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ORIGINAL ARTICLE

Morphine Potentiates Dysbiotic Microbial and Metabolic Shifts in Acute SIV Infection

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Abstract

Human Immunodeficiency Virus (HIV) pathogenesis has been closely linked with microbial translocation, which is believed to drive inflammation and HIV replication. Opioid drugs have been shown to worsen this symptom, leading to a faster progression of HIV infection to Acquired Immunodeficiency Syndrome (AIDS). The interaction of HIV and opioid drugs has not been studied at early stages of HIV, particularly in the gut microbiome where changes may precede translocation events. This study modeled early HIV infection by examining Simian Immunodeficiency Virus (SIV)-infected primates at 21 days or less both independently and in the context of opioid use. Fecal samples were analyzed both for 16S analysis of microbial populations as well as metabolite profiles via mass spectrometry. Our results indicate that changes are minor in SIV treated animals in the time points examined, however animals treated with morphine and SIV had significant changes in their microbial communities and metabolic profiles. This occurred in a time-independent fashion with morphine regardless of how long the animal had morphine in its system. Globally, the observed changes support that microbial dysbiosis is occurring in these animals at an early time, which likely contributes to the translocation events observed later in SIV/HIV pathogenesis. Additionally, metabolic changes were predictive of specific treatment groups, which could be further developed as a diagnostic tool or future intervention target to overcome and slow the progression of HIV infection to AIDS.

Keywords HIV pathogenesis · Opioids · Intestine microbiome · Intestine metabolism · Non-human primates · SIV

SR and ShB contributed equally to the senior authorship of this paper.

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Background

Over the last two decades, with the ongoing development of more effective multi-drug cocktails for the treatment of human immunodeficiency virus (HIV), HIV has transitioned from a fatal, untreatable disease to a chronic, manageable disease in patients with access to these therapeutics. With therapy, patient plasma viral loads can be dramatically reduced and maintained below the level of detection. However, as treated patients continue to live longer, there has also been an increased prevalence of age-related co-morbidities including cardiovascular disease (Grunfeld et al. 2009; Lorenz et al. 2008; Sico et al. 2015), kidney disease (Kalayjian et al. 2012; Ryom et al. 2014), osteoporosis (Brown and Qaqish 2006; Chitu-Tisu et al. 2016) and neurocognitive disorders (Antinori et al. 2007; Heaton et al. 2011; Tozzi et al. 2007). Underlying these pathologies is chronic immune activation attributed to a combination of sources including persistent, low-level viral replication, immune dysregulation, illicit drug abuse and microbial translocation (Lederman et al. 2013). In fact, it has been recognized that the intensity of chronic immune activation, not the level of viral replication, is the primary factor in determining disease progression (Giorgi et al. 1999; Hazenberg et al. 2003; Jiang et al. 2009). A decade ago, Brenchley et al. published a seminal perspective paper that put forward the idea that HIV infection leads to gut mucosal immune dysfunction and subsequent microbial translocation across the damaged epithelial barrier; postulating this as a key process in HIV infection driving chronic systemic immune activation which ultimately enhances disease progression(Brenchley et al. 2006). This proposition has been extensively studied and largely confirmed in recent years (Brenchley et al. 2006; Dandekar et al. 2010; Mudd and Brenchley 2016). However, mucosal immune dysfunction can also manifest in the context of other factors including illicit drug abuse (Meng et al. 2015).

There is a strong correlation between the abuse of illicit drugs, particularly intravenous drugs including opiates, and the acquisition and progression of HIV disease (Ancuta et al. 2008; Bokhari et al. 2011; El-Hage et al. 2008; Gurwell et al. 2001). It is well established that opiates such as morphine have immunomodulatory functions (Mellon and Bayer 1998; Roy et al. 2011). They inhibit cell-mediated immune responses by enhancing the expression of IL-4 while at the same time, suppressing the expression of IFN-y – two key cytokines regulating these responses (Roy et al. 2005; Wang et al. 2003). Therefore, it is not surprising that chronic morphine abuse in the context of HIV infection can exacerbate disease progression by attenuating the immune responses necessary for antiviral protection. Furthermore, chronic morphine exposure alone has been shown to reduce pathogen clearance and induce microbial translocation across the gut barrier providing another possible avenue for morphine-mediated enhancement of HIV disease progression (Banerjee et al. 2016a, b; Hilburger et al. 1997b; Meng et al. 2013).

The gut is colonized with a diverse population of billions of microorganisms termed the gut microbiome which is essential for maintaining homeostasis within the gut (O'Hara and Shanahan 2006). The gut microbiome serves several important functions including defense against colonization of pathogenic strains by displacement, production of bacteriocins and nutrient competition; maintenance of gut epithelial barrier; induction of mucosal secretory IgA to neutralize bacterial penetration; facilitating absorption of nutrients by microbial metabolism of indigestible carbohydrates and promoting systemic immune maturation and functions (Thaiss et al. 2016; Turner 2009). In light of these fundamental functions, the role of the gut microbiome in setting the stage for inflammatory manifestations is becoming increasingly recognized (Cabrera-Perez et al. 2016; Stecher 2015). Microbial dysbiosis is a hallmark of interrupted gut homeostasis and has been observed in numerous diseases(Pflughoeft and Versalovic 2012). Dysbiosis can occur as a direct effect of a pathogen outcompeting commensal bacteria, such as in *C. difficile* (Khoruts et al. 2010) or indirectly as a result of altered host immunity, as in inflammatory bowel disease (Nell et al. 2010). Microbial dysbiosis in HIV/SIV infections have been shown to be correlated with chronic immune activation in late stages of disease progression (Dillon et al. 2014; McHardy et al. 2013; McKenna et al. 2008; Moeller et al. 2013; Mutlu et al. 2014; Vujkovic-Cvijin and Dunham 2013). Furthermore, HIV patients treated with probiotics that restore non-pathogenic populations have demonstrated diminished inflammation and improved outcomes elucidating the integral role the microbiome exerts in the process of chronic inflammation (d'Ettorre et al. 2015). However, most studies examining changes in the gut microbiome involve cases of chronic SIV/HIV; this study seeks to determine if early changes are present and if so, could these changes predispose one to microbial translocation through immune dysregulation in gut mucosal tissues that ultimately accelerate disease progression.

Understanding the interplay between the gut microbiome, HIV and morphine is critical for the development of new therapies to ameliorate the chronic immune activation found in these patients. In this study, we utilize the SIV macaque model of HIV pathogenesis to assess for alterations in gut microbial composition and diversity as well as changes in bacterial associated metabolites in the presence or absence of acute SIV infection with or without chronic morphine dependence. Our results show that an acute 3-week SIV infection is sufficient to induce microbial metabolic changes even at this early time point. However, chronic morphine exposure produces more dramatic changes in the microbial community and related metabolites. Our data demonstrates that morphine and not SIV, at least at this early time point, has a greater impact on the induction of dysbiosis and associated changes in metabolites. Together, SIV infection in the context of chronic morphine dependence results in significant changes in the gut

microbiome providing potential mechanisms to support the immune dysregulation and enhanced pathogenesis found in chronically infected patients.

Methods

Availability of Data and Material NextGen sequencing files from the 16S microbiome analysis are available in the University of Minnesota "Data Repository for U of M" (DRUM) database and can be accessed at the following link: https://doi.org/10.13020/D6GC76

Metabolic data was processed and analyzed by Metabolon (http://www.metabolon.com/). The resulting data is presented in the S2_table.pdf attached with this submission. In addition, the final analysis provided by Metabolon can be requested from the authors on reasonable request.

Ethics Statement

All animal protocols were approved by the local animal care committee (IACUC, #2012–2070) at the University of Kansas in accordance with the Guide for the Care and Use of Laboratory Animals.

NHP Housing and Sampling

Animals used in this study were male, Indian-origin rhesus macaques between 3 and 4 years of age. All animals tested negative for tuberculosis, herpes B virus and simian retrovirus prior to enrollment in this study. Animals were individually housed in steel holding cages, in two dedicated rooms within the AAALAC-approved animal facility at the University of Kansas Medical Center. The NHPs were exposed, daily, to 12-h light-dark cycles and given laboratory chow and water ad libitum. All animals had access to numerous environmental enrichments. All animal protocols were approved by the local animal care committee (IACUC) at the University of Kansas in accordance with the *Guide for the Care and Use of Laboratory Animals*. Feceal samples were collected at the time points indicated (S1 Fig). Pre-treatment samples were obtained from all 14 male rhesus macaques (Pre). Four animals were given morphine alone (Ms; blue), with collections at days 21, 64, and 84 in addition to a pre sample (S1 Fig, top panel). Four animals were treated solely with SIV (SIV; red), and samples were collected 3, 8, 15, and 22 days post infection in addition to a Pre sample (S1 Fig, middle panel). Finally, SIV + morphine animals were treated with morphine (Ms; blue) for 70 days prior to receiving SIV infection (SIVMs;green) (S1 Fig, bottom panel). For this final group, samples were collected at 21 and 64 days prior to SIV treatment, and were analyzed as morphine alone. Following SIV infection, samples were collected at 73 days morphine/3 days SIV, 78 days morphine/8 days SIV, 84 days morphine/14 days SIV, and 92 days morphine/22 days SIV. The collections with SIV in these animals mirror the time points of fecal collections in SIV alone animals following infection.

Morphine Treatment

NHPs were injected intramuscularly three times daily (8 h interval) with morphine; the dose was ramped up during the first two weeks from 2 mg/kg (week 1) to 3 mg/kg (week 2) to the final dose of 4 mg/kg which was continued for the duration of the study (D84 for Morphine alone, D92 for Morphine + SIV). Morphine administration was maintained throughout the study to minimize withdrawal effects. NHPs co-infected with SIV were pretreated with opioids for 70 days prior to SIV infection to establish a chronic use period. Animals not receiving morphine were intramuscularly injected with saline in parallel with morphine administration. Pharmaceutical grade morphine (50 mg/mL) was purchased from the University of Kansas Medical Center Pharmacy.

SIV Infection

SIVmacR71/17E (Raghavan et al. 1999), an SIVmac239-derived virus, was originally prepared from pooled brain homogenates from macaques infected with R17 and R71E both of which cause encephalitis. Virus stock was prepared in CD8 + T cell depleted, ConA-activated macaque peripheral blood mononuclear cells (PBMC) and assayed for infectivity in CEMx174 cells and by plaque assay in GHOST Hi5 cells. Animals were inoculated intravenously via the saphenous vein with approximately 10^4 plaque forming units (PFU) of virus.

Necropsy

All monkeys were monitored daily for evidence of distress or disease including changes in attitude, appetite or behaviors suggestive of illness (lack of movement, withdrawn behavior, etc). At study endpoint (after 12 weeks of morphine or 3 weeks SIV infection) animals were euthanized. In preparation for necropsy, the animals were deeply anesthetized with ketamine (3 mg/kg) and medetomidine (0.15 mg/kg) given IM. Laparotomies were then performed, and the animals exsanguinated from the descending aorta and perfused transcardially with normal saline. A comprehensive sample collection was conducted throughout the body including the ileum. Samples were placed into cassettes and fixed in 4% paraformaldehyde or 10% formalin. These cassettes were then processed for routine histopathological analysis.

Viral Loads in Plasma

Viral loads in the plasma were assessed by Real-time RT-PCR. Briefly, plasma were centrifuged at 20,000 g for 1 h at 4 °C and pellets were resuspended in PBS. RNA was isolated from the pellets using the QIAgen RNA mini-kit (Qiagen). Real-time PCR was performed using SIV gag-specific primers and a Taqman probe (Applied Biosystems) and analyzed on the ABI Prism 7700 sequence detection system. Thermal cycling conditions were as follows: 50 °C (2 min), 60 °C (30 min), 95 °C (10 min), 95 °C (15 s; 44 cycles) and 60 °C (30 s; 44 cycles). Viral loads were calculated as Gag copies/million GAPDH copies. The minimum level of detection was 18 copies.

16S Sequencing

Feces were collected from animals and frozen in liquid nitrogen. The fecal matter was lysed using glass beads in MagnaLyser tissue disruptor (Roche) and total DNA isolated using Power-soil/fecal DNA isolation kit (Mo-Bio) as per manufacturer's specifications. All samples were quantified via the Qubit® Quant-iT dsDNA Broad-Range Kit (Invitrogen, Life Technologies, Grand Island, NY) to ensure that they met minimum DNA concentration and mass requirements and submitted to the University of Minnesota Genomic Center for microbiome analysis as follows: To enrich the sample for the bacterial 16S V4 rDNA region, DNA was amplified utilizing fusion primers designed against the surrounding conserved regions which are tailed with sequences to incorporate Illumina (San Diego, CA) flow cell adapters and indexing barcodes. Each sample was PCR amplified with two differently bar coded V4 fusion primers and were advanced for pooling and sequencing. For each sample, amplified products were concentrated using a solid-phase reversible immobilization method for the purification of PCR products and quantified by electrophoresis using an Agilent 2100 Bioanalyzer®. The pooled 16S V4 enriched, amplified, barcoded samples were loaded into the MiSeq® reagent cartridge, and then onto the instrument along with the flow cell. After cluster formation on the MiSeq® instrument, the amplicons were sequenced for 250 cycles with custom primers designed for paired-end sequencing. Using QIIME (Caporaso et al. 2010), sequences were quality filtered and demultiplexed using exact matches to the supplied DNA barcodes. Resulting sequences were then searched against the Greengenes reference database of 16S sequences, clustered at 97% by UCLUST (closed-reference OTU picking).

Mass Spectrometry Analysis

Fecal samples were collected as for 16S DNA analysis. Samples were shipped on dry ice to Metabolon, Inc., where samples were prepared and analyzed using a 548 named biochemical compounds screen. Briefly, samples were divided into 4 fractions for analysis: one for UPLC-MS/MS with positive ion mode electrospray ionization, one for analysis by UPLC-MS/MS with negative ion mode electrospray ionization, one for analysis by UPLC-MS/MS polar platform (negative ionization), one for analysis by GC-MS. Raw data was analyzed with peak-identification and quality control, processed, and normalized using proprietary hardware and software, utilizing a library of authenticated standards.

16S Analysis and Statistics

OTU tables were rarefied to the sample containing the lowest number of sequences in each analysis. QIIME 1.8 was used to calculate alpha diversity (alpha_rarefaction.py), summarize taxa (summarize_taxa_through_plots.py), and compare variability within and between groups (compare_distance_boxplots.py). A repeated measures ANOVA was used to compare Observed OTU and Shannon Diversity Index values between groups and over time with subject included in the error term. PERMANOVA analysis was done using the pseudo-F statistic within each time point and group independently (time points: Ms., $N = 4$; SIV, $N = 5$; SIVMs = 7) using 999 permutations. OTU table output was visualized and analyzed using Statistical Analysis of Metagenomic Profiles, or STAMP, software (Parks et al. 2014). Principal Coordinate Analysis was done within this program using observation ID level. Heatmaps were generated using family level (L5) taxonomic data, which included microbes that were significantly changed ($p < 0.05$) between groups compared: Pre vs Ms. timepoints (Fig. 2), Pre vs SIV timepoints (Fig. 3), and Pre vs SIV + Ms. timepoints (Fig. 4). Indicator species analysis was performed in R using the IndicSpecies package with combined taxonomic output from the summarize taxa command above (De Cáceres and Legendre 2009). The relative abundances of all OTUs were compared across each group (Pre, Ms., SIV, SIVMs). A complete list is provided in S4 Table.

Mass Spectrometry Analysis and Statistics

Metabolites were log transformed and ANOVA contrasts and paired t-tests examined metabolic differences between treatment groups and time points. Random forest analysis provides an unbiased and supervised classification based on decision trees, and identifies features (metabolites) that predict sample group membership. The top 30 biochemicals are presented.

Results

In order to look for short-term changes in the presence of SIV, animals were observed for 21 days post-infection with SIV.

The infection paradigm was carried out in both control animals as well as animals that were given chronic morphine (S1 Figure) in order to detect whether opioids alter early pathogenesis of SIV, particularly on the microbiome of the intestine. SIV infection was validated with real-time PCR on plasma samples which all confirmed positive infection of SIV animals (S3 Table). Fecal samples were collected at regular intervals to assess changes in the microbiome over this early time period.

Microbial Diversity Is Not Altered by SIV and Morphine

Microbial diversity did not differ significantly between groups (Fig. 1a – Observed OTUs; $F1,72 = 1.59$, $p = 0.211$; Shannon Diversity Index; $F1,72 = 0.298$, $p = 0.743$).

Each of the four treatment groups were then stratified over the time points collected to see if any changes occurred over time. There were no significant time-associated changes observed in Ms. (Fig. 1b), SIV (Fig. 1c), or SIVMs (Fig. 1d) samples compared to matched Pre samples in each group (SIV + Ms. $F_{6,35} = 1.044$, $p = 0.414$; SIV only $F_{4,15} = 0.63$, $p = 0.649$; Morphine only $F_{3,12} = 0.4$, $p = 0.755$). This suggests that changes in microbial diversity are not a hallmark of ongoing exposure to infection or drug exposure at the early time points observed.

Morphine and SIV Significantly Alter Gut Microbial Composition

Microbial composition was compared between groups and time points. Morphine caused a shift in microbial composition evident in beta-diversity (weighted UniFrac) plots (Fig. 2a). PERMANOVA analysis indicated a significant difference in microbial composition pre and post morphine induction (pseudo-F: 4.027, p value: 0.004). Post-morphine induction, microbial composition remained stable and did not change significantly over time (PERMANOVA pseudo-F: 0.537, p value: 0.944). Differentially abundant taxa included *Methanobacteriaceae* of the domain *Archaea* which was enriched in many of the post-morphine induction Ms. samples, and *Streptococcaceae* and *Ruminococcaceae* family microbes which were enriched in the pre-morphine samples (Fig. 2b).

Microbial composition was not significantly altered by SIV infection alone, although many samples appeared to be trending away from Pre samples (Fig. 3a). A PERMANOVA analysis of the five SIV sample time point groups (pseudo-F: 1.4354, p value: 0.131) show that the microbial composition is not significantly altered between groups. Despite this, a heatmap of the significantly altered families shows clustering among many SIV samples apart from Pre samples, though the

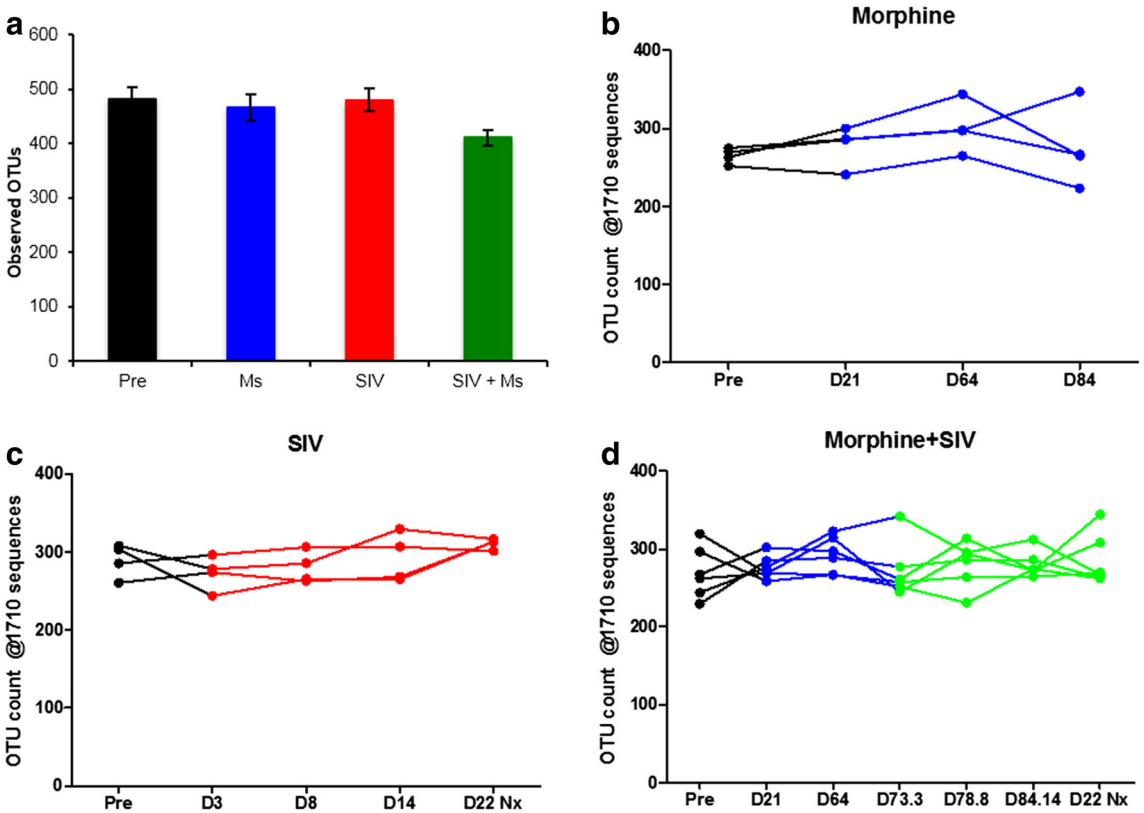

Fig. 1 Morphine and/or SIV does not change the number of species over time. The number of species (alpha diversity) was calculated up to 1710 sequences, which was the minimum sequence number in all samples. (**a**) No significant differences were observed in microbial diversity across groups. At 1710 sequences, there was no change in at any time point in Ms. (**b**), SIV (**c**) or SIVMs (**d**) treated animals

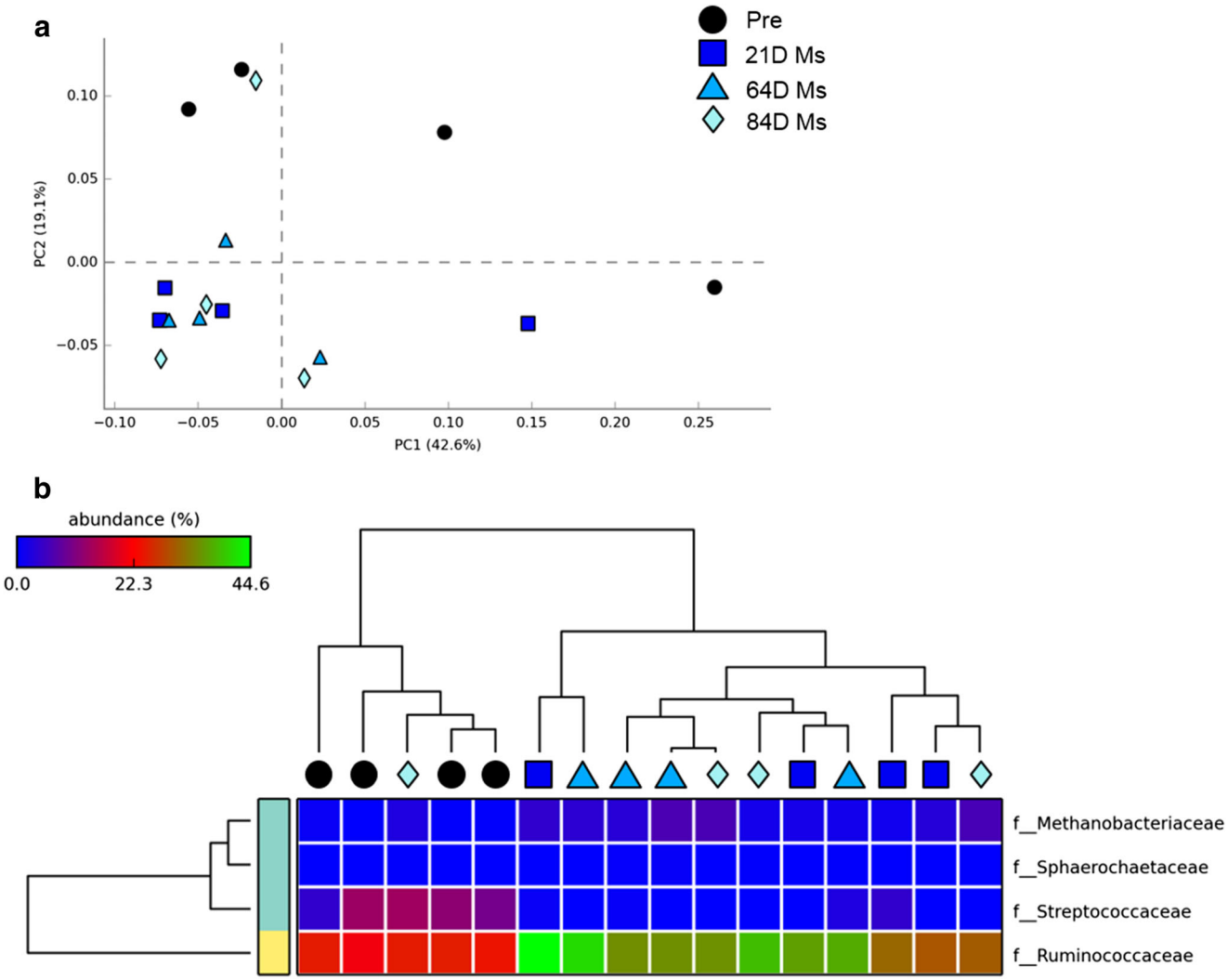

Fig. 2 Morphine induces dysbiosis in nonhuman primates which is stable over time. (**a**) Weighted PCoA of Ms. (various shades of blue) altered the microbial signature of animals from matched Pre (black) samples at the earliest (21D) time point, which was stable over time. (**b**) A heatmap of significantly changed families ($p < .05$) between time points showed that Pre (black) was distinctly clustered from Ms. samples, aside from one animal at 84D morphine

Pre samples themselves form two clusters (Fig. 3b). *Ruminococcacea* family microbes were enriched post-SIV infection (Fig. 3b). Notably, the SIV samples that cluster closely with Pre samples (black) are animal matched, suggesting that SIV did not induce early changes unilaterally, but may have greater effects in a subset of animals.

SIVMs (green) samples showed a shift in microbial composition similar to the microbial community shift seen in Ms. (blue) time points (Fig. 4a), again indicating that morphine has a greater effect on the microbiota than SIV at this early point infection. PERMANOVA analyses indicated that there was a significant change in the microbial community between Pre samples and all samples collected after morphine treatment began (pseudo-F: 6.839, p value = 0.001). However, there was no significant difference in the microbial communities of monkeys treated with morphine and monkeys treated with morphine and SIV (pseudo-F = 0.956, p value = 0.421. This was further explored by comparing weighted UniFrac distances within and between groups (S3 Fig. Distances within Pre samples were significantly shorter than distances between Pre and Ms. samples and between Pre and SIVMs samples (Pre vs. Pre / Pre vs Ms. Bonferroni-corrected $p = 0.023$, Pre vs. Pre / Pre vs. SIVMs Bonferroni-corrected $p = 0.009$, t-test). However, distances within Ms. samples and between Ms. samples and SIVMs samples was not significantly different (Bonferroni-corrected $p = 1$, t-test). These results indicate that the microbial community shifts after morphine introduction but does not change significantly when SIV is subsequently introduced. Differential abundance analysis revealed that, similar to morphine alone, *Methanobacteriaceae* family microbes were enriched and *Streptococcaceae* family microbes were diminished in morphine and SIV + morphine samples as compared to Pre samples (Fig. 4b).

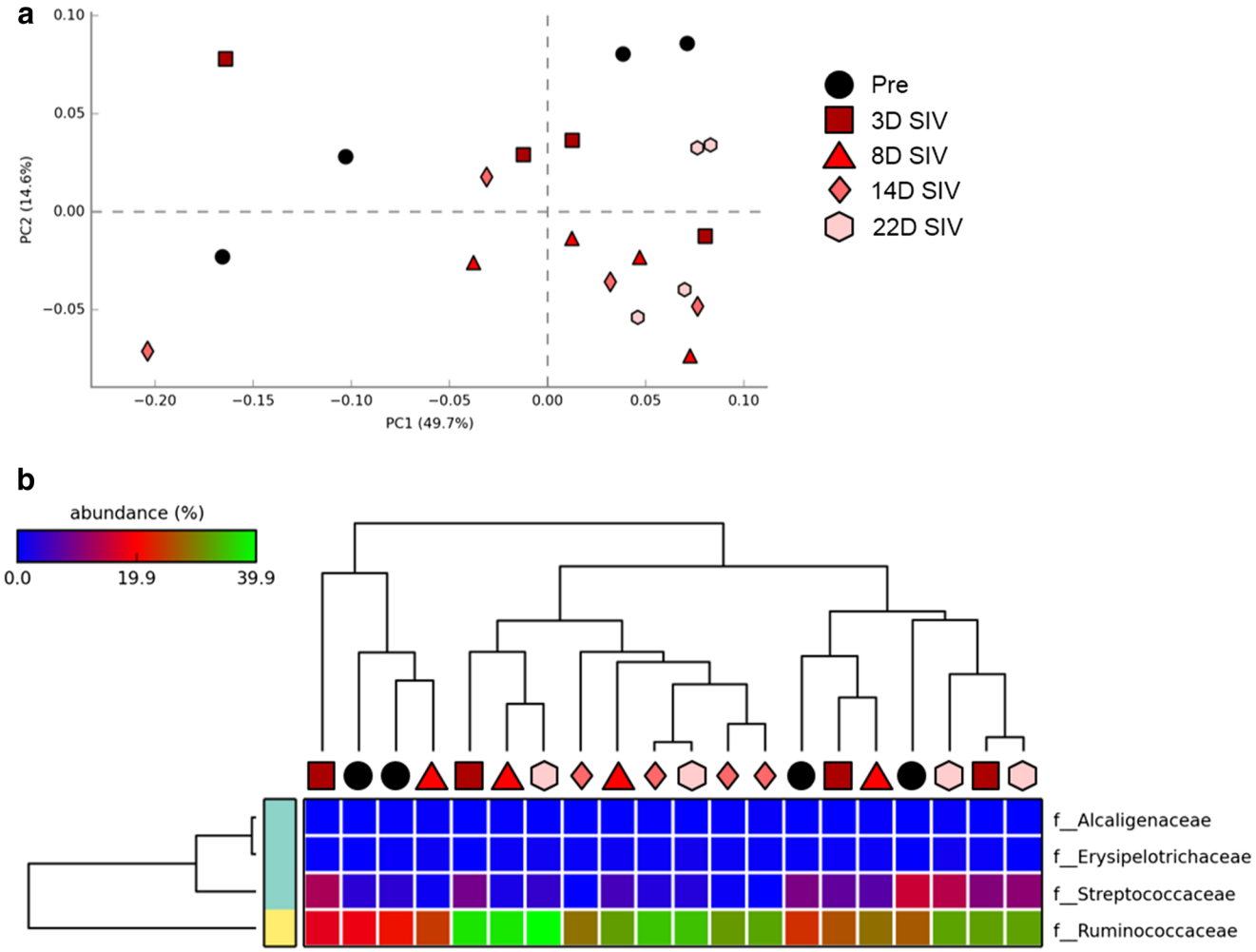

Fig. 3 SIV does not significantly alter total microbial composition, but certain families are significantly altered at early timepoints. (**a**) Weighted PCoA of SIV (various shades of red) altered the microbial signature of animals from matched Pre (black) samples. However, the earliest (3D, dark red) time point was not changed greatly. (**b**) A heatmap of significantly changed families ($p < .05$) between time points showed disparity between Pre (black) samples. SIV samples that associated closely with Pre samples were from the same animal, implying that SIV had no induced dysbiosis at that point. Despite this, a large number of SIV samples clustered distinctly as a middle branch from Pre

The species composition of all treatments was compared as a dendogram (S2 Fig). The dendogram corroborates evidence of shifts observed with morphine treatment in Ms. (blue) and SIVMs (green), which cluster distinctly from matched Pre samples (black). SIV (red) clusters more closely with matched Pre samples than samples with morphine (blue/green), again showing that changes due to SIV at this early stage of infection are less significant than the dysbiosis induced by morphine.

Specific Microbial Taxa Are Linked with Morphine Treatment or SIV Infection

To understand if particular taxa were driving the clustering in the species composition, an indicator species analysis was performed on the Pre and terminal collection time points (84D morphine, 22D SIV, 92D morphine/22D SIV) for each group (S1 Table). All three treatment groups showed a reduction in the *Leuconostocaceae* family compared with Pre samples. Interestingly, despite not showing significant beta diversity alterations, SIV was associated with increases in *Veillonellaceae, Fibrobacteraceae fibrobacter, Veillonellaceae Megasphaera, Ersipelotrichaceae RFN20,* and *BS11*. SIV was also associated with decreases in the *TM7–3* order along with *Paraprevotellaceae YRC22*. Morphine was associated with decreases in *Streptococcaceae* streptococcus as well as *Pasteurellaceae Aggregatibacter*. Finally, SIVMs samples showed a decrease in *Veillonellacea dialister, Pasteurellacea actinobacillus, Pasteurellacea haemophilus,* and *Methanobacteriacea methanosphaera*.

Histological data from these each group show infiltration of immune cells into the lamina propria however

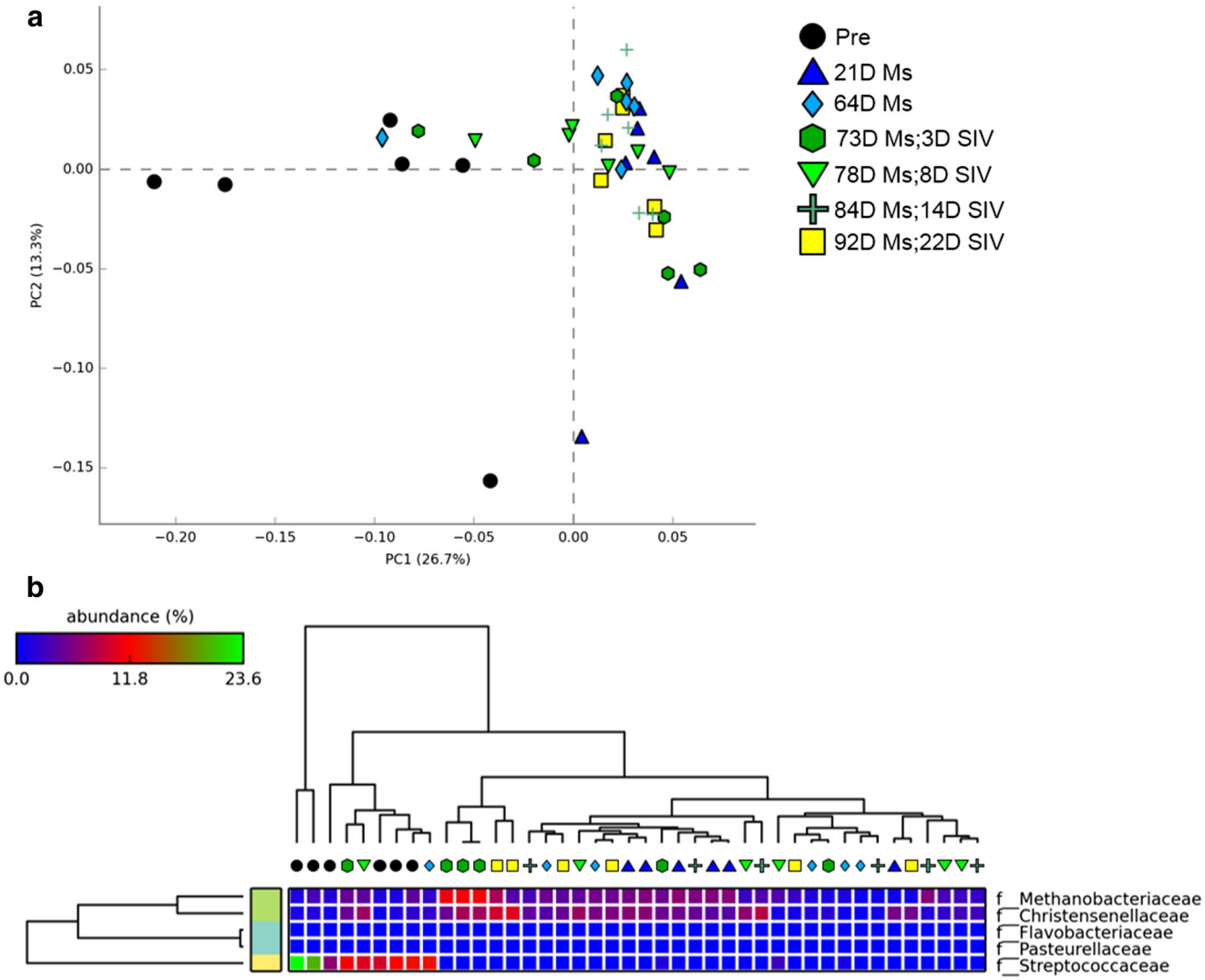

Fig. 4 Morphine induces dysbiosis that is not altered further with SIV infection. (**a**) Weighted PCoA of NHP infected with SIV and co-treated with morphine. As with Ms. animals, morphine (various shades of blue) altered the microbial signature of animals from their pretreatment (black) samples at the earliest (21D) time point, which was stable over time even after the animals were infected with SIV (various shades of green). (**b**) A heatmap of significantly changed families ($p < .05$) between time points showed that pre-treatment (black) was distinct from the majority of treatment samples (various shades of blue and green). Treatments (matched Ms. vs SIVMs) or time points did not cluster distinctly

no significant difference was observed between the SIV and the morphine or morphine+ HIV groups (S4 Figure). Overall, the species highlighted in this analysis are again supportive of microbial dysbiosis and contributing to the overall inflammatory environment observed in patients with SIV/HIV infections and chronic opioid abusers.

Microbial Metabolites Are Significantly Altered with SIV Infection and more So when Combined with Chronic Morphine

In order to capture the functions associated with each microbial community, we examined the metabolic profile of Pre samples and terminal points (22D SIV, 64D morphine, and 92D morphine+ 22D SIV). We selected these time points because limited differences were observed at intermittent time points within a treatment group. A significant number of metabolites were altered in each treatment group (D64 morphine, 22D SIV, 92D + 22D Morphine+SIV) compared with pre-treatment samples, indicating that the treatments drastically altered the metabolic profile. To this end, the metabolic profile for each treatment group was loaded into a forest prediction matrix and each had strong predictive capabilities: D64 morphine vs pre-samples (85% predictive accuracy, Fig. 5a), D22 SIV vs pre-samples (87% predictive accuracy, Fig. 5b), and 64D morphine vs 92D Morphine +

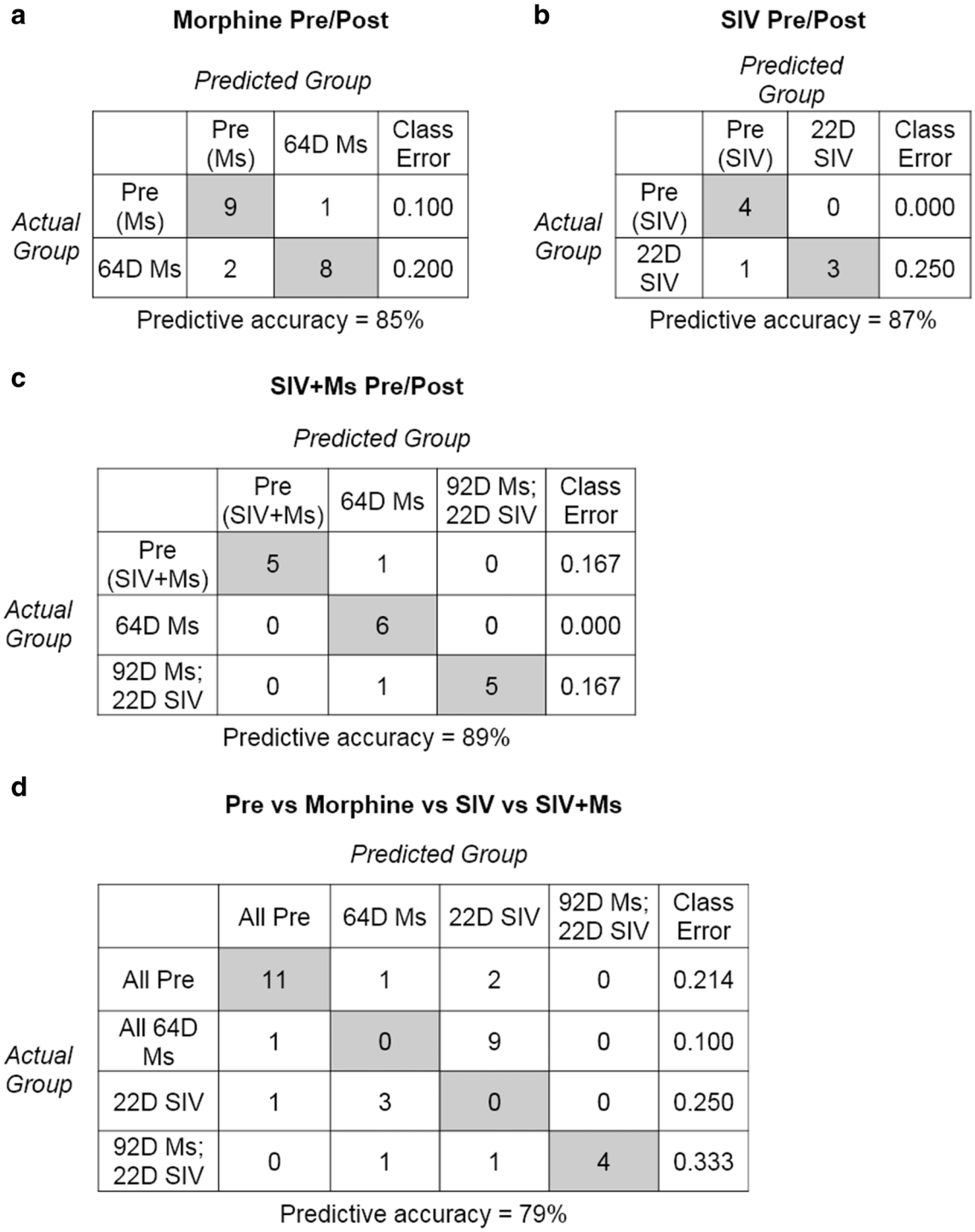

Fig. 5 Forest Matrix analysis of metabolic profile predicts treatment groups high accuracy against matched pre-samples. The forest matrix assessment was used to predict sample treatments based on their metabolic profile. **a**) There was a predictive accuracy of 85% between matched NHP treated with morphine (64D, $n = 10$, class error = .2) vs pre-treated samples (n = 10, class error = .1). **b**) There was a predictive accuracy of 87% between matched NHP infected with SIV (22D, $n = 4$, class error = .25) vs pre-treated samples (n = 4, class error = 0). **c**) There was a predictive accuracy of 89% between matched NHP treated with morphine (64D, $n = 6$, class error = 0), NHP treated with morphine and infected with SIV (92D Ms/22D SIV, n = 6, class error = .167), and pre-treated samples (n = 6, class error = .167). **d**) There was a predictive accuracy of 79% between NHP treated with morphine (64D, n = 10, class error = .1), NHP treated with SIV alone (22D, n = 4, class error = .25), NHP treated with morphine and infected with SIV (92D Ms/22D SIV, n = 6, class error = .333), and pre-treated samples ($n = 14$, class error = .214)

22D SIV vs pre-samples (89% predictive accuracy, Fig. 5c). When all treatment groups were considered together, the model still maintained a 79% predictive accuracy amongst all treatment groups (Fig. 5d). Metabolites that defined each prediction were prioritized to see which played a major role (S4 Fig).

The primates treated with morphine alone exhibited significant alterations in several fecal metabolites (S2 Table). The most notable alterations in this group involved biochemicals linked to microbiome function, oligosaccharide metabolism, and amino acid and lipid homeostasis. Compared with pre-treated controls, NHP treated with morphine showed a decrease in primary bile acids including cholate (0.48 fold change, $p < .0031$) and glycocholate (0.48 fold change, $p < .0015$); conversely, several secondary bile acids increased, including 7-ketolithocholate (1.35 fold change, $p < .0166$), 12-dehydrocholate (3.62 fold change, $p < .0015$), taurocholenate sulfate (6.33 fold change, $p < .0388$) and 3b-hydroxy-5-cholenoic acid (2.4 fold change, $p < .0201$). Additionally, morphine showed an increase in sphingolipid metabolites, including sphinganine (6.68 fold change, $p < .0211$) and sphingosine (3.8 fold change, $p < .0056$). These changes may correlate with differences in dietary intake and absorption or with changes in fatty acid and triglyceride metabolism within the morphine-treated subjects which will need to be examined in a future study.

Similar to microbiota analysis, the SIV-infected group displayed significantly fewer metabolite alterations than morphine when it was compared to its control group. Serotonin metabolites and microbial metabolites were among the biochemicals most strongly altered in NHP infected with SIV (S4B Fig). When the D22 SIV group was compared to the Pre SIV group, significant increases were observed in serotonin (4.03 fold change, $p < .0002$) and its acetylated derivative N-acetylserotonin (7.93 fold change, $p < .0081$).

The number of statistically significant metabolite differences arising from the SIV infection rose dramatically, however, when morphine was already on board (Table 1). When the day 92 Morphine+SIV group was compared to the day 64 Morphine+SIV group, significant alterations were observed in some of the biochemicals affected in the day 22 SIV-infected group. Significant examples included serotonin (SIV: 4.03 fold change, $p < .0002$; SIVMs: 10.49 fold change, $p < 6.86E-07$), N-acetylserotonin (SIV: 7.93, $p < .0081$; SIVMs: 14.84 fold change, $p < 3.93E-06$), N-acetylkynurenine (SIV: 3.92 fold change, $p < .077$; SIVMs: 7.99 fold change, $p < .0006$), tricarballylate (SIV: 7.26 fold change, $p < 0.359$; SIVMs: 2.57 fold change, $p < .0325$), and secondary bile acids such as deoxycholate (SIV: 7.17 fold change, $p < .0198$; SIVMs: 7.46 fold change, $p < .0027$). These metabolites appear to track therefore with SIV infection status.

Discussion

This study shows for the first time that morphine induces microbial dysbiosis in NHP, and that dysbiosis associated with SIV begins developing within 3 weeks (22 days) of infection. Significant changes were observed in the bacterial composition of morphine- treated animals, particularly in partnership with SIV infection, compared with pre-samples. Changes in bacterial composition has been a hallmark for negative outcomes in other studies, including HIV (Gómez-Hurtado et al. 2011; Tamboli et al. 2004; Vujkovic-Cvijin et al. 2013). This study also examined functional changes by characterizing fecal metabolic profiles, which were also significantly altered by both morphine and SIV infection. Combined, this study supports that SIV infection alters the gut microenironment beginning at very early time points of infection, and opioids like morphine are a dominant force that will exacerbate SIV-mediated alterations early in infection, which has important ramifications for opioid abusers who become infected with HIV.

The link between microbiota and immune activation and inflammation has been well established (Belkaid and Hand 2014). The effects of morphine on the microbiota have not been examined in NHP or humans previously, but have been observed in mice using a slow-release pellet (Santanu Banerjee et al. 2016a, 2016b). The morphine-associated dysbiosis observed in the present study by morphine was consistent over the time course of

Table 1 Significantly changed metabolites between treatment groups

	Morphine only	SIV only	Morphine + SIV
Comparison groups - paired t-test	64D Ms Pre	22D SIV Pre	92D Ms./22D SIV 64D Ms
Total biochemicals ($p < 0.05$)	163	49	164
Biochemicals ($p < 0.05$) # up | # down	67 | 96	29 | 20	100 | 64
Total biochemicals ($0.05 < p < 0.10$)	67	46	53
Biochemicals ($0.05 < p < 0.10$) # up | # down	39 | 28	20 | 26	32 | 21

drug administration (D21, D64, D84). While the mechanism for these changes was not assessed, opioid-induced immune modulation has previously been demonstrated in the gut, which results in bacterial translocation and altered tolerance to bacteria-mediated inflammation(Santanu Banerjee et al. 2013; Feng et al. 2006; Hilburger et al. 1997a, b; Meng et al. 2013; Ninković and Roy 2012). Another potential mechanism is through constipation, which has also been shown, independent of morphine, to alter intestinal microbiota (Zhu et al. 2014). It is likely that both contribute to an extent, and as this study did not directly control for constipation, it will need to be addressed in future work. Dysbiosis with morphine was maintained through long-term exposure to morphine, which suggests that the microbial shift is not limited by the development of tolerance. However, with this study design it is unclear how long these changes are stable and whether they would persist in the absence of escalating doses that are generally observed in chronic opioid users.

In this study, SIV infection was examined at time points earlier than previous studies of SIV- or HIV-induced microbial dysbiosis (Dillon et al. 2014; McHardy et al. 2013; McKenna et al. 2008; Moeller et al. 2013; Mutlu et al. 2014; Vujkovic-Cvijin and Dunham 2013), During the infection window, a gradient is visible in beta diversity from pre-samples to D22, though the composition changes did not reach statistical significance. Specifically, SIV animals had an enrichment in the genera *Megasphaera* and family *Erysipelotrichaceae which have been associated with immune activation in previous studies of HIV* (Neff et al. 2018; Vázquez-Castellanos et al. 2015). Additionally, the metabolic profile of SIV-infected animals was altered. This suggests that microbial function changes before changes in microbial composition are observed in association with SIV.

Previous studies of HIV have shown a link between dysbiosis and bacterial translocation, (Dinh et al. 2015; Gómez-Hurtado et al. 2011; Wu et al. 1998); therefore, dysbiosis and metabolic shifts may be a precursor to the bacterial translocation thought to drive HIV replication (Brenchley et al. 2006). Bacterial translocation or serum levels of LPS was not measured as part of this study design, and are not believed to occur early in infection (Brenchley et al. 2006). While it is clear that dysbiosis begins at this early stage of SIV infection, future studies with intermediate time points will be necessary to fully map the dysbiosis to the onset of bacterial translocation observed in late-stage SIV- or HIV-infections. This would allow for interventional therapies to be developed, as the microbial shift that occurs is likely to play a role in disrupting gut homeostasis that helps drive HIV replication.

NHP infected with SIV after treatment with morphine showed significantly altered bacterial composition and metabolite profiles, which likely underlie the relatively quick pathogenesis observed in HIV patients who abuse opioids (Meng et al. 2015; X. Wang et al. 2011). A specific example we observed was alterations of microbes within the *Methanobacteriacea* family in these samples, which have been shown to play an essential role in the production and adsorption of short-chain fatty acids (Pimentel et al. 2012). We observed changes in sphingolipid metabolism in morphine samples, which may play a role in the hosts ability to function properly. Morphine has been shown to greatly increase HIV and SIV pathogenesis (Donahoe and Vlahov 1998; Rivera-Amill et al. 2010). Thus, we expected that SIV with morphine would induce dysbiosis earlier than SIV alone, but this was not supported by our data. Animals co-treated with SIV and morphine did not cluster distinctly from animals treated solely with morphine, suggesting that SIV has a relatively minor impact on the microbiome compared with morphine at these early time points of infection. However, future studies will be needed to investigate intermediate time points of SIV infection to see if dysbiosis occurs more overtly. Additionally, different paradigms of opioid use will need to be tested in the future. Infecting the animals prior to morphine exposure or looking at the effects of withdrawal within early time points of SIV infection would also be interesting avenues to pursue.

Consequences from microbial dysbiosis include functional shifts in the metabolic profile. Metabolite shifts can contribute to host pathologies and disruption of gut homeostasis, including further changes to the gut microbiome (Asselin and Gendron 2014; Russell et al. 2013). Not surprisingly, altered metabolism of gut microbiota has been shown to be a factor in chronic immune activation of HIV patients (Vázquez-Castellanos et al. 2014). The metabolic environment is clearly shifting in early SIV infection as well as in the context of morphine. This was observed through the forest matrix analysis, which accurately predicted treatments of the samples at or above 79%. In addition, numerous individual metabolites were significantly shifted.

Of the metabolites shifted, bile acids were notable because of their established role in preventing bacterial overgrowth and translocation (Boesjes and Brufau 2014; Hofmann and Eckmann 2006; Lorenzo-Zúñiga and Bartoli 2003). Select primary bile acids were decreased when morphine was present, whereas secondary bile acids were increased by both morphine and SIV. Thus, the deregulation of bile acid metabolism may contribute to microbial dysbiosis, and the eventual bacterial translocation observed in SIV and HIV infections. In previous experiments, we observed that morphine deregulated both primary and secondary bile acids in mice (Santanu Banerjee et al. 2016a, b). As this study focused on bile acids in feces, future studies will need to examine the host contribution to the bile acid cycle, including liver production, to see how morphine influences the pathway in NHP. If it is an early step in bacterial translocation, which drives HIV replication and may occur earlier in HIV-infected individuals who abuse opioids, bile acids may be a therapeutic target to limit HIV replication.

Other metabolic shifts observed have functional potential as well. Samples treated with morphine, but not infected with SIV, displayed an increased number of sphingolipids. This is interesting, as sphingolipids have been shown in other studies to be increased with both morphine and HIV. These papers generally associated sphingolipids with negative effects on neurological symptoms (Bae et al. 2014; Bandaru et al. 2011; Muscoli et al. 2010), which generally occurs chronically with HIV and faster when opioids are present (Bell et al. 1998; Donahoe and Vlahov 1998; Hauser et al. 2007).

Additionally, serotonin was increased in association with SIV infection. This correlates with an increase in *Clostridia,* which emerged as a significant indicator of SIV-infected animals, and have been shown to produce serotonin(Yano et al. 2015). Aside from functioning as a neurotransmitter, serotonin also has roles in modulating gut motility and immune responses (Gershon 2004; Khan and Ghia 2010; Spiller 2007). The increase in serotonin metabolites may correlate therefore with alterations in intestinal function and/or with alterations in the immune system of NHP infected with SIV. Interestingly, changes in serotonin receptors have been described in NHP infected with SIV are reported to express differential levels of the serotonin receptor SERT, which occurred as early as 7 days after infection in peripheral blood mononuclear cells (Yu et al. 2010).

This study shows that morphine induces microbial dysbiosis and functional shifts in the metabolic profile that can interact at early time points with SIV infection. In combination with SIV infection, morphine-induced significant microbial and metabolic dysbiosis, which supports the worsened pathogenesis observed in co-morbid patients. Altered metabolites, such as bile acids, sphingolipids, and serotonin, can have detrimental impacts on the host, particularly for immune responses that require tight regulation and ultimately drive HIV replication. This study was conducted using time points of SIV-infection much earlier than has previously been studied with microbial dysbiosis in SIV/HIV. Ultimately, this study highlights potential changes that may be advantageous to intervene with therapeutically as early as possible to delay or prevent the onset of AIDS, particularly in opioid abusing patients.

Conclusion

To our knowledge, this is the first time that the effects of opioids on the intestinal microbiome have been studied at early stages of HIV/SIV infection. In late stage HIV, it is well accepted that bacterial translocation results in circulating LPS that helps to drive HIV replication through systemic inflammation (Brenchley et al. 2006). Given the importance of bacterial translocation in the pathogenesis of HIV, we believe that the observed dysbiosis in bacterial communities and metabolites by morphine at early stages of SIV infection demonstrates an important role of opioids in promoting systemic LPS in HIV patients. Having an altered bacterial community, as well as a disrupted bacterial metabolite profile, contributes to increased or unregulated inflammation that can help drive HIV replication (Gómez-Hurtado et al. 2011). Given that opioids promote these changes, as observed in this study with early time points of SIV infection, we believe that this is a mechanism to help explain the increased progression of HIV to AIDS in the opiate-using population. By understanding the onset of HIV to AIDS in this vulnerable population, we can hopefully design better therapies to abate the disease and generate better long-term outcomes.

Authors' Contributions GS, ShB, and SR conceived the idea for the project. GS and VH analyzed and arranged figures from the bioinformatics data. SC, RH, and PC cared for the animals, delivered treatments, and handled sample collection. GS, SB, JM processed samples for 16S sequencing and metabolic analysis. FV performed histology staining and analysis. GS wrote the initial draft of the paper. GS, SC, ShB, and SR were the primary editors and reworked the manuscript. All authors have read and approved the final manuscript.

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Compliance with Ethical Standards

Ethics Approval All animal protocols were approved by the local animal care committee (IACUC, #2012–2070) at the University of Kansas in accordance with the Guide for the Care and Use of Laboratory Animals.

Consent for Publication Not applicable.

Competing Interests The authors declare that they have no competing interests in this section.

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