



Immunophenotypic Characterization of ART-naive, HIV Infected Adults in Kombewa Sub-county, Kisumu: A Cross Sectional Study of Rural Population in Western Kenya

Jew Ochola^{1,3*}, Jackson C. Korir³, Walter Otieno², Julie Ake⁴, Christina Polyak⁴ and David H. Mulama³

Abstract

Reliable information on clinical immunophenotypic biomarkers is critical in the management of Hepatitis B and C infections especially in ART naive HIV positive adults. CD4 cell counts and the level of liver function bio-molecules can be monitored to assess the body status and treatment response to interventions. This was a cross sectional laboratory investigation study aimed at investigating the immunological phenotypes in HIV positive adults from Kisumu West Sub-county, so that overall patient care, participation of individuals in clinical trials and evaluation of adverse events can be improved. Median ALT values in Hepatitis C positive HIV positive ART Naive participants were higher than in the Hepatitis C negative participants ($p=0.0494$). The study underscores the need for monitoring liver enzymes levels in ART naive HIV positive patients as well, screening for viral hepatitis before initiation of Anti-Retro Viral therapy as these diseases pathologically alters the body's immune biomarkers.

Keywords

Immunophenotypic; Hepatitis C; ART naive; HIV positive; CD4 cell counts

Introduction

Proper management of common public health issues more so in developing countries where medical infrastructure is undeveloped is urgently needed. This calls for availability and proper understanding of appropriate biomarkers that can be readily accessed for monitoring disease progression, therapeutic intervention as well as prognosis that can be applied in treatment and clinical studies for reference purposes. These values are urgently needed in Sub-saharan African that is ravaged by both parasitic diseases such as malaria and Schistosomiasis, bacterial diseases as well as viral diseases such as HIV Aids [1]. Human Immunodeficiency Virus (HIV) infection and Acquired Immunodeficiency Syndrome (AIDS) are twin global health challenges [2]. Infected subjects are usually susceptible to multiple infections including hepatitis B and C. The common hepatitis

infection in HIV infected subjects is due to hepatitis B (HBV) and hepatitis C (HCV) [3]. The complications associated with HBV and HCV are the leading cause of chronic liver diseases especially in and immunological compromised patients [4]. Global HIV prevalence is estimated at 40 million while, chronic HBV and HCV infection accounts for an estimated 370 million, and 130 million respectively. It is well known that infections with HBV and/or HCV have a severe and invasive impact on the health of millions of people around the world, as these infections are often asymptomatic [5].

Reliable information on clinical immunophenotypic biomarkers is critical in the management of these diseases. CD4 cell counts and the level of liver function bio-molecules can be monitored to assess the body status and treatment response to interventions. World Health Organization (WHO) advises that appropriate mechanisms be applied when certain immunologic or clinical features are observed [6]. For instance, the WHO recommends initiation of HAART in HIV patients co-infected with hepatitis B or hepatitis C irrespective of value of CD4 count. The implication in HIV patients who have CD4 counts above 350 cells/ μ l but who may be positive for Hepatitis B and/or Hepatitis C is not factored in the treatment regimen of Highly Active Anti-Retroviral Therapy (HAART) until they get to end-stage liver damage [7]. There is need to establish reference values for liver enzymes to provide a guideline in monitoring of patients who may be prone to liver toxicity.

Although data on the epidemiology of these infections in the general population in Kenya exists, there is limited information on clinical biomarkers and immuno-phenotypic characteristics that may be putative in their management [8]. There is relatively scanty information regarding liver enzyme levels (hepatic biomarker characteristics) and immuno-phenotypic characteristics among ART naive HIV positive adults with or without viral hepatitis. Proper determination of the levels of clinical biomarker analytes in HIV positive ART naive adults with or without Hepatitis B and C is important in clinical management.

To date no study has been done to characterize the level of clinical markers and immunophenotypes in the vulnerable population within Kombewa. The aim of this study was to determine the levels of CD4 immunophenotypes in HIV positive adults from Kisumu West Sub-county so that overall patient care, participation of individuals in clinical trials and evaluation of adverse events can be improved.

Methods

Study site

The study was conducted in Kombewa Sub-Country, which is part of the Kisumu County, Kenya. The study site lies at 34°45' E 0°10' S, average elevation 1,289 m above sea level, near the northeast shore of Lake Victoria and 40 km northwest of the County capital of Kisumu town. This is a predominantly a rural population and mainly of the Luo ethnic group. The main economic activity of the inhabitants is fishing on Lake Victoria, the second fresh water lake in the world as well as other fresh rivers that drain into it. The stable diet is maize meal, sorghum, millet, cassava, fish and local vegetables. Farming,

*Corresponding author: Jew Ochola, HJF Medical Research International, Kisumu, Kenya, Tel: 0708987930; E-mail: ochojew@yahoo.com

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mostly of food crops, is done during the two rainy seasons; the “long rainy season” between March and May and the “short rainy season” between October and December, in a tropical climate, which are usually accompanied by spikes in malaria intensity. Basically, this region is a malaria holo-endemic region as well as other parasitic diseases such as Schistosomiasis.

Study population and participant recruitment

The Kombewa Center operates a Health and Demographic Surveillance System which conducts biannual demographic and syndromic surveillance surveys in addition to collecting population data (including in- and out-migrations, births, deaths and verbal autopsies to assign cause of death) for participating households [9]. The HDSS study area population of approximately 150,000 (a 369 square km area); serves as the catchment area for most epidemiologic and research studies conducted at the CRC in Kombewa. Every building within the HDSS area has been marked and, therefore has an address. The KHDSS is a longitudinal population registration system designed to track the evolving demographic and health status of the study populations over time. Various studies nested on this platform take advantage of the sampling frame inherent in the HDSS, whether at individual, household/compound or regional levels. This study comprised of adults aged 18 years and above who were enrolled in a larger cohort study to assess the impact of clinical practices, biological factors and socio-behavioral issues on HIV infection and disease progression in an African context.

Ethical considerations and approval

This study was approved by the KEMRI Ethics Review Committee (ERC) and the Walter Reed Army Institute of Research (WRAIR) Institutional Review Boards. The study was conducted in accordance with Good Clinical Practice (GCP), the Declaration of Helsinki and local rules and regulations of the Kenya Government Expert Committee on Clinical Trials of the Pharmacy and Poisons Board. The researcher requested an approval for the use of the data for this sub-study from the sponsor’s protocol chair and the study Principal Investigator. Written and signed informed consent was sought from study participants before samples were drawn.

Disclaimer

The investigators have adhered to the policies for protection of human subjects as prescribed in AR 70–25.

Blood collection, immunological analysis and quality control

Blood samples were collected by trained phlebotomist, by use of veni-puncture procedures from participants who obtained a written consent. Blood samples for the Hepatitis B and C and biochemical analysis were collected aseptically into 10 ml vacutainer tubes (Becton Dickson, New Jersey USA). Blood samples for CD4 analyses were collected in EDTA anticoagulated tube. Serum samples for serological assays for hepatitis B and C markers were stored at -20°C until testing time. The procedure for Hepatitis B Surface antigen ELISA summarily involved addition of 100 μl of the controls or specimens to the appropriate wells of the microwell plate. Two Positive Controls, two Low Positive Controls, and three Negative Controls were assayed on each plate. The plate was then covered with a plate sealer to minimize evaporation and then incubated for 60 to 65 minutes at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ using a dry-heat static incubator. At the end of the incubation period, the plate cover was removed and the fluid aspirated from each well

into a biohazard container. The plate was then washed a minimum of five times with a Wash Solution (at least 400 μl /well/wash), with a soaking duration of 30 to 60 seconds between each wash. The wash solution was aspirated after each wash and after the last wash; the inverted plate was blotted on clean, absorbent paper towels. 100 μl of Working Conjugate Solution was then added to each well containing a specimen or control. The plate was then covered with a plate sealer to minimize evaporation and then incubated for 60 to 65 minutes at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ using a dry-heat static incubator. At the end of the incubation period, the plate was then washed as in the steps above. 100 μl of the Working TMB Solution was then added to each well containing a specimen or control and incubated for 30 min to 33 min at room temperature (15°C - 30°C) in the dark. 100 μl of Stopping Solution was then added to each well to terminate the reaction and then the results of the absorbance read within 30 minutes after adding the Stopping Solution, using the 450 nm filters with 615 nm to 630 nm as the reference and results evaluated for acceptability.

ELISA procedure for determination of anti-HCV antibodies summarily involved addition of 200 μl of specimen diluents to all wells. 20 μl of the controls, calibrators or specimens were then added to the appropriate wells. The microwell strip holder was then covered with a plate sealer and incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 60 min \pm 5 min. The sample solutions were then aspirated from the micro wells then filled completely with wash buffer and washed for 5 times. The plate was inverted and firmly tapped on a clean paper towel to remove excess wash buffer, if necessary. 200 μl of conjugate was then added to all wells and then the micro-well strip holder was covered with a new plate sealer and then incubated at 37°C for 60 min \pm 5 min. The plate was then washed as above. The substrate solution, that contains OPD tablets, was then added to the plate and then incubated at room temperature in the dark for 30 min \pm 1 min. 50 μl of 4 N sulphuric acid was then added to all wells and the plate was read at a wavelength of 490 nm with a reference wavelength of 620 nm or 630 nm within 60 min following the addition of sulphuric acid. The results were then evaluated for acceptability.

CD4 determinations were done by use of FACS Count Flow cytometer analyser. These samples were analysed in whole blood using the standard operating procedures and as per the manufacture instructions. Serum Biochemistry levels were assessed the same day of blood collection by use of C111 biochemistry analyser. The serum biochemistries were evaluated using the standard procedures and as per the instrument manufacturer instructions to determine the serum levels for the clinical biomarker characteristics.

Data management and statistical analysis

The collected data was entered and organized into excel spread sheet and analysed using Graphpad prism V5 software. Both descriptive and inferential statistics were used in the analysis. Non-parametric methods were used for data that is not normally distributed. Categorical variables were analysed by use of fisher exact test. Spearman’s rank correlations were used in bi-variate analysis for non-normally distributed data. Due to data distribution and/or sample size, Mann Whitney test were used in comparison of categorical variables. $P < 0.05$ was considered statistically significant fewer than two tailed tests.

Results

The study investigated 57 (n=57) adult study participants from Kisumu West Sub-County who were ART naive HIV positive having or not the hepatitis B and hepatitis C. Residents of this locality are mainly of the Luo ethnic group and their economic mainstay is subsistence farming and fishing. The study had data set of 57 participants from Kisumu West Sub-County (n=57) that was above the calculated minimum sample size of 45 study participants. Majority (45.6%) of the study participants were from the age group 21 to 30 years, while 21.0% of the study participants were illiterate. More than 96% of the population was from the Luo ethnic group (Table 1).

Table 1: Socio-demographic characteristics of the study participants in the immunophenotypic arm of the study.

Characteristics	Gender		Total (%)
	Males n (%)	Females n (%)	
Age bracket (years)			
21 to 30	7(26.9)	19(73.1)	26(45.6)
31 to 40	12(54.5)	10(45.5)	22(38.6)
41 to 50	2(33.3)	4(67.7)	6(10.5)
51 to 60	1(33.3)	2(67.7)	3(5.3)
Marital status			
Single	8(32)	17(68)	25(43.9)
Married	9(64.3)	5(35.7)	14(24.6)
Divorced	2(67.7)	1(33.3)	3(5.3)
Widowed	3(60)	2(40)	5(8.8)
Religion			
Christian	22(39.3)	34(60.7)	56(98.2)
Muslims	0(0.0)	1(100.0)	1(1.8)
Tribe/ethnicity			
Luo	21(38.2)	34(61.8)	55(96.5)
Luhya	1(50.0)	1(50.0)	2(3.5)
Other	0(0.0)	0(0.0)	0(0.0)
Education			
Certificate and above	5(45.5)	6(54.5)	11(19.3)
High school	6(33.3)	12(67.7)	18(31.6)
Primary school	7(43.8)	9(56.2)	16(28.1)
Illiterate	4(33.3)	8(67.7)	12(21.0)

Socio-geodemographic characteristics of study participants showing age, marital status, religion and tribe. The percentages are shown in the parenthesis.

Elevated liver enzymes clinical biomarker in HIV positive ART naive adults Co-infected with Hepatitis

Initiation of anti-retroviral therapy requires a proper understanding of the health status of HIV infected individuals, as these drugs have been known to lead to liver toxicity and pathology. The study examined the levels of liver enzymes (ALT and AST) in HIV sero-positive individuals who were either infected with hepatitis B and hepatitis C.

Effect of Hepatitis B on liver enzymes

To investigate if Hepatitis B infection affected Liver enzymes in the study participants from Kisumu West, the levels of AST and ALT were compared in HIV patients. It was observed that there were no significant differences in the median levels of ALT and AST. The median ALT value in hepatitis B positive individuals was 20.35 IU/L compared to the median value in hepatitis B negative individuals which was 20.3 IU/L ($p=0.6282$). For AST values, the median AST value in hepatitis B positive individuals was 30.0 IU/L compared to the median values in hepatitis B negative individuals which was 26.2 IU/L ($p=0.7786$). Figure 1 shows the levels of clinical biomarkers in hepatitis B positive adults compared to hepatitis B negative adults.

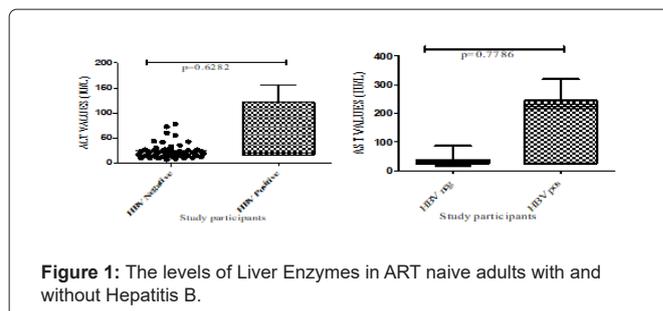


Figure 1: The levels of Liver Enzymes in ART naive adults with and without Hepatitis B.

Effect of Hepatitis C on liver enzymes

The study found that there were significantly high differences in the levels of liver enzymes in the HIV positive participants who were hepatitis C positive compared with those who were negative. The median ALT value in hepatitis C positive individuals was 30.8 IU/L compared to the median values in Hepatitis C negative individuals which was 19.8 IU/L ($p=0.0494$). For AST values, the median AST value in hepatitis C positive individuals was 42.4 IU/L compared to the median values in Hepatitis C negative individuals which was 29.4 IU/L ($p=0.3630$) (Figure 2).

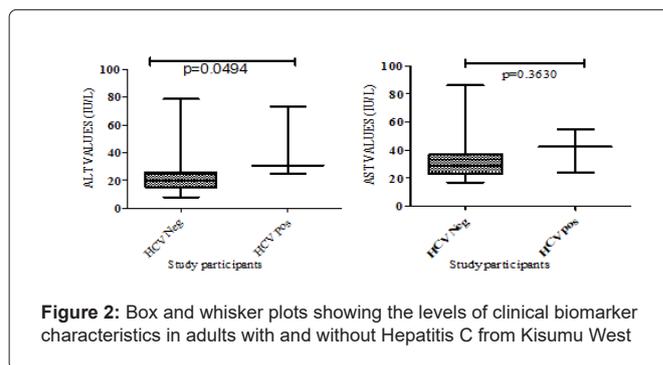


Figure 2: Box and whisker plots showing the levels of clinical biomarker characteristics in adults with and without Hepatitis C from Kisumu West

Table 2 below shows a summary of the clinical biomarker levels in the different groups of the participants in the study. This table shows the summary of median levels of clinical biomarker characteristics in HIV positive ART Naive adults from Kisumu West (Table 2).

Table 2: Summary of the median levels of the clinical Biomarker characteristics in HIV positive ART Naive individuals in the study.

	Median Levels of clinical Biomarker Characteristics (IU/L)					
	Hepatitis B status			Hepatitis C status		
	HBV Neg	HBV Pos	P values	HCV Neg	HCV Pos	P values
ALT	20.3	20.35	0.6282	19.8	30.8	0.0494
AST	26.2	30	0.7786	29.4	42.4	0.363

Immunophenotypic characterization of HIV infected ART naive adults with hepatitis B and C co-infections

The study also examined the levels of CD4 immuno-phenotypes in individuals with and without hepatitis B and hepatitis C co-infections in HIV positive ART naive adults.

Hepatitis infection and immunophenotypes

There were no significant differences in the median levels of CD4 in the HIV positive ART naive adults who had hepatitis B positive against those who didn't have hepatitis B. the median CD4 value in hepatitis B positive individuals was 519.5 cells/μl compared to the median values in Hepatitis B negative individuals which was 378 cells/μl (p=0.6732).

Similarly for hepatitis C, there were no significant differences in the median levels of CD4 in hepatitis C positive individuals compared to the median levels in hepatitis C negative individuals (p=0.2918) (Figure 3).

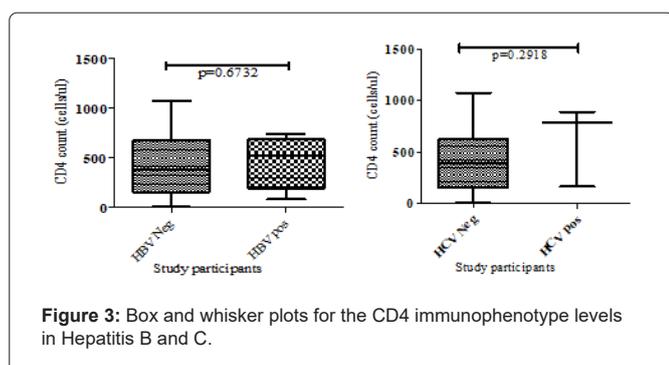


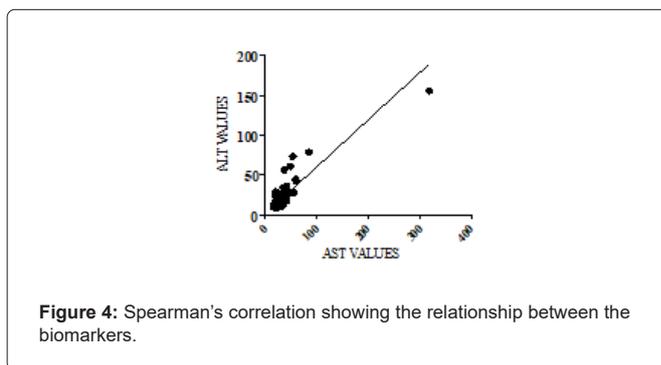
Table 3 shows the summary of the median levels of the immunophenotypes in HIV positive ART naive individuals in the study.

Table 3: Summary of the median levels of CD4 immunophenotypes in HIV positive ART naive adults in the study.

	Median Levels of CD4 immunophenotypes					
	HBV Neg	HBV Pos	P values	HCV Neg	HCV Pos	P values
CD4 levels	378	519.5	0.673	392.5	784	0.292

The relationship between the immunophenotypes and liver enzyme biomarker characteristics

To investigate the relationship between the biomarkers, spearman's correlation analysis was used to do this. The rationale for studying this is that for example, liver dysfunction is a real challenge in the management of HIV infected patients. It is important to monitor trend of these liver functions especially before introduction of anti-retrovirals in order to evaluate effects that could result. It was observed that there was a positive correlation between where a rise in ALT was correlated with a rise in AST. Figure 4 shows the correlation of the clinical biomarkers and the immunophenotypes.



Discussion

This study looked at the immuno-phenotypic and hepato-biomarker characteristics of HIV positive adults' not on anti-retroviral drugs that were infected or not with hepatitis B and C in Kisumu West Sub-County. Most studies have focused on different aspects of HIV positive group on ART since they are the majority and they are mostly accessible. As some modes of exposure/transmission to HIV virus are almost the same as in viral hepatitis relating blood infections. This necessitates studying the immune-phenotypic and hepato-biomarker characteristics in this group of ART naive individuals. The study also assessed the relationship between the immunophenotypic and hepato-biomarker characteristics.

Characterization of HIV positive ART naive adults with hepatitis B and C co-infections

In this study, regarding liver enzymes, there was a significant increase in the serum liver enzymes ALT in HIV-HCV co-infected people as compared to hepatitis C negative individuals. Although the study participants who were co-infected with the viral hepatitis and HIV were few, the study supports other studies conducted by Gui et al., who reported that many people with chronic hepatitis have elevated liver enzymes levels [10].

ALT is produced in hepatocytes, the major cell type in the liver. ALT is often inaccurately referred to as a liver function test. The level of ALT in the blood may be elevated in conditions in which hepatocytes are damaged or die. As cells are damaged, ALT leaks out into the bloodstream. All types of hepatitis (viral, alcoholic, drug-induced, etc.) cause hepatocyte damage that can lead to elevations in the serum ALT activity. The ALT level may also be elevated in cases of liver cell death resulting from other causes, such as shock or drug

toxicity. The level of ALT may correlate roughly with the degree of cell death or inflammation, however, this is not always the case. An accurate estimate of inflammatory activity or the amount cell death can only be made by liver biopsy [11].

AST is an enzyme similar to ALT but less specific for liver disease as it is also produced in muscle and can be elevated in other conditions (for example, early in the course of a heart attack). AST is also inaccurately referred to as a liver function test by many physicians. In many cases of liver inflammation, the ALT and AST activities are elevated roughly in a 1:1 ratio. In some conditions, such as alcoholic hepatitis or shock liver, the elevation in the serum AST level may be higher than the elevation in the serum ALT level. (Jens et al., web page). In a similar research in Northern Nigeria, Otegbayo et al. reported a high mean level of ALT among HIV patients co-infected [12]. Increased levels of liver enzymes observed among HIV patients co-infected with HCV in this present research may be because of the asymptomatic nature of HCV that leads to late diagnosis. At this stage, the patient might have developed most symptoms and thus, in chronic stage of the infection since 70-80% of people infected with HCV develop chronic infections [13]. Even though the normal levels of AST and ALT vary depending on the individual, the range for normal AST is reported between 10-40 U/L and ALT between 7-56 U/L. More specifically, the recommended AST values are 14-20 U/L for males, and 10-36 U/L for females. 44 (77.19%) participants had AST values within the recommended range while 52 (91.23%) of the participants had ALT values within the recommended range.

Liver enzyme elevations are a frequent finding in HIV-infected patients as a consequence of several risk factors, however the analysis of these events is limited as precise etiology is rarely clearly defined. Abnormalities in liver function tests could be produced exclusively by direct inflammation in hepatocytes, caused by the HIV virus. Although the mechanisms by which HIV causes hepatic damage are still unknown, studies have shown that it may be as result of apoptosis (induced by caspases 2,7 and 8) and mitochondrial dysfunction with decreasing mitochondrial DNA in several tissues. Another injury mechanism is permeability alteration in mitochondrial membrane by HIV proteins which stimulate an inflammatory response [14]. Alanine aminotransferase (ALT) is a hepatic enzyme that may be used as a marker of hepatocellular injury. However, the impact of viral hepatitis on the immune system and liver enzymes needs further studies in both on HAART and HAART naive HIV positive patients.

The results for this study showed an overall median CD4 count of 433 cells/mm³ and median of 407 cells/mm³ for the 57 adult participants studied. CD4 levels were studied in adults who had hepatitis B and C against those who were negative for hepatitis B and C. In this study there were no significant differences in the median CD4 levels in the HIV positive ART naive adults from Kisumu West who are either co-infected or not with hepatitis B and C infections. This was inconsistent with a study done by Mohammad that reported that within a NVS cohort, individuals without chronic HCV had a statistically significant elevation in mean CD4 count compared to those NVS with chronic HCV. The correlation of CD4 count with HCV status in the study was statistically significant for earliest recorded CD4 count and there was a trend toward significance with the most recent CD4 count as well [15]. In this study there was a similarly statistically significant elevation in the mean CD4% and mean CD4/CD8 ratio in those without chronic HCV compared to the chronic HCV group.

However, in the current study from Kisumu West, the researcher acknowledges a limitation of low numbers of Hepatitis positive cases partly due to low prevalence of Hepatitis in the area thus limiting adequate conclusions to be drawn from this.

In this study of 22 males and 35 females, there was also difference on the mean CD4 values in relation to gender. The median CD4 value in the females was slightly higher than males (437 cells/mm³ in females compared to 427 cells/mm³ in males). Similar findings as for this were observed in studies that have been conducted in Nigeria [16]. The higher CD4 values in females compared to males could probably be due to biological factors. It has been speculated that gender and age-related variations within the immune system parameters may contribute to the pathogenesis of several gender and age-related diseases such as autoimmune disorders in female patients [17,18]. Further, it has been shown that there are gender differences in the generation of CD8 cells during HIV-1 infection, due to increased immune activation compared to men [19]. Since the number of subjects with Hepatitis B and C were few, comparison of the positive Hepatitis B and C individuals in terms of gender could not be done to effectively yield statistical significant results.

Globally, the recommended CD4 range is 400 –1600 cells/mm³. Some people would be initiated to medication when their CD4 counts reaches 350 cells/mm³. On the other hand, according to AIDS.gov, one of the qualifications for an AIDS diagnosis is CD4 count less than 200 cells/mm³. Almost half of the patients (49.1%) had CD4 count less than 400 cells/mm³. Those who required to be initiated on medication were 42.10%, while 28.07% had CD4 counts less than 200 cells/mm³.

In this study, again it was not possible to characterize the immune and biomarker characteristics in the viral hepatitis positive individuals. This is because even in the general population prevalence of the viral hepatitis is low and so getting higher numbers to be studied sometimes is hard. This study only had four individuals who were positive for hepatitis C and four who were positive for Hepatitis B, one of these who had Hepatitis B and C co-infection. Characterizing these on their own would not provide statistical significant results, and thus causality could not be inferred.

The relationship between the phenotypic and clinical biomarkers in HIV positive ART naive adults with or without Hepatitis B and C

To investigate the relationship between the biomarker analytes correlation analysis was used to do this. Rationale for this is that for example, liver dysfunction is a real challenge in the management of HIV infected patients, thus evaluation of the relationship of the liver enzymes levels is necessary. Especially a proper understanding on how this is depicted in HIV positive ART naive patients is required. This is due to the fact that the use of ART has completely modified the pattern of hepatic events in HIV infection resulting in significant decrease in morbidity and mortality among HIV infected patients [14]. As seen above, ALT could be used as markers of hepatocellular injury. Liver enzyme elevations are frequent in human immune deficiency virus (HIV)-infected patients which may be caused by the HIV virus in those without other risk factors for liver damage. Patients with high ALT should be evaluated for early start of antiretroviral treatment in those without risk factor for liver damage regardless of the CD4+ cell count, especially where facility for estimating viral load is not available.

Limitations

Further, similar study of clinical characterization and immunophenotypes that would utilize higher number of hepatitis B and C positive individuals and with higher number of individuals in general could be warranted for proper characterization of the immuno-phenotypic and hepato-biomarker characteristics. The sample size could have been small for this study due to the criteria of limited data that was only accessible in ART naive individuals.

Conclusion

There were significant differences in regard to the median levels of the ALT in hepatitis C infected group compared to hepatitis C non infected participants. For the CD4 immuno-phenotypes and clinical biomarker analytes (ALT and AST enzyme levels) there were no significant differences in both the HIV positive ART naive adults with or without Hepatitis B and C infections. However, before arriving at a conclusion, a study with more respondent who tested positive for Hepatitis B or C would give a correct picture. Screening for viral hepatitis should also be performed alongside HIV testing in order to detect it early for effective therapy and good prognosis.

Competing interests

The authors declare no financial or personal relationships that would influence them in developing the article.

Author contributions

Authors CP and JA conceived and designed the experiment. Author JO conducted the laboratory aspects of the study, performed data analysis and drafted the manuscript. Author JK and DHM helped in professional guidance, data analysis and reviewing the manuscript. All authors read and approved the final manuscript.

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Author Affiliations

Top

¹HJF Medical Research International, Kisumu, Kenya

²U.S. Army Medical Research Directorate – Africa, Kisumu, Kenya

³Department of Biological Sciences, Masinde Muliro University of Science and Technology, Kakamega County Kenya

⁴Henry M Jackson Foundation for the Advancement of Military Medicine, Bethesda, USA

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