Gut Microbiome Signatures of Risk and Prodromal Markers of Parkinson Disease

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Objective: Alterations of the gut microbiome in Parkinson disease (PD) have been repeatedly demonstrated. However, little is known about whether such alterations precede disease onset and how they relate to risk and prodromal markers of PD. We investigated associations of these features with gut microbiome composition.
Methods: Established risk and prodromal markers of PD as well as factors related to diet/lifestyle, bowel function, and medication were studied in relation to bacterial α-/β-diversity, enterotypes, and differential abundance in stool samples of 666 elderly TREND (Tübingen Evaluation of Risk Factors for Early Detection of Neurodegeneration) study participants.
Results: Among risk and prodromal markers, physical activity, occupational solvent exposure, and constipation showed associations with α-diversity. Physical activity, sex, constipation, possible rapid eye movement sleep behavior disorder (RBD), and smoking were associated with β-diversity. Subthreshold parkinsonism and physical activity showed an interaction effect. Among other factors, age and urate-lowering medication were associated with α- and β-diversity. Physical inactivity and constipation were highest in individuals with the *Firmicutes*-enriched enterotype. Constipation was lowest and subthreshold parkinsonism least frequent in individuals with the *Prevotella*-enriched enterotype. Differentially abundant taxa were linked to constipation, physical activity, possible RBD, smoking, and subthreshold parkinsonism. Substantia nigra hyperechogenicity, olfactory loss, depression, orthostatic hypotension, urinary/erectile dysfunction, PD family history, and the prodromal PD probability showed no significant microbiome associations.
Interpretation: Several risk and prodromal markers of PD are associated with gut microbiome composition. However, the impact of the gut microbiome on PD risk and potential microbiome-dependent subtypes in the prodrome of PD need further investigation based on prospective clinical and (multi)omics data in incident PD cases.

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The presence of gastrointestinal pathological α-synuclein deposits and constipation in prodromal and clinically established Parkinson disease (PD) suggests an integral role of the gut–brain axis for the early pathogenesis of PD.[1–3] The synucleinopathy is hypothesized to ascend via the vagal nerve from peripheral neurons of the

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gastrointestinal tract to the brain.[4] Moreover, increased intestinal permeability,[5] elevated stool inflammatory cytokines,[6] and colonic wall inflammation[7] have been shown in PD patients, and may also represent key gastrointestinal processes in prodromal PD. Mice overexpressing α-synuclein show aggravated motor dysfunction when colonized with intestinal microbiota from PD patients.[8] However, the specific role of gut microbiota for PD and the factors modulating such processes along the microbiota–gut–brain axis are still largely unknown. The most consistently shown PD-related changes of gut microbial composition include an increase in the relative abundances of *Verrucomicrobiaceae* and *Akkermansia* and a decrease in *Prevotellaceae* and *Prevotella*.[9,10] The latter has also been associated with progressive PD motor symptoms over 2 years[11] and with rapid eye movement sleep behavior disorder (RBD),[12] a highly specific prodromal marker of PD. Prospective evidence of several PD risk markers (indicating an increased PD risk) and prodromal markers (indicating an already initiated neurodegenerative process) confirm the concept of a prodromal phase preceding clinically established PD by years or even decades.[13] Based on this evidence, it is possible to define specific predictive values for these markers and to calculate individual prodromal PD probabilities based on age and marker profiles.[14,15] Age[16] and several markers established in the International Parkinson and Movement Disorder Society (MDS) research criteria for prodromal PD[14,15] have also been associated with gut microbial changes; these include risk markers such as male sex,[17] diabetes,[18] nonsmoking, and nonuse of caffeine[16,17] and prodromal markers, such as RBD,[12] constipation,[illegible] and depression.[illegible] Moreover, physical inactivity, a risk marker for PD[15] and for most major chronic diseases occurring frequently in old age,[20] has been reported to affect microbial β-diversity in elderly males[21] as well as various inflammatory and immune processes.[22] However, these studies focused on microbial associations with single or few PD-related markers, or studied very small samples considering the multitude of other markers and confounding factors that may modulate the gut microbiome. For instance, factors related to diet, bowel function, and disease/medication often exert effects on microbial composition,[16–18] and may thus bias findings of marker associations. The present study therefore assessed gut microbial diversity, enterotypes, and taxonomic composition and investigated their associations with a comprehensive set of PD risk and prodromal markers, the overall prodromal PD probability, and a wide range of other potential confounders in a large sample of elderly individuals.

Patients and Methods

All subjects were participants of the prospective Tübingen Evaluation of Risk Factors for Early Detection of Neurodegeneration (TREND) study. The cohort has been enriched regarding an increased PD risk by partly recruiting participants based on the presence of olfactory loss, depression, and/or possible RBD. Biennial comprehensive assessments in 1,202 individuals have been performed over the past 10 years (www.trend-studie.de/english). Stool samples were collected at the third follow-up of the study and associated with markers/factors assessed at the corresponding wave of assessments. Stool was sampled using collection tubes containing a DNA stabilizer (PSP Spin Stool DNA Plus Kit; STRATEC Molecular, Birkenfeld, Germany), provided using postal services and frozen and stored at −80°C immediately upon arrival. Samples were available for 745 participants. After excluding individuals taking antibiotic medication (n = 47), patients with PD (n = [illegible]) or atypical/secondary parkinsonism (n = 2), incident cases of PD (n = 3), and individuals with missing dietary and medication data (n = 16), data from 66[illegible] individuals were included in the analyses.

In total, 9 risk and 9 prodromal markers as defined by the recently updated MDS research criteria for prodromal PD were selected a priori and investigated for associations with microbial measures.[15] We assessed the PD risk markers male sex, substantia nigra hyperechogenicity (transcranial ultrasound), nonsmoking, no consumption of coffee (cups per day), positive PD family history (first-degree relatives), physical activity (ie, low activity levels as measured using hours of sport/wk), type 2 diabetes (diagnosis), and occupational solvent and pesticide exposure (self-reported). Prodromal markers comprised olfactory loss (Sniffin' Sticks, 16 SS), depression (acute or lifetime diagnosis), constipation (Rome III criteria, sum score),[23] possible RBD (RBD screening questionnaire), excessive daytime somnolence, erectile and urinary dysfunction, and symptomatic orthostatic hypotension (the latter 3 assessed using the Unified Multiple System Atrophy Rating Scale questionnaire as described previously).[24] Moreover, motor deficits indicating subthreshold parkinsonism were assessed using the MDS Unified Parkinson's Disease Rating Scale part III[25] (score > 6 after excluding action/postural tremor items; scores from 3 to 6 were defined as borderline motor deficit).[14] Furthermore, 90 additional variables were considered comprising:

- Eighty variables that were screened as potential confounders for adjustment in the statistical analyses (see statistical approach below)

    - Seven generic/physiological variables (eg, age, body mass index [BMI], education, and exhaustion from climbing 3 levels of stairs [no, yes, not capable])
    - Five variables related to bowel function (eg, irritable bowel syndrome, bloating, vomiting/diarrhea)
    - Sixteen diet-related variables (assessing weekly meat, vegetables, dairy, protein, carbohydrate, and alcohol consumption)
    - One variable on gout
    - Fifty-one variables related to medication
- Exploratory variables that measure further aspects of prodromal PD or represent alternative (yet less established)[14] groupings/definitions of risk/prodromal markers
    - Prodromal PD probability values, and diagnoses of possible (>50% probability) and probable prodromal PD (>80%) as calculated based on age and comprehensive individual marker profiles according to the MDS research criteria for prodromal PD[14]
    - Exploratory alternative groupings/definitions of risk/prodromal markers comprised 3 variables on (functional) constipation, 2 variables on smoking status/history, and 2 variables on motor ratings

For the complete list of the 18 risk and prodromal markers and the 90 additional variables, their specific assessment methods, variable definitions, and descriptive statistics, see the Supporting Material. The study was approved by the local ethical committee (Medical Faculty, University of Tübingen; 444/2019BO2). All participants provided written informed consent.

Stool Sample DNA Extraction, Library Preparation, and Sequencing

DNA extraction, library preparation, and sequencing were performed at the DNA Sequencing and Genomics Laboratory of the Institute of Biotechnology, University of Helsinki. All samples were stored at −80°C and randomized in a −20°C room before bulk DNA extraction with the PSP Spin Stool DNA Plus Kit (STRATEC Molecular). One blank was added per extraction batch to assess potential contamination. After extraction, DNA concentrations were measured with NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). V3–V4 regions of the bacterial 16S ribosomal RNA gene were amplified in Verity 96-well Thermal Cyclers (Thermo Fisher Scientific) using a previously described polymerase chain reaction (PCR) protocol.[11] The amount of template DNA used for the first PCR ranged between 11.25 ng and 1311.26 ng. Each PCR batch included blank samples for assessment of potential contamination. Dual indexes were used in the second PCR; these had been selected using BARCOSEL[26] to allow pooling and sequencing of all samples in 1 pool run on 3 separate runs on a MiSeq (Illumina, San Diego, CA; v3 600 cycle kit, forward/reverse read length 328/278 bases). Thus, each sample was sequenced 3 times among all the other samples, reducing possible batch effects.

Sequence Analysis

The raw sequence data contained 48,782,168 sequence reads (availability: ENA accession number PRJEB32920). We combined the sequence reads from the 3 sequencing runs, and then trimmed primers and low-quality sequences with cutadapt (v1.8.3,[27] parameters q = 30 and m = 160). Merging paired reads, alignment to a reference database (SILVA, v132), chimera removal, taxonomic classification (reference database: Ribosomal Database Project (RDP), v16, 16S rRNA reference (PDS)), and operational taxonomic unit (OTU) clustering ("cluster.split" approach) were run with mothur (v1.40.0),[28] following the standard operating procedure (SOP) for MiSeq data.[29] Parameters differing from the SOP were maxlength = 500 and maxhomop = 8 for the first "screen.seqs," start = 2 and end = 17012 for the second "screen.seqs," diffs = 4 for "pre.cluster," cutoff = 70 for "classify.seqs," and keeping archaea sequences in "remove.lineage." Additionally, singleton sequences were removed prior to "classify.seqs" using "split.abund" with cutoff = 1. After excluding data from blanks and all OTUs with ≤10 sequence reads, the final dataset consisted of 25,390,744 sequence reads (34,082 ± 4,785 per sample).

Microbial Measures

All microbiota analyses were performed using genus-level data. α-Diversity was estimated with the inverse Simpson index (R package: phyloseq).[30] This index summarizes richness (number of different taxonomic units) and evenness (abundance distribution of taxonomic units) for each sample. The measure chosen for β-diversity was Bray–Curtis dissimilarity (R package: vegan). It quantifies the intersample compositional dissimilarity based on both presence/absence of taxonomic units and their abundances. Nonmetric multidimensional scaling (NMDS; R package: vegan) was applied to produce an ordination based on rank orders in the Bray–Curtis dissimilarity matrix, and to plot compositional dissimilarities between samples (and groups of samples) in a 2-dimensional ordination space.

Microbial enterotypes[31] were determined using the algorithm provided at http://enterotypes.org/. All unclassified taxa (genus level) were excluded from α-diversity, enterotype, and differential abundance analyses. For differential abundance, analyses were also performed for the family level and OTU level.

Statistical Analyses

The present study investigates possible associations of 18 single risk and prodromal markers with different microbial measures. To investigate this primary objective, potentially confounding effects of 80 different factors were considered for adjustment of risk and prodromal marker effects in the statistical analysis. Moreover, 10 variables measuring additional aspects of PD (eg, prodromal PD probabilities) or alternative marker groupings/definitions entered the analyses as exploratory variables (mentioned above and listed in the Supporting Material). The study used a 2-stage statistical analysis approach. First, in a prescreening step, each of the 18 risk and prodromal markers as well as each of the 90 additional variables were tested separately (for specific tests, see below) for associations with microbial measures (α-diversity, β-diversity, enterotypes). Here, markers/variables showing effects with $p < 0.1$ were selected for subsequent multifactorial statistical modeling. From the multiple measures of constipation, smoking, and motor function, the variable with the lowest p value was selected. Results of the prescreening step are shown in the Supporting Material. In a second step, all selected risk and prodromal markers and additional confounding adjustment factors were entered into multifactorial statistical models, and model selection was performed with final models only comprising markers/factors showing marginal effects with $p < 0.1$ (bolded p values in the Supporting Material: single variable association). Because the primary objective of the study was association testing for the 18 risk and prodromal markers (which were tested at least in the prescreening step), Bonferroni corrections for n = 18 tests were applied in the multifactorial tests, and effects of the 18 markers with a corrected threshold of $p < 0.003$ were considered significant. For adjustment factors, no correction for multiple testing was applied ($p < 0.05$). In enterotype analyses, differences between all 3 enterotype groups were statistically tested post hoc pairwise, and accordingly effects with $p < 0.0009$ were considered significant after correction for multiple testing for n = 18 × 3 = 54 tests.

Linear multiple regressions of α-diversity were performed. Associations of markers/factors with β-diversity were tested using PERMANOVAs (single variable: *adonis*, multifactorial: *adonis2,* both commands from the R package vegan). Moreover, α- and β-diversity were tested for interaction effects between physical activity and other risk and prodromal markers of PD. Marker/factor differences between enterotypes were tested using Fisher exact tests and Kruskal–Wallis tests, and subsequent multifactorial analyses using multinomial logistic regressions with enterotype as dependent variable.

For differential abundance analysis, we used DESeq2, a method based on generalized linear models with negative binomial distributions (sequence count data). The DESeq2 model included all covariates accounted for in the final PERMANOVA model for β-diversity, and p values were adjusted for multiple testing using false discovery rate (FDR) corrections accounting for the number of different taxa tested. We used R (v3.5.1) for all analyses and figures. TREND study data were collected and managed using REDCap electronic data capture tools hosted at the University of Tübingen.

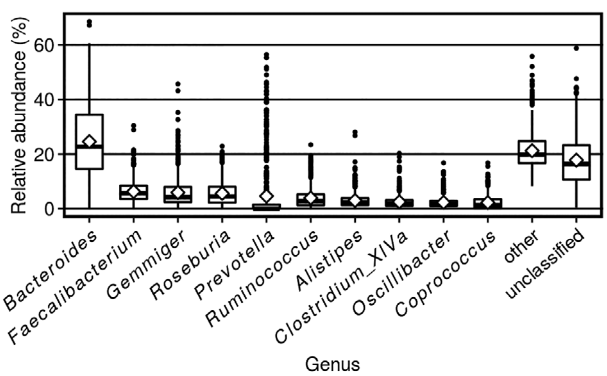

FIGURE 1: Relative abundances of the 10 most common bacterial genera.

Results

Descriptive Statistics

The TREND study sample (n = 666) had a mean age (±SD, range) of 68.4 ± 6.3 years (53–86). Risk and prodromal marker variables showed the following descriptive statistics: male sex (52.7%), substantia nigra hyperechogenicity (21.6%), positive PD family history (14.7%), physical inactivity (no activity, 25.7%; <1 h/wk, 30.9%; 1–2 h/wk, 24.2%; 2–4 h/wk, 8.3%; >4 h/wk, 10.4%), nonsmoking (never, 6.8%; former, 46.7%; current smoker, 46.5%), nonuse of coffee (14.4%), type 2 diabetes (8.3%), occupational solvent exposure (10.8%), occupational pesticide exposure (1.3%), olfactory loss (19.7%), depression (31.5%), constipation (severity sum score = 3.1 ± 3.9, range = 0–25), possible RBD (12.6%), excessive daytime somnolence (3.8%), erectile dysfunction (in males, 21.5%), urinary dysfunction (5.1%), symptomatic orthostatic hypotension (4.7%), and regarding motor functions, no motor deficit (90.5%), borderline motor deficit (6.0%), and subthreshold parkinsonism (3.5%). Based on prodromal PD probabilities (3.8 ± 11.4%, 0.0–89.1%), 15 individuals (2.3%) were diagnosed with possible prodromal PD and 5 individuals (0.8%) with probable prodromal PD. The most abundant bacterial genus in the subjects' stool samples was *Bacteroides*, followed by *Faecalibacterium, Gemmiger, Roseburia, Prevotella,* and *Ruminococcus* (Fig 1).

TABLE 1. Association of α-Diversity with Risk and Prodromal Markers of Parkinson Disease and Additional Factors

	Estimate	SE	*p*
Intercept	4.05	1.24	0.00113[a]
Risk markers			
Physical activity (h sport/wk)	−0.23	0.09	0.00765[b]
Occupational solvent exposure (no, yes)	−0.91	0.32	0.00401[b]
Prodromal markers			
Constipation (number of fulfilled Rome III criteria items of functional constipation)	0.23	0.12	0.04540[b]
Subthreshold parkinsonism (no, borderline, yes)	1.06	0.56	0.05930
Physical activity × subthreshold parkinsonism	−0.33	0.19	0.08769
Additional adjustment factors			
Age (yr)	0.03	0.02	0.04757[b]
Exhaustion from climbing stairs (no, yes, not capable)	−0.75	0.21	0.00038[b]
Urate-lowering medication (no, yes)	−1.25	[illegible]	0.00812[b]
Thyroid medication (no, yes)	[illegible]	0.24	0.00951[b]
Total dairy consumption (score)	[illegible]0.20	0.11	0.07369

[a]Significant effects of risk and prodromal markers in multiple regression models after Bonferroni correction for multiple testing ($p < 0.003$).

[b]Effects with an uncorrected $p < 0.05$.

SE = standard error.

α-Diversity

α-Diversity on the genus level as indicated by the inverse Simpson index showed associations ($p < 0.05$) with 2 risk markers, 1 prodromal marker, and 4 additional adjustment factors (Table 1). Overall, the multiple regression model explained 8.0% of α-diversity variance (adjusted R^2). Constipation severity and age were positively associated with α-diversity, whereas physical activity and occupational solvent exposure as well as intake of thyroid medication, urate-lowering medication, and exhaustion from walking stairs were inversely associated with α-diversity (Fig 2). None of the selected variables showed an interaction ($p > 0.1$) with physical activity on α-diversity. When entering motor deficits into the regression, no association with α-diversity was observed ($p > 0.1$), and the interaction of motor deficits and physical activity was nonsignificant ($p = 0.088$).

β-Diversity

Intersample differences in microbial composition as indicated by β-diversity showed significant associations with multiple risk and prodromal markers of PD (Table 2). In addition to age, physical activity, constipation, and BMI explained most of the variance (R^2; Fig 3). Moreover, sex, smoking, possible RBD, different medications, and dark bread consumption showed associations with β-diversity. Although motor deficits showed no effect, the interaction between physical activity and motor deficits explained variance in β-diversity ($p = 0.002$).

Enterotypes

Bacteroides-enriched (70.9% of samples) microbiomes were more frequent compared to *Prevotella*-enriched (21.5%) and *Firmicutes*-enriched (7.7%) microbiomes. Enterotypes differed in several risk and prodromal markers and additional factors (Table 3). For instance, the lowest physical activity levels as well as most severe constipation were observed for the *Firmicutes*-enriched enterotype (Fig 4). Higher physical activity levels were linked to the *Bacteroides*-enriched enterotype. Constipation severity was lowest and subthreshold parkinsonism least frequently observed in individuals with the *Prevotella*-enriched enterotype.

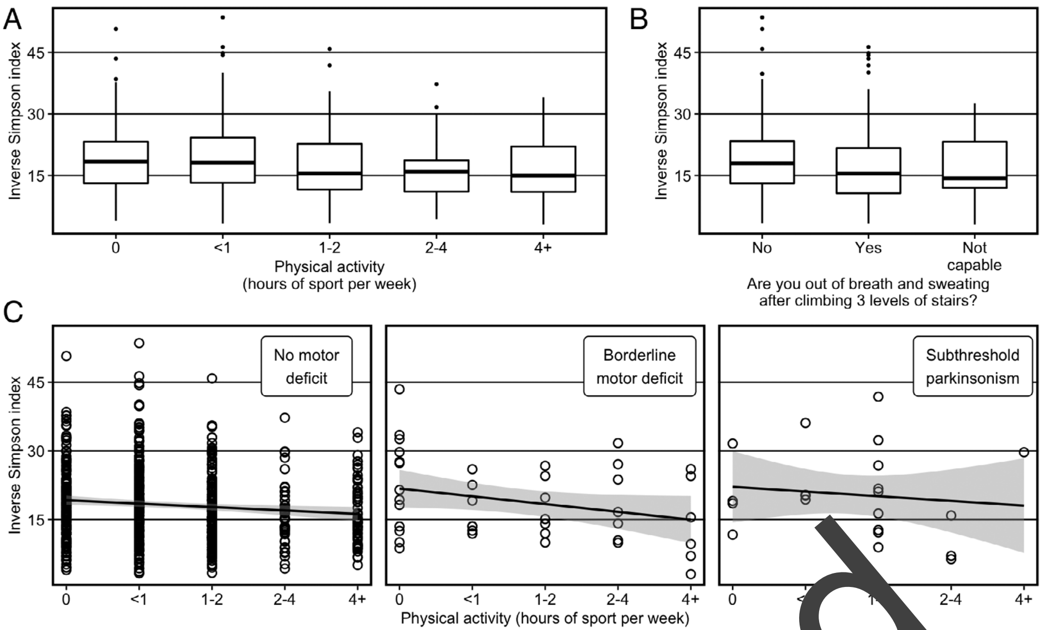

FIGURE 2: α-Diversity and physical motor measures. Magnitude of α-diversity by (A) levels of weekly physical activity, (B) physical exhaustion, and (C) physical activity levels in different motor deficit groups.

TABLE 2. Associations of β-Diversity with Risk and Prodromal Markers in Parkinson Disease and Additional Factors

	R^2	F	p
Risk markers			
Physical activity (h sport/wk)	0.014	9.09	0.001[a]
Male sex (no, yes)	0.006	3.59	0.003[a]
Smoking (pack-years)	0.004	2.36	0.020[b]
Prodromal markers			
Constipation (sum score of Rome III criteria items of functional constipation)	0.009	5.48	0.002[a]
Possible RBD (no/yes)	0.003	2.14	0.037[b]
Subthreshold parkinsonism (no, borderline, yes)	0.002	1.45	0.159
Physical activity × subthreshold parkinsonism	0.006	3.56	0.002[a]
Additional adjustment factors			
Age (yr)	0.008	4.88	0.001[b]
Diabetes medication (no, yes)	0.004	2.66	0.011[b]
Urate-lowering medication (no, yes)	0.004	2.43	0.016[b]
Beta-blocker medication (no, yes)	0.003	1.91	0.060
Dark bread (no, yes)	0.003	2.19	0.034[b]
BMI (kg/m^2)	0.013	8.41	0.001[b]

[a]Significant effects of risk and prodromal markers in permutational multivariate analysis of variance models after Bonferroni correction for multiple testing ($p < 0.003$).
[b]Effects with an uncorrected $p < 0.05$.
BMI = body mass index; RBD = rapid eye movement sleep behavior disorder.

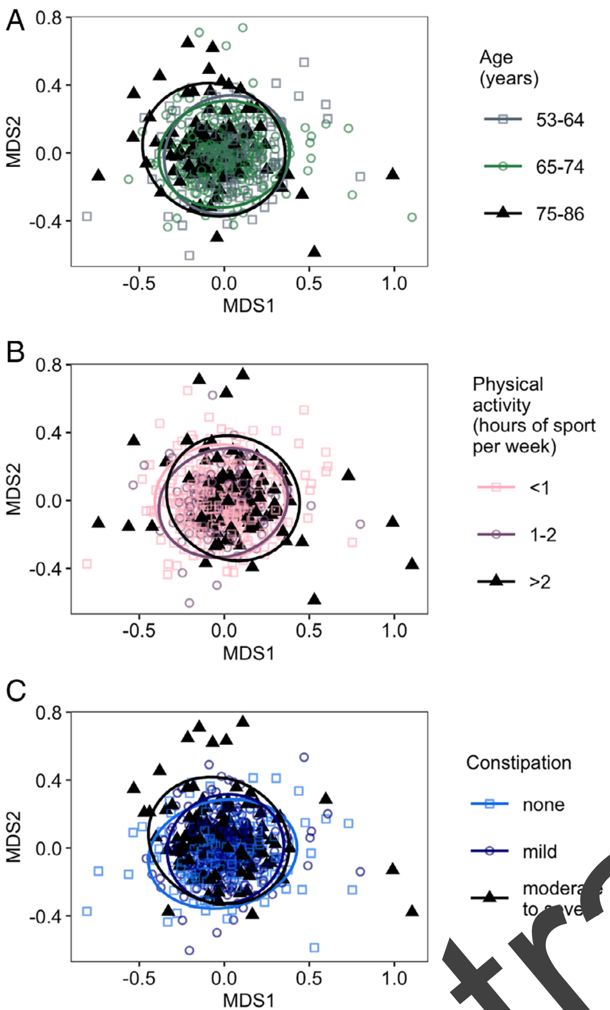

FIGURE 3: Nonmetric multidimensional scaling (NMDS) plots in 2-dimensional space (MDS1 and 2) of factors showing significant Bray–Curtis distance differences. Differences in microbial composition between (A) age groups, (B) groups with different physical activity levels, and (C) groups stratified by severity of constipation are shown. Circles indicate 95% confidence intervals.

Differential Abundance

Several of the variables associated with β-diversity were also linked to the abundances of specific taxa (Supporting Material; all *p* values FDR-corrected). The variable with the most differentially abundant taxa was constipation (Table 4). Of taxa previously associated with PD on the genus level,[10] increased constipation severity was significantly associated with decreased abundance of *Faecalibacterium* (p = 0.022) and *Roseburia* (p = 0.008), physical exhaustion with a decrease in *Bifidobacterium* (p = 0.039), and possible RBD with a decrease in *Lactobacillus* (p = 0.023). Further, motor deficits indicating subthreshold parkinsonism were associated with a decrease in *Odoribacter* (p = 0.031). Possible RBD was further associated with a decrease in *Faecalicoccus* (p = 0.017) and *Victivallis* (p = 0.017), and an increase in the abundance of *Haemophilus* (p = 0.003). Urate-lowering medication was associated with higher abundance of *Clostridium III* (p = 0.005) and *Parasutterella* (p = 0.032). The taxa *Prevotella and Akkermansia* were not statistically significant in any of the differential abundance comparisons.

Discussion

The present study investigated associations between gut microbial composition, risk markers and prodromal markers of PD, subthreshold parkinsonism, and a wide range of potential confounders in 666 elderly individuals. Among these markers, particularly physical activity, constipation, possible RBD, smoking, and subthreshold parkinsonism were associated with alterations in microbial community composition. Moreover, age, sex, occupational solvent exposure, and an interaction of subthreshold parkinsonism and physical activity were associated with different microbial measures. As expected, effect sizes of individual markers and factors (≤1% explained variance) and multifactorial models (~8%) were small.[16,17] None of the microbial measures was associated with substantia nigra hyperechogenicity, olfactory loss, depression, orthostatic hypotension, urinary/erectile dysfunction, family history of PD, or the overall prodromal PD probability calculated based on marker profiles of individuals.

The relative abundances of bacterial taxa were mostly similar to other samples of healthy elderly individuals.[32] Lower relative abundance of, for example, *Bacteroides* compared to previous findings may be explained by differences in cohort composition due to recruitment, demographic, regional, and/or lifestyle factors.

Age, constipation, and low physical activity were associated with increasing and occupational solvent exposure with decreasing within-sample α-diversity. Higher α-diversity in PD patients compared to controls has been reported,[33–35] yet overall evidence is inconsistent,[10] and it is unclear whether α-diversity and PD are linked. Similar to our results, older age in an elderly population has been associated with higher α-diversity.[36] However, statistical modeling of such effects is complicated by the diseases, medications, and lifestyle changes often associated with advanced age. Regarding constipation, higher α-diversity has been linked to harder stool consistency[16] and functional constipation.[37] Autonomous dysfunction related to prodromal PD may contribute to these α-diversity effects, which however need to be disentangled from other potentially important factors, such as diet, stool water content and transit time, and bacterial growth rates.[38] Physical inactivity increases the risk of many chronic diseases[20] including PD[15]; conversely, being active may lead to lower prevalence of prodromal PD markers such as

TABLE 3. Associations of Enterotypes with Risk and Prodromal Markers of Parkinson Disease and Additional Factors

	Bacteroides* vs *Firmicutes			***Bacteroides* vs *Prevotella***			***Firmicutes* vs *Prevotella***		
	Estimate	**SE**	***p***	**Estimate**	**SE**	***p***	**Estimate**	**SE**	***p***
Intercept	−1.44	0.44	0.00092[a]	−0.28	0.26	0.28121	1.17	0.47	0.01299[b]
Risk markers									
Physical activity (h sport/wk)	−0.58	0.16	0.00031[a]	−0.25	0.09	0.00313[b]	0.33	0.17	0.05763
Male sex (no, yes)	−0.60	0.34	0.08162	0.25	0.20	0.22016	0.85	0.38	0.02355[b]
Prodromal markers									
Constipation (sum score of Rome III criteria items of functional constipation)	0.07	0.03	0.02404[b]	−0.07	0.03	0.04502[b]	−0.14	0.04	0.00133[b]
Subthreshold parkinsonism (no, borderline, yes)	0.43	0.30	0.14722	−0.36	0.30	[illegible]	−0.79	0.40	0.04628[b]
Additional adjustment factors									
Legumes (score)	−1.08	0.36	0.00285[b]	0.07	[illegible]	[illegible]	[illegible]	0.39	0.00274[b]
Functional bloating (no, yes)	0.95	0.45	0.03235[b]	−0.59	0.46	0.20014	−1.54	0.59	0.00906[b]
Vegetarian (no, yes)	1.05	0.51	0.04091[b]	0.38	[illegible]	[illegible]	−0.68	0.65	0.29823

[a]Significant effects of risk and prodromal markers in logistic regressions after Bonferroni correction for multiple testing (also considering 3 group comparisons, $p < 0.0009$).
[b]Effects with an uncorrected $p < 0.05$.
SE = standard error.

constipation.[39] The effects of inactivity could be related to the processes observed in PD, that is, increased chronic inflammation,[7] immune responses,[6] and intestinal barrier permeability.[5] Exhaustion from climbing stairs, an indicator of low cardiorespiratory [illegible] was associated with a reduction in α-diversity, in line with earlier studies indicating lower diversity in lower fitness[40] and frailty.[41] This apparently contradictory result may be partly explained by differences between physical activity and capability. Moreover, the PD risk marker occupational solvent exposure[15] was associated with a decrease in α-diversity. Strengths and directions of effects of different risk and prodromal markers on microbial composition may vary based on the underlying pathophysiological pathways, potentially explaining why the overall prodromal PD probability calculated based on comprehensive marker profiles showed no significant association with α-diversity.

β-Diversity (between samples) has been consistently shown to differ between PD patients and controls.[10] Several PD risk and prodromal markers were associated with β-diversity even in multifactorial models while considering potential confounders. These findings suggest that microbial composition might already be altered in the prodromal phase of PD. Although α- and β-diversity are not directly related, constipation and physical inactivity were again among the variables explaining most of the variance. While showing a nonsignificant interaction effect for α-diversity (p = .088), physical activity and motor deficits indicating subthreshold parkinsonism showed a significant interaction effect for β-diversity, and thus between-sample dissimilarities in microbial composition depend on the interaction of both markers. It remains speculative to what degree these associations play a specific role in prodromal PD. Links between β-diversity and possible RBD, sex, and smoking further support the concept of microbial changes preceding PD. While replicating effects of antidiabetic and beta-blocker medication,[17] our study showed the most consistent associations of α- and β-diversity and differential abundance for urate-lowering medication. Urate is a powerful antioxidant linked to reduced PD risk,[14] and our results may implicate gut microbiota in this context. Dark bread was the only dietary factor associated with β-diversity. Dark bread can be seen as an indicator of high fiber consumption, and thus presumably better intestinal barrier integrity and short-chain fatty acid (SCFA) production.[42] Because both have been suggested

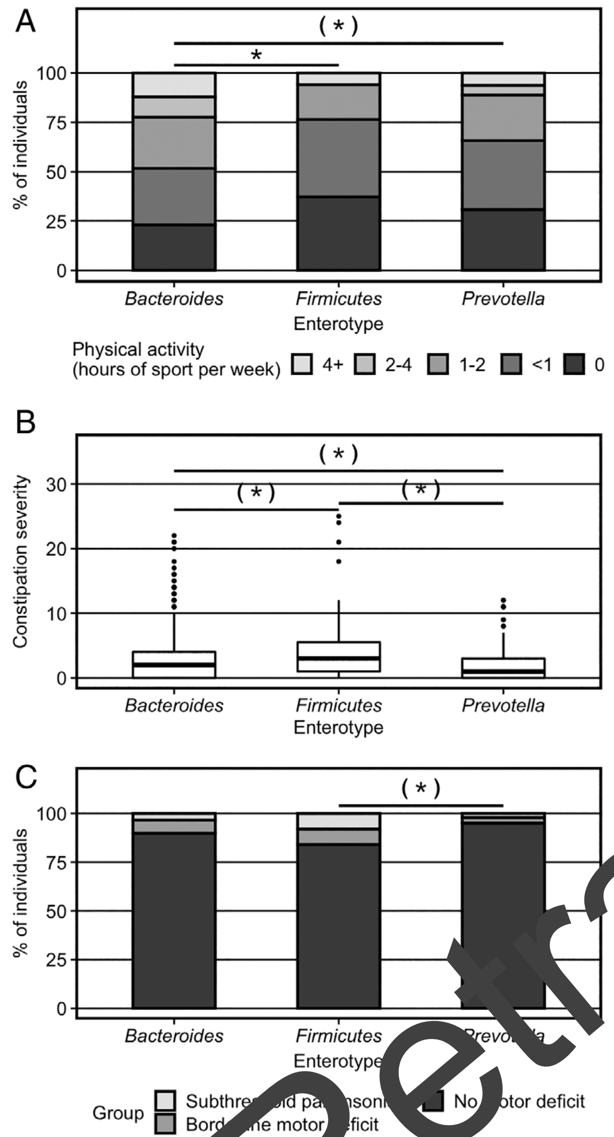

FIGURE 4: Enterotype group differences regarding (A) proportions of levels of physical activity, (B) severity of constipation, and (C) proportions of individuals with motor deficits indicating subthreshold parkinsonism. *Significant after Bonferroni correction (p < 0.0009); (*) indicates effects with an uncorrected p < 0.05.

to be impaired in PD[5,43] and to play a role in several processes along the microbiota–gut–brain axis,[42] dietary factors might also be important in prodromal PD. After physical activity, BMI explained the most variance in β-diversity. For some dietary factors linked to BMI, independent effects might have been too small to reach the threshold for statistical significance.

Subthreshold parkinsonism was least frequently present in individuals with the *Prevotella*-enriched compared to the *Firmicutes*-enriched enterotype. This finding is in line with the reduced abundance of *Prevotella* in PD,[10] in more severely progressing PD,[11] and in RBD[12] compared to controls. Thus, the present study supports the relevance of *Prevotella* in prodromal PD. Microbial changes due to constipation are often argued to confound microbiome analysis of PD patients. The *Prevotella*-enriched enterotype was also the least common in individuals with high constipation severity scores. Subjects with high scores typically had the *Firmicutes*-enriched enterotype in accordance with previous findings.[44] Viewing constipation in PD as being linked to a disturbed enteric nervous system showing similar cellular changes to those of affected brain structures may suggest common relevance of *Prevotella* for prodromal dysautonomic and motor deficits. In this light, constipation may not be confounding, but may reflect a common pathogenic process. Although constipation may exert effects on microbial composition via several plausible mechanisms, such effects may be different in diseased individuals as compared to healthy subjects, as recently demonstrated in PD for the association between α-diversity and stool consistency.[11] Physical inactivity was more frequently observed in individuals with the *Firmicutes*-enriched enterotype, whereas the *Bacteroides*-enriched enterotype was more frequent in active subjects. Although consistent with some evidence gained from human[45] and mouse studies,[46] processes underlying the links between physical activity, other prodromal markers in PD,[39] obesity, nutrition, and microbial composition are complex and need to be further investigated.

The results of differential abundance analyses were partly consistent with previous findings, but some taxa reported for PD[10] and RBD[12] showed no association with risk and prodromal markers. In contrast with enterotype analyses, no significant differential abundance effect was observed for *Prevotella*, which may be partly explained by differences in covariates considered. Among other candidate genera, *Akkermansia* showed no significant association with any of the risk and prodromal markers. However, possible RBD showed several differentially abundant taxa, which have not been previously associated with PD[10] or RBD[12] (*Faecalicoccus, Victivallis,* and *Haemophilus*). *Lactobacillus* was decreased in individuals with possible RBD, whereas an increase in PD patients has previously been shown repeatedly.[10] It is possible that subtypes of prodromal PD exist with varying involvement of the gut (eg, RBD representing a subtype with early autonomic denervation).[47] Also, the microbiome may (differentially) change over time, that is, from prodromal to clinically established PD. Such complexity may hamper the identification of (prodromal) PD-related microbiome signatures. However, these signatures may allow for early stratification of individuals based on their microbiome (and underlying or inherent pathologies), and early and targeted therapeutic interventions.

TABLE 4. Number of Differentially Abundant Taxa in Association with Risk and Prodromal Markers of Parkinson Disease and Additional Factors in Multifactorial Models

	Family Level	Genus Level	OTU Level
Risk markers			
Physical activity (h sport/wk)	4	5	7
Smoking (pack-years)	1	1	12
Prodromal markers			
Constipation (sum score of Rome III criteria items of functional constipation)	10	25	51
Possible RBD (no, yes)	4	4	4
Subthreshold parkinsonism (no, borderline, yes)	0	1	4
Additional adjustment factors			
Age (yr)	0	2	5
Exhaustion from climbing stairs (no, yes)	3	5	9
Urate-lowering medication (no, yes)	3	2	5
Antihypertensive medication (no, yes)	1	1	1
Diabetes medication (no, yes)	2	5	9

OTU = operational taxonomic unit; RBD = rapid eye movement sleep behavior disorder.

Constipation severity was significantly associated with decreased abundance of *Faecalibacterium* and *Blautia*, SCFA-producing taxa that can exert positive effects on the intestinal mucosa[48] and are decreased in PD.[10] The finding of a decrease in *Odoribacter* in individuals with subthreshold parkinsonism has potential relevance for prodromal PD. *Odoribacter* [illegible] involved in SCFA production and tryptophan metabolism. It might be relevant for gastrointestinal integrity and serotonergic bowel dysfunction (prolonged transit time) as well as central nervous dysfunction (anxiety) as suggested by findings in an autism mouse model.[49]

The present study has several limitations. (1) Some markers, and dietary and medication data, were assessed using self-reports. Although those assessments were structured and highly standardized, quantitative measures or medical records might be more accurate, for example, for assessing physical activity, diets and medication. (2) Interaction analyses were limited to those involving physical activity, given its relevance as a PD risk marker[15] and for prodromal PD markers.[39] Further research on marker interactions and clusters as well as factors underlying potential biases is needed to better model the complexity of microbial associations. (3) Stool samples were not frozen immediately after defecation but after postal delivery, and the delay may constitute a technical confounder. However, collection tubes contained a DNA stabilizer, and the impact of delayed freezing on microbial composition should therefore be minimal.[11,50] (4) Some risk and prodromal markers were present at lower frequency, and lower statistical power might have contributed to some non-significant findings.

In conclusion, several risk and prodromal markers in PD, in particular markers related to motor aspects and constipation, were associated with altered microbial α- and β-diversity, enterotypes, and bacterial abundance. Constipation, physical activity, possible RBD, urate levels, smoking, and subthreshold parkinsonism might be particularly linked to the prodromal microbiome in PD. However, many other markers predictive of PD and overall prodromal PD probability values showed no significant association with any microbial measure. Prodromal microbial changes might only be observable in subgroups with specific marker constellations. The functional roles of these markers and associated microbiota for intestinal permeability, stool immune mediators, colonic inflammation, and the systemic interactions with the host organism need further investigation. The prodromal microbiome(s) in PD, temporal dynamics of microbiota toward PD diagnosis, and etiological relevance in prodromal PD should be investigated based on prospective marker profiles and (multi)omics data in incident PD cases.

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Author Contributions

S.H.e., K.B., G.W.[illegible], W.M., [illegible]D.B., P.A., and F.S. contributed to the conception and design of the study; S.H.e., S.H.a., V.T.E.A., L.P., [illegible]C.D., C.S., K.B., U.S., A.-K.v.T., L.P., P.A., and F.S. contributed to the acquisition and analysis of data; S.H.e., V.T.E.A., W.M., P.A., and F.S. contributed to drafting the text and preparing the figures.

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Data Availability

TREND data are available upon request. Microbiome sequence raw data are available under the European Nucleotide Archive accession number PRJEB32920.

Potential Conflicts of Interest

V.T.E.A., L.P., P.A., and F.S. are inventors on granted patents related to the use of microbiota analysis in the diagnosis of PD (FI127671B, US10139408B2) and pending patents related to the use of microbiota in the diagnosis and treatment of PD (US16/186,663, EP3149205). These patents/patent applications are currently assigned to NeuroInnovation Oy and licensed to NeuroBiome. NeuroInnovation Oy provides clinical neurological services for patients and health care providers as well as consultant services in the field of neurology and microbiota. NeuroBiome pursues the development and commercialization of diagnostic and therapeutic applications of microbiota for PD. Currently, no such products are marketed by NeuroBiome or NeuroInnovation Oy. F.S. owns 85% of shares of NeuroInnovation Oy and 100% of shares of NeuroBiome. None of the abovementioned inventors has received any fees or royalties from these companies related to microbiota-related products, but they may do so in the future if development and commercialization are successful. F.S. is a member of the scientific advisory board of and has received fees and stock options from Axial Biotherapeutics, a company that is developing gut–brain axis–related therapeutics for PD and autism spectrum disorders.

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