Gut microbial metabolites in Parkinson’s disease: Association with lifestyle, disease characteristics, and treatment status

Robin M. Voigt\textsuperscript{a,b,c,*}, Zeneng Wang\textsuperscript{d,e}, J. Mark Brown\textsuperscript{d,e}, Phillip A. Engen\textsuperscript{a}, Ankur Naqib\textsuperscript{b}, Christopher G. Goetz\textsuperscript{f}, Deborah A. Hall\textsuperscript{f}, Leo Verhagen Metman\textsuperscript{f}, Maliha Shaikh\textsuperscript{a}, Christopher B. Forsyth\textsuperscript{a,b,c}, Ali Keshavarzian\textsuperscript{a,b,c,g}

\textsuperscript{a}Rush Center for Integrated Microbiome and Chronobiology Research, Rush University Medical Center, Chicago, IL, United States

\textsuperscript{b}Department of Internal Medicine, Rush University Medical Center, Chicago, IL, United States

\textsuperscript{c}Department of Anatomy and Cell Biology, Rush University Medical Center, Chicago, IL, USA

\textsuperscript{d}Department of Cardiovascular & Metabolic Sciences, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, United States

\textsuperscript{e}Center for Microbiome & Human Health, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, United States

\textsuperscript{f}Department of Neurological Sciences, Rush University Medical Center, Chicago, IL, United States

\textsuperscript{g}Department of Physiology, Rush University Medical Center, Chicago, IL, United States

Abstract

There is growing appreciation of the importance of the intestinal microbiota in Parkinson’s disease (PD), and one potential mechanism by which the intestinal microbiota can communicate with the brain is via bacteria-derived metabolites. In this study, plasma levels of bacterial-derived metabolites including trimethylamine-N-oxide (TMAO), short chain fatty acids (SCFA), the branched chain fatty acid isovalerate, succinate, and lactate were evaluated in PD subjects (treatment naïve and treated) which were compared to (1) population controls, (2) spousal / household controls (similar lifestyle to PD subjects), and (3) subjects with multiple system atrophy (MSA). Analyses revealed an increase in the TMAO pathway in PD subjects which...
was independent of medication status, disease characteristics, and lifestyle. Lactic acid was
decreased in treated PD subjects, succinic acid positively correlated with disease severity, and
the ratio of pro-inflammatory TMAO to the putative anti-inflammatory metabolite butyric acid was
significantly higher in PD subjects compared to controls indicating a pro-inflammatory shift in the
metabolite profile in PD subjects. Finally, acetic and butyric acid were different between PD and
MSA subjects indicating that metabolites may differentiate these synucleinopathies. In summary,
(1) TMAO is elevated in PD subjects, a phenomenon independent of disease characteristics,
treatment status, and lifestyle and (2) metabolites may differentiate PD and MSA subjects.
Additional studies to understand the potential of TMAO and other bacterial metabolites to serve as
a biomarker or therapeutic targets are warranted.

Keywords
Parkinson’s disease; PD; Microbiota; Metabolites; TMAO; MSA

1. Introduction

Parkinson’s disease (PD) is a progressive neurodegenerative disorder with a diverse and
largely unknown etiopathogenesis (Jankovic and Tan, 2020). Genetic factors influence PD,
but most cases of PD are idiopathic without a clear genetic component, indicating that other
factors such as environment and lifestyle are important. Many environment and lifestyle
factors that influence risk of PD and PD progression (e.g., diet, physical activity) (Jackson
et al., 2019; Fang et al., 2018) also influence the intestinal microbiota, suggesting that the
microbiota could be one mechanism by which environment and lifestyle influence PD.

The intestinal microbiota is a term for the total collection of micro-organisms that live in
the intestine. Data from our group and others show that PD-associated intestinal microbiota
are distinct from age-matched controls, an observation that is true in both treatment naïve
(i.e., not using PD medications) and treated PD patients (Keshavarzian et al., 2015; Sun
and Shen, 2018; Huang et al., 2021; Hill-Burns et al., 2017; Mertsalmi et al., 2017; Tetz et
al., 2018; Aho et al., 2019; Barichella et al., 2017; Li et al., 2019a; Li et al., 2019b; Ren
et al., 2020; Scheperjans et al., 2015). It is not clear if the abnormal intestinal microbiota
precedes PD diagnosis or is a consequence of PD; however, the PD-associated microbiota
is sufficient to promote PD-like behaviors in genetically susceptible animals (Sampson
et al., 2016). Our group has demonstrated that colonizing a genetic rodent model of PD
(alpha-synuclein overexpressing mice) with stool from PD patients promotes the PD-like
phenotype, an effect that is not observed with stool from non-PD controls (Sampson
et al., 2016). These intriguing data suggest that the microbiota (whether a cause or consequence
of PD) can influence pathological processes underlying PD and beg the question of what
specific features of the PD-associated microbiota might influence PD pathogenesis. One
factor that is believed to be important in mediating microbiota-brain communication are
bacterial-derived metabolites.

This study sought to evaluate bacterial-derived metabolites in PD patients with a focus
on the trimethylamine-N-oxide (TMAO) pathway and other metabolites implicated in PD
including short chain fatty acids (SCFA), the branched chain fatty acid isovaleric acid, lactic acid, and succinic acid. The hypothesis was that TMAO would be elevated in PD subjects compared to controls. This hypothesis was based on literature demonstrating that TMAO is a key contributor to many age-associated diseases (atherosclerosis, obesity), including disorders associated with increased risk of PD (insulin resistance/metabolic syndrome, cardiovascular disease) (Wang et al., 2011; Koeth et al., 2013; Zhu et al., 2016; Zhu et al., 2017; Skye et al., 2018; Barrea et al., 2018; Potashkin et al., 2020). Little is known about TMAO in PD. Three recent studies evaluated TMAO levels in PD patients: one reported an increase in plasma TMAO in PD patients (Chen et al., 2020), and two others a decrease in plasma (Chung et al., 2021) and stool TMAO (Tan et al., 2021) of PD patients. These conflicting results may be due to the heterogeneity of PD patients (disease duration, disease severity, treatment status, lifestyle). Therefore, we conducted a comprehensive analysis of PD subjects based on medication status (treatment naïve vs treated PD patients) and disease characteristics (PD duration, severity) and compared them to two different control groups: (1) population controls and (2) spousal / household controls (who share many lifestyle/environmental factors with PD patients). Additionally, treated PD subjects were compared to subjects with multiple system atrophy (MSA) to understand similarities and differences in metabolites between these two distinct synucleinopathies.

2. Materials and methods

2.1. Sample procurement & subjects

All subjects signed an Institutional Review Board approved informed consent form (ORA #10062805) prior to providing samples to the Rush Center for Integrated Microbiome and Chronobiology Research Tissue Repository (Rush University Medical Center, Chicago, IL). This study utilized plasma samples from 86 PD subjects (n = 22 treatment naïve PD (nPD), n = 64 treated PD (tPD)), 7 MSA subjects (1 MSA-C, 6 MSA-P), 24 spousal / household PD controls (spC), and 20 population (i.e., non-spousal, non-household controls) (pC). Population, non-household control subjects were matched to PD subjects based on age, sex, race, and BMI. Subject characteristics are detailed in Table 1.

Population controls were recruited from the Gastroenterology Clinic at Rush University Medical Center. PD, spousal / household controls, and MSA subjects were recruited from the Movement Disorders Clinic and the Parkinson’s Disease Gastroenterology Clinic at Rush University Medical Center. A neurologist specializing in movement disorders examined and confirmed the diagnosis of PD and MSA patients based on clinical diagnostic criteria (without neuropathological confirmation). PD was characterized according to the United Kingdom Parkinson’s Disease Society Brain Bank diagnostic criteria, Hoehn & Yahr (H&Y) staging scale, and the Movement Disorders Society Revision of the Unified Parkinson’s Disease Rating Scale (MDS-UPDRS) I-IV (Goetz et al., 2004; Goetz et al., 2008). Treated PD subjects were taking levo-dopa, in addition to other PD medications, while treatment naïve PD subjects were not taking medication for Parkinsonian symptoms. MSA was diagnosed according to consensus guideline criteria (Gilman et al., 2008).

Inclusion criteria for PD and MSA subjects: current diagnosis of PD or MSA, age (>40), and able to consent. Inclusion criteria for population controls and spousal controls: (1) 40–80
years of age, (2) normal physical exam, complete blood count (CBC), and comprehensive metabolic profile; (3) no gastrointestinal complaints, symptoms, or documented chronic gastrointestinal chronic disease; (4) no neurodegenerative disease; and (5) for spousal controls (spC) living in the same household as a PD subject. Exclusion criteria for all subjects: (1) prior intestinal resection, (2) history of gastrointestinal disease except for hiatal hernia, GERD, hemorrhoids, (3) severe renal disease defined by creatinine more than 2.5 times normal, (4) markedly abnormal liver function defined by ALT/AST over 4 times normal levels or elevated bilirubin, (5) antibiotic use in the 12 weeks prior to sample collection, (6) unstable dietary habits over the 12 weeks prior to sample collection, (7) consumption of probiotics or prebiotics within the four weeks prior to sample collection.

Gastrointestinal symptoms were assessed using Patient-Reported Outcomes Measurement Information System (PROMIS) gastrointestinal symptoms scale of which the constipation subscale was used to understand the relationship between gastrointestinal motility and metabolite levels (Spiegel et al., 2014).

2.2. Sample collection

Blood was collected via antecubital venipuncture into an EDTA vacutainer for plasma separation (#368589; Becton, Dickinson and Company, NJ USA). Whole blood was then centrifuged at 4 °C, 1920 ×g, for 15 min. Plasma was aliquoted into cryovials and stored at −80 °C until analysis and were subjected to a single freeze-thaw cycle.

Stool and mucosal-associated microbiota communities were assessed in a subset of PD (n = 32) and population control (n = 13) subjects. These data were previously reported as part of larger cohort of PD and control subjects (Keshavarzian et al., 2015). Stool samples were collected at home using an anerobic collection method (BD Gaspak, Becton Dickinson and Company, Sparks, MD) as described previously (Keshavarzian et al., 2015). Mucosal biopsies were collected from sigmoid colon via limited, unprepped sigmoidoscopy as described previously (Keshavarzian et al., 2015). Briefly, suction was not used during advancement of the scope (Olympus America Inc., Center Valley, PA) and biopsy forceps were not taken out of the channel until sample collection. Biopsies were taken from pink mucosa without visible feces at the sigmoid colon (Keshavarzian et al., 2015). Samples were collected within four weeks of each other and were stored at −80 °C until use.

2.3. Measurement of plasma metabolites

2.3.1. TMAO pathway co-metabolites—TMAO is generated by a metaorganismal pathway (i.e., microbe and host). Micronutrients in high fat foods (e.g., phosphatidylcholine, L-alpha glycerylphosphorylcholine, choline, L-carnitine) are metabolized by gut microbial enzymes (TMA-lyase enzymes (CutC/D) andCntA/B (YeaW/X)) to generate the metabolite TMA (Brown and Hazen, 2015; Brown and Hazen, 2018; Craciun and Balskus, 2012; Wang et al., 2021; Zhu et al., 2014; Wang et al., 2015) which is then further metabolized by the host enzymes (flavin-containing monoxygenases (FMO) largely FMO3) to produce TMAO (Cashman and Zhang, 2006; Akerman et al., 1999; Bennett et al., 2013). Betaine and gamma-butyrobetaine (butyrobetaine) are precursors to TMAO (Koeth et al., 2014). Stable isotope dilution high performance liquid chromatography with on-line tandem mass
spectrometry (LC-MS/MS) was used for quantification, as previously described (Wang et al., 2014). Their d9(methyl)-isotopologues were used as internal standards. LC–MS/MS analyses were performed on a Shimadzu 8050 triple quadrupole mass spectrometer. TMAO and its d9(methyl)-isotopologue, along with other metabolites and their corresponding d9(methyl)-isotopologues were monitored using multiple reaction monitoring of precursor and characteristic product ions as follows: m/z 76.0 → 58.1 for TMAO; m/z 85.0 → 66.2 for d9-TMAO; m/z 60.2 → 44.2 for TMA; m/z 69.0 → 49.1 for d9-TMA; m/z 104.0 → 60.1 for choline; m/z 113.1 → 69.2 for d9-choline; m/z 118.0 → 58.1 for betaine; m/z 127.0 → 66.2 for d9-betaine; m/z 162.0 → 103.0 for carnitine; m/z 171.0 → 103.0 for d9-carnitine; m/z 146.0 → 87.0 for butyrobetaine; m/z 155.0 → 87.0 for d9-butyrobetaine. Series concentrations of standard mix were mixed with internal standard mix to prepare calibration curves.

2.3.2. Short chain fatty acids, branched chain fatty acids, and other metabolites—SCFA (predominantly acetic, butyric, propionic acid), lactic acid, and succinic acid are the products of catabolism of carbohydrates (Rios-Covian et al., 2016; Oliphant and Allen-Vercoe, 2019). Production of SCFA and the branched chain fatty acid, isovaleric acid are the consequence of protein fermentation (Oliphant and Allen-Vercoe, 2019; Yao et al., 2016). Quantitation was performed using isotope dilution GC–MS/MS by using MRM mode. The absolute quantity of each SCFA was determined using calibration curves measured for each analyte using methods we previously described (Zhang et al., 2021; Lieber et al., 2019). Samples were analyzed by using the Thermo TSQ-Evo triple quadrupole in tandem with the Trace 1310 gas chromatograph (Thermo Fisher Scientific). Chromatographic separation was achieved by using an HP-5MS fused-silica capillary column (30 m × 0.250 mm × 0.25 μm; Agilent Technologies, Santa Clara, CA, USA) coated with 5% pheny-methyl siloxane. Each extract (1 μl) was injected in split mode (10:1). Helium as carrier gas flow was 1 ml/min. The GC oven temperature program was as follows. The initial temperature of 40 °C was held for 2 min after injection before it was increased up to 50 °C at 3 °C/min, followed by increase to 110 °C at 5 °C/min, then 250 °C at 30 °C/min and 310 °C at 70 °C/min, and then held at 310 °C for 3 min. Argon was used as collision gas. The injector, transfer line, and ion source temperature were set at 260, 290, and 230 °C, respectively. The mass spectrometer was tuned to an electron impact ionization energy of 70 eV in the MRM mode with the following parent to daughter ion transitions: m/z 61.0 → 43.0 for acetic acid, m/z 63.0 → 45.0 for [13C2]-acetic acid, m/z 71.0 → 41.0 for butyric acid, m/z 78.1 → 46.1 for D7-butyric acid, m/z 85.1 → 57.1 for isovaleric acid, m/z 87.1 → 59.1 for D2-isovaleric acid, m/z 135.1 → 45.1 for lactic acid, m/z 138.1 → 48.0 for D3-lactic acid, m/z 75.1 → 57.0 for propionic acid, m/z 77.1 → 59.0 for D2-propionic acid, m/z 101.1 → 55.0 for succinic acid, and m/z 105.1 → 57.0 for D6-succinic acid.

2.3.3. Bacterial metabolite ratios—TMAO is broadly considered to be a pro-inflammatory metabolite and the SCFA butyrate is largely (although not exclusively) considered to be anti-inflammatory (Hakhamaneshi et al., 2021; Brunt et al., 2021; Zhao and Wang, 2020; Siddiqui and Cresci, 2021; Sivaprakasam et al., 2016). In this study the relative ratio of putative pro-inflammatory (i.e., TMAO) versus anti-inflammatory (i.e., SCFA) metabolites has been used to provide information about the “inflammatory
potential” of the microbiota. A similar analysis approach has been used to assess the ratio of pro-inflammatory microbiota communities (e.g., Firmicutes / Bacteroidetes) and other metabolites (e.g., kynurenine / tryptophan) (Keshavarzian et al., 2015; Reus et al., 2015; Baranyi et al., 2013).

2.4. Microbiota assessments & analysis

Microbiota data were collected in a previous study and a portion of those data were used in the current evaluation (Keshavarzian et al., 2015). In brief, high-throughput amplicon sequencing of the V4 variable region of the microbial 16S ribosomal RNA gene was performed on genomic DNA extracted from stool and colonic mucosal biopsy tissue. Pearson correlation analysis was conducted between TMA and absolute abundance of taxa at the genus level in PD subjects (n = 32). In silico community functional predictions were performed using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013) and significant differences in Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog pathway abundances between groups (PD n = 32 versus pC n = 13) were evaluated (false discovery rate-corrected p value <0.05) (Kanehisa and Goto, 2000).

2.5. Statistical analysis

Outliers were identified in each group as samples more than two standard deviations from the mean and outliers were excluded from the analyses. If a subject was an outlier for one metabolite, they were excluded from all analyses. Similarly, if a PD subject with a spouse or their respective spousal / household control subject was an outlier, their respective partner was also excluded from analyses. The final number of samples included in each analysis are indicated in each figure.

Fisher’s Exact Test, Chi-Square Analysis, or Student’s t-test, or Mann-Whitney test were used to compare demographic features and disease characteristics between group comparisons of interest. A combination of Student’s t-test, one-way analysis of variance (ANOVA) with Tukey’s post-hoc test, and correlation analysis were used to assess between group differences. Pearson’s or Spearman correlation (as appropriate) were used to evaluate relationships between metabolites and continuous disease characteristics (i.e., duration). Values provided represent average ± standard deviation of the mean unless otherwise noted. Statistical analyses were performed in GraphPad Prism (v9.0, GraphPad Software LLC San Diego California). Sample size for this study was calculated based on primary outcome and thus selected to be powered to detect differences in TMAO levels between regular non-household controls and PD subjects (α = 0.5, >95% power). Statistics were corrected for multiple testing (FDR) using two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli f (FDR) with adjusted p-values (q-values) considered significant when q<0.05.

3. Results

3.1. TMAO biosynthesis in PD subjects

Population controls were compared with PD subjects (treatment naïve and treated combined) which were statistically indistinguishable based on demographic characteristics (Table 1: Neurobiol Dis. Author manuscript; available in PMC 2022 August 01.
This primary analysis supported the study hypothesis and revealed that PD subjects had significantly higher levels of TMAO pathway constituents than controls including betaine, butyrobetaine, TMA, and TMAO (Fig. 1A). The increase in TMA was not accounted for by elevated dietary substrate, as there were no differences in carnitine or choline between population controls and PD subjects.

To understand the potential contribution of the intestinal microbiome to TMA levels, absolute abundance of stool and mucosal-associated microbiota were correlated with plasma TMA levels (the metabolite directly produced by the microbiota) in a subset of study subjects (PD n = 32, pC n = 13). Significant positive correlations included: 

- *Faecalibacterium* (stool: p = 0.02, r = 0.44), Roseburia (stool: p = 0.03, r = 0.41), and Unclassified bacteria in the family Lachnospiraceae (mucosa: p = 0.02, r = 0.43) (all phylum Firmicutes, class Clostridia).
- *Collinsella* (stool: p = 0.04, r = −0.40) (phyla Firmicutes, class Clostridia) negatively correlated with plasma TMA. However, no correlations were significant after correcting for multiple comparisons (q < 0.05, data not shown). PICRUSt analysis was conducted to determine if pathways associated with TMA production were differentially abundant between population controls and PD subjects (e.g., choline-TMA lyase, choline monooxygenase, carnitine monooxygenase, glycine betaine reductase, TMAO reductase). No significant between group differences were noted between PD and control subjects (data not shown).

To account for the possibility that environment and lifestyle contribute to differences in TMA and TMAO, PD subjects with a spouse (n = 19) were compared to their spouse who lived in the same household (n = 19). These groups were statistically indistinguishable based on demographic characteristics except for sex. PD subjects were predominantly male and spouses predominantly female (p = 0.01) reflecting that spousal / household controls in this study were all opposite sex spouses. TMA was significantly higher in PD subjects compared to the spousal / household control subjects (Fig. 1B) suggesting the increase in TMA in PD subjects is likely not environment or lifestyle dependent. The apparent increase in TMAO in PD subjects was not significant compared to their spousal / household controls but based on a power calculation, a sample size of 39 would be required for this comparison to be appropriately powered. Betaine and butyrobetaine were nor dietary precursors of TMA (carnitine, choline) were not different between the groups and did not account for the elevated levels of TMA or TMAO.

### 3.2. TMAO biosynthesis: medication use, disease duration, and disease severity

The PD group was heterogeneous in terms of medication status, disease duration, and disease severity; therefore, to determine if these factors influenced TMA and TMAO levels, a subgroup analysis was performed. Treated PD (tPD) and treatment naïve PD (nPD) groups were significantly different in terms of disease duration (nPD 3.1 ± 0.4 vs tPD 9.1 ± 0.9, p < 0.01) and clinical severity (MDS-UPDRS: nPD 19.7 ± 1.3 vs tPD 27.0 ± 1.7, p = 0.02; H&Y Stage (median (range)): nPD 2.0 (1.0–2.0) vs tPD 2.0 (1.0–3.0), p = 0.04) with treated PD subjects having longer duration and more severe disease. Other demographic characteristics were not different between groups (all p > 0.05, data not shown).
Population controls, treatment naïve, and treated PD subjects were significantly different for TMAO pathway constituents including betaine, butyrobetaine, TMA, and TMAO (Fig. 2). Post hoc analysis showed that TMA was significantly higher in treated PD subjects compared to population controls and treatment naïve PD subjects, whereas TMAO was significantly higher in both treatment naïve and treated PD subjects compared to population control subjects (Fig. 2). Dietary precursors of TMA (carnitine, choline) were not different between the groups and did not account for the elevated levels of TMA or TMAO in PD subjects.

### 3.3. Other metabolites

In addition to TMA, bacteria produce numerous other metabolites that can influence host physiology. In this study, SCFA (acetic, butyric, and propionic acid), the branched chain fatty acid isovaleric acid, succinic acid, and lactic acid were evaluated. There were no differences in these metabolites when comparing population controls to PD subjects (combined treatment naïve and treated PD subjects), nor when comparing PD subjects to their respective spousal / household controls (all \( p > 0.05 \), data not shown). However, comparing treatment naïve and treated PD patients to populations controls revealed between group differences in lactic acid. Specifically, treated PD subjects had less lactic acid than treatment naïve PD subjects (Fig. 3, \( q < 0.05 \)). This appeared to be driven by a single high sample in the treatment naïve group (not a statistical outlier) nonetheless, these data suggest low lactic acid levels may be a feature associated with treated PD subjects.

### 3.4. Disease characteristics

To account for within group variability in characteristics including disease duration, disease severity, and constipation (common complaint in PD patients), correlation analyses was conducted between disease characteristics and bacterial metabolites (three PD subjects had confirmed PD by a movement disorders specialist did not have disease characteristics recorded and were omitted from the correlation analyses, leaving \( n = 72 \)). Disease duration and disease severity assessed by H&Y Stage did not correlate with any bacterial metabolite (all \( q > 0.05 \), data not shown). However, three significant correlations were identified. Succinic acid negatively correlated with disease severity assessed by MDS-UPDRS, whereby more succinic acid was associated with lower PD severity (\( p < 0.01, r = -0.33, q = 0.04 \)) (Fig. 4). Constipation (assessed via PROMIS) positively correlated with succinic acid (\( p = 0.01, r = 0.35, q = 0.04 \)) and negatively associated with butyric acid (\( p < 0.01, r = -0.51, q = 0.01 \)) (Fig. 4).

### 3.5. Inflammatory metabolite balance

To understand the inflammatory potential of the metabolites, the ratio of pro-inflammatory bacterial metabolite (TMA) to the putative anti-inflammatory SCFA butyrate was assessed. This analysis was limited to TMA which is produced by the microbiota (TMA is subsequently converted to TMAO by mammalian enzymes). Analysis revealed that there was a proinflammatory shift in bacterial-derived metabolites when comparing PD (treatment naïve and treated combined) to population control subjects. The ratio of TMA/Butyrate was significantly higher in PD subjects compared to population control subjects (Fig. 5A, \( q = 0.01 \)). The increase in the pro-inflammatory potential of the microbiota was also apparent.
when PD and their spousal / household controls (n = 19 pairs) were compared (Fig. 5B, q = 0.01). Medication treatment status significantly impacted the ratio of TMA/Butyrate ratio (Fig. 5C, q = 0.01) with post hoc analysis revealing that treated PD subjects had a higher ratio of TMA/Butyrate compared to treatment naïve PD and population controls (Fig. 5C). Additionally, an increased ratio of TMA to other SCFA were also noted including acetic acid and propionic acid (all q ≤ 0.01, Fig. 5A–C) supporting the idea of a pro-inflammatory shift in bacterial metabolites.

3.6. TMAO biosynthesis: PD versus multiple system atrophy (MSA)

Like PD patients, MSA patients have an abnormal intestinal microbiota compared to age matched controls (Engen et al., 2017). To understand if differences in bacterial metabolites may distinguish between PD and MSA, metabolite levels were compared between these groups. MSA is generally a more disabling disease that is less treatment-responsive than PD, and disease severity was different between treated PD and treated MSA groups (MDS-UPDRS: tPD 27.0 ± 1.7 vs MSA 44.6 ± 8.9, p < 0.00; H&Y Stage (median (range)): tPD 2.0 (1.0–3.0) vs MSA 3.0 (2.5–5.0), p < 0.00), but disease duration and other demographic characteristics were not different between groups.

The comparison between PD and MSA subjects was exploratory in nature and this assessment was not sufficiently powered (MSA n = 7), yet significant between group differences were observed. The SCFA acetic acid was significantly higher in MSA compared to treated PD subjects (Fig. 6A: MSA 246.7 ± 54.0 vs tPD 174.8 ± 5.0) and butyric acid was significantly lower in MSA compared to treated PD subjects (Fig. 6B: MSA 4.42 ± 0.68 vs tPD 6.40 ± 0.28). Analysis between PD and MSA subjects revealed no between group differences in the TMAO pathway, the branched chain fatty acid isovaleric acid, the SCFA propionic acid, lactic acid, succinic acid, nor the ratio of butyrate/TMA (p > 0.05, data not shown).

4. Discussion

There are a plethora of data demonstrating that the microbiota is altered in PD patients (Keshavarzian et al., 2015; Sun and Shen, 2018; Huang et al., 2021; Hill-Burns et al., 2017; Mertsalmi et al., 2017; Tetz et al., 2018; Aho et al., 2019; Barichella et al., 2017; Li et al., 2019a; Li et al., 2019b; Ren et al., 2020; Scheperjans et al., 2015). Whether the microbiota is a factor that triggers PD pathogenesis or is a consequence of PD is unknown; however, in either case, the microbiota could be an important factor contributing to neuro-inflammation and neurodegeneration. There are many ways that the microbiota can influence the brain, one of which is bacterial-derived metabolites (Tan et al., 2021; Vascellari et al., 2020; Li et al., 2021; Fernandez-Veledo and Vendrell, 2019; Unger et al., 2016; Aho et al., 2021; Shin et al., 2020), and in the current study, the TMAO pathway and other bacterial-derived metabolites were evaluated in subjects with PD and in another synucleinopathy, MSA.

Recent conflicting studies report altered levels of TMAO pathway constituents in PD patients: one demonstrates an increase in plasma TMAO in PD patients, one a decrease in plasma TMAO, and another a decrease in stool TMA (Chen et al., 2020; Chung et al., 2021; Tan et al., 2021). The heterogeneity in PD populations (disease duration, treatment
status, lifestyle) likely contribute to the differences in these studies. As hypothesized, the current study showed that PD subjects had higher plasma levels of TMA and/or TMAO than controls. The increase in the TMAO pathway, was not accounted for by increased levels of dietary precursors (i.e., carnitine, choline) nor were disease characteristics (duration, clinical severity) able to convincingly account for the increase in the TMAO pathway in PD subjects. Therefore, the elevated TMAO pathway constituents appear to be a trait of PD, rather than reflecting disease state (disease duration, clinical severity). The observation that constipation impacts the levels of butyrate and succinic acid is notable and understanding how gastrointestinal motility contributes to the PD-associated microbiota should be investigated in the future. Additionally, (though admittedly this study was not adequately powered to analyze lifestyle factors), lifestyle does not appear to contribute to the increase in TMAO pathway, as the increase in TMA is also observed in PD subjects when compared to their spousal / household controls. The lack of significance in TMAO may be attributed to (1) small sample size (the study was powered to detect changes in population controls and PD subjects with $n = 40$/group) or (2) the observation that spousal / household control subjects had higher levels of TMAO compared to population controls which could reflect PD-associated lifestyle, caregiver stress (which is known to influence the intestinal microbiota), and/or that inhabitants of the same household tend to share similar microbiome communities (Song et al., 2013). Additionally, the lack of significance in TMAO levels between PD and spousal controls indicates the importance of gene-environment interactions insomuch as high levels of TMAO are not sufficient to promote PD without an underlying genetic susceptibility (e.g., propensity to alpha-synuclein aggregation or neuroinflammation).

Reports by our group and others demonstrate pro-inflammatory shifts in the intestinal microbiota of PD patients compared to age matched controls (Keshavarzian et al., 2015; Sun and Shen, 2018; Huang et al., 2021; Hill-Burns et al., 2017; Mertsalmi et al., 2017; Tetz et al., 2018; Aho et al., 2019; Barichella et al., 2017; Li et al., 2019a; Li et al., 2019b; Ren et al., 2020; Scheperjans et al., 2015). In this study, we evaluated the relationship between the intestinal microbiota and plasma TMA levels in a subset of population controls and PD subjects. Actino-bacteria, Proteobacteria, and Firmicutes (class Clostridia) are reported to possess enzymes capable of converting dietary precursors to TMA (CutC/D for choline, CntA/B for carnitine) (Martinez-del Campo et al., 2015; Rath et al., 2017; Falony et al., 2015; Romano et al., 2015). Although they did not meet the stringent level of significance in this study ($q < 0.05$), plasma TMA was negatively associated with Collinsella (phylum Firmicutes, class Clostridia) and positively correlated with Faecalibacterium, Roseburia, and unclassified bacteria in the family Lachnospiraceae (phylum Firmicutes, class Clostridia). These bacteria are consistently reported to be decreased in PD subjects compared to controls (Keshavarzian et al., 2015; Sun and Shen, 2018; Huang et al., 2021; Hill-Burns et al., 2017; Mertsalmi et al., 2017; Tetz et al., 2018; Aho et al., 2019; Barichella et al., 2017; Li et al., 2019a; Li et al., 2019b; Ren et al., 2020; Scheperjans et al., 2015) and are typically thought to produce beneficial metabolites such as SCFA and their relationship to TMA in this analysis is counter-intuitive. The relationship between these bacteria and TMA could
reflect: (1) varied function of the bacteria under different conditions (e.g., SCFA under some conditions, TMA under other conditions) or (2) that the bacteria are a biomarker of a metabolic function related to TMA production or absorption into the systemic circulation. Clearly, additional studies will be required to better understand the relationship between the intestinal microbiota and TMA. It is important to note that even a small increase in the abundance of TMA-producing bacteria as a percent of the total community can have a meaningful impact on production of TMA from dietary sources (Martinez-del Campo et al., 2015). In fact, there are robust associations between foods / dietary patterns enriched in TMA precursors (choline, carnitine) and risk of PD. For example, consumption of full fat dairy products is associated with increased risk of PD (Chen et al., 2007; Chen et al., 2002; Park et al., 2005) and consumption of the Mediterranean diet (which limits dairy and red meat - both have high levels of TMA precursors) are associated with reduced risk of PD (Mischley et al., 2017; Gao et al., 2007; Alcalay et al., 2012). Taken together, small changes in the abundance of TMA-producing bacteria could have detrimental consequences when coupled with dietary habits. Diet was not evaluated in the current study and future evaluations should evaluate diet to begin to understand the interplay between microbiome, diet, TMA / TMAO levels. The lack of robust associations between the microbiota and TMA levels in the current study can be interpreted in several ways: (1) the subset of microbiota analyzed (n = 32 PD, n = 13 pC) may not be sufficiently powered or (2) microbiota-derived TMA may play a less important role than other factors like absorption of dietary TMA (found in red meat, eggs, dairy, fish), efficiency of conversion of TMA to TMAO (FMO3), and/or elimination of TMA or TMAO (urinary excretion). The contribution of these alternative mechanisms will require additional investigation.

TMAO is a risk factor for many age-associated diseases but perhaps none has been as well characterized as cardiovascular disease. It stands to reason that if TMAO contributes to cardiovascular disease and PD then cardiovascular disease would be a risk factor for PD. Currently available data on the relationship between cardiovascular disease and PD are mixed with some studies suggesting a positive and others a negative association (Potashkin et al., 2020; Savica et al., 2012; de Lau et al., 2006; Powers et al., 2009; Vikdahl et al., 2015; Qiu et al., 2011; Scigliano et al., 2006; Park et al., 2020; Hong et al., 2018). Differences in specific methodologies may contribute to the varied results but an additional factor that is likely important is the heterogenous pathophysiology of PD reflecting the unique combination of genetic and environmental factors that culminate in PD. In other words, individual variations in susceptibility to the detrimental effects of TMAO (increased inflammatory tone or propensity for alpha-synuclein aggregation) based on genetic background or other environmental factors are important and should be investigated further.

The exact mechanisms of how TMAO promotes disease are relatively mysterious. TMAO may contribute to PD pathogenesis by promoting inflammation, oxidative stress, and the expression of pro-inflammatory cytokines (e.g., TNF-α, IL-6, IL-18) (Hirsch and Hunot, 2009; Tansey and Goldberg, 2010; Glass et al., 2010; Salter and Stevens, 2017; Schlachetzki et al., 2014). TMAO can promote neuroinflammation by disrupting the blood brain barrier (Subramaniam and Fletcher, 2018), permitting increased entry of pathogenic and pro-inflammatory substances into the brain leading to microglia activation (a hallmark...
characteristic of PD and thought to be important in dopaminergic degeneration in PD) (Kim and Joh, 2006). Additionally, TMAO is associated with increased assembly and activation of the NOD-like receptor family, pyrin domain containing 3 (NLPR3) inflammasome and subsequent increased production of IL-1β and IL-18 (Boini et al., 2017; Saco et al., 2014; Yue et al., 2017; Sun et al., 2016; Chen et al., 2017). Knocking down the NLRP3 inflammasome, blunts TMAO-induced effects including production of IL-1β and IL-18 indicating that the effects of TMAO are (at least partially) mediated via the NLRP3 inflammasome (Yue et al., 2017; Sun et al., 2016; Chen et al., 2017). TMAO-induced effects on the NLRP3 inflammasome may be an important mechanisms of how TMAO could promote neuroinflammation and influence PD (Haque et al., 2020; Lee et al., 2019). Additionally, TMAO can dose-dependently induce folding of human alpha-synuclein (Uversky et al., 2001).

Other metabolites have also been investigated in PD including lipids, vitamins, amino acids, and other organic compounds, with mixed results (Tan et al., 2021; Vascellari et al., 2020; Li et al., 2021; Fernandez-Veledo and Vendrell, 2019; Unger et al., 2016; Aho et al., 2021; Shin et al., 2020). This study investigated SCFA (acetic, butyric, propionic acid), the branched chain fatty acid isovaleric acid, lactic acid, and succinic acid. Lactic acid was reduced in treated PD subjects. Reduced lactic acid in treated PD subjects is consistent with reduced abundance of lactate-producing bacteria in the PD-associated microbiota reported in some studies but it should be noted that many different bacteria produce lactic acid and not all lactic acid-producing bacteria are reduced in PD (Keshavarzian et al., 2015; Sun and Shen, 2018; Huang et al., 2021; Hill-Burns et al., 2017; Mertsalmi et al., 2017; Tetz et al., 2018; Aho et al., 2019; Barichella et al., 2017; Li et al., 2019a; Li et al., 2019b; Ren et al., 2020; Scheperjans et al., 2015; Hopfner et al., 2017). Despite the differences reported in the literature, a recent study demonstrates that administration of a lactate producing bacteria (Lactobacillus plantarum) has beneficial effects on motor symptoms in PD patients which suggests that a deficit in lactic acid may be important in PD (Lu et al., 2021). An additional complicating factor is that lactic acid is produced by both mammalian cells and by the microbiota therefore, the origin of the decreased lactic acid cannot be directly attributed to the microbiota in this study. Another bacterial metabolite of note was succinic acid which negatively correlated with PD severity (MDS-UPDRS). This finding is consistent with a recent report indicating that fecal succinic acid levels are decreased in PD patients (and may be related to disease severity) (Fernandez-Veledo and Vendrell, 2019). No between group differences were noted for isovaleric acid or SCFA (acetic, butyric, and propionic acid). Our group and others previously reported reduced levels of SCFA-producing bacteria in the PD intestinal microbiota (Keshavarzian et al., 2015; Unger et al., 2016; Aho et al., 2021), and yet the current study did not find significant differences between levels of plasma SCFA in PD and controls. While most studies report reductions in SCFA, reduced levels of SCFA in PD patients is not universally observed as recent studies report no changes in SCFA levels or increased levels of SCFA in PD patients (Vascellari et al., 2020; Shin et al., 2020). These differences likely reflect the heterogeneous nature of PD populations in terms of disease characteristics and lifestyle and may also reflect differences in the levels of stool versus systemic SCFA.
There was a shift in the ratio of pro-inflammatory (i.e., TMA, TMAO) to anti-inflammatory (i.e., butyrate) metabolites in PD subjects in this study. This shift in the inflammatory potential of the PD-associated microbiota is consistent with the pro-inflammatory changes in the microbiota reported in PD patients (e.g., shift in Firmicute / Bacteroidetes ratio) (Keshavarzian et al., 2015; Sun and Shen, 2018; Li et al., 2021; Unger et al., 2016; Nuzum et al., 2020; Bullich et al., 2019; Gerhardt and Mohajeri, 2018; Lin et al., 2019). The shift to a proinflammatory profile in PD subjects could account (at least in part) for why transplanting stool from human PD patients into a genetically susceptible mouse model of PD promotes the PD-like phenotype. Additional studies will be needed to confirm this assertion.

As an exploratory assessment, differences between PD and MSA subjects were evaluated. The small sample size of MSA subjects (n = 7) was not sufficiently powered to detect significant differences and yet acetic acid and butyric acid distinguished PD and MSA. The difference in these SCFA is in agreement with another study demonstrating altered levels of SCFA including plasma propionic acid (lower in MSA compared to PD) (He et al., 2021). No statistical differences were noted for TMA or TMAO indicating that an increased TMAO pathway is a feature of both PD and MSA. Future studies with larger sample size including an evaluation of MSA subtypes (MSA-P and MSA-C) and post-mortem brain pathology and/or brain imaging to confirm diagnosis may provide further resolution. Taken together, this exploratory assessment suggests that bacterial metabolites (e.g., acetic or butyric acid), may be potential biomarkers for differentiating MSA and PD patients, but the diagnostic accuracy of metabolites will require additional studies.

In conclusion, there is a building collection of literature indicating a relationship between bacterial metabolites and PD including the TMAO pathway, SCFA, and other metabolites. Recent publications highlight the importance of the microbiota-gut-brain axis and it is conceivable that these data can be leveraged clinically in two ways. First, bacterial-derived metabolites could be utilized as a biomarker of disease and second, modifications of the microbiota (and the production of microbiota-derived metabolites) could be a viable treatment approach for PD. New treatment approaches for PD are desperately needed and utility of microbiota-modifying approaches or the use of CutC/D inhibitor to limit production of TMA by the microbiota should be further investigated, perhaps as an adjunct therapy for existing treatment strategies.

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**Abbreviations:**

- **ANOVA** analysis of variance
- **CutC/D** choline TMA-lyase enzymes

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Fig. 1. TMAO Biosynthesis in PD Subjects.
(A) PD subjects (treatment naïve and treated combined) were compared to population controls. (B) PD subjects with a spouse (spPD) were compared to their spousal, household controls. pC = population (non-spousal, non-household) control, PD = treatment naïve and treated PD subjects combined, spC = spousal, household control, spPD = PD subjects with a spouse pair. Student’s t-test (p value) followed by multiple comparison correction (q value): *q < 0.05, **q < 0.01, ****q < 0.0001.
Fig. 2. TMAO Biosynthesis Pathway: PD Treatment Status.
PD subjects were divided into treatment naïve and treated and were compared to population controls. pC = population (non-spousal, non-household) control, nPD = treatment naïve PD subjects, tPD = treated PD subjects. One-way ANOVA (p value) followed by multiple comparison correction (q value), post hoc Tukey values shown on graph: **p < 0.01, ***p < 0.001.

|                | pC Subjects (n=20) | nPD Subjects (n=19) | tPD Subjects (n=55) | p Value | q Value | Post hoc |
|----------------|--------------------|---------------------|---------------------|---------|---------|----------|
| Carnitine (µM) | 36.04 ± 2.20       | 39.38 ± 1.51        | 35.45 ± 1.29        | 0.27    | 0.22    | n/a      |
| Choline (µM)   | 17.36 ± 1.16       | 18.95 ± 0.97        | 18.01 ± 0.79        | 0.66    | 0.44    | n/a      |
| Betaine (µM)   | 39.71 ± 3.17       | 55.73 ± 5.31        | 48.16 ± 2.01        | 0.01*   | 0.01*   | pC v nPD* |
| Butyrobetaine (µM) | 0.26 ± 0.003 | 0.28 ± 0.004       | 0.27 ± 0.002        | 0.01*   | 0.01*   | pC v nPD* |
| TMA (µM)       | 0.57 ± 0.05        | 0.56 ± 0.05         | 1.02 ± 0.08         | <0.01*  | <0.01*  | pC v tPD* pNP v tPD* |
| TMAO (µM)      | 1.75 ± 0.14        | 3.14 ± 0.26         | 2.83 ± 0.17         | <0.01*  | <0.01*  | pC v nPD* pC v tPD* |
Fig. 3. Metabolite Profile in PD Subjects.

PD subjects were divided into treatment naïve and treated and compared to population controls. pC = population (non-spousal, non-household) control, nPD = treatment naïve PD subjects, tPD = treated PD subjects. One-way ANOVA (p value) followed by multiple comparison correction (q value) with post hoc Tukey values shown on graph: **p < 0.01.
Fig. 4. The Association between disease characteristics and Metabolites in PD Subjects.
Correlation analysis was used to evaluate the relationship between disease severity (assessed via MDS-UPDRS) and gastrointestinal complaint of constipation (assessed via PROMIS).
Fig. 5. The ratio of Pro-Inflammatory to Anti-Inflammatory Metabolites in PD Subjects.
(A) PD subjects (treatment naïve and treated) were compared to population, non-spousal, household healthy controls. (B) PD subjects with a spouse were compared to their spousal / household controls. (C) PD subjects were divided into treatment naïve and treated and compared to population control subjects. Number of subjects in each group indicated on the x-axis. pC = population control, PD = treatment naïve and treated PD subjects combined, spC = spousal / household control, spPD = PD subjects with a spouse pair, nPD = treatment naïve PD subjects, tPD = treated PD subjects. (A & B) Student’s t-test (p value): *p < 0.05, **p < 0.01. (C) One-way ANOVA (p value) with post hoc Tukey values shown on graph: **p < 0.01, ***p < 0.001.
Fig. 6. PD and MSA Subject had Different Metabolite Profiles. Metabolites were compared between treated PD and treated MSA subjects. Number of subjects in each group indicated on the x-axis. tPD = treated PD subjects, MSA = multiple system atrophy subjects. Student’s t-test (p value): *p < 0.05, ****p < 0.0001.
Table 1

Subject demographics.

|                                      | Population Controls | Household Controls | PD Subjects | MSA |
|--------------------------------------|---------------------|--------------------|-------------|-----|
| N                                    | 20                  | 24                 | 86          | 7   |
| Race (n, %)                          |                     |                    |             |     |
| African American / Black             | 2 (10%)             | 1 (4%)             | 0 (0%)      | 0 (0%) |
| Asian                                | 0 (0%)              | 0 (0%)             | 1 (1%)      | 0 (0%) |
| Caucasian / White                    | 18 (90%)            | 23 (96%)           | 84 (98%)    | 7 (100%) |
| Other                                | 0 (0%)              | 0 (0%)             | 1 (1%)      | 0 (0%) |
| Sex (n, %)                           |                     |                    |             |     |
| Male                                 | 13 (65%)            | 7 (29%)            | 57 (66%)    | 6 (86%) |
| Female                               | 7 (35%)             | 17 (71%)           | 29 (34%)    | 1 (14%) |
| Age (years)                          |                     |                    |             |     |
| Ave ± SEM                            | 61.6 ± 1.7          | 63.0 ± 1.7         | 62.6 ± 1.0  | 68.3 ± 8.8 |
| Range                                | 51–80               | 48–77              | 45–82       | 53–77 |
| BMI                                  |                     |                    |             |     |
| Ave ± SEM                            | 27.7 ± 1.4          | 27.0 ± 1.3         | 26.5 ± 0.5  | 27.2 ± 2.7 |
| Range                                | 19–40               | 20–35              | 18–48       | 13–34 |
| Disease Duration (years)             |                     |                    |             |     |
| Ave ± SEM                            | n/a                 | n/a                | 7.6 ± 0.8   | 5.9 ± 1.2 |
| Range                                | 0.5–54              | 1–9                |             |     |
| MDS-UPDRS                            |                     |                    |             |     |
| Ave ± SEM                            | n/a                 | n/a                | 25.3 ± 1.3  | 44.6 ± 8.9 |
| Range                                | 0–67                | 20–88              |             |     |
| HY Stage                             |                     |                    |             |     |
| Median                               | n/a                 | n/a                | 2.0         | 3.0  |
| Range                                | 1.0–3.0             | 2.5–5              |             |     |
| PROMIS (median, range)               |                     |                    |             |     |
| Constipation Subscale                | n/a                 | n/a                | 6 (0–32)    | n/a |
| Medication (n, %)                    |                     |                    |             |     |
| Dopamine precursor                   | 53 (62%)            | 2 (29%)            |             |     |
| Dopamine agonist                     | 13 (15%)            | 0 (0%)             |             |     |
| Glutamate agonist                    | 11 (13%)            | 1 (14%)            |             |     |
| Anticholinergics                     | 1 (1%)              | 0 (0%)             |             |     |
| COMT inhibitors                      | 2 (2%)              | 0 (0%)             |             |     |
| MAO-B inhibitors                     | 9 (10%)             | 0 (0%)             |             |     |
| Antidepressant                       | 1 (1%)              | 0 (0%)             |             |     |
| Muscle relaxer                       | 2 (2%)              | 0 (0%)             |             |     |
| OTC Medications (not PD)             | 47 (55%)            | 4 (57%)            |             |     |