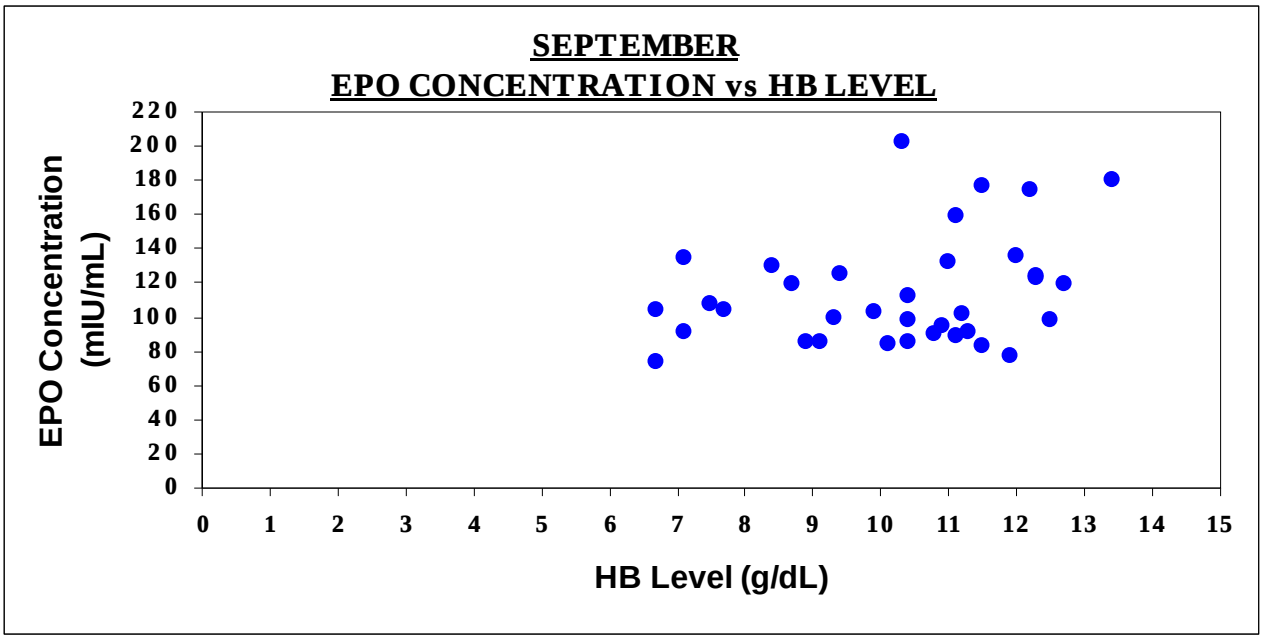


Figure 4.12 EPO concentration versus HB levels in the month of September

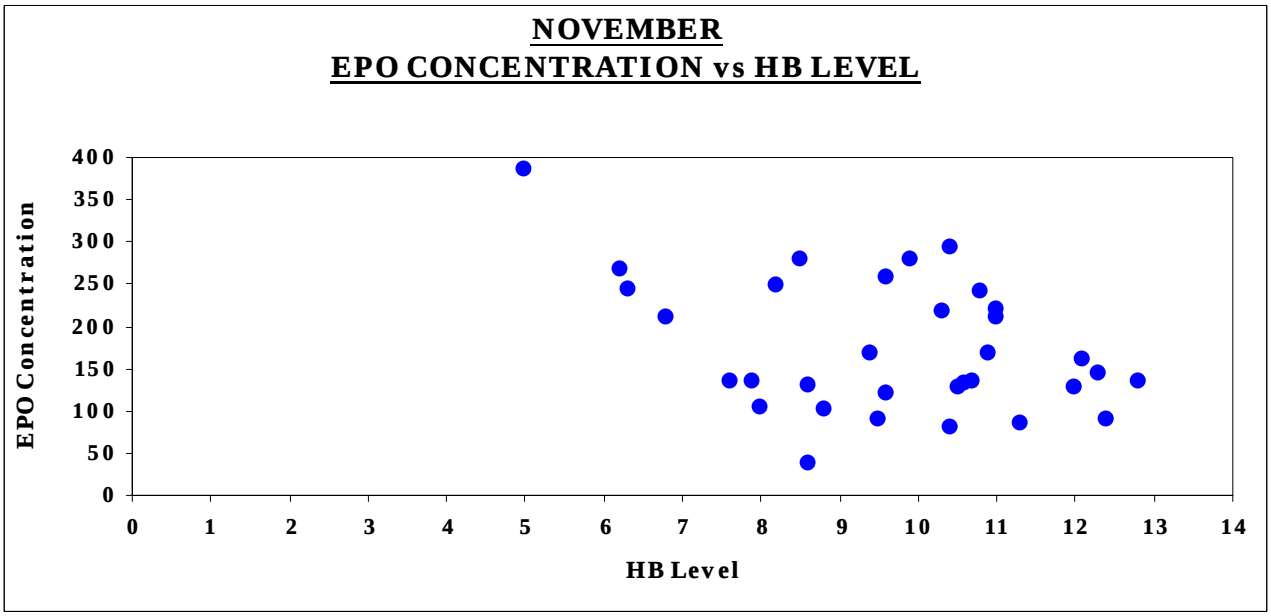


Figure 4.13 EPO concentration versus HB levels in the month of November

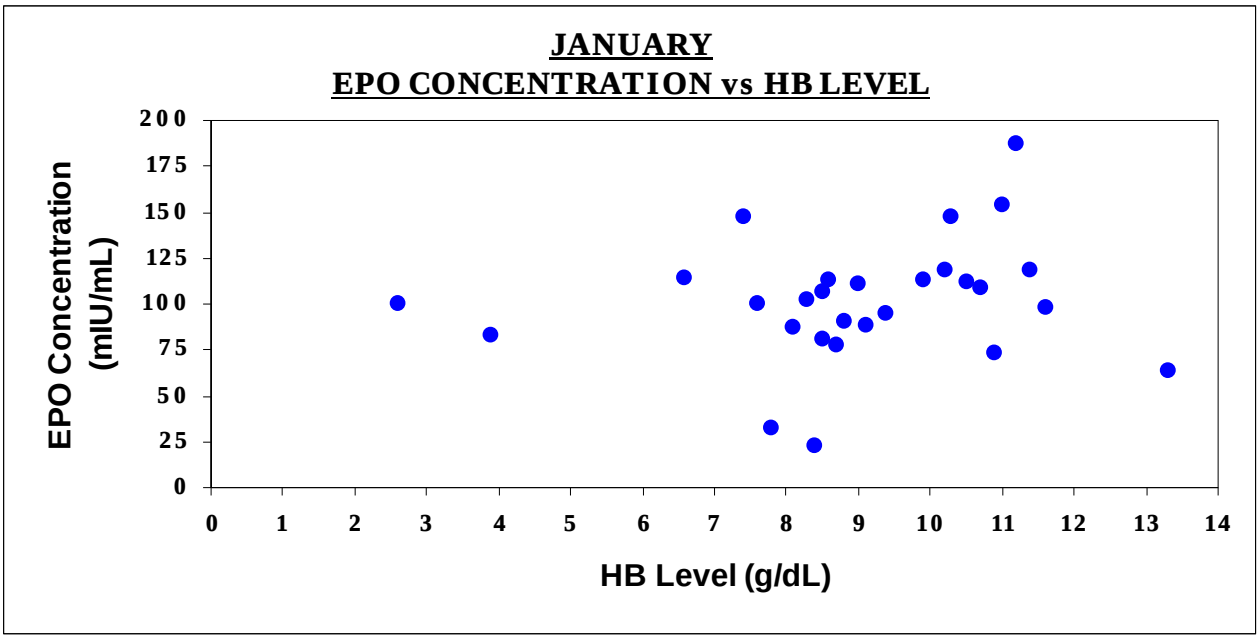


Figure 4.14 EPO concentration versus HB levels in the month of January

4.3.2 Antibody levels versus haemoglobin levels over six months

The antibody levels versus the haemoglobin levels were also scattered. From the months of August 2006 to January 2007 of the trial (Figure 4.15 to Figure 4.18), there had been a correlation between the antibody levels and the haemoglobin levels. In the month of December (Figure 4.17) the correlation was very significant R=0.1 as compared to the other months .This was a significant trend in the haemodialysis patients receiving RHuEPO, an increase in the antibody levels. An increase in the antibody levels corresponded with a decreased HB level. The remaining months presented the correlation of HB levels and AB levels which were not significant.

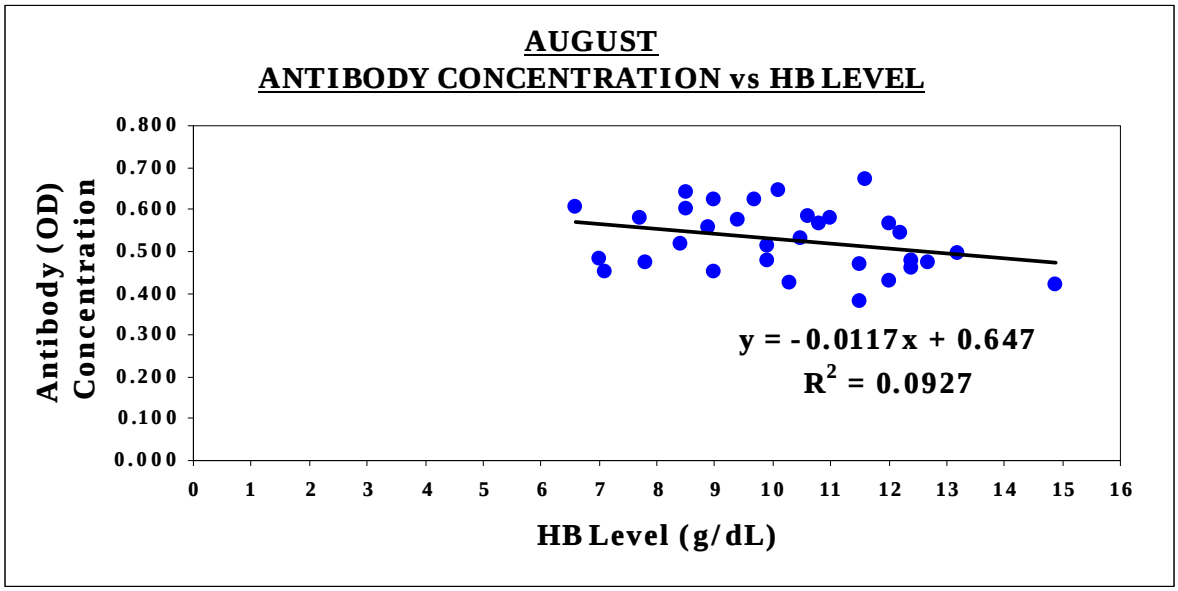


Figure 4.15 Antibody levels versus HB levels in the month of August

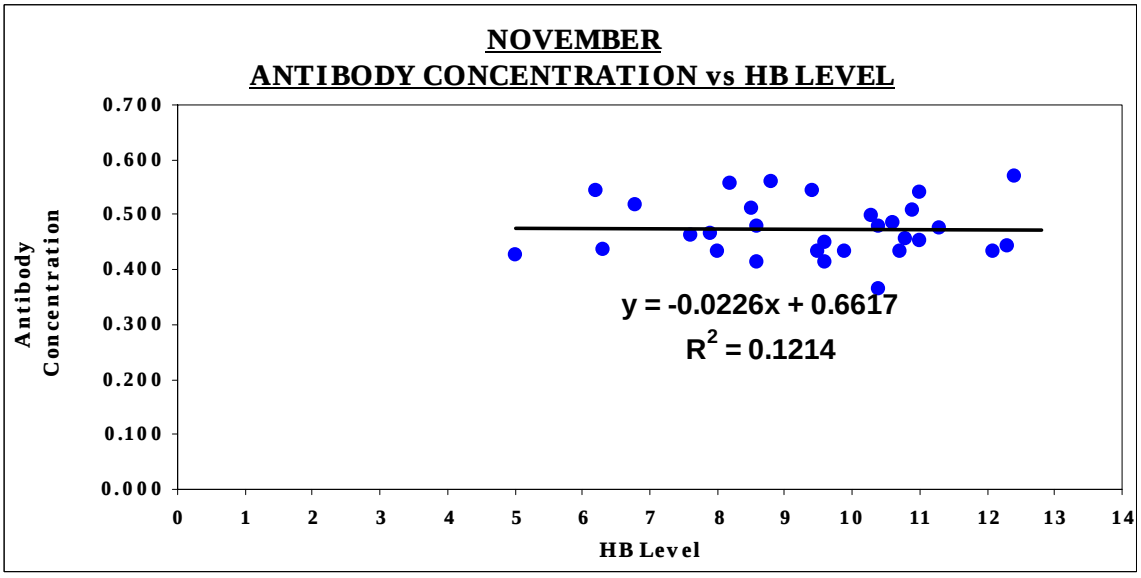


Figure 4.16 Antibody levels versus HB levels in the month of November

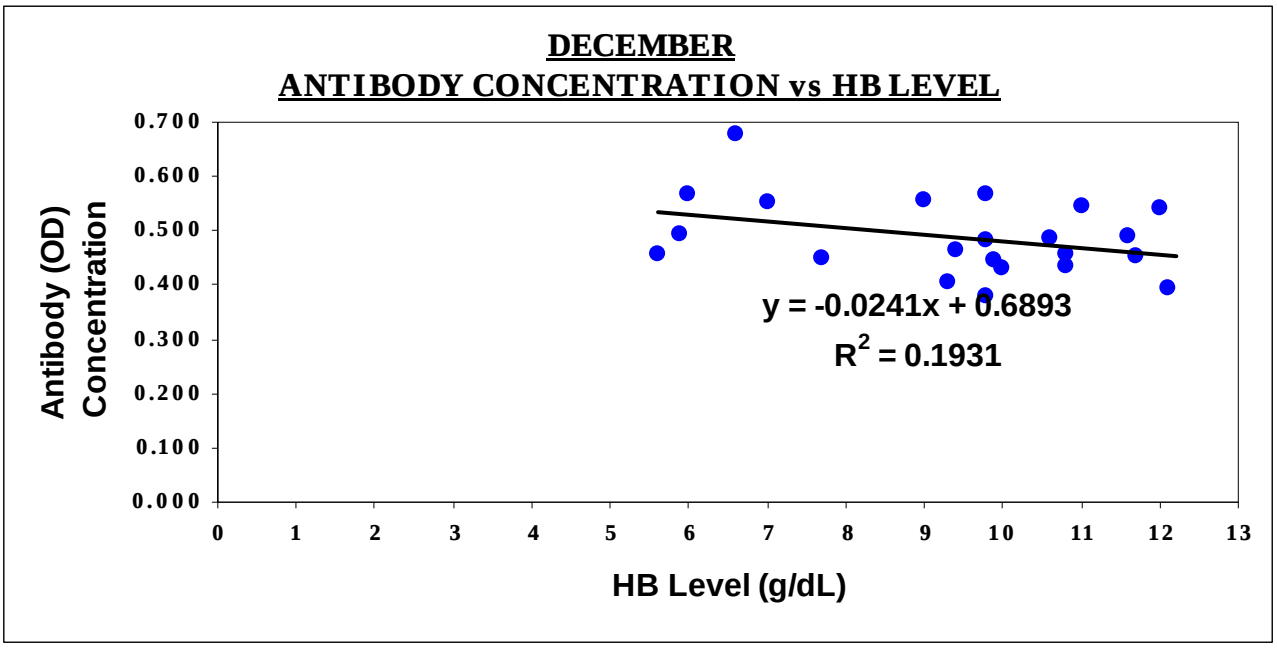


Figure 4.17 Antibody levels versus HB levels in the month of December

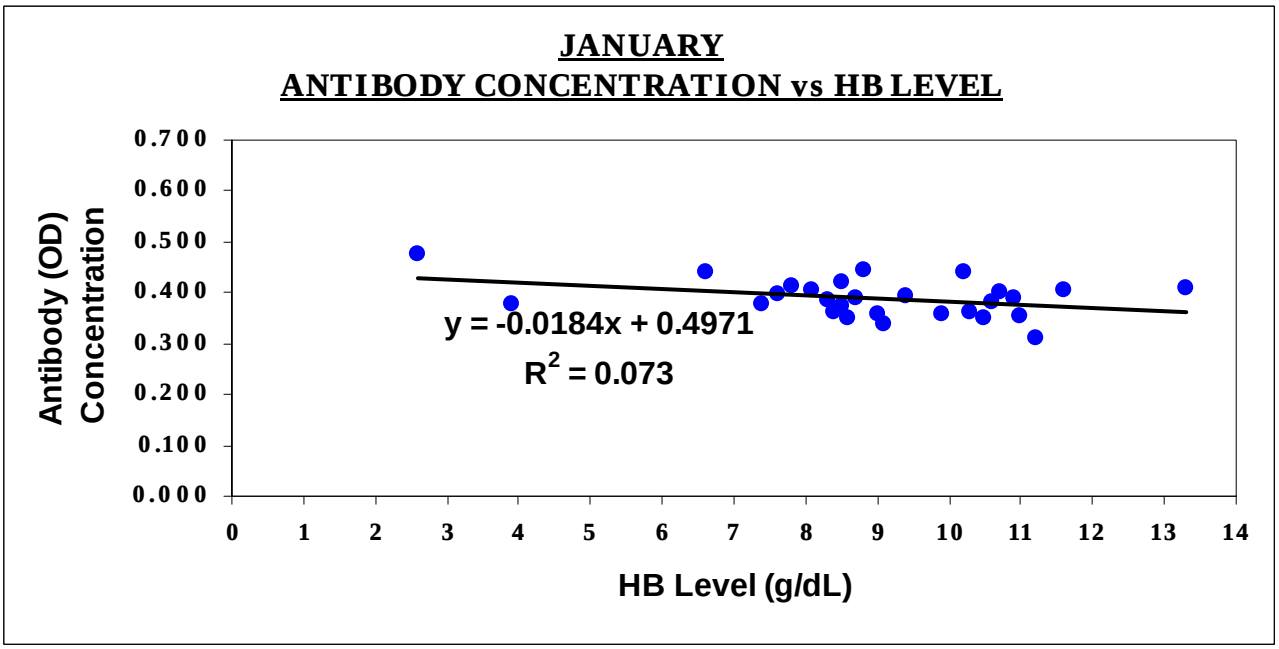


Figure 4.18 Antibody levels versus HB levels in the month of January

4.3.3 Erythropoietin concentration versus antibody levels in HD patients over six months

The EPO concentration was stable during the study period (132.84). The antibody to EPO levels varied more widely between 80-140 or 50-400 OD. When compared to the normal individuals, significantly high antibody levels were found in few hemodialysis patients receiving EPO. No correlation had been observed with the antibody levels and the erythropoietin levels in the HD patients receiving RHuEPO throughout the trial.The correlation between the EPO concentration and the AB levels were insignificant, R= < 1.

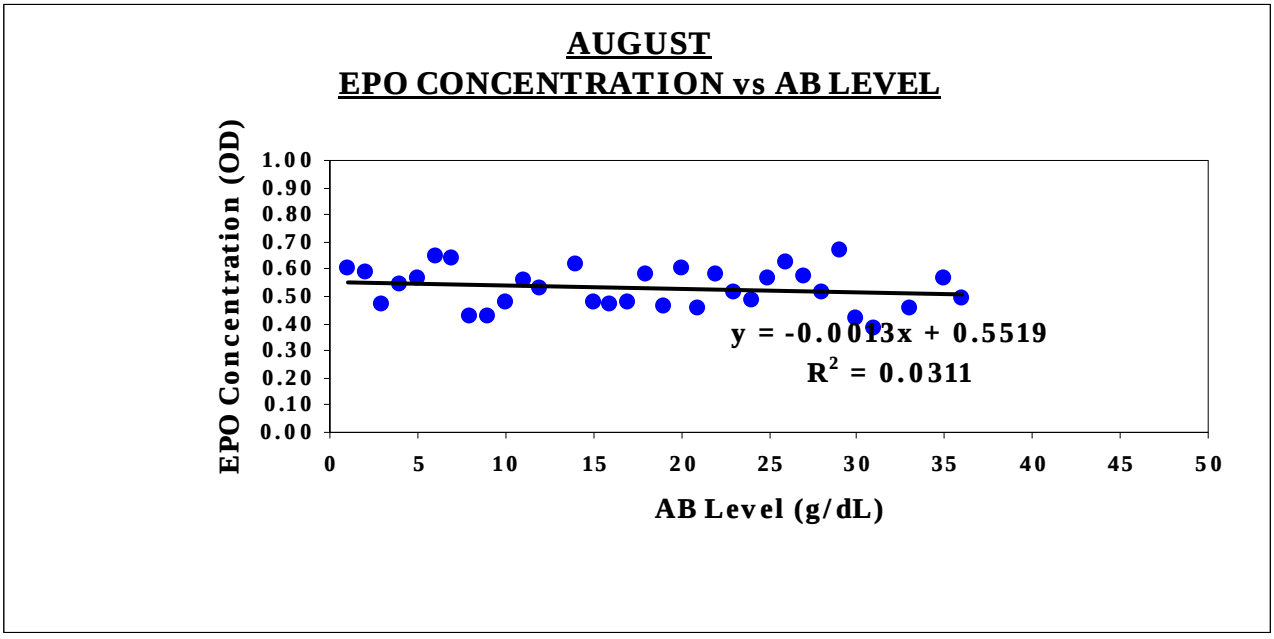


Figure 4.19 EPO concentration versus AB levels in the month of August

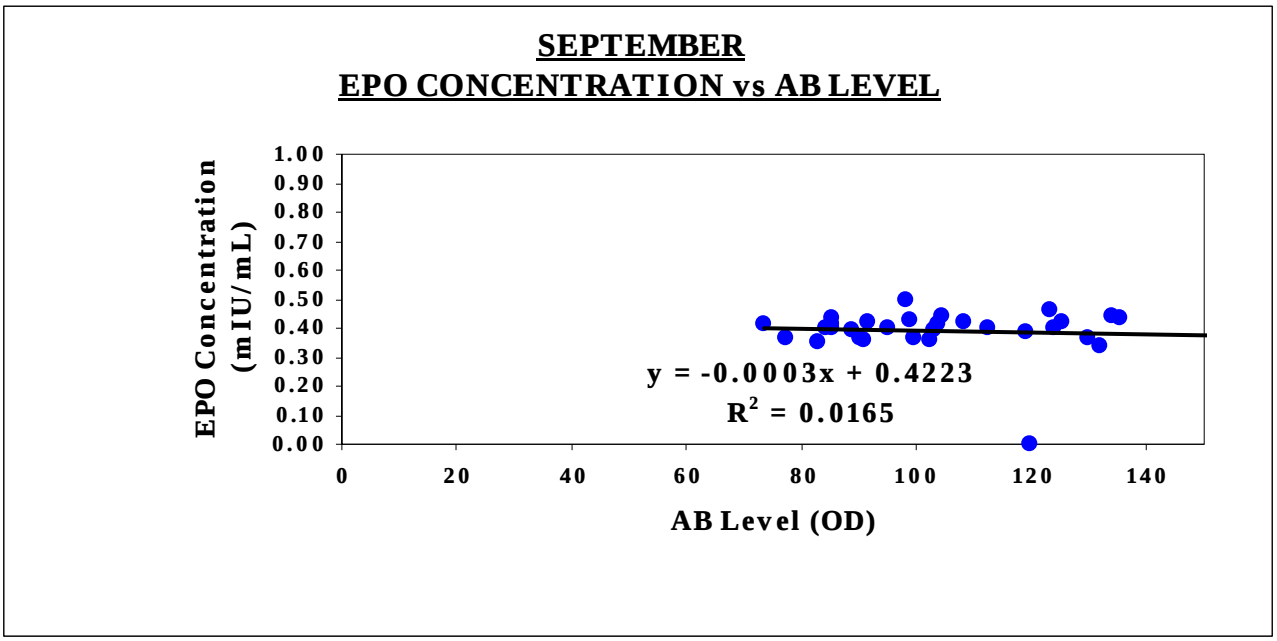


Figure 4.20 EPO concentration versus AB levels in the month of September

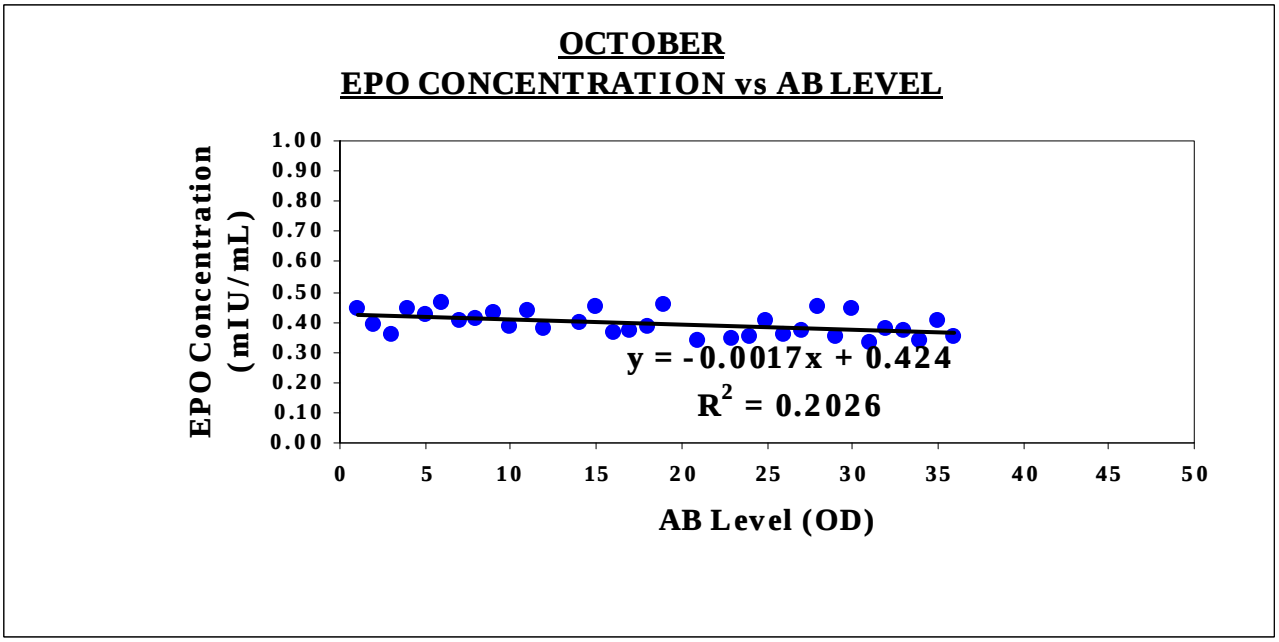


Figure 4.21 EPO concentration versus AB levels in the month of October

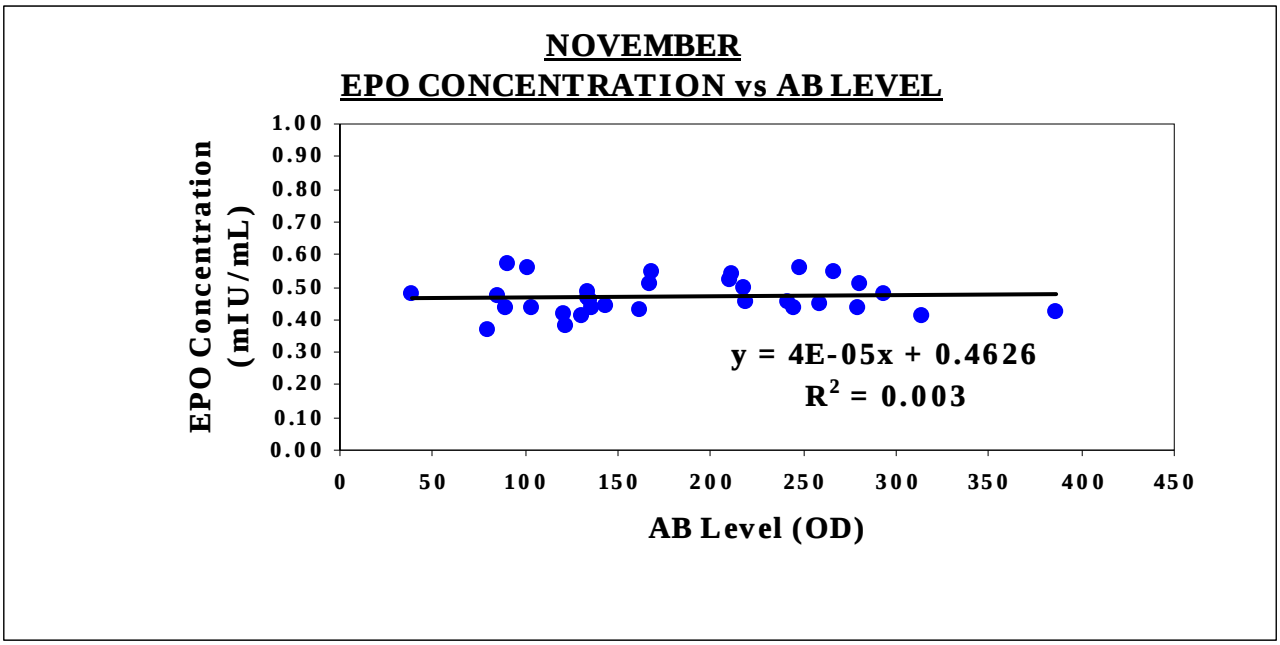


Figure 4.22 EPO concentration versus AB levels in the month of November

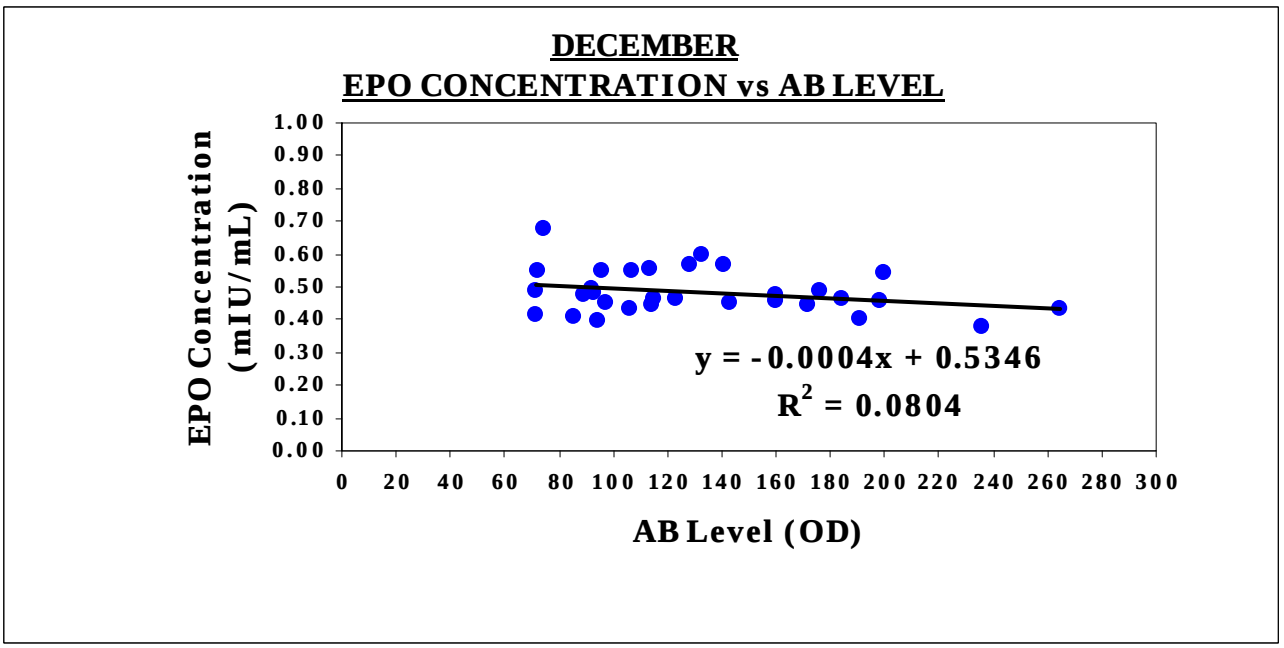


Figure 4.23 EPO concentration versus AB levels in the month of December

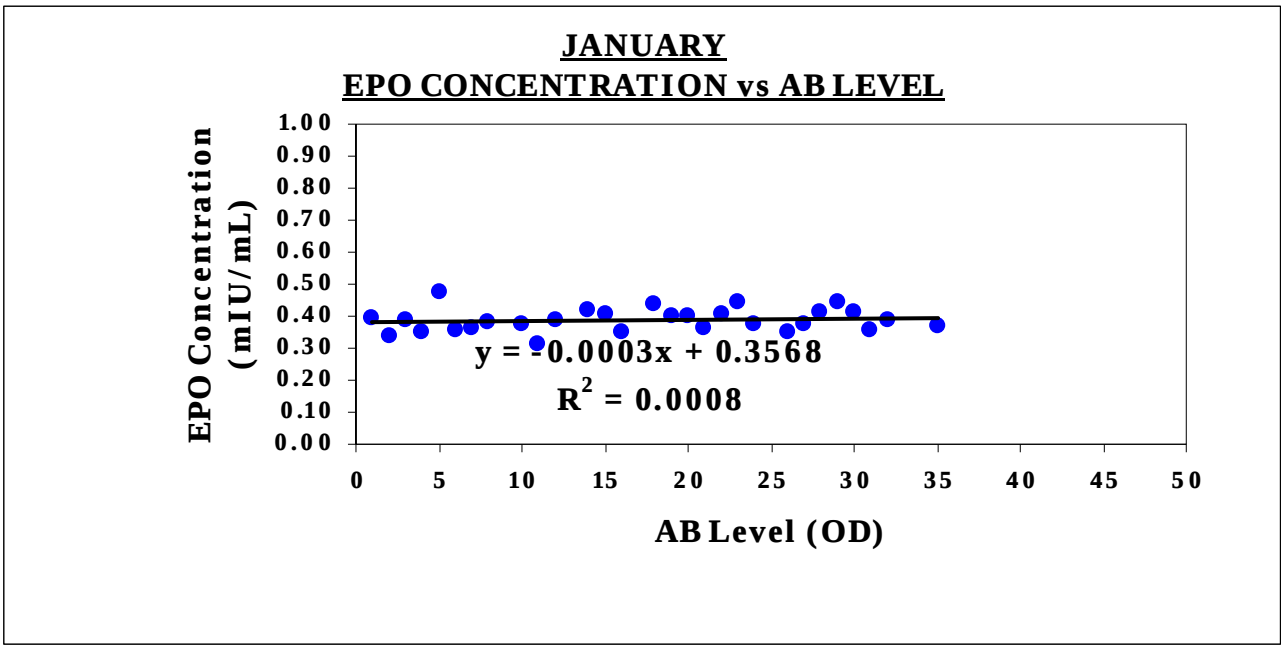


Figure 4.24 EPO concentration versus AB levels in the month of January

4.4 The blood levels of the healthy individuals not on haemodialysis

The blood levels of the two groups of healthy individuals (Table 4.1) represent the EPO concentration, AB levels, and the HB levels. The mean male EPO concentration (93.59, ±11.5Miu/mL) was lower than that of the female mean EPO concentration (213.19, ±24.6 Miu/mL). There was no significance between the two means ($p > 0.05$).

The antibody levels ranged between 0.16 and 0.36 in the both groups of healthy individuals, although these individuals were not on the RHuEPO treatment and not on HD (Table 4.4). The mean AB levels in females (0.23, ±0.03) were higher than in the males (0.19, ±0.01 There was no significance between the two means in the male and female groups ($p>0.05$).

The mean haemoglobin levels (Table 4.1) in the healthy male individuals (15.2, ±0.32 g/dL) were higher than the HB levels in the female individuals (12.1, ±0.14g/dL). The comparison between the two groups presented a significant difference ($p<0.05$).

Table 4.4 All blood levels of healthy individuals

Male HB (g/d L)	**Male EPO conc (mIU/mL)**	**Male AB (OD)**		**Female HB (g/dL)**	**Female EPO conc (mIU/mL)**	**Female AB (OD)**
15.3	75.74947	0.41		12.2	98.77763	0.421
13.9	77.18873	0.433		12.1	75.74947	0.386
15.1	73.59058	0.478		12.4	141.95543	0.354
15.6	130.44135	0.319		12.3	213.19884	0.371
15.7	111.01134	0.495		11.6	94.45985	0.397
Means						
15.12	93.596	0.427		12.12	124.828	0.385
SEM± \						
0.323	11.508	0.030		0.139	24.609	0.011
Male and Female HB P(T<=t) one- tail	**Male and Female EPO conc P(T<=t) one- tail**	**Male and Female AB P(T<=t) one- tail**				
1.86	0.14	0.12				

4.5 THE COMPARISON OF THE HEALTHY INDIVIDUALS AND THE HD PATIENTS ON RHuEPO TREATMENT

4.5.1 The comparison of the healthy male individuals and the haemodialysis male patients on RHuEPO treatment

The mean EPO concentration of the healthy male individuals (93.59, ±11.50 mIU/mL) being almost similar in the male HD patients (94.09, ±7.54 mIU/mL) receiving RHuEPO treatment (Table 4.4). treatment. There was no significance between these two mean values ($p > 0.05$).

The mean HB levels of the healthy male individuals (15.12 ±0.32 g/dL) was significantly higher than the HD male patients (9.87, ±0.26 g/dL) receiving RHuEPO treatment (Table 4.4). This was a significant difference between the mean values ($p < 0.05$).

The mean AB levels of the healthy male individuals (0.42, ±0.03) were also very close to the AB levels of the male HD patients (0.45, ±0.01 on RHuEPO (Table 4.4). There was no significant difference between the two means ($p > 0.05$).

4.5.2 The comparison of the healthy female individuals and the haemodialysis female patients on RHuEPO treatment

The mean EPO concentration of the healthy female individuals (124.82mIU/mL, ± 24.60) was lower than the female HD patients (132.84, ±15.24mIU/mL) on RHuEPO treatment (Table 4.5). There was no significance between these means ($p > 0.05$). The

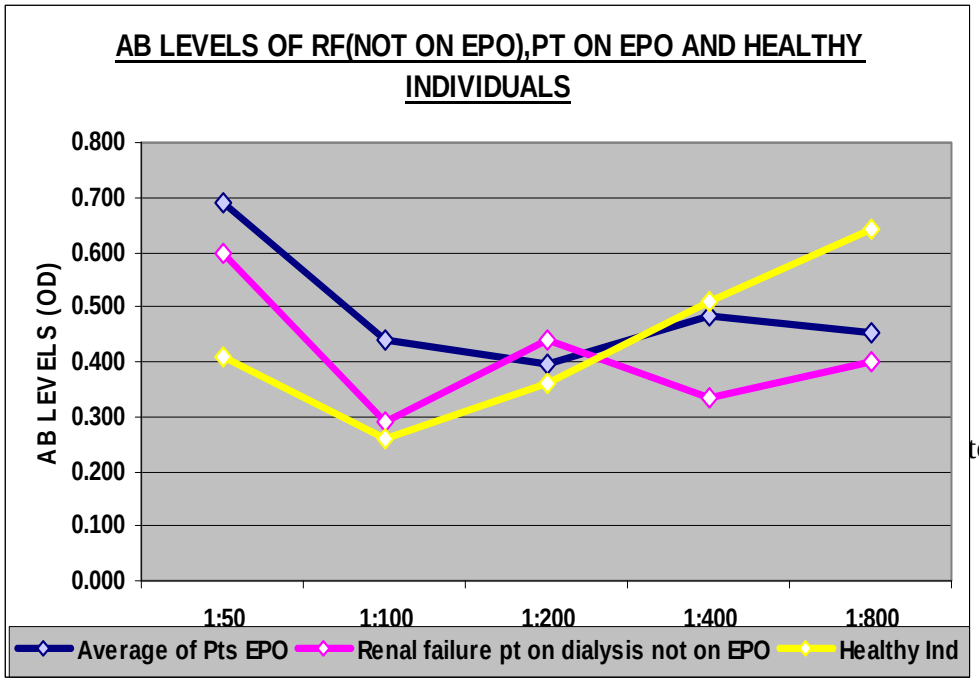
AB concentration of the healthy female individuals (0.38, ±0.01) was slightly lower than the female HD patients (0.43, ± 0.01) on RHuEPO (Table 4.5) RHuEPO. There had been no significance between the means ($p>0.05$). The mean HB levels of the female healthy individuals (12.12, ±0.13 g/dL) were higher than the HD female patients (9.99, ±0.27 g/dL) on RHuEPO (Table 4.5). There had been a significant difference between these two mean values ($p< 0.05$).

Table 4.5 The comparison of the healthy individuals and the HD patients on RHuEPO

	Male healthy individual	**Male HD-RHuEPO**		**Female healthy individual**	**Female HD-RHuEPO**
EPO conc (mIU/mL)					
Means	93.596	94.092		124.82	132.84
SEM ±	11.508	7.549		24.60	15.24
P(T=t) one-tail	0.491				
AB levels					
Means	0.427	0.454		0.385	0.434
SEM ±	0.030	0.011		0.011	0.011
P(T=t) one-tail	0.234				
HB levels (g/dL)					
Means	15.12	9.87			
SEM ±	0.323	0.264			
P(T=t)one-tail	0.000				

4.6 The antibody dilutions of 1:50 to 1:800 in the sample population

The three different groups of patients which included (HD patients on RHuEPO, HD patients not on RHuEPO, and the healthy individuals) were selected for the AB dilutions of 1:50 to 1:800. In all groups of the population, the AB levels were the highest in the 1:50 dilution (Figure 4.25). Although the other dilutions were acceptable the 1:50 were consistently higher in all groups of sample population. Therefore the 1:50 dilution for the AB detection ELISA test was selected.



to

Table 4.6 General distribution of patient numbers to the antibody levels

	AUG	**NOV**	**JAN**
AB LEVELS (OD)	Pt no's	Pt no's	pt no's
<399	3	3	18
0.400-0.499	19	21	13
0.500-0.599	12	8	nil
0.600-0.699	nil	nil	nil
0.700-0.799	nil	nil	nil
Healthy Individuals AB levels	**0.319-0.421**	**0.386-0.495**	**0.390-0.478**
Positive AB Levels >500	**Negative AB Levels <499**		

4.7 Distribution of the antibody concentrations

The antibody concentrations were the highest in the ranges of <399 in the months of September, October, and January (Table 4.6). These consisted of an average of 18 patients whom fell in this group during these months. An average of 14 patients during the trial period was in this group, which we considered as the background. Every month patients were in this AB concentration group (Table 4.6). Fifteen positive serum samples were seen in the month of August. During the months of November and December were eight

patients whom were tested positive using 1:50 dilutions. All patients with AB levels <499 was included as the AB that were tested negative as according to our background levels. All patients that had AB levels >500 OD were considered as our positive AB group.

Table 4.7 Distribution of patients according to antibody levels to RHuEPO

	AUG	**NOV**	**JAN**	
NEGTIVE ABS < 499				
MEANS HB (g/dL)	10.67	9.67	9.26	**P value >0.10**
MEANS EPO (mIU/mL)	280.36	316.16	280.36	**P value >0.10**
MEANS AB (OD)	0.454	0.440	0.388	**P value >0.10**
POSITIVE ABS > 500				
MEANS HB	9.89	9.21	Nil	**P value >0.10**
MEANS EPO	376.645	316.67	Nil	**P value >0.10**
MEANS OF AB	0.584	0.531	Nil	**P value >0.10**

4.8 Distribution of the patients according to high AB levels

Patients were grouped according to the antibodies being either positive or negative. The means of the HB levels and erythropoietin levels showed no statistical significance. In January there were no patients that had AB levels > 500.There was no significant difference between the positive and negative AB groups, for both of the HB and EPO levels

Table 4.8 A Summary of key results of selected patients with high AB levels

	AUGUST		**NOVEMBER**		**JANUARY**		**WKLY**
# 1							12000
HB (g/dL)	7.8		7.9		6.6		
EPO(mIU/L)	8.82		134.04		113.89		18000
AB (OD)	0.475		0.464		0.442		
# 2							
HB (g/dL)	11.6		5.0		7.8		18000
EPO(mIU/L)	318.5		385.91		378.5		
AB (OD)	0.671		0.426		0.412		
# 3							
HB (g/dL)	6.6		6.3		3.9		2000
EPO(mIU/L)	61.35		244.86		82.95		4000
AB (OD)	0.606		0.435		0.376		
# 4							
HB (g/dL)	9		10.3		8.2		18000
EPO(mIU/L)	203.4		266.45		No sample		
AB (OD)	0.625		0.543		No sample		
# 5							
HB (g/dL)	12.2		11		10.9		6000
EPO(mIU/L)	136.19		167.14		73.59		
AB (OD)	0.543		0.541		0.389		
# 6							
HB (g/dL)	8.9		10.9		8.5		8000
EPO(mIU/L)	136.19		167.14		80.78		
AB (OD)	0.558		0.508		0.372		

4.9 Selected patients with high AB levels

Six patients were selected with high AB titer / levels from three different months of the trial period (Table 4.8). The HB levels and EPO concentration was closely monitored. The weekly doses of RHuEPO were recorded accordingly over the selected months. A significant decrease in HB levels was observed in the above selected patients (#1 to #6). In some of the selected patients there was an increase in the EPO concentration over the months as in #1 and #4 with a decrease in HB levels. The remaining samples (#6, #5, #3, and #2) however had an inconsistent trend in the EPO concentration with a decreasing HB

levels (Table 4.8). In the one patient (#4), no ELISA was done for EPO and AB due to certain constraints. Patients from #2 to #5 were in our positive AB group ranging with AB levels >500 OD. Patient #1, however had AB levels <499 OD and were in our negative AB group.

CHAPTER FIVE: DISCUSSION

Erythropoietin levels

The EPO levels of our patients were measured using the ELISA over a period of six months. The EPO levels over the months increased in the last month as compared to the first month of the investigation. It was significant to note that in the third month there was an increase in the EPO level which settled in the last month of the trial. The grand total mean value of the EPO concentration of the female groups over the period of the trial was 132.84, ±15.24 mIU/mL as compared to the male groups of a mean EPO concentration of 94.09, ±7.55 mIU/mL. The means in our female groups presented with higher EPO levels as compared with the means of the male groups over the period of the trial. The p value =0.01.

Whereas, the healthy individuals means of the females EPO levels was 124.83 mIU/mL and males was 93.60 mIU/mL. This was not statistically significant as the (p value = 0.14). The healthy individuals EPO levels in the male group were compared to that of the HD male group receiving EPO treatment. It was not statistically significant (p = 0.49). Although the means in the males, the healthy individuals not receiving EPO was 93.60, ± 11.51 mIU/mL and the means in the male receiving EPO was 94.09, ±7.55 mIU/mL.

Weekly erythropoietin doses

The groups were further divided into their weekly beta EPO doses respectively. The EPO levels were fairly consistent in all groups of the weekly epoetin beta doses from the (0, 2000iu, 4000iu, 6000iu, 8000iu, 12000iu, 18000iu) in the haemodialysis

patients. However the number of females per group presented with the higher means of EPO levels as compared with the males in all epoetin groups. The mean values of the male HD patients not receiving EPO (0 doses) over the trial period was 123.16mIU/mL and the female group 132.29mIU/mL. The females receiving 2000iu EPO weekly doses had means of 135.36 mIU/mL as compared to the males of 61.36 mIU/mL. The females receiving 6000iu weekly doses had means of 120.20 mIU/mL and males had 84.39 mIU/mL. The female group in the 8000iu weekly dose had means of 143.23 mIU/mL as to the males of 139.04 mIU/mL. The females in the 12000iu weekly dose had means of 147.38 mIU/mL as to the males of 104.08 mIU/mL. The females in the 18000iu weekly dose had means of 141.40 mIU/mL as to the males of 122.87 mIU/mL. Mean values of the EPO levels during the trial period had increased in all groups of EPO doses. The haemodialysis patients receiving RHuEPO treatment presented with higher mean EPO levels as compared with the haemodialysis patients not receiving RHuEPO and the healthy individuals not in chronic renal failure. In the first month of the trial the total sample population of both male and females together presented in the 8000iu weekly dose, with a mean in the EPO levels of 61.58, ± 21.01 mIU/mL and the p value >0.01.

The erythropoietin levels presented were seen to be lower in the patients who did not receive RHuEPO than patients whom were receiving RHuEPO. Similar findings of higher EPO levels in paediatric peritoneal dialysis patients were observed. The residual renal function could clear larger solutes and produce erythropoietin therefore the higher EPO levels (Beckman et al., 1988).

The study carried out by Ifudu showed that women in end stage renal disease on HD required a higher dose of EPO to attain an equivalent haematocrit equivalent to that

of men (Ifudu, 2002). If any clinical implications, of gender associated differences in haemaocrit and RhuEPO dose are unclear. There are no gender differences in the severity of anaemia- induced end organ injury. It has been noticed that healthy women and those with end stage renal disease, the lower haematocrit may be protective as blood donation in men with normal renal function is associated with reduced incidence of acute myocardial infarction (Tuomainen et al., 1997). End stage renal disease therapy of the target haematocrit and RHuEPO was varied according to gender. The more critical concern is because of the higher dose of RHuEPO requirements in women; women on HD may be given i.v. iron to treat low haematocrit (Ifudu et al., 2000).

Earlier studies usually included a limited number of patients mostly individual cases were EPO was measured occasionally. This phenomenon received less attention because of efficient RHuEPO therapy and highly infectious hepatitis B virus (HBV) (Brown et al., 1980; Klassen et al., 1990). Apart from the kidneys, the liver has some potential to produce EPO. The stimulation of hepatic EPO production had been considered an explanation for the decreased anaemia in haemodialysis (HD) patients with the hepatitis virus infection (Meyrier et al., 1981; Klassen et al., 1990). Increased serum EPO levels in haemodialysis patients were seen in experimental animals, extra renal production of EPO was at maximum during liver regeneration (Brox et al., 1996). The rise in circulating EPO could be due to the prolonged life span of the hormone or to an increase in its production. Although the hepatocytes remove desialylated through their galactose receptors, the physiological significance of EPO catabolism is by the liver is doubted (Piroso et al., 1991). Therefore the hepatic synthesis of EPO may be stimulated along with that of acute phase proteins (Abel et al., 1996). RHuEPO dose requirement may be reduced in patients with

ESRD who develop hepatitis. This finding is attributed to elevated serum endogenous EPO produced in the liver or the reduced catabolism of EPO ((Ifudu et al., 2001; Klaasen et al., 1990).

As seen in the data (Table 4.5), the mean erythropoietin levels were higher in the haemodialysis patients receiving EPO as compared to the control groups. The HB levels had a decrease in the mean values over the six months in the haemodialysis patients receiving EPO as compared to the control groups were it had been fairly stable. The concentration of EPO depends not only on the HB concentration of the blood but also on the disease causing the anaemia. This however relates to the findings of tissue hypoxia which is the primary stimulus for the production of EPO. In severe anaemia the concentration of EPO increases hundred fold or more in blood (Cotes, 1989; Erslev, 1987). The concentration of EPO depends not only on the haemoglobin concentration of the blood but also on the disease causing the anaemia. Patients' suffering from aplastic anaemia's had an increase serum EPO concentration than patients of other anaemia's (De Klerk, 1980).

The erythropoietin therapy retards the progression of chronic kidney disease. Transplant recipients with the evidence of chronic allograft dysfunction benefit from erythropoietin therapy. The decline in renal function had decelerated during the EPO treatment. The glomerular filtration rate (GFR) appeared to stabilise in the EPO treated patients which had accounted for the change in renal function (Becker et al., 2002). Patients who have undergone a previous kidney transplant require higher doses of EPO than those whom have never undergone a kidney transplant (Almond et al., 1994). It is unclear why the high EPO dose requirement persists in patients after the transplant nephrectomy (Ifudu, 2002).

Hemoglobin levels

The HB level is affected by nutritional status, inflammation, and the availability and effectiveness of the RHuEPO, therapy as well as the degree of kidney function (Levin, 2002). The question arises on what factor or environment is being enhanced to not allow for the increasing stimulation of erythropoietin. The increased EPO levels have an underlying phenomenon of the environment that is affecting the process of erythropoietin as been seen in the results obtained. The environment in the HD patients on EPO treatment is not the same as in the healthy individuals.

Roman et al., 2004, in their current study which is part of the ongoing effort to elucidate the value of raising the haemoglobin target in dialysis patients. Partial correction of anaemia by erythropoietin improves haemodialysis (HD)-associated immunosuppression. The study compared the immune function of HD patients with congestive heart failure or ischemic heart disease on erythropoietin therapy randomized to normal versus anaemic blood haemoglobin concentration. Delayed-type hypersensitivity, CD4 and CD8 counts, anti–tetanus toxoid antibody levels taken after vaccination, erythrocyte complement receptor 1 expression, and lymphocyte proliferate responsiveness were taken as measures of their immune status. The observation period was 1 yr, and the trial was open label. Their data suggest that certain aspects of immune function, particularly delayed-type hypersensitivity, may be improved in HD patients by normalization of haemoglobin through the administration of increased doses of erythropoietin (Roman et al., 2004).

Recombinant erythropoietin had considerable effects on the immune system. Pfaeffl et al., 1998, found a significant decrease in the CD3, CD4, CD8, after 18 month of administration of RHuEPO compared to their control group values although the

CD4, CD8 ratio was increased. Other studies showed that RHuEPO had some effects on both humoral and cellular immunity as an immunodulating factor (Barany et al., 1992; Imiela et al., 1993).

The increasing rate of RHuEPO administration in dialysis patients was shown in the high AB titers (Friedlaender et al., 1996; Gharavi et al., 2001). Other experiments showed that RHuEPO affected the immune cells and the cytokine production. The administration dose (Imiela et al., 1993), length of treatment (Imiela et al., 1993; Trzonkowski et al., 1996) and the rate of presensitization of the patients with renal deficiency could have influenced the RHuEPO effects on the immune system as well as the antihuman leukocyte Ag (HLA) titer. The reduction of anti HLA Ab titer was observed. Vella et al., 1998 reported that RHuEPO could not decrease anti HLA titer in patients who had been highly sensitised by multiple pregnancy, blood transfusions or previous transplantation. The lower reduction in the AB titer in their group was caused by the activation of their immune system. The RHuEPO was not effective on the reduction of the Lymphocytotoxic AB titer (Vahdati, 2005).

In our study there was no statistical significant change in the haemoglobin levels over the six month period from the patients receiving RHuEPO. The comparison of the male and female groups on HD receiving EPO treatment was as follows: the mean values of the HB levels in the males were 9.89, ± 0.26 g/dL, and in the females the mean HB was 9.99, ± 0.27 g/dL. The (p value = 0.38) which was statistically insignificant in the comparison of the HD patients in the male and female groups receiving EPO treatment. Whereas compared to the healthy individuals the comparison of the HB levels in the male and female groups are as follows the mean HB in the male group was 15.12, ± 0.32 g/dL and the mean HB in the female was

12.12, ± 0.13 g/dL. The (p value = 0.00) which was statistically significant with the comparison of the male and female mean HB levels in the healthy individuals.

The decrease in the mean HB values of both male and female groups was observed with the different weekly doses of epoetin beta. The decrease in the haemoglobin levels were seen in the different epoetin weekly doses. No correlation had been seen with the high EPO levels and the decreased HB levels during the course of the trial. However the healthy individuals had higher HB levels. The HB levels were also maintained throughout the trial in the haemodialysis patients not receiving RHuEPO. A degree of EPO resistance had been observed in the haemodialysis patients receiving RHuEPO as compared the control groups. The observation from the results obtained that the healthy individuals had adequate EPO levels produced by the kidney which had a positive effect on the haemoglobin blood levels. However this had not been noticed in the renal patients receiving RHuEPO. This has been established in renal anaemia that the resistance to RHuEPO has been defined as the failure to attain target haemoglobin while receiving high doses of RHuEPO to maintain the target haemoglobin.

In the oncology or haematological setting the resistance to RHuEPO therapy had been regarded as no satisfactory haemoglobin increase despite high weekly doses of RHuEPO (Ng et al., 2003). The inadequate response of EPO treatment was seen in the absolute and functional deficiency as well as elevated C-reactive protein (CRP) levels which the author suggests to screen patients for other causes of inadequate responses to EPO therapy (Horl et al., 2000).

High plasma PTH concentrations have been associated with resistance to epoetin, but treatment with active forms of vitamin D may counteract it (Carozzi et al., 1990). Tarng et al., treated 12 patients resistant to EPO, iron overloaded with ascorbic acid. After 8 weeks of treatment an increase in the haematocrit and transferrin was noticed which allowed a decrease in the monthly dose of RHuEPO? Possible explanations for this effect of ascorbic acid include the increased iron absorption, mobilization of iron from tissue stores and increased iron utilization in the erythron (Tarng et al., 1999).

The means of the HB levels in the total sample population during the period of the trial of six months varied in the different groups. The HD patients not on EPO treatment in first month had a mean HB of 11.57g/dL and the last month mean HB was 10.83g/L. Patients on 2000 iu/wk EPO in the first month presented with mean HB of 11.20 and last month dropped to 9.40g/dL. Patients on 6000 iu/wk EPO in the first month the mean HB 12.30gd/L and the last month was 10.0g/dL. Patients on 8000iu/wk EPO in the first month was 10.27gd/L and the last month was 8.84gd/L. Patients on the 18000 if/wk EPO in the first month the mean HB was 9.60 and the last month dropped to 7.40g/dL. These patients presented with no response to the higher doses of EPO treatment.

Patients who do not respond to high doses of EPO have been referred to as hyporesponsive or EPO resistant and these were in keeping with the results found by Dreuke, 1990; Stivelman, 1989). Despite high doses of epoetin, the Europoean Survey on Anaemia Management (ESAM) showed patients with low HB levels. The epoetin effect being dose dependent, the administration of higher doses could overcome the inhibitory effect of circulating cytokines on erythropoietin hence

overcoming EPO resistance to a degree (Horl et al., 2000). The findings of Horl et al., (2000) was the achieved HB levels of patients with neoplasia or hepatitis were the same as his entire population sample of HD patients, this was in accordance with another study which the excessive cytokine production reduces the epoetin response (Horl, 1999).

Conclusion

The increase of EPO levels and the presence of antibodies with a decreased HB had been clearly observed. The role of the immune system in the control of the production of erythropoietin is poorly understood. The levels of circulating immunoreactive erythropoietin, tumour necrosis factor alpha, interleukin-1 beta and interleukin-6 was determined in 10 septic patients for up to 4 days following the admission to an internal intensive care unit. The data show that the production of erythropoietin was not suppressed despite an increase in the levels of proinflammatory cytokines. Circulating erythropoietin and interleukin-6 greatly increased in the 6 nonsurviving patients. The pattern of the serum erythropoietin level in the nonsurvivors resembled that of acute phase proteins and was independent of the blood haemoglobin concentration. Similar to interleukin 6, abnormally high serum erythropoietin levels appear to be a negative prognostic indicator in patients suffering from septic shock (Abel et al., 1996).

Chronic inflammation influences renal anaemia and reduces EPO effectiveness. Chronic kidney disease and haemodialysis induce elevated cytokine and C- reactive protein (CRP) levels at variable extents. The presence of the IL-6 allele -174G has an influence on the higher doses of epoetin in chronic HD patients. The polymorphism

of the anti-inflammatory interleukin -10 (IL-10) does not influence the epoetin doses, it acts against the systemic inflammation. This was probably due to that cytokines had inhibitory effects on haematopoiesis, in addition to its beneficial effects on inflammation (Girndt et al., 2007).

The optical density of the antibody levels compared in the male and female groups in the healthy individuals were insignificant ($p = 0.14$). The mean OD of antibody levels (AB) values in the males was 0.19, ± 0.02. The mean value in the females was 0.24, ± 0.03. However the HD patients receiving EPO treatment in the male and female groups were as follows; the mean AB values in the females were 0.25, ± 0.02. The mean AB values in the males were 0.19, ±0.01. The difference in the results between males and females had a (p value= 0.01) which was statistically significant in the comparison of the optical density of AB in the HD patients male and female groups receiving EPO treatment.

The means of the antibody levels, optical density (OD) in the trial period of six months in the HD patients with no EPO treatment (0 doses) was 0.42. The patients with 2000 iu/wk EPO presented with mean AB levels of 0.45. The patients with 6000 iu/wk presented with mean AB levels of 0.44. The patients with 8000 iu/wk presented with mean AB levels of 0.43. The patients with 18000 iu/wk presented with mean AB levels of 0.46. The mean AB levels were averaged in all our patient groups, therefore all our patients presented with antibodies.

Throughout the course of the trial there had been a correlation between the antibody titer and the haemoglobin levels. In the month of December only, the correlation was very significant were R=0.1 as compared to the other months .This was a significant

trend in the haemodialysis patients receiving RHuEPO, were an increase in the antibody(AB) titer correlated with the decrease in the haemoglobin levels throught the trial. An increase in the antibody titer corresponded with a decreased HB level. However there was a lack of correlation with the AB titer and HB. These results obtained showed some significance as compared with the results observed in the following studies.

There is some reason to discuss the presence of antibodies found in our study. This antibody titer corresponded with studies that were done in the past. Patients treated with RHuEPO to correct anaemia developed anti erythropoietin antibodies. These antibodies did not neutralize erythropoietin, but may have been low affinity antibodies (Castelli et al., 2000).

After an initial normal response to RHuEPO, patients from another study became severely anaemic and required repeated blood transfusions. The presence of strong, neutralizing antierythropoietin antibodies was demonstrated in all the patients. The antibodies recognized the core protein of the endogenous erythropoietin after complete deglycosylation of the molecule. The high affinity binding sites also bound denatured EPO, which suggested that these AB were directed against a linear epitope of the EPO molecule. In each of the cases the level of immunopreciptating antibodies was able to neutralize all EPO molecules produced by the patients, fully inhibiting erythropoiesis (Casadevall, 2002). We did not test whether the antibodies found in our results were neutralizing or not.

No correlation had been observed with the antibody levels and the erythropoietin levels in the HD patients receiving RHuEPO throughout the trial. As been observed

an increase in the EPO levels presented a fairly constant antibody titer. This however showed that the EPO level had no effect on the AB titer. In the healthy individuals a different trend was observed where the antibody titer did not correlate with the HB levels. These individuals had antibodies present, but they presented with higher HB levels in both of the groups which were the male and the female. In the second control group, the HD patients not receiving RHuEPO presented with antibodies but with fairly consistent HB levels thought the study. It is not clear. However they were not on RHuEPO for at least six months prior to our study.

The antibody titer was monitored according to the doses of RHuEPO in the relevant groups of the weekly epoetin beta doses from the (0, 2000iu, 4000iu, 6000iu, 8000iu, 12000iu, and 18000iu). No correlation of the antibody titer and the EPO levels was observed from our data. However an increase in the EPO beta doses had no increasing effect on the antibody titer as been seen in the HB levels. However the results obtained are supported that other factors other than the degree of divergence between the endogenous and the recombinant molecule could influence the immunogenicity of the therapeutic protein (Schellekens, 2002). Whereas patients do not generally produce anti- EPO antibodies because of the immunologic tolerance to self proteins (Schellekens, 2002).

The results obtained from our various groups of patients which included the haemodialysis patients receiving RHuEPO, the haemodialysis patients not receiving RHuEPO and the healthy individuals were reviewed. The antibody dilution of 1:50 was selected. The AB levels were the most appropriate in the 1:50 dilution for the AB detection.

The concentration levels of antibodies were grouped accordingly as positive AB's >500 and negative AB'S <499. The healthy individuals had AB levels in the ranges of 0.319 - 0.478 OD. These AB levels in the healthy individuals were used as the background. Three months were selected (August, November and January), majority of the patients had negative AB's. The results we found were similar to patients whom were receiving RHuEPO ten patients with AB levels that tested negative (Urra et al., 1997). However in August and November there were twelve (35%) and eight (25%) patients respectively that presented with positive AB's. Our results showed no positive AB's present in the month of January. We found no specific reason for this finding. The AB levels that tested positive from our results ranged from 500-599 OD.

There was no statistical difference in the HB levels and the EPO levels between the two groups. The positive and negative groups were categorised according to those who were tested positive and negative to RHuEPO. Although the mean HB levels were higher in the AB groups that tested negative, this was statistically insignificant ($p>0.10$). The EPO levels were higher in the positive AB group and it also presented to be statistically insignificant ($p>0.10$). However, the positive and negative AB groups had varying values of their EPO levels, HB levels and AB levels. These were all statistically insignificant ($p>0.10$).

The selection of patients with high AB levels was observed over a selected period of months. The AB levels and the HB levels dropped significantly during the months selected. The respective patients required blood transfusions accordingly. We can speculate that these patients could have positive AB's or neutralising AB's present. Further studies can be pursued. These patients were on continuous weekly RHuEPO therapy and were required and increase in their doses was made accordingly. The

ferritin levels in all patients were monitored during the period of the trial. All patients who required their weekly doses of iron were given accordingly. Iron supplementation was strictly monitored during the trial to ensure adequate iron levels were maintained. All the ferritin levels were within the target range for haemodialysis. In most cases the ferritin levels were above the target ranges and treated accordingly.

The % Saturation remained above 30% in all the groups of the patients with all the different weekly EPO beta doses. This represented adequate iron levels in the patient population number. Transferrin saturation (TSAT) was a parameter to measure circulating transferrin loaded with iron. Erythropoiesis becomes iron deficient when TSAT falls less than 20%. Treatment with higher ESA doses can result in a decrease in TSAT levels even under conditions of iron storage. These observations had been already been established (Horl et al., 2007).

The transferrin levels were monitored over the period of the trial, and the means of the different groups were fairly consistent throughout the months. In the male and female groups there had been no significant differences in the mean values of the transferrin levels. However a slight increase in the males was noted as compared to the females. Patients iron status was being maintained. Transferrin levels are affected by the visceral protein generation rate but are primarily influenced by iron status, with iron deficiency increasing, and iron excess reducing hepatic transferrin synthesis (Kuhlmann et al., 2007).

CHAPTER SIX: CONCLUSION

The results of the study showed that there were higher levels of erythropoietin concentration in the haemodialysis patients than the control groups selected. The healthy individuals who were not receiving RHuEPO had the presence of EPO concentration as well as the presence of an AB titer. This had no effect on their HB levels which were maintained. The antibodies present were in all groups of the sample population selected. However there was a lack of correlation with the increasing antibody titer and the HB levels in the haemodialysis patients receiving RHuEPO.

The antibodies however were not neutralizing further. The EPO levels were higher than normal levels as compared to the healthy individuals and the one other control group with lower levels of HB concentrations. For further investigation individual patients should be selected with positive antibodies and be screened. There were low HB levels observed despite the presence of antibodies. This allows us to further investigate on which antibodies were present. We measured the EPO levels, the AB levels and the HB levels. The AB levels were not correlating directly. However neutralizing antibodies could bind to the portions of the drug molecules involved in receptor binding or cell activation, thereby blocking the therapeutic effect of the drug (Zhong et al., 1998).

Patients selected with high AB levels were observed over a few months of the trial. The EPO levels and HB levels had significantly dropped during the time observed. Subsequent blood transfusions were required. We can speculate that these patients can have positive AB's present. In the study the HB levels were not increased over

the trial period with higher RHuEPO doses. Higher doses of RHuEPO therapy showed no clear increase in haemoglobin levels. From a total of forty patients, twelve patients (35%) in the months of August and eight (25%) in the month of November were tested positive for antibodies to RHuEPO. The serum of these patients needs to be further tested by the bioassay. These studies need to be pursued.The cohort of this study need to be further studied and monitored to detect those who may carry neutralising AB's. The bioassay may be useful to confirm the results found especially in the patients with increased AB levels and decreased HB levels. They may require assessing the functionality of the bone marrow. A comparison of the HB levels and EPO levels in the two groups with those positively tested AB's can be further studied.

The screening method using the bioassay would help those patients presenting with low EPO levels and increased EPO doses and the correlation of the antibody to EPO be determined. The bioassays are one of the exclusive assays that can quantitatively measure neutralizing activity against therapeutic proteins. Alteration of protein structure or blocking of receptor binding sites or other functional sites by antibodies can inhibit cell responses to cytokines and other therapeutic proteins (Sytkowski and Donahue, 1987).

Further investigations could be done to see whether the antibodies neutralize their growth. We can further investigate at EPO neutralizing, serum of these patients can be selected to analyse what resistance is to EPO for the cell to grow. There is that undefined question that some factor is reacting to the EPO for the growth .This is contributed with the bioassays which are used to quantify EPO neutralizing antibodies, including EPO stimulated growth of erythroid precursors in plasma clot

cultures of mononuclear blood cells or bone marrow cells (Casadevall et al., 1996; Lacombe et al., 1984). Bioassays are proven to be useful for the measuring the immunogenic potential of proteins expressed in different cells (Whadhwa et al., 1999) and studying the binding stoichiometries of antibodies with their target proteins (Grossberg et al., 2001). The biocore assay has been the most versatile surface for repeated assays (Swanson et al., 2004). 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 Biocore assay is recommended to be used for the further investigation of the measuring 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). Although there is a positive correlation between the antibody level and haemoglobin, the screening test way may have to be done to have clarity. Individual patients with suspected neutralizing antibodies will be assessed with the bioassay.

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CHAPTER SEVEN: REFERENCES

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CHAPTER SIX: APPENDICES

APPENDIX A

FLOW CHART

FEBRUARY – NOV 2005 (9 MONTHS)

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

DEC 2005 – JULY 2006 (8 MONTHS)

Consent to notify patients of study
Consent from staff in units and collection
Selection of patients (study population)

AUG 2006 – FEBRUARY 2007 (8 MONTHS)

Lab tests
Collection of samples
Checking parameters
Checking blood levels

MARCH 2007 -JULY 2007 (5 MONTHS)

Analysing
Presentation of results

AUG 2007 – OCT 2007 (3 MONTHS)
Bringing all information together

NOV 2008 – FEB 2008 (4 MONTHS)
Final write up
Correction of all information

Literature review and project write up is ongoing.

APPENDIX B

DURBAN UNIVERSITY OF TECHNOLOGY

CONSENT FOR PARTICIPATION OF STUDY

TITLE:

The assessment of antibodies to Erythropoeitin in haemodialysis patients

INTRODUCTION:

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.

PURPOSE OF THIS STUDY

Renal patients have a decreased production in 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.
This study will monitor your haemoglobin levels on monthly basis and monitor your body's response to maintain the production of red blood cells.

REQUIREMENTS OF THE PATIENT

You are required to attend every dialysis session and receive your treatment (injection) which will be given in your dialysis line as normal. Blood will be drawn from your dialysis lines and tested (10mls only) on a monthly basis for a period of six months.

PATIENTS RIGHT TO PARTICIPATE

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

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.

Informed Consent Form

Date :

Title of research study : The Assessment of Native Erythropoeitin And Antibodies to Recombinant Erythropoeitin in Haemodialysis Patients

Names of supervisors : Professor A.G Assounga, Dr J.K. Adam
Telephone : (031) 2401325 (031) 2085291

Name of research student : Sherilene C Benjamin
Cellphone : 073 508 0362

PLEASE CIRCLE THE APPROPIATE ANSWER:

1. Have you read the research information sheet? YES/NO

2. Have you had the opportunity to ask questions regarding this study? YES/NO

3. Have you received satisfactory answers to your questions?

YES/NO

4. Have you had the opportunity to discuss this study?

YES/NO

5. Have you received enough information about this study?

YES/NO

6. Do you understand the implications of your involvement in this study?

YES/NO

7. Do you understand that you are free to withdraw from this study?

a) At any time? YES/NO
b) Without having to give a reason for withdrawing? YES/NO
c) Without affecting your future health cares? YES/NO

8. Do you agree to voluntarily participate in this study?

YES/NO

9. Whom have you spoken to? _____

Please ensure the researcher completes each section with you.
If you have answered NO to any of the above, please obtain the necessary information before signing.

Please print in block letters:

PATIENT Name _____ Signature _____

WITNESS Name _____ Signature _____

RESEARCH STUDENT Name _____ Signature _____

APPENDIX C

DURBAN UNIVERSITY OF TECHNOLOGY

Isivumelwano Sokungenela Ucwaningo

ISIHLOKO:

Ukuhlolwa kwezivikeli mzimba kusetsheniziswa i Erythropoeitin ezigulini elilashelwa inzinso.

ISINGENISO

Uyamenywa ukuba uvolontiye ocwaningweni liwalesifundo.
Ulazi olukulenewadi iuzokwenza wazi ukuthi oluewaningo lumayelane nani, nokuthi uzozuza kanyani njengoba ulashwa ngomshini.Uma kunemibuzo uyacelwa ukuthi ubuze kunoma imuphi umucwaningi noma umsebenzi wase kilinki yezinso.

INHLOSO YALDLUCWANINGO

Iziguli zezinso zikhiqiza izinhlayiyane ezibomvu zegazi ezincane/bese zidinga ukulekelewa ngokuthi zifakelwe igazi elidingwa umzimba njalo ngasuku.

OKUDINGEKAYO EZIGULINI

Udingeka ukuthi uzenjalo ekiliniki yezinso ngezinsuku ozinikiwe.Ukuze uthole ukulashwa,umjovo ozonikwa ulandelana ngezinsuku zekilinike.

ILUNGELO LOKUNGENELA KWEZIGULI

Ukungenela lolucwaningo uyazi volontiyela.Uma uyeka noma ngasiphi isikhathi alipheli ilungelo lakho lokulashwa.

KUYIMFIHLO

Lonke ulwazi oluzotholakala ubuzwa luzoba imfihlo.Ulwazi oluyoqokelelwa kwabanye abantu lufakwe emiqulwini yocwaningo, angeke luqukethe ulwazi olobe lusho wena njengesiguli.

IFOMU LOKUZIVUMELA

Isihloko socwaningo Ukuhlolwa kwezivikeli mzimbazeErythrpoeitin e Ezigulini ezihamba umshini wezinso

Igama ukasuphavaza **:** Professor A.G Assounga, Dr J.K Adam
Ucingo : (031) 2401325 (031) 2085391

Igama lomfundi ocwaningayo : Sherilene C Benjamin
Ucingo : 073 508 0362

Ucelwa Uukuba Ufake Isikokela Kanje Empendulweni efanele:

1.Ulifundile iphepha olunolwazi locwaningo?

YEBO/CHA

2. Ubenalo ukuneliseka ezimpendulweni ithuba lokubuza imibuzo mayelana nalolucwaningo?

YEBO/CHA

3.Utholile ukuneliseka ezimpendulweni ozinikiwe?

YEBO/CHA

4. Ubenalo yini ithuba lokuthi niluxoxe lolucwaningo?

YEBO/CHA

5. Ulutholile ulwazi olwanele ngalolucwaningo?

YEBO/CHA

6. Uyayiqonda imiphumela yokuthi ungenele lolucwaningo?

YEBO/CHA

7. Uyaqonda ukuthi ukhululekile ukuthi ungaluyeka lolucwaningo:

a)Noma ngasiphi

YEBO/CHA

b) Ngaphandle kokuthi uthole isizathu

YEBO/CHA

c)Ngaphandle kokuthi ulahlekelwe amalungelo akho okulashwa

YEBO/CHA

8. Uyavumelana nokuthi uzivumele ukungenela lolucwaningo

YEBO/CHA

Uyacelwa ukuthi uqiniseke ukuthi unesi/umcwaningi uligcwalisa nawe lolucwaningo.
Uma uphendule ngo Cha kulokhu ekungenhla.Uyacelwa ukuthi uthole ulwazi olwanele ngaphandi kokuthi usayine.

Ucelwa ukuti ubhale ngamagama amakhulu

UFAKAZI IGAMA _____SAYINA _____

ISIGULI IGAMA _____SAYINA _____

UMGWANINGI/UMFUNDI IGAMA _____SAYINA _____

APPENDIX D

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 perioxidase – conjugated goatantihuman IgG

Horseradish perioxidase – conjugated goatantihuman IgG 5ul

PBS 11.5 ml

Preparation of substrate

The substrate was preapared 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
Tetramethyldenzidine	11ml

The solution was vortexed.

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

Stop Solution (2N Sulphuric acid)

Supplied from the ELISA kit

APPENDIX E

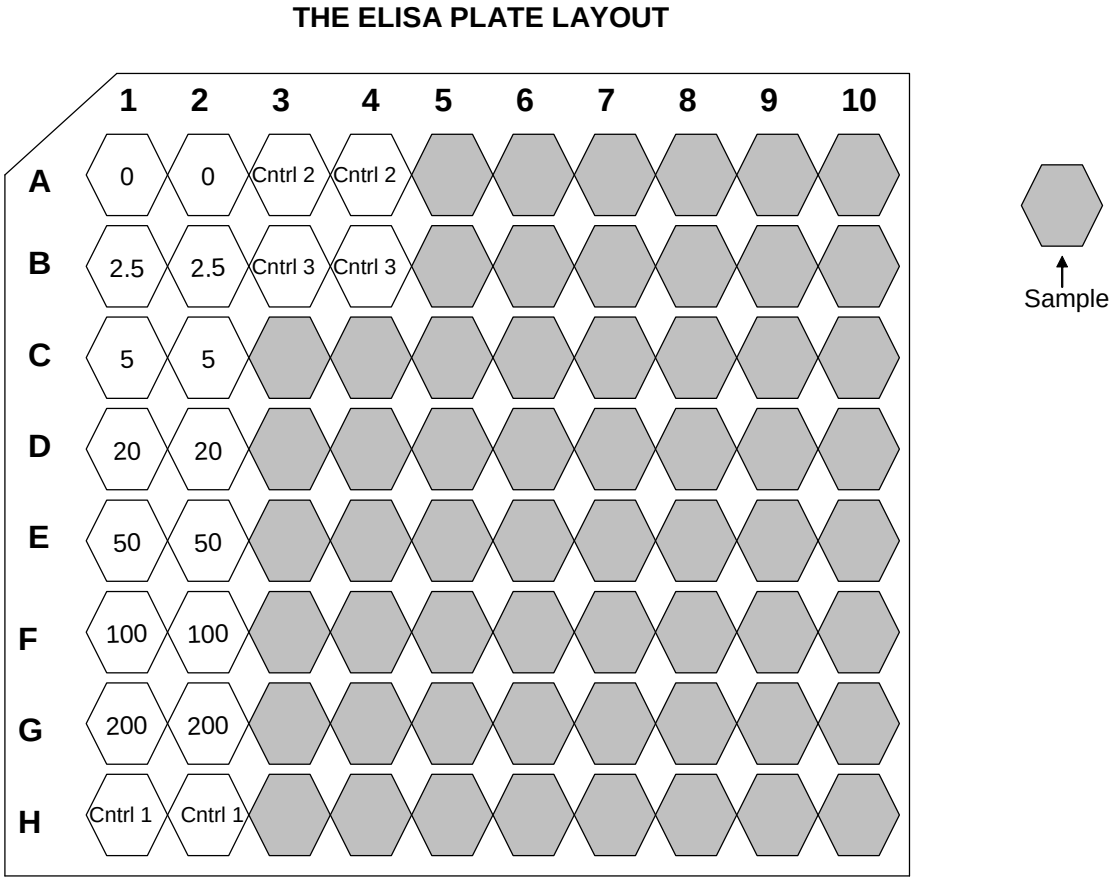

Fig 3.3 Plan of ELISA plate

APPENDIX F

FLOW CHART- SUMMARISING THE RESEARCH PROCESS