Associations of Cocaine Use and HIV Infection With the Intestinal Microbiota, Microbial Translocation, and Inflammation

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ABSTRACT. Objective: HIV and illicit drug use have been associated with altered nutrition, immune function, and metabolism. We hypothesized that altered composition and decreased diversity of the intestinal microbiota, along with microbial translocation, contribute to nutritional compromise in HIV-infected drug users. **Method:** We enrolled 26 men and 6 women, 15 HIV infected and 17 HIV uninfected, in this exploratory, cross-sectional study; 7 HIV-infected and 7 HIV-uninfected participants had used cocaine within the previous month. We examined the independent effects of cocaine use and HIV infection on the composition and diversity of the intestinal microbiota, determined by 16S rRNA gene pyrosequencing. Using dietary records, anthropometrics, and dual x-ray absorptiometry, we examined the additional effects of nutritional indices on the intestinal microbiota. We compared markers of inflammation and microbial translocation between groups. **Results:** Cocaine users had a

higher relative abundance of Bacteroidetes ($M \pm SD = 57.0\% \pm 21$ vs. $37.1\% \pm 23$, $p = .02$) than nonusers. HIV-infected individuals had a higher relative abundance of Proteobacteria (Mdn [interquartile range] = 1.56% [0.5, 2.2] vs. 0.36% [0.2, 0.7], $p = .03$), higher levels of soluble CD14 and tumor necrosis factor- α , and lower levels of anti-endotoxin core antibodies than uninfected subjects. HIV-infected cocaine users had higher interferon- γ levels than all other groups. Food insecurity was higher in HIV-infected cocaine users. **Conclusions:** We identified differences in the relative abundance of major phyla of the intestinal microbiota, as well as markers of inflammation and microbial translocation, based on cocaine use and HIV infection. Nutritional factors, including alcohol use and lean body mass, may contribute to these differences. (*J. Stud. Alcohol Drugs*, 75, 347–357, 2014)

COMMENSAL MICROORGANISMS in the human gastrointestinal tract are essential to health because they produce vitamins, maintain mucosal integrity, and facilitate a regulated immune response (Hattori and Taylor, 2009). These microbes promote nutrition by extracting nutrients from components of our diet that our digestive enzymes are unable to metabolize (Barcenilla et al., 2000; Gill et al., 2006). Several recent studies have also demonstrated that the intestinal microbiota have systemic effects on fat deposition and glucose metabolism (Bäckhed et al., 2005; Turnbaugh et al., 2006). Alterations of the microbiome may cause metabolic instability (Walker et al., 2005), resulting in less efficient nutrient extraction as well as an augmented osmotic load in the colon, leading to diarrhea. Intestinal disorders such as inflammatory bowel disease (Manichanh et al., 2006) and

recurrent *Clostridium difficile* infections (Chang et al., 2008) have been associated with reduced diversity of the intestinal microbiota. Both drug use and HIV infection result in health compromises that could lead to alterations in the intestinal microbiota.

Drug use, in particular cocaine use, has been shown to be associated with impaired nutritional status (Quach et al., 2008) as well as with an increased risk of HIV and hepatitis C infection (Howe et al., 2005; McQuillan et al., 2006). Cocaine use affects body composition, irrespective of diet and lifestyle choices, resulting in a lower percentage of body fat compared with non-drug users (Forrester et al., 2000, 2005). Compromised nutritional status can weaken intestinal integrity, alter mucin glycoproteins, and modify the composition of the intestinal flora (Deplancke and Gaskins, 2001; Gordon et al., 2012). Cocaine use is associated with gastrointestinal symptoms such as anorexia, nausea, vomiting, and diarrhea, which could also perturb the intestinal microbiota, as would use of antibiotics to treat infections associated with drug use (Dethlefsen et al., 2008; Young and Schmidt, 2004). Drug-user lifestyle factors, nutritional hygiene, and dietary choices could also affect intestinal flora. Finally, chronic cocaine use disturbs systemic cytokine levels and causes immune activation (Pellegrino et al., 2001; Ruiz et al., 1998; Xu et al., 1999); chronic elevation in tumor necrosis factor (TNF)- α levels result in cachexia. We propose that in the setting of drug use, the convergence of metabolic derangements, nu-

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tritional deficits (including poor quality diet and low body mass index [BMI]), gastrointestinal symptoms, and immune activation may significantly affect the microbiota, which in turn could alter metabolic capability, intestinal integrity, bacterial translocation, and immune balance, initiating a vicious cycle of nutritional compromise.

Cocaine users are also at increased risk for HIV infection, which results in many of the same outcomes (compromised nutrition, altered metabolism, and immune activation) (Hendricks et al., 2008) as well as HIV enteropathy and weight loss. HIV-infected persons have been shown to have increased markers of microbial translocation, including lipopolysaccharide (LPS) and its co-receptor, soluble CD14, compared with uninfected persons (Brenchley et al., 2006). Similarly, levels of bacterial 16S rRNA gene levels in the plasma of HIV-infected persons have been noted to be higher compared with uninfected persons, and to correlate with plasma LPS levels (Jiang et al., 2009). Last, anti-LPS antibody (anti-endotoxin-core antibodies [EndoCAB]) levels have been found to be lower in HIV-infected individuals compared with uninfected persons, indicating attempted neutralization of increased circulating LPS (Brenchley et al., 2006). These observed differences suggest a potential mechanism by which alterations in gut microbial flora lead to loss of intestinal mucosal integrity, translocation of bacterial products across the intestinal barrier, and persistent immune activation and chronic inflammation in HIV-infected persons.

We evaluated the microbiota of cocaine users with and without HIV infection to detect differences in composition and diversity of the microbiota in these groups and to determine if there is an association with nutritional status. We hypothesized that the diversity of the intestinal microbiota would be reduced with both cocaine use and HIV infection, and that HIV-infected cocaine users would have the least diverse intestinal microbiota. We hypothesized that this reduced diversity would be associated with microbial translocation and inflammation, as well as impaired nutrition. Previous studies have evaluated the microbiota in HIV-infected individuals using a variety of methods, including culture and quantitative polymerase chain reaction (PCR) (Ellis et al., 2011; Gori et al., 2008); however, few have used 16S rRNA gene pyrosequencing to evaluate all higher-order taxa identifiable by this method. To our knowledge, this is the first study to compare the composition and diversity of intestinal microbiota between HIV-infected cocaine users and nonuser controls.

Method

Clinical and laboratory data

We enrolled 7–10 participants in each of four groups: (a) cocaine users with HIV, (b) cocaine users without HIV,

(c) non-cocaine users with HIV, and (d) non-cocaine users without HIV. HIV-infected participants were recruited from our unit's longitudinal cohort for the CARE Study (Mangili et al., 2011), community health and infectious diseases clinics, and the community at large; uninfected participants were recruited from community health clinics, Tufts Medical Center, and the community at large. Exclusion criteria included factors identified as having potential confounding effects on the intestinal microbiota: namely, age younger than 20 years or older than 60 years, BMI less than 20 kg/m² or greater than 30 kg/m², antibiotic use in the previous 8 weeks, probiotic use in the preceding 4 weeks, and gastrointestinal morbidity (including irritable bowel syndrome, inflammatory bowel disease, history of gastrointestinal cancer or surgical resection, or acute, severe gastrointestinal symptoms requiring medical attention). Pregnancy, lactation within the past 6 months, and opportunistic infection were additional exclusion criteria. Participants with hepatitis C antibody or hepatitis B surface antigen or core antibody were excluded based on the potential effects of chronic viral hepatitis on mucosal immunity (Agaugué et al., 2007). Last, to limit confounding by prior cocaine use or current use of other illicit substances, criteria for exclusion included recent use of heroin or methamphetamines as well as use of cocaine within the past 5 years but not in the last month. Cocaine use was defined as use of cocaine by any route within the last month. All participants were tested for HIV antibody or, if HIV infected, presented clinical or laboratory documentation of HIV infection. The Tufts Health Sciences Campus Institutional Review Board approved this study, and signed informed consent was obtained from all participants.

Laboratory data collected included high-sensitivity C-reactive protein (CRP), aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and total bilirubin, as well as HIV viral load and CD4+ count for HIV-infected participants. Anthropometrics—including height, weight, triceps skin folds, and waist, hip, and arm circumferences—were measured by a trained study coordinator. Dual x-ray absorptiometry (DXA) scan was performed to determine total, appendicular, and truncal lean mass and fat mass body composition. Dietary data were collected via a 24-hour dietary recall and then analyzed using Nutrition Data System for Research software, version 2010, developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN (Schakel et al., 1988). Healthy Eating Index scores (from 0 to 100) were calculated from these data (Kennedy et al., 1995). Drug/alcohol/tobacco use, food security, and behavioral/lifestyle data were obtained via an Audio Computer Assisted Self-Interviewing (ACASI) program (Tufts ACASI/CASI/CAPI Systems, <http://acasi.tufts.edu>). Food security was assessed using a scale from 0 to 6 adapted from the Radimer/Cornell Measures of Hunger and Food Security Scale (Radimer et al., 1990) and defined as a

binary variable (secure = score 0–1, insecure = score >1) or as a multichotomous variable (none = score 0–1, mild–moderate = score 2–4, or severe = score >4).

16S rRNA amplicon pyrosequencing

Stool samples were collected in sterile plastic containers either at the study site or at home by participants and then placed on ice in polystyrene coolers; they were delivered within 24 hours of collection and then frozen immediately at -80°C . After enzymatic digestion with lysozyme, lysostaphin, mutanolysin, and proteinase K, followed by bead-beating at 4°C , DNA was extracted from stool samples using the QIAamp DNA Stool Minikit (Qiagen, Germantown, MD) according to the manufacturer's protocol. For each sample of extracted DNA, PCR amplicons of the 16S rRNA gene were generated using a primer set based on the variable region V3-5. PCRs were carried out in triplicate in parallel with a barcode-specific negative control; reactions yielding no amplicons or those in which the negative controls were positive were repeated. The DNA concentration of pooled amplicons was determined with a Quant-iT assay (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Amplicons were then pooled in equimolar concentrations, purified using Agencourt Ampure XP beads (Beckman-Coulter, Beverly, MA), and sequenced on a Roche Genome Sequencer GS FLX+ using the Lib-L Sequencing kit according to Roche protocols at the Tufts University Genomic Core facility.

Computational analyses were performed using the open source software platform QIIME Version 1.5 (<http://qiime.org>) (Caporaso et al., 2010). Sequences were de-noised to remove chimeras and low-quality reads and then sequences were assigned to samples by barcode. Similar sequences were clustered into operational taxonomic units (OTUs) based on a minimum identity of 97%. The most frequent sequence within each OTU was used for alignment with the online ribosomal database Greengenes (<http://greengenes.secondgenome.com/>) and for construction of a phylogenetic tree; taxonomy was assigned using the Ribosomal Database Project classifier (<http://rdp.cme.msu.edu>). The classification data were used to generate comparisons between samples of percent relative abundance at selected taxonomic levels. To determine the amount of diversity contained within communities (alpha diversity), QIIME normalizes the number of sequences per sample and then generates phylotype-based measures, including equitability (evenness of distribution of phylotypes), number of observed species, Shannon diversity index (a measure of richness and evenness based on uncertainty in predicting the identity of a randomly selected species from a community) (Tuomisto, 2010), Chao-1 (a nonparametric estimator of species richness based on the number of times each species is observed in a community) (Hughes et al., 2001),

and phylogenetic distance (total branch length of phylotypes in a sample from a phylogenetic tree of the entire sample pool).

Microbial translocation and inflammatory markers

Blood samples were handled and processed under sterile, endotoxin (LPS)-free conditions, and plasma was frozen at -80°C . Plasma bacterial 16S rDNA levels were quantified by real-time PCR, as described by Jiang et al. (2009). LPS was measured by a commercially available Limulus Amebocyte lysate assay (Associates of Cape Cod), plasma cytokines (TNF- α , interleukin [IL]-1 β , interferon [IFN]- γ , and IL-6) were measured by the Meso Scale Discovery 4-plex immunoassay (Rockville, MD), and EndoCAB (R&D, Minneapolis, MN) and soluble CD14 (Hycult Biotech, Plymouth Meeting, PA) by enzyme-linked immunosorbent assays according to the manufacturer's instructions.

Statistical exploratory analysis

Demographic information, social history, clinical characteristics, and percent relative abundance of phyla and genera in stool were compared between groups using Student's *t* test or analysis of variance (ANOVA) for normally distributed variables and Wilcoxon rank-sum or Kruskal-Wallis test for non-normally distributed variables. Several of the alpha diversity parameters were normally distributed in our sample; however, given the small number, we compared these parameters by Wilcoxon-rank sum and Kruskal-Wallis tests. Body composition by DXA scan was separated by gender, expressed as continuous variables (lean/fat body mass and percent body fat), and compared between groups by Student's *t* test or ANOVA. Dietary components were expressed as continuous variables, log transformed where indicated, and compared between groups by Student's *t* test or ANOVA, or Wilcoxon rank-sum and Kruskal-Wallis if non-normal despite transformation. Food security was analyzed as a binary variable using chi-square test with Fisher's exact method where indicated. Spearman's correlation was used to determine correlations between markers of inflammation and microbial translocation. Comparisons were made (a) between HIV-infected ($n = 15$) and HIV-uninfected ($n = 17$) groups, (b) between cocaine users ($n = 14$) and nonusers ($n = 18$), and (c) between all four groups.

Linear regression analysis was performed to determine factors associated with relative abundance of the two predominant phyla, Bacteroidetes and Firmicutes, as the distributions for the other phyla were highly skewed because of many zero values. The following factors were evaluated due to their reported effect on the microbiota and/or their potential as confounders: age, gender, BMI, Healthy Eating Index, CRP (natural log), food security, alcohol use (binary variable [yes/no]), current smoking, and lean body mass

TABLE 1. Baseline characteristics

Variable	HIV negative (n = 17)	HIV positive (n = 15)	Cocaine nonusers (n = 18)	Cocaine users (n = 14)	HIV- negative cocaine nonusers (n = 10)	HIV- negative cocaine users (n = 7)	HIV- positive cocaine nonusers (n = 8)	HIV- positive cocaine users (n = 7)	p value group
Demographics									
Age, in years, <i>Mdn</i> (IQR)	44.9 (35, 50)	50.7 (46, 53) ^a	45.6 (42, 52)	49.1 (46, 51)	42.2 (34, 46)	48.5 (44, 50)	51.5 (47, 55)	49.8 (46, 53)	.09 ^c
Male, n (%)	13 (76)	13 (87)	15 (83)	11 (79)	8 (80)	5 (71)	7 (88)	6 (86)	.9
Smokers, n (%)	6 (35)	8 (53)	3 (17)	11 (79) ^b	2 (20)	4 (57)	1 (13)	7 (100)	.001
Alcohol use, n (%)	16 (94)	12 (80)	15 (83)	13 (93)	9 (90)	7 (100)	6 (75)	6 (86)	.7
Food secure, n (%)	15 (88)	6 (40) ^a	15 (83)	6 (43) ^b	9 (90)	6 (86)	6 (75)	0 (0)	<.001
Healthy Eating Index, <i>M</i> ± <i>SD</i>	53.6 ± 17	61.7 ± 14	62.4 ± 12	50.9 ± 15 ^b	59.5 ± 13	45.2 ± 19	66.1 ± 11	56.6 ± 16	.07 ^c
Clinical and laboratory data^d									
BMI (kg/m ²)	26.8 (25, 28)	25.2 (24, 28)	26.3 (25, 28)	25.6 (22, 28)	27.1 (25, 28)	26.5 (20, 29)	25.5 (25, 27)	24.6 (22, 28)	.6 ^c
CRP (mg/L)	1.1 (0.7, 3.7)	2.1 (0.9, 4.4)	1.7 (0.8, 4.7)	1.7 (0.7, 4.1)	1.7 (0.8, 4.7)	0.7 (0.4, 1.9)	1.7 (0.8, 3.8)	2.1 (1.5, 4.4)	.2 ^e
HIV parameters									
CD4 count, cells/μl ^d	—	701 (534, 840)	693 (537, 887)	701 (429, 806)	—	—	694 (537, 887)	701 (429, 806)	.8 ^e
CD4 percentage ^d	—	35.7 (27, 40)	33.6 (28, 37)	39.1 (19, 47)	—	—	33.6 (28, 37)	39.1 (19, 47)	.4 ^e
HIV viral load, cp/ml	—	<75 (all)	<75 (HIV+)	<75 (HIV+)	—	—	<75 (all)	<75 (all)	—
Using HAART, n (%)	—	15 (100)	8 (44)	7 (50)	—	—	8 (100)	7 (100)	—
Using NNRTI, n (%)	—	9 (60)	6 (40)	3 (20)	—	—	6 (75)	3 (43)	.3
Using PI, n (%)	—	6 (40)	2 (13)	4 (27)	—	—	2 (25)	4 (57)	.3
Using PI and NNRTI, n (%)	—	2 (13)	2 (13)	0 (0)	—	—	2 (25)	0 (0)	.5
DXA measurements									
Men	(n = 13)	(n = 13)	(n = 15)	(n = 11)	(n = 8)	(n = 5)	(n = 7)	(n = 6)	
Lean body mass, kg ^d	60.1 (56, 62)	58.7 (56, 62)	60.1 (56, 62)	56.2 (56, 67)	58.7 (56, 61)	60.1 (56, 65)	60.1 (56, 62)	56.1 (44, 67)	0.9 ^e
Percentage of body fat ^d	19.8 (17, 22)	18.4 (15, 23)	21.7 (17, 26)	16.2 (12, 20) ^f	22.1 (20, 26)	16.2 (15, 20)	18.4 (15, 26)	17.2 (12, 20)	0.1 ^e
Women	(n = 4)	(n = 2)	(n = 3)	(n = 3)	(n = 2)	(n = 2)	(n = 1)	(n = 1)	
Lean body mass, kg ^d	46.8 (42, 52)	44.7 (40, 50)	40.9 (40, 44)	49.8 (50, 54)	42.4 (41, 44)	52.0 (50, 54)	39.8	49.6	—
Percentage of body fat ^d	36.1 (32, 38)	39.9 (35, 45)	36.5 (29, 45)	35.6 (35, 39)	32.5 (29, 37)	37.2 (36, 39)	44.7	35.1	—

Notes: **Bold** indicates statistical significance. IQR = interquartile range; BMI = body mass index; CRP = C-reactive protein; HAART = highly active anti-retroviral therapy; NNRTI = nonnucleoside reverse transcriptase inhibitor; PI = protease inhibitor; DXA = dual X-ray absorptiometry. ^a*p* < .05 for the difference between HIV positive and HIV negative groups; ^b*p* < .05 for the difference between cocaine users and nonusers; ^cstatistical test analysis of variance; ^ddata are expressed as median (interquartile range); ^estatistical test Wilcoxon rank-sum; otherwise, Fisher's exact test; ^f*p* = .05 for the difference between cocaine users and nonusers.

measured by DXA scan. First, a univariate model was generated for each outcome with HIV infection or cocaine use as the sole predictor. Then, a multivariate model was generated for each predictor variable, controlled for HIV status and cocaine use; the lean body mass model was additionally controlled for gender. Given the number of predictors and small sample size, multivariate analyses with more than three predictors were not performed due to model instability. For alpha diversity statistics, linear regression was performed for the normally distributed outcome variables as above. For each non-normally distributed variable, a binary variable was created with the median as the threshold. Logistic regression analyses were performed to determine predictors of a diversity score above the median. Similarly, binary variables (at/below the median or above the median) were created for the 30 most prevalent genera identified in the entire sample pool. In the event that the median was zero, the genus outcome was defined as zero or nonzero. Logistic regression models were performed with individual confounders controlled for HIV infection and cocaine use. Statistical significance was defined by a *p* value of less than .05. Analyses were performed using SAS software, version 9.2 (SAS Institute, Cary, NC).

Results

Demographics and clinical information

Thirty-two participants completed the study: 26 men and 6 women, whose mean age was 47.0 years ± 8 and mean BMI was 25.8 kg/m² ± 3. Of the 60 participants who met the study criteria by telephone interview and had a screening visit, 28 were excluded: 2 for antibiotic use, 2 for BMI, 1 for HIV-2 infection, 21 for laboratory evidence of viral hepatitis, and 2 as a result of inadvertent combination of specimens. Baseline characteristics of the study population by group are shown in Table 1. HIV-infected participants were older on average than uninfected participants (50 years ± 5 vs. 44 years ± 9, *p* = .02). Cocaine users were more likely to smoke than nonusers (79% vs. 17%, *p* < .001); however, for those who smoked, pack-year history of smoking did not differ significantly between groups (data not shown). More HIV-infected participants were food insecure (60%) than uninfected participants (12%) (*p* = .008). Similarly, more cocaine users were food insecure (57%) than nonusers (17%) (*p* = .03). All of the HIV-infected cocaine users were food insecure. When food insecurity was further categorized

as none, mild–moderate, or severe, HIV infection was present in four fifths (80%) of those with mild–moderate food insecurity and in five sixths (83%) of those with severe food insecurity ($p = .01$). There was no difference in the use of nonnucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), or PI/NNRTI combinations between HIV-infected cocaine users and nonusers. HIV-infected participants had similar absolute and percentage CD4+ cells, as well as HIV viral load, regardless of current cocaine use.

Body composition and anthropometry

Among females ($n = 6$), average lean body mass by DXA was higher for cocaine users than for nonusers ($51.2 \text{ kg} \pm 3$ vs. $41.5 \text{ kg} \pm 2$, $p = .008$). Among males, ($n = 26$), cocaine users had both lower mean percentage of body fat ($16.7\% \pm 5$ vs. $21.5\% \pm 5$, $p = .03$) and lower mean total body fat by DXA scan than nonusers ($12.2 \text{ kg} \pm 4$ vs. $17.1 \text{ kg} \pm 5$, $p = .01$; data not shown).

Dietary intake

Based on data from 24-hour recall, HIV-infected participants consumed on average more calories from polyunsaturated fatty acids than did uninfected subjects ($12.1\% \pm 7$ vs. $7.2\% \pm 4$, $p = .02$). More alcohol was consumed by the HIV-uninfected group than by the HIV-infected group (Mdn [interquartile range {IQR}] = 0.68 g [0, 16] vs. 0 g [0, 0], $p = .01$). Cocaine users consumed more alcohol than nonusers (Mdn [IQR] = 4.7 g [0, 37] vs. 0 g [0, 0.2], $p = .04$). The HIV-uninfected, cocaine-using group consumed more alcohol

than all other groups, with a median consumption of 15.9 g [8, 59] daily (Mdn of all other groups = 0 , $p = .001$). Mean vitamin E and K consumption was slightly greater among HIV-infected participants compared with uninfected subjects (natural log vitamin E = $3.0 \text{ mg} \pm 0.7$ vs. $2.4 \text{ mg} \pm 0.9$, $p = .04$; natural log vitamin K = $4.9 \mu\text{g} \pm 1$ vs. $4.2 \mu\text{g} \pm 0.9$, $p = .04$). Cocaine users had a lower mean Healthy Eating Index than nonusers (50.9 ± 18 vs. 62.4 ± 12 , $p = .04$).

Microbial translocation and inflammatory markers

Thirty participants had available data on microbial translocation and inflammatory markers. Markers of microbial translocation and inflammation by HIV infection and cocaine use are shown in Table 2. As expected, soluble CD14 was significantly higher ($p = .01$) and EndoCAb lower ($p < .01$) in HIV-infected participants. There were no differences in soluble CD14 or EndoCAb by cocaine use. IFN- γ was higher among cocaine users than nonusers (trend only, $p = .06$), and TNF- α was higher among HIV-infected participants than uninfected participants ($p = .03$). There were no differences in the levels of IL-1 β , IL-6, 16S rRNA gene, or LPS by HIV status or cocaine use. Among these markers, TNF- α levels correlated significantly with IFN- γ ($p < .01$) and IL-6 ($p = .04$) levels (Table 3).

Intestinal microbiota analyses

The sequencing run resulted in 163,820 sequences after trimming for quality control and chimeras. The average number of sequences per sample was $4,405 \pm 1,367$.

TABLE 2. Markers of microbial translocation and systemic inflammation by HIV infection and cocaine use

Variable	HIV negative (<i>n</i> = 16)	HIV positive (<i>n</i> = 14)	<i>p</i> HIV	Cocaine nonusers (<i>n</i> = 17)	Cocaine users (<i>n</i> = 13)	<i>p</i> cocaine
Soluble CD14, pg/ml × 10 ⁶	1.3 (1.2, 1.5)	1.7 (1.6, 1.9)	.01	1.5 (1.3, 1.7)	1.6 (1.4, 1.9)	.7
EndoCAb, MMU/ml	31.3 (23, 42)	17 (11, 27)	.006	23 (18, 33)	27 (17, 38)	.9
LPS, EU/ml	0 (0, 0)	0 (0, 0)	.7	0 (0, 0)	0 (0, 0)	.4
IFN-γ, pg/ml	0.54 (0.5, 1.0)	1.2 (0.5, 1.6)	.05	0.55 (0.4, 1.0)	1.2 (0.5, 1.6)	.06
IL-6, pg/ml	0.65 (0.4, 1.0)	0.69 (0.5, 1.2)	.4	0.62 (0.4, 1.2)	0.75 (0.6, 1.2)	.2
IL-1β, pg/ml	0.002 (0.0, 0.04)	0.01 (0, 0.1)	.3	0.005 (0, 0.03)	0.006 (0, 0.1)	.8
TNF-α, pg/ml	2.5 (1.9, 3.0)	3.2 (2.8, 4.2)	.03	2.8 (2.6, 4.0)	2.9 (1.9, 3.3)	.8
16S rRNA gene copies/μl ^a	14 (0, 77)	0 (0, 0.8)	.08	0 (0, 2.9)	0.6 (0, 38)	.5
	HIV-negative nonusers (<i>n</i> = 9)	HIV-negative cocaine users (<i>n</i> = 7)	HIV-positive nonusers (<i>n</i> = 8)	HIV-positive cocaine users (<i>n</i> = 6)	<i>p</i> group	
Soluble CD14, pg/ml × 10 ⁶	1.3 (1.2, 1.4)	1.5 (1.1, 1.7)	1.7 (1.6, 1.9)	1.7 (1.6, 1.9)	.03	
EndoCAb, MMU/ml	29.2 (22, 41)	38.3 (27, 60)	20.0 (12, 30)	15.6 (11, 19)	.01	
LPS, EU/ml	0 (0, 0)	0 (0, 0.2)	0 (0, 0)	0 (0, 0)	.2	
IFN-γ, pg/ml	0.55 (0.5, 0.9)	0.53 (0.5, 1.2)	0.67 (0.4, 1.2)	1.6 (1.5, 2.5)	.009	
IL-6, pg/ml	0.62 (0.4, 1.2)	0.72 (0.4, 0.9)	0.60 (0.4, 0.9)	0.96 (0.7, 2.5)	.3	
IL-1β, pg/ml	0.003 (0, 0.03)	0 (0, 0.07)	0.01 (7.8 × 10 ⁻⁴ , 0.04)	0.06 (0, 0.3)	.6	
TNF-α, pg/ml	2.8 (2.2, 3.0)	1.9 (1.8, 2.9)	2.8 (2.7, 4.1)	3.4 (3.0, 6.9)	.04	
16S rRNA gene copies/μl ^a	1.5 (0, 37)	38.2 (0, 80)	0.2 (0, 2)	0 (0, 0.8)	.2	

Notes: Wilcoxon rank-sum is statistical test for binary comparisons; Kruskal-Wallis for group comparisons. All statistics are presented as Mdn (interquartile range). **Bold** indicates statistical significance. EndoCAb = anti-endotoxin core antibody; MMU = IgM median-units; LPS = lipopolysaccharide; EU = endotoxin units; IFN = interferon; IL = interleukin; TNF = tumor necrosis factor. ^aFor 16S, $n = 32$: HIV negative, nonusers, $n = 10$; HIV positive cocaine users, $n = 7$.

TABLE 3. Spearman's correlation (*p*) between markers of microbial translocation and systemic inflammation

Variable	CRP	Soluble CD14	IFN- γ	IL-6	IL-1 β	TNF- α	EndoCAb
CRP	—	0.2 (0.3)	0.1 (0.5)	0.1 (0.5)	-0.04 (0.8)	0.3 (0.1)	-0.3 (0.2)
Soluble CD14		—	0.05 (0.8)	0.1 (0.5)	0.3 (0.08)	0.1 (0.5)	-0.3 (0.09)
IFN- γ			—	0.3 (0.07)	0.2 (0.4)	0.5 (0.008)	-0.2 (0.2)
IL-6				—	0.02 (0.9)	0.4 (0.04)	-0.05 (0.8)
IL-1 β					—	0.006 (1.0)	-0.2 (0.2)
TNF- α						—	-0.2 (0.3)
EndoCAb							—

Notes: **Bold** indicates statistical significance. CRP = C-reactive protein; IFN = interferon; IL = interleukin; TNF = tumor necrosis factor; EndoCAb = anti-endotoxin core antibody.

Relative abundance of phyla. The relative abundance of the four predominant phyla—Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria—and the remaining phyla, categorized as “other,” were compared between groups based on HIV infection and cocaine use. HIV-infected individuals had a higher relative abundance of Proteobacteria than HIV-uninfected subjects (*Mdn* [IQR] = 1.6% [0.5, 2.2] vs. 0.36% [0.2, 0.7], *p* = .03). Cocaine users had a higher mean relative abundance of Bacteroidetes than nonusers (57.0% $\pm$ 21 vs. 37.1% $\pm$ 23, *p* = .02). Conversely, there was a trend in cocaine users toward a lower mean relative abundance of Firmicutes than nonusers (40.8% $\pm$ 21 vs. 57.2% $\pm$ 26, *p* = .06). When all four groups were compared, the relative abundance of Proteobacteria was significantly different between groups (highest in HIV-infected cocaine users). There were no differences in the relative abundance of Actinobacteria, Fusobacteria, or other phyla. The relative abundance of the major phyla is shown in Table 4. Figure 1 demonstrates the median percentage of relative abundance of each phylum by HIV infection and cocaine use.

Model estimates for Bacteroidetes and Firmicutes are shown in Table 5. In a model of HIV infection and cocaine use only, cocaine use was significantly associated with the relative abundance of Bacteroidetes in stool (estimate = 20.2, *SE* = 8.0, *p* = .02). After we controlled for HIV infection

and cocaine use, only alcohol use was also associated with the relative abundance of Bacteroidetes in stool (estimate = 26.0, *SE* = 11.6, *p* = .03). Cocaine use had an approximately equal and opposite effect on the relative abundance of Firmicutes in the stool when controlled only for HIV status, but this only trended toward statistical significance (estimate = -16.3, *SE* = 8.7, *p* = .07). After HIV infection and cocaine use were controlled for, only alcohol use was associated with the percentage relative abundance of Firmicutes in the stool (estimate = -30.8, *SE* = 12.4, *p* = .02). Therefore, we found that cocaine and alcohol use were associated with an increase in the relative abundance of Bacteroidetes and a decrease in the relative abundance of Firmicutes.

Relative abundance of genera. The abundance of each of the 30 most frequent genera represented by the OTU sequences generated was compared between groups. The genus *Succinivibrio* was found in only three HIV-infected participants who were not cocaine users (*p* = .02 for difference by group). The *Alistipes* genus was more common in HIV-uninfected participants than in HIV-infected participants (*Mdn* [IQR] = 11 OTU [7, 31] vs. 2 OTU [0, 7], *p* = .03). There were no statistically significant differences in the percentage of relative abundance of any other genera between groups. The percentage of relative abundance of the 10 most abundant genera among the four groups is shown in Figure

TABLE 4. Relative abundance of major phyla

Variable	Bacteroidetes	Firmicutes	Proteobacteria ^a
HIV negative (<i>n</i> = 17)	47.6 $\pm$ 23	50.8 $\pm$ 23	0.36 (0.2, 0.7)
HIV positive (<i>n</i> = 15)	43.8 $\pm$ 26	49.1 $\pm$ 28	1.56 (0.5, 2.2)
<i>p</i>	.7	.8	.03
Cocaine nonusers (<i>n</i> = 18)	37.1 $\pm$ 23	57.2 $\pm$ 26	0.51 (0.3, 1.1)
Cocaine users (<i>n</i> = 14)	57.0 $\pm$ 21	40.8 $\pm$ 21	0.91 (0.2, 1.9)
<i>p</i>	.02	.06	.4
HIV-negative nonusers (<i>n</i> = 10)	39.9 $\pm$ 23	58.9 $\pm$ 22	0.26 (0.2, 0.4)
HIV-negative cocaine users (<i>n</i> = 7)	58.5 $\pm$ 19	39.3 $\pm$ 18	0.82 (0.4, 1.9)
HIV-positive nonusers (<i>n</i> = 8)	33.6 $\pm$ 24	55.0 $\pm$ 31	1.4 (0.8, 1.9)
HIV-positive cocaine users (<i>n</i> = 7)	55.6 $\pm$ 25	42.3 $\pm$ 25	1.8 (0.09, 2.5)
<i>p</i>	.1	.3	.03

Notes: *M* $\pm$ *SD* with comparison by *t* test (binary) or analysis of variance unless otherwise noted. **Bold** indicates statistical significance. ^aMedian (interquartile range), with comparison by Wilcoxon rank-sum (binary) and Kruskal-Wallis.

FIGURE 1. Relative abundance (%) of major phyla by HIV infection and cocaine use (median). * $p = .03$ for difference in Proteobacteria by HIV infection and by group; † $p = .02$ for the difference in Bacteroidetes by cocaine use.

2; despite perceptible differences on the figure, there were no statistically significant differences between groups among these genera. The genus *Succinivibrio* and the genus *Alis-tipes* are not represented on the figure because their relative abundance was so small compared with the other genera.

Alpha diversity

Five alpha diversity indices were compared between groups based on HIV infection and cocaine use. Sequences

were rarified to 2,060 in order to calculate alpha diversity indices. Each parameter was calculated five times and the numerical average of each parameter was used for statistical analysis. There were no statistically significant differences between groups. In a model of HIV infection and cocaine use only, neither was associated with the equitability index, number of observed species, or Shannon diversity index. None of the variables predicted Chao-1 or phylogenetic distance indices above the median when controlled for HIV and cocaine use.

TABLE 5. Linear regression estimates for major intestinal bacterial phyla, adjusted for cocaine use and HIV infection

Variable	Bacteroidetes		Firmicutes	
	Estimate (SE)	<i>p</i>	Estimate (SE)	<i>p</i>
HIV infection	-4.8 (8)	.5	-0.9 (9)	.9
Cocaine use	20.2 (8)	.02	-16.3 (9)	.07
Age	0.8 (0.6)	.2	-0.9 (0.6)	.1
Female gender	-5.2 (11)	.6	6.8 (11)	.5
BMI	-0.1 (1.6)	.9	-0.5 (1.7)	.8
Healthy Eating Index	-0.1 (0.3)	.7	0.1 (0.3)	.7
CRP (natural log)	-1.9 (4)	.6	4.4 (4)	.6
Food security	-0.7 (10)	1.0	-6.6 (12)	.6
Alcohol use	26.0 (12)	.03	-30.8 (12)	.02
Current smoker	-6.6 (9)	.5	10.5 (11)	.4
Lean body mass ^a	-7×10^{-4} (7×10^{-4})	.3	8×10^{-4} (7×10^{-4})	.3

Notes: **Bold** indicates statistical significance. BMI = body mass index; CRP = C-reactive protein. ^aAdditionally adjusted for gender.

FIGURE 2. Relative abundance (%) of most abundant genera by HIV infection and cocaine use (median)

Discussion

Prior studies have identified differences in the intestinal microbiota between HIV-infected and uninfected persons but are difficult to compare given the wide variety of sampling methods. For example, *Pseudomonas aeruginosa* was found by quantitative PCR in 92% of fecal samples from HIV-infected persons who had not received highly active antiretroviral therapy, compared with only 20% of uninfected subjects (Gori et al., 2008). Ellis et al. (2011) found a trend for higher prevalence of the order Enterobacteriales in HIV-infected subjects than in controls using 16S rDNA PCR with specific primers for this order. In addition, the relative abundance of Enterobacteriales correlated with a lower duodenal CD4+ count, and the relative abundance of Bacteroidales correlated with activated CD8+ T-cells in peripheral blood (Ellis et al., 2011). In a mouse model of inflammation of various etiologies, the abundance of some phylotypes was reduced but the growth of others was fostered, specifically Enterobacteriaceae, of the Proteobacteria phylum (Lupp et al., 2007). In this study, we found an increased relative abundance of the phylum Proteobacteria (which includes the Enterobacteriales order and the species *Pseudomonas*

aeruginosa) among HIV-infected subjects compared with uninfected subjects. In addition, we found only HIV-infected individuals had members of the *Succinivibrio* genus, also a member of the Proteobacteria phylum. These data suggest that certain groups of phylotypes are associated with HIV infection and may contribute to inflammation in this population; however, larger studies are needed to confirm these findings and to determine if these differences have clinical relevance.

Cocaine use appears to affect the relative abundance of the two major phyla, Bacteroidetes and Firmicutes. The altered abundance of Bacteroidetes relative to Firmicutes has been seen in other studies. Specifically, the intestinal microbiota of children from Burkino Faso was enriched with Bacteroidetes compared with that of children from the European Union; this difference was postulated to result from dietary differences (De Filippo et al., 2010). Similarly, obesity has been associated with an increased relative abundance of Firmicutes compared with leanness; as obese humans lost weight, the abundance of Bacteroidetes in their intestines increased (Turnbaugh et al., 2006). Elderly persons (ages 70–85 years) have also been shown to have less Firmicutes and more Bacteroidetes compared with younger

persons (Mäkivuokko et al., 2010). We did observe that cocaine users had a lower Healthy Eating Index than nonusers; additionally, food security was lower among cocaine users. Food insecurity may reduce the diversity of the diet and could affect the types and variety of substrates presented to the gut microbiota.

Among females, cocaine users had higher lean body mass, whereas male cocaine users had lower fat mass than male nonusers. Lower fat mass was previously noted in female HIV-infected drug users compared with nonusers (Forrester et al., 2000) and in male HIV-infected drug users compared with nonusers despite similar energy intake (Forrester et al., 2005). As we did not note any associations between dietary or body composition measures with the relative abundance of phyla, it may be that in the setting of adequate dietary intake of nutrients, other factors associated with cocaine use and/or HIV may be the predominant determinants of the intestinal microbiota.

A study in mice suggested that in the context of immune dysfunction, the presence of the microbiota may encourage metabolic compromise in order to preserve immunity (Shulzhenko et al., 2011); this could also be viewed as a compromise that preserves the mutualism between the intestine and its microbiota (Costello et al., 2012). Bacterial genes can also affect metabolic reactions, including the transformation of bile acids and production of short-chain fatty acids (Nicholson et al., 2012). Bile acid metabolites serve as ligands for hormone receptors, such as the Farnesoid X receptor, which in turn affect lipid and carbohydrate metabolism, as well as the regulation of intestinal immunity (Nicholson et al., 2012). In addition, other metabolites of intestinal bacteria are linked to a variety of metabolic or homeostatic mechanisms, such as the endocannabinoid system, which is involved in appetite (Nicholson et al., 2012). Therefore, not only the presence of the microbiota but also its composition and metabolites can affect metabolism. This may be of particular importance in a population exposed to the various neuroendocrine effects of cocaine.

In concordance with prior studies, we observed elevated levels of soluble CD14 and decreased levels of anti-LPS antibodies in HIV-infected persons. Differences in inflammatory cytokines by HIV infection and cocaine use are of interest and, if confirmed in larger cohorts, could provide a mechanistic link between the composition of the intestinal microbiota, microbial translocation, and systemic inflammation in these groups: a disordered intestinal microbiota might impair local intestinal immunity, resulting in increased microbial translocation, upregulation of inflammatory cytokines, and resultant systemic inflammation. Therefore, although health outcomes cannot be directly linked to specific differences in the intestinal microbiota at present, these data suggest that with further metagenomics studies, the influence of these alterations at the molecular level could be determined.

In models predicting either Firmicutes or Bacteroidetes abundance, the effect size of alcohol use was unexpected. Eighty-eight percent of participants reported any alcohol use, although few subjects reported consuming alcoholic beverages in the last 24 hours. Thus, this effect is not likely mediated by participants' use of alcohol as a food substitute. When both heavy episodic drinking (five or more drinks in a row for men, four or more drinks in a row for women) and hazardous drinking (defined by the National Institute on Alcohol Abuse and Alcoholism [2005]) were plotted against the percentage of abundance of the two major phyla, the standard deviations around the means were much greater than for the alcohol use variable, although the direction of the effect was the same. With a larger sample size, the effect of the quantity of alcohol may have been clearer. Other investigators recently found that a subgroup of alcoholics whose mucosa-associated microbiota was classified as dysbiotic (using length heterogeneity PCR and multitag pyrosequencing) had increased abundance of Proteobacteria and decreased abundance of Bacteroidetes (Mutlu et al., 2012). These data are in contrast to our findings, but both the methods used and the defined groups are markedly dissimilar.

This study is limited by a small sample size; however, it was designed as a preliminary investigation to evaluate possible associations and to guide further research. Because of the sample size, we were unable to control for duration or type of antiretroviral therapy or duration and intensity of cocaine use. Our HIV-infected participants were all treated and had suppressed viral loads and high median CD4⁺ counts. This likely contributed to the applicability of the study results but may have limited our ability to detect differences that would be more pronounced in a less immune-competent sample. On average, HIV-infected participants were older than HIV-uninfected participants. However, all participants were younger than 60 years of age, which should limit the age-related changes in the intestinal microbiota. By limiting subject BMI to reduce possible confounding, we likely selected a narrower range of body composition values among participants, which may have limited our ability to detect associations between these measures and the intestinal microbiota. Nevertheless, we were able to detect differences in lean body and fat mass between cocaine users and nonusers despite this BMI restriction. Although there were few differences in dietary intake between groups, notably alcohol use, these differences could directly affect the intestinal microbiota. Larger studies could further evaluate the independent effects of cocaine use and HIV infection after adjusting for alcohol and diet through multivariable analysis.

Future research in this area could further explore the effect of cocaine use, of varying intensity and route of administration, on the intestinal microbiota. Similarly, the effects of cigarette smoking and alcohol use on the intestinal microbiota have not been widely studied in any population, and our preliminary results demonstrate a potential impact of

alcohol on microbiome composition. The intestinal microbiome in other populations with nutritional compromise, such as other substance users, hospitalized patients, and patients with liver disease or cancer, could also be evaluated with respect to composition, diversity, translocation, and systemic inflammation.

In conclusion, we identified differences in the relative abundance of major phyla of the intestinal microbiota, as well as markers of inflammation and microbial translocation, based on cocaine use and HIV infection. Cocaine users had a higher relative abundance of Bacteroidetes whereas HIV-infected individuals had a higher relative abundance of Proteobacteria. HIV-infected persons had higher levels of soluble CD14 and TNF- α and lower levels of anti-endotoxin core antibodies; HIV-infected cocaine users had higher IFN- γ levels than all other groups. Nutritional factors, including alcohol use, food insecurity, and lean body mass, may contribute to these differences. Larger studies are necessary to clarify the role of the intestinal microbiota in HIV infection and cocaine use.

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