The Fecal Microbiome and Metabolome of Pitt Hopkins Syndrome, a Severe Autism Spectrum Disorder

**ABSTRACT** Alterations to the gut microbiome have been reported between children with autism spectrum disorders (ASDs) and typically developing (TD) children. Characterizing these differences has led to the proposal of new treatments for ASD, such as probiotic interventions and fecal matter transplants. However, no study to date has characterized the gut microbiome or metabolome in Pitt Hopkins syndrome (PTHS), a severe ASD with a high incidence of gastrointestinal (GI) disturbances such as constipation. Here, we surveyed the gut microbiome and metabolome in a cohort of PTHS individuals and their unaffected parents. We focused our analysis on *Clostridium bolteae*, a microbe previously associated with ASD known to chemically modify bile acids in the gut. PTHS individuals carry a higher load of *C. bolteae* than their parents as well as both ASD and non-ASD individuals from the American Gut Project cohort. Specific metabolites were associated with PTHS, including bile acids and sphingosines. With a metadata reanalysis tool, we found that PTHS-associated metabolites have previously been identified in inflammatory bowel disease and obesity patients. These results suggest microbial involvement in PTHS, but further research must be performed to clarify the exact mechanisms through which microbes may act. Furthermore, new associations between PTHS-specific metabolites and other conditions may lead to additional therapeutic options for PTHS individuals.

**IMPORTANCE** GI disturbances in ASD such as severe constipation can be medically significant and often require medication. This is especially true for individuals with PTHS, suggesting that the gut microbiome may be involved in PTHS’s pathology. Revealing associations between specific gut microbes and PTHS may allow the development of new therapeutics or the application of existing therapeutics to ease day-to-day challenges encountered by PTHS individuals. In this study, we characterized an association between *C. bolteae* and PTHS, in addition to metabolites linked to both PTHS and *C. bolteae*. We also identified other microbiome-involved medical conditions where PTHS-associated metabolites have been isolated. Utilizing common metabolites to identify conditions with similar phenotypes may suggest new therapeutic options for GI-related symptoms.
Autism spectrum disorders (ASDs) are a heterogeneous group of neurodevelopmental disorders characterized by deficits in communication and social interaction and repetitive stereotyped behaviors (1). No single etiology of ASD has been identified, and current hypotheses suggest both genetic and environmental contributions (2, 3). On the environmental side, alterations in the gut microbiome are reported in individuals with ASD compared to unaffected children (4). Furthermore, both gnotobiotic animal and probiotic studies suggest a potential causative role for the gut microbiome in behavioral and neuropathological endophenotypes in human ASD (5–8).

Pitt Hopkins syndrome (PTHS) (9, 10) is a rare and extreme form of ASD that is caused by a pathogenic variant of the TCF4 gene found on chromosome 18q21.2 (11–13). PTHS is characterized by severe intellectual disability and psychomotor delay, facial dysmorphism, hyperventilation-apneic spells, stereotypic movements, and seizures. Gastrointestinal (GI) disturbance, especially severe constipation, is the most common extraneurological manifestation of PTHS; it can cause significant pain and often requires medication. The pathology of GI abnormalities in ASD appears to be at least partially related to the gut microbiome. Despite substantial evidence of its alterations and potential therapeutic value in ASD, no study to our knowledge has examined the composition of the fecal microbiome or its resultant metabolites in PTHS. Thus, the purpose of this study was to characterize the gut microbiome and metabolome of 39 children with PTHS compared to 46 unaffected family members to understand how each contributes to clinical pathology (see Table 1 for demographic and clinical information). This characterization is critical for targeting interventions to improve the quality of life of these individuals.

Our analysis narrows in on Clostridium bolteae, a microbe that is significantly more abundant in ASD children’s stool and is associated with abdominal infections (14, 15). C. bolteae’s ability to conjugate bile acids in the gut has been thoroughly characterized (16). Since dysregulated bile acid metabolism has been associated with GI dysfunction in ASD model mice and GI problems are a hallmark of PTHS, we were interested in whether C. bolteae and bile acids would be more common in the gut of PTHS individuals (17).

Metagenomics and 16S rRNA gene amplicon results. It has previously been reported that gut microbiome alpha diversity is lower in ASD than in typically developing (TD) individuals (18), so we extended this comparison to include PTHS. Specifically, we measured Faith’s phylogenetic diversity among PTHS, ASD, and non-ASD individuals by combining our 16S rRNA V4 sequencing data with American Gut Project (AGP) data (Fig. 1A). We restricted the analysis to individuals under the age of 20 years to mitigate age-related changes in gut microbial diversity, as most PTHS individuals surveyed are young. Consistent with previous observations, we observed lower alpha diversity in

| TABLE 1 | Demographic and clinical characterization of the 39 Pitt Hopkins syndrome patients and their 46 unaffected family membersa |
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| Metric | Value for group |
| | Pitt Hopkins syndrome patients (n = 39) | Unaffected family members (n = 46) |
| Mean age (yrs) ± SD (no. of individuals) | 10.18 ± 8.50 (39) | 39.42 ± 9.78 (38) |
| % (no.) of females | 60.5 (38) | 60.5 (43) |
| % (no.) of Caucasian individuals | 87.2 (39) | 90.0 (40) |
| % (no.) of individuals with USA as country of residence | 92.3 (39) | 90.9 (44) |
| % (no.) of individuals with antibiotic use in the last yr | 51.3 (39) | 38.5 (39) |
| % (no.) of individuals with at least weekly use of probiotic | 48.6 (37) | 17.1 (41) |
| % (no.) of individuals with normal bowel movement quality | 44.7 (38) | 90.0 (40) |
| % (no.) of individuals in overweight or obese BMI category | 8.57 (35) | 56.1 (41) |

aNotably, a large share of PTHS patients have used antibiotics within the last year and take probiotics regularly compared to their family members. Furthermore, PTHS patients are less likely to be overweight or obese and are more likely to have abnormal stool quality. BMI, body mass index.
both PTHS and ASD individuals (PTHS versus AGP ASD, $U = 3,668$ and $P = 0.013$; PTHS versus AGP non-ASD, $U = 11,372$ and $P = 0.001$; AGP ASD versus AGP non-ASD, $U = 124,957$ and $P = 0.039$ [by a Mann U test]). We assessed differences in beta diversity among PTHS, ASD, and non-ASD (from the AGP) individuals using permutational multi-variate analysis of variance (PERMANOVA), observing significant PTHS-versus-ASD ($\text{pseudo}-F = 4.823; P = 0.003$) and PTHS-versus-non-ASD ($\text{pseudo}-F = 4.086; P = 0.007$) differences in weighted UniFrac distances (see Table S2 in the supplemental material).

We then narrowed our focus to *C. bolteae*, a microbe with known associations with ASD (14, 15, 18). Shotgun metagenomics found *C. bolteae* to be elevated relative to the highly prevalent reference organism *Ruminococcus obeum* in PTHS individuals compared to their unaffected parents (Fig. 1B) ($t = 4.015; P = 1.49e^{-4}$ [by a $t$ test]).

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Furthermore, within affected individuals younger than 20 years of age, we observed a negative correlation between the log ratio of \textit{C. bolteae} to \textit{R. obeum} and age (Fig. 1C) (Pearson \(r = -0.56; P = 5.90e-4\)). To disentangle whether this correlation is an artifact of age, we compared the relative abundance of \textit{C. bolteae} in PTHS individuals under 20 years of age (\(n = 34\)) to those in individuals with a self-reported medical diagnosis of ASD (\(n = 315\)) and non-ASD individuals also younger than 20 years of age (\(n = 1,047\)) from the AGP cohort. A single 16S amplicon sequence variant (ASV) had an exact match to one of the \textit{C. bolteae} genomes observed in the shotgun assessment. The 16S data indicate that a significantly larger proportion of PTHS individuals have high relative abundances of \textit{C. bolteae} than either ASD or non-ASD individuals (Fig. 1D and E) (PTHS versus ASD, odds ratio = 2.69 and \(P = 0.017\); PTHS versus non-ASD, odds ratio = 3.65 and \(P = 7.62e-4\) [by Fisher’s exact test]). This suggests that the higher relative abundance of \textit{C. bolteae} in PTHS individuals than in their parents is more related to their PTHS status than to the differences in the ages of the cohorts. We further examined the relationship between \textit{C. bolteae} and age in the 16S data. For a compositional additive log ratio (ALR) transform, we used the \textit{C. bolteae} ASV with 100% sequence identity to the genome record from the shotgun data and the top 5 most prevalent 16S ASVs and correlated this log ratio with the age of the individual. PTHS and ASD were nonsignificant, whereas the non-ASD individuals exhibited a negative correlation (Pearson \(r = -0.07; P = 0.0160\)) (Fig. S1).

**Metabolomics results.** Unlike in the metagenomic results, we observed no significant differences in metabolite alpha diversity between PTHS individuals and their parents (Fig. 2A) (Kruskal-Wallis statistic = 3.324; \(P = 0.0683\)). However, we observed a significant difference in metabolite beta diversity (Fig. 2B) (PERMANOVA statistic = 2.06076; \(P = 0.004\)). To identify metabolites associated with PTHS, we performed a differential analysis with the Songbird multinomial model (19); we found that these differentials correlated with mmvec (20) PC2 (Pearson \(r = 0.45; P = 1.37e-17\)) (Fig. 2C; see also Text S1 in the supplemental material for more background on Songbird and mmvec). Furthermore, given the association between \textit{C. bolteae} and PTHS, we employed mmvec to identify metabolites associated with \textit{C. bolteae}. No association was found between metabolite Songbird differentials and their mmvec conditional probabilities for \textit{C. bolteae} genomic operational taxonomic unit (gOTU) G000371705 (Fig. 2D) (Pearson \(r = 0.09; P = 0.113\)), but those metabolites with high conditional probabilities were analyzed further (Table S1). The top Songbird and mmvec metabolites were examined with a metadata reanalysis tool (21), which showed that they are often isolated from patients with irritable bowel disease (IBD), obesity, and Crohn’s disease, among other conditions (Fig. 2E). Finally, as we are interested in the association between \textit{C. bolteae} and bile acids, we examined all level 3-annotated bile acids by feature-based molecular networking (22, 23) (Fig. 2F and Table 2). Many bile acids appear to be enriched in PTHS patients relative to their parents, particularly those that are chemically modified, suggesting microbial involvement in PTHS.

**Discussion.** Multi-omic microbiome assessments are increasingly common thanks to the prospect of a more detailed understanding of the role of microbes and molecules in an environment. Here, we utilized the strength of each data layer: 16S for comparison against a large publicly available data set, shallow shotgun sequencing for the identification of species-level features, and metabolomics for the characterization of molecular features. These data layers were then used in combination: resolving a putative ASV linked to \textit{C. bolteae} allowed us to compare it to public 16S data, and an integrative method gave us the ability to predict which microbes and molecules are probabilistically related.

The combination of these -omic strategies suggests an increased burden of \textit{C. bolteae} within individuals with PTHS and provides evidence of a unique molecular repertoire with microbial relationships. Chronic gastrointestinal issues are a hallmark of PTHS, and many individuals suffer from frequent seizures anecdotally related to the extent of constipation. \textit{C. bolteae}’s modification of bile acids has experimentally been shown in murine models (16), and while the same conjugated bile acids were not observed in this study, associations between PTHS and
bile acids and between \textit{C. bolteae} and bile acid precursors were observed. Alterations to bile acid metabolism have previously been linked to constipation (24), and in canines, bile acid tests are recommended if seizures are observed as a proxy for liver function (25).

These results are not mechanistic, and further work is necessary to determine the exact relationships between bile acids and \textit{C. bolteae}. Specifically, our bile acid conclusions are limited by our lack of standard-matched level 1 annotations (22). We are able to detect several bile acids with level 3 annotations, but future studies should look to characterize individual bile acids in PTHS with specific standards. Furthermore, as Pitt Hopkins syndrome is a rare disease, the overall sample size considered in this study is limited, with reduced statistical power. Based on -omics observations, we advocate for assessments of microbial involvement in TCF4 mutation murine models and whether
alterations to the microbiome (e.g., humanizing and antibiotics, etc.) mitigate PTHS-like GI disturbances (26) and behaviors (27).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, PDF file, 0.2 MB.
FIG S1, TIF file, 0.2 MB.
TABLE S1, PDF file, 0.1 MB.
TABLE S2, PDF file, 0.03 MB.

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**TABLE 2 Bile acid spectral matches**

| Feature ID | m/z   | RT (s) | Annotation                                                                 |
|------------|-------|--------|---------------------------------------------------------------------------|
| 1281       | 375.2895 | 4.31   | (R)-4-((5R,8R,9S,10S,13R,14S,17R)-10,13-Dimethyl-3-oxohexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoic acid |
| 1422       | 355.2630 | 3.56   | Cholic acid                                                               |
| 1589       | 389.2688 | 3.32   | (4R)-4-((3R,5R,6S,7R,9S,10R,12S,13R,14S,17R)-3,6,7,12-Tetrahydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoic acid |
| 1825       | 371.2581 | 4.05   | (R)-4-((3S,5S,7R,8R,9S,10S,13R,14S,17R)-3,7-Di hydroxy-10,13-dimethyl-2-oxohexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoic acid |
| 2037       | 407.2793 | 3.30   | (R)-4-((1R,3S,5S,7S,8S,9S,10S,12S,13R,14S,17R)-1,3,7,12-tetrahydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoic acid |
| 4291       | 431.2769 | 3.57   | (R)-4-((1S,3S,5S,6S,7R,8R,9S,10S,13R,14S,17R)-3,7,12-Trihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoic acid |
| 4346       | 357.2794 | 7.04   | (4R)-4-((5S,7S,9S,10S,12R,13R,14S,17R)-7,12-di hydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoic acid |
| 4356       | 466.3164 | 3.25   | (R)-4-((3R,5S,7S,8R,9S,10S,12S,13R,14S,17R)-3,7,12-Trihydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoic acid |
| 4725       | 405.2642 | 3.17   | (R)-4-((3R,5S,7S,8R,9S,10S,13R,14S,17R)-3,7-Di hydroxy-10,13-dimethyl-7,12-dioxohexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoic acid |
| 5864       | 500.3051 | 4.03   | 2-((R)-4-((3S,5S,7S,8R,9S,10S,12S,13R,14S,17R)-3,7-Di hydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamido)ethane-1-sulfonic acid |
| 6289       | 375.2891 | 5.46   | (R)-4-((3R,5S,8R,9S,10S,13R,14S,17R)-10,13-Dimethyl-3-oxohexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoic acid |
| 6859       | 359.2939 | 5.15   | (R)-4-((3S,5R,8R,9S,10S,13R,14S,17R)-3-Hydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoic acid |
| 6866       | 375.2888 | 3.94   | (R)-4-((3R,5R,8R,9S,10S,13R,14S,17R)-10,13-Dimethyl-3-oxohexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoic acid |
| 7601       | 375.2895 | 4.24   | (R)-4-((3R,5R,8R,9S,10S,13R,14S,17R)-10,13-Dimethyl-3-oxohexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanoic acid |
| 7602       | 516.2991 | 3.49   | 2-((R)-4-((3S,5S,7S,8R,9S,10S,12S,13R,14S,17R)-3,7-Di hydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamido)ethane-1-sulfonic acid |
| 7607       | 522.2861 | 4.03   | 2-((R)-4-((3S,5S,7S,8R,9S,10S,13R,14S,17R)-3,7-Di hydroxy-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanamido)ethane-1-sulfonic acid |

*Feature information for all compounds annotated as bile acids is shown. The feature identifiers outlined here match up to the compounds in the molecular network in Fig. 2F. The annotations are all level 3, as defined by the Metabolomics Standards Initiative (19).

RT, retention time (seconds).
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