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## Evaluation of serum erythropoietin levels in chronic kidney disease patients in Federal Medical centre, Umuahia, Nigeria.

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### Abstract

Erythropoietin (Epo) is a glycoprotein hormone that regulates erythropoiesis. It is produced by interstitial fibroblasts in the kidney in close association with peritubular capillary and tubular epithelial tubule. The study was done to determine the levels of erythropoietin (Epo) in some patients diagnosed with renal diseases in Federal Medical Centre, Umuahia, Abia State. A total of one hundred and eighty (180) subjects (90 males and 90 females) were recruited for the study. One hundred and twenty subjects were recruited from those confirmed with renal diseases from the Urology Department of the hospital. Sixty (60) subjects (30 and 30 females) were predialysis chronic kidney disease patients, sixty (60) subjects (30 and 30 females) were chronic kidney disease patients on dialysis (D), and sixty (60) subjects (30 and 30 females) were the apparently healthy controls. Venous blood samples were collected from the subjects using aseptic standard method from the antecubital fossa. Serum samples were used for erythropoietin assay. Erythropoietin was determined by sandwich ELISA method. The results were statistically analysed using student t-test and statistical significance set at  $P < 0.05$ . The result showed significant decrease ( $P < 0.05$ ) in Erythropoietin levels of predialysis subjects ( $23.3 \pm 3.2$  iu/l) compared to Controls ( $48.6 \pm 24.0$  iu/l) respectively and showed significant increase ( $P < 0.05$ ) in erythropoietin level of patients on dialysis ( $71.4 \pm 14.7$  iu/l) compared to controls ( $48.6 \pm 24.0$  iu/l). The result also showed significant decrease ( $P < 0.05$ ) in Erythropoietin levels of predialysis subjects ( $23.3 \pm 3.2$  iu/l) compared to dialysis subjects ( $71.4 \pm 14.7$  iu/l) respectively. The study shows that Epo differs in predialysis patients. The difference could be due to the administration of recombinant erythropoietin to dialysis patients.

**Keywords:** Erythropoietin, Anaemia, chronic kidney disease, Predialysis, Dialysis

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### Introduction

Erythropoietin (Epo), is a glycoprotein hormone that regulates erythropoiesis. It is a cytokine for erythrocyte precursors in the bone marrow. Human erythropoietin has a molecular weight of 30.4 kDa [1]. It is produced by interstitial fibroblasts in the kidney in close association with peritubular capillary and tubular epithelial tubule. It is also produced in perisinusoidal cells in the liver. While liver production predominates

in the fetal and perinatal period, renal production is predominant during adulthood. In addition to erythropoiesis, erythropoietin also has other known biological functions. For example, it plays an important role in the brain's response to neuronal injury [1]. EPO is also involved in the wound healing process [2].

Exogenous erythropoietin is produced by recombinant DNA technology in cell culture. Several different pharmaceutical agents are available with a variety of glycosylation patterns, and are collectively called erythropoiesis-stimulating agents (ESA). The specific details for labelled use vary between the package inserts, but ESAs have been used in the treatment of anemia in chronic kidney disease, anemia in myelodysplasia, and in anemia from cancer chemotherapy. Boxed warnings include a risk of death, myocardial infarction, stroke, venous thromboembolism, and tumor recurrence. Exogenous erythropoietin has been used illicitly as a performance-enhancing drug; it can often be detected in blood, due to slight differences from the endogenous protein, for example, in features of posttranslational modification[2].

The primary role of erythropoietin is an essential hormone for red cell production. Without it, definitive erythropoiesis does not take place. Under hypoxic conditions, the kidney will produce and secrete erythropoietin to increase the production of red blood cells by targeting CFU-E, proerythroblast and basophilic erythroblast subsets in the differentiation. Erythropoietin has its primary effect on red blood cell progenitors and precursors by promoting their survival through protecting these cells from apoptosis[2].

Erythropoietin has a range of actions including vasoconstriction-dependent hypertension, stimulating angiogenesis, and inducing proliferation of smooth muscle fibers. It can increase iron absorption by suppressing the hormone hepcidin [3].

Multiple studies have suggested that erythropoietin improves memory. This effect is independent of its effect on hematocrit [4,5]. Rather, it is associated with an increase in hippocampal response and effects on synaptic connectivity, neuronal plasticity, and memory-related neural network [6]. Erythropoietin may have effects on mood [4].

Erythropoietin has been shown to exert its effects by binding to the erythropoietin receptor (EpoR)[7,8].

Erythropoietin binds to the erythropoietin receptor on the red cell progenitor surface and activates a JAK2 signaling cascade. Erythropoietin receptor expression is found in a number of tissues, such as bone marrow and peripheral/central nervous tissue. In the bloodstream, red cells themselves do not express erythropoietin receptor, so cannot respond to erythropoietin. However, indirect dependence of red

cell longevity in the blood on plasma erythropoietin levels has been reported, a process termed neocytolysis[8].

Erythropoietin levels in blood are quite low in the absence of anemia, averaging at around 10 mU/ml. However, in hypoxic stress, erythropoietin production may increase 1000-fold, reaching 10,000 mU/ml of blood. Erythropoietin is produced mainly by peritubular capillary lining cells of the renal cortex, which are highly specialized, epithelial-like cells. It is synthesized by renal peritubular cells in adults, with a small amount being produced in the liver. Regulation is believed to rely on a feedback mechanism measuring blood oxygenation [9]. Constitutively synthesized transcription factors for erythropoietin, known as hypoxia-inducible factors, are hydroxylated and proteosomally digested in the presence of oxygen. Erythropoietin is a 30.4 kD glycoprotein and class I cytokine consisting of 165 amino acids [10]. Erythropoietin has four acidic oligosaccharide side chains (3 N-linked and 1 O-linked) and contains up to 14 sialic acid residues. Its carbohydrate portion contributes 40% of its molecular weight [10]. The N-linked polysaccharide side chains appear to be important for the biosynthesis and secretion of erythropoietin, enhance its stability in blood, and limit hepatic clearance, thus facilitating the systemic transit of erythropoietin from kidney to bone marrow [11]. The variable nature of the sialic acid content gives rise to erythropoietin isoforms with differences in charge. As the number of sialic acid groups on the carbohydrate portion of erythropoietin increase, so does its serum half-life, whereas receptor-binding capacity decreases [8,12-14]. Clearance, however, appears to have a stronger influence on *in vivo* activity than receptor-binding affinity.

Each erythropoietin molecule has two erythropoietin receptor (EpoR) binding sites. There are two affinities of the EpoR for erythropoietin in solution: one of high and one of low affinity (needs 1,000 times the concentration of erythropoietin for activation) [14]. Diseases of the kidney are diverse, but individuals with kidney disease frequently display characteristic clinical features. Common clinical conditions involving the kidney include the nephritic and nephrotic syndromes, renal cysts, acute kidney injury, chronic kidney disease, urinary tract infection, nephrolithiasis, and urinary tract obstruction. Many other disease cases have adverse effects on the kidney which is the major site of production of erythropoietin such hypertension, diabetes, HIV/AIDS[14].

The kidneys secrete a variety of hormones, including erythropoietin, and the enzyme renin. Erythropoietin is released in response to hypoxia in the renal circulation. It stimulates erythropoiesis in the bone marrow[14].

Anemia can develop well before the onset of uremic symptoms due to end-stage renal disease (ESRD). Although anemia due to renal dysfunction generally develops when the glomerular filtration rate (GFR) declines to <30 mL/min, it can also be observed in those with markedly higher GFRs (such as 60 mL/min) and tends to occur at higher levels of GFR in African Americans than whites[14].

If left untreated, the anemia of chronic kidney disease is associated with several abnormalities. These include deterioration in cardiac function, decreased cognitive function and mental acuity, fatigue, and other signs and symptoms. There are also associations with an increased risk of morbidity and mortality, principally due to cardiac disease and stroke[14].

## Aim

The aim of this study is to estimate erythropoietin levels in Patients with chronic kidney diseases at the Federal Medical Centre, Umuahia.

## Materials and Methods

### Study area

The study was conducted at the Federal Medical Centre, Umuahia. Umuahia is the capital of Abia State, with a population of 264,662 and covers a land area of about 245KM<sup>2</sup>. It lies on Latitude 5.52627(decimal degree) North and Longitude 7.48959(decimal degree) East with an elevated altitude of 152 meters [15].

Umuahia is inhabited by the three major tribes of Nigeria but dominated by the Igbo speaking people. They are more of traders, Technical workers, civil servants and farmers. About two Universities are located there. They have few government hospitals which the most respected is Federal Medical Centre, Umuahia. They patronise drug dealers without consulting medical doctors, take a lot of NSAIDS, use herbal medicines, high prevalence of UTI and have few markets to buy better food stuffs.

## Advocacy, Pre-survey contacts and ethical considerations

With a letter of introduction from the Department, the secretary, Health Research and Ethical Committee of the Health Institution was met with a well detailed research proposal, after which an ethical approval was obtained for the research work.

### Study population and enrolments

The formula of Araoye [16] was used to determine the sample size.

$$n = \frac{Z^2 \cdot X \cdot P(q)}{d^2}$$

where n = sample size  
 z = confidence interval 95% (1.96)  
 p = prevalence rate  
 q = 1 - p  
 d = degree of freedom 5% (0.05)

Prevalence rate 7.7% according to Egbi *et al.* [17].

$$n = \frac{(1.96)^2 \times 0.077 \times 0.923}{(0.05)^2}$$

Minimum sample size = 100 but for the study sample size of 180 was used.

The study group (patients) were recruited from the Urology Department of Federal Medical Centre, Umuahia by the help of some nurses who helped in retrieving the patients' folders after they gave their consents. A total of one hundred and eighty (180) subjects were recruited for the study whose age ranges from 35-71 years. Sixty (60) subjects (30 males and 30 females) were chronic renal disease patients on predialysis, sixty (60) subjects (30 males and 30 females) were chronic kidney subjects on dialysis. Also, sixty (60) subjects (30 males and 30 females) were the controls who were apparently healthy individuals.

### Inclusion criteria

Written and oral consents were obtained from the subjects and other information which was helpful for the study. The subjects were selected based on those who were confirmed of having different renal diseases such as chronic kidney disease on predialysis and chronic kidney disease on dialysis. The control subjects were selected on ground that they were apparently healthy.

**Exclusion criteria**

For the study group, the subjects showing normal renal function, HIV/AIDS patients, patients with urinary tract infection, hypertensive patients, those on nephrotoxic drugs such as NSAIDs were excluded from the study.

For the controls, subjects showing any clinical conditions of any disease at all were excluded for the study.

**Sample Collection**

Sterile 5ml syringe was used to collect venous blood aseptically from the antecubital vein of each subject by standard technique. About 5ml of blood was dispensed into plain tubes for Erythropoietin assay. The blood samples for serum were allowed to clot for 2 hours at room temperature before centrifugation for 20 minutes at approximately 1000Xg.

**Laboratory investigations**

All reagents and kits were commercially purchased from reputable company whose standard operating procedures were strictly followed. Human Epo (Erythropoietin) ELISA kit was purchased from Elabscience with catalog No: E-EL-H0066c. The erythropoietin was bought from Elabscience Biotechnology Co.Ltd, Wuhan.

**Principle of Erythropoietin (Sandwich-Elisa Method) of Elabscience**

The ELISA kit uses sandwich-ELISA as the method. The micro ELISA plate provided in this kit was coated with an antibody specific to Erythropoietin. Standard or samples are then added to the appropriate micro ELISA plate wells and combined to the specific antibody. Then a biotinylated detection antibody specific for Erythropoietin and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Erythropoietin, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour turns yellow. The optical density (OD) is proportional to the concentration of Erythropoietin. You can calculate the

concentration of Erythropoietin in the sample by comparing the OD of the sample to the standard curve.

**Assay procedure**

All the reagents were allowed to reach room temperature, mixed thoroughly by gently swirling before pipetting.

**1. Add sample:** 100µL of standard, blank, or sample was added per well. The blank well was added with reference standard and sample Diluent. Solutions were added to the bottom of micro ELISA plate well, mixed gently and covered the plate with sealer and incubated for 90 minutes at 37°C.

**2. Biotinylated detection antibody:** The liquid of each well was removed. 100µL of biotinylated Detection Antibody working solution was added immediately to each well and covered with plate sealer. The plate was gently tap to ensure thorough mixing and then incubated for 1 hour at 37°C.

**3. Wash:** Each well was aspirated and washed 3 times. It was washed by filling each well with wash buffer (approximately 350 µL). At the last wash the remaining wash buffer was removed. The plate was inverted and pat against thick clean absorbent.

**4. HRP Conjugate:** 100 µL of HRP conjugated working solution was added to each well and covered with the plate sealer and incubated for 30 minutes at 37°C.

**5. Wash:** The wash process was repeated 5 times as in step 3.

**6. Substrate:** 90 µL of substrate solution was added to each well and covered with a new plate sealer and was incubated for about 15 minutes at 37°C.

**7. Stop:** 50 µL of stop solution was added to each well and colour turned to yellow immediately.

**8. OD Measurement:** The optical density (OD) of each well was determined at once using a microplate reader set to 450nm.

**Statistical analysis**

The data were presented as mean values and  $\pm$  standard variation ( $\pm$  SD) in tables. The data were analysed using t-test and Pearson Product moment method. Statistical significance was set at  $P < 0.05$

## Results

**TABLE 1: Comparison of erythropoietin (Epo) Levels in patients on predialysis and control**

Parameters	PD (60)	Control (60)	Level of significance
Epo(iu/l)	23.3±3.2	48.6±24.0	P<0.05

PD=Predialysis

Table 1 showed significant decrease (P<0.05) in Erythropoietin levels of predialysis subjects (23.3±3.2 iu/l) compared to Control subjects (48.6±24.0 iu/l) respectively.

**TABLE 2: Comparison of erythropoietin(Epo) in patients with dialysis to control**

Parameters	D (60)	Control (60)	Level of significance
Epo(iu/l)	71.4±14.7	48.6±24.0	P<0.05

D= Dialysis

Table 2 showed significant increase (P<0.05) in Erythropoietin level of dialysis subjects (71.4±14.7 iu/l) compared to control subjects (48.6±24.0 iu/l).

**TABLE 3: Comparison of Erythropoietin in Patients on predialysis and on dialysis**

Parameters	PD (60)	D (60)	Level of significance
Epo(iu/l)	23.3±3.2	71.4±14.7	P<0.05

PD=Predialysis

D=Dialysis

Table 3 showed significant decrease (P<0.05) in Erythropoietin a levels of predialysis subjects

(23.3±3.2 iu/l) compared to dialysis subjects (71.4±14.7 iu/l) respectively.

## Discussion

The study was done to estimate the levels of erythropoietin in patients with anaemias of renal diseases on predialysis and dialysis at the Federal Medical Centre, Umuahia. Anaemia is one of the clinical and laboratory manifestations of chronic kidney disease. Relatively little is known about the development and progression of anaemia in patients with chronic kidney disease. As kidney function declines and in patients with more advanced chronic kidney disease stages, the incidence and prevalence of anemia increased. There is an exponential relationship between glomerular filtration and anaemia .

Table 1 showed significant decrease (P<0.05) in Erythropoietin levels of predialysis subjects (23.3±3.2 iu/l) compared to Control subjects (48.6±24.0 iu/l) respectively. A relative lack of erythropoietin (Epo) is considered to be the main cause of the development of renal anaemia. The main stimulus for elevated

synthesis of erythropoietin is tissue hypoxia, which normally leads to an exponential increase in serum erythropoietin levels .This feedback is affected in patients with pathological condition involving the kidneys and the developing anaemia is not adequately compensated by a sufficient increase in the erythropoietin production[18].The kidneys of the subjects on predialysis might have been affected to cause the reduction in the erythropoietin level when compared to the control subjects who were apparently healthy but the level can still sustain the subject although, the subject will be anaemic. The result obtained here is in accordance with research done by Fehr *et al.*[19] which showed low erythropoietin in the study group.

Table 2 showed significant increase (P<0.05) in Erythropoietin level of dialysis subjects (71.4±14.7 iu/l) compared to control subjects (48.6±24.0 iu/l).

This might be as a result of gradual improvement in the damaged fibroblasts of the renal cortex particularly the renal medulla at the outer layer between the basolateral membrane of the proximal tubules and peritubular capillaries. Hybridisation techniques have confirmed that erythropoietin is not produced for storage, but an increase in its production related to hypoxic stimulus is caused by exponential growth in the number of specialised cells [20]. This observation could be attributed to many of the dialysis patients are always palced on recombinant erythropoietin therapy. The feedback mechanism is highly sensitive because an increase in the activity of specific mRNA can be demonstrated within two hours of the onset of hypoxic stimulus [21].

Normochromic normocytic anaemia can occur in various chronic diseases, and it appears that dysregulation of iron homeostasis and inflammatory processes act as the main mediators [22,23]. Although, anaemia in diabetic chronic kidney disease is likely multifactorial, involving erythropoietin deficiency as well as iron dysregulation and inflammation [24]. Absolute erythropoietin deficiency can be caused by diminished erythropoietin production as well as by erythropoietin-sensing errors [25]. The study did not find support for absolute erythropoietin deficiency, because erythropoietin levels stayed within a considerably normal range even though significantly lower ( $P < 0.05$ ) than the control subjects.

Table 3 showed significant decrease ( $P < 0.05$ ) in Erythropoietin levels of predialysis subjects ( $23.3 \pm 3.2$  iu/l) compared to dialysis subjects ( $71.4 \pm 14.7$  iu/l) respectively. This could be as a result of improvement in the fibroblasts of the kidneys. This showed that dialysis has a good effect on the repair of the damaged renal tissues by increasing the level of erythropoietin which prevents apoptosis of the cells.

## Conclusion

The study showed that there was decrease in Epo levels in the renal diseases studied. The depression of erythropoietin level were more pronounced in chronic kidney disease subjects before dialysis (predialysis) and improved on dialysis. These subjects need urgent attention to restore the function of the kidneys.

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