Parkinsonism and Related Disorders 65 (2019) 124–130

Contents lists available at ScienceDirect

Parkinsonism and Related Disorders

journal homepage: www.elsevier.com/locate/parkreldis

Dysbiosis of gut microbiota in a selected population of Parkinson's patients

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ARTICLE INFO

Keywords:
Parkinson's disease
Dysbiosis
Gut microbiota
Predictors
Functional pathways
16S rRNA

ABSTRACT

Introduction: In recent years the hypothesis that gut microbiota associates with Parkinson's disease (PD) has gained importance, although it has not been possible to define a specific microbiota composition as a predictive biomarker of this disease. We have investigated dysbiosis of gut microbiota in a selected population of PD patients from Central Italy, and examined the weight of specific confounders and predictors, in order to identify potential correlations with clinical phenotypes.

Methods: 152 fecal samples were collected from 80 patients and 72 healthy controls. Patients were enrolled according to tight inclusion criteria. Microbiota composition was studied through 16s ribosomal RNA gene amplicon sequencing analysis in combination with data on dietary/life habits. Age, loss of weight, and sex were recognized as confounding factors, whereas PD-status, age, Body Mass Index, “eat cereals”, “gain of weigth” and “physical activity” as predictors. The presence of *Lactobacillaceae*, *Enterobacteriaceae* and *Enterococcaceae* families was significantly higher in feces from PD patients compared to healthy controls, while *Lachnospiraceae* were significantly reduced. Lower levels of *Lachnospiraceae* and higher levels of *Enterobacteriaceae* families also correlated with increased disease severity and motor impairment (Hoehn & Yahr stage, MDS-UPDRS Part III). Predictive metagenomics indicated a significant variation of genes involved in the metabolism of short chain fatty acids and amino acids, and in lipopolysaccharide biosynthesis.

Conclusions: PD showed a distinctive microbiota composition. Functional predictions suggest changes in pathways favoring a pro-inflammatory environment in the gastrointestinal tract, and a reduction in the biosynthesis of amino acids acting as precursors of physiological transmitters.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide, with a prevalence of 1% of the population above 60 years [1]. The standard diagnosis is based on clinical observations of symptoms, such as resting tremor, bradykinesia, rigidity, and postural instability, which reflects the loss of dopaminergic neurons in the substantia nigra [2], and more recently nuclear imaging biomarkers [3]. Asides from this core motor syndrome, PD is a multisystem disorder involving also the gastrointestinal tract. Recent studies highlighted the role of gastrointestinal microbiota in the regulation of the nervous system, and in the pathogenesis and progression of nervous system-related diseases [4]. Consistently, studies on germ-free mice and antibiotic-treated pathogen-free mice have shown impairment in hippocampal neurogenesis [5].

The bidirectional connection between the gut and the central nervous system (CNS), the gut-brain axis, is influenced by the gut microbiota. Alterations of the microbiota-gut-brain axis have been associated with CNS diseases such as multiple sclerosis, Alzheimer's disease and Parkinson's disease [4]. Indeed, α-syn aggregates, the neuropathological hallmark of PD, are found in the enteric nervous system, and gastrointestinal symptoms such as constipation and colonic inflammation are reported in over 80% of PD cases, and they precede the motor signs by several years [6]. Scheperjans' group provided a pioneering study in support of upregulation of *Verrucomicrobia* and down-regulation of *Prevotellaceae* in PD patients [7]. So far at least 10 studies have

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https://doi.org/10.1016/j.parkreldis.2019.06.003
Received 15 February 2019; Received in revised form 23 May 2019; Accepted 2 June 2019

investigated the implication of gastrointestinal dysfunction and microbiota imbalance on PD pathogenesis. Some studies on PD patients found increased levels of members of the *Lactobacillaceae* family and of the related genus *Lactobacillus* [7–10], and reduced levels of the *Lachnosphiraceae* family and the related genera *Blautia* and *Roseburia* [8,10–15]. Conflicting data have emerged, such as the decreased levels of *Prevotella*, recently questioned by Pezzoli's group [10]. Hence additional data are required to understand the correlation between gut microbiota and PD.

The current study presents an analysis of the fecal microbiota composition on a large cohort of PD patients from Central Italy. We selected patients with disease stages ranging from *de novo* to the latest and compared their microbiota compositions with their caregivers' or partners' ones. Compared to controls, patients showed a distinctive microbiota composition, that could be associated to a pro-inflammatory environment in the gastrointestinal tract.

2. Methods

2.1. Recruitment of patients and clinical characteristics

Recruited patients had a diagnosis of idiopathic PD according to the UK Parkinson's Disease Society Brain Bank criteria [16] with a disease duration (measured as the time following the first diagnosis) longer than 6 months. Patients were required to meet the following criteria: 1) no cognitive impairment, as defined by a Montreal Cognitive Assessment (MoCA) score above 25; 2) no chronic gastro-intestinal (GI) disease, including malabsorption; 3) no clinical history of gastric lesions, gastroresection or major intestinal surgery; 4) complete agreement with the study design. All patients were clinically classified using Hoehn & Yahr staging (H&Y) and Movement Disorder Society Unified Parkinson's Disease Rating Scale Part III (MDS-UPDRS Part III) score during ON time, to estimate disease progression and motor impairment. Patients were divided into three subgroups according to Kang et al. guidelines [17]: Akinetic-rigid (AR) ($n = 30$), Tremor dominant (TD) ($n = 30$), and Mixed (M) ($n = 19$), based on different onset motor presentations.

Exclusion Criteria were: 1) concomitant neurologic and/or psychiatric diseases; 2) systemic and/or neurologic infectious, inflammatory, or autoimmune diseases; 3) atypical parkinsonism syndromes and vascular parkinsonism; 4) acute GI phlogosis or GI disease in the last 4 weeks; 5) use of domperidone, or any drug potentially affecting gastrointestinal motility and integrity; 6) use in the last 4 weeks of pre-probiotics or therapy based upon steroids, nonsteroidal anti-inflammatory drugs or antibiotics; 7) anamnesis suggestive of GI cancer pathology.

Selected patients were recruited for the study during scheduled visits at the Outpatient Clinic for Movement Disorders of the Neurological Clinic of the University of "Tor Vergata", between January 2017 and May 2018. For all patients, a reliable and beneficial response to submaximal doses of levodopa was established. A careful control of daily intake of dopaminergic agents was crossed-checked with the partner and/or caregiver. All patients gave written informed consent after receiving an extensive disclosure of the study purposes and risks. The trial was approved by the local ethics committee (RS 73/18).

As a control group, we considered a population of healthy volunteers mainly represented by patient spouses or people who share patients' habits of life. The exclusion criteria for controls were the presence of a neurological disease, acute or chronic GI diseases and the use of prokinetic drugs, or any drug potentially affecting gastrointestinal motility and integrity.

Stool samples were collected at home by all subjects. Information about subject's life and diet habits were obtained through anamnestic interview collecting 31 variables divided into five categories: 1) general data (age, sex and cesarean section), 2) weight: Body Mass Index (BMI), 3) dietary habits, 4) life habits and 5) PD medications and clinical features.

2.2. Sequencing and bioinformatic analysis of 16S rRNA amplicons

Fecal samples were collected using the Pre-analytical Sample Processing (PSP) stool collection tubes containing 8 ml of Stool DNA Stabilizer, which allows the storage of the sample at ambient temperature for at least three months. All samples were processed within the timeframe suggested by the manufacturer's instruction. DNA extraction from stool samples was performed with PSP Spin Stool DNA Kit Plus (Stratec Molecular). Stool samples were lysed under denaturing conditions at 95 °C and centrifuged for removing PCR inhibitors and cell debris contained in the feces. Samples were then treated with Proteinase K at 70 °C, and DNA was purified through a spin column system, eluted and quantified using a NanoDrop spectrophotometer ND1000 (Termofisher). 16S rRNA amplicon (V3–V4 regions) sequencing analysis was performed using an Illumina MiSeq 2x300bp.

Raw sequencing data (forward and reverse reads) were merged using PEAR 0.9.6, with a minimum overlap of 100 base pairs (bp) [18]. Merged sequences were quality-checked and trimmed using Trimmomatic 0.36, discarding sequences with an average quality below 30 and a final length below 400 bp [19]. Reads were analyzed using QIIME 1.9.1 [20] and clustered in Operational Taxonomic Units (OTUs) using Usearch 6.1 and GreenGenes 13.8 at 97% similarity, removing low frequency OTUs [21]. QIIME was used to build a phylogenetic tree using a midpoint approach to choose the tree root.

2.3. Statistical analysis

Statistical analyses were performed with R 3.4.4, using phyloseq 1.22.3 and vegan 2.4–5. In our analysis we included all variables that were potentially associated with the outcome (predictors) and those that were likely associated both with the outcome and each predictor (confounders). We evaluated the effect of our target predictors both marginally (through non-parametric testing) and conditionally on confounders (through a general linear model (GLM)). More details on statistical methods are given below. The GLM multivariate approach, alongside marginal comparisons, allowed us to evaluate the effects of target predictors as if groups were balanced with respect to confounders.

We collected information on 21 variables common to Parkinson's Disease patients and Healthy Controls (HC), testing the association with the PD status (PD vs HC) using a univariate logistic regression model. Variables with $p < 0.05$ were considered as potential confounders.

Only OTUs present in at least 10% of samples were analyzed. Taxa were first tested using the Wilcoxon-Mann-Whitney test, as discussed above. Samples were normalized using DESeq2 [22]. α-Diversity was computed at the species level using three metrics (Chao1, Shannon, Simpson). β-Diversity was computed using four metrics: Bray-Curtis and weighted, unweighted and generalized Unifrac metrics [23]. Microbiota predictors were identified using the PERMANOVA test with 9999 permutations, assessing the marginal effect of each variable. For all the four metrics, predictors were identified using a stepwise regression through manual backward elimination. Variables statistically significant in at least one metrics were inserted in the model as predictors. Differential abundance of each taxon (families and genera) was evaluated using a generalized linear model. We defined a "full" GLM that describes a taxon using all identified predictors and confounders:

$$\text{Taxon} \sim \text{PD-status} + \text{Sex} + \text{Age} + \text{Loss\_5 kg} + \text{BMI} + \text{Gain\_5 kg} + \text{Yoghurt} + \text{Physical\_Activity}$$

Data were fitted using five distributions: negative binomial, zero-inflated negative binomial, Poisson, zero-inflated Poisson and hurdle distribution; using R packages: stats 3.4.4, MASS 7.3–50 and pscl 1.5.2. For each taxon, the model with the lowest Bayesian information criteria score was selected.

For each taxon we created a "nested" GLM, using all predictors and confounders, except PD-Status:

Table 1
Differential abundance analysis on bacterial families.

Phylum	Family	(A) PD cases vs HC				(B) PD cases (excluding iCOMT users) vs HC			
		Pc-GLM	Pc-WC	Abundance (%)		Pc-GLM	Pc-WC	Abundance (%)	
				PD	*HC*			*PD*	*HC*
Proteobacteria	Enterobacteriaceae	3.15E-04	4.45E-04	4.81	1.39	4.14–03	1.51E-03	4.78	1.39
Firmicutes	Lactobacillaceae	3.69E-03	3.02E-03	1.10	0.30	1.61E-03	5.83E-03	1.04	0.30
Firmicutes	Enterococcaceae	4.36E-03	4.98E-03	0.06	0.03	4.48E-02	6.99E-02	0.04	0.03
Firmicutes	Lachnospiraceae	8.30E-03	1.34E-02	11.53	15.67	2.29E-02	2.77E-02	11.83	15.67

(A) PD cases vs HC, only bacterial Families with a significative p-values detected by two methods (Generalized Linear Models and Wilcoxon-Mann-Withney test) are reported.
(B) PD cases excluding patients taking iCOMT vs controls. Pc-GLM = p-value corrected for multiple testing using the GLM approach; Pc-WC = p-value corrected for multiple testing using the Wilcoxon-Mann-Withney test; %Abundance = relative abundance of taxa in PD and HC samples.

Taxon ~ Sex + Age + Loss_5 kg + BMI + Cereals + Gain_5 kg + Physical_Activity

We compared full and nested GLMs with the anova function in R and corrected the p-values for multiple testing using the Benjamini-Hochberg procedure. Only taxa confirmed by both GLM and the Wilcoxon-Mann-Whitney test were reported and discussed. The effect of PD clinical features (PD phenotype, medications, and disease duration) in patients' microbiota were tested using the PERMANOVA test with 9999 permutations.

2.4. Functional analysis

An OTU normalized by DESeq table was used to infer microbiota metabolic functions using PICRUSt [24]. The table included OTUs only from differentially abundant families between PD and HC. OTU tables were normalized by copy number; functions were predicted using KEGG orthologs, grouped at level 3. Differences between PD and HC were tested using DESeq2 1.18.1 [25] and p-values were corrected for multiple testing using Benjamini-Hochberg. Only metabolic pathways were analyzed. Pathways were considered significant when the corrected p was < 0.05 and the differences in mean proportion was > 0.02.

3. Results

3.1. Study cohort and confounders

The analyses were carried out on 80 PD patients and 72 healthy controls (HC). Table S1 shows the list of variables used to describe each

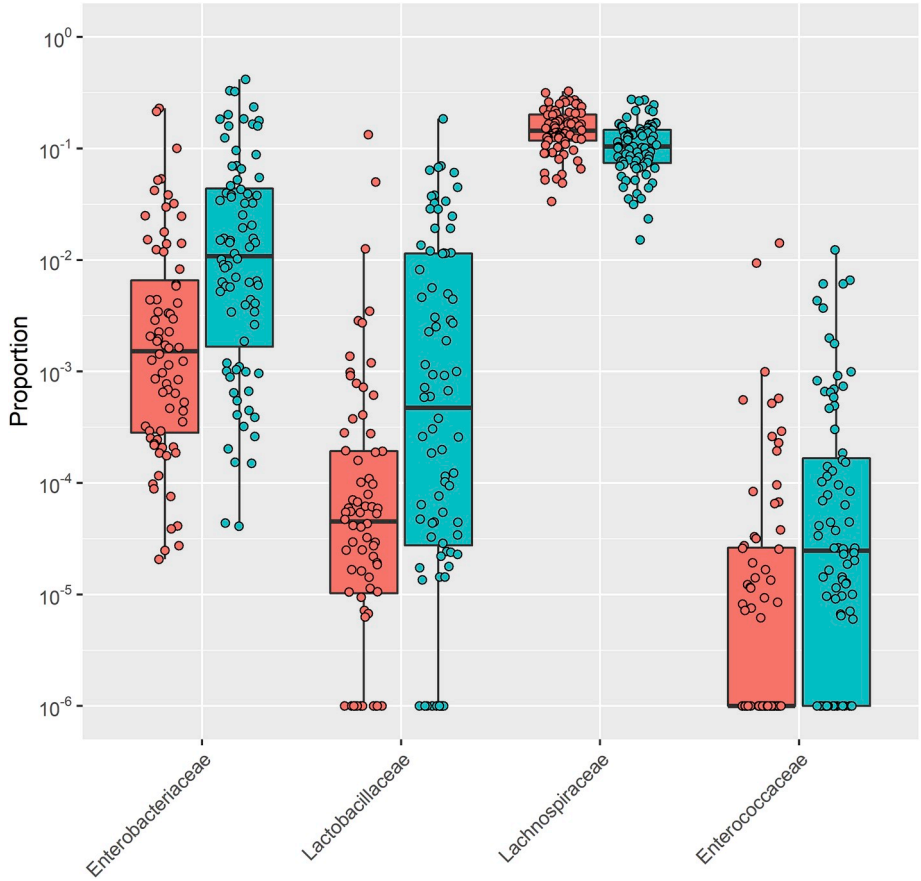

Fig. 1. Bacterial families in feaces of PD patients (cyan) compared to HC (pink). The relative abundance is plotted in log10 on the y-axis. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

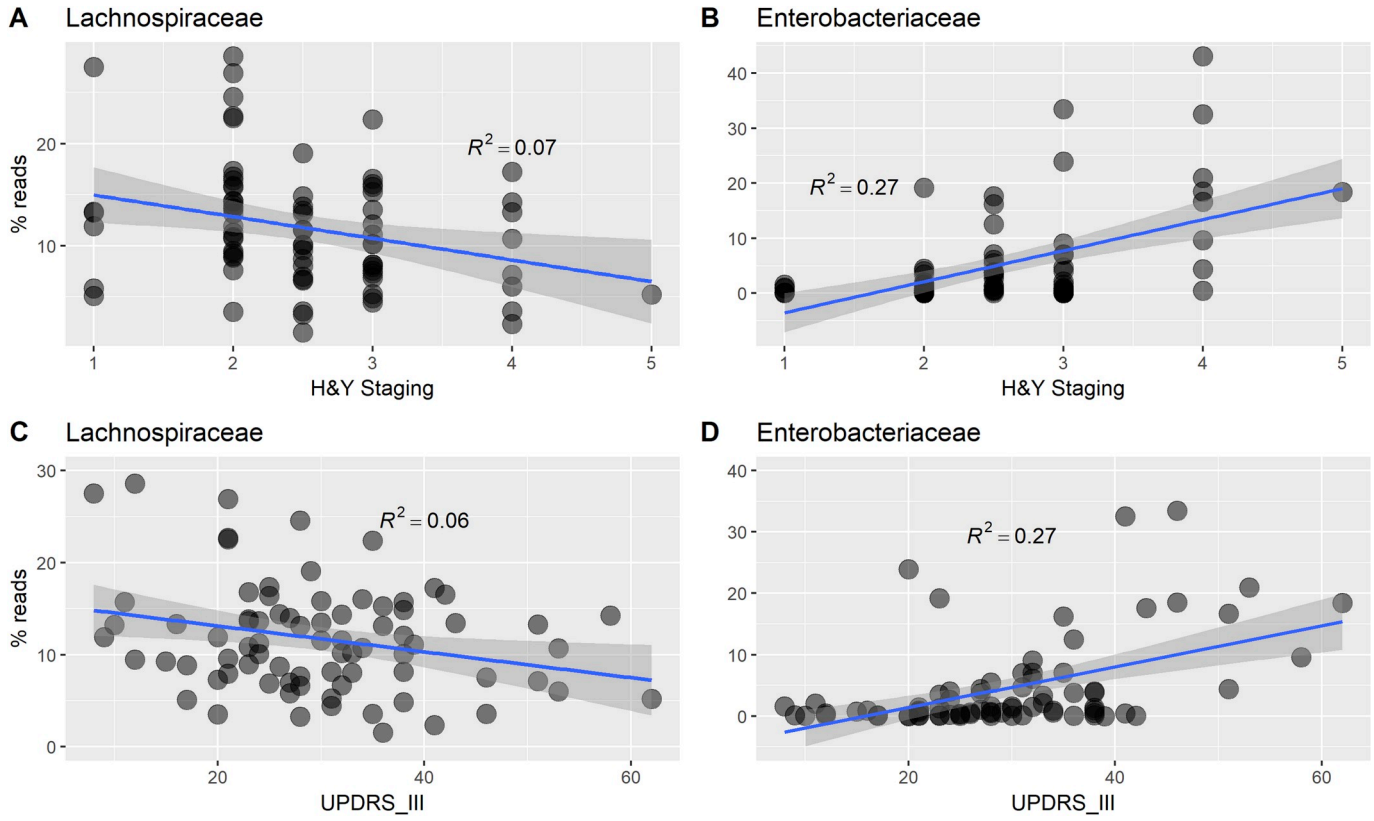

Fig. 2. Plot of the frequency of *Lachnospiraceae* and *Enterobacteraceae* families as a function of the staging, expressed with the Hoehn and Yahr scale (A,B) and motor impairment, expressed with the UPDRS part III score (C,D). The straight line is a fit through a regression analysis showing a decreasing and an increasing trend for *Lachnospiraceae* and *Enterobacteraceae* families respectively. The models are statistically significant (*Lachnospiraceae* p-value < 0.005, *Enterobacteraceae* p-value < 0.005).

participant. A specific anamnestic interview was provided to each participant, to gather information about their life and diet habits, since the microbiota can be affected, beside pathological status, also by environmental factors. Three variables, namely "Age," "Sex" and "Lost 5 kg in the last year", were unbalanced between PD and HC, and were therefore considered possible confounders. Controls were 3.6 years younger than patients (p = 0.0166); men were prevalent in PD cases and women in HC cases. Sex skewness was included as a possible confounder although the p is slightly higher than the threshold (p = 0.0626). Finally, PD patients reported more weight loss (p = 0.0003).

3.2. Sequencing data and diversity of fecal microbiota

Sequencing of 152 fecal samples provided 27,631,096 reads, that were taxonomically assigned. The dataset included bacteria from 2809 OTUs, 204 species, 106 genera, 57 families, 36 orders, 25 classes, and 14 phyla. No statistically significant differences between PD and HC were found for the three alpha diversity indices (p = 0.1725 for Chao1, p = 0.7343 for Shannon, p = 0.9779 for Simpson).

A PERMANOVA test, considering only PD-status as a grouping variable, indicated that PD-status was a statistically significant predictor of gut microbiota composition for all the metrics (Table S2A). Another PERMANOVA test showed that the microbiota difference between PD and HC was still significant when considering all the variables reported in Table S1. The analysis, carried out following the procedure reported in Supplemental Tables S3, S4, S5 and S6, showed that the PD-status was statistically significant even when considering all the possible variables and independently of the applied metrics. Variables describing PD medications and clinical features were not included in this analysis. The analysis identifies five variables as significant predictors that were predicted by at least one metrics (Table S2B).

3.3. Differential abundance analysis of PD and HC taxa

The role of PD status on taxa abundance was evaluated using the Wilcoxon-Mann-Whitney test and comparing two generalized linear models: 1) the "full" model based on a linear combination of the PD status and confounders and predictors, and 2) the "nested" model based on a linear combination excluding the PD-Status.

Table 1 and Fig. 1 show the frequencies of bacterial families showing significative differences between PD and HC, as confirmed by the two statistical methods. In Table 1A all the patients are considered, while in Table 1B the patients taking catechol-O-methyl transferase (COMT) inhibitors are excluded from the analysis, since assumption of COMT inhibitors affects the microbiota composition (Table S2B). Levels of *Lactobacillaceae*, *Enterobacteriaceae* and *Enterococcaceae* families are higher in feces of PD patients compared to HC controls, while *Lachnospiraceae* were reduced.

Table S7A and Fig. S1 show the frequencies of bacterial genera with the most substantial differences between PD and HC. *Citrobacter*, *Enterococcus*, *Lactococcus*, *Klebsiella*, *Salmonella*, *Shigella* and an unclassified *Enterobacteriaceae* were more abundant in PD, whilst the *Roseburia* genus was more abundant in HC (Table S7A). However, the differences between PD and HC observed for *Citrobacter, Shigella, Enterococcus, Salmonella* and *Roseburia* becomes not significant after excluding the eight patients taking COMT inhibitors (Table S7B).

3.4. Gut microbiota in PD patients

Table S2C shows that disease duration, staging and PD medications affect the microbiota composition, whilst no significant differences

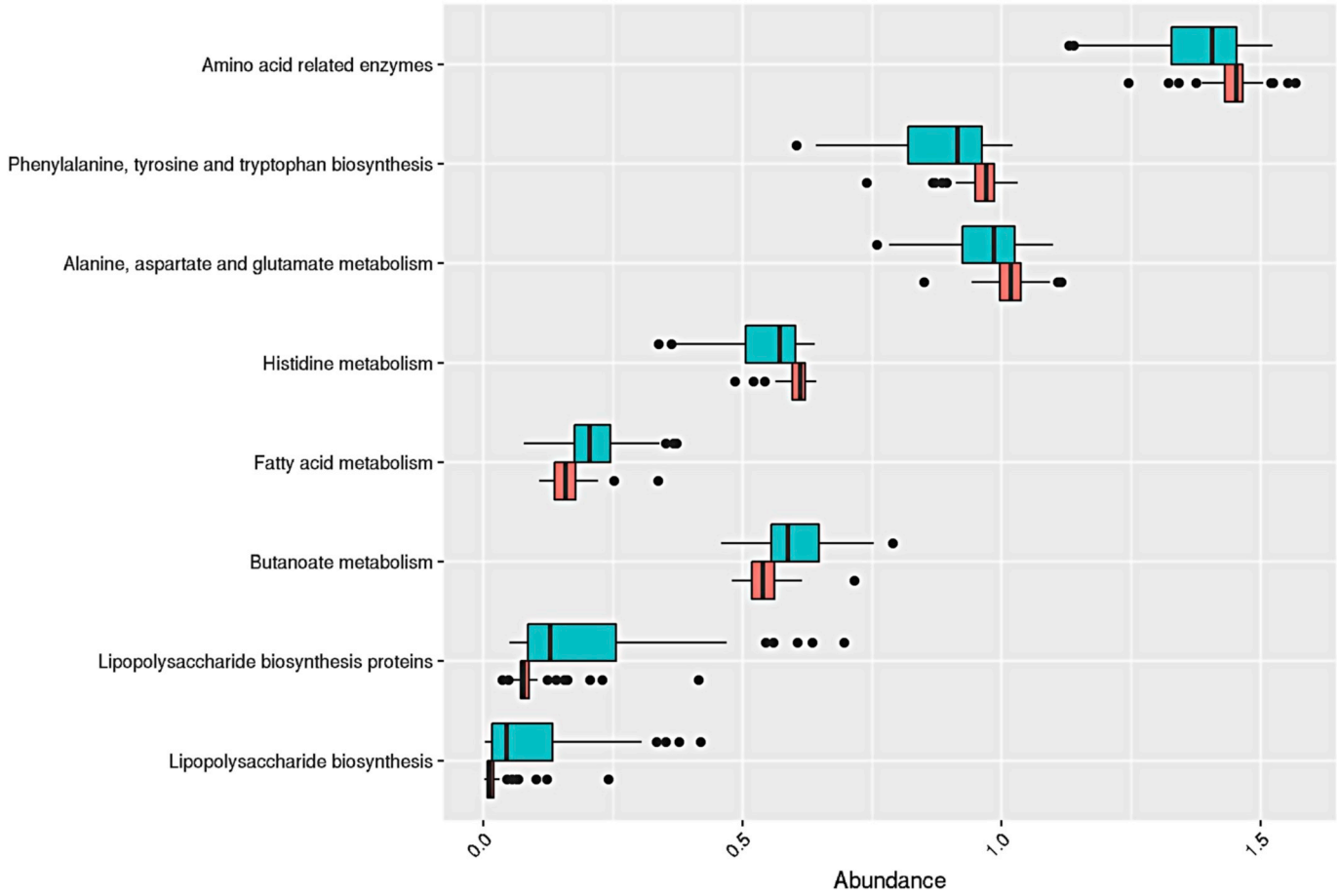

Fig. 3. Main Biochemical pathways differing in PD (cyan) and HC (pink) cases. Only metabolic pathways at KEGG hierarchical level 1 were investigated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were observed among the different PD phenotypes.

A regression analysis, carried out to examine the correlation between the frequency of the bacterial families and the severity of the disease (H&Y staging) showed a decreasing trend for *Lachnospiraceae* and an increasing trend for *Enterobacteriaceae* (Fig. 2A–B). In line with these results, *Lachnospiraceae* and *Enterobacteraceae* show the same trend for the motor impairement (UPDRS III scale) (Fig. 2C–D). These results are in agreement with the findings that *Lachnospiraceae* and *Enterobacteriaceae* levels are, respectively, reduced and increased in PD in comparison to HC samples (Table 1).

Table S2C also shows that the disease duration significantly affects the whole microbiota composition, nevertheless the regression analysis does not show any significant effect on single bacterial families.

The PERMANOVA analysis used to identify the clinical features affecting the PD patients gut microbiota is reported in Supplemental Tables S8, S9, S10 and S11.

3.5. Functional prediction

The bacterial families with altered abundances between PD cases and HC were analyzed using PICRUSt, to identify the differences in the functional pathways (Fig. 3). Pathways involved in amino acid metabolism, such as the biosynthesis of phenylalanine, tryptophan and tyrosine, were reduced in PD samples, as well as those involved in the metabolism of alanine, aspartate, glutamate and histidine. The biosynthesis of lipopolysaccharides and lipopolysaccharide proteins, as well as the metabolism of fatty acids and butanoate, were increased in PD patients.

4. Discussion

The connection between gut microbiota and Parkinson's disease was analyzed comparing 80 PD and 72 HC samples by 16S rRNA gene sequencing using a statistical approach to detect potential confounders and/or predictors. We detected a significant effect for PD and identified age, loss of weight and sex as confounders, and Body Mass Index and “eat cereals”, “gain of weight” and “physical activity” as predictors. The α-Diversity was not affected by the pathological status. We have not found significant correlations with specific motor phenotypes (Table S2).

The abundance of several taxa differed between PD cases and controls. *Enterobacteriaceae*, *Lactobacillaceae* and *Enterococcaceae* families were more abundant in PD samples whilst *Lachnospiraceae* were less abundant in PD samples (Fig. 1, Table 1A), confirming results found in other studies [2,8,10,12,14,27]. Notably, analysis carried out excluding patients who take COMT inhibitors reduces the significance of *Enterococcaceae* when using the Wilcoxon-Mann-Whitney test suggesting that its abundance can be modulated by iCOMT assumption.

Interestingly, patients with severe PD show a decrease in *Lachnospiraceae* and an increase in *Enterobacteriaceae* abundance, (Fig. 2A–D), suggesting that the frequencies of these two families are correlated to disease progression and motor impairment, although additional data are needed in order to confirm this hypothesis.

Results concerning the levels of *Lactobacillaceae* and *Enterococcaceae* in PD are conflicting. For *Lactobacillaceae,* we found an increased level in PD patients compared to HC, in accordance with other studies [7–10], but one study reports a decrease in PD patients [27]. Concerning the *Enterococcaceae,* we found increased levels in PD patients, as reported in two previous studies [9,12], differently from a study

reporting reduced levels in PD patients [27]. We observed an increased level of the *Verrucomicrobiaceae* family in PD patients, but only using the GLM approach (data not shown), in line with previous studies [2,7,8,10,15].

Several genera are altered between PD patients and controls (Table S7A). The significance of five genera (*Citrobacter*, *Shigella*, *Enterococcus*, *Salmonella* and *Roseburia*) dropped when iCOMT users were excluded from the analysis, suggesting that the abundance of these genera is influenced by the drug. A debated genus is *Prevotella,* found at lower levels in PD in some studies, but not in others [7,10,13]. We do not find any decrease of *Prevotellaceae* and *Prevotella* in PD. A possible explanation for the different results may be attributed to differences in inclusion criteria or different dietary habits since *Prevotella* is extremely sensitive to fiber intake [28,29].

The functional prediction analysis revealed that pathways involved in the synthesis of lipopolysaccharide (LPS), an endotoxin produced by Gram-negative bacteria, are higher in PD versus HC (Fig. 3). This association is likely due to an increase in the level of *Enterobacteriaceae*, associated with gut inflammation in various diseases [30]. LPS disrupts the homeostasis, inducing the production of pro-inflammatory cytokines that can enter the bloodstream and reach the blood-brain barrier, leading to neuroinflammation and neuronal death [2,6,27]. The prediction analysis revealed an increase in fatty acid and butanoate metabolism in PD, likely correlated to a reduction of the SCFA-producing *Lachnospiraceae* family (Fig. 1). Interestingly, SCFAs, in particular butanoate, have a beneficial effect on gastrointestinal motility and in the maintenance of the intestinal barrier integrity and homeostasis [31].

Overall, these results suggest that the alteration of the composition of the microbiota is associated with a pro-inflammatory environment in the gastrointestinal tract. Accordingly, a recent study that analyzes stool samples from 156 individuals with Parkinson's diseases revealed the presence of higher levels of inflammatory factors, indicating gastrointestinal inflammation in PD [32]. Whether the inflammation leads to the alteration of the composition of microbiota or vice versa is still a debated question.

Interestingly, functional prediction analysis indicates a lower level of genes involved in the biosynthesis of phenylalanine, tryptophan and tyrosine, precursors of serotonin, dopamine, and norepinephrine neurotransmitters in PD. This finding deserves further attention due to the essential role of these neurotransmitters in the communication between gut and brain.

5. Conclusion

This work explores the microbiota composition of PD in comparison to HC subjects. We identified families and genera altered in PD patients. The main altered families are in line with the results of studies from other countries, although our samples were collected only in Central Italy.

The altered families are involved in gut inflammation as suggested by the functional analysis. This result is in line with a recent work measuring the levels of inflammatory factors in stool samples of PD patients [32].

It is interesting that patients affected by immune-mediated inflammatory diseases, such as multiple sclerosis, show altered bacterial families and genera, which differs from those identified in PD patients, although inflammation appears to be a common symptom [33].

Competing interest

None.

Funding

This work was partially supported by “Progetto Regione Lazio NEUROMICRO” to AD and by Fondazione Baroni to NBM and AS.

Author roles

1) a) conception and design of the study; b) acquisition of data; c) DNA extraction and sequencing; d) bioinformatic analysis; e) Statistical analysis; f) Analysis and interpretation of data.
2) a) drafting the article; b) revising the article;
3) a) final approval to the version to be submitted;

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M Pierantozzi: 2b),3a)
NB Mercuri: 2b),3a)
S Biocca: 1a),2b),3a)
A Stefani: 1a),2b),3a)
A Desideri: 1a),2b),3a)

Research data

The dataset will be released in the NCBI's Sequence Read Archive repository (PRJNA510730) on date 06-30-2019.

Acknowledgements

We acknowledge Davide La Rocca for helping in data processing and Professor Jens Z. Pedersen for his linguistic consultancy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2019.06.003.

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