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ORIGINAL ARTICLE

Seroconversion of T helper cytokines and circulating antibody isotype directly correlate the amelioration of HIV infection during HAART therapy

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ABSTRACT
HIV infection disrupts and abrogates the overall immune system and thus makes the infected victims vulnerable to array of opportunistic infection and makes them succumb to very serious health conditions including death. One of the hallmarks of HIV infection is drastic declination of CD4 T cells (also known as T helper cells). One of the serial sabotages of reduction in T helper cell number is the skewed cytokine niche especially elevated Th-2 cytokine microenvironment in patients which can promote viral persistence and pathological consequences. Therapy with HAART had been shown to restore the AIDS by improving CD4 number however it is not known whether it was due to overall restoration of T cell number or by improving their function such as the secreting protective cytokines and/or improving the B cell function. Previous studies have reported that a preponderance of Th-1 cytokine niche promoted an IgG isotype, IgG2 and increased Th-2 cytokine microenvironment promoted IgG1 antibody isotype. The current study was aimed to study the cytokine status of HIV infected individuals prior to treatment and after. In addition to that our study also focused on the IgG isotye determination in their plasma before and after HAART treatment. To conduct these evaluations we registered 186 HIV positive individuals which consisted of 108 HIV patients who were treated for more than 2 years with HAART ($HAART^{Rx+}$) and 78 recently diagnosed patients who were about to be treated with HAART ($HAART^{Rx-}$). HIV positivity of the patients were confirmed for viral load by RT-PCR and their CD4 number were measured by flowcytometry. Plasma samples obtained were subjected for Th-1 cytokines (IFN γ and IL 2) and Th-2 cytokine (IL 4 and IL 10) by ELISA. Same plasma samples were used for estimation of IgG isotypes namely IgG1 and IgG2. This study revealed that HIV infected individuals ($HAART^{Rx-}$) had a non-protective type of cytokine, Th-2 as revealed a three-fold escalation of IL-4 and IL-10. Interestingly these patients had a predominance of IgG1 over IgG2 which support the notion that Th-2 cytokines promote IgG1 isotype antibody. Ironically but positively the $HAART^{Rx+}$ patients had the seroconversion from Th-2 to Th-1 which revealed by a seven to ten fold increase of IFN γ and IL 2. Convincingly these patients had a seroconversion from IgG2 suggesting that HIV infection skew the immune response to non-protective Th-2 response which was restored to protective Th-1 type by HAART therapy. Besides the improvement of T cell number we wanted to reiterate that HAART therapy ameliorate the function of T cells especially CD4 T cells.
KEYWORDS: *T helper cells, cytokines, HAART, antibody isotype, human immunodeficiency virus.*

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INTRODUCTION

Human immunodeficiency virus (HIV) is a positive sense RNA genome CD4 T cell tropic virus causes acquired immunodeficiency virus (AIDS), a sexually transmitting disease (STD) affecting the people for the past three decades [1]. Black days caused by HIV pandemic is unforgettable though an unprecedented pandemic caused by SARS-CoV-2 out shadowed all the other disease entities but it gave way for the human intelligence today [2]. As of now the global SARS-CoV-2 cases is dwindled down to negligible number thanks to corona vaccines, therapies and international humanitarianism[3]. Such a scenario is still

in the horizon for HIV because this virus had a death toll of 35 million people and about 40 million people are still infected with this virus. As per 2022 statistics of WHO, 1.3 million people globally newly acquire the disease and 630,000 deaths annually [4, 5]. In India alone there are 190 HIV new cases every day and 8 new infections every hour and 43000 deaths annually [6]. Anti HIV vaccines are still in infancy and none of the vaccines are approved by FDA yet. In contrary, anti HIV drugs advanced dramatically though no drug can offer a pristine cure. The confounding reason for the lack of vaccine or drug for HIV is the poor understanding of the HIV pathogenesis and the right time to intervene it During HIV infection there is a drastic declination of CD4 T cells which paves way for the array of opportunistic infections and death [7, 8]. Similarly Highly active antiretroviral therapy improves the CD4 number and eventually eliminating the circulating viruses however the intracellular viruses or proviruses making the situation vulnerable for viral reactivation [9, 10]. Research indicates a shift from Th1 to Th2 cytokines during HIV infection, with consequences such as decreased IL-2 and IFN-γ, and elevated IL-4 and IL-10 levels playing a role in the journey from HIV to AIDS [11, 12]. Human antibodies are essential for protecting against pathogens and diseases. Different antibody classes have specific roles, targeting various pathogens and activating immune cells [13]. The production of antibodies involves a dominant pathway, but switching between IgG subclasses follows distinct temporal paths influenced by cytokines and epigenetic factors [14]. Among the four IgG subclasses (IgG1, IgG2, IgG3, and IgG4), IgG1 and IgG3 are potent mediators of Fc and complement functions, especially for protein antigens, while IgG2 is less efficient, mainly responding to polysaccharide antigens[15]. In HIV infection, IgG1 is often the most prevalent subclass, showing high reactivity to antigens, followed by IgG3 and IgG2 [16]. Elevated IgG1 levels can stem from conditions promoting subclass switching, increased IgG1-specific cell frequencies, and high IgG1 secretion [17]. HAART involves a combination of at least three antiretroviral drugs like NRTI, NNRI, and INSTI, leading to significant clinical and immunological improvement by reducing viral load and increasing CD4+ T cell counts [18]. Although the precise effect of HAART on plasma cytokine levels remains unclear, previous research indicates that cytokine variations before HAART treatment may accelerate immunodeficiency progression, while successful HAART has been shown to elevate plasma IFN γ levels and decrease IL 10 levels [19]. The efficacy of HAART could be influenced by pro and anti-inflammatory cytokines, impacting immune regulation in HIV infection and, in turn, affecting IgG isotypes, which have implications for humoral response functions [20]. In this backdrop the current study was done to evaluate the status of immune system before and after HAART and examined the correlation with improvement of AIDS. We found that HIV patients prior to treatment (HAART $^{Rx-}$) had a doomed immune status i.e. preponderance of Th-2 cytokines namely IL-10 and IL-4 and IgG1 antibody isotype. On the contrary people subjected to anti-retroviral regimens (HAART^{Rx+}) were found to have a restored immune status i.e. overwhelming mighty powered Th-1 cytokines (IFN γ and IL 2) and IgG2 isotype. Though there is no correlation in terms of gender or age there was a distinct progress in CD4 number and viral load especially there was a positive correlation with Th-1 cytokine and IgG2 and reduced viral load and improved CD4 T cells among HAART^{Rx+} patients. An opposite immune status was observed among the treatment naïve patients. In this paper we categorically proved that upon infection with HIV it clearly poisonously stings on the immune system.

MATERIAL AND METHODS

Study Population:

This study was conducted at the Department of Microbiology, Dr. ALM Post Graduate Institute of Basic Medical Sciences, and University of Madras. A total of 236 study subjects from an antiretroviral therapy centre (ART centre) in Chennai, India were recruited for this study. The study population included 78 newly diagnosed HIV-positive HAART naïve patients (HAART $^{Rx-}$) that comprised the test group and 108 HIV-positive HAART experienced patients (HAART $^{Rx+}$) that served as the comparison group. The comparison group included patients who had been receiving HAART $^{Rx+}$ for over 2 years since diagnosis. 50 HIV seronegative healthy subjects served as the control group. This study was a comparative cross-sectional study and appropriate ethical clearance was obtained from the Institutional Human Ethical Committee (IHEC no: PGIBMS/Co/Tara/Human Ethical Com/Micro/12/150).

Sample collection:

Informed consent forms were obtained from all the subjects before sample collection and patient bio-data and medical history was collected using a questionnaire that. 5 ml of blood was drawn by venipuncture from all the subjects in the study. The blood samples were aliquoted in EDTA tubes for plasma cytokines and IgG1 and IgG2 antibody analysis while whole blood was used for the enumeration of CD4+ T cells.

CD4+ T cell count:
Whole blood was used for the enumeration of CD4+ T cells. 20µl of blood was incubated with CD4+ antibody and CD4+ T cell count was carried out by flow cytometry using Fluorescent Activated Cell Sorter (FACS count) system (BD™ Biosciences, USA) according to the manufacture's protocol [21].

Viral load measurement:
0.2 ml of plasma was used for HIV-1 RNA quantitation, viral load was determined by using the Abbott Real Time HIV-1 assay (Abbott Molecular Inc, USA). The HIV-1 quantitation standard was used to quantify the HIV RNA in each sample through reverse transcriptase amplification and detection. A range of 40 copies/ml (1.6 $\log_{10}$) to 10 million copies/ml (7.0 $\log_{10}$) was set as the viral load limit detection [22].

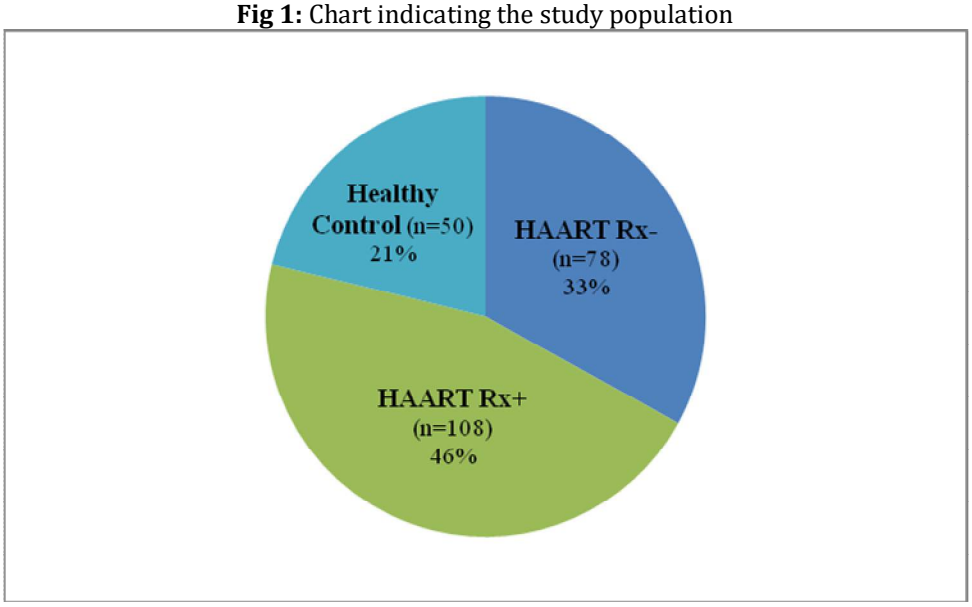
Quantification of cytokines:
Quantitative determination of cytokines in the plasma (Th1 subset: IFN γ and IL 2, Th2 subset: IL 4 and IL 10) was carried out by ELISA with ELISA MAX™ Deluxe Sets (Biolegend, USA) according to the manufacturer's instructions. 100µl of capture antibody specific to human IFN γ, IL 2, IL 4, and IL 10 was coated onto the 96-well microtitre plate and incubated overnight at 4˚C. Following incubation, the wells were blocked with 200µl of 1x Assay diluents for 1 h at RT (18˚C-25˚C). 100ul of plasma samples or standard was added to each well and incubated for 2 h at RT. Next, 100µl of detection antibody specific to human IFN γ, IL2, IL4, and IL10 was added to the wells followed by 1 h incubation at RT. Thereafter, 100µl of Avidin Horseradish peroxidase (HRP) was added and incubated for 30 mins at RT. The microtiter plate was washed and 100µl of TMB substrate solution was added and incubated in the dark for 30 min at RT. Lastly, stop solution was added to each well and the absorbance was read at 450 nm using a microplate reader (iMARK™ Microplate reader, Bio-Rad, USA). The lowest detection limit was set at 0.45ng/ml and plasma cytokine concentrations were calculated and expressed as pg/ml. The resulting data were analyzed using Graph Pad Prism version 8.0 for Windows (Graph Pad, CA, USA). Cytokine levels are presented as mean ± standard deviation. The difference in the cytokine concentrations between newly diagnosed HIV-positive patients HAART $^{Rx-}$ and HIV-positive patients HAART $^{Rx+}$ was analyzed using Student's *t-test* and significant levels were considered at $P < 0.05$.

Quantification of IgG1 and IgG2 antibody:
IgG1 and IgG2 antibody levels in patient plasma was quantified using ELISA with IgG1 human ELISA kit and IgG2 human ELISA kit (Invitrogen,USA - Cat No: BMS2092, BMS2093) in accordance to the manufacture's protocol. 80µl of 1x assay buffer was added to each well followed by 20µl of plasma samples or standard. 50µl of HRP conjugated antibody was added to each well and incubated for 1 hr at RT. Following incubation, the wells were washed and 100µl of TMB substrate solution was added and further incubated in the dark for 30 min at RT. Lastly, 100µl of stop solution was added to the wells and the absorbance was read at 450 nm using a microplate reader (iMARK™ Microplate reader, Bio-Rad, USA). The lowest detection limit was set at 0.32ng/ml for IgG1 and 0.25ng/ml for IgG2, the antibody levels were calculated and expressed as mg/ml. The resulting data were analyzed using Graph Pad Prism version 8.0 for Windows (Graph Pad, CA, USA) and the IgG1 and IgG2 antibody levels were presented as mean ± standard deviation. The difference in the antibody levels between newly diagnosed HIV-positive patients HAART $^{Rx-}$ and HIV-positive patients HAART $^{Rx+}$ was analyzed using Student's *t-test* and significant levels were considered at $P < 0.05$.

RESULT

Sociodemographic characteristics:
A total of 236 individuals participated in the study, of which 78 were newly diagnosed HIV positive HAART $^{Rx-}$, 108 were HIV positive HAART $^{Rx+}$ patients while 50 healthy individuals were the control group. Males constituted 44% (n=105) of the study population, while female participants made up 56% (n=131) as shown in Figure 1. The average age of the study respondents was 39.2 years. The mean HAART time period for HIV infected HAART $^{Rx+}$ patients was 2 years from diagnosis.

Fig 1: Chart indicating the study population



CD4+ T cells recovery after initiation of HAART:
Majority of the patients in the test group (76%) had CD4+ T cell count ranging from 200-600 cells/µl while the healthy control (24%) had more than 850 cells/µl (Table 1). The mean CD4+ T cell count in HIV infected HAART $^{Rx-}$ patients was less than 250 cells/µl while the mean of CD4+ T cell count in HIV infected patients who HAART $^{Rx+}$ for over 2 years was found to be more than 600 cells/µl.

Table 1: CD4+ T cell levels in HIV positive HAART $^{Rx-}$ patients and HIV positive HAART $^{Rx+}$ patients

CD4+ T cell count (cells/µl)	HAART $^{Rx-}$ n=78	HAART $^{Rx+}$ n=108
≤ 250	52	-
250 - 400	15	-
400-600	11	24
400-600	-	78

Viral load suppression after initiation of HAART:
Among the HAART $^{Rx-}$ test group 79% of the patients had viral load ranging from 7$\log_{10}$ copies/ml to 5$\log_{10}$ copies/ml (1,000,000 to 12589 copies) while the rest 21% of the patients had viral load ranging between 4 $\log_{10}$ copies/ml to 3 $\log_{10}$ copies/ml (12,589 to 1,259 copies). Following HAART initiation, 91% of the test group displayed low undetectable viral load between 2 $\log_{10}$ copies/ml and 1$\log_{10}$ copies/ml (126 to 13 copies), whereas the other 9% displayed viral load between 4$\log_{10}$ copies/ml and 3 $\log_{10}$ copies/ml.

Table 2: Viral load levels among the HIV positive HAART $^{Rx-}$ patients and HIV positive HAART $^{Rx+}$ patients

Viral load ($\log_{10}$ copies/ml)	HAART $^{Rx-}$ n=78	HAART $^{Rx+}$ n=108
7- 5	62	-
4 - 3	16	10
2 – 1	-	98

T helper cytokines concentration levels in HAART $^{Rx+}$ patients:
The mean plasma concentration of Th1 cytokines IFN γ and IL 2 among HAART $^{Rx+}$ patients was found to be 123.9 pg/ml and 113.3 pg/ml. In contrast, mean levels of the Th2 cytokines IL 4 and IL10 were discovered to be 15.7 pg/ml and 9.8 pg/ml, respectively, in the plasma of HAART $^{Rx+}$ patients. Significant differences were noted in the plasma levels of Th1 cytokines – IFN γ (123.90 ± 7.5 pg/ml) and IL 2 (113.35 ± 2.4pg/ml) and Th2 cytokines – IL 4 (15.70 ± 5.3pg/ml) and IL 10 (9.89 ± 3.1pg/ml) (Fig 2).

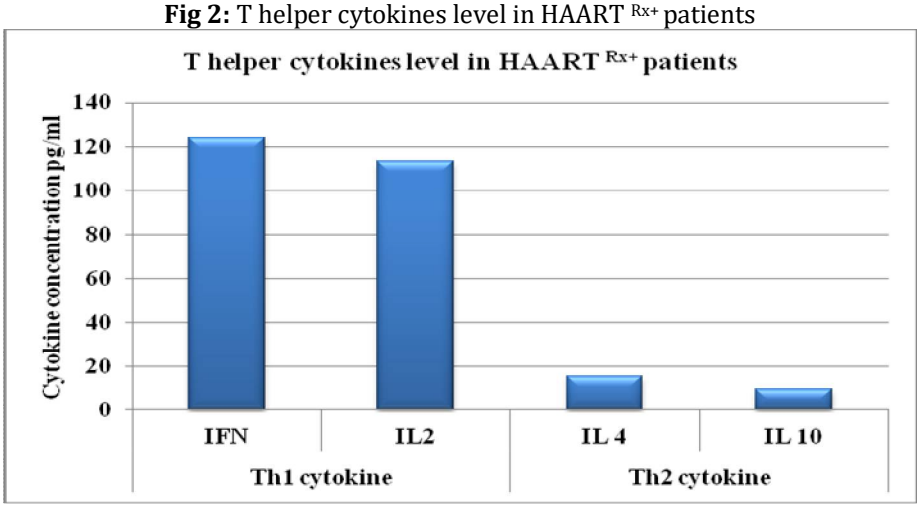
Group comparisons in Table 3 shows that Th1 pro-inflammatory cytokines level was significantly higher compared to level of Th2 anti-inflammatory cytokines in HAART $^{Rx+}$ patients.

Table 3: T helper cytokine levels in HIV positive HAART $^{Rx+}$ patients

Study groups	Th1 cytokine (pg/ml) Mean ± SD		Th2 cytokine (pg/ml) Mean ± SD	
	IFN γ	IL 2	IL 4	IL 10
HAART $^{Rx+}$	123.90 ± 7.5^{a}	113.35 ± 2.4^{b}	15.70 ± 5.3^{c}	9.89 ± 3.1^{d}
Healthy control	7.40 ± 1.4	4.51 ± 2.9	5.06 ± 4.3	7.88 ± 4.8

*{a}vs.{c}P<0.05, {a}vs.{d}P<0.05, {b}vs.{c}P<0.05 and {b}vs.{d}P<0.05

Fig 2: T helper cytokines level in HAART $^{Rx+}$ patients



T helper cytokines concentration levels in HAART $^{Rx-}$ patients:

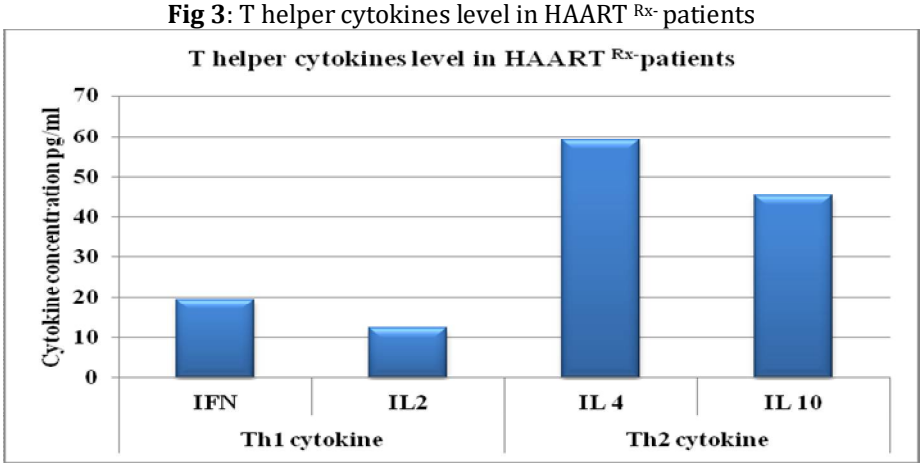
Among HAART $^{Rx-}$ patients, the mean plasma concentrations of the Th1 cytokines IFN γ and IL 2 were determined to be 19.4 pg/ml and 12.3 pg/ml, respectively. Corresponding to this, HAART $^{Rx-}$ patients' mean plasma Th2 cytokines IL 4 concentration was 60.2 pg/ml and IL10 45.3 pg/ml. Significant variations were found in the plasma levels of Th2 cytokines - IL4 (60.2 ± 6.8 pg/ml) and IL10 (45.26 ± 2.6 pg/ml) and Th1 cytokines – IFN γ (19.4 ± 2.9 pg/ml) and IL 2 (12.32 ± 2.2 pg/ml) (Fig 3). Group comparisons in Table 4 reveal that in HAART $^{Rx-}$ patients, the level of Th2 anti-inflammatory cytokines was significantly greater than the level of Th1 pro-inflammatory cytokines.

Table 4: T helper cytokine levels in HIV positive HAART $^{Rx-}$ patients:

Study groups	Th1 cytokine (pg/ml) Mean ± SD		Th2 cytokine (pg/ml) Mean ± SD	
	IFN γ	IL 2	IL 4	IL 10
HAART $^{Rx-}$	19.4 ± 2.9^{a}	12.3 ± 2.2^{b}	60.2 ± 6.8^{c}	45.3 ± 2.6^{d}
Healthy control	7.4 ± 1.4	4.5 ± 2.9	5.1 ± 4.3	7.9 ± 4.8

*{a}vs.{c}P<0.05, {a}vs.{d}P<0.05, {b}vs.{c}P<0.05 and {b}vs.{d}P<0.05

Fig 3: T helper cytokines level in HAART $^{Rx-}$ patients



IgG1 and IgG2 antibody levels and HAART:

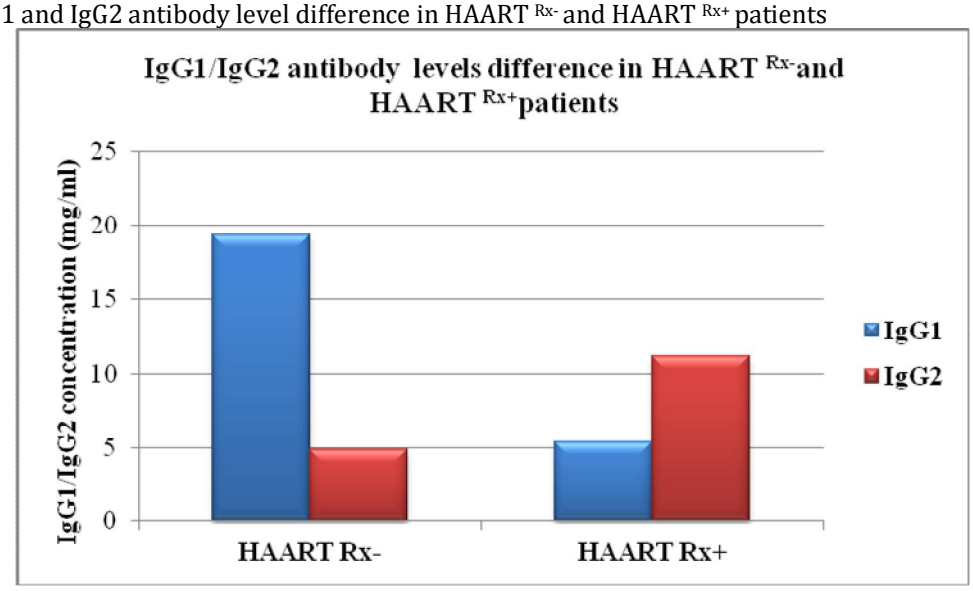
In HAART $^{Rx+}$ patients, the mean plasma IgG1 antibody concentration was 4.86 mg/ml, while in HAART $^{Rx-}$ patients, it was 19.3 mg/ml. In contrast, the mean plasma IgG2 concentration was found to be 5.32 mg/ml in HAART $^{Rx-}$ patients and 11.17 mg/ml in HAART $^{Rx+}$ patients (Fig 4). Group comparisons in Table 5 amongst HAART $^{Rx-}$ patients, there were significant differences in plasma IgG levels (19.3 ± 3.3 mg/ml) and IgG2 levels (5.32 ± 2.1 mg/ml) similarly significant variations were noted in HAART $^{Rx+}$ patients IgG1 levels (7.6 ± 2.5 mg/ml) and IgG2 levels (13.8 ± 2.6 mg/ml) compared to the healthy control.

Table 5: IgG1 and IgG2 antibody levels in HIV positive HAART $^{Rx-}$ patients and HIV positive HAART $^{Rx+}$ patients.

Study group	IgG 1 (mg/ml)	IgG 2 (mg/ml)
HAART $^{Rx-}$	19.3 ± 3.3^{a}	5.32 ± 2.1^{d}
HAART $^{Rx+}$	4.86 ± 4.5^{b}	11.17 ± 4.1^{e}
Healthy control	7.6 ± 2.5^{c}	13.8 ± 2.6^{f}

*Data given as Mean±SD {a}vs.{b} P<0.05, {a}vs.{c} P<0.05, {b}vs.{c} P<0.05, {d}vs.{e}= P<0.05, {d}vs.{f} P<0.05and {e}vs.{f} P<0.05.

Fig 4: IgG1 and IgG2 antibody level difference in HAART $^{Rx-}$ and HAART $^{Rx+}$ patients



Correlation between T helper cytokine and IgG1/IgG2 antibody levels in HIV positive HAART $^{Rx+}$ and HAART $^{Rx-}$ patients:

The comparative study on levels of Th1/Th2 cytokines along with IgG1/IgG2 antibody levels in the plasma of HIV infected HAART $^{Rx-}$ and HAART $^{Rx+}$ patients showed an increased level of Th2 anti inflammatory cytokines IL 4 (58.9 pg/ml) and IL 10(45.2 pg/ml) along with increased levels of IgG1 antibody levels (19.3 mg/ml) in HAART $^{Rx-}$ patients. Among the HAART $^{Rx+}$ patients, higher levels of Th1 pro-inflammatory cytokines IFN γ (123.9 pg/ml) and IL 2 (113.3 pg/ml) was noted along with increased levels of IgG2 (11.17 mg/ml) antibody demonstrating the switch from Th2 to Th1 immune response in HIV positive HAART $^{Rx+}$ patients.

DISCUSSION

The study analyzed the sociodemographic characteristics, CD4+ T cells recovery, viral load suppression, T helper cytokine concentrations, and IgG1 and IgG2 antibody levels in HIV positive patients who were HAART $^{Rx-}$ and those who were HAART $^{Rx+}$, as well as healthy individuals. The findings of this study suggests that patients undergoing HAART experience significant recovery of CD4+ T cells and rapid decline in viral load thus demonstrating immune reconstitution as described by earlier studies [23-25. Plasma cytokine levels in HAART $^{Rx-}$ patients and HAART $^{Rx+}$ patients were quantified and it was found that in comparison to Th1 plasma cytokines IFN γ and IL 2, our study found higher levels of Th2 plasma cytokines IL 4 and IL 10 in HIV-infected patients who are yet to commence HAART. These results are in line with those of a related study conducted elsewhere[26]. Similarly higher plasma concentrations of IFN γ and IL 2 in patients undergoing HAART for over 2 years since diagnosis in comparison with IL 4 and IL 10

was observed. This is consistent with research [27, 28] showing changes in pro-inflammatory and anti-inflammatory cytokines after HAART regimen that patients undergoing HAART have low plasma levels of IL 4 and IL 10. The mean plasma IgG1 antibody concentration was lower in HAART $^{Rx+}$ patients than in HAART $^{Rx-}$ patients, while the mean plasma IgG2 concentration was higher in HAART $^{Rx+}$ patients than in HAART $^{Rx-}$ patients. Significant variations were found in the plasma levels of IgG1 and IgG2 in both the HAART $^{Rx+}$ and HAART $^{Rx-}$ groups compared to the healthy control, these findings are consistent with a study of a similar nature when examining factors associated with the development of broadly neutralizing antibodies (bNAbs) in HIV-1 patients, it was observed that IgG subclass responses are regulated differently, leading to distinct patterns of neutralizing and non-neutralizing antibodies. Parameters such as viral load, viral diversity, infection duration, and ethnicity significantly influence the rate of neutralization, predominantly in antigen-specific antibody responses driven by selective antigens and dependent on IgG subclasses [16]. In the newly diagnosed HIV-positive HAART $^{Rx-}$ patients, HIV immune activation is accompanied by the release of pro-inflammatory and anti-inflammatory cytokines by T cells, macrophages, and monocytes [29, 30]. Increased production of IL 4 and IL 10 triggers a Th2 immune response, which in turn activates B cells and produces antibodies, creating a positive feedback loop that further drives the Th2 response and inhibits Th1 immunological activity [31, 32]. Our findings that there were substantial variations between Th1 (IFN γ and IL 2) and Th2 (IL 4 and IL 10) plasma cytokine levels in HAART $^{Rx-}$ patients confirm the aforementioned observation. HIV disease progression is significantly slowed down by HAART induced immunological reconstitution, notably the Th2 to Th1 shift [33]. Our study found higher levels of Th1 plasma cytokines (IFN γ and IL 2) in patients on HAART $^{Rx+}$, which was supported by earlier studies of a similar type [34, 35].

CONCLUSION

Our findings imply that HIV infection is characterized by dysregualtion of cytokine production and decline in CD4+ T cells. While significant levels of Th2 cytokines were found in HAART $^{Rx-}$ patients, suggesting a switch from Th2 to Th1 cytokines indicative of a rapid progression to AIDS, our data show that HAART $^{Rx+}$-induced immunological reconstitution resulted in increasing levels of Th1 cytokines. In conclusion, this study examined various factors in HIV-positive patients, including sociodemographic characteristics, CD4+ T cell recovery, viral load suppression, T helper cytokine concentrations, and IgG1 and IgG2 antibody levels. The findings indicate that patients undergoing highly active antiretroviral therapy (HAART $^{Rx+}$) experience significant recovery of CD4+ T cells and a rapid decline in viral load, demonstrating immune reconstitution. The levels of plasma cytokines differed between HAART $^{Rx-}$ patients and those undergoing HAART $^{Rx+}$ with higher levels of Th2 cytokines (IL 4 and IL 10) in HAART $^{Rx-}$ patients and higher levels of Th1 cytokines (IFN γ and IL 2) in HAART $^{Rx+}$ patients. These findings are consistent with previous research. Furthermore, significant variations were observed in the plasma levels of IgG1 and IgG2 in both groups compared to healthy controls. The study also highlighted the impact of HAART on immune activation and the shift from a Th2 to Th1 immune response. Overall, the results support the notion that HAART $^{Rx+}$ -induced immunological reconstitution plays a significant role in slowing down HIV disease progression.

Overall, further research on pro and anti-inflammatory cytokines is needed to maximize their value as indicators of HIV disease progression and treatment in HIV-infected individuals. For a more accurate assessment of how HAART affects cytokine levels, measurement of these cytokines might continue after prolonged treatment.

Ethical Approval: Approved

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Conflict of Interest: The authors have no conflicts of interest regarding this investigation**.**

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