Original Article

Evaluation of Pro-inflammatory and Coagulation Biomarkers among PLHIV on Highly Active Antiretroviral Therapy with Effect of Viral Suppression on them

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Abstract

Background : HIV infection considered Chronic Inflammatory Disease carries risk of Cardiovascular Disease (CVD) events and cancer not related to AIDS, even after initiation of Antiretroviral Therapy (ART) due to increased levels of highly sensitive C-reactive Protein (hs-CRP), Interleukin-6 (IL-6) and D-dimers persisting due to various causes.

Aims and Objectives: Present study aimed at evaluating these biomarkers, both baseline and after ART initiation, with effect of viral suppression and immunological status upon them.

Materials and Methods : Prospective observational study was conducted on newly diagnosed HIV positive patients started on ART. Measurement of IL-6, hs-CRP and D-dimer by immuno-enzymatic assay was carried out both at start of therapy and after six months of ART. CD4+ cell count was measured by Fluorescent Activated Cell Sorter (FACS) method. Frequency distribution, paired T-test and cross tabulation was used to create summary tables and compare items.

Results : Mean value of hs-CRP and D-dimer was 19.06 mg/ l and 728.03 ng/ ml before ART which decreased to 16.09 mg/ l and 554.09 ng/ ml after ART initiation respectively. Mean value of biomarkers in patients on ART with HIV viral load as Target Not Detected (TND) and higher CD4+ cell count was less, compared with those having higher viral load count of <150 copies/ ml and it was statistically significant. Majority (69.14%) of the study participants had HIV -1 viral load as TND after six months of ART initiation.

Interpretation and Conclusion : Timely and proper ART initiation is instrumental in decreasing overall mortality and morbidity. Development of risk stratification system involving these biomarkers will further help predict beforehand and quantify any undetected clinical risk in PLHIV.

Key words : ART, CD4+, CVD, D-dimer, hs-CRP, IL-6, PLHIV.

HIV is now considered a Chronic Inflammatory Disease, rather than a fatal one, in countries where ART is available. It results in chronic immune activation causing rise of pro-inflammatory cytokines such as Interleukin-6 (IL-6) and high sensitivity C-reactive Protein (hs-CRP). Coagulation biomarkers like Tumor Necrosis Factor (TNF) may also be elevated leading to development of a hypercoagulable state due to rise in levels of factor VIII and decreased levels of protein S. These cytokines also down regulate the expression of several proteins which are required for fibrinolysis. HIV replication has been shown to be a major factor causing up-regulation of coagulation pathways along with inflammatory and thrombotic activity for Cardiovascular Disease (CVD) risk[1]. Chronic inflammation among PLHIV may be as a result of activation of dendritic cells and lymphocytes, injury to endothelial lining and mucosal barrier, along with other factors related to HIV replication[1,2].

Editor's Comment :

- HIV infection has a tendency to increase various inflammatory bio-markers.
- Timely ART initiation with its proper compliance leads to lower levels of these bio-markers and drastically reduces HIV viral load causing overall reduction in mortality and morbidity among PLHIV.

Currently available ART drugs cannot eradicate HIV virus from the human body because a pool of latently infected CD4+ (cluster of differentiation 4) cells is established during the earliest stages of acute HIV infection. It persists within the organs/ cells and fluids (eg, Brain, Liver and Lymphoid Tissues) despite prolonged suppression of plasma viraemia by ART to <50 copies/ml. The primary goal of ART is maximal and sustained reduction of plasma viral levels and restoration of immunological functions. This reduction in viral load also leads to an overall reduced transmissibility thereby reducing number of new infections[3].

ART has been found to be associated with lower levels of these biomarkers, so interrupting or delaying ART because of unpleasant side effects or they becoming ineffective over time, further increases the risk of non-AIDS related illnesses like neurocognitive disorders, CVD, Metabolic

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Syndrome, Bone abnormalities and non-HIV associated cancers (in particular, Kaposi sarcoma) in the long term[3-5].

Enormous literature is available Worldwide regarding evaluation of various biomarkers among PLHIV but currently there is lack of sufficient Indian literature, emphasizing the importance of various inflammatory and coagulation biomarkers like D- dimer, hs-CRP and IL-6 in assessing progress of HIV infection to chronic inflammatory stage and various other accompanying diverse clinical associations. Present study conducted in PGIMS Rohtak was aimed at evaluating these biomarkers among PLHIV and their response to ART initiation, along with effect of viral suppression and immuno-logical status upon these biomarkers.

Material and Methods

Study Setting and Ethical Approval : With prior permission from Institutional Biomedical Research Ethics Committee (BREC) vide their letter no. BREC/19/TH/Micro-02 dated 26.12.2019, present study was carried out in Department of Microbiology, over a period of one year from January, 2020 to February, 2021.

Study Design : Prospective observational study was conducted on newly diagnosed HIV patients started on ART.

Study Population : Total of 35 pre-diagnosed HIV patients enlisted from ART Center of Medicine Department were enrolled for this study after obtaining written informed consent from them prior to sample collection.

Inclusion Criteria : Patients of either sex and above 18 years of age with pre- diagnosed HIV infection.

Exclusion Criteria :

(i) Refusal to participate / not providing consent.

(ii) Pregnant and lactating females.

(iii) Patients with pre-diagnosed malignancy, history of Tuberculosis, previous history of CVD, Myocardial Infarction, Coronary Artery Disease, Stroke, Peripheral Vascular Disease, or Congestive Heart Failure.

(iv) History of previous ART or refusal to start ART

Sample size : Required for study was calculated as:

$$N = (Z_{1-\alpha/2} + Z_{1-\beta})^2 / \Delta^2$$

where n= sample size required. $Z_{1-\alpha/2}$ is level of significance at 95% confidence interval = 1.96. $Z_{1-\beta}$ is assuming 80% power of the study = 0.84. $\Delta^2 = 0.5$.

Now, $N = (1.96+0.84)^2 / (0.5)^2 = 8/0.25 = 32$.

Considering loss to follow-up and dropout rate of 10%, the required sample size came out to be 32. Hence, minimum of 35 subjects were registered for this study.

Sampling Technique :

(i) Blood was collected by venipuncture using all aseptic precautions and serum/ plasma separation was done.

(ii) Specimens were stored at -20°C. Repeated freezing and thawing of samples was avoided[6].

Test Methodology : Demographic profile of the enrolled cases was assessed and subjected to detailed physical and clinical examination along with routine investigations. CD4+ cell count, D-dimer, IL-6 and hs-CRP were measured both at start of therapy and after six months of ART. HIV-RNA was measured only after six months of ART according to the National AIDS Control Organization (NACO) guidelines[7].

Measurement of Proinflammatory and Coagulation Biomarkers : All serum samples were processed for measurement of D-dimer, IL-6, and hs-CRP. IL-6 levels were measured by immuno-enzymatic assay following manufacturer's instructions of DIAsource® IL-6 ELISA kit (DIAsource® Louvain-la-Neuve, Belgium) based on monoclonal antibodies directed against distinct epitopes of IL-6. Testing for hs-CRP was done by DIAsource® hs-CRP ELISA kit (DIAsource® Louvain-la-Neuve, Belgium) based on monoclonal antibodies directed specific for different regions of CRP following a typical two-step capture or sandwich type ELISA. D-dimer was detected by using kit based on sandwich ELISA for the quantitative determination of D-dimer in plasma based on monoclonal antibodies. (Kit- Technozym® D-dimer ELISA, Technoclone Herstellung von Diagnostica, Vienna, Austria).

Measurement of CD4+ Count : One ml of blood was collected in EDTA vacutainer and was processed by Fluorescent Activated Cell Sorter method as per kit instructions (Kit- Sysmex CD4% easycount, Equipment – Sysmex Partec GmbH CyFlow Counter, Kobe, Japan).

Measurement of HIV RNA Levels : Five ml of blood sample has to be collected in EDTA vacutainers and plasma was separated. HIV RNA levels were measured by quantitative Polymerase Chain Reaction (PCR) (Kit - Abbott Real Time HIV-1 reagents, Equipment's- Automated m2000sp system and Abbott m2000rt, Libertyville Township, IL, USA). The amplification cycle at which a specific fluorescent signal was detected was proportional to the amount of HIV-1 RNA present in the original sample. The assay had a lower limit of detection of around 80- 150 HIV-1 RNA copies/ ml as being reported as <150 copies/ml for 0.2 ml volume of plasma sample. Specificity of test was 99.28 to 100 %.

Statistical Analysis : Data was entered into Microsoft Excel and further statistical analysis was done using Statistical Package for Social Sciences (SPSS), version 20. Frequency distribution, paired T-test and cross tabulation was used to create summary tables and

compare items within and across categories. The quantitative variables in patients were expressed as mean ± SD and compared using paired t-test. The association was tested using categorical data and analyzed using chi-square test. p value of <0.05 was considered statistical significant.

Disposal of Waste : All biomedical waste generated in the laboratory in this study was discarded as per the Biomedical Waste Management and Handling Rules, 2016 guidelines.

Results

Demographic Details of Cases : Total of 35 pre-diagnosed HIV patients were enrolled, who were willing to be part of study and start ART. Out of these 35 participants, 27 (77.1%) were male and rest 8 (22.9%) were female. Their age range was 18-59 years of age with mean age of 36 years. The youngest among study population was 22 years of age while oldest was of 53 years of age. Majority of the patients were married (62.9 %) with literacy level of secondary schooling and above (51.4%) (Table 1).

Mean value of hs-CRP and IL-6 was higher in male participants as compared to females. However, the mean difference before and six months after ART initiation decreased in both genders. Mean value of D-dimer post ART initiation was higher in males as compared to females. In contrast, the mean value of CD4+ before and after six months of ART was higher among female patients as compared to males. The mean difference pre and post ART initiation also increased after six months implying that CD4+ cell count increased more in females as compared to males (Table 2).

The mean difference between hs- CRP value, D- dimer and CD4+ levels before and after six months of ART initiation was statistically significant (Table 3). Mean value of hs-CRP, IL-6, D-dimer in patients having higher HIV viral load of <150 copies/ ml was significantly higher than in those with HIV-1 viral load as TND. However, the CD4+ count in patients with viral load followed opposite pattern, in which patients with HIV viral load as TND had more CD4+ cells/ ml as compared to the group having higher viral load. The mean difference for all the parameters was statistically significant (Table 4).

Table 1 — *Demographic details of study Participants (n=35)*

Demographic factors	Frequency	Percentage
Gender :		
Male	27	77.10
Female	8	22.9
Age Group (in years) :		
18-29	12	34.3
30-39	7	20
40-49	12	34.3
50-59	4	11.4
Marital Status :		
Married	22	62.9
Unmarried	9	25.7
Divorced	1	2.9
Separated	2	5.7
Widow	1	2.8
Highest Education :		
Illiterate	5	14.3
Primary school	12	34.3
Secondary school	10	28.5
College and above	8	22.9
Risk Factors :		
Heterosexual	8	22.9
MSM	2	5.7
Injectable drug users/ unsafe injections	8	22.9
Blood Transfusions history	2	5.7
Unknown	7	20
Sex worker	2	5.7
Migrant labor	2	5.7
Trucker	4	11.4

MSM - men having sex with men

Discussion

In present study, value of inflammatory markers (hs-CRP and IL-6) was found above the normal range before the initiation of ART. Mean value of hs-CRP in participants being 19.06 mg/l before ART, which was above the normal

Table 2 — *Comparison of mean of pro-inflammatory biomarkers, coagulation biomarker and CD4+ cell count, before and after six months of ART between Male and Female participants*

Parameter	Gender	Mean
Hs-CRP (mg/l) Pre- ART	Male	19.59
	Female	17.25
Hs-CRP Post- ART	Male	16.28
	Female	15.44
IL-6 (pg/ml) Pre- ART	Male	95.11
	Female	63.38
IL-6 Post- ART	Male	93.67
	Female	69.25
D-dimer (ng/ml) Pre-ART	Male	724.81
	Female	738.88
D-dimer Post-ART	Male	558.19
	Female	540.25
CD4+ (cells/ ml) Pre- ART	Male	356.89
	Female	383.88
CD4+ Post- ART	Male	375.22
	Female	428.25

Hs-CRP, high sensitivity C-reactive protein; ART, antiretroviral therapy; IL-6, interleukin-6; CD4+, cluster of differentiation 4

Table 3 — *Association between mean value of pro-inflammatory biomarkers, coagulation biomarker and CD4+ cell count, before and after six months of ART in study participants as paired differences (Paired sample 't' test)*

Paired differences	Mean	P- value
Hs CRP (mg/l)Pre- ART - hs CRP Post ART	2.97	0.001*
IL-6 (pg/ml)Pre-ART - IL-6 Post ART	-0.23	0.946
D- dimer (ng/ml) Pre-ART - D- dimer Post ART	173.94	0.002*
CD4+ (cells/ml) Post ART- CD4+ Pre-ART.	24.29	0.043*

P≤0.05; Hs-CRP : High sensitivity C-reactive Protein; ART : Antiretroviral Therapy; IL-6 : Interleukin-6; CD4+ : Cluster of Differentiation 4

Table 4 — *Association between mean value of pro-inflammatory biomarkers, coagulation biomarker and CD4+ cell count Post ART with HIV-1 Viral Load in study participants (Independent sample t-test)*

Biomarker	Mean difference	p-value
hs-CRP (mg/l)	-15.51	0.001*
IL-6 (pg/ml)	-133.82	0.001*
D-dimer (ng/ml)	-841.78	0.001*
CD4+ (cells/ml)	212.70	0.007*

P≤0.05; Hs-CRP, high sensitivity C-reactive protein; ART, antiretroviral therapy; IL-6, interleukin-6; CD4+, cluster of differentiation 4

range of 0-3 mg/l as per laboratory range of expected normal values. The mean value came down to 16.09 mg/l after ART. The mean of hs-CRP among males (19.59 mg/l) was higher than females (17.25 mg/l) before ART initiation and after six months of treatment, mean value of hs-CRP among males (16.28 mg/l) was still higher than in females (15.4 mg/l). Overall there was reduction in hs-CRP after 6 months of ART. In our study, hs-CRP of four patients increased, six patients had constant values, while in 21 patients the value decreased after six months of ART. The mean value of IL-6 in all 35 participants was 87.86 pg/ ml at baseline which was above normal range of 0-45 pg/ml set by laboratory, as derived from serum samples of 30 apparently healthy persons.

The baseline mean value of D-dimer was 728.03 ng/ ml which decreased significantly to 554.09 ng/ ml after six months of ART, considering normal laboratory reference range of less than 250 ng/ml and this decrease was statistically significant (<0.005). Several studies have indicated that ART reduces the level of D-dimer in HIV patients[5,8-11]. Still potential benefits of ART in relation to decreasing inflammatory and coagulation biomarkers differ by specific class or drug, emphasizing the need for anti-inflammatory treatment apart from ART to achieve a greater success rate[12-14].

In present study, mean value of CD4+ count in 35 patients before ART was 363.06 cells/ml, which increased to 387.34 cells/ ml after ART. Similar findings were observed in various other studies[5,9,15]. In contrast late ART initiation, late diagnosis, poor body response to ART are among few factors causing poor CD4+ recovery as seen in some other studies[16-18].

Majority (69.14%) of the study participants had HIV-1 viral load as Target not Detected (TND) after six months of ART initiation, indicating that viral load suppression was appropriate in these patients. It shows importance of timely starting ART and properly adhering to it, along with monitoring of inflammatory bio-markers so as to get good results and that ultimate goal of HIV target not getting detected can be persistently achieved. Mogosetsi, *et al*[19], conducted a study on South African population and included 98 HIV infected patients. Viral load levels were measured after six months of ART. Total of 90 out of 98 patients achieved viral load suppression. The reason for high prevalence of viral suppression, could be due to high adherence to medication, age of the PLHIV patients, better healthcare delivery system and ART regimen followed.

The mean value of findings from some studies suggest that despite patients being on ART, some of them still have raised inflammatory biomarkers. The reason can be attributed to residual immune activation which can be due to prevailing low levels of HIV replication[14-18,20]. Factors like ART regimen used, ethnical variability, duration of treatment and underlying inflammatory changes could be another reason for this variability.

Conclusion

Overall the present study, suggests that timely and proper ART initiation is instrumental in decreasing both pro-inflammatory and coagulation biomarkers values in both genders. It shall help in building a risk stratification system or screening tool for PLHIV, thereby decreasing the overall morbidity and mortality among them. Also these bio markers can be utilized to monitor disease prognosis during ART particularly in resource poor setting where regular HIV-1 viral load monitoring facility is still unavailable.

Recommendation :

Since this study involved comparatively less number of subjects, it is also recommended that a cohort study using larger population with longer follow up be designed to help quantify any clinical event risk associated with fluctuations in these pro-inflammatory biomarkers overtime in PLHIV. Also, a risk stratification system should be developed for HIV patients, involving all these biomarkers, to reduce overall mortality and morbidity among PLHIV.

Limitations :

Present study had few limitations. Firstly, it was single centered study that included PLHIV who visited the hospital during study period. Secondly, patients were followed for maximum six months for the outcomes, limiting the scope to know more about changes in these biomarkers with accompanying quantification of clinical risk associated during the course of life in PLHIV. Also since this study was conducted the same time COVID-19 pandemic was reported, possibility of some baseline increase in inflammatory and coagulation biomarkers among study participants cannot be ruled out.

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Conflicts of Interest : None.

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