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Prospective Changes in Serum Levels of Some Proinflammatory Cytokines and Erythropoietin among Anaemic HIV-infected Patients Attending Kenyatta National Hospital Comprehensive Care Centre

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Abstract

Between 70 to 80% of HIV infected patients develop anaemia which is a major complication in advanced HIV infection. The multifactorial etiology of the HIV-associated anaemia requires extensive studies on its unique pathophysiology as a step towards improving therapeutic options and disease management.The objective of this study is to monitor changes in serum levels of erythropoietin (Epo), Tumor necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), C-Reactive Protein (CRP) and anaemia in HIV infected patients over six months' period. This study is Longitudinal descriptive study and it was conducted at Kenyatta National Hospital, Kenyatta National Hospital can be considered as Comprehensive Care Centre.

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The study used one hundred and eighty-four (184) seropositive adults aged 18 to 60 years.The results shows that Blood cells exhibited pathologies ranging from: Rouleaux formation, round macrocytes, microcytic hypochromic cells and target cells in frequencies that decreased with increase in CD4+ cells. Normochromic, macrocytic normochromic and dimorphic anaemias were observed. Bicytopenia (erythrocytopenia and leucopenia), reactive thrombocytosis with giant platelets, neutrophil and eosinophil hypersegmentations were also observed. Persistent increase in Epo and CRP levels were demonstrated among subjects throughout the study period. Increases in TNF-α levels without corresponding increase in IL-6 levels were observed. Persistence anaemia in presence of high Epo levels is suggestive of hyposensitivity to Epo by erythroid precursors. Asynchronized increases in TNF-α and IL- 6 levels may have deprived the duo the synergy required to effectively inhibit Epo production further facilitating the escalating levels of Epo observed. High levels of CRP observed indicate enhanced inflammation processes associated with HIV infection. Iron studies to rule out the role of iron-restricted erythropoiesis in the development microcytic, dimorphic anaemias and the granulocytic hypersegmentations noted are recommended. Studies on the possibility of Epo hyposensitivity derailing the effectiveness of recombinant human Epo in the management of HIV – associated anaemia are recommended.

Key words: Proinflamatory cytokines; erythropoietin; anaemia; Human immunodeficiency Virus.

1. Introduction

Among the haematological abnormalities in HIV-infected patients, anaemia is the most common and has been associated with disease prognosis and poor clinical outcomes. According to World Health Organization (WHO), anaemia is said to occur when haemoglobin (Hb) levels fall below 12 g/dl and 13 g/dl in females and males, respectively. The development of HIV-associated anaemia has numerous contributors including: myelosuppressive drugs especially zidovudine (AZT), the virus itself, haemopoiesis – inhibiting soluble serum factors, antibodies against red blood cells (RBCs), infection-related gastrointestinal bleeding and hypogonadism in men [1,2,3,4,5]. This multifactorial origin of HIV- associated anaemia complicates not only the definition of its etiology but also its diagnosis and choice of therapeutic measures. With HIV- associated anaemia posing serious complications including poor quality of life, association with disease progression and poor clinical outcomes the recognition of studies of the unique pathophysiology of this anaemia as an important step towards improving therapeutic options and disease management cannot be underscored [6]. Erythropoietin (Epo), a glycoprotein hormone with a molecular weight of, 39 KDa and produced mainly in cells of kidney in adults has serum levels that are directly associated with those of Hb [7,8]. Human TNF-α is a glycosylated protein of 17 KDa and a length of 157 amino acids. Interleukin 6 (IL-6) is a pleiotropic, α-helical, 22-28 KDa phosphorylated and variably glycosylated cytokine and plays important roles in the acute phase reaction, inflammation, haematopoiesis, bone metabolism, and cancer progression. C-reactive protein is synthesized in the liver and is a marker of inflammation. During active inflammation its blood levels rise and this rise is always accompanied by decrease in CD4+ cell counts in HIV infected patients, [9]. High levels of TNF-α correlate with increased risk of mortality and interacts with IL-6 to inhibit Epo production [10,11]. It has been observed that levels of serum immunoreactive erythropoietin in HIV-infected patients in various stages of illness fail to rise commensurately with increasing anaemia, suggesting that insufficient amounts of erythropoietin may be one cause of anaemia in

this setting [12]. Changes in the levels of Epo, TNF-α, IL-6 and CRP in active HIV/AIDS have been demonstrated in populations from industrialized countries. With Africa being home for approximately 70% of the global AIDS victims and Kenya in particular harboring about 1.5 million HIV infected adults studies on the changes in serum levels of these proteins associated with anaemia is paramount. In this study changes in serum levels of Epo TNF-α, IL-6, CRP and Hb among HIV infected adults in CD4+ clusters of < 200, 200-499 and ≥ 500 cells/mm^3 were monitored at intervals of three months for six months.

2. Materials and Methods

The study protocol was approved by Kenyatta National Hospital/University of Nairobi (KNH/UoN) ethics and review committee. A total of 184 HIV seropositive adults aged between 18 and 60 years were recruited consecutively as they consented. Both ARV- treated and ARV-naïve patients were recruited. Also similarly recruited were 101 HIV - seronegative blood donors as referents. Clinical and social demographic characteristics of the study participants were recorded. The study subjects were grouped into CD4+ < 200 (n = 22), 200-499 (n = 86) – and ≥ 500 cells/mm^3 group based on the CD4 counts in the blood sample at recruitment. The CD4+ groups were further grouped into males and females: CD4+ < 200 (13 males & 9 females), 200-499 (43 males & 43 females) - and ≥ 500 (9 males & 67 females) cells/mm^3 groups. A total of 5 mls blood samples were obtained from each study subject at recruitment (F_0), after 3 months (F_1) and after 6 months (F_2) during the study; while blood sample were obtained from the referent group at the recruitment stage only. The blood samples were then dispensed into EDTA vacutainers and plain tube in appropriate portions.

The EDTA samples were used for CD4+ cell counts and haemogram determinations; while sera from blood samples in plain vacutainers were used for: Epo, TNF-α, IL-6 and CRP, assays. Haemogram was done on a Cell TAC F Model 822® analyzer with tri level (normal, low and high) commercial controls were used for quality control; while peripheral blood films were stained with Leishman's stain. The CD4+ counts were done on a FACS CALIBUR® machine that used commercial controls set at zero, low, medium and high concentrations for quality control. CRP assays were carried out in a fully automated instrument, Humastar 600® that was calibrated with normal and pathological controls and with 2 levels of "Turbidos" control. Erythropoietin, TNF-α, IL-6 assays were done using Thermo Scientific® quantitative sandwich enzyme immunoassay technique on HumaReader HS® Elisa reader. The concentrations of the tests were read off curves constructed using serially diluted commercial standards. Parameters that demonstrated significant differences between male and female means among the referents were analyzed under the separate genders in HIV positive respondents; where no such differences were demonstrated the parameters were analyzed without regard to gender.

The raw data obtained was recorded into laboratory note book, entered into excel computer data base then after cleaning and verification transported into statistical package for the social sciences (SPSS) version 21 and analysis done there-in. Means, medians, standard error of the mean, minimum and maximum ranges were determined. Comparison of the means between males and females; between HIV negative and HIV positive respondents was done using student - t test. HIV negative respondents' 95% confidence interval (CI) was used as reference ranges to determine frequencies of increased or decreased parameters of HIV positive respondents. Peripheral blood films were microspically examined for pathologies and their frequencies determined and

tabulated.

3. Results

Demographic characteristics of the respondents is presented in Table 1. For the HIV negative control group, the mean age was 30.2 years, 74.3 % were male, 63.4% had tertiary education, 45.5% were students, 95 % were non-smokers and 75.2% did not consume alcohol. For the HIV positive group, the mean age was 39.7, 64.7% were females, 43.5% had secondary education, 51.1% were employed, 98.9 % were non-smokers and 88% were teetotaler.

Table 1: Demographic characteristics of the respondents**.**

		HIV - positive			HIV- negative			
		Mean±	Min-	95% CI	Mean±	Min-	F(%)	95% CI
Variable		SEM	Max		SEM	Max	N = 101	
Age (yr)		39.7±0.63	19-59	26.1-53.3	30.2±0.96	18-56		28.3-32.1
Education								
	None			12.3-22.9			1 (1%)	0.2-5.3
	Primary			17.8-29.8			10 (9.9%)	3.4-17.1
	Secondary			36-50			26 (25.7%)	18.9-35.8
	Tertiary			27-40.3			64 (63.4%)	54.7-72.9
Occupation								
	None			0.1-2.0			1 (1%)	1.2-5.3
	House wife			2.9-9.5			0 (0%)	0.2-5.3
	Business			37-51			24 (23.8%)	15.2-32.3
	Employed			42.7-56.8			30 (29.7%)	22.1-39.1
	Student			1.1- 6			46 (45.5%)	35.4-54.3
Smoking								
	Yes			0.5- 4.6			5 (5%)	2.7-12.1
	No			95.4-99.5			96 (95%)	87.9-97.3
Alcohol								
	Yes			8.5-17.6			25 (24.8%)	17.9-34.4
	No			82.4-91.8			76 (75.2%)	65.6-82.1

The CD4+ levels for all the respondents was determined at the commencement of the study and also on the third and sixth months, for the HIV infected individuals. Baseline data indicated that the CD4+ counts for the HIV negative control group averaged 780.9 ±31.2 cells /mm^3 while that of the HIV positive individuals was 333.0 ± 19.7 cells / mm^3. Follow up data on the HIV positive respondents indicated that the CD4+ levels at three and six months were elevated to 501.6±25.4 and 497 ± 19.7 cells / mm^3, respectively.

When the HIV respondents were categorized into three groups, according to WHO guidelines, it was found that in the group with less than 200 cells/mm^3 it averaged 119 ± 15.1 cells/mm^3; for CD4+ 200 – 499 cells/mm^3 group it was 376.5 ±19.9 cells/mm^3; while in CD4+ >500 cells/mm^3 the group average was 723.8 ± 32.2 cells/mm^3. Of the respondents 152 (82.6%) were ARV-treated and 32 (17.4 %) were ARV- naïve with their CD4+ cell counts of 502.7 ± 23.5 and 562.7± 68.6 cells/mm^3, respectively (Table 2).

Table 2: Baseline levels of CD4 counts among the HIV positive respondents.

Respondent group		Number	CD4+ cell counts (Mean ± SEM)		
			Baseline	3 months	6 months
HIV positive	All	184	333±19.7	501.6 ± 25.4	497.1 ± 19.7
	CD4+ < 200 cell/mm^3	22	119 ± 15.1	261.4 ± 54.7	279.4 ±59.6
	CD4+ 200 - 499 cell/mm^3	86	376.5 ± 19.9	402.8 ±38.7	363.8± 9.2
	CD4+ ≥ 500 cell/mm^3	76	723.8 ± 32.2	666.7± 49.1	720.8 ±27.5
	ARV treated	152	502.7± 23.5	548 ± 29	538 ± 22
	ARV naive	32	562.7± 68.6	279 ± 19	301 ± 24

4. Blood cell pathologies

The referents displayed normal red blood cells with diameters almost the same size as that of the nucleus of a small mature lymphocyte. While the tests groups showed pathology that ranged from microcytic hypochromic, round macrocytes, rouleaux formation to target cells. The frequencies of these pathology is presented in Table 3. In the group with CD4+ levels less than 200 cells/mm^3 the baseline distribution of the pathology was as follows: rouleaux formation (27.3%), macrocytosis (22.7%), hypochromasia (13.6 %) and targeting (9.1%), all of which exhibited a decline in frequency six months later (Table 3). In the CD4 200–499 cells/mm^3 group the macrocytosis frequency was 10.4% at the screening stage and this continuously dropped to 2.3 % and 1.1% after the third and sixth month, respectively. While the rouleaux frequency of 2.3% that was observed at the screening stage was found to stabilize at 1.1% in the follow up samples. For hypochromasia the frequency of 2.3% that was recorded during screening was found to disappear after three months but it re-immerged after six months at a higher frequency of 4.7%. In the CD_4, 200–499 cells/mm^3 group, the target cells (2.3%) were only observed in the sixth month of the study. For the CD4+ ≥ 500 cells/mm^3 group the macrocytosis of 10.5 % observed during screening was found to stabilize at 5.3 % in follow up samples; for rouleaux formation it was stable at 3.9% in the first three months before it declined to 2.3% at the end of the study. However, for target cells there was mixed results (Table 3).

In the referent group and those that were HIV positive with CD4+ levels of < 200cells/mm^3 no white cell pathology was reported in the six months of study. However, HIV positive respondents in CD4+ levels of 200 – 499 and ≥ 500 cells/mm^3 groups demonstrated the hypersegmentation of neutrophil and eosinophils. For the group with CD4+ levels at 200 – 499 the neutrophil hypersegmentation dropped from 11.6% to 0 % after three months, then rose to 2.3% after the six month; while that of eosinophil dropped from11.6% to 0% after three months. In CD4+ ≥ 500 cells/mm^3 group neutrophil and eosinophil hypersegmentation frequency decreased from 2.6% to 1.3 % in the six months of the study. There was no pathology observed in the HIV negative control. However, HIV – infected respondents showed giant thrombocytes. In the group with CD4+ 200 – 499 the platelet giant cells were only observed in the baseline sample (1.2%) while in the group that had CD4+ levels ≥ 500 cells/mm^3 the pathology was observed at inception and in the first three months (1.3%) (Table 3).

Table 3: Frequency (%) of observed blood cell changes in the respondents.

Morphological Change	CD4 + < 200 cells/mm^3			CD4+ 200 - 499cells/mm^3			CD4+ ≥ 500 cells/mm^3		
	N = 22			N = 86			N = 76		
	F_0	F_1	F_2	F_0	F_1	F_2	F_0	F_1	F_2
Rouleaux formation	6 (27.3%)	3 (13.6%)	1 (4.5%)	2 (2.3%)	1 (1.1%)	1 (1.1%)	3 (3.9%)	3(3.9%)	2 (2.3%)
Target cells	2 (9.1%)	1 (4.5%)	0 (%)	0 (0%)	0 (0%)	2 (2.3%)	0 (0%)	2 (2.3%)	1 (1.3%)
Microcytic hypochromic cells	3 (13.6%)	4 (18.2%)	1 (4.5%)	2 (2.3%)	0 (0%)	4 (4.7%)	0 (0%)	6 (7.9%)	3 (3.9%)
Round macrocytes	5 (22.7%)	1 (4.5%)	1 (4.5%)	9 (10.4%)	2 (2.3%)	1 (1.1%)	8 (10.5%)	4 (5.3%)	4 (5.3%)
Neutrophil hypersegmentation	0 (0%)	0 (0%)	0 (0%)	10 (11.6%	0 (0%)	2 (2.3%)	2 (2.6%)	2 (2.3%)	1 (1.3%)
Eosinophil hypersegmentation	0 (0%)	0 (0%)	0 (0%)	10 (11.6%)	0(0%)	0 (0%)	2 (2.6%)	1 (1.3%)	1 (1.3%)
Giant platelets	0 (0%)	0 (0%)	0 (0%)	1 (1.2%)	0 (0%)	0 (0%)	1 (1.3%)	1 (1.3%)	0 (0%)

F_0 = baseline stage; F_1 = Follow up at 3 months; F_2 = Follow up at 6 months

4.1. Differences in haematological parameters in HIV negative respondents

Significant differences between male and female HIV negative respondents (referents) were observed in: red blood cells (4.9±0.08 x 10 ^{12}/l versus 4.5±0.12 x10 ^{12}/l, p = 0.008), haemoglobin (13.9±0.22 g/dl versus 12.2±0.31 g/dl, p < 0.001), mean cell volume (86.8± 0.64 fl versus 82.4±1.69 fl, p = 0.019), white blood cells (5.2±0.14x10^9 /l versus 6.5±0.25x10^9/l, p < 0.001), neutrophils (2.3±0.9x10^9/l versus 3.2±0.22x10^9 /l, p < 0.001), lymphocytes (2.1±0.08x10^9 /l versus 2.5±0.12x10^9 /l, p = 0.007), CD4+ (703.1±32.2 cells/mm^3 versus 542±59.4 cells /mm^3, p < 0.001) and platelets (248.4±9.6 x10^9 /l versus 302.9 ±17.61 x 10^9/l , p = 0.009) as shown in table 4.

Table 4: Differences in haematological parameters in HIV negative respondents

		Respondent group						
		HIV Negative all (N=101)			Males(n=75)	Females(n = 26)		
	Parameter	Mean (SEM)	95% CI	Mean(SEM)	95% CI	Mean (SEM)	95% CI	P-value
	RBC x 10^{12}/l	4.8 (0.06)	4.6- 4.9	4.9 (0.08)	4.7- 5.0	4.5 (0.12)	4.2- 4.7	0.008**
	Hb g/dl	13.5 (0.21)	13.1-13.9	13.9 (0.22)	13.5-14.4	12.2 (0.31)	11.6-12.9	< 0.001**
	MCV Fl	85.7 (1.12)	84.4- 87	86.8 (0.64)	85.6-88.1	82.4(1.69)	78.9-85.9	0.019*
	MCH g/l	28.5 (0.46)	27.9-29	28.8 (0.29)	28.2-29.3	27.6 (0.67)	26.2-28.9	0.111
	MCHC g/l	33.2(0.16)	32.9-33.5	33.1 (0.20)	32.7-33.5	33.4 (0.25)	32.9-33.9	0.334
	WBC x 10^9/l	5.5 (0.14)	5.2- 5.8	5.2 (0.14)	4.9- 5.4	6.5 (0.25)	6.0-7.1	< 0.001**
	Nx10^9/l	2.5 (0.01)	2.3- 2.7	2.3 (0.09)	2.1-2.5	3.2 (0.22)	2.8-3.6	< 0.001*
	Lx10^9/l	2.2 (0.07)	2.0-2.3	2.1 (0.08)	1.9-2.2	2.5 (0.12)	2.2-2.7	0.007**
	Mx10^9/l	0.4 (0.04)	0.4-0.5	0.4 (0.02)	0.3-0.5	0.5 (0.04)	2.2-2.7	0.05
	Ex10^9/l	0.2 (0.02)	0.2-0.3	0.2 (0.02)	0.2-0.3	0.2 (0.02)	0.1-0.2	0.19
	CD4+ /mm^3	780.9 (31.22)	718.9-842.8	703.1(32.22)	683.8-767.5	542 (59.44)	882.7-1127.6	< 0.001**
	Px10^9/l	262.4 (8.71)	245.1-279.7	248.4 (9.56)	229.4-267.4	302.9 (17.61)	266.6-339.2	0.009**

WBC (white blood cells), N (neutrophil), L (lymphocyte), E (eosinophil), RBC (red blood cell), MCV (mean cell

volume), MCH

(mean cell haemoglobin), MCHC (mean cell haemoglobin), P (platelet). ** Significant difference at $p < 0.01$ (one-tailed),

* Significant difference at $P < 0.05$ (two-tailed) using t-test.

4.2. Frequency of haematological abnormalities among HIV positive male respondents

The frequency of erythrocytopenia (RBC < 4.7 x 10^{12}/l) decreased progressively from 92.3 % to 84.6% then to 61.5% in baseline, third and sixth months of the study respectively in the CD4+ cells < 200 cells / mm^3 group. In CD4+ 200 – 499 cells /mm^3 group however, the frequency decreased from the baseline frequency level of 61.5% to 48.8% then increased to 51.2% in the third and sixth months respectively; while the frequency remained constant at 22.2 % throughout the study period in CD4+ ≥ 500 cells / mm^3 group. The highest frequency of anaemia (Hb < 13.5 g/dl) was observed in CD4+ < 200 cells / mm^3 group and decreased from 69.2% to 53.8% then to 38.5% in the baseline, third and sixth months of the study respectively. In the CD4+ 200 -499 cells/mm^3 group the frequency of anaemia decreased from the baseline level of 30.2% to 23.3% then to 14 % in the third and sixth months of the study respectively. The frequency of anaemia decreased by three times in the third month (from baseline level of 33.3% to 11. 1%) then doubled (to 22.2%) in the sixth month of study in the CD4+≥ 500 cells / mm^3 group (Table 5).

The frequency of macrocytosis (MCV > 88.1 Fl) increased from the baseline level of 69.2% and stabilized at 76.9% in the third and sixth months of the study in the CD4 < 200 cells/mm^3 group. The level of the abnormality decreased from a stable frequency level of 74.4%, observed at baseline and three months' periods to 72.1% in the sixth month of the study in CD4+ 200 – 499 cells / mm^3 group; while it remained at 100% throughout the study period in CD4+ ≥ 500 cells / mm^3 group. Leucopenia (WBC < 4.9 x 10^9/l) demonstrated the highest frequency level of 76.9% in CD4+ < 200 cells / mm^3 group throughout the study period. The abnormality decreased in frequency from baseline level of 51.2% to 39.5% in the third month of the study, then increased to 58.1 % during the sixth month of the study in the CD4+ 200 – 499 cells / mm^3 group. This abnormality decreased from the baseline level of 33.3% to 22.2% in the third month and then decreased further to 11.1% in the sixth month of the study in the CD4+ ≥ 500 cells /mm^3 group (Table 5)

Neutropenia (N < 2.2 x 10^9/l) levels decreased from the baseline frequency level of 53.8% to 46.2% in the third month then increased again to 61.5% in the sixth month of the study in CD4+ < 200 cells/mm^3 group. The abnormality however, demonstrated a steady increase from the baseline frequency level of 41.9% to 51.2 % then to 62.8% during the third and sixth months respectively in the CD4+ 200 - 499 cells/mm^3 group. In the CD4+ ≥ 500 cells /mm^3 group, neutropenia frequency levels decreased from the baseline level of 33.3% to 22.2 % then to 11.1% in the third and sixth months respectively during the study. Lymphocytopenia (L < 1.9 x 10^9/l) decreased from the baseline level of 84.6% and stabilized at 53.8% in the third and sixth months of the study in the CD4+ < 200 cells / mm^3 group. The abnormality decreased from the baseline frequency level of 67.4% to 41.9% then to 2.3% in the

third and sixth months of the study respectively in CD4+ 200 – 499 cells / mm^3 group. This abnormality increased from the baseline frequency level of 22.2% to 33.3% in the third month of the study and then completely resolved to 0 % by the sixth month of the study in the CD4+ ≥ 500 cells /mm^3 group. During the study, the highest frequency level of thrombocytosis of 61.5% was observed at baseline and in the third month which then decreased to 46.2% in the sixth month in CD4+ < 200 cells / mm^3 group. The frequency of this abnormality increased from the baseline level of 46.5% to 48.8% in the third month, then decreased to 39.5% in the sixth month of the study in the CD4+ 200 – 499 cells / mm^3 group; while it remained at the baseline frequency level of 55.6% during the first three months of the study and then decreased to 33.3% in the sixth month (Table 5).

Table 5: Frequency of haematological abnormalities among HIV positive male respondents

			Respondent group								
	HIV positive Males (n=75)		HIV positive males (n = 65)								
			CD4+ < 200 cells / mm^3			CD4+ 200 – 499 cells /mm^3 (n=43)			CD4+ ≥ 500 cells /mm3 ((n= 9)		
Parameter	95% C.I		F_0	F_1	F_2	F_0	F_1	F_2	F_0	F_1	F_2
RBC x 10^{12}	4.7 - 5.0	Frequency (%) of									
		Erythrocytopenia									
		RBC < 4.7 x 10^{12}/l)	12 (92.3)	11 (84.6)	8 (61.5)	21 (48.8)	22 (51.2)	19 (44.2)	2 (22.2)	2 (22.2)	2 (22.2)
Hg g/dl	13.5 - 14.4	Frequency (%) of									
		anaemia									
		(Hb < 13.5g/dl)	9 (69.2)	7 (53.8)	5 (38.5)	13 (30.2)	10 (23.3)	6 (14)	3 (33.3)	1 (11.1)	2 (22.2)
MCV Fl	85.6 - 88.1	Frequency (%) of									
		Macrocytosis									
		(MCV > 88.1 fl)	9 (69.2)	10 (76.9)	10 (76.9)	32 (74.4)	32 (74.4)	31 (72.1)	9 (100)	9 (100)	9 (100)
WBC x 10^9/	4.9 - 5.4	Frequency (%) of									
		Leucopenia									
		(WBC< 4.4 x 10^9/l)	10 (76.9)	10 (76.9)	10 (76.9)	22 (51.2)	17 (39.5)	25 (58.1)	3 (33.3)	2 (22.2)	1 (11.1)
N x 10^9/l	2.1- 2.5	Frequency (%) of									
		Neutropenia									
		(N< 2.1x x10^9/l)	7 (53.8)	6 (46.2)	8 (61.5)	18 (41.9)	22 (51.2)	27 (62.8)	3 (33.3)	2 (22.2)	1 (11.1)
L x 10^9/l	1.9 - 2.2	Frequency (%) of									
		Lymphocytopenia									
		(L < 1.9 x 10^9/l)	11 (84.6)	7 (53.8)	7 (53.8)	29 (67.4)	18 (41.9)	21 (2.3)	2 (22.2)	3 (33.3)	0 (0)
P x 10^9/l	229.4 - 276.4	Frequency (%) of									
		Thrombocytosis									
		(P > 276.4 x 10^9/l)	8 (61.5)	8 (61.5)	6 (46.2)	20 (46.5)	21 (48.8)	17 (39.5)	5 (55.6)	5 (55.6)	3 (33.3)

F_0 = baseline stage; F_1 = Follow up at 3 months; F_2 = Follow up at 6 months

4.3. Frequency of haematological abnormalities among HIV positive female respondents

The frequency levels of erythrocytopenia (RBC < 4.2x 10^{12} /l) increased from the baseline level of 44.4 % to a stable level of 77.8% up to the end of the study period in CD4+ < 200 cells / mm^3 group. The abnormality decreased from the baseline level of 51.2 % to 46.1% in the third month and then increased to 48.8% in the sixth month in the CD4+ 200-499 cells /mm^3 group. In CD4+ ≥ 500 cells/mm^3 group, erythrocytopenia levels decreased from the baseline frequency level of 52.2% to 47.8% in the third month and then increased to 53.7% in the sixth month. The

level of anaemia (Hb < 11.6 g/dl) increased from the baseline frequency level of 66.7% to 88.9% in the third month then decreased to 66,7% in the sixth month in the CD4+ < 200 cells/mm^3 group. The abnormality increased from the baseline frequency level of 25.6 % to 27.9% in the third month then increased further to 37.2% in the sixth month in CD4+ 200 – 499 cells/mm^3 group. In CD4+ ≥ 500 cells/mm^3 group, the level of anaemia increased from the baseline frequency level of 35.8% to 40.3% in the third month, then decreased to 35.8% in the sixth month. The frequency level of macrocytosis (MCV >85.9 fl) remained at 55.6% during the entire study period in CD4+ < 200 cells / mm^3; while in CD4+ 200 – 499 cells /mm^3 group the frequency levels increased from the baseline level of 62.8% to 65.1% in the third month then increased further to 67.4% in the sixth month during the study.

The baseline macrocytosis frequency level of 73.1% decreased to 68.7% in the third month and increased to 71.6% during the sixth month of the study in CD4+ ≥ 500 cells / mm^3 group (Table3c). In the CD4+ < 200 cells / mm^3 group's baseline leucopenia (WBC < 6.0 x 10^9/) frequency level of 88.9% decreased after three months and stabilized at 77.8% for rest of the study period. Leucopenia increased from the baseline frequency level of 69.8% to 74.4% and then to 79.1% in the third and sixth months of the study in CD4+ 200 – 499 cells / mm^3 group. In CD4+≥ 500 cells / mm^3 group the baseline leucopenia frequency level of 59.7% decreased to 50.7% in the third month and then increased marginally to 53.7% in the sixth month of the study. Baseline neutropenia (N < 2.8 x 10^9 /l) frequency level of 66.7% decreased to 55.6% in the third month then increased to 77 8% in the sixth month in CD4+ < 200 cells / mm^3 group during the study. In CD4+ cells 200 – 499 cells / mm^3 the baseline neutropenia frequency level of 51.2 % increased to 58.1 % in three months and then increased further to 69.8% in the sixth month of the study. The baseline neutropenia frequency level of 61.2 % decreased markedly to 11.9%, then increased markedly to 62.7% in the third and sixth months respectively.in CD4+≥ 500 cells /mm^3 group (Table 6).

The baseline lymphocytopenia (L < 2.2 x 10^9/l) frequency level of 100% observed in CD4+ < 200 cells /mm^3 group decreased drastically to 11.1 % and then increased markedly to 66.7% in the third and sixth months respectively during the study. The frequency level of the abnormality in CD4+ 200 – 499 cells /mm^3 group decreased from the baseline level of 72.1% to 62.8% then increased to 67.4% in the third and sixth months respectively during the study. In the CD4+ ≥ 500 cells /mm^3 group, lymphocytopenia baseline frequency level of 44.8% decreased to 28.4% in the third month then increased to 41.8% in the sixth month. Finally, the frequency levels of thrombocytosis (P >339.3 x 10^9 /l.) observed in the CD4+ < 200 cells / mm^3 increased from the baseline level of 22.2% to 44.4% then decreased again to 22.2% in the third and sixth months of the study respectively.

In CD4+ 200 – 499 cells /mm^3 group the frequency level of thrombocytosis at baseline was 41.9% and remained unchanged in the next three months but decreased to 6.5% in the sixth month of the study. The frequency level of thrombocytosis demonstrated marginal changes in the CD4+ ≥ 500 cells /mm^3 group with a decrease from the baseline level of 37.3% to 28.4% in three months followed by marginal increase to 29.9% in the sixth month of the study (Table 6).

Table 6: Frequency of haematological abnormalities among HIV positive female respondents

			Respondent group								
HIV negative			HIV positive females (N = 119)								
	Females		CD4+ < 200 cells/mm^3			CD4+ 200 - 499 cells/mm^3			CD4+ ≥ 500 cells/mm^3		
Parameter	95% CI	Abnormality	F_0	F_1	F_2	F_0	F_1	F_2	F_0	F_1	F_2
RBC x 10 ^{12}/l	4.2 - 4.7	Frequency (%) of									
		Erythrocytopenia									
		RBC < 4.2x10^{12}/l	4 (44.4)	7 (77.8)	7 (77.8)	22 (51.2)	20 (46.1)	21 (48.8)	35 (52.2)	32 (47.8)	36 (53.7)
Hb g/dl	11.6-12.9	Frequency (%) of									
		Anaemia									
		(Hb < 11.6g/dl)	6 (66.7)	8 (88.9)	6 (66.7)	11 (25.6)	12 (27.9)	16 (37.2)	24 (35.8)	27 (40.3)	24 (35.8)
MCV fl	78.9-85.9	Frequency (%) of									
		macrocytosis									
		MCV > 85.9 Fl	5 (55.6)	5 (55.6)	5 (55.6)	27 (62.8)	28 (65.1)	29 (67.4)	49 (73.1)	46 (68.7)	48 (71.6)
WBCx10^9/l	6.0 - 7.1	Frequency (%) of									
		Leucopenia									
		(WBC<6 x 10^9/l)	8 (88.9)	7 (77.8)	7 (77.8)	30 (69.8)	32 (74.4)	34 (79.1)	40 (59.7)	34 (50.7)	36 (53.7)
Nx109/l	2.8-3.6	Frequency (%) of									
		Neutropenia									
		(N < 2.8x10^9/l)	6 (66.7)	5 (55.6)	7 (77.8)	22 (51.2)	25 (58.1)	30 (69.8)	41 (61.2)	8 (11.9)	42 (62.7)
Lx10 9 /l	2.2 - 2.7	Frequency (%) of									
		Lymphopenia									
		(L < 2.2 x10^9/l)	9 (100)	1 (11.1)	6 (66.7)	31 (72.1)	27 (62.8)	29 (67.4)	30 (44.8)	19 (28.4)	28 (41.8)
P x10 9/ l	266.6-339.2	Frequency (%) of									
		Thrombocytosis									
		(P >339.2 x 10^9/l)	2 (22.2)	4 (44.4)	2 (22.2)	18 (41.9)	18 (41.9)	20 (53.5)	25 (37.3)	19 (28.4)	20 (29.9)

F_0 = baseline stage; F_1 = Follow up at 3 months; F_2 = Follow up at 6 months

4.4. Frequency of abnormalities of Epo, TNF- α, IL-6 and CRP parameters among the HIV positive respondents

The frequency of increased serum Epo levels (Epo >368.9 pg./ml) decreased from the baseline level of 90.9% to stable level of 77.3% at the third month to the end of the study period in CD4+ < 200 cells /mm^3 group. In CD4+ 200 – 499 cells / mm^3 group the Frequency levels of increased serum Epo decreased from the baseline level of 41.9% to 15.1 then increased to 37.2 in the third and sixth months respectively; while in the CD4+ ≥ 500 cells / mm^3 group the baseline frequency level of 76.3% decreased to 73.7% and then increased to75% in the third and sixth months respectively during the study. The frequency of increased serum levels of tumor necrosis factor – alpha (TNF-α >7.0 pg./ml) decreased from the baseline level of 72.7% to 31.8 % in the third month then increased two-fold to 63.6% in the sixth month in CD4+ < 200 cells / mm^3 group during the study. In CD4+ 200 – 499 cells /mm^3 group, the baseline frequency of increased TNF-α serum level of 41.9% decreased to 15.1% in the third month and then increased to 37.2 % by the sixth month of the study; while in CD4+≥ 500 cells / mm^3 group the baseline frequency level 28.9% decreased to 11.8 % by the third month then increased to 25% in the sixth month of the study (Table 7).

The frequency levels of increased serum interleukin – 6 levels (IL-6 >2.6 pg. /ml) was 9.1% in CD4+ < 200 cells / mm^3 group and 9.3% in CD4+ 200 – 499 cells / mm^3 group throughout the study period. In CD4+ ≥ 500 cells / mm^3 group however, the baseline frequency levels of 7.9% increased to 17.1% in the third month and then decreased to 9.2 % in the sixth month of the study. The frequency of increased C- Reactive Protein serum levels (CRP > 4.0 mg /ml) increased from the baseline level of 77.3% to 81.6 % in the third month and then decreased to 63.6% by the sixth month in CD4+ < 200 cells / mm^3 group during the study. In CD4+ 200 – 499 group the abnormality demonstrated an increase from the baseline frequency level of 54.7% to 62.8% by the third month a further increase to 70.8% by the sixth month of the study period. Baseline frequency levels of 55.3% increased to 67.1 % in the third month and then decreased to 56.6% by the sixth month in CD4+≥ 500 cells / mm^3 group during the study period (Table 7).

Table 7: Frequency of abnormalities of Epo, TNF- α, IL-6 and CRP parameters among the HIV positive respondents

	Respondent group										
	HIV negative		HIV positive all (n = 184)								
	All (n = 101)		CD4+< 200 cells/mm^3 (n=22)			CD4+ 200 - 499 cells/mm^3 (n= 86)			CD4+ ≥ 500 cells/mm^3 (n= 76)		
Parameter	95%CI	Abnormality	F_0	F_1	F_2	F_0	F_1	F_2	F_0	F_1	F_2
EPO pg/ml	294.3 -368.9	Frequency (%)									
		of increased levels									
		(EPO > 368.9 pg/ml)	20(90.9)	17(77.3)	17(77.3)	74(86)	62(72.1)	63(73.3)	58(76.3)	56(73.7)	57(75)
TNF-α pg/ml	5.-7.0	Frequency (%) of									
		increased levels									
		(TNF-α > 7.0 pg/ml)	16(72.7)	7(31.8)	14(63.6)	36(41.9)	13(15.1)	32(37.2)	22(28.9)	9(11.8)	19(25)
IL-6 pg/ml	1.6 - 2.6	Frequency (%) of									
		increased levels									
		(IL-6 > 2.6 pg/ml)	2(9.1)	2(9.1)	2(9.1)	8(9.3)	8(9.3)	8(9.3)	6(7.9)	13(17.1)	7(9.2)
CRP mg/ml	2.7 - 4.0	Frequency (%)									
		of increased levels									
		(CRP > 4.0 mg/ml)	17(77.3)	18(81.8)	14(63.6)	47(54.7)	54(62.8)	61(70.9)	42(55.3)	51(67.1)	43(56.6)

F_0 = baseline stage; F_1 = Follow up at 3 months; F_2 = Follow up at 6 months

5. Discussions

Anaemia occurs in both ARV-treated and ARV-naïve HIV patients with multifactorial etiology. The complications associated with anaemia in advanced stages of HIV/AIDS requires concerted research studies to understand the unique pathophysiology of HIV- associated anaemia. This study was designed to prospectively monitor changes in: blood cell morphologies, proteins and cytokines that influence blood cell development in order to morphologically classify the observed anaemia as well as determine the accompanying changes in serum levels of Epo, TNF- α, IL-6 and CRP.

The significantly higher WBC mean values in females than in males has not been previously documented. The various erythrocyte pathologies that included: rouleaux formation, round macrocytes and microcytic hypochromic changes observed among the HIV positive respondents in the study have been previously reported to characterize HIV-associated anaemia [13]. The etiology of microcytic hypochromic features has been linked to: iron deficiency, chronic disease and congenital haemoglobinopathies including thalassemia; while that of macrocytic normochromic changes has been associated with: alcoholism, liver disease, hypothyroidism and side effects of certain medications such as those used to treat cancer, seizures and autoimmune disorders [14,15]. In this study there was no evidence of congenital haemoglobinopathies from the blood film evaluations and 88% of the respondents were teetotaler from the information on the questionnaire filled in by the study respondents. Moreover, 152 out of the total 184 (82.6%) HIV positive respondents were on antiretroviral therapy. Therefore, microcytic hypochromic features observed were possibly due to chronic illness and /or iron deficiency; while round macrocytes were associated with antiretroviral therapy. Indeed, macrocytic anaemia has been reported to arise from reduced erythropoiesis complicated by drug therapy [16,17]. Rouleaux formation has been associated with anaemia, hypovolemia and acute - phase proteins, particularly fibrinogen and low albumin levels [18,19]. The other erythrocyte pathology observed in the study was presence of target cells. Target cells have been associated with haemoglobinopathies and liver diseases [20]. In this study however, their presence may be attributed to possible liver disorder secondary to HIV infection. The frequency levels of rouleaux formation, target cells and microcytic hypochromic cells were highest in CD4+ < 200 cells / mm^3 and decreased in the higher CD4+ cell count groups (CD4+ 200 - 499- and ≥ 500 cells / mm^3). Reduction in the levels of these red cell changes with increases in CD4+ cell counts signifies decreases in the degree of HIV- associated pathology since changes in CD4+ cell counts have been reported as markers for HIV disease progress [21].

Highest levels of erythrocytopenia were observed in CD4+ < 200 cells/mm^3 group. This is suggestive of suppressed erythropoiesis by HIV infection whose degree is dependent on the severity of the disease. Previously it has been reported that there is inhibition of haemopoiesis by HIV infection [22]. The level of observed anaemia decreased with increases in CD4+ cell counts in both males and females but generally anaemia levels were higher in females than in males. Higher levels of anaemia in females than in males may be explained partly by the fact that there is increased iron needs in women of child-bearing age [23,24,25]. The level of observed macrocytosis increased with increases in CD4+ cell counts; while microcytic hypochromic cells observed in microscopy spread across all the CD4+ groups. Morphologically three types of anaemia were observed: normocytic normochromic, macrocytic and dimorphic with normocytic normochromic dominating in CD4+ < 200 cells/mm^3 group, macrocytic dominating in both CD4+ 200 -499- and CD4+ ≥ 500 cells/mm^3 groups and dimorphic anaemia spread in all the CD4+ cell groups. have Normocytic normochromic (40.4%), dimorphic anaemia (18.8%) and microcytic hypochromic anaemia (7.2%) in HIV antiretroviral (ARV) naïve patients has been reported [26]; while in HIV infected patients on ARV therapy they demonstrated anaemia in the following distribution: normochromic anaemia (63.88%), microcytic, hypochromic anemia (19.44%), macrocytic anemia (2.8%) and dimorphic anemia (13.88%) [26]. It has also been reported that treatment with zidovudine (AZT) inferred macrocytic anaemia of 34.62% [27]. These levels of red cell pathologies were not derived from respondents classified into CD4+ cell groups and so

could not be directly compared with those of this study. All these observations show that both HIV infection and ARV therapy singly or in combination can cause anaemia in HIV/AIDS.

Other blood cell changes observed in this study included: neutropenia, lymphocytopenia and monocytopenia. These observations agree with other studies that have associated pancytopenia with HIV infection arising from suppression of haemopoiesis by the virus itself and by haemopoiesis - inhibiting soluble serum factors [2,28,29]. High levels of neutropenia observed in this study compare well with that of 70% reported before [30]. Moreover, high levels of observed lymphocytopenia with levels that generally decreased with increases in CD4+ counts compare well with the levels previously reported: 84% [26], 28% [31], and 23% [32]. Lymphocytopenia in HIV infection has been reported to arise from lymphocyte depletion through antibody-dependent cytotoxicity mechanisms [33]. Decrease in the levels of lymphocytopenia with increases in CD4+ counts show that lymphocytes are markers of changes in CD4 levels. This supports previous reports that have recommended the use of absolute lymphocyte counts in place of CD4+ counts in resource-limited settings [34,35].

Neutrophil and eosinophil hypersegmentations of slight to moderate levels were observed in this study. Previously these pathologies have been reported to be classically pathognomonic of the megaloblastic anaemias, often caused by vitamin B_{12} or folate deficiencies, or DNA-replication poisons and manifesting with presence of oval macrocytes in peripheral blood film [15]. Recent reports however, have strongly associated neutrophil hypersegmentations with iron deficiency anaemia [35,36,37,38,39]. Peripheral blood film examination is an indispensable tool to the haematology practice and is interpreted together with results of automated blood cell counts [40]. Blood film evaluations in this did not show any oval macrocytes to support the association of vitamin B_{12} or folate deficiencies, or DNA-replication poisons with the macrocytic changes observed. This observation therefore supports the reports that associate the granulocytic hypersegmentation with iron deficiency.

Slight thrombocytosis observed in this study was accompanied by giant platelets. Other reports have however, observed thrombocytopenia in HIV infection [26,36], possibly as a component of pancytopenia that is said to characterize the disease [28,29]. Conditions that give rise to reactive or secondary thrombocytosis include: haemorrhage, acute infections, iron deficiency, cancer and splenectomy or hyposplenism [41,42]. In HIV infection thrombocytosis is reported to arise from compensatory thrombopoietin production [43]. The actual cause of the observed thrombocytosis in this study could not be fully explained but thrombopoietin assay could possibly give clear inferences.

In this study there was no gender-based significant differences observed in mean values of erythropoietin (Epo), tumor necrosis factor-alpha (TNF- α), interleukin-6 and C - Reactive Protein (CRP) in HIV negative respondents, although gender-based reference ranges for the parameters have been reported elsewhere among the Caucasians [44]. Nevertheless, this is in keeping with the reports that there are differences in reference values between people of African descent and Caucasians [45,46].

Among the HIV positive respondents there were markedly increased levels of erythropoietin in all the CD4+ cell groups. Increases in Epo levels in HIV infection have been previously reported [7,8]. However, some reports have attributed HIV-associated anaemia to inadequate levels of Epo arising from circulating auto-antibodies against endogenous Epo [12,47]. These antibodies have been reported to arise from molecular mimicry between Epo and the HIV-1 p17 protein [47]. This study's findings however, disagree with the reports that associate anaemia in HIV with Epo deficiency and instead propose hyposensitivity to Epo or Epo resistance as the cause of HIV-associated anaemia. This is in line with the in vitro studies that have shown that progenitor cells from HIV infected patients were intrinsically refractory to the growth effects of Epo, resulting in accumulation of colonies at a relatively undifferentiated stage of erythroid development [6]. There was slight to marked increases in TNF- α distributed in all the CD4+ cell groups. Increases in TNF- α in HIV infection has been reported previously [49,50]. In addition, there were slight increases in interleukin - 6 levels distributed in all the CD4+ cell groups. Previous studies have reported that increases in TNF- α and IL-6 in HIV synergistically inhibit Epo synthesis [51]. The frequency levels of increases in TNF- α observed in this study were much higher than those of IL-6 increases and possibly because of this imbalance, the two cytokines could not inhibit Epo synthesis resulting in the observed Epo increases. There were moderate to marked increases in CRP levels which decreased with increases in CD4+ cells. Increases in CRP as a proinflammatory protein in HIV has already been reported [52,53].

6. Conclusion

Among HIV negative respondents significantly higher WBC mean values in females than in males were established. HIV positive respondents demonstrated anaemia levels that reduced with increases in CD4+ cells; the anaemia was classified morphologically into normocytic normochromic, macrocytic normochromic and dimorphic. Also demonstrated was bicytopenia (erythrocytopenia and leucopenia) which was thought to be due to the suppression of haemopoiesis by the virus itself and / or by haemopoiesis- inhibiting soluble serum factors. The observed thrombocytosis was thought to be associated with compensatory thrombopoietin production. However, thrombopoietin assay would have provided inferences.

Increased levels of Epo were observed in all the CD4+ groups. High levels of anaemia in increased Epo levels observed was probably due to either Epo hyposensitivity or Epo resistance. Increased levels of TNF-α of mixed frequencies across the CD4+ cell groups were observed. Minimal increases in IL-6 were also observed. It was thought that increases in the levels of the two cytokines were inadequate to effectively inhibit Epo synthesis. The observed increases in CRP in all the CD4+ cell groups were thought to be due to active inflammation associated with HIV infection or due to chronic infection. Establishment: of gender – based reference ranges for WBC values and of African - based reference values for Epo, TNF-α, IL-6 and CRP parameters was recommended. Levels of rouleaux formation and targeting of erythrocytes together with CD4+ cell counts as markers of HIV/AIDS disease severity was recommended. Further studies on the observed changes over longer study periods was recommended. Clinical trial-based studies on the effect of Epo hyposensitivity on use of recombinant human erythropoietin in managing anaemia in HIV were recommended. Monitoring the observed changes in haematological parameters, the

proinflammatory proteins and Epo in ARV –treated and ARV-naïve HIV patients separately was recommended

7. Study Limitations

The recruitment of study respondents was not randomized and this may have reduced the strength of making population inferences of the studied characteristics. The details of antiretroviral therapy regimens and therapies for HIV- coinfections were not put into account in this research work. The reagents employed for Epo, TNF and IL-6 assays were recommended for research use only. Limited work has been done in Sub-Saharan Africa on Epo, TNF-α, IL-6 and CRP parameters and this hindered inter-laboratory comparison of the results obtained.

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