Association between the gut microbiome and organic acid profiles in a Japanese Population with HIV Infection

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Abstract

Introduction: An increased incidence of metabolic syndrome has been observed in human immunodeficiency virus (HIV)-infected individuals. In contrast, gut dysbiosis is involved in various pathogeneses, including vascular endothelial disorders. Organic acids, including short-chain fatty acids (SCFAs), are essential for maintaining gut homeostasis. Therefore, this study aimed to explore the gut microbiome profile and organic acids in a Japanese population infected with HIV.

Methods: Forty-nine patients with HIV infection on combination antiretroviral therapy (cART) were enrolled and divided into the high and low CD4 groups based on a CD4 cutoff of 350 cells/μL. Stool samples were analyzed by 16S ribosomal RNA next-generation sequencing and high-performance liquid chromatography. The association between the gut microbiome, including bacterial taxa and organic acids, was statistically analyzed.

Results: The fecal microbial community composition was significantly different between HIV patients with CD4 counts above and below 350 cells/μL. The relative abundance of *Roseburia*, *Prevotella*, *Prevotella_9*, and *[Clostridium]_methylpentosum_group* were significantly enriched in the high CD4 group. Fecal succinic acid tended to be more abundant in the low CD4 group, and acetic, propionic, and butyric acids tended to be more abundant in the high CD4 group. *Roseburia* was positively correlated with butyric acid levels. *Prevotella_9* and *Prevotella* were negatively correlated with succinic acid levels and positively correlated with acetic and propionic acid levels.

Conclusions: This study showed intestinal dysbiosis bordering on a CD4 count of 350 in patients with HIV infection undergoing cART. These findings might help in understanding intestinal damage and systemic inflammation in HIV infection.

Keywords: HIV, gut microbiota, *Roseburia*, *Prevotella_9*, short-chain fatty acid, succinic acid

Abbreviations: combination antiretroviral therapy, cART; human immunodeficiency virus, HIV; Short-chain fatty acids, SCFAs; amplicon sequence variants, ASVs; high-performance liquid chromatography, HPLC; men who had sex with men, MSM; operational taxonomic units, OTUs; principal coordinate analysis, PCoA; people living with HIV, PLWH.

Introduction

In the current era of effective combination antiretroviral therapy (cART), human immunodeficiency virus (HIV) infection is a chronic, manageable disease. Combination ART has dramatically improved the health of patients with HIV, increased their life expectancy, and reduced the risk of HIV transmission. However, HIV-infected individuals are more likely to have complications — especially cardiovascular, musculoskeletal, kidney, liver, neurological diseases, and cancer [1, 2] — than non-HIV-infected individuals of the same age [3]; this is called 'non-AIDS morbidity.'

In clinical cohort studies, the mortality ratios of HIV patients on cART with CD4 counts lower than 350 cells per μL were much higher than those with CD4 counts above 350 cells per μL. Besides, the mortality rate of HIV patients with CD4 counts above 500 cells per μL was close to that of the general population [4, 5]. Evidence indicates that many markers of inflammation are higher in antiretroviral-treated adults than in age-matched healthy individuals [6, 7]. Currently, causes of inflammation have been explained by ongoing HIV production; high levels of other co-pathogens, including cytomegalovirus; cART toxicity; traditional risk factors; irreversible damage to the immunoregulatory system; and the translocation of microbial products across damaged mucosal surfaces [8].

The gut microbiome is important for maintaining intestinal homeostasis and plays a vital role in maintaining the mucosal barrier function and regulation of innate and adaptive immune responses [9, 10]. Recently, cross-sectional studies have demonstrated changes in the gut microbiota of patients with HIV-1 but have focused mostly on Western populations. Some studies have shown that higher CD4 counts correlate with higher bacterial diversity in the guts of patients with HIV [11, 12], and CD4 counts also alter bacterial diversity [13, 14]. However, few studies have focused on Asian populations, such as the Japanese population [15-17].

With a better understanding of the gut microbiome, it is now known that in addition to the intestinal flora itself, its metabolites are also involved in the immune system [18]. Short-chain

fatty acids (SCFAs) (mainly acetic, propionic, and butyric acids) are produced by fiber fermentation by gut bacteria, particularly by members of the Firmicutes phylum [19]. SCFAs are an important link between the flora and the immune system; they involve different molecular mechanisms and cellular targets, are essential for the maintenance of intestinal homeostasis, and also play a role in HIV infection [20]. Additionally, other organic acids, such as lactic and succinic acids, produced by gut bacteria are important SCFA precursors that may play a relevant role in health and disease. Succinic acid has attracted considerable attention as a proinflammatory mediator in intestinal inflammation [21]. It is already known that there is a depletion of colonic producers in HIV-positive patients [22], but the correlations among bacteria species, organic acids, and CD4 counts have not been explored yet.

Therefore, intestinal organic acid profiles and microbiome dysbiosis are HIV infection highlights and are likely to be related to the morbidity and mortality of complications associated with HIV infection. Despite advances in the field, the relationship between HIV infection and organic acids remains unclear. Hence, we sought to understand the association among the gut microbiome, CD4 status, and intestinal organic acids in patients with long-term suppression of HIV viremia.

Materials and methods

-Study participants and sample collection

An observational study of HIV-1-infected individuals was conducted in the Outpatient Department of Infectious Diseases at Nara Medical University Hospital, Kashihara, Nara, Japan. All participants provided written informed consent in accordance with the Declaration of Helsinki. This study was approved by the Ethics Committee of Nara Medical University (reference no.1040). In total, 49 patients with HIV on cART were enrolled and divided into the high and low CD4 groups based on a CD4 cutoff of 350 cells per μL because CD4 counts below 350 are associated with higher mortality and non-AIDS complications [5, 23].

-DNA extraction from stool samples

Stool samples were collected and stored at -80 ℃ until further use. Samples were processed and DNA was extracted as previously described with minor modifications [24-26]. Briefly, bacterial DNA was isolated and purified by enzymatic lysis using lysozyme (Sigma-Aldrich Co. LCC., Tokyo, Japan) and achromopeptidase (Wako, Osaka, Japan). The suspension was treated with 1% (wt/vol) sodium dodecyl sulfate and 1 mg/ml proteinase K (Merck, Tokyo, Japan) and incubated at 55℃ for one hour. The lysate was treated with phenol/chloroform/isoamyl alcohol (Life Technologies Japan Ltd., Tokyo, Japan). The DNA samples were purified by treatment with RNase A (Wako, Osaka, Japan), followed by precipitation with 26% PEG solution (PEG6000 in 1.6 M NaCl). The DNA was pelleted by centrifugation, rinsed with 75% ethanol, and dissolved in TE buffer.

-PCR amplification and analysis of 16S rRNA sequences

The 16S rRNA gene sequencing was performed as previously described with minor modifications [27]. The V3–V4 regions of the 16S rRNA gene were amplified by PCR using TaKaRa Ex Taq® Hot Start Version (Takara Bio Inc., Shiga, Japan) and the Illumina forward primer 5'-AATGATACGGCGACCACCGAGATCTACAC (adaptor sequence) plus barcode (eight bases) plus ACACTCTTTCCCTACACGACGCTCTTCCGATCT (sequence primer) plus NN (sequence for improved cluster separability) plus CCTACGGGNGGCWGCAG-3' (341F) and the Illumina reverse primer 5'-CAAGCAGAAGACGGCATACGAGAT (adaptor sequence) plus barcode (eight bases) plus GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT (sequence primer) plus NN (sequence for improved cluster separability) plus GACTACHVGGGTATCTAATCC-3' (805R) to the hypervariable V3–V4 region of the 16S rRNA gene; they contain the Illumina index and adapter overhang sequences. The amplicons generated from each sample were purified using

SPRISelect (Beckman Coulter, Brea, CA, USA). Purified amplicons were quantified using a Quantus Fluorometer and ONEdsDNA System (Promega, Madison, WI, USA) and pooled at approximately equal concentrations. Mixed samples were sequenced using the MiSeq Reagent Kit V3 (600 cycles) and MiSeq sequencer (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The 16S rRNA gene sequence data generated from the MiSeq sequencer were processed using the quantitative insights into the Microbial Ecology 2 (QIIME2 October 2019) pipeline [28]. The paired-end raw sequence reads were quality-filtered, denoised, merged, and chimeras were removed using QIIME2's DADA2 plugin [29]. Taxonomic classification of amplicon sequence variants (ASVs) obtained from DADA2 was performed using the feature-classifier classify-sklearn plugin [30] in the SILVA database v138.1. A phylogenetic tree was generated by the "phylogeny align-to-tree-mafft-fasttree" plugin and diversity analysis was performed using the "diversity core-metrics-phylogenetic" plugin.

-Organic acid measurement using high-performance liquid chromatography (HPLC)

Feces (200 mg) were placed in a 2.0 mL tube and suspended in 800 μL of 1× PBS. The suspensions were vortexed for 1 min, kept on ice for 5 min, and centrifuged at 10,000 × *g* for 5 min at 4 ℃. The supernatants were filtered through a 0.45-μm filter (Merck Millipore, MA, USA). Organic acid measurements were conducted using high-performance liquid chromatography (Prominence, SHIMADZU, Kyoto, Japan) as previously described [31].

-Statistical analysis

We performed statistical methods such as the following and considered $p < 0.05$ statistically significant. The Mann-Whitney U test was used for the comparison of continuous variables between two groups. Spearman's correlation analysis was conducted to identify relationships between fecal microbiome abundance and organic acids. These tests were performed using R software version 4. 1. 1.

Results

-Study populations

Among the 49 patients with HIV, 31 were men who had sex with men (MSM), and 46 had been treated with cART for > 16 months. The participants' characteristics are listed in Table 1. The characteristics of participants in both groups were similar, with the following exceptions: hypertension (8.6% in the high CD4 group and 35.7% in the low CD4 group; $p = 0.02$) and body mass index (median 34.2 in the high CD4 group and 22.4 in the low CD4 group; $p = 0.043$).

-Fecal microbiome diversity

Rarefaction analysis of operational taxonomic units (OTUs) indicated that sufficient sequencing depth was achieved to avoid biases from unequal sample sizes.

In the low CD4 group, we found decreased alpha diversity (estimated observed OTUs and chao1: Figure 1A) compared to the high CD4 group. Species richness values based on the Shannon index were lower in the low CD4 group than in the high CD4 group, although the differences between groups were not statistically significant ($p = 0.293$).

The fecal microbiota was dominated by five phyla: Firmicutes, Actinobacteriota, Bacteroidota, Proteobacteria, and Fusobacteriota (Supplementary Table 1). There was no statistical difference between the high and low CD4 groups at the phylum level (Supplementary Figure 1). The bacterial taxa in the genus level in each category are listed in Table 2. *Blautia*, *Bifidobacterium*, and *Faecalibacterium*, which are known to produce SCFAs, were dominant in both groups. *Prevotella_9*, *Catenibacterium*, *Megamonas*, and *Megasphaera* were listed in the top 20 genera in the high CD4 group, whereas *Enterococcus*, *Anaerostipes*, *Coprococcus*, and *Ruminococcus* were listed in the top 20 genera in the low CD4 group. There was a positive correlation between chao1 and CD4 counts or nadir CD4

counts (Figure 1B). Consistent with this, a permutational multivariate analysis of the variance test of beta diversity based on unweighted UniFrac distance revealed that the bacterial communities were significantly different between the low and high CD4 groups. Intragroup dissimilarity between the two groups was supported by principal coordinate analysis (PCoA) based on the unweighted UniFrac results (Figure 1C).

Furthermore, several taxa were significantly enriched in the high CD4 group, including *Roseburia*, *Prevotella*, *Prevotella_9*, and *[Clostridium]_methylpentosum_group*, which produce SCFAs elsewhere [32-36] (Figure 2, Supplementary Figure 2).

-Intestinal organic acid measurement

Organic acid measurements in the feces revealed no statistically significant difference between the low and high CD4 groups. However, acetic acid, propionic acid, and butyric acid were enriched in the high CD4 group. In contrast, succinic acid was enriched in the low CD4 group (Figure 3A). Next, we investigated the relationships between the amounts of organic acids in feces and the relative abundance of SCFA-producing bacterial taxa at the genus level, which were enriched in the high CD4 group. The results of the above four SCFA-producing bacteria are shown in Supplementary Table 2. The organic acids that showed significant correlations with at least one of these species were succinic acid, acetic acid, propionic acid, and butyric acid. *Roseburia* was positively correlated with butyric acid levels. *Prevotella_9* and *Prevotella* were negatively correlated with succinic acid and positively correlated with acetic and propionic acid levels. In contrast, *[Clostridium]_methylpentosum_group* showed no correlation with the amount of organic acids in feces (Figure 3B/C/D/E).

Discussion

We report the gut microbiome of patients with HIV who received successful cART in Japan. Moreover, we also revealed the association between fecal organic acids and the gut microbiome

in people living with HIV (PLWH).

In the present study, Firmicutes, Actinobacteriota, and Bacteroidota occupied 97.6% at the phylum level of the gut microbiome, and *Blautia* and *Bifidobacterium* were dominant at the genus level. In the healthy Japanese group, Firmicutes, Actinobacteriota, and Bacteroidota were ranked in order of abundance, and at the genus level, *Bifidobacterium* and *Blautia* were dominant compared to other countries [26]. By contrast, in a Chinese study, Firmicutes, Bacteroidota, and Proteobacteria were dominant at the phylum level in both PLWH and healthy controls, whereas *Bacteroidetes* and *Prevotella* were dominant at the genus level in both PLWH and healthy controls [37]. The abundance of the gut microbiome was similar regardless of HIV infection but might depend on dietary habits or area of residence. Thus, it is difficult to compare our data with data from other countries, including among HIV-positive or -negative populations. This report is important because only a few studies have examined the gut microbiome of PLWH in Japan.

Our findings showed a statistically significant association between low CD4 count, low nadir CD4 count, and decreased alpha diversity in the gut microbiome of patients with HIV. Ishizaka et al. [16] also reported a decreased alpha diversity in the low CD4 group, especially in those with CD4 counts below 250 cells per μL, compared with that of those with CD4 counts above 250 cells per μL and healthy controls; they also reported that alpha diversity was restored upon treatment initiation. In our study, a similar reduction in alpha diversity with lower CD4 counts was observed; however, the new results suggest that even 350 cells per μL can be a cutoff CD4 count. Our findings showed that nadir CD4 counts were positively correlated with the alpha diversity of the gut microbiome in patients with HIV. Guillén et al. [38] reported that nadir CD4, female sex, Caucasian race, non-MSM status, and HIV status were associated with low gene richness in the gut microbiome. In this study, the nadir CD4 count might have been a confounding factor for the current CD4 count.

Herein, there were many similar taxa at the genus level compared with Ishizaka et al. [16],

and *Prevotella_9, Megamonas, Catenibcaterium, Roseburia, Prevotella,* and *Romboutsia* spp. were relatively abundant (Supplementary Figure2). Among these, *Prevotella_9*, *Rosenburia*, and *Prevotella* spp. have been reported to be SCFA-producing bacteria.

In our study, fecal succinic acid tended to be more abundant in the low CD4 group, and acetic, propionic, and butyric acids tended to be more abundant in the high CD4 group, although the metabolomic analysis did not reveal any significant differences. This lack of clear differences between the two groups in organic acids may be due to the fact that *Blautia*, *Bifidobacterium*, and *Faecalibacterium*, which have been reported to produce SCFAs, were abundant in both groups.

In contrast, we found positive correlations between *Roseburia* and butyric acid as well as *Prevotella* and acetic and propionic acids. We also found a negative correlation between *Prevotella* and succinic acid. Generally, a healthy intestinal microbiome is dominated by obligate anaerobic bacteria whose fermentation increases SCFAs. One of the major SCFAs is butyrate, which maintains an anaerobic environment in the intestine by facilitating oxygen consumption by epithelial cells and improving the intestinal barrier function [39]. SCFAs play an important role in the maintenance of intestinal and immune homeostasis and are predominantly related to anti-inflammatory effects (butyric > propionic > acetic acids) [40]. The role of gut dysbiosis in chronic HIV infection suggests that reducing intestinal inflammation and increasing the gut barrier function may effectively improve the prognosis of patients with HIV. The HIV-associated microbial profile that was previously observed was similar to ours with regard to the enrichment of facultative anaerobic bacteria and a decline in butyrate-producing bacteria [16, 41].

We have shown that fecal succinic acid tends to be more abundant in the low CD4 group and has a negative correlation with *Prevotella* and *Prevotella_9* in the feces of people with chronic HIV infection. Succinic acid is an important immunoregulatory metabolite that can modulate the immune response and inflammation in a variety of ways. Specifically, microbiota-derived

succinic acid was shown to initiate a type II immune response [42, 43]. Liu et al. [44] have shown that notable depletion of Blautia and elevated succinic acid may underlie hepatic inflammation in IgG4-related sclerosing cholangitis. To the best of our knowledge, few studies have examined the association between fecal succinic acid, CD4 count, and bacterial taxa. Our study might help in understanding intestinal damage and systemic inflammation in HIV infection.

This study had certain limitations. It lacked healthy controls; therefore, we could not compare the HIV-positive and -negative groups. In addition, there have been some reports of gut microbiome alterations associated with sexual behavior [35, 45], but we were unable to compare the MSM and non-MSM groups.

Collectively, our study shows that gut dysbiosis is associated with HIV infection, despite successful cART. These observations will enable a better understanding of the correlation between HIV, the gut microbiome, and organic acids, and the design of new microbiome-based therapies for HIV infection. Further studies are required to evaluate the long-term consequences of alterations in the microbiota following HIV infection and treatment.

Conclusions

This report provides a new understanding of the changes in the gut microbiota and enriches the knowledge on the dysbiotic features in PLWH in Japan. Additionally, it demonstrates the importance of studying specific changes in our population and highlights the necessity for deeper and longer studies with different HIV groups.

Ethical considerations:

Patient consent was obtained to publish the medical data. Patient privacy was fully protected, and personal information was handled to ensure that the patient could not be identified. The study protocol was conducted in accordance with the Declaration of Helsinki.

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Conflicts of interest:

All authors declare no conflict of interest.

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Authors' contributions

All authors meet the ICMJE authorship criteria. AN, KU, MK, and HY conceived and designed the experiments. KU, MK, NH, YO, TO, KM, and KK coordinated the sample collection. NH, AN, YS, SH, and RN performed the experiments. SH, SE, TA, AM, KO, and MT analyzed the data. NH and UK wrote the paper. All authors reviewed and/or edited the manuscript. All authors have seen and approved the manuscript, and the corresponding author has full access to the data and accepts final responsibility for the decision to submit for publication.

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Figure Captions

Figure 1A: Alpha diversity (observed OTUs and chao1) in the high and low CD4 groups

Figure 1B: Correlation between alpha diversity (chao1) and CD4 or nadir CD4 in HIV patients

Figure 1C: Beta diversity comparisons between the low and high CD4 groups (Unweighted UniFrac : $p = 0.002$)

Figure 2: Heat map of significantly different bacterial flora composition between the low and high CD4 groups. Color intensity indicates row-scaled (z-score) relative abundance.

Figure 3A: The amount of organic acid in feces in the low and high CD4 groups.
The number above each bar is the *p*-value between the two groups.

Figure 3B: Correlation between *Roseburia* and Succinic/Acetic/Propionic/Butyric acids

Figure 3C: Correlation between *Prevotella_9* and Succinic/Acetic/Propionic/Butyric acids

Figure 3D: Correlation between *Prevotella* and Succinic/Acetic/Propionic/Butyric acids

Figure 3E: Correlation between *[Clostridium]_methylpentosum*_group and Succinic/Acetic/Propionic/Butyric acids

Supplementary Figure 1: Correlation between bacteria taxa at the phylum level and CD4 counts

Supplementary Figure 2: The boxplot of bacterial taxa treated in Figure 2 between the low and high CD4 groups