TOTAL LYMPHOCYTE COUNT AS A PROGNOSTIC MARKER FOR CD4 COUNT IN RESOURCE LIMITED SETTINGS

Chukwuma G.O.

Department of Medical Laboratory Science, Faculty of Health Sciences & Technology, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, Anambra State, Nigeria

Ezeifeka G. O.

Department of Veterinary Medicine and Animal production, Federal University of Agriculture, Umudike, Abia State, Nigeria

Ahaneku G.I.

Department of Medicine, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, Anambra State, Nigeria

Chukwuma O.M.

Department of Medical Laboratory Science, Faculty of Health Sciences & Technology, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, Anambra State, Nigeria

Nwaokorie F.O.

Nigeria Institute for Medical Research (NIMR) Yaba , Lagos State, Nigeria

Kalu S.O.

Nnamdi Azikiwe University Teaching Hospital Nnewi, Anambra State Nigeria

Ndembi N.

Institute for Human Virology Abuja, Nigeria

Manafa P.O

Department of Medical Laboratory Science, Faculty of Health Sciences & Technology, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, Anambra State, Nigeria

Abstract

Understanding the Total lymphocyte count (TLC) and CD4 relationship could aid design predictive instruments for making clinical decisions during antiretroviral therapy. The aim of this study was to determine the predictive ability of TLC for CD4 count less than or equal to 350cells/mm^3.

A cross sectional study involving 432 HIV-I infected persons randomly

recruited from the HIV Clinics of Nnamdi Azikiwe University Teaching Hospital (NAUTH) Nnewi, NAUTH Oba and St Charles Borromeo Hospital Onitsha was conducted. Ethical approval was obtained and blood samples were collected. The following were determined; HIV- screening and confirmation using serial testing algorithm with ELISA rapid test kits, CD4 count using Cyflow partec machine and Haematological profile using Sysmex KX21N. Data analysis was carried out using SPSS version 17.

Out of the 432 HIV patients involved in this study, females dominated with a population of 274(63.4%) against the males numbering 158(36.6%). The relationship between the TLC cut offs and CD4 count ≤350 cells/mm^3 were all statistically significant (P<0.05) except for a TLC value of 2600cells per mm^3. At a value of 1200 cells/mm^3, TLC had a sensitivity of 25% and specificity of 96% while at 2400 cells/mm^3, TLC had a sensitivity of 76% and specificity of 39% for CD4 count of ≤350cells/mm^3.TLC was found to have the most significant relationship with CD4 count of all parameters tested using regression analysis. Finally, TLC could serve as a surrogate for CD4 count for monitoring treatment in resource poor areas where facilities for CD4 count may not be available.

Introduction

The Total lymphocyte count are associated with disease progression in patients with AIDS (Aupril et al, 2008) and the World Health organization has suggested that they could be used as markers to initiate highly active antiretroviral therapy (HAART) in resource- poor settings(WHO, 2004) .Plasma HIV RNA levels and CD4+ lymphocyte counts are established markers of HIV disease progression and quantify an individual's risk for AIDS and death. Obtaining CD4+ cell counts and HIV RNA levels may be impractical for countries with scarce resources, due to the expense of assays and technical factors such as access to the proper equipment(Beck et al, 1996). Therefore there is a need for prognostic markers that can be used in resource limited settings. One marker that may fulfill this need is the Total lymphocyte count (TLC). It is relatively inexpensive and straightforward to measure. TLC has been shown to correlate with CD4+ cell counts, particularly among symptomatic individuals (Beck et al, 1996) and single measurements have been shown to have some prognostic information for survival and progression to AIDS (Schechter et al, 1994). Furthermore, world health organization guidelines for developing countries (WHO, 2002) suggest the use of TLC for disease monitoring if CD4+ cell counts are not known. Currently the WHO has adjusted to a CD4 count level of

350cells/mm^3 before initiating treatment(WHO, 2010) ,there was need therefore to revise the TLC cut-off for CD4 Count <350cell/mm^3. In Nigeria, the Federal Ministry of Health now commences ART in all patients with HIV infection who have a CD4+cell count ≤350 cells/mm3 including pregnant women irrespective of clinical symptom. Inaddition, ART is started in all patients with WHO clinical stage 3 or 4 irrespective of CD4 count(FMH, 2010)..

Materials and Methods

Study Design

A cross sectional study was conducted among 432 HIV-I infected persons (158 males and 274 females), randomly recruited from the HIV Clinics of Nnamdi Azikiwe University Teaching Hospitals Nnewi, Oba and St Charles Borromeo hospital Onitsha. Of these, 181 were drug naïve and 251 drug experienced . Ethical consideration was obtained from the Ethical committee of both hospitals. Informed consent was obtained from the patients after which they were served questionnaire. Questionnaires were accurately filled and the following data obtained; personal factors like age, gender, type of antiretroviral drug administered and number of years on drug. Five milliliters of blood samples were collected using EDTA anticoagulant bottles The following parameters were performed for each participant; HIV- screening and   confirmation, CD4 count and Haematological profile.

Inclusion Criteria

1. HIV-I infected on HAART therapy for atleast I year period (for drug experienced group)

2. HIV infected but not on drugs (for drug naïve participants)

3. Age range of 15-70 years

Exclusion Criteria

1. Patients were excluded if they test negative to HIV-I.

2. If they are drug experienced but duration is less than 1 year.

3. Pregnant women (After performing a pregnancy test for those not visibly pregnant and excluding those visibly pregnant).

Study Area

The study area was Nnewi, Oba and Onitsha all in Anambra state Nigeria.These towns are located within the tropical rain forest. They are both commercial towns. While Onitsha is located beside the popular river Niger, Oba lies 7kilometers south of Onitsha and Nnewi is located east of river Niger and 22kilometers from Onitsha.

Calculation Of Sample Size

Sample size was calculated using the minimum sample size for simple proportion with 5% margin of error and 95% level of confidence as shown below. Equally, a monthly prevalence rate (P) was obtained from the

HIV clinic of the Nnamdi Azikiwe University Teaching Hospital Nnewi and St Charles Borromeo hospital Onitsha.

$N = Z^2 PQ/D^2$

Where Z= Standard normal deviation at 1.96(which corresponds to 95% confidence interval).

P= Monthly Sero prevalence of HIV infection in HIV clinic attendees =16%

Q=1-P

D=Degree of accuracy/ precision expected = 0.05%

Substituting for the above formulae

$N = 1.96^2 \times (0.16 \times 0.84)/(0.05)^2 = 206$.

Sampling Procedure And Sample Size

The sample size for the study was 432 patients. The patients were recruited using stratified random sampling technique by drawing the sample frame of the patients which consist of antiretroviral drug experienced stratum and antiretroviral drug naïve stratum; 251 and 181 patients were randomly selected from each stratum respectively using probability by replacement method.

HIV Counselling And Testing Procedure

The nature of the research was explained to each participant after which informed consent was sought. Subsequently, the participants were presented with the informed consent form and they consented to it. Only patients who gave their consent were included in the study and they were assured of confidentiality and freedom of withdrawal. HIV testing was performed after pre test counseling and also post test counseling took place after the testing by trained HIV counselors, according to National AIDS programs guidelines. The investigation was performed according to standard laboratory practice and followed guidelines approved by National HIV Rapid test Algorithm. Rapid testing was done using Determine, Unigold and Stat Pak. The results obtained were analyzed statistically using the SPSS version 17.

Methods

HIV Rapid Testing

The rapid HIV test kits consisting of 3 different antigens were used for the diagnosis of HIV infection by serial testing algorithm. The first test was performed using Determine kit (Abbott Laboratories, USA) (screening test) and if positive a second test is performed using Unigold(Trinity Biotech, Ireland), if positive the patient is considered positive for HIV but if negative, the third test which acts as tie breaker is carried out using Stat pak (Chembio Diagnostic System, USA).

CD4 Count

The CD4+ T cell count was done to determine the level of immune function. The Cyflow Partec machine (Partec GmbH, Munster, Germany) were used. The Cyflow detects and identifies fluorochrome- labeled (phytoerythrin, PE) cells as they pass through an optical detecting system by detecting the angle of scatter of incident laser and the wavelength of fluorescence emitted. Twenty microlitres of patient's blood was added to 20µl of PE antibody in Rohren tube. This was adequately mixed and incubated in the dark for 15mins at room temperature. Exactly, 800µl of the buffer was then added to the tube and mixed adequately. The sample tube was plugged onto the sample port of the Cyflow machine for counting of the CD4 + T cells. The monitor displayed the result of the counting and this was recorded as the number of cells/µl of blood.

Assessment Of Haematological Profile Using The Sysmex Kx 21n Auto Analyzer

White Blood Cell/ Haemoglobin Analysis

Here,the volume of WBC and HB in the blood were measured . Blood was aspirated from the sample probe into the sample rotor valve. Six microlitre of blood measured by the sample rotor valve was transferred to the WBC transducer chamber along with 1.994ml of diluents. At the same time, I added 1.0ml of WBC /HB lyse to prepare 1:500 dilution of sample. The solution was made to react in this status for approximately 10 seconds, RBC was then hemolysed and platelet shrunk, with WBC membrane held as they are. At the same time, haemoglobin was converted into red coloured methaemoglobin. Of the diluted/ hemolysed sample in the WBC transducer chamber, approximately 1ml was transferred to the HB flow cell. Five hundred microlitre of sample in the WBC transducer was aspirated through the aperture. The pulses of the blood cells when passing through the aperture were counted by the DC detection method. In the HB flow cell, 555nm wavelength beam irradiated from the light emitting diode(LED) was applied to the sample in the HB flow cell. Concentration of this sample was then measured as absorbance. This absorbance was compared with that of the diluents alone that was measured before addition of the sample, thereby calculating HB .

Red Blood Cell And Platelet Analysis

In RBC and platelet analysis, RBC and Platelet count in the blood were measured. Here, I aspirated blood   from the sample probe into the sample rotor valve. Then 4uL of blood measured by the sample rotor valve was diluted into 1:500 with 1.996ml of diluents and brought to the mixing chamber as diluted sample . Out of the 1:500 dilution sample, 40uL was measured by the sample rotor valve, diluted into 1:25000 with 1.960ml of diluent, then transferred to the RBC/ platelet transducer chamber.

Subsequently, 250uL of the sample in the RBC/platelet transducer was aspirated through the aperture. At this time, RBC and platelet are counted by the DC detection method. At the same time, haematocrit value was calculated by the RBC pulse height detection method.

Results

Table 1: Age Distribution Of Hiv Infected Individuals According To Sex And Drug Use

AGE YEARS	DRUGS		P-VALUE	SEX		P-VALUE
	EXPERIENCED NO (%)	NAÏVE NO (%)		MALE NO (%)	FEMALE NO (%)	
<25	49(19.5)	32(17.7)		27(17.1)	54(19.7)	
25-44	88(35.1)	87(48.1)		62(39.2)	113(41.2)	
45-64	112(44.6)	62(34.3)	0.032	67(42.4)	107(39.1)	0.239
>64	2(0.8)	0(0)		2(1.3)	0(0)	
TOTAL	251(100)	181(100)		158(100)	274(100)	

Results are presented as Means ± S.D. P is significant at P≤0.05

Table 2: Sensitivity, Specificity, Positive And Negative Predicitive Values Of Tlc Cut Offs For Cd4 Count ≤ 350 Cells/Mm^3

CD4 COUNT (CELLS/MM^3) N=432	TLC CUT OFF	P-VALUE	SENSITIVITY (%)	SPECIFICITY (%)	PPV (%)	NPV (%)
	1200	0.000	25	96	89	48
≤350	1400	0.000	33	89	81	49
	1600	0.000	43	83	78	51
	1800	0.000	52	77	76	53
	2000	0.000	62	64	70	55
	2200	0.000	69	49	65	54
	2400	0.001	76	39	63	53
	2600	0.83	80	27	60	49

Results are presented as Means ± S.D. P value is significant at P<0.05

- PPV refers to positive predictive value
- NPV refers to negative predictive value

TLC=1767.388+1.002CD4 (P-VALUE=0.000)

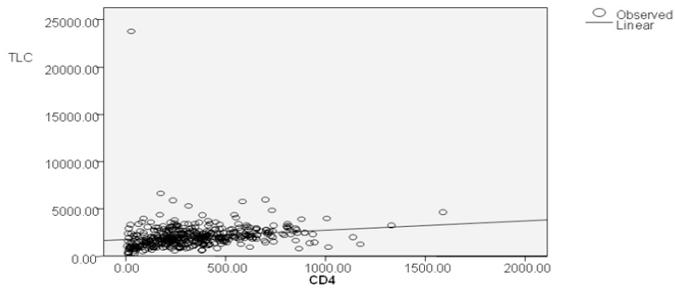FIG 1:REGRESSION LINE GRAPH BETWEEN TLC AND CD4
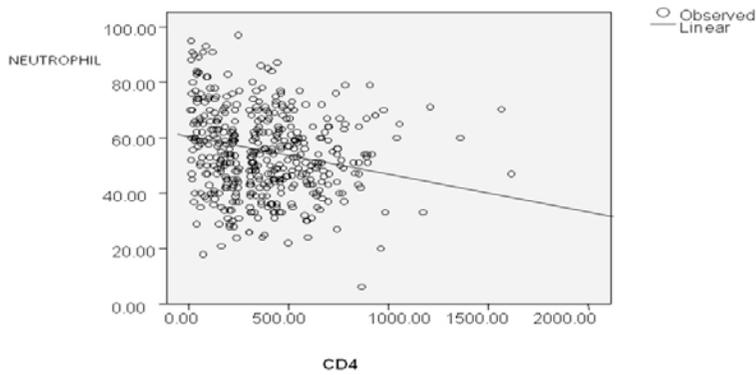
NEUTROPHIL=57.735+(-0.013)CD4 (P-VALUE=0.000)

FIG 2: REGRESSION LINE GRAPH BETWEEN NEUTROPHIL AND CD4

HB=10.265+0.002CD4 (P-VALUE=0.000)

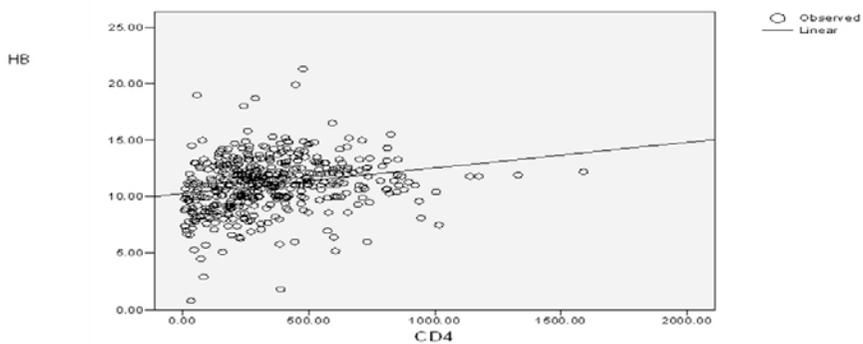FIG 3: REGRESSION LINE GRAPH BETWEEN HB AND CD4

69

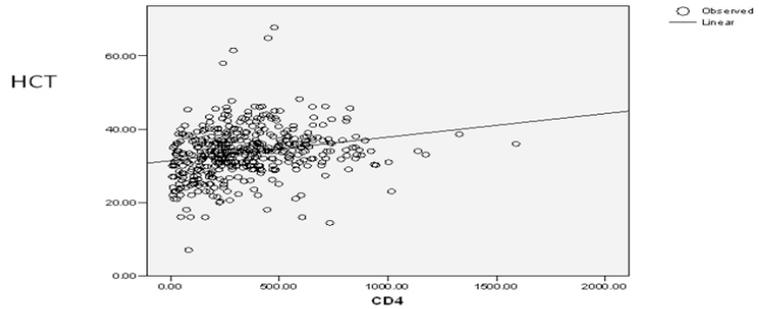$$HCT = 31.421 + 0.006\,CD4 \; (P = 0.000)$$

FIG 4: REGRESSION LINE GRAPH BETWEEN HCT AND CD4

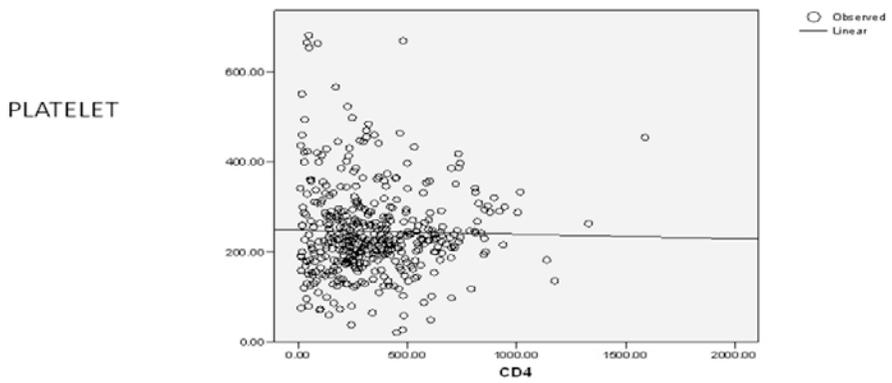$$PLATELET = 248.564 + (-0.010)CD4 \; (P\text{-}VALUE = 0.636)$$

FIG 5: REGRESSION LINE GRAPH BETWEEN PLATELET AND CD4

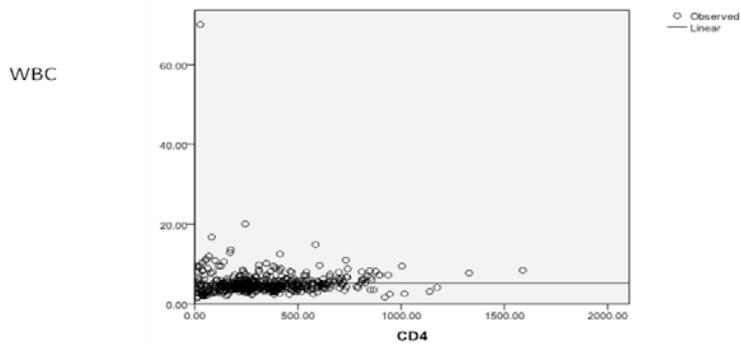$$WBC = 5.291 + 2.680\,CD4 \; (P\text{-}VALUE = 0.972)$$

FIG 6:REGRESSION LINE GRAPH BETWEEN WBC AND CD4

Discussion

CD4 testing is the recognized gold standard used to stage HIV/AIDS, guide treatment decisions for HIV-infected persons and evaluate effectiveness of therapy. The need for a less expensive surrogate marker that can be used in resource-limited setting is however necessary. This study which consisted of 432 participants with 181 drug naïve and 251 drug experienced sought to assess the suitability of Total lymphocyte count (TLC) as a surrogate marker for CD4 count in resource-limited localities. An attempt to use TLC as a surrogate for CD4 count was made in table two. The WHO has advised that in resource poor areas where routine CD4 count is not readily available, the TLC can be used as an alternative (WHO,2004).The World Health Organization (WHO) currently recommends that adults living with HIV start antiretroviral therapy (ART) when their CD4 counts fall below 350 cells/µl as against 200cells/mm^3 (WHO, 2010). There was need therefore to revise the TLC cut-off for CD4 Count ≤350cell/mm^3. Here, various values of CD4 cut offs were tested against a CD4 count ≤350 cells/mm^3. These includes TLC of (1200, 1400, 1600, 1800, 2000, 2200, 2400 and 2600) cells/mm^3. The relationship between the different TLC cut offs and CD4 count ≤350 cells/mm^3 were all statistically significant with P<0.05 except at TLC level of 2600cells/mm^3 with P>0.05. At a value of 1200 cells/mm^3, TLC had a low sensitivity of 25% and the highest specificity of 96%. It also had the highest positive predictive value of 89% and lowest negative predictive value of 48% at the 1200 cells/mm^3 cut off for TLC. The sensitivity of the different TLC cut offs have an inverse relationship with the specificity. Therefore at a TLC value of 2400 cells/mm^3, TLC had a high sensitivity of 76% and specificity of 39%. At a TLC level of 2600cells/mm^3 there was no significant relationship between TLC and CD4 count of ≤350cells/mm^3 (P>0.05). This shows that TLC values of 2600cells/mm^3 and greater may not be useful in monitoring CD4 count of ≤350cells/mm^3 .The present study shows that a TLC range of 1200-2400 cells/mm^3 could predict CD4 ≤350 cells mm^{-3} but this will serve a better purpose in the management and monitoring of HIV patients if a calibration cut-off could be established in our local settings. This in addition to WHO clinical stage of disease, haemoglobin and weight could serve as useful model in resource poor setting.The value of TLC as a surrogate for CD4 in monitoring HIV disease in the absence of viral loads and CD4 counts has been argued (Crowe et al, 2003) but current WHO guidelines only commit to using TLC in conjunction with clinical data as a criterion to initiate HAART in resource poor settings (WHO,2006). In a work carried out by Mwangi et al, 2011, in Kenya, they found that Of 362 HIV positive pregnant women, 160(44.5%) had CD4 count <350 cells/mm^3. Using linear regression optimal cut-off points for TLC, HB, BMI were 850cell/mm^3,

8.4g/dl and 15.5kg/m^2 respectively. These cut-off points were highly specific but with very low sensitivity. The best cut-off point using generated sensitivity and specificity values was TLC≤2200 with Sensitivity of 68% and Specificity of 51%.

Regression line graphs of predictive parameters for CD4 count are shown in the figures above. In this assessment, a regression line graph was drawn to ascertain the level of relationship between CD4 count and WBC, HB, TLC, Neutrophil, HCT and Platelet. TLC was found to have the most significant relationship with CD4 count (Fig. 1), with P value of 0.000 and for every unit increase in TLC, there is a 1.002 increase in CD4 count. WBC (Fig 6) and Platelet (Fig 5) had no significant relationships with CD4 count with P values of 0.972 and 0.636 respectively. HB, Neutrophil and HCT also gave significant relationships with CD4 count with P values of 0.000, 0.000, 0.000 and 0.000 respectively .This shows that TLC compares most favorably with CD4 count and may serve as a surrogate for it. In a similar pattern, a linear regression model was conducted by Owiredu et al, 2011, to assess the ability of some selected study parameters to predict CD4 counts in HAART-naïve patients and those on HAART. It was observed that for unit increases in CD4 as an independent variable, there were corresponding unit increases in TLC, lymphocyte count, haemoglobin and weight. There was no linear relationship between total white blood cell count and CD4 count and platelet count showed a negative relationship. The ability of TLC, lymphocyte count, haemoglobin and weight to predict CD4 count in both HAART-naïve patients and those on HAART suggests that these parameters which are relatively inexpensive and easily available compared to techniques for assaying CD4 and viral load could serve as accurate tools that can be used for monitoring the patients' immune status during therapy in addition to determining when patients should start antiretroviral therapy. Mwamburi *et al.*, 2005, reported a similar finding and suggested modification of the models to suit specific needs for use in underserved areas. Finally, TLC could serve as a surrogate for CD4 with effective clinical staging of disease, inclusion of haemoglobin level, weight values and appropriate calibration of TLC cutoffs to suit local requirement.

References:
Aurpil L., Puthanakit T., Sirisanthana T.,(2008). Haematological changes after switching from stavudine to zidovudine in HIV-infected children receiving highly active antiretroviral therapy.*HIV Med*; **9**: 317-321.
Beck E.J., Kupek E.J., Gompels M.M., Pinching A.J.,(1996). Correlation between total and CD4 lymphocyte counts in HIV infection: not making the good an enemy of the not so perfect. *Int J STD & AIDS*; **7**:422–428.

Crowe S., Turnbull S., Oelrichs R., Dunne A.,(2003). Monitoring of human immunodeficiency virus infection in resource-constrained countries. *Clin Infect Dis*;**37**(1):25–35.

Federal Ministry Of Health Nigeria(2010). National Guidelines For Hiv/Aids Treatment And Care. Www.Who.Int/Hiv/Pub/Guidelines/Nigeria_Art.Pdf. Assessed 9th April, 2013.

Mwamburi D.M., Ghosh M., Fauntleroy J., Gorbach S.L., Wanke C.A., (2005). Predicting CD4 count using total lymphocyte count: a sustainable tool for clinical decisions during HAART use. *Am J Trop Med Hyg*;**73**(1):58–62.

Mwangi W.W., M'imunya J.M.,Kiarie J.,Kinuthia J., Kudoyi W., Spitzer R.,Mabeya H., Mukoko D., Mutai K., Jepchirchir J., (2011). Utility of an Algorithm of Surrogate Markers for CD4 Count to Determine Eligibility for HAART among HIV Infected Pregnant Women. Available at https://idsa.confex.com/idsa/**2011**/.../Handout/.../Poster%20for%20IDSA.pdf . Assessed 9th April, 2013.

Owiredu W.K.B.A., Quaye L., Amidu N., Addai-Mensah O.,(2011). Prevalence of anaemia and immunological markers among Ghanaian HAART-naïve HIV-patients and those on HAART. *Afr Health Sci*; **11**(1): 2–15.

Schechter M., Zajdenverg R., Machado L.L., Pinto M.E., Lima L.A., Perez M.A., (1994). Predicting CD4 counts in HIV-infected Brazilian individuals: a model based on the World Health Organization staging system. *J Acquir Immune Defic Syndr*; **7**:163–168.

World Health Organization., (2002)Scaling up antiretroviral therapy in resourcelimited settings. Guidelines for a publichealthapproach.Available at:http://www.who.int/HIV_AIDS. Accessed July 1, 2002.

World Health Organization (WHO)., (2004): scaling up antiretroviral therapy in resource limited settings: treatment guideline for a public health approach. Geneva: WHO, 2004; pp 1-68 (available at http:/www.who.int/3by5 /publications/documents/arv_guidelines/en/index

WHO: HIV/AIDS Programme.,(2006). Antiretroviral therapy for HIV infection in adults and adolescents: recommendations for public health approach. http://www.who.int/hiv/pub/guidelines/artadultguide lines. pdf

World Health Organization., (2010). Antiretroviral therapy for HIV infection in adults and adolescents: Recommendations for a public health approach: 2010 revision. Available at:http:// www.who.int/hiv/pub/arv/adult2010/en/. Assessed 9th April, 2013.