ISOLATIONS AND CHARACTERISATION OF SOME GUT MICROBIOMES IN HIV POSITIVE INDIVIDUALS IN JOS, NIGERIA

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ABSTRACT

Background: The human gut microbiota has an important implication in the maintenance of human health and disease pathogenesis. Recent research has shown that gut microbial imbalance, or dysbiosis, may lead to microbial gut translocation and chronic inflammation in HIV-infected individuals, further enhancing HIV progression, and potentially towards the development of AIDS. The isolation and characterization of some gut microbiomes in HIV positive individuals in Jos Nigeria as well as the comparison of the blood parameters would improve patient management since gut microbiota in HIV individuals can have an over-representation of pro inflammatory Proteobacteria, associated with mucosal and systemic immune activation.

Methodology: This research investigated the gut microbiome using feacal samples and compare the Haematological, blood chemical, immunological and virological parameters of 5 naive HIV-infected patients, 10 treated HIV-infected patients (less than 10years on ART and more than 10 years on ART) and five (5) samples from healthy individual which served as the controls.

Result: Results showed that faecal samples from both HIV negative controls and HIV-infected individuals had dominant taxa from the phyla Firmicutes, Bacteroidetes and Proteobacteria. There was relative abundance of Firmicutes (**42.2**%), Bacteroidetes (**57.7).** The analysis shows a significant decrease in the Bacteroidetes (coliforms) count while a significant riseof the abundance of Firmicutes (clostridia) with HIV progression. The fecal microbiota of individuals on ART for more than ten years exhibited significantly higher relative abundances of Clostridium cluster compared to HIV negative individuals. The total bacteria count had the highest m abundance (**27.7%**), followed by Bacteroides counts (**24.4%**), Clostridia counts (**19.8%**), coliforms counts (**18.2%**) while the lowest was Lactic Acid Bacteria counts (**9.9%**). Bacteroides counts was high among HIV patients on drugs for more than ten years. The Bacteroidetes (coliforms) counts was highest in HIV-negative controls while the Firmicutes (clostridia) count was highest among HIV patient who were on drugs for more than ten years. The magnitude of divergence from HIV-negative microbiota samples does not correlated with CD4+ T cell count or plasma HIV-1 RNA viral load (all Spearman correlation p-values > 0.73). There was significant increase in the Haemoglobin, packed cell volume and platelets count with HIV progression due to the dysbiosis in the gut and likely bacteria translocation that invade the gut with a significant increase of monocytes with HIV progression.

Conclusion: This study is important for public health because it provided new insights into intestinal microbiome symbiosis related to HIV-1 infection. Immune status and ART were the key factors interactively affecting the gut microbiome. It suggests that microbiome composition influences the progression of HIV infection.

INTRODUCTION

The mechanism that HIV over activates the immune system is still unclear to researchers. The leaky gut theory may be associated with this immune over activation. This theory implies that bacteria/bacterial products such as lipopolysaccharides (LPS), translocate out of the gastrointestinal tract (GIT), due to an increased permeability of the GIT and overall decreased mucosal barrier integrity (e.g., tight junctions decline), and into the blood, causing a systemic chronic immune activation (Wu S *et al.,* 2015). Chronic immune activation is detrimental to individuals infected with HIV. Increased T cell turnover creates an imbalance in the immune homeostasis and results in T-cell half-life decrease, T cell clonal exhaustion, and possibly depletion of memory T cell pools; additionally, chronic immune activation leads to constant T cell generation, and subsequently driving viral replication (Doitsh *et al.,* 2014)

A study conducted in China evaluated uninfected and chronically HIV-infected human stool samples for alpha (diversity within samples) and beta diversity (diversity between samples) and discovered an increase in the phyla Firmicutes and Proteobacteria in chronic HIV-infected patients, in comparison to non-HIV infected controls (Yang *et al.,* 2016). In the same study, an increase of Bacteroides and Arabacteroides were also observed in chronically infected patients. There have been conflicting results on the changes of the microbiome regarding HIV infection. Several studies, (Vazquez-Castellanos *et al.,* 2015) showed an increase of Prevotella and a loss of Bacteroides in HIV infected individuals; whereas, other studies have shown the opposite effects (Nowak *et al.,* 2015) or no difference in these two genera (McHardy *et al.,* 2013).

HIV disrupts the overall immune system by destroying CD4+ T cells and allowing for opportunistic infections to occur, eventually leading to the development of AIDS. However, it is not fully understood what makes an individual susceptible to developing AIDS or the exact sequence of pathogenesis from HIV towards the development of AIDs. This study helps to further develop an understanding of the isolation and characterization of microbial composition and microbial products influencing the pathogenesis of progressive HIV infection. Recent research has shown that during HIV infection, Gut microbiota modifications have recently been associated with inflammation and microbial translocation in HIV-infected individuals, further enhancing HIV progression, potentially towards the development of AIDS. It is based on the facts above that this study investigated the usefulness of gut microbiome since many microbes in the community are as yet unidentified in our present study population.

This study will help further develop an understanding of the gut bacterial changes and their characterization profile in HIV-positive individuals in Jos and how microbial composition and products influence the pathogenesis of progressive HIV infection.

This study aims to determine the microbiome community in HIV patients accessing treatment alongside the effect of ART on the microbiome community and to compare the Haematological, blood chemistry, immunological and virological parameters of HIV patients with gut microbiome and normal individuals within Jos Metropolis.

MATERIALS AND METHODS

Study location: The study was carried out among HIV positive patient's adult above 18years attending APIN JUTH, Jos Nigeria. Exclusion criteria included antibiotic treatment within the three previous months, not having TB and body mass index

Ethical Clearance: Ethical approval was gotten from the Jos University Teaching Hospital Research and Ethics Committee.

Study population: The study was carried out on HIV positive patient's adult above 18years attending APIN JUTH, Jos Nigeria. Exclusion criteria included antibiotic treatment within the three previous months, not having TB and body mass index <18.5 or ≥25 kg/m2.

Preparation of volunteers: The HIV positive patients were encouraged to provide aliquot of stool samples for analysis while blood samples were collected for

Haematology, Blood Chemistry and Plasma Viral load.

Sample size/ Collection of Samples

A total of twenty (20) stool and blood samples each were collected. Fifteen (15) samples were collected from HIV positive patients while five (5) samples from healthy individuals which served as the controls. Stool samples were collected from all patient and analyzed for gut bacterial micriobiome community and their blood samples assessed for Haematological indices, Blood Chemistry, CD4 and Plasma Viral load.

General Asepsis: All methods of sample collection were carried out under aseptic conditions. Workbenches were made aseptic by cleaning with sterilizing reagents, flaming the environment via a lit gas burner.

Stool Samples: All of the faecal samples were properly handled and collected in disposable plastic sterile dung cups. All samples arrived the laboratory within 24h and were immediately frozen at -20 °C and stored until analyzed.

Fecal Characteristics: Fecal characteristic such as color, texture, presence of blood and worms were observed in the feces of HIV/AIDs patients. The faecal samples were collected from fifteen (15) patients who have HIV infection and five (5) healthy individuals and stored in -80 freezer.

Blood Samples: Blood samples were collected from HIV/AIDS patients attending Jos University Teaching Hospital for Haematological indices, Blood Chemistry, CD4, and Plasma Viral load.

Collection of Blood: Blood samples were collected with care and adequate safety precautions to ensure test results are reliable, contamination of the samples was avoided and infection from blood transmissible pathogens was prevented. Protective gloves were worn when collecting and handling blood samples. Needles, and syringes were sterile, and dry, and blood collecting materials were discarded safely to avoid injury from needles.

Analysis of Blood sample for Blood Chemistry: Venous blood was drawn into plain-filled tubes and was analyze using COBAS C311 autoanalyzer. Samples were allowed to clot and the serum separated and slotted into analyzer, Results were copied from the displayed on an LCD screen and/or in printed copy

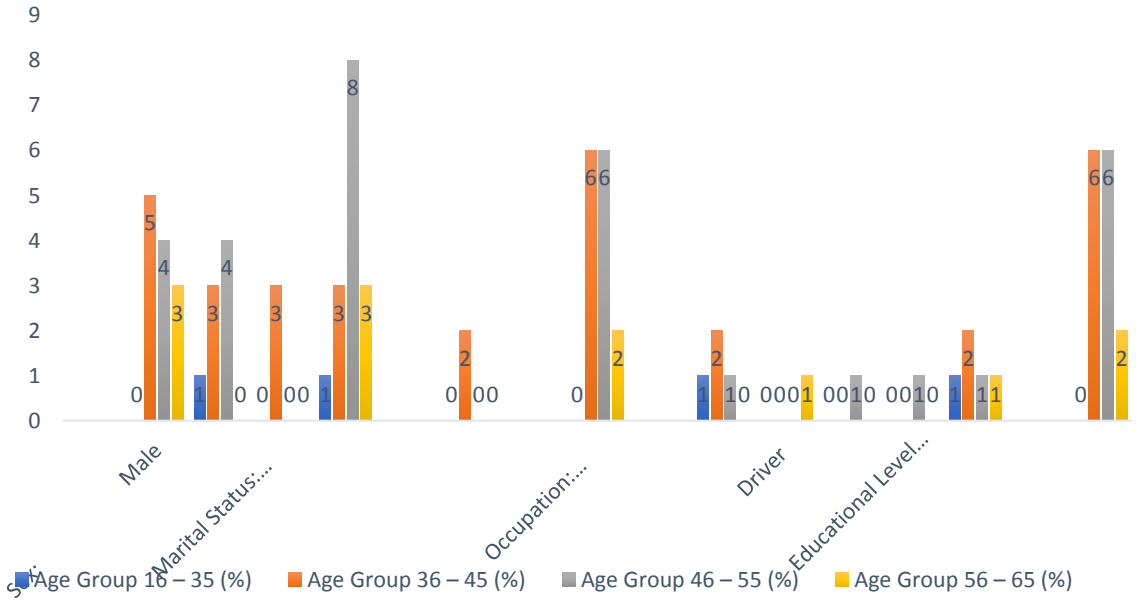
Analysis of Blood sample for CD4: Venous blood was drawn into EDTA-filled tubes and was analyzed using Cyflow counter autoanalyzer. Devices have inbuilt test processes with compartment for reagents and wash solutions. Samples were slotted into analyzer or aspirated, Results was copied from the displayed on an LCD screen and/or in printed copy

STATISTICAL ANALYSIS

Numerical Data was obtained from the experiment and these data were analyzed using MS--Excel and the data presented as means and standard deviations. The Significant difference between means were analyzed using ANOVA and Regression analysis with a significance level of $p<0.05$

Table 1: Demographic Characteristics of the study population of HIV patients receiving Antiretroviral Drugs (ART) in Jos University Teaching Hospital based on Age

Characteristics		Age Group				(N=20)
		16 – 35	36 – 45	46 – 55	56 – 65	
Sex:	Male	0	5	4	3	12 (60)
	Female	1	3	4	0	8 (40)
Marital Status:	Single	0	3	0	0	3 (15)
	Married	1	4	7	3	15 (75)
	Divorced	0	2	0	0	2 (10)
Occupation	C/Servant	0	6	6	2	14 (70)
	Business	1	2	1	0	4 (20)
	Driver	0	0	0	1	1 (5)
	Farmer	0	0	1	0	1 (5)
Educational Level	Primary	0	0	1	0	1 (5)
	Secondary	1	2	1	1	5 (25)
	Tertiary	0	7	5	2	14 (70)



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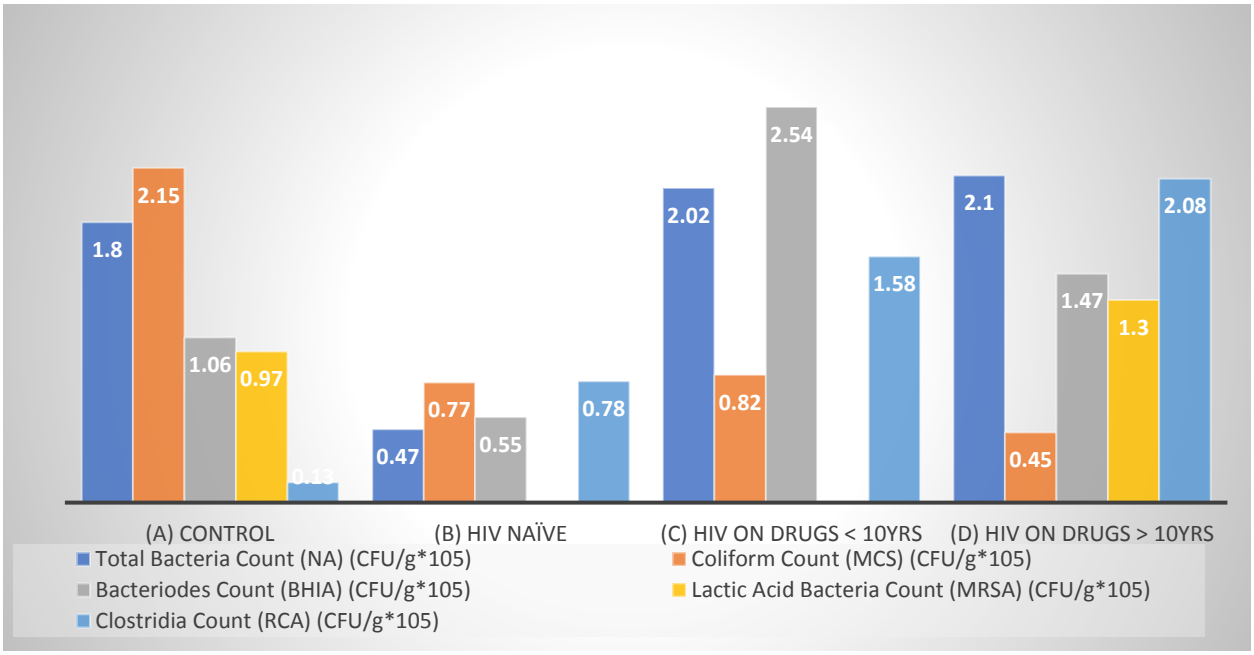

Figure 2: Relative Normal Abundance (Bacterial Load) of Gut Microbiota of HIV patients receiving Antiretroviral Drugs (ART) in Jos University Teaching Hospital

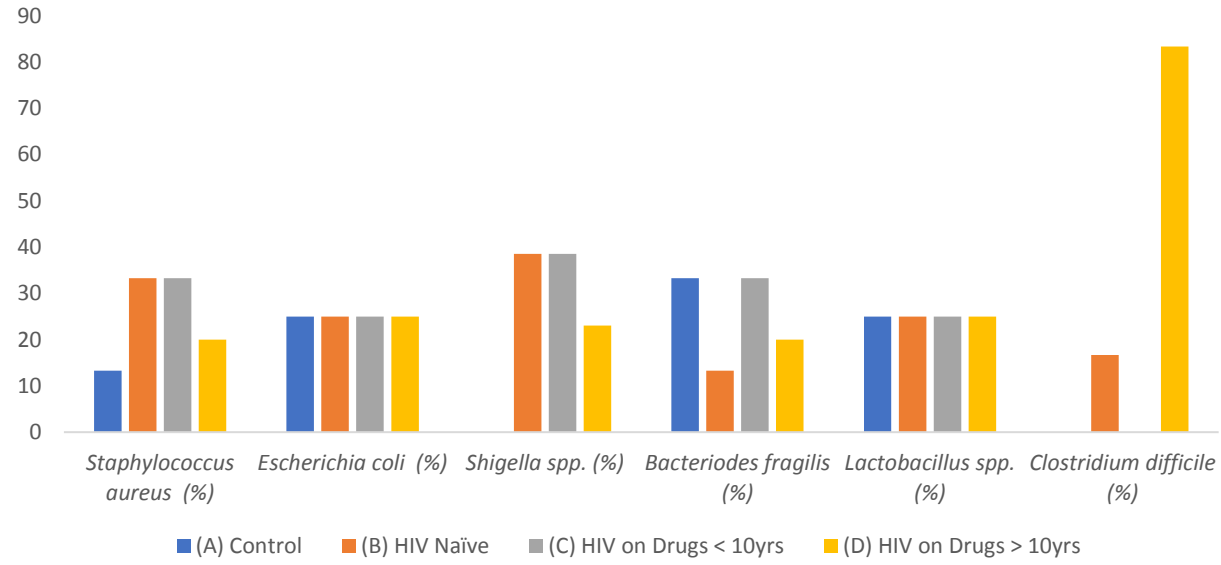

Figure 3: Frequency of occurrence of Bacterial isolate from stool sample of some HIV patients receiving Antiretroviral Drugs (ART)

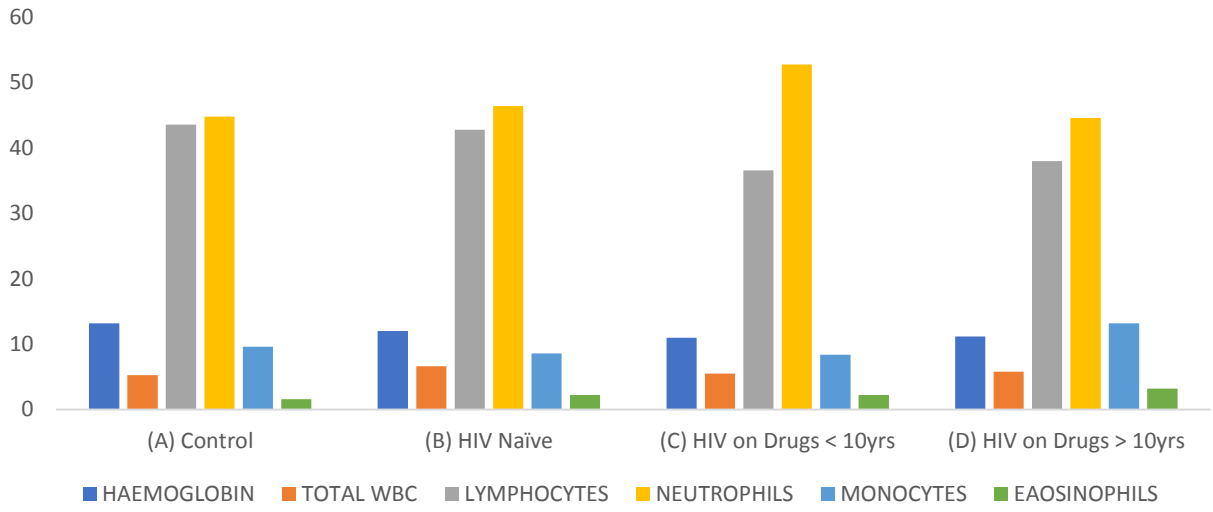

Figure 4: Haematological parameters of some HIV patients receiving Antiretroviral Drugs (ART Biochemical, immunological and Virological parameters of some HIV patients with respect to parameter tested.

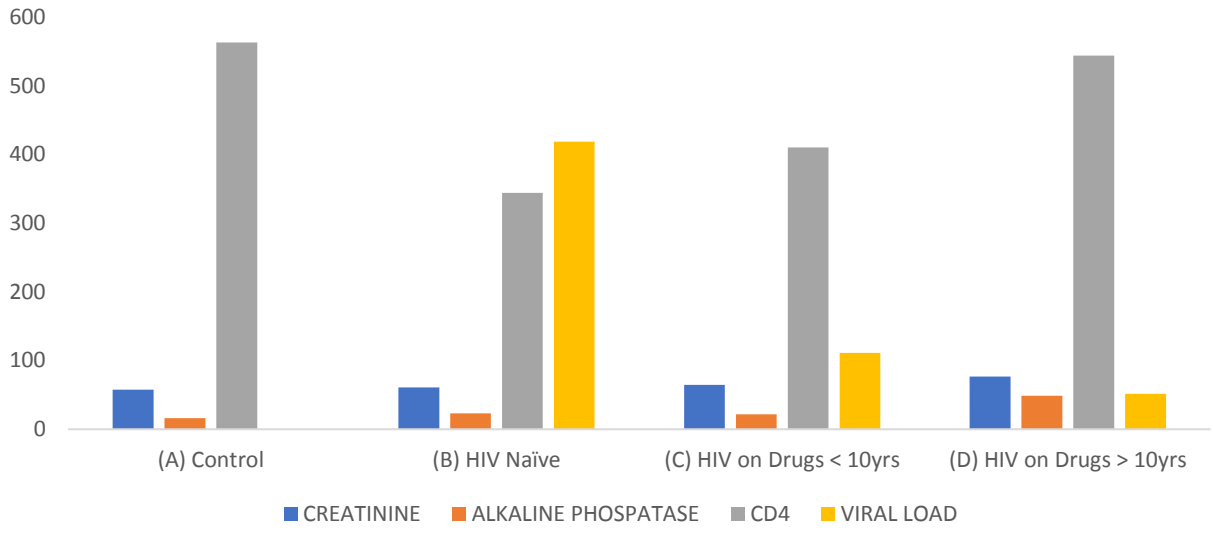

Figure 5: Biochemical, immunological and Virological parameters of some HIV patients with respect to parameter tested

Table 2: Statistical analysis of the Bacteria counts using ANOVA

Group of Patients	Number	Total Bacteria Count (NA) (CFU/g*10^5)	Coliform Count (MCS) (CFU/g*10^5)	Bacteriodes Count (BHIA) (CFU/g*10^5)	Lactic Acid Bacteria Count (MRSA) (CFU/g*10^5)	Clostridia Count (RCA) (CFU/g*10^5)
(B) HIV Naïve	5	0.244	0.163	0.841	0.252	0.154
(C) HIV on Drugs < 10yrs	5	0.810	0.087	0.610	0.249	0.422
(D) HIV on Drugs > 10yrs	5	0.479	0.029 [a]	0.660	0.689	0.023 [a]

a. The mean difference is significant at the 0.05 level.

Table 3: Statistical analysis of the Biochemical, Immunological and Virological Parameters counts using ANOVA

GROUP OF PATIENTS	CREATININE	ALKALINE PHOSPATASE	CD4	VIRAL LOAD
(B) HIV Naïve	0.689	0.550	0.429	0.044*
(C) HIV on Drugs < 10yrs	0.373	0.644	0.062	0.570
(D) HIV on Drugs > 10yrs	0.026*	0.012*	0.011*	0.012*

*. The mean difference is significant at the 0.05 level.

Table 4: Statistical analysis of the Hematological Parameters counts using ANOVA

GROUP	HAEMO GLOBIN	T.WBC	LYMPHO CYTES	NEUTRO PHILS	MONOC YTES	EAOSINO PHILS	BASOP HILS	PLATE LETS
(B) HIV Naïve	0.209	0.101	0.879	0.213	0.569	0.379	0.379	0.249
(C) HIV on Drugs < 10yrs	**0.029***	0.798	0.193	0.975	0.496	0.379	0.379	0.208
(D) HIV on Drugs > 10yrs	**0.044***	0.525	0.293	0.799	**0.033***	**0.028***	0.379	0.113

*. The mean difference is significant at the 0.05 level.

DISCUSION:

In this study, we focused on the differences in microbial composition among healthy non-HIV infected controls and those who had HIV, as well as any microbial compositional changes between AIDS development time points with respect to age and year of ART management.

We compared the faecal bacterial microbiome, hematological, biochemical, immunological and viral load parameters among age-matched and period of individuals been on ART. We found that both healthy controls and HIV-infected were colonized by 3 main phyla: Bacteroidetes, Firmicutes and Proteobacteria. This confirmed previous gut phyla characterizations in HIV infection (Mahowald *et al.,* 2009). Within these

main phyla we saw the greatest microbial abundance among the gram-negative genera Prevotella, Bacteroides, and Ruminococcus in both our healthy controls and HIV patients. The Bacteroidetes number (57.7%) while the Firmicutes number (42.2%). The fecal microbiota of individuals on ART for more than ten years exhibited significantly higher relative abundances of Clostridium cluster compared to HIV negative (Fig. 2, 3, Table 2). HIV negative individuals had increased Bacteroidaceae (Bacteroides), (Fig. 2, 3, Table 2).

In HIV negative controls, we saw a high total bacterial count but reduced bacteria count in naïve HIV patients with very little changes in patients who had been on drugs. The total bacteria count had the highest (27.7%), Bacteroides counts (24.4%), Clostridia counts (19.8%), coliforms counts (18.2%) while the Lactic Acid Bacteria counts (9.9%). Bacteroides counts was high with HIV patient on drug more than ten years. The Bacteroidetes (coliforms) counts was highest in HIV negative control while the Firmicutes (clostridia) count was highest in HIV patient who are on drugs for more than ten years. The analysis shows a significant decrease in the Bacteroidetes (coliforms) count with HIV progression while a significant rise with Firmicutes (clostridia) with HIV progression. Mouse experiments have supported that a Bacteroides-poor microbiota in the context of a Western diet may have negative health effects (Gauffin Cano *et al.*, 2012).

We enrolled 15 HIV-infected patients naïve and treated with HAART to identify any microbial differences among HIV-negative patients, HAART-treated individuals and healthy controls. The results showed that microbial diversity was increased after HAART; these effects were most apparent as increased levels of Bacteroides, Blautia and Faecalibacterium (Fig. 2 and 3). Since HIV-positive individuals suffer from increased incidence of diarrhea in the absence of obvious enteric pathogens and increased intestinal inflammation (Bjarnason *et al.*, 1996; Brenchley and Douek, 2008), we had expected an expansion of bacteria that increase with other chronic intestinal inflammatory diseases.

The faecal microbiota of HIV-1-infected patients was not completely restored after therapy, no chronically infected individual sampled on ART exhibited a strong shift towards the HIV-negative individuals (Fig. 2, 3), indicating that short-term ART was insufficient to restore the microbiota. However, the microbiota of individuals treated on ART above ten years for whom we did collect stool and blood samples showed a closer resemblance to HIV-negative individuals than on HIV patients less than ten years of infection (Fig.2, 3). HIV infected individuals on ART less than ten years had significantly higher bacterial count compared to the naïve subjects, the Negative subject and ART patients more than ten years were not significantly different from each other (Table 2).

It is important to note that although the HIV negative individuals presented microbial composition, the bacterial count and blood parameters were extremely low compared to the positive control. It is believed that the concentration is too low to influence the data. Overall, our descriptive study revealed that HIV negative individuals' microbiome showed an increase in the genera Bacteroides, and Ruminococcus compared to HIV naïve and those on drugs.

Consistent with an important role for adaptive immunity in modulating interactions between intestinal bacteria and blood parameters. The analysis of the blood samples also revealed a lot of investigations. There was significant increase in the Haemoglobin, packed cell volume and platelets count with HIV progression. Due to the dysbiosis in the gut and likely bacteria translocation that invade the gut, there was also a significant increase of monocytes with HIV progression. To investigate whether disease severity impacted gut microbiota in HIV infection, we determined whether microbiota diversity correlated with peripheral CD4+ T cell count or plasma HIV-1 RNA viral load in 15 individuals with naïve and those who were on ART. As an estimate of divergence from healthy, we calculated the correlation between each HIV microbiota sample and HIV negative control samples. The magnitude of divergence from HIV-negative microbiota samples does not correlated with CD4+ T cell count or plasma HIV-1 RNA viral load (all Spearman correlation p-values > 0.73).

Several studies have suggested that HIV infection results in increased gut permeability and translocation

of microbial products into circulation (Dinh *et al.*, 2015).

The effect of HIV infection on the innate arm of the immune system is not well-understood. Acute HIV infection is associated with a dramatic increase in inflammatory (non-classical CD16+) monocytes (Campbell *et al.*, 2014). This population has been shown to remain elevated throughout the first year of HAART treatment (Han *et al.*, 2009). Monocytes are susceptible to HIV infection and can serve as a reservoir for latent HIV (Campbell *et al.*, 2014). The reduction in circulating monocyte numbers could potentially be due to low levels of viral reactivation (Han *et al.*, 2009).

Recently it was shown that innate lympoid cells regulate CD4+ T cell responses to intestinal bacteria (Hepworth *et al.*, 2013). CD4+ T cell-microbe interactions are perhaps best understood for Bacteroides fragilis, which is in a genus that consistently and dramatically decreases with HIV infection. Because HIV targets central players of innate and adaptive immunity including CD4+ T cells, monocytes, and macrophages (McMichael *et al*., 2010; Mogensen *et al.*, 2010), changes in gut microbiota with infection supports that the immune system plays an important role in shaping composition. The CD4+ count was noticed to have increased with HIV progression alongside the markers for the liver (ALT) and kidney (Creatinine) while the viral load decreases. Given these interactions between Bacteroides species and CD4+ T cells, it is of interest that Bacteroides decrease in HIV-positive individuals when CD4+ T cell populations are compromised, suggesting that CD4+ T cell interactions may be essential for persistence of Bacteroides in the gut. Even within the overall compromised T-cell populations of HIV positive subjects, we show that proliferative responses to Bacteroides species are preferentially depleted compared to other species tested.

Microbiota changes with HIV-infection can have several underlying causes including 1) a compromised ability of the innate and/or adaptive immune system to control commensal bacteria, 2) the indirect selection of inflammation-tolerant versus sensitive bacteria resulting from a chronic inflammatory state or 3) a loss of interaction with CD4+ T cells that produce regulatory responses that promote tolerance of beneficial microbes. Although the immunologic driving factors of microbiota changes in HIV-infection are likely to be complex, we began to explore drivers of compositional differences by examining CD4+ T cell and viral load proliferative response to bacteria highly related microbiomes that differed with HIV infection status.

A persistence of an HIV-associated microbiota in some individuals on long-term ART is also consistent with the observation that ART treatment generally does not completely restore CD4+ T cells in blood cell (Estes *et al.*, 2008). Discordance between CD4+ T cell counts in and the periphery may explain why we did not observe a correlation between peripheral CD4+ T cell count and the degree of divergence in fecal microbiota composition from HIV-negative

The failure of ART to consistently restore the gut microbiota to a state resembling HIV negative individual is consistent with persistence of gastrointestinal diseases with ART. HIV positive subjects on successful ART remain at greater risk of multiple inflammatory diseases, including atherosclerosis (Hsue *et al.*, 2009). The changes we saw in our study are not broadly definitive primarily due to the relatively small sample size. There are still many questions that need to be addressed and further studied. Are the changes we see in the HIV patients due to the changes of integrity in the gut? Are these changes driving HIV progression or dissemination? Or is HIV infection driving these changes?

Conclusions

Understanding gut microbiota alterations associated with HIV-infection and factors that drive these alterations may help explain gut-linked diseases prevalent with HIV. Collectively, the results of our study showed that both healthy controls and HIV-infected were colonized by 3 main phyla: Bacteroidetes, Firmicutes and Proteobacteria. These findings implicated the interactive roles of immunodeficiency and ART for affecting gut microbiota in HIV-1-infected individuals, providing new insights into intestinal microbiome symbiosis related to HIV-1 infection. Immune status and ART were the key factors interactively affecting the gut

microbiome in HIV-1-infected individuals with the most of the blood cell marker correlating with the counts of gut microbiota. Future studies will look at molecular profiling of the microbiota among HIV patient would also help in the management of HIV patients.

Limitation of Study:

The sample size is not large enough because of the procedures involve and cost implications since it is a grey area of research.

The molecular profiling of the microbiota among HIV patient would also help in the management of HIV patients.

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