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Reduced Gut Microbiome Diversity in People With HIV Who Have Distal Neuropathic Pain

Ronald J. Ellis,* Robert K. Heaton,† Sara Gianella,‡ Gibraan Rahman,§ and Rob Knight¶

*Departments of Neurosciences and Psychiatry, †Department of Psychiatry, ‡Department of Medicine, §Bioinformatics and Systems Biology Program, ¶Department of Pediatrics, University of California, San Diego, California

Abstract: **Objective:** Gut dysbiosis, defined as pathogenic alterations in the distribution and abundance of different microbial species, is associated with neuropathic pain in a variety of clinical conditions, but this has not been explored in the context of neuropathy in people with HIV (PWH).

**Methods.** We assessed gut microbial diversity and dysbiosis in PWH and people without HIV (PWoH), some of whom reported distal neuropathic pain (DNP). DNP was graded on a standardized, validated severity scale. The gut microbiome was characterized using 16S rRNA sequencing and diversity was assessed using phylogenetic tree construction. Songbird analysis (https://github.com/mortonjt/songbird) was used to produce a multinomial regression model predicting counts of specific microbial taxa through metadata covariate columns.

**Results:** Participants were 226 PWH and 101 PWoH, mean (SD) age 52.0 (13.5), 21.1% female, 54.7% men who have sex with men, 44.7% non-white. Among PWH, median (interquartile range, IQR) nadir and current CD4 were 174 (21, 302) and 618 (448, 822), respectively; 90% were virally suppressed on antiretroviral therapy. PWH and PWoH did not differ with respect to microbiome diversity as indexed by Faith’s phylogenetic diversity (PD). More severe DNP was associated with lower alpha diversity as indexed by Faith’s phylogenetic diversity in PWH (Spearman’s $\rho = .224$, $P = 0.0007$), but not in PWoH (Spearman’s $\rho = .032$, $P = .748$). These relationships were not confounded by demographics or disease factors. In addition, the log-ratio of features identified at the genus level as Blautia to Lachnospira was statistically significantly higher in PWH with DNP than in PWH without DNP ($t$-test, $P = 1.01e-3$). Furthermore, the log-ratio of Clostridium features to Lachnospira features also was higher in PWH with DNP than in those without ($t$-test, $P = 6.24e-5$).

**Conclusions:** Our results, in combination with previous findings in other neuropathic pain conditions, suggest that gut dysbiosis, particularly reductions in diversity and relative increases in the ratios of Blautia and Clostridium to Lachnospira, may contribute to prevalent DNP in PWH. Two candidate pathways for these associations, involving microbial pro-inflammatory components and microbially-produced anti-inflammatory short chain fatty acids, are discussed. Future studies might test interventions to re-establish a healthy gut microbiota and determine if this prevents or improves DNP.

**Perspective:** The association of neuropathic pain in people with HIV with reduced gut microbial diversity and dysbiosis raises the possibility that re-establishing a healthy gut microbiota might ameliorate neuropathic pain in HIV by reducing proinflammatory and increasing anti-inflammatory microbial products.

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**Key words:** HIV, microbiome, neuropathic pain, gut dysbiosis.
Gut Dysbiosis: Links to Pain Phenotypes

Gut dysbiosis, defined as pathogenic alterations in the distribution and abundance of different microbial species, has been shown in individuals with various pain conditions. As an example, in fibromyalgia—a syndrome considered by many to be one of neuropathic pain as it shares with sensory polyneuropathies symptoms of burning pain, pricking and touch-evoked allodynia, and in which small fiber pathology has been found—alterations in the gut microbiota also have been reported. 

Another study found that the abundance of the Bifidobacterium and Eubacterium genera, which include microbes that participate in the metabolism of neurotransmitters, was significantly reduced in fibromyalgia patients who experience pain with neuropathic qualities.

Neuropathic pain associated with peripheral nerve injury also is linked to the gut microbiota. For example, germ-free mice and those pretreated with antibiotics demonstrated reduced oxaliplatin-induced mechanical hyperalgesia. Providing evidence that neuroinflammation is critical to these relationships, the dorsal root ganglia of antibiotic-treated mice showed reduced infiltration of macrophages, and lower levels of IL-6 and TNF-α compared to mice fed with water. In another report, reciprocal gut microbiota transfers between C57BL/6 (B6) and 129SvEv (129) mice as well as antibiotic treatments demonstrated reduced mechanical hyperalgesia and allodynia.

Microglia proliferated in the spinal cords of paclitaxel treated mice harboring a pain-sensitive gut microbiota. A third study found that an abnormal composition of the gut microbiota contributed to neuropathic pain susceptibility induced by spared nerve injury in rats. Fecal microbiota transplantation from rats resilient to spared nerve injury pain resulted in reduced pain in pseudo-germ-free mice.

The Burden of DNP in PWH

Distal sensory polyneuropathy (DSP) was assessed using a validated neurological examination administered by trained nurses. Similarly, DNP and its severity were evaluated using a validated self-report tool. Evaluations included clinical examination for neuropathy signs (bilateral distal vibration, sharp and touch loss) and self-reported neuropathy symptoms (pain, numbness/sensory loss, paresthesias). Moderate or worse neuropathy was defined as two or more clinical signs of neuropathy from the list above. DNP was defined as burning, aching, or shooting symptoms and classified into five categories of clinician-rated pain severity: none, slight (occasional, fleeting), mild (frequent), moderate (frequent, disabling), and severe (constant, daily, disabling, requiring analgesic medication or other pain medication). Participants were characterized as men who have sex with men (MSM) based on self report. MSM status was of particular interest since it has been shown previously to be strongly associated with differences in the gut microbiome.

Characterization of the Gut Microbiome

Stool was collected according to a standardized protocol. Participants unable to provide specimens at the on-site visit were provided with a kit to collect and freeze stool off site and return it within 24-hours. Stool samples are aliquoted into 5 equal parts, one gram was homogenized and processed in a nucleic acid preservation tube at −80°C for 16S DNA sequencing. Gut microbial diversity was characterized using 16S rRNA sequencing. Gut microbial diversity was indexed by Faith’s phylogenetic diversity (PD) and greater dependence in activities of daily living than those who remained pain-free. DNP contributes to the burden of polypharmacy in older PWH. These findings highlight the increasing burden of DSP and DNP in the growing population of older long-term PWH survivors despite viral suppression on cART.

We sought to characterize potential alterations, including differences in gut microbial diversity and dysbiosis in PWH with DNP.

Methods

Participants

We recruited PWH and PWoH from community sources at a single site in San Diego, CA for studies of neurological complications of HIV, including DSP and DNP. Inclusion criteria were HIV positive or negative confirmed by serology. Exclusion criteria were active neurological illnesses other than those related to HIV, and active psychiatric (eg, psychosis) or substance use disorder that might interfere with completing study evaluations. All participants signed an IRB-approved written consent and the study was approved by the UCSD Human Subjects Protection Committee (IRB).

Clinical Examination

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diversity calculations were performed using robust Aitchison principal components analysis on the unrarefied table and PERMANOVA through the Adonis method in QIIME2.1,26,30

**rRNA Gene Sequencing**

DNA extraction and 16S rRNA amplicon sequencing were done using Earth Microbiome Project (EMP) standard protocols (http://www.earthmicrobiome.org/protocols-and-standards/16s).6 DNA was extracted with the Qiagen MagAttract PowerSoil DNA kit as previously described.25 Amplicon PCR was performed on the V4 region of the 16S rRNA gene using the primer pair 515f to 806r with Golay error-correcting barcodes on the reverse primer. Amplicons were barcoded and pooled in equal concentrations for sequencing. The amplicon pool was purified with the QIAGEN DNeasy UltraClean Microbial Kit (GmbH, Hilden, Germany) and sequenced on the Illumina MiSeq sequencing platform (Illumina, San Diego, CA). Sequence data were demultiplexed and minimally quality filtered using the Qiita defaults. Sequence data were demultiplexed and minimally quality filtered using the QIIME 1.9.1 script split_libraries_fastq.py, with a Phred quality threshold of 3 and default parameters (per Qiita recommendations)4 to generate per-study FASTA sequence files. Data generated in this study has been deposited on Qiita under study ID 11135. Sequencing data associated with this study have been deposited to EBI/ENA with accession number ERP122366. Phylogenetic tree construction was performed using the align_to_tree_mafft_fasttree command in the QIIME2 phylogeny plugin. Taxonomic assignment of microbial features was done through QIIME2 (https://doi.org/10.1186/s40168-018-0470-z) using a Naive Bayes classifier trained on the GreenGenes 13.8 99% OTU database (https://doi.org/10.1038/ismej.2011.139).

**HIV Disease and Treatment Characteristics**

Clinical and laboratory data were ascertained via comprehensive neuromedical evaluations consisting of a structured clinician-administered interview, physical and neurological examinations and standard laboratory assays as previously described.16 Levels of HIV RNA in plasma were measured via reverse transcriptase-polymerase chain reaction (Amplicor, Roche Diagnostics, Indianapolis, IN) and were considered undetectable below the lower limit of quantitation of 50 copies/mL. As is the convention in the literature and as has been previously validated,10 nadir CD4+ T-cells were by self-report and current CD4 by flow cytometry in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory.

**Statistical Analyses**

Group differences in background characteristics (ie, demographics, neuropsychiatric and neuromedical characteristics) and microbiome alpha diversity were examined using analysis of variance (ANOVA), Wilcoxon/Kruskal-Wallis tests, and Chi-square statistics as appropriate. Variables were log10-transformed as needed to ensure normality of the distributions. We conducted one primary comparison, comparing microbiome diversity to DNP severity, stratified by HIV serostatus. The comparison was done using Spearman’s $\rho$ with DNP severity as the independent variable. All other analyses were secondary. Covariates examined included demographics and MSM status; the latter has been shown previously to be strongly associated with differences in the gut microbiome.25 Multivariable models including these covariates were performed using standard least squares regression. Analyses were conducted using JMP Pro version 15.0.0 (SAS Institute Inc., Cary, NC, 2018). Multinomial regression was performed through Songbird on the full feature table excluding microbial features present in fewer than 10% of samples.26 Model construction included DNP, HIV status, & MSM status. The interaction of DNP and HIV status was included in addition to their individual effects. We found that adding additional parameters such as sex did not result in better fit models quantified by calculating the pseudo Q2 value compared to a null model. Differential coefficients from this regression were used to determine log-ratios of taxa abundances that differentiate between groups through t-tests.

**Results**

**Demographics and HIV Disease and Neuropathy Characteristics**

Participants were 226 PWH and 101 PWoH (Table 1). In the 2 groups combined, the mean (SD) age was 52.0 (13.5); 21.1% were females, 54.7% were men who have sex with men (MSM) and 44.7% were non-white. Among PWH, median (interquartile range, IQR) nadir and current CD4 were and 174 (21, 302) and 618 (448, 822), respectively; 90% were virally suppressed on antiretroviral therapy. DSP was more frequent in PWH than PWoH (26.2 vs 9.9%). DNP was more common (34.1% vs 28.2%), respectively; 90% were virally suppressed on antiretroviral therapy.

<table>
<thead>
<tr>
<th>Variable</th>
<th>PWoH N (%)</th>
<th>PWH N (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years (mean SD))</td>
<td>51 ± 16.7</td>
<td>52.4 ± 11.8</td>
<td>.238</td>
</tr>
<tr>
<td>Sex female – N (%)</td>
<td>41 (23.3%)</td>
<td>28 (12.4%)</td>
<td>&lt;.001</td>
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<td>MSM – N (%)</td>
<td>23 (22.8%)</td>
<td>154 (69.4%)</td>
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<td>Ethnicity Black – N (%)</td>
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<td>44 (19.5%)</td>
<td>.858</td>
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<tr>
<td>Hispanic</td>
<td>20 (19.8%)</td>
<td>50 (22.1%)</td>
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</tr>
<tr>
<td>Non-Hispanic White</td>
<td>57 (56.4%)</td>
<td>124 (54.9%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>4 (4.0%)</td>
<td>8 (3.5%)</td>
<td></td>
</tr>
<tr>
<td>Distal sensory polyneuropathy</td>
<td>10 (9.9%)</td>
<td>66 (26.2%)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Distal neuropathic pain – N (%)</td>
<td>12 (11.9%)</td>
<td>77 (34.0%)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>
Microbiome Diversity and HIV Neuropathic Pain

Among PWH, there was a dose-response relationship such that worse pain was associated with a stepwise reduction in alpha diversity as indexed by Faith’s PD (Spearman’s $r = .224$, $P = .0007$), with a monotonic decrease in diversity for each increase in pain severity. This was not the case for PWoH ($r = -.146$, $P = .0283$ and $r = .180$, $P = .0069$, respectively). These relationships were not confounded by demographics, sexual orientation, or HIV disease factors. In particular, sex, which differed between PWH and PWoH, was non-significant in multivariable models.

Participants self-identifying as MSM had higher Faith’s PD than non-MSM (13.0 ± 3.94 vs 11.3 ± 3.65; $P = 1.07e-4$). Rates of DSP (56.1% vs 67.9%, $P = .023$) and sex ($r = .317$, $P = .0080$) were both significantly associated with microbiome diversity (full model $P = .0051$). Older females (a surrogate for menopausal status) had higher diversity than younger (r = .317, $P = .0080$). Among female PWH, age did not confound the relationship between DNP and microbiome diversity (age $P = .0109$; whole model $P = .0012$).

Figure 2 shows a robust Aitchison PCA beta diversity ordination that demonstrates a microbial separation of samples by the various levels in this study. PERMANOVA of all samples show that beta diversity differences were highly statistically significant for HIV status, MSM status, and the interaction of HIV status and DNP but not for DNP by itself (Table 2). Thus, accounting for the effects of other terms in the multivariable model, HIV emerged as significantly associated with diversity, indicating that confounding between HIV and DNP (DNP more

Figure 1. Among PWH, there was a dose-response relationship such that worse pain was associated with a stepwise reduction in alpha diversity as indexed by Faith’s PD (Spearman’s $r = .224$, $P = .0007$). Among PWoH, there was no significant relationship (Spearman’s $r = .032$, $P = .748$). Box plots show for each group the median (central white line), 25th and 75th percentiles (box) and 5th and 95th percentiles (whiskers). Values to the left of each box plot are the medians.
frequent in PWH than PWoH; odds ratio, 95% confidence interval 3.83 [1.98, 7.43]) and between HIV and MSM (more PWH than PWoH were MSM; OR 10.5 [6.03, 18.2]) was responsible for the lack of difference in the univariable analysis. A second robust Aitchison PCA beta diversity analysis on only MSM subjects showed a statistically significant difference between those with DNP and those without ($P = .028$).

Songbird analysis of the full cohort was used to produce a multinomial regression model predicting counts of specific microbial taxa through metadata covariate columns. Including both DNP and HIV status in this model resulted in regression coefficients indicating individual microbial taxa associations with DNP and HIV status relative to all other taxa. Taxonomic assignments of microbes were summarized according to genus-level identification in GreenGenes for downstream differential abundance analysis.

DNP:HIV+ coefficients from the regression were averaged for each genus and sorted to determine which genera were most associated with DNP and HIV status relative to all other microbes while taking into account prevalence and abundance. We used these genus rankings to choose numerator and denominator taxa rather than testing all possible combinations of microbial features to reduce the risk of reporting false positives. We noted *Lachnospira* as a genus that had a low average coefficient and high average prevalence, indicating that these microbes are relatively less associated with DNP: HIV+ status than other microbes — as such we use it as the denominator in log-ratio comparisons. Log-ratio of sets of taxa are compared rather than relative abundance to avoid issues of compositionality inherent to sequencing data (https://doi.org/10.1038/s41467-019-10656-5). Genera that were highly associated for use in the numerator were chosen the same way as the denominator. The log-ratio of features identified at the genus level as *Blautia* to *Lachnospira* was statistically significantly higher in PWH with DNP than in PWH without DNP (t-test, $P = 1.01e-3$) while retaining 89% of samples (Fig 3). Furthermore, the log-ratio of *Clostridium* features (2) to *Lachnospira* features also was higher in PWH with DNP than in those without (t-test, $P = 6.24e-5$). There were not enough samples from PWoH with DNP to make any meaningful statistical conclusions with this cohort.

**Discussion**

Our findings suggest that gut dysbiosis may contribute to prevalent DNP in PWH or may otherwise modulate the clinical phenotype of distal sensory polyneuropathy in HIV. These results are concordant with findings in other neuropathic pain conditions. There was no evidence of confounding by measured covariates. In particular, MSM PWH, who had a more diverse gut microbiome, as shown in previous studies, did not show a significant association between generalized pain severity and gut microbial diversity. In other words, the DNP-gut microbiome diversity association appears to be specific for HIV-related DNP as compared to other pain conditions. The lack of a similar association in PWoH may reflect their qualitatively different microbiomes (independent of diversity) as shown in previous studies, or may be due to the low frequency of DNP in this group.

The specific mechanism by which the gut microbiome influences pain is not known. However, we offer several points in this regard. First, our findings are consistent
with observations in other neuropathic pain conditions. Second, our findings have plausible pathophysiological underpinnings based on inflammatory microbial components, neuroprotective microbially produced short chain fatty acids (SCFAs), reciprocal communications between the gut and the brain (the gut-brain axis), and commonalities in the brain regions influenced by the gut microbiome and those involved in pain processing.\textsuperscript{5,19,28} We observed higher relative abundances of \textit{Blautia} and \textit{Clostridium} species in the DNP group. This is in agreement with a study of a neuropathic pain model in rats, chronic constriction injury, where rats with neuropathic pain had significantly increased \textit{Blautia} \textit{compared to controls}. Similarly, some \textit{Clostridium} species (eg, \textit{C. scindens}), were found in higher abundance in fibromyalgia patients with neuropathic pain.\textsuperscript{27} Finally, \textit{Lachnospira} produce SCFAs\textsuperscript{48} which are neuroprotective\textsuperscript{36} and anti-inflammatory.\textsuperscript{49} Thus, relative reductions in \textit{Lachnospira} abundance might reduce SCFAs, exacerbating inflammation and neural injury.

An additional mechanism by which the gut microbiome and DNP may be linked is through the autonomic nervous system (ANS). One key component of the gut-brain axis is the vagus nerve, which comprises large numbers of small, lightly myelinated and unmyelinated fibers, many regulating ANS function. Modulation of the CNS by the gut microbiome is mediated through the vagus by neurotransmitters and neuroendocrine mechanisms.\textsuperscript{5,42,45,50} Sensory polyneuropathy in PWH includes a prominent component of small fiber injury, leading to ANS dysfunction.\textsuperscript{23} Evidence from animal models supports that gut microbiota changes correlate with dysfunction of the ANS.\textsuperscript{38} In addition, small fiber injury frequently manifests as neuropathic pain.\textsuperscript{17} Thus, the composition and diversity of the gut microbiota may influence small fiber injury that causes both pain and ANS dysfunction.

Pathways linking the gut microbiome to pain processing neural pathways via the gut-brain axis could mediate pain perception in neuropathy. There is overlap between brain regions and neurotransmitters affected by the gut microbiota and those that process pain. For example, chronic treatment with \textit{Lactobacillus rhamnosus} JB-1 induced region-dependent alterations in GABA mRNA in the cingulate.\textsuperscript{39} Indeed, the anterior cingulate cortex appears to be particularly important in processing the emotional and cognitive aspects of pain.\textsuperscript{20,51}

Most research on gut microbiome changes related to antiretroviral treatment has focused on the impact of immune recovery, rather than on the direct effects of different antiretroviral drugs. One small study of 16 patients showed different patterns of microbial changes in patients starting an efavirenz-containing regimen versus a protease inhibitor-based regimen.\textsuperscript{34} Our analyses did not show effects of specific antiretroviral drugs or classes on microbial diversity. A potential limitation in interpreting these results is that DNP may have been incorrectly attributed to DSP. We showed here that those with DNP were much more likely to have DSP than those without DNP. Additionally, we have previously reported that when examined with more sensitive measures of neuropathy including electrophysiology, the great majority of those with DNP in fact do have DSP.\textsuperscript{35} Furthermore, we carefully elicited reports of pain that are typical for DSP-related DNP, rather than other neuropathic pain conditions. The number of PWoH with DNP was very small, limiting statistical power in this group. An additional limitation is the correlational nature of the study design, which precluded causal associations. However, other studies have shown that manipulation of the gut microbiome reduces susceptibility to neuropathic pain after nerve injury and chemotherapy, suggesting that the gut microbiome-pain connection is common to a variety of pain condition and is a causal factor. Finally, we acknowledge that the observed correlations might reflect the influence of unobserved confounding variables.

Future studies should evaluate the potential for interventions to re-establish a healthy gut microbiota, such as fecal transplantation or pre- or probiotics, to improve or prevent neuropathic pain.

**Author Contributions**

Ronald J. Ellis conceived the research question, conducted the analyses and wrote the manuscript. Robert K. Heaton was PI of the grant that funded this work and edited drafts of the manuscript. Sara Gianella assisted with interpretation of the findings and edited drafts of the manuscript. Gibraan Rahman performed additional analyses assisted with interpretation of the findings and edited drafts of the manuscript. Rob Knight assisted with interpretation of the findings and edited drafts of the manuscript.

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