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Comparative Study of Immune Activation and Inflammatory Markers IL-6 and IL-4 in HIV Patients

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Abstract: ***HIV Human Immunodeficiency Virus significantly impacts the immune system, leading to AIDS Acquired Immune Deficiency Syndrome. This study evaluates the levels of inflammatory markers IL-6 and IL-4 in HIV patients. It was an observational case-control study conducted at the Department of Biochemistry and General Medicine ART Unit Sardar Patel Medical College Bikaner, Rajasthan. Fifty HIV patients and fifty healthy controls were included. The study analyzed serum interleukins IL-6 and IL-4. Results showed significantly higher levels of IL-6 and IL-4 in newly diagnosed HIV ART-nave patients compared to follow-up patients after one year of ART treatment and healthy subjects. The findings indicate that IL-6 and IL-4 levels could be used in combination with CD4 counts to monitor disease progression and treatment efficacy.***

Keywords: HIV, Interleukins, Inflammatory Markers, IL-6, IL-4

1. Introduction

HIV infection gradually weaken immune system by invading and destroying CD4+-cells. Being a causative agent, it weakens the immunes system, leadings to AIDS (Acquired Immune Deficiency Syndrome).1 In Rajasthan prevalence of adult HIV and individuals living with HIV infection as calculated by NACO and Ministry of Health and Family Welfare and Indian Government in year 2021 was 0.10% and 67, 186 people respectively.2

Both type of immune response, innate response and adaptive responses are elevated at the time of HIV infection. $CD4^+$ cells plays an essential role in developing immunity[3] and after triggered by infection due to HIV, they started secreting cytokines, [4] which regulates the immune responses.3

Cytokines can be categorized as either pro-inflammatory or anti-inflammatory, and believed to play a crucial role as initiators and mediators of infection generated inflammation. An HIV infection led to the secretion of cytokines by infected cells as well as cells participates in generating immune response. Such secreted cytokines regulate the immunological function and affects viral replication.3

Being inflammatory cytokines, IL-6 (pro-inflammatory) and IL-4 (anti-inflammatory) are used as biomarker. IL-6 functions as growth factor for B-cell and CD4 T-cell proliferation. In course of acute infection in newly diagnosed HIV+ patients, proportionately increased secretion of IL-6 occurred, it helps in activation of T-cells, B-cells and release of other hormones in association with other cytokines. This type of response by IL-6 during acute infection is very helpful to fight against virus. However, continuous production of IL-6 may enfeeble the immunity for the long-term. It is because, elevated level of IL-6 may lead to premature destruction of cells of immunity, and made susceptible to other non-AIDS defining events.5 IL-4 helps in regulation of two most important receptors (CCR5 and CXCR4) of HIV by upregulating CXCR4 expression and downregulating CCR5 receptor expression in CD4+ T-cells. In addition to this, IL-4 also stimulated HIV expression by mechanism of transcription activation. As results IL-4 act as a key regulator of HIV infection and plays a crucial role in controlling viral evolution.6

Alterations in level of cytokine during HIV infection can affects the different functions performed by immune system that directly controlling the replication and progression of virus.3 Antiretroviral therapy (ART) is capable of controlling viral load (VL), but later on a long-term effective treatment results in low grade chronic inflammation and activation of immune system which is coupled with a number of life-threatening non-AIDS defining events[7]. As a consequence of continuous low-grade inflammation and activated immune system, a level of inflammatory biomarkers generally rises significantly.8

The role played by pro-inflammatory and anti-inflammatory cytokines in prognosis and pathophysiology of infection due to HIV is not yet well understood. Evaluating cytokines levels in serum can depict the rate of viral replication and the extent of damage or recovery to immune system, especially during ART.

2. Material and Methods

Total 100 age and sex matched subjects were chosen for the study. Both patient and control group provided written consent for participation and information confidentiality was

guaranteed. Two groups were created from study participants. **HIV+ patients:** Total 50 newly diagnosed HIV+ patients having no pre-existing AIDS symptoms and had no previous exposure of ART were taken in this group. **Healthy Subjects:** Contained 50 healthy HIV seronegative individuals. **Inclusion Criteria:** Newly diagnosed, clinically confirmed seropositive patients aged above 18 years. **Exclusion Criteria:** Below 18 years, patients having pre-existing symptoms of AIDS as stated by WHO at the start of ART therapy, having previous exposure to ART**,** having co-morbidities like diabetes mellitus, secondary infections other than HIV, inflammatory conditions, diarrhea, rheumatoid arthritis, cancer, etc., having obesity, allergic reactions or pregnancy etc, and patients who were on drugs that are known to affect concentration of IL-6, and IL-4.

A detailed history of patients and controls, including age, gender, education, occupation, monthly income, residential area, mode of transmission of viral infection, weight, type of addiction smoking, tobacco chewing, alcohol, etc., socio-economic status decided by the modified Kuppuswamy Scale, and marital status, was collected. Sample from patients and controls were collected after taking an informed consent. Interleukin-6, Interleukin-4 were measured.

Sampling Procedure

10 ml of blood sample were drawn by puncturing vein from both HIV+ and healthy individuals under general aseptic precautions. After collecting blood samples of blood were aliquoted into EDTA vial for measuring CD4 count, plain gel vial (to minimize risk of hemolysis) for serum separation to measure cytokines (Il-6 and IL-4). After centrifugation serum was separated and transferred to aliquots with proper identification number and stored at ≤-20 °C. At time of measurement of parameters, frozen sample was thawed at room temperature. And, estimation was done. And, IL-6 and IL-4 were estimated using Merilyzer | EIAQuant™ Elisa Reader.

Statistical Analysis

The mean values of different parameters in various studied groups were compared and analysed using appropriate statistical methods. Pearson correlation were employed in analysing the relationship between the cytokines and other parameters. Receiver operating characteristic curve were done to calculate cut-off values. Kappa coefficient of Agreement was used for measuring the degree of agreement among variables. Chi square test is used for qualitative variables. SPSS statistical software (version 24.0) was used for data analysis. Significance were considered at $P < 0.05$.

3. Observation

The average age of HIV+ patients was 35.34 years and of healthy subjects was 34.48 years Overall male preponderance was present in both groups. Most common mode of infection transmission was sexual method (92%) followed by 8% through injection. According to modified Kuppuswamy Scale majority of patients and healthy subjects from lower and upper lower class followed by lower middle class (Table: 1).

Table 1: Demographic Details of HIV+ patients and Healthy subjects.

	HIV+ Patients (N=50)	Healthy Subjects (N=50)	P-value
Age			
(Mean±SD)	35.34±10.36	34.48±9.46	0.6656
Gender			
Male	38 (76%)	43 (86%)	0.2024
Female	12 (24%)	7 (14%)	
Probable Mode of Transmission			
Sexual	46 (92%)	- -	- -
Injection	4 (8%)	- -	
Socio economic scale			
Lower	7 (14%)	6 (12%)	0.2263
Upper Lower	34 (68%)	27 (54%)	
Lower Middle	8 (16%)	12 (24%)	
Upper Middle	1 (2%)	5 (10%)	

We observed that mean CD4+ count in HIV+ patients before initiation of ART was 178.32±83.93 cell/µl and after 1-year ART was 293.12±102.24 cell/µl. This rise in CD4+ count in HIV+ patients after 1-year ART was statistically significant as compared to CD4+ count before initiation of therapy (p-value <0.0001). The mean IL-4 and IL-6 in HIV+ ART naïve patients was 10.91 pg/ml and 13.71pg/ml respectively followed by after 1-year therapy mean IL-4 and IL-6 level becomes 2.45 pg/ml and 4.53 pg/ml respectively. In healthy subject group mean IL-4 and IL-6 level was 1.97 pg/ml and 3.47 pg/ml respectively. Here, we found a statistically significant difference in level of IL-4 and IL-6 before and after therapy compared to each other and with healthy subjects (p-value <0.0001) (Table: 2).

Table 2: comparison of IL-4 and IL-6 amongst HIV+ ART naïve, with 1-year ART and healthy subjects

	IL-6 (pg/ml)	IL-4 (pg/ml)	CD4+ count cell/µl
HIV+ART naïve (a)	13.71±4.27	10.91±2.96	178.32±83.93
HIV+ with 1-year ART (b)	4.53±1.82	2.45±1.54	293.12±102.24
Healthy Subjects (c)	3.47±1.33	1.97±0.31	- -
P-value			
(a) Vs (b)	<0.0001	<0.0001	<0.0001
(a) Vs (c)	<0.0001	<0.0001	- -
(b) Vs (c)	0.0012	0.032	- -

A Pearson correlation of IL-6 and IL-4 with CD4+ count in newly diagnosed patients and after follow-up was calculated. We found that in HIV+ ART naïve and after 1-year ART IL-6 and IL-4 shows an inverse correlation with CD4+ count (r=-0.583, r=-0.519;-0.510,-0.498 respectively). And, IL-6 shows direct correlation with IL-4 before and after (r=0.598; r=0.517) (Table: 3)

Table 3: Pearson Correlation between IL-6, IL-4 ADA and CD4+ Count in HIV+ ART naïve and 1-year follow-up patients.

Pearson Correlation		CD4+	IL-4
IL-6	HIV+ ART naïve	- 0.583	0.598
	HIV+ 1-year therapy	- 0.519	0.517
IL-4	HIV+ ART naïve	- 0.510	
	HIV+ 1-year therapy	- 0.498	

The ROC curve at 95 CI was utilized to evaluate the accuracy of IL-6 and IL-4 and to determine cut-off points above which IL-6 and IL-4 shows further weakening of immune system. The cut-off value for IL-6 and IL-4 in HIV+ ART naïve patients is 11.3 pg/ml at AUC 0.89 and 9.2 pg/ml at AUC 0.824. And, At this cut-off in HIV+ ART naïve patients, IL-6 and IL-4 was found to be powerful predictor of CD4+ cell count ≤200 cell/µl with sensitivity of 82.35%,; 85.29% specificity 75%; 68.75%%, and accuracy 80%; 80% respectively. After 1-year ART the cut-off value for IL-6 and IL-4 is 6.75 pg/ml at AUC 0.92 and 3.36 pg/ml at AUC 0.909. And, At this cut-off in HIV+ patients after 1-year follow up, IL-6 and IL-4 was found to be powerful predictor of CD4+ cell count ≤200 cell/µl with sensitivity of 71.4%,; 85.71% specificity 90.7; 83.72%, and accuracy 88%; 84% respectively (**Table: 4, Fig: 1**).

Table 4: Receiver operative curve analysis for evaluating Prognostic accuracy of IL-6 and IL-4 against CD4+ count

	IL6		IL-4	
	ART naïve	With 1 – year ART	ART naïve	With 1 – year ART
AUC	0.89	0.92	0.824	0.909
p-value	<0.0001	0.002	<0.0001	0.003
Cut off	11.3 pg/ml	6.75 pg/ml	9.2 pg/ml	3.36 pg/ml
Sensitivity	82.35%	71.4%	85.29%	85.71%
Specificity	75%	90.7%	68.75%	83.72%
PPV	87.5%	55.56%	85.29%	46.15%
NPV	66.67%	95.12%	68.75%	97.3%
Accuracy	80%	88%	80%	84%

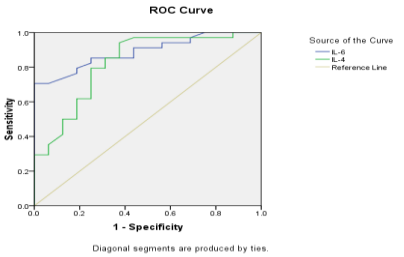

Figure 1a: Receiver operative curve analysis for evaluating Prognostic accuracy of IL-6 and IL-4 against CD4+ count in HIV+ ART naïve patients

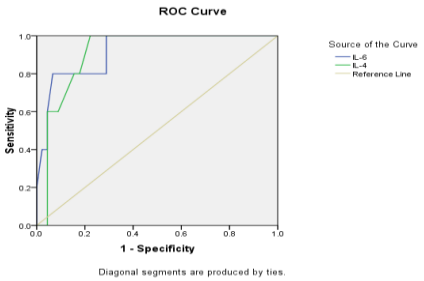

Figure 1b: Receiver operative curve analysis for evaluating Prognostic accuracy of IL-6 and IL-4 against CD4+ count in HIV+ with 1-year ART patients.

The Kappa coefficient of agreement between CD4+ count and IL-6 and IL-4 before start of ART is 0.555 and 0.540 respectively which shows a moderate agreement between the two tests. And, after 1-year ART treatment is found 0.703 and 0.438 which shows a substantial agreement and fair agreement between the two tests (Table: 5).

Table 5: Quantify agreement with kappa results

		Kappa Coefficient (95 CI)	
		IL-6	IL-4
CD4+ Count	Before	0.555 (0.312-0.797)	0.540 (0.290-0.790
	After	0.703 (0.425 to 0.916)	0.438 (0.167 to 0.709)

4. Discussion

Cytokines are the important moderator of the working of the immunity and therefore function as specific markers of response an antigen induces in the host.[9]

Here, we assessed the inflammatory response of cytokine (IL-4 and IL-6) in HIV positive patients with a categorization as ART naïve patients and patients who had an ART therapy from last 1-year. Additionally, these were compared with, healthy subjects. This longitudinal assessment revealed that serum pro-inflammatory cytokines interleukin-6 and anti-inflammatory cytokines interleukin-4 level was markedly high in newly diagnosed HIV+ ART naïve patients compared to follow-up patients who had taken 1-year ART treatment ($p < 0.0001$) and from healthy subjects also ($p < 0.0001$). Along with this, when level of IL-6 and IL-4 in follow-up patients had 1-year therapy were compared to healthy subjects, a statistically significant deviation observed between follow-up patients and controls.

In our study the prevalence of HIV infection is very common among 3rd and 4th decade of life with male preponderance. **Bossonario et al[10]** in their study shows that a significant occurrence of HIV infection among youth or younger age group may be suggestive of multiple sexual relationships and partners. However, initiation of sexual activity at younger age and without the use of condoms during intercourse are some of the predisposing factors of HIV infection and increase vulnerability to infection. Another possible cause for high occurrence of infection among young generation is migration for work from one place to another. And, this preponderance of males over females may be due to the influence of the populations, medical seeking behaviors of female, gender bias and the extent of stigma.[11]

A significantly low CD4+ count in HIV+ patients before ART shows that CD4+ cells are the primary target of HIV. Concordance to this our study results shows significantly low CD4+ cells in HIV+ patients before ATR. And this level rises after ART. In **Essien-Baidoo S et al[12]** study mean baseline CD4 counts and after 1-year ART treatment were 220.8 ± 10.83 cells/ mm3, and 592.3 ± 15.27 cells/mm3, respectively. **Musa et al[13]** reported that ART naive HIV+ patients had significantly lower CD4+ T-cell counts in comparison to healthy controls ($p>0.01$). **Shebl et al[14]** reported that $CD4^+$ count significantly declined in infection due to HIV. CD4+ T-cells plays an important role in coordinating both type of cellular immunity and humoral immunity responses against

foreign invaders and are maintained at constant levels in the human body through homeostatic mechanisms. On the surface of T-helper cells HIV binds with CD4 molecule and its replication occurs within them, resulting in the death of these T-helper cells and a gradual decline in their numbers.[15]

Here, we found that level of IL-6and IL-4 was significantly high in ART naïve patients compared to their level after 1-year and healthy subjects. We also found that level of IL-6 and IL-4 in HIV+ patients after 1-year ART was also high compared to healthy subjects. It is observed that induction of IL-6 occurs instantly during early stages of infection in monocytes/macrophages. Thus, expression of IL-6 is increases subsequently.1[6] **Velazquez et al**[17] and **Rose-John**[18] **S** study results also favored that rise in IL-6 may be supported by the facts that, it is a pleotropic cytokine which is produced in reciprocation to inflammation, damage or infection in a tissue. Biological function of IL-6 constitutes regeneration and activation of immune system.1[9] **Nosik et al**[20] study revealed that concentration of IL-6 in serum of ART naïve HIV+ patients with mono-infection was significantly high compared to healthy controls. They also reported that serum concentration of IL-6 gradually decreases in HIV+ patients which were on ART. This decrease in concentration of IL-6 is comparable to concentration in healthy subjects. **Conesa-Buendía et al**[21] study observed a significant reduction in IL4 in patients who had taken treatment for 12 months when compared to baseline. **Szymanska et al**[22] reported that concentration of IL-4 in plasma of HIV+ patients before start of initiation of ART was found to be twice as much as in healthy subjects. **Osuji et al**[9] assessed the serum concentration of anti-inflammatory cytokines in HIV+ patients. They found statically significant difference in level of interleukin-4 before start of ART therapy, after 6 months and after 1-year of therapy. But, they found non-significant difference in level of HIV+ patients after 1-year therapy with respect to controls.

An inverse correlation of IL-6 and IL-4 with CD4+ cells was found in our study. In concordance with our results **Musa et al**[13] observed that IL-4, and IL-6 correlated inversely with CD4 T cell count ($P<0.0001$). **Keating et al**[23] reported that during HIV infection, high levels of inflammatory cytokines are linked with elevated viraemia and reduced CD4+ T-lymphocyte counts. **Iketleng et al**[24] suggest that cytokines have been considered to be a better marker for CD4+ T-cell declined during infection. **Lu et al**[25] reported that serum levels of the pro-inflammatory cytokines were elevated in the CD4 count < 200 cells/μL group compared to CD4 count > 200 cells/μL group.

The results in our study shows that prognostic value of IL-6 and IL-4 against CD4+ count in ART naïve patients was maximum (AUC=0.89 and 0.824). And, in patients after 1-year ART was maximum (AUC=0.92 and 0.909). We also calculated Kappa coefficient agreement among CD4+ count and IL-6 and IL-4 before and after 1-year ART treatment. Kappa coefficient value of 0.555; 0.703 and 0.540; 0.438 shows a moderate, substantial agreement and moderate, fair agreement between two tests respectively. This means that we may depend on IL-6 and IL-4 for the prognosing the effect of ART therapy on HIV positive patients.

5. Conclusion

Infection with HIV is associated with immune system activation and altered cytokine production. Antiretroviral therapy increases CD4 cell counts and decreases the levels of cytokines such as IL-4 and IL-6. Our study found significantly higher levels of IL-6 and IL-4 in ART-nave HIV patients compared to those on ART for one year and healthy controls. This study highlights the importance of IL-6 and IL-4 as biomarkers for monitoring the immune response in HIV patients. The findings suggest that these markers, alongside CD4 counts, can provide valuable insights into the effectiveness of ART treatment and the progression of the disease.

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