ASSOCIATION BETWEEN THROMBOPOIETIN AND HAEMATOLOGICAL PARAMETERS IN WHOLE BLOOD TRANSFUSED SUBJECTS WITH CHRONIC KIDNEY DISEASE ATTENDING UNIVERSITY OF PORT-HARCOURT TEACHING HOSPITAL

Mbeera B. S.[*], Ken-Ezihuo S. and Eze E. M.

Dept. of Medical Laboratory Science, Rivers State University, Port Harcourt, Rivers State, Nigeria.

*Corresponding Author: Mbeera B. S.

Dept. of Medical Laboratory Science, Rivers State University, Port Harcourt, Rivers State, Nigeria.

ABSTRACT

Thrombopoietin (TPO) is the key hematopoietic growth factor regulating the production of platelets from bone marrow megakaryocytes and maintaining platelet hemostasis. It is not clear whether whole blood transfusion in chronic kidney disease subjects affect changes in thrombopoietin and hematological parameters. Anaemia is known to be a major complication in CKD. The aim of the study was to assess the association between thrombopoietin and some haematological parameters in subjects with chronic kidney disease (CKD) who previously had or had not received multiple blood transfusion. A total of one hundred and fifty two (152) subjects were recruited for this study. One hundred and twenty two (122) subjects were recruited from those confirmed with renal diseases from the Urology Department of the hospital. Thirty eight (38) subjects were non-transfused with blood and eighty four (84) subjects were multitransfused with blood. Thirty subjects were apparently healthy controls. Thrombopoietin (TPO) was determined by sandwich ELISA method while the full blood count was determined using haematology autoanalyser, Mindray BC-5800. The results were statistically analysed using GraphPad prism version 5.0 and statistical significance set at P<0.05. A significant increase (p<0.0001) in TPO in the multitransfused 5.85±3.58 pg/ml compared to non-transfused and control subject 1.13±0.52 pg/ml, 1.15±0.36 pg/ml was observed. The significant increase in thrombopoietin may probably have been triggered by the transfusion of blood in the subjects that took blood transfusion. There was a positive and non-significant correlation between TPO and PCV (r = 0.011; p = 0.921), Hb (r = -0.066; p = 0.866), MCHC (r = 0.063; p = 0.564), MCV (r = 0.055; p= 0.618), PLT (r = 0.125; p = 0.257) respectively. It is concluded that transfusion enhances stimulation of thrombopoietin production but does not improve anaemia in CKD subject. It is therefore recommended that thrombopoietin assessment be properly checked in all CKD patients as this shall further aid the diagnosis of haematological characteristics in CKD since a known inverse relationship exist between thrombopoietin (TPO) and platelets.

KEYWORDS: Thrombopoietin, haematological parameters, *Chronic Kidney Disease, Blood Transfusion, Port-Harcourt.*

INTRODUCTION

Anemia, as an inevitable and frequent complication of chronic kidney disease (CKD), is often accompanied by a wide range of clinical symptoms, such as impaired physical capacity, decreased neurocognitive function and poor quality of life both in nondialysis and dialysis patients (Astor *et al.,* 2002). Recently, it has been appreciated that anemia begins to develop early in the course of CKD, and the prevalence of anemia in stage 3–5 CKD was 12.0% (Regidor *et al.,* 2011) The prevalence of CKD worldwide is estimated at 8–16% varying substantially across countries and regions (Arogundade and Barsoum, 2008). Even within Nigeria, there are variations in the reported prevalence of CKD, varying between 6 and 12% (Ulasi *et al*., 2013). Renal diseases

are associated with a variety of haemopoietic changes. Anemia is the most commonly reported hematologic abnormality present in CKD and its most important cause is failure of renal erythropoietin secretion. The prevalence of anemia in patients with CKD varies between 77.5 and 94%, depending on the stage of the disease (Ijoma *et al*., 2010; Shittu *et al*., 2013). The cardiovascular disease burden in CKD is further increased in the presence of anemia, particularly in the progression of CKD to end stage renal disease (ESRD) and frequent hospitalization (Silverberg *et al*., 2009). The other detrimental effects of anemia include fatigue, depression, reduced exercise tolerance, and cardiovascular consequences, such as left ventricular hypertrophy and left ventricular systolic dysfunction.

These effects tend to worsen morbidity and lead to poor clinical outcome in the patients (KDOQI, 2002).

The number and function of platelets and white cells may also be affected in CKD resulting in complications such as increase in susceptibility to infections and coagulopathy (Dorglalaleh *et al*., 2013; Kato *et al.,* 2008; Kaw and Malhotra, 2006). Reduced lymphocyte number may also be a marker of malnutrition in CKD (Kovesdy *et al.,* 2009). Elevated total white cell counts and granulocyte counts are associated with the increased progression of CKD, cardiovascular morbidity, and mortality (Bash *et al.,* 2009).

The differentiation, multiplication and growth of precursor cells and the activity of matured blood cells are regulated by a group of at least twelve (12) glycoprotein hormones, collectively known as haematopoietic growth factors. These include: erythropoietin (EPO), granulocyte-colony stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF), granulocyte-monocyte colony stimulating factor (GM-CSF), thrombopoietin (TPO), interleukin-3 (IL-3), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7) and tissue necrosis factor (TNF) (Hoffbrand and Paul, 2016a). The aim of the study was to assess the association between thrombopoietin and some haematological parameters in subjects with chronic kidney disease (CKD) who previously had or had not received multiple blood transfusion.

MATERIALS AND METHODS
Study Area
The study was carried out in Port Harcourt, the capital city of Rivers State, Nigeria. Port Harcourt situated within geographical coordinates 4°49′27″N 7°2′1″E. Port Harcourt features a tropical wet climate with lengthy and heavy rainy seasons and very short dry seasons. Only the months of December and January truly qualifies as dry season months in the city.

Study Population
A total study population of one hundred and fifty two (152) subjects were randomly collected. This comprised of 84 CKD subjects (case population) with chronic kidney disease that have received one or more of blood transfusion, 38 CKD subjects (case population) that have not been transfused. The remaining 30 subjects (control) are apparently healthy subjects who were confirmed medically free from kidney disease. Both the case and control subjects were age-matched (18 to 60 years old). The study population were recruited consecutively within a three-month period from the nephrology outpatient clinic, medical outpatient clinics, as well as medical inpatients (with CKD) in UPTH. Consent was obtained from each participant prior to blood collection. Their demographical information was collected from their hospital folder.

Sample Size
Purposive sampling and randomized method were used in the selection of subjects, taking into consideration, the total number of patient attending the nephrology clinic in University of Port-Harcourt Teaching Hospital, Rivers State.
The sample size was calculated using Cochran's sample size formula as shown below (Cochran, 1977).

$$N = \frac{Z^2 pq}{d^2}$$

Where   N = the desired sample size
Z = the Standard Normal deviate usually set at 1.96 corresponding to the 95%
Confidence level
p = the prevalence of target population
q = 1- p
d = degree of accuracy desired set at 0.05
Therefore N = $\frac{(1.96)^2 \times 0.078 \times (1\text{-}0.078)}{(0.05)^2}$

p = 7.8% or 0.078
N = 110
By adding 10% of non-respondent, the sample size will be 121.

Sample Collection
Ten milliliters (10) of venous blood sample was drawn from the peripheral vein in the upper limb of subjects; 5mls was transferred into EDTA bottles for the analysis of haematological parameters within 4 hours of sample collection and the remaining5mls was transferred into plain plastic bottle containing no additives or anticoagulant for the analysis of erythropoietin and thrombopoietin. The clotted blood was centrifuged at 1500 r.p.m. for 15 min at 20-22°C and the obtained serum was stored at -20°C until assayed. Assays was carried out on the serum sample thawed only once.

Experimental Analyses
Estimation of Thrombopoietin Using ELISA
Thrombopoietin (TPO) solid-phase, sandwich ELISA (enzyme-linked immunosorbent assay) is designed to measure and detect the amount of the target bound between a matched antibody pair. A target-specific antibody has been pre-coated in the wells of the supplied microplate. Samples, standards, or controls are then added into these wells and bind to the immobilized (capture) antibody.

Experimental Procedure
Serum TPO level was determined by a solid-phase enzyme-linked immunosorbent assay (Quantikine R & D). Briefly, a murine monoclonal antibody specific for thrombopoietin was precoated onto a microplate. Assay diluent composed of a buffered protein base with preservative was added to each well. Standards and samples were pipetted into wells and incubated for 3hours at 2–8°C. After aspiration and washing four times with a buffer, monoclonal antibody against TPO conjugated with horseradish peroxidase was added to

each well and incubated for 1 h at 2–8°C. Aspiration and washing were repeated four times, and a mixture of hydrogen peroxide and tetramethylbenzidine was added to the wells and incubated for 30 min at room temperature. The reaction was stopped by addition of 2N sulphuric acid. Optical density of each well was determined within 30 min by using a microplate reader set at 450 nm. TPO concentrations were extrapolated from a standard curve. The minimum detectable dose of TPO was less than 15 pg/ml.

Estimation of Haematological Parameters

Haematological parameters: red cell counts (RBC), white blood cell counts (WBC), lymphocyte counts (LYM), granulocyte counts(GRA), mean cell volume(MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), Hematocrit (HCT), Haemoglobin concentration and platelet count (PLT) were analysed using an automated system, Mindray BC-6800 Haematology analyser (Shenzhen, 2017). It is a five-part machine that analyses 33 parameters. To activate the cell counter, prime button was pressed. The tube of well- mixed EDTA blood was placed under the whole blood aspirator tip (inserted at least 1 inch into the blood), and whole blood button was then pressed. When wipe was displayed on the indicator, the blood sample was removed and aspirator tube was wiped with a piece of gauze moistened in diluents. After which result were then produced and printed out within minutes.

Statistical Analysis

Data collected for this study were subjected to statistical analysis using Graph pad Prism version 5.0. The sample population was grouped among healthy individuals (control), diseased patients who have been transfused with whole blood and those who are naïve to blood transfusion (subject). Quantitative data were analyzed using one-way analysis of variance (ANOVA) and a probability value (p-value) of < 0.05 was considered significant.

RESULTS

The aim of the study was to assess the association between thrombopoietin and some haematological parameters in subjects with chronic kidney disease (CKD) who previously had or had not received multiple blood transfusion in Port Harcourt. A total of one hundred and fifty two (152) subjects consisting 94 (61.8%) and 58 (38.2%) of males and females respectively were recruited for this study. One hundred and twenty two (122) subjects comprising 76 (62.3%) males and 46 (37.7%) females were recruited from those confirmed with renal diseases from the Urology Department of the hospital. Thirty eight (38) subjects 26 males and 12 females (68.4% and 31.6% respectively) were non-transfused with blood and eighty four (84) subjects which is made up of 50 males and 34 females (59.5% and 40.5% respectively) were multitransfused with blood. Thirty (30) subjects of which 18 (60%) and 12 (40%) were males and females respectively, were apparently healthy controls. The demographical information of the participants are shown in table 4.1.

Table 4.2 shows the mean trombopoietin values of CKD subjects who have received multiple whole blood transfusion (case 1), those that are naïve to blood transfusion (case 2), and apparently healthy individuals (control). On comparing the mean values of trombopoietin values with control, statistically significant difference was observed in thrombopoietin (TPO) values F = 57.52; p = <0.0001). The mean TPO control subjects, case 1 and case 2 (1.15±0.36, 1.13±0.52 and 5.85±3.58) respectively. However, Tukey's multiple comparison test of TPO case 2 versus TPO control showed no statistically significant difference. Other comparisons of TPO case 1 versus TPO control and TPO case 1 versus TPO case 2 showed statistically significant difference.

Table 4.3 shows the correlation coefficient between thrombopoietin and haematological parameters of chronic kidney disease subjects who have been transfused (case 1). Using Pearson correlation between thrombopoietin and haematological parameters of chronic kidney disease subjects who have been transfused (cases1), Thrombopoietin showed no statistically significant correlation with haematological parameter values: PCV (r = 0.011; p = 0.921), Hb (r = 0.066; p = 0.551), MCHC (r = 0.063; p = 0.570), MCH (r = -0.085; p = 0.440), MCV (r = 0.055; p= 0.618), Platelets (r = 0.125; p = 0.257), RBC (r = -0.110; p= 0.317).

Table 4.4 shows the correlation coefficient between thrombopoietin and haematological parameters of chronic kidney disease subjects who have not been transfused (case 2). Using Pearson correlation between thrombopoietin and haematological parameters of chronic kidney disease subjects who have not been transfused (cases2), Thrombopoietin showed no statistically significant correlation with haematological parameter values:PCV (r = 0.033; p = 0.844), Hb (r = -0.027; p = 0.874), MCHC (r = 0.055; p = 0.742), MCH (r = 0.205; p = 0.217), MCV (r = -0.266; p= 0.107), Platelets (r = 0.077; p = 0.644).

Table 4.1: Shows the Demographic Information of the Participants.

Characteristics	N%	Control N (%)	Non-Transfused N (%)	Multi-transfused N (%)
Overall	152 (100)	30 (19.7)	38 (25)	84 (55.3)
Age (Mean±SD)	33.17±10.20	30.43±9.24	33.97±11.63	35.11±9.73
Age Group (Years) **Males:** <21 21-30 31-40 41-50 51-60 60+	7 (4.6) 12 (7.8) 24 (15.8) 16 (10.5) 18 (11.8) 17 (11.3)	2 (6.7) 3 (10.0) 6 (20.1) 2 (6.7) 3 (10.0) 2 (6.7)	2 (5.3) 2 (5.2) 5 (13.2) 6 (15.8) 7 (18.4) 4 (10.5)	3 (3.6) 7 (8.3) 13 (15.5) 8 (9.5) 8 (9.5) 11 (13.1)
Age Group (Years) **Females:** <21 21-30 31-40 41-50 51-60 60+	3 (2.0) 7 (4.6) 10 (6.6) 11 (7.3) 18 (11.8) 9 (5.9)	1 (3.3) 1 (3.3) 1 (3.3) 4 (13.3) 4 (13.3) 1 (3.3)	0 (0) 2 (5.2) 2 (5.2) 2 (5.2) 4 (10.5) 2 (5.2)	2 (2.4) 4 (4.8) 7 (8.3) 5 (6.0) 10 (11.9) 6 (7.1)
Age group of males	94 (61.8)	18 (60)	26 (68.4)	50 (59.5)
Age Group of females	58 (38.2)	12 (40)	12 (31.6)	34 (40.5)

Table 4.2: Thrombopoietin Values of Control, Chronic Kidney Disease Subjects Who Have Received Multiple Transfusion (Case 1) and Subjects Who Have Not (Case 2).

Variables	Control n=30	Case 2 n=38	Case 1 n=84	p-value	F-value
TPO (pg/ml)	1.15 ± 0.36	1.13 ± 0.52	5.85 ± 3.58	<0.0001	57.52
POST Hoc: Tukey's Multiple Comparison Test **Comparison Remarks**					
TPO Case1 (pg/mL) vs TPO Case 2 (pg/mL)			Significant***		
TPO Case 1 (pg/mL) vs TPO Control (pg/mL)			Significant***		
TPO Case 2 (pg/mL) vs TPO Control (pg/mL)			Not significant		

Table 4.3: Correlation Coefficient between thrombopoietin and Haematological Parameters of Chronic Kidney Disease Subjects Who Have Been Transfused.

Correlation Coefficient	PCV (%)	Hb (g/dl)	MCHC (g/dl)	MCH (pg/cell)	MCV (fl)	PLT (x10^9/L)	RBC (x10^6/ul)
TPO (mIU/L)	0.011	0.066	0.063	-0.085	0.055	0.125	-0.110
p-value	0.921	0.866	0.564	0.790	0.618	0.257	0.317
Remark	NS	NS	NS	NS	NS	NS	NS

Table 4.4: Correlation coefficient between thrombopoietin and Haematological Parameters of Chronic Kidney Disease Subjects who have not been Transfused.

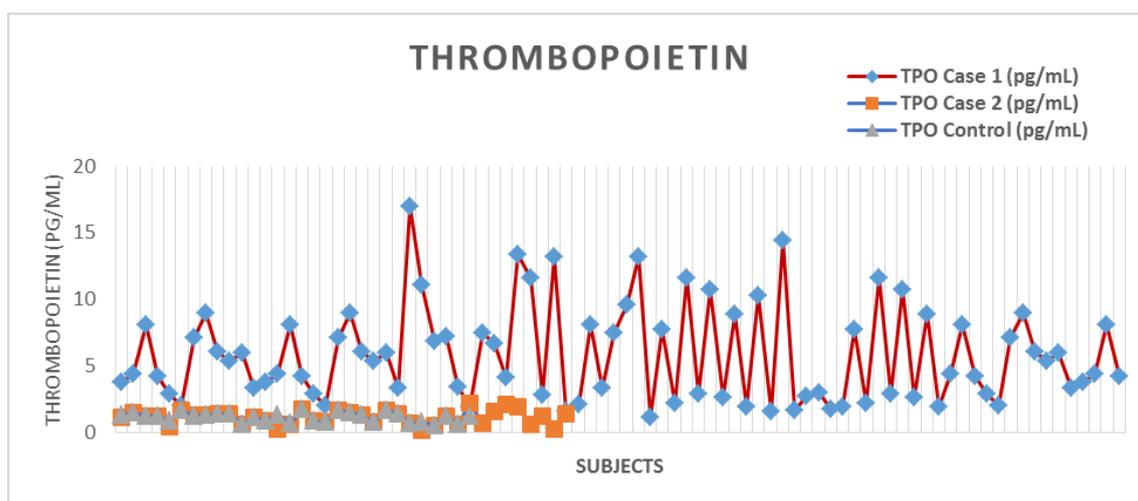| Correlation Coefficient	PCV (%)	Hb (g/dl)	MCHC (g/dl)	MCH (pg/cell)	MCV (fl)	PLT (x10^9/L)
TPO (mIU/L)	-0.033	-0.027	0.055	0.205	-0.266	0.077
p-value	0.844	0.874	0.742	0.217	0.107	0.644
Remark	NS	NS	NS	NS	NS	NS

Figure 4.2. Line Graph of Thrombopoietin levels of Cases 1, 2 and Control Subjects.

DISCUSSION
The aim of the study was to assess the association between thrombopoietin and some haematological parameters in subjects with chronic kidney disease (CKD) who previously had or had not received multiple blood transfusion in Port Harcourt. A significant elevation of thrombopoietin (TPO) in multiple transfused patients compared with nontransfused and control was observed in this study, a reflection of thrombocytopenia. This is supported by Joseph *et al.*, (2012). This may be as a result of the impairment of thrombopoietin which is the primary regulator of platelet production (Kaushansky, 1998) due to the malfunctioning of the kidney in the diseased condition. Thrombopoietin levels in the blood are inversely related to the number of platelets in the blood and megakaryocytes in the bone marrow. Interleukin (IL)-3, IL-6, IL-11, granulocyte-macrophage colony-stimulating factor, and c-KIT ligand increase megakaryocyte or platelet counts *in vivo* and *in vitro*. TPO binds to its receptor MPL (formerly known as c-MPL), enhances megakaryocyte colony formation, and increases the size, number, and ploidy of megakaryocytes, and platelet production (Joseph *et al.*, 2012). TPO is also required to maintain the viability of hematopoietic stem cells. The renal production of TPO is inducible by inflammation, and the concentration of TPO to which megakaryocytes are exposed is also determined by the platelet concentration. Platelets, bearing TPO receptors, remove the hormone from the circulation, at least partially accounting for the diverse relationship between TPO and platelet levels. In addition, lower platelets results in higher TPO, which stimulates the differentiation of megakaryocytes into platelets (Joseph *et al.*, 2012).

In keeping with the negative correlation, a positive correlation of TPO and PCV, Hb, MCHC MCV and PLT in non-transfused and multiple transfused was observed which is consistence with the report of (Abdulrahaman *et al*., 2013). A known inverse relationship exist between thrombopoietin (TPO) and platelets count which signify increase in TPO leads to decrease in platelets count and vice versa. This is could be a further reflection of the damaged fibroblast of the renal cortex particularly the renal medulla at the aoter layer. Elevated levels of thrombopoietin has been observed in several bone marrow failure diseases such as aplastic anemia (AA) that is characterized by pancytopenia and bone marrow hypocellularity, (Kojima *et al.,* 1992; Feng *et al.,* 2011). It is a well-demonstrated fact that TPO is involved in stimulating the growth of committed megakaryocyte progenitors, with progressive maturation of megakaryocytes, and proplatelet formation, (Horie *et al., 1997).*

Several aspects of relationship between platelet concentration and serum TPO levels have been studied but with no conclusive evidence, with some studies indicating the platelets to contain TPO receptors that efficiently bind and remove TPO from circulation, (Kuter, 1996) hence, suggesting that circulating levels of TPO are inversely related to platelet mass. Several others opine that TPO is constantly produced in vivo, primarily in the liver and kidneys, (Stoffel *et al.,* 1996) and that the circulating TPO levels are regulated by platelet concentration, (Kuter & Rosenberg, 1995).

CONCLUSION
It is concluded that transfusion enhances stimulation of thrombopoietin production and recommended that thrombopoietin assessment be properly checked in all CKD patients as this shall further aid the diagnosis of haematological characteristics in CKD since a known inverse relationship exist between thrombopoietin (TPO) and platelets.

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