

## Impact of Cytokines on Hepatotoxicity Induced by Antiretroviral Therapy in HIV-1 Infected Individuals in Northwest Cameroon

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### **Abstract**

**Keywords:** *Cytokines, HAART, HIV-1, Hepatotoxicity, Alanine aminotransferases and aspartate aminotransferases.*

The advent of highly active antiretroviral therapy (HAART) has enabled HIV-1 infected people to live long and fruitful lives. Since drug and viral mediated toxicities are hallmarked by a modulation of patient's cytokine profiles, we assessed the impact of ART on plasma cytokine profiles of HIV-1 patients. Blood samples were collected from 68 HAART naïve HIV-1 patients at baseline (D0), 30 days (D30) and 180 days (D180) following HAART initiation. Serum levels of Alanine aminotransferases (ALT) and aspartate aminotransferases (AST) was analysis enzymatically. Human Th1/Th2/Th17 cytokines were measured using a cytometric bead array assay. Data were analyzed using Graph pad prism 6 and SSPS. There was a significant increased ( $p < 0.001$ ) in the mean serum levels of both ALT and AST corresponding with the treatment duration. A negative correlation was observed between CD4<sup>+</sup> T cell counts and serum levels of either ALT ( $-0.522$ ,  $p = 0.000$ ) or AST ( $-0.505$ ,  $p = 0.000$ ). The prevalence of hepatotoxicity increased significantly ( $P = 0.000$ ) and was found to be 0(0.0%), 34(50.0%) and 47(69.1%) at D0, D30 and D180 respectively. Mean IL-2, IL-6, IL-17A and TNF- $\alpha$  cytokines were higher in patients with hepatotoxicity compared to patients with no hepatotoxicity at D30 and D180 with a significant difference ( $p < 0.05$ ) seen only in IL 17-A and IL 6. The prevalence of hepatotoxicity increased with treatment duration and was associated with modulations in the human Th1/Th2/Th17 cytokine profile. IL-6 and IL-17A seem to play a significant role in the pathophysiology of hepatotoxicity. As such they might be used either alone or with other biomarkers to assess HAART induced hepatotoxicity in our context.

### **Introduction**

The advent of highly active anti-retroviral therapy (HAART) has allowed more people infected with the human immunodeficiency virus type 1 (HIV-1) to live normal and productive lives [1, 2]. As of 2016, 17 million people were accessing HAART [1]. HAART functions through suppressing HIV-1 viral load thereby restoring the immune system [3, 4]. However sustained use of HAART is associated with liver disease (hepatotoxicity) which is reported to be the most common non-AIDS related cause of death among HIV-1 infected patients accounting for well over 14% of all deaths [5- 8]. The era of 'test and treat' with increased availability of different HAART regimes including various nucleoside/nucleotide analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs), necessitates a close monitoring of their toxicities to ensure sustainable long term management of HIV-1 infection in especially low income countries [6, 9,10]. Liver disease is accompanied by biochemical abnormalities in liver functions which is marked by elevated serum activity of the two commonly used liver enzymes; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) that are involved in breakdown of amino acids [9, 10].

Even with the most effective HAART regiment, a key consequent of HIV-1 infection is maintaining the immune system in perpetual activation. Cytokines are inflammatory mediators that serve important roles in the pathogenesis of many acute and chronic diseases [11] and include T helper type 1 (Th1), Th2 and Th17 cytokines [12]. Th1 cytokines include the interleukin-2 (IL-2), IL-12, IL-18, interferon (IFN)- $\gamma$  and tumor necrosis factor-alpha (TNF- $\alpha$ ); Th2 type cytokines include (IL-4, IL-6, IL-10, IL-13) and Th17 cytokines (IL-17, IL-22, IL-23, IL-1). A number of studies highlight a critical role of cytokines in the pathogenesis of HIV-1 often culminating in cytokine profile

dysregulation both *in vivo* and *in vitro* [13-16]. Studies addressing these issues included those looking at the impact of different cytokine on HIV-1 replication [16-18], comparing the cytokine expression profile between HIV-infected and uninfected donors [15, 19], measuring cytokine production from *in vitro* infected cells [17, 20] or analysing cytokines with respect to diagnosis, treatment and vaccine development [19, 21].

Cytokines are produced by virtually all nucleated cell in the body including the Kupffer cells of the liver [11]. A few studies reveal that there is possible correlation between cytokines and liver damage [12, 22, 23]. Thus an imbalance in cytokine production plays a key role in the development of liver damage, necro-inflammation, and subsequent fibrosis [24, 25]. These cytokines activate several pathways such as the apoptotic pathways which result in liver damage [21, 26]. Examples of the cytokines associated with liver disease include IL-1, IL-6, IL-12, IL-17, IL-18, IFN- $\gamma$ , and TNF- $\alpha$  [22-24, 26 and 27]. However the role of cytokine in HAART induced hepatotoxicity must be clarified to improve patient care. In this study we investigated the relevance of cytokine modulation amongst HIV-1 patients on HAART with and without hepatotoxicity. Results from this study may allow improve therapeutic approaches involving the modulation of cytokines to attenuate the drug induced hepatotoxicity during antiretroviral treatment of HIV-1 infected people.

## Materials and method

### Study Population and Site

This was a longitudinal study comparing Th1/Th2/Th17 cytokines in HIV-1 treated patients with or without hepatotoxicity in five HIV-1 outpatient treatment centers in the Northwest Region (NWR) of Cameroon. These centres were; Ndop, Santa, Bali and Bafut District Hospitals and the Regional Hospital Bamenda. These sites were chosen because they represent the different population background of the entire region. Ndop, Bali and Bafut are rural areas from the Northern, Eastern and Western areas of the NWR. Bamenda is an urban area close to Santa which is a rural area and share boundaries with the western region of Cameroon. This study received ethical clearance from the Cameroon National Ethical Committee (No 2016/01/689/CE/CNERSH/SP) and administrative authorization from the Ministry of Public Health of Cameroon. Screened and recruited participants were followed for up for a period of 24 weeks from February 2016 to November 2016 with blood being collected at initiation (D0), 30 (D30) and 180 (D180) days after antiretroviral initiation. These patients were initiated on first line HAART consisting of 2 nucleoside reverse transcriptase inhibitor in combination with a non nucleoside reverse transcriptase inhibitor. This include either; Zidovudine/Lamivudine/Efavirenz (AZT+3TC+EFV) or Zidovudine/Lamivudine/Nevirapine (AZT+3TC+NVP) or Tenofovir/lamivudine/Efavirenz (TDF+3TC+EFV) and co-trimoxazole based on the Ministry of Public Health ART initiation criteria guidelines as proposed by WHO [28]. All drugs were provided free of charge from the Cameroon National AIDS control program.

### Blood Samples

Venous blood was collected by trained institutional phlebotomist dedicated for research. Eight ml of venous blood was collected in uniquely coded tubes. Then 3ml and 5ml were transferred into dry and ethylenediaminetetraacetic acid (EDTA) test tubes to obtain serum and plasma respectively. Assays for serologic markers of HIV-1, hepatitis B virus (HBV) and hepatitis C virus (HCV) were determined using an ELISA technique. Serum transaminases were measured same day and the harvested plasma was stored at -80°C for cytokine measurement.

### Measurement of Transaminases (AST and ALT)

The liver transaminases enzymes; were measured using spectrophotometer as described by International Federation of Clinical Chemistry (IFCC) protocol of 2002 [29] using SPINREACT commercial kits (SPAIN) guided by the controls [30].

### Cytokines levels

Cytokines were measured by the Cytometric Bead Array (CBA) flow cytometry using the Human Th1/Th2/Th17 CBA kit (Cat. No. 560484, BD Biosciences, California) as described by [18]. This allowed for the simultaneous detection of IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ , IFN- $\gamma$  and IL-17A.

### Statistical Analysis

Data was analyzed using SPSS version 20 (Armonk, NY, USA.) and Graph Pad Prism 6 (GraphPad Software, San Diego, CA, USA). SPSS was used to determine the hepatotoxicity proportions of categorical data using the chi square test and ANOVA test to determine the difference in mean transaminases. Mean differences between two

groups were determined using Mann-Whitney (U) of unpaired t test data while comparison among the 3 groups of treatment duration was done using non-parametric Kruskal Wallis test (H).  $P < 0.05$  was considered statistically significant.

## Results

### Study Population characteristics:

100 ARV naïve HIV-1 infected people were screened and a total of 68 (68%) participants were recruited in this study. Amongst the participants 35(51.1%) were males with mean aged 35.4 (IQR; 18-61 – 42.50) years old and 33(48.5%) female with mean aged 36.2 (IQR; 18-57) years. The majority of participants received TDF+3TC+EFV drug regimen ( $n = 48$ , 70.6%). The median helper CD4 T cell counts were not significantly higher in the participants who were placed on AZT+3TC+NVP therapy 214.9 (18-498). Helper CD4<sup>+</sup> T cell categorization included a majority of participants 39(IQR; 57.3%) with severe immunosuppression ( $>200$  cells/mm<sup>3</sup>). The median BMI in kg/m<sup>2</sup> counts were significantly higher in the female participants (31.1 kg/m<sup>2</sup>) than their male counterpart (24.1 kg/m<sup>2</sup>). Baseline mean (SEM) ALT and AST were higher in males than in females. However all values were within the normal ranges (Table 1).

*Table 1: Demographic data of patients by degree of immunosuppression*

Variable	Participants (n=68)		
	Severe immunosuppression {<200 cells/mm <sup>3</sup> } (40:58.8)	Advanced immunosuppression {200-349 cells/mm <sup>3</sup> } (16:23.5)	Mild immunosuppression {350-499 cells/mm <sup>3</sup> } (12:17.7)
degree of immunosuppression (n : %)			
Gender :Female (33:48.5) Male (35:51.5)	15(45.5) 25(71.4)	11(33.3) 5(14.3)	7(21.2) 5(14.3)
Age :<35years (33:48.5) >35years (35:51.5)	16(48.5) 24(68.6)	10(30.3) 6(17.1)	7(21.2) 5(14.3)
<b>ART type</b>			
TDF+3TC+EFV (48:70.6)	29(60.4)	11(22.9)	8(16.7)
AZT+3TC+EFV (12:17.6)	7(58.3)	3(25.0)	2(16.7)
AZT+3TC+NVP (8:11.8)	4(50.0)	2(25.0)	2(25.0)
Median age in years (IQR)	36.5 (18-61)	33.3 (18-56)	37.3 (26-57)
Median ALT in U/L (IQR)	24.2 (4.1-39.6)	25.9 (16.0-35.3)	24.9 (10.7- 39.9)
Median AST in U/L (IQR)	27.0 (6.9-38.0)	27.6 (10.5-34.7)	25.2 (10.0-31.8)
Median BMI in kg/m <sup>2</sup> (IQR)	24.8 (14.5-43.0)	32.3 (17.2-88.3)	28.9 (21.7-57.3)
Median CD4 count in cells/mm <sup>3</sup> (IQR)	91.8 (8-191.)	278.8 (216-341)	410.1 (356-498)

### Prevalence of Hepatotoxicity:

Antiretroviral therapy is usually associated with liver injury marked by a corresponding increase in liver enzymes which could be used to track hepatotoxicity [31]. In this study AST and ALT levels were used with severe hepatotoxicity considered as degree 3 and 4 changes in AST or ALT levels [31, 32].

In figure 1a, a significantly inverse correlation is observed between helper CD4<sup>+</sup> T cell counts and ALT (-0.522,  $p = 0.000$ ) and AST (-0.505,  $p = 0.000$ ). On the other hand, mean AST values were higher than ALT values at D30 and D180. Mean ALT ( $F = 9.95$ ;  $p = 0.000$ ) and AST increased significantly ( $F = 11.60$ ;  $p = 0.000$ ) with the duration of treatment (figure1b).

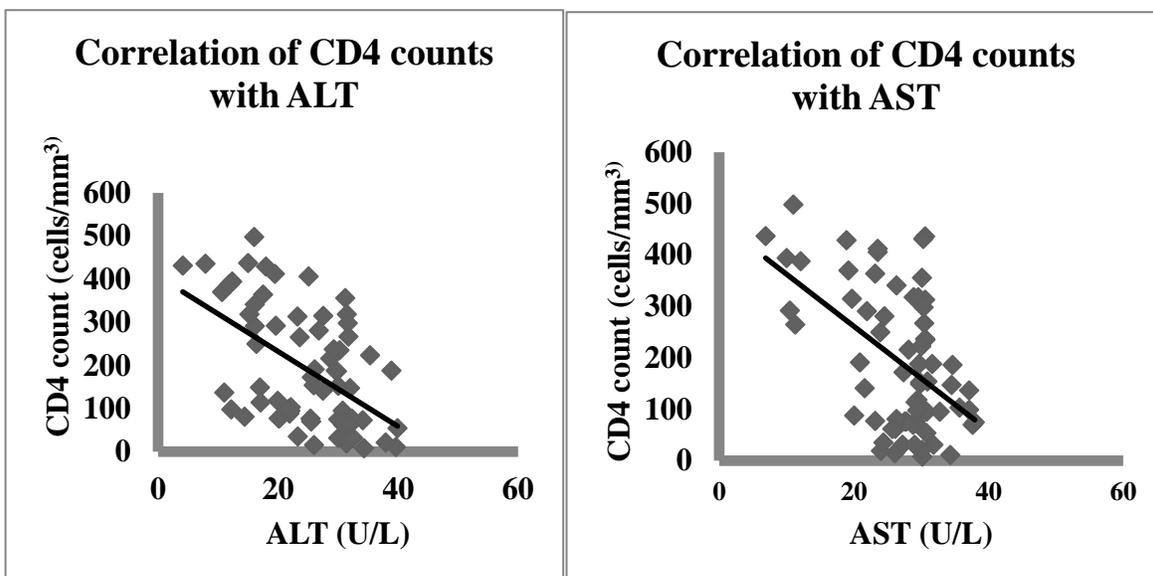


Figure 1a: Correlation of CD4 T-cell counts with ALT and AST at D0

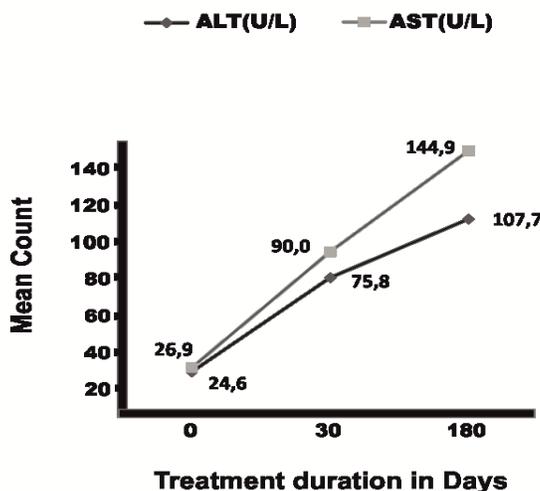


Figure 1b: Variation of mean ALT (U/L) and AST (U/L) during the study duration

The prevalence of hepatotoxicity using ALT was 23(33.82%) and 35(51.47%) and that of AST was 24(35.28%) and 37(54.41%) at D30 and D180 respectively. In figure 2a and figure 2b below, Grades 1, 2, 3 and 4 hepatotoxicity as classified using ALT and AST respectively, were highest at D180 compared to Day30 and this differences were statistically significant ( $p < 0.001$ ). Based on either ALT or AST or both, the prevalence of hepatotoxicity increased significantly ( $\chi^2 = 41.93$ ,  $P = 0.000$ ) with treatment duration and was found to be 0(0.0%), 34(50.0%) and 47(69.1%) at D0, D30 and D180 respectively. Severe hepatotoxicity that requires close clinical follow up (defined by Grades 3 and 4 toxicity of either ALT or AST or both) was found to be 15(22.1%) and 27(39.7%) at D30 and D180 respectively. Clients with severe immunosuppression registered the highest prevalence of hepatotoxicity (Grade 1 to 4) compared to those with mild immunosuppression using ALT and AST as shown on figure 2c and 2d respectively. However this difference were not significant (ALT;  $\chi^2 = 2.5$ ,  $P = 0.87$  and AST;  $\chi^2 = 4.6$ ,  $P = 0.59$ ).

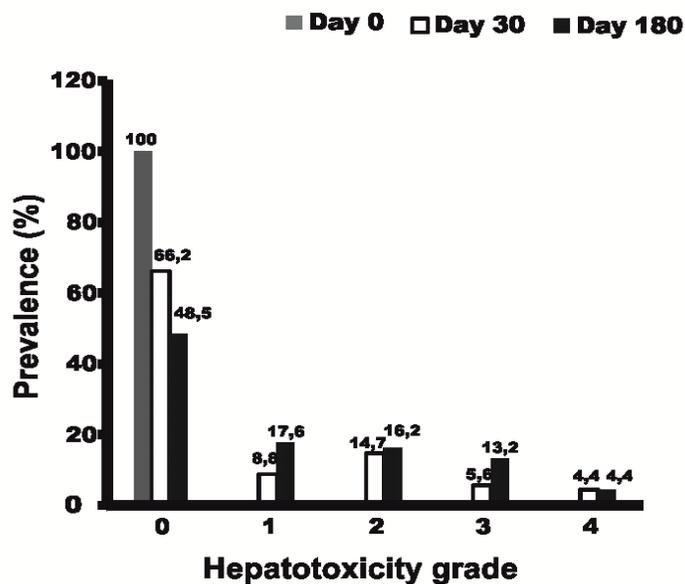


Figure 2a: Prevalence of Hepatotoxicity Grade as classified using ALT

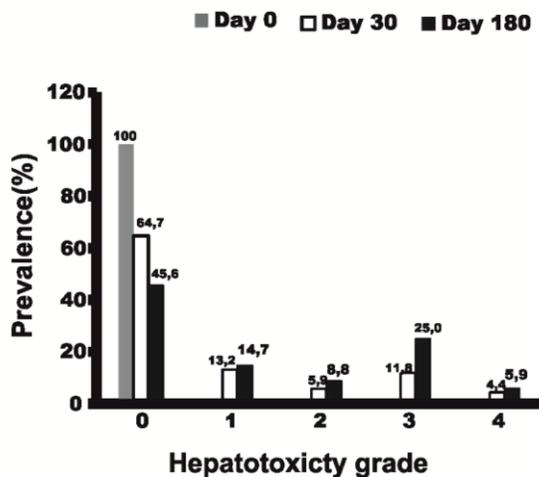


Figure 2b: Prevalence of Hepatotoxicity Grade as classified using AST

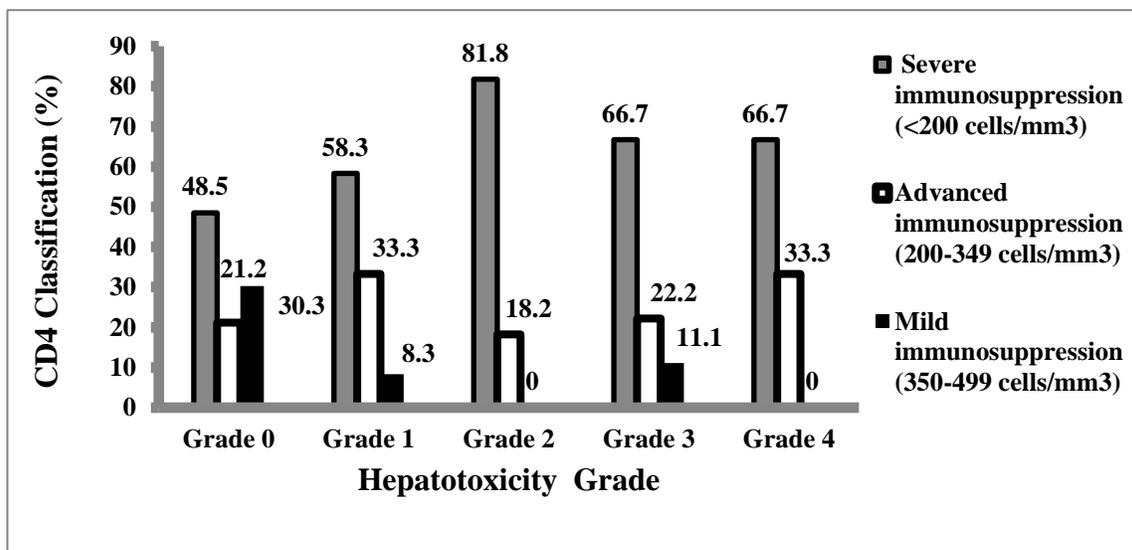


Figure 2c: Assessing degree of immune suppression with hepatotoxicity grade as classified by ALT at D180

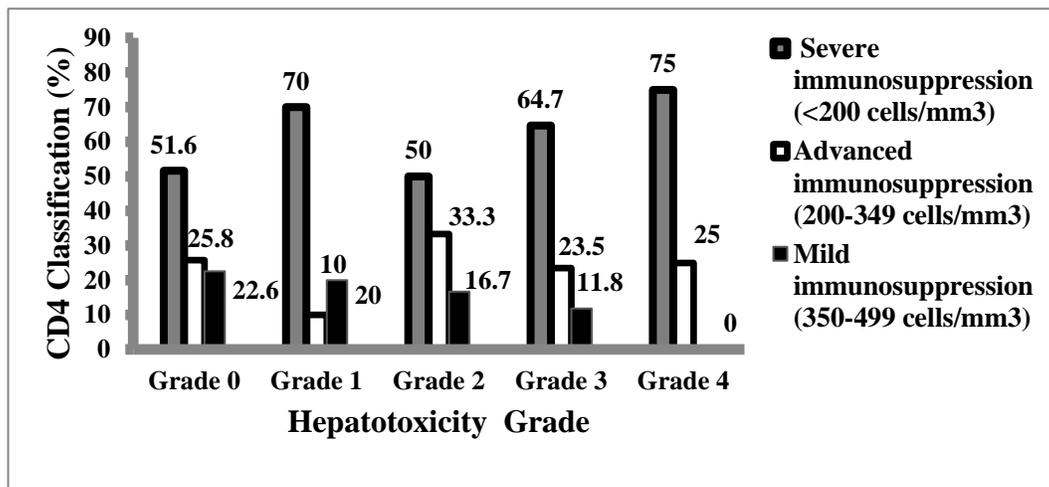


Figure 2d: Assessing degree of immune suppression with hepatotoxicity grade as classified by AST at D180

**Variation of mean Cytokines during the entire study duration**

A three-by-three Kruskal Wallis test was used to assess normal concentration of mean IL-2, IL-4, IL-6, IL-10, IL-17A, IFN- $\gamma$  and TNF- $\alpha$  cytokines levels at different treatment durations (D0, D30 and D180). IL-4 was undetectable in any of the samples. When mean cytokine levels at baseline and D180 were compared, a non significant ( $p > 0.05$ ) negative Pearson correlation ( $-r$ ) was seen in mean IL-2, IL-6, IFN- $\gamma$  and TNF- $\alpha$  while a positive Pearson correlation was seen with IL-10 and IL-17A. However, only IL-17A showed a significant difference  $p = 0.04$  (Table 2).

Table 2: Pearson correlation of cytokine at D0 and D180

CYTOKINE	IL2	IL6	IL10	IL17	TNF- $\alpha$	IFN- $\gamma$
Pearson correlation	-.036	-0.109	0.123	0.049	-0.239	-0.032
P value	0.77	0.38	0.32	0.04	0.69	0.80

Comparison between treatment duration showed no statistical significant difference ( $p > 0.05$ ) with IFN- $\gamma$ , TNF- $\alpha$ , IL-6 and IL-2 while a statistical significant difference ( $p < 0.05$ ) was seen with IL-10 and IL-17A. Analysis of this figure also showed that IFN- $\gamma$  and IL-17A values decrease with increase in the duration of treatment while cytokines IL-2, IL-6 and TNF- $\alpha$  decreases at D30 and later on increases by D180. In addition IL-10 increases at D30 and later on decreases by D180 (Figure 3).

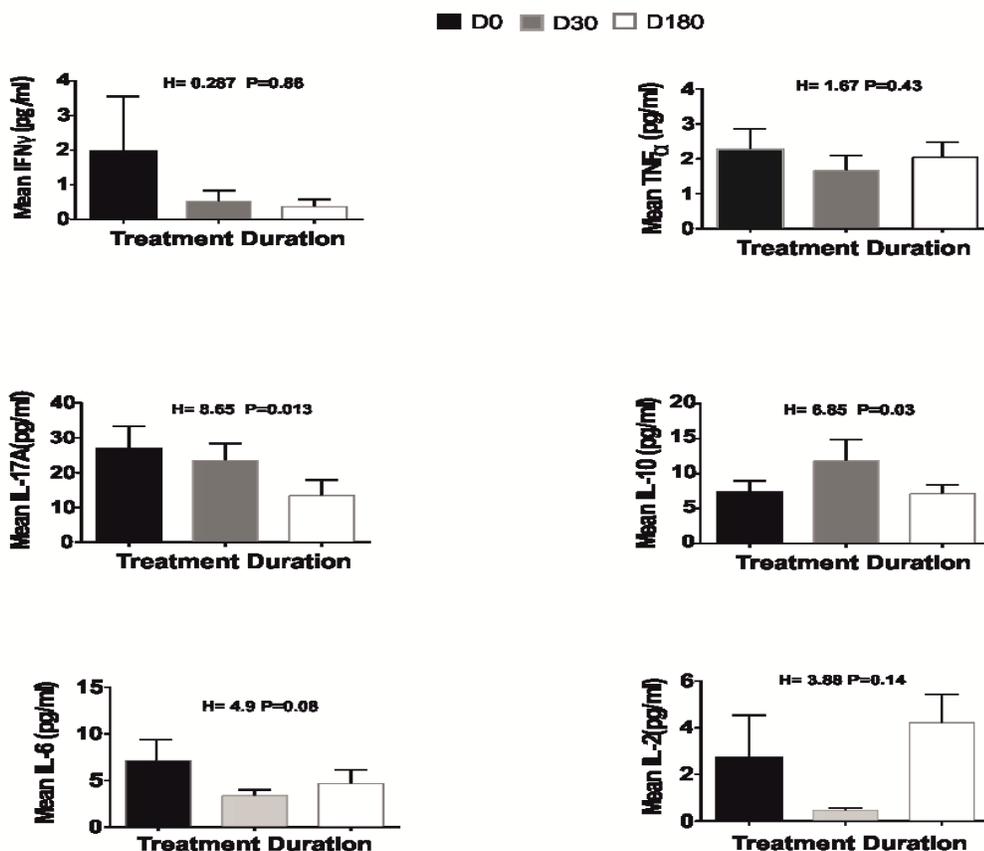


Figure 3: Influence of treatment duration on Mean cytokine variation

**Comparison of mean Cytokines between patients with and without hepatotoxicity**

Mean plasma concentrations of TNF- $\alpha$ , IFN- $\gamma$ , IL-17A, IL-6 and IL-2, were higher in HIV-1 patients presenting with hepatotoxicity compared to those without hepatotoxicity at D30 (Figure 4a). On the other hand, mean plasma concentrations of TNF- $\alpha$ , IL-17A, IL-10, IL-6 and IL-2 cytokines increased in HIV-1 patients presenting with hepatotoxicity compared to those without hepatotoxicity at D180 (Figure 4b). With the exception of 17-A and IL 6 cytokines that showed significant difference ( $p < 0.05$ ) at D30 and D180, these differences were not statistically different.

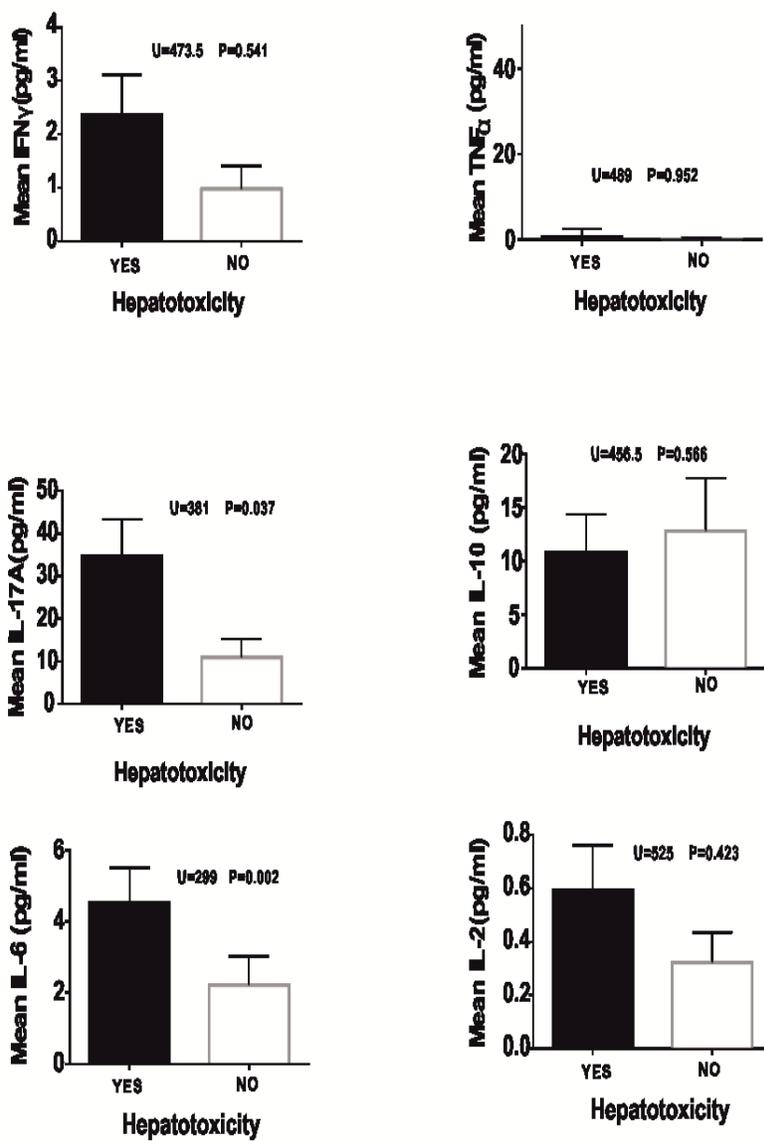


Figure 4a: Mean cytokine variation between patients presented with and without hepatotoxicity at day 30

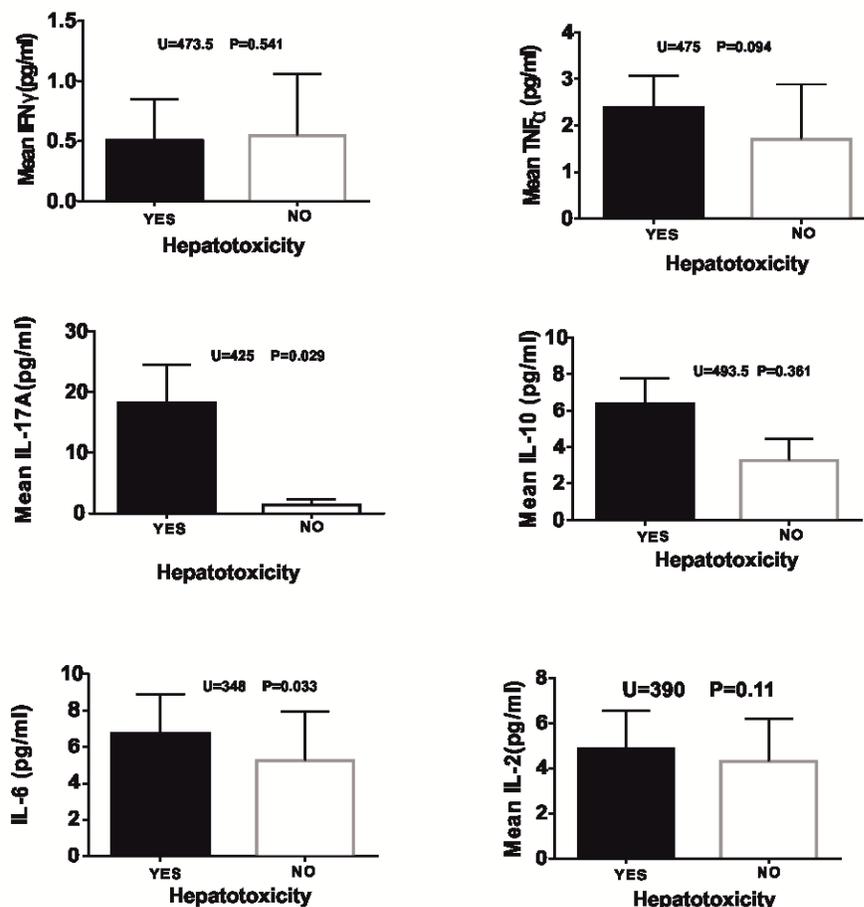


Figure 4b: Mean cytokine variation between patients presented with and without hepatotoxicity at day 180

## Discussion

Liver disease is an emerging challenge in the clinical management of HIV-1 infection especially with an ever increasing HAART coverage. The majority of antiretroviral drugs have been associated with liver injury leading to elevated liver enzyme and consequent hepatotoxicity [31-34].

In order to eliminate confounding factors that could effects the cytokine profile participants reporting any home or hospital base treatment for fever were excluded. Participants taking alcohol or with concurrent infections like HBV, HCV and malaria were excluded from the study. Thus during screening and recruitment efforts were made to reduce potential bias arising from other previously reported sources of inflammatory cytokines [35-43].

In this study the transaminases (ALT and AST) as previously reported [31- 34] increased with treatment duration. Toxicity linked to HAART as revealed by an increased in transaminases levels is time dependent [33-37]. Abnormal levels of liver enzymes may be caused by multiple factors, including toxic medication, coinfection with hepatitis C virus (HCV) or hepatitis B virus (HBV) [38, 39, 44-48].

ARV drugs induced direct toxicity to the liver through drug metabolism via the cytochrome pathways, idiosyncratic polymorphisms of the enzymatic complexes, hypersensitivity reactions, mitochondrial toxicity or by activating death receptors and intracellular stress pathways [36, 49,50].The severity of hepatotoxicity ranges from transient increase (grade 1 and grade 2) to severe increase in levels (grade 3 and grade 4) that may lead to hepatic failure and death [6, 9, 33]. The increased prevalence of hepatotoxicity from 0(0.0%) at D0 to 34(50.0%) at D30 and 47(69.1%) at D180 is evidence that the use of HAART causes hepatotoxicity in HIV patients [32, 34, 38].

Evaluation of CD4 count level is a vital tool in the management of HIV-1 infection. With the progressive loss of CD4<sup>+</sup>T cells in HIV infection, the dysfunction in the T cells compartment is reflected by cytokine expression [13]. The negative correlation between CD4<sup>+</sup> T cell count and transaminase levels is probably due to the fact that a reduction in CD4<sup>+</sup> lymphocytes results to an increased in protein (e.g lipopolysaccharide) turnover which act as a pro-inflammatory and pro-fibrotic substance on the liver resulting in a rise in the liver enzymes [9].

The negative correlation observed between treatment duration and levels of IL-2, IL-6, IFN- $\gamma$  and TNF- $\alpha$  probably indicate that increased cytokine production was a consequent of chronic stimulation of T cells by HIV. A decrease in INF- $\gamma$  seen at the end of this study is similar to previous studies carried elsewhere [51, 52]. Treatment with HAART is known to significantly inhibit viral replication and consequently decrease production of INF- $\gamma$  [52]. Considering participants with hepatotoxicity, mean IFN- $\gamma$  levels were higher at D30 compared to D180. This down regulation therefore shows no correlation between IFN- $\gamma$  and hepatotoxicity. This result is similar to those carried out in patients with chronic hepatitis B which is the leading cause of liver disease [23, 53]. Studies in rats and humans have shown that, IFN- $\gamma$ , TNF- $\alpha$  and IL-6, stimulate hepatocytes leading to activation of transduction pathways which are implicated in hepatocellular death during liver diseases and injuries [11, 26, 27]. However, there is need to examine the roles of these cytokines during HAART induced hepatotoxicity.

In this study mean plasma TNF- $\alpha$  decreased at D30 and later increases at D180. In addition we recorded an insignificant increase in TNF- $\alpha$  values in participants with hepatotoxicity which was similar to previous reports [26, 54, 55]. This is most probably because TNF- $\alpha$  regulates viral replication. Although our study did not demonstrate any significant changes in TNF- $\alpha$  levels between participants with and without hepatotoxicity, the high levels of TNF- $\alpha$  in people with hepatotoxicity is supportive of the fact that TNF- $\alpha$  may also induce hepatotoxicity. Further investigations is necessary to determine if TNF- $\alpha$  is expressed on hepatic cells considering that TNF- $\alpha$  is an inflammatory cytokine which triggers the production of additional cytokines such as IL-6 and has been shown to be significantly higher in patients with hepatotoxicity [11, 56].

A significant decrease ( $p=0.001$ ) in mean IL-17A levels study duration, and between participants with and without hepatotoxicity at D30 and D180 contradicts previous studies [57, 58]. The decrease in IL-17A is because Th17 cells that produce IL-17A are highly susceptible to HIV and thus are depleted with the use of HAART [59]. The significant increase seen in patients with hepatotoxicity is because depletion of Th17 cells may impair the mucosal protection that leads to increase amount of bacterial products, such as lipopolysaccharide (LPS) getting into the general circulation. LPS has been shown to elicit significant inflammatory response that mediates Kupffer cells activation and thus causes liver damage [8, 24, 27]. Thus this result indicates that IL-17A is associated with hepatotoxicity. Previous studies have shown that elevated IL-10 levels are associated with disease progression, increase viral load and decrease in CD4<sup>+</sup> T-cells [16, 18]. The decrease in IL-10 level seen at D180 confirms the relevance of high levels of IL-10 in regulating viral replication and inhibiting disease progression thus boosting the effect of HAART [60, 61]. Although the difference was not significant, increase level of IL-10 seen at D180 can be attributed to a reduction hepatotoxicity. It has been reported that the liver is the main source of IL-10 which probably plays a major role in chronic liver diseases [55, 61, 62]. Furthermore the positive correlation observed between IL-10 and AST in patients with chronic hepatitis B (CHB) is a clear indication that IL-10 can also be secreted in patients with HAART induced hepatotoxicity [23]. As such future studies using a larger population with longer treatment duration needs to be carried out to confirm these findings. Decrease plasma levels of IL-6 on D30 and an increase by D180 can be attributed to hepatotoxicity since the prevalence of hepatotoxicity was highest at D180. The high level of IL-6 might be due to the fact that IL-6 is a key factor in liver regeneration [24]. In addition other studies have also demonstrated the importance of IL-6 during the course of the liver infection [11, 26, 54]. Our data also reveal that IL-2 increases in participants with hepatotoxicity compared to those without hepatotoxicity at D30 and D180. During treatment, viral replication is significantly inhibited and thus subsequent reduction of IL-2 [51, 63]. Thus the high prevalence of hepatotoxicity at D180 might contribute to the observed high level of IL-2. Thus there is a probability that IL-2 can affect hepatic cells implying that it determination might be useful in the management of HAART induced hepatotoxicity.

## Conclusions and Recommendation

Data from this study supports the fact that increased prevalence of hepatotoxicity during the course of HAART is associated with modulation of plasma levels of Human Th1/Th2/Th17 cytokines. Increasing levels of some Human Th1/Th2/Th17 cytokines including IL-2, IL-6, IL-10, and TNF- $\alpha$ , and IL-17A probably suggests liver damage. On the other hand IL-6 and IL-17A seem to play a significant role in the pathophysiology of hepatotoxicity. Thus

plasma cytokine profile associated with HAART induced hepatotoxicity might be used either alone or with other biomarkers to assess HAART induced hepatotoxicity.

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### Abbreviations

ALT: Alanine aminotransferases, AST: Aspartate aminotransferases, AZT+3TC+NVP: Zidovudine/Lamivudine/Nevirapine, AZT+3TC+EFV: Zidovudine/Lamivudine/Efavirenz, BMI: Body Mass index, CBA: cytometric bead array, D: day, HAART: highly active antiretroviral therapy, IL: interleukin, (IFN)- $\gamma$ : interferon gamma, SEM: standard error of the mean TDF+3TC+EFV: Tenofovir/Lamivudine/Efavirenz, TNF- $\alpha$ : tumor necrosis factor-alpha

**Conflict of Interests:** The authors declare no conflict of interests regarding the publication of this paper.

### Ethics approval and consent to participate

This study protocol was reviewed and approved by the National Ethics Committee of Cameroon approved the study protocols (N<sup>o</sup>2016/01/685/CF/CNERSH/SP), and all patients gave their written consent.

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