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Preliminary Evaluation of Association of Cluster Differentiation-4⁺ Cells with Interleukin-6 Levels in HIV-Infected Patients on ART in a Tertiary Health Facility in North Eastern Nigeria

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Abstract

Immunity status of HIV-Infected patients is physiologically compromised which can be inferred from the Cluster of differentiation four (CD4⁺) cell count which is also used as a parameter in health and immune status in the management of HIV-Infected patients on antiretroviral therapy (ART). Higher CD4 counts typically signify healthier immune systems. However, IL-6 has a multiple biological effects on immune and proinflammatory responses including enhancing and proliferation of and maturation of B cells as well as immunoglobulin production, and to activate T lymphocyte function. In this descriptive case-control prospective study, blood samples of selected HIV-infected patients on ART were collected during one of their periodic visit. Those with CD4 count of 100 to 200 cells/ul and those with ≥ 400 cells/ul blood were tested for Interleukin-6 level along with healthy controls by cytoflow and ELISA technique respectively. Results revealed IL-6 levels of 272.93 ± 250.84 in those with CD4 count of 100 to 200 cells/ul, while 301.99 ± 261.23 was obtained in patients with ≥ 400 cells/ul, while the apparently

healthy controls have 145.98 ± 156.36 within the standard range of 6.5 – 200 pg/ml. There was significant increase in the IL-6 levels when compared to the healthy controls. In HIV-Infected patients on ART, the higher the CD4 count, the higher the IL-6 cytokine level with the females generally having higher IL-6 cytokine values than the males.

1. Introduction

Human immunodeficiency virus (HIV) infection, the cause of acquired immunodeficiency syndrome (AIDS), is a global pandemic and one of the most destructive diseases ever faced by mankind [1]. HIV is still a major cause of death worldwide [2]. Accompanying it are profound socio-economic and public health consequences. Even though HIV pandemicity is heterogeneous within regions with some countries more afflicted than others, Sub-Sahara Africa is far worst affected with an estimated infection rate of 22.9 million in 2010, making up 68% of total global HIV infections [3]. A number of HIV prevention and treatment strategies introduced had scaled down deaths from 2.2 million in 2005 to 1.8 million deaths in 2010 [3]. A considerable prolongation of survival in HIV infection has been achieved but it is however unfortunately accompanied by an increased frequency of non-HIV-related co-morbidities [4]. Cluster differentiation four (CD4) T-lymphocytes and other lymphocytes synchronise the immune system's response to pathogens [5]. In HIV-un-infected individuals, CD4⁺ cell count provides a picture of immune system health, with higher CD4 counts typically signifying healthier immune systems. Children have high CD4⁺ cell counts, which decline slowly through adolescence and then plateau [6].

As HIV-patient care improves, there are growing concerns that among HIV-positive adults with high cluster differentiation-4⁺ (CD4⁺) cell counts on effective anti-retroviral therapy (ART), serious non-AIDS conditions are the primary cause of severe morbidity and mortality [7]. Recently, more HIV immunological biomarkers are being investigated for possible association with already established ones (such as the CD4⁺ cells count) which could possibly serve as independent predictor (s) of HIV-related non-AID complications. The associations of biomarkers such as pro- and anti-inflammatory cytokines, acute phase protein, C-reactive protein (CRP), as well as CD4 counts of HIV-positive subjects are being compared among various populations [8].

Interleukin-6 (IL-6), a Pro-inflammatory cytokine, is a multifunctional cytokine that regulates various physiological processes [9], including playing a key role in the acute phase response and in the transition from acute to chronic inflammation [10, 11]. A growing body of evidence is emerging that overproduction of pro-inflammatory cytokines such as IL-6 plays a central role in the pathogenesis of infection with HIV [12]. HIV infection has before now, been shown to modulate the expression and secretion of IL-6 by

monocytes and macrophages [13]. HIV-positive patients on effective anti-retroviral therapy (ART) have significantly higher plasma levels of IL-6 than matched uninfected subjects [14, 15]. Activated inflammation, as demonstrated by persistently higher IL-6 levels, may have profound and far-reaching clinical implications [9]. Even though the reasons HIV infection is associated with a chronic inflammatory state are not entirely understood, some recent reports involving HIV-infection have associated increased plasma IL-6 levels to a variety of adverse clinical outcomes, including anemia, cancer, cardiovascular disease and death [16 – 20].

The HIV infection of targets cells requires viral attachment and entry via CD4 protein (receptor) on the target cells, which has high-affinity interaction with a coat (envelope) protein of HIV named gp 120 [21]; in addition to a co-receptor which most frequently is either chemokine receptor CCR5 or CXCR4. The CD4⁺ cells migrate to the lymphoid tissue where the virus replicates and then infect new CD4⁺ cells. The CD4⁺ (T-helper) cells count is a crucial part standard treatment guideline in the administration of anti-retroviral therapy. The hallmark of untreated HIV infection is an inexorable CD4 T-cell count reduction in the most of patients while improved to normal counts are encountered in effective anti-retroviral therapy [22]. However, severe morbidity and mortality are still observed in HIV-positive adults with high CD4⁺ cell counts on effective anti-retroviral therapy (ART) and serious non-AIDS conditions are thought to be the primary cause [7]. Hence, there is glaring need for investigation into the associations of various biomarkers to establish independent predictors of morbidity and mortality in HIV-positive subjects. Therefore, this study is to investigate the plasma IL-6 cytokine level in HIV-Infected patients on ART with defined range of CD4⁺ counts in comparison with apparently healthy control subjects in the same community.

2. Materials and Methods

2.1. Study Area

This study was carried out between January 2012 and March 2012 in the University of Maiduguri Teaching Hospital (UMTH), Maiduguri, Nigeria. UMTH is a major referral medical center in the North eastern Nigeria, with 500 bed size and sub-specialties in medicine and training of other health care professionals. Maiduguri is the capital of Borno state, which lies on latitude 11.5°N and longitude 13.5°E, and occupies an area of 50,778 square kilometers.

2.2. Study Subjects

A total of seventy five (75) adults subjects were prospectively recruited into this study. Fifty (50) HIV-infected subjects on ART in UMTH and twenty five (25) non-HIV infected apparently healthy were recruited as study and control subjects, respectively. Study and control subjects were age-gender matched.

2.3. Study Design

This is a descriptive case-control prospective study.

2.4. Ethical Clearance

Ethical approval was received from the Ethics and Research Committee of the University of Maiduguri Teaching Hospital, Maiduguri.

2.5. Inclusion Criteria and Exclusion Criteria

Inclusion criteria

- Subjects with a confirmatory test of HIV infection
- Patients aged 18 - 65
- Patients on ART for at least 1 year
- Both Males and Females

Exclusion criteria

- Pediatric and elderly patients
- HIV patients on ART with confirmed Cancer
- HIV patients with confirmed TB.
- HIV patients with confirmed HBsAg
- HIV female patients with confirmed pregnancy

2.6. Sample Collection

Blood sample

Five milliliters of blood was obtained by venipuncture into Ethylene Diamine Tetraacetic Acid (EDTA) bottles from the HIV- Infected patients and apparently healthy subjects as controls, transported to the laboratory. CD4⁺ cell counts of all samples were done within 3 hours of collection. After the CD4⁺ cell count assay, the plasma was aspirated into nunc tubes and stored at -70°C and stored until tested for IL-6 cytokine.

2.7. Laboratory Testing

2.7.1. Cluster of Differentiation 4 Count Assay Procedure

PRINCIPLE

This is based on the principle of fluorescence or emission of light resulting from the release of energy gained through the absorption of light at different wavelength. Monoclonal antibodies specific for desired cell-surface proteins can each be coupled to a fluorescent dye called fluorochrome that will fluoresce with a defined spectrum of light when excited by light at a certain wavelength (532nm). The flow cytometer can detect cells labeled with such fluorochrome conjugated antibodies as they pass in a liquid stream through a beam of laser light of defined wave-length. As each cell passes through the 46 laser beam, the laser light is scattered and excites any dye molecules bound to the cell, causing it to fluoresce. Sensitive photomultiplier tubes can detect the scattered light and the fluorescence emissions respectively providing information on each cells granularity and extent to which it bound a given fluorescence dye.

Partec Cyflow counter for CD4⁺ cell count was used for the assay. Into partec test tube (Rohren tube) containing 20 µl CD4 antibody, 20 µl of well mixed whole blood was added. The solution was mixed gently and incubated in the dark for

15 minutes at room temperature. A total of 800 µl of CD4 buffer was added and mixed gently. Tube was plugged on to the counter and allowed to run ensuring CD4⁺ cells, monocytes and noise are well separated and gated. Results were automatically displayed in the screen in counts/µl.

2.7.2. Testing for Interleukin-6 Cytokine

Sample analysis was carried out by Sandwich ELISA method, using commercially produced enzyme-linked immunosorbent assay (ELISA) 6.25-200 pg/ml from ELI-Pair (Diacclone Besancon Cedex, France). All samples and standards were run in duplicates and the average value considered after reading at optical density (OD) of 450 nm. The recommended IL-6 standard range was 6.25-200 pg/ml. Using standard wells values, each standard OD (Y axis) versus the corresponding standard concentration (x-axis). A standard curve was drawn on linear graph paper manually to obtain the best linear/linear curve to give the most accurate results. Concentrated samples (i.e. >200 pg/ml) are diluted with standard diluent buffer and the OD result obtained was multiplied by the corresponding dilution factor.

2.7.3. Reference Curve for Human IL-6

Optical Density (OD) against Concentration (pg/ml).

Tests were based on the test standard and dwarfed standard curve to calculate the IL-6 value in specimens.

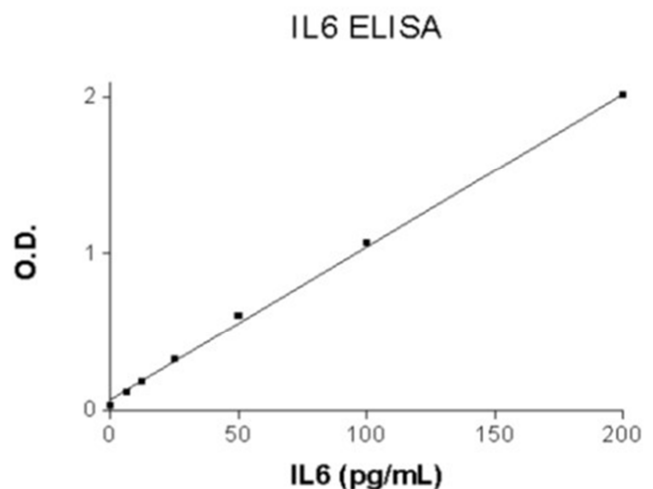


Figure 1. IL-6 Standard Curve ranging from 6.25 to 200 pg/ml.

2.8. Statistical Analyses

Statistical package for social sciences (SPSS IBM) version 20.0 software (Chicago, Illinois, USA) was used for statistical analyses. Descriptive data was presented as mean \pm standard deviation and percentage (%). Comparison of means of the study groups were carried out using paired sample t-test, taking 95% confidence interval. Box-plot was used in presenting some data. P -values ≤ 0.05 was considered as statistically significant.

3. Results

A total of seventy five (75) adults subjects were

prospectively recruited into this study. Fifty (50) of the subjects who were HI-Infected on ART in UMTH, consisting of twenty five (25) HIV-infected subjects with CD4 counts of 100 - 200 cells/ul of blood and twenty five (25) HIV-infected subjects with CD4 counts of ≥ 400 cells/ul of blood. Twenty five (25) apparently healthy subjects were recruited as control. Study and control subjects were age-gender matched. Table 1 showed Gender distribution of HIV-infected Patients on ART and Control Subjects. Table 2 showed the mean age of the HIV-infected patients on ART and Control Subjects. The mean Interleukin-6 concentration of HIV-infected patients on ART based on CD4 levels as compared with the control subjects were shown in table 3. The mean \pm standard deviation of Interleukin-6 concentration of HIV-infected

patients on ART with CD4 counts of 100 - 200 cells/ul of blood (272.93 ± 250.84) was significantly higher than that of control subjects (135.11 ± 145.20) with P-value 0.00. The mean \pm standard deviation of Interleukin-6 concentration of HIV-infected patients on ART with CD4 counts of ≥ 400 cells/ul of blood (301.99 ± 261.23) was also significantly higher than that of control subjects (135.11 ± 145.20) with P-value 0.00. Figures 1, 2 and 3 showed boxplots of Interleukin-6 concentrations of control subjects, HIV-infected patients on ART with CD4 counts of 100 - 200 cells/ul of blood and ≥ 400 cells/ul of blood against the genders of control subjects, HIV-infected patients on ART with CD4 counts of 100 - 200 cells/ul of blood and ≥ 400 cells/ul of blood, respectively.

Table 1. Gender distribution of HIV-Infected Patients on ART and Control Subjects.

Gender	Control Subjects	Patients with 100-200 cells/ul	Patients with ≥ 400 cells/ul
Male	10 (40%)	14 (56%)	12 (48%)
Female	15 (60%)	11 (44%)	13 (52%)
Total	25 (100%)	25 (100%)	25 (100%)

Table 1. Shows the gender distributions of the HIV-Infected patients on ART and the healthy controls.

Table 2. The Mean Age of the HIV-Infected Patients on ART and Control Subjects.

	Number of subjects (n)	Mean \pm Standard deviation	Standard error
Age of the Control Subjects	25	30.56 ± 11.04	2.208
Age of HIV-Patients with CD4 count of 100-200 cells/uL	25	38.24 ± 11.48	2.297
Age of HIV-Patients with CD4 count ≥ 400 cells/uL	25	35.80 ± 11.64	2.328

Table 2. Shows the mean age of the HIV-Infected patients on ART and the healthy controls.

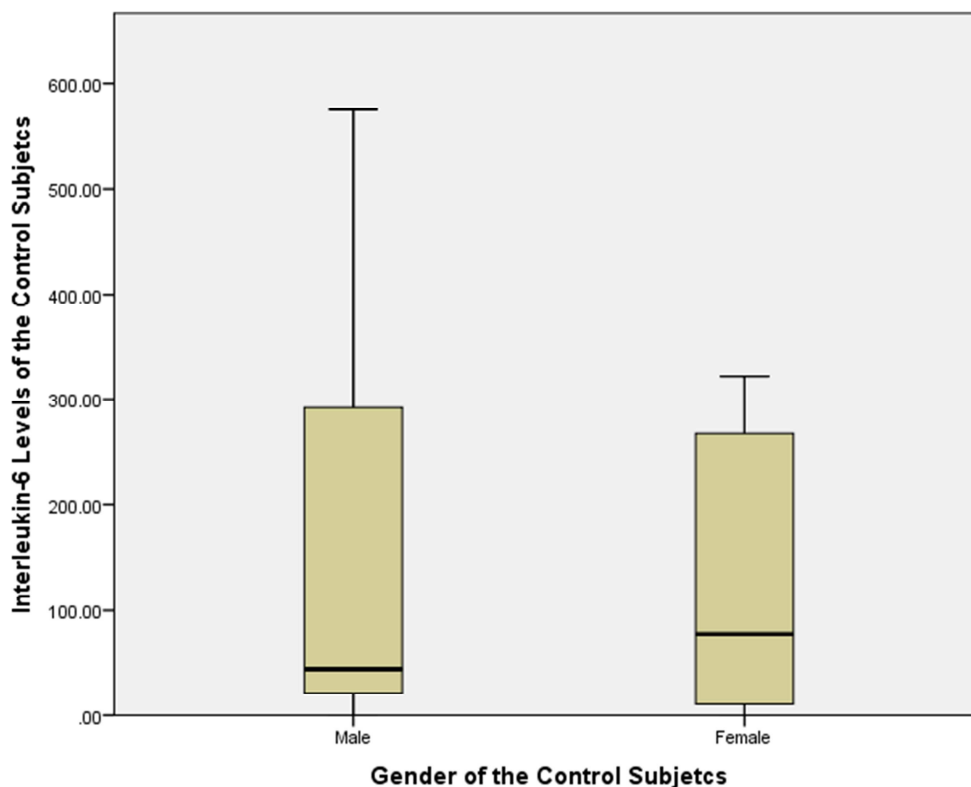


Figure 2. A box plot showing the plasma Interleukin-6 concentration according to gender healthy controls. There is a significant difference in the female subjects higher than in males.

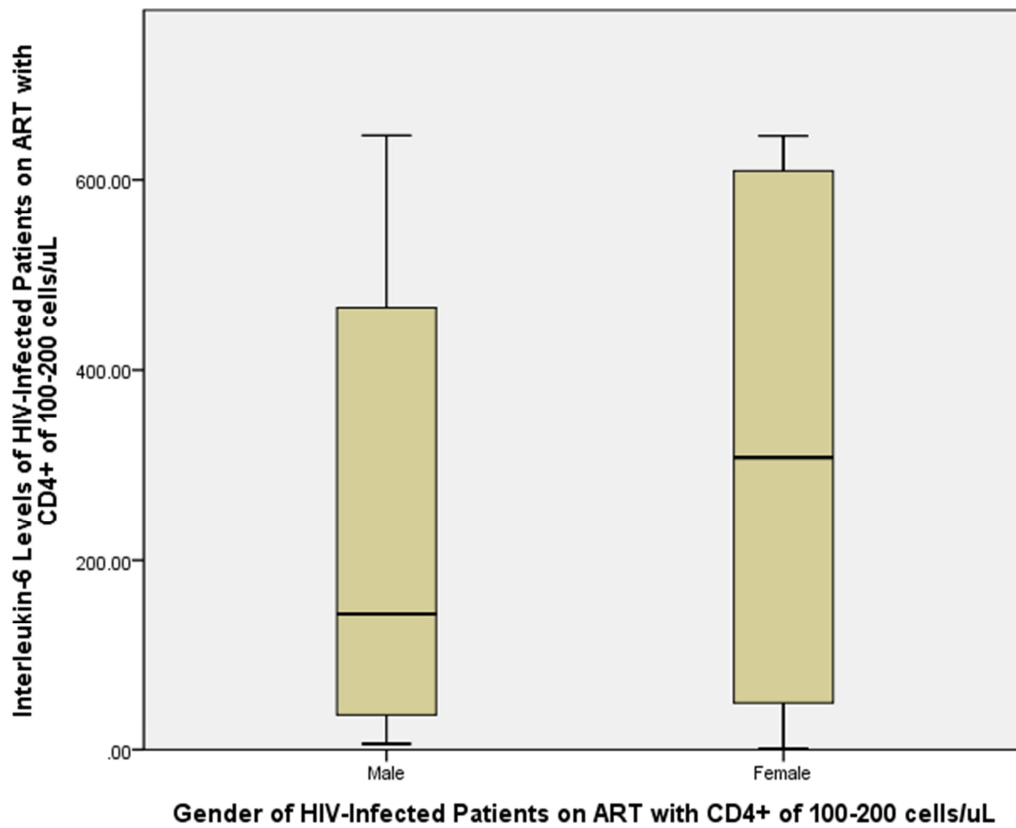


Figure 3. A box plot showing the plasma Interleukin-6 concentration in HIV-Infected patients on ART with CD4⁺ count of 100 – 200 cells/uL according to gender. The Interleukin-6 concentration is significantly higher in female than in males.

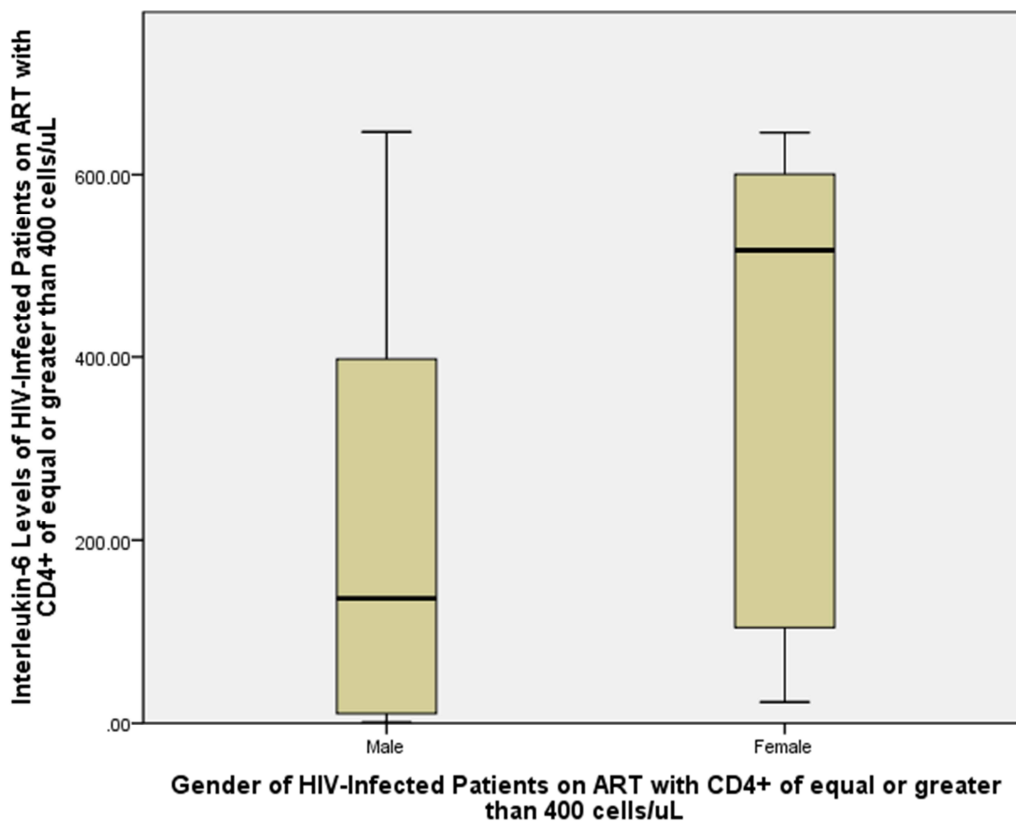


Figure 4. A box plot showing the plasma Interleukin-6 concentration in HIV-Infected patients on ART with CD4⁺ count of ≥ 400 cells/uL according to gender. The Interleukin-6 concentration is significantly higher in female than in males.

Table 3. Mean Interleukin-6 Concentration in HIV-Infected Patients on ART and Control Subjects in relation to CD4⁺ Cell count.

	Control Subjects (Mean±SD)	Patients with 100 – 200 cells/ul (Mean±SD)	Level of Significance	Patients with ≥400 cells/ul (Mean±SD)	Level of Significance
Interleukin-6 (IL-6) Concentration (pg/ml)	145.98 ± 156.36	272.93 ± 250.84	0.028	301.99 ± 261.23	0.021

Table 3. Shows the mean plasma IL-6 cytokine concentration in HIV-Infected patients on ART in relation to CD4 count.

4. Discussion

The result of this study demonstrates the utility of IL-6 cytokine in the evaluation of HIV-infected patients on ART in correlation with CD4⁺ cell count as compared in apparently healthy controls which falls within standard range of 6.5 - 200 pg/ml. Further studies will need to correlate individual CD⁺ count with IL-6 cytokine levels. This study results correlates with Breen *et al.*, (1990) where chronic HIV infection is associated with overproduction of proinflammatory cytokines including IL-6 which may also play a role in the development of the inflammatory response observed at clinical presentation of immune restoration disease IRD and may provide a marker of persistent immune activation.

Antiretroviral therapy (ART) entails treatment with a combination of two nucleoside reverse transcriptase inhibitors and a potent protease or non-nucleoside reverse transcriptase inhibitors, and has been taken as the gold standard for management of HIV patients [23]. Studies have shown that, most patients who just start the ART, and some of those in other treatment stages, and those with Tuberculosis/ Mycobacterium infection and Hepatitis B *e.t.c.*, mostly have low CD4 counts and battles with IRD.

Maini (1996) reported that CD4 count of 1- 200 cells /ul level higher in females than males in healthy subjects (1996) which is also revealed in this study (Figure 2 – 4). It is interesting to note that it is the same in HIV-Infected patients on ART as observed in this study. However, the higher the CD4 count, the higher the IL-6 cytokine levels.

This study population falls in a bracket adult age (18-65 years), age was insignificantly related to CD4⁺ cell counts and IL-6 cytokine levels. It has been reported by Tuomola *et al.* (1997) that CD4⁺ cell count is highest during the early years of life; it declines steadily to a stable adult value s and its lowest in the elderly. The IL-6 level is generally higher in females than males (Figure 2 – 4). Regarding the higher proinflammation in females, studies have shown that sexes differ in the intensity, prevalence, and pathogenesis of infections caused by viruses, bacteria, parasites, and fungi. Males and females of species ranging from humans to horses and rodents differ in their responses to and the outcome of diverse pathogenic infections [26, 27]. For each infectious diseases, there are numerous and diverse ways in which sex and gender can impact differential susceptibility between males and females.

Males and females differ in their innate immune responses, suggesting that some sex differences are germ line encoded. Innate detection of nucleic acids by pattern recognition

receptors (PRRs) differs between sexes [28]. Females tend to generate stronger innate and adaptive immune response than males [29, 30], which may raise discrimination in IL-6 levels in females. High IL-6 levels may result in an increased risk of functional disability and functional decline [31]. Further studies will be required to know if IL-6 cytokine levels in HIV-Infected patients on ART can be used as a substitute parameter in place of CD4 counts in their management. This will enhance patient care in developing countries as IL-6 cytokine test is less costly and required lesser high-technology to perform.

5. Conclusion

This study has shown that in HIV-Infected patients on ART, the IL-6 cytokine increases as the CD4⁺ cells count increases. In addition, female patients generally have a higher CD4⁺ cell count than males with corresponding increase in IL-6 cytokine.

Recommendations

Deductions from the result of this study require the need to routinely monitor CD4⁺ cell count and IL-6 cytokine concentrations in HIV-Infected patients on ART. There is need to conduct a similar study, but inclusive of confirmed HIV-Infected patients with confirmed Mycobacterium Tuberculosis infection, HBsAg, Hepatitis C on ART for comparison and CD4 counts and IL-6 cytokine levels for comparison and correlation for treatment application and mathematical reference.

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