Oral dysbiosis and inflammation in Parkinson's disease

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Supplementary Material

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Supplementary Material A

Methods

Inclusion criteria

Selection criteria were a diagnosis of PD based on United Kingdom Parkinson's Disease Society Brain Bank Criteria [1] and a PD diagnosis made after 50 years of age to minimize the chance of recruiting patients with monogenic parkinsonism.

Exclusion criteria

Exclusion criteria included active smoking, extensive periodontal therapy within the last month, oral, respiratory or digestive diseases within the last 3 months, presence of full dentures or removable dental prosthesis or presence of less than 16 natural teeth, dementia, major active pathologies, any drug abuse, antibiotic treatment within the last 3 months, use of probiotics within the last month, radiation therapy within the last 6 months, oral antiseptic use within the last month, presence of a first degree relative with PD or more than one relative (any degree) with PD.

Controls were excluded if they presented with symptoms associated with premotor PD, such as hyposmia (Hyposmia Rating Scale score <24/30) [2] and Rapid Eye Movement (REM) sleep Behavior Disorder (RBD) diagnosis (RBD-Screening Questionnaire >4/13) [3].

Analysis of oral microbiota

The bacterial community composition in the saliva and the subgingival fluid were assessed using the culture-free approach.

DNA extraction

DNA was extracted using Extract-N-Amp Plant PCR Kit (Sigma-Aldrich). Forty µL of Extract-N-Amp Plant Extraction solution was added to the tube containing paper points. The tubes were briefly vortexed, spun down and incubated at 95°C for 10 min. After adding 40 µl of Extract-N-Amp Plant Dilution solution to stop the reaction, the samples were vortexed and spun down. Thawed saliva samples were homogenized by vortexing. Twenty-five µL saliva were mixed with 100 µL Extract-N-Amp Plant Extraction solution, briefly vortexed, spun down and incubated at 95°C for 10 min. The reaction was stopped by adding 100 µL Extract-N-Amp Plant Dilution Solution, brief vortex-mixing and spinning down. All extracts were stored at –20°C for a maximum of one week. Three negative extraction controls (NECs) were processed in parallel with clinical samples by omitting the addition of biological material in Extract-N-Amp Plant PCR Kit reagents (NEC for saliva samples); two additional NECs also included the paper points (NEC for plaque samples).

qPCR assay

Bacterial load was determined by qPCR experiment targeting the V3 region of the bacterial 16S rRNA gene as described previously [4], using 1 µL of 10-fold diluted DNA extracts. One pg of *Escherichia coli* strain DH5-alpha, used to construct a reference curve, corresponds to 1,493 copies of the 16S rRNA gene.

Amplicon sequencing

The V3–4 region of the bacterial 16S rRNA genes (*E. coli* positions 341-805) was amplified using 5 µL of DNA extract in a 20 µl volume of KAPA 2G Robust HotStart ReadyMix containing each of 0.4 µM forward primer 341F 5’-CCTACGGGNGGCWGCAG-3’ and reverse primer 805R 5’-

GACTACHVGGGTATCTAAKCC-3'. The PCRs were carried out with an initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 60 s, and a final extension at 72°C for 10 min. Duplicate PCRs of each sample were combined and run on a 2100 Bioanalyzer (Agilent Technologies) for quality analysis and quantification. The amplicon barcoding/purification and construction of the sequencing library was performed as described previously [5]. The sequencing was carried out for 2 × 300 cycles on an Illumina MiSeq instrument using MiSeq v3 Reagent Kit at LGC Genomics (Berlin, Germany).

Sequencing data were submitted to the European Nucleotide Archive (ENA; http://www.ebi.ac.uk/ena) under study number PRJEB39870.

Sequence analysis

Paired reads were quality filtered and joined using PEAR (-m 470 -n 390 -t 150 -v 10 -q 33 -p 0.0001 -u 0) [6]. Merged sequence reads were clustered into zero-radius operational taxonomic units (zOTUs) using UNOISE3 [7] from USEARCH v.10.0.240, which resolves differences of as little as one nucleotide and provides taxonomic resolution superior to conventional 97% OTUs [8]. This pipeline also removes out putative chimeric sequences.

From the sample dataset we removed zOTUs matching any of the following criteria: 1) presented <90% identity to reference EzBioCloud 16S database [9] sequences (downloaded on the 19 August 2019) as revealed by USEARCH [10] (-id 0.90 -query_cov 0.99); 2) were represented by ≤10 counts; and 3) had an R-OTU [5] value >0.1 in saliva and/or plaque dataset. zOTUs were classified using EzBioCloud 16S database via MOTHUR's [11] command classify.seqs (method=wang cutoff=80).

Ecological indices

Ecological indices (diversity, richness) were calculated from the relative abundance of zOTUs in PRIMER.

Supplementary Material B. Comorbidities and medications

	Percentage of PD patients	Percentage of healthy controls	*p* *
Comorbidities			
Diverticulosis (asymptomatic)	5	0	1
Myocardial infraction	10	0	0.487
Coronary artery disease	5	0	1
Hypercholesterolemia	10	10	1
Arterial hypertension	15	20	1
Cancer (treated)	20	5	0.342
Osteoporosis	10	10	1
Pulmonary embolism	5	0	1
Heart arrhythmia	10	0	0.487
Endometriosis	5	0	1
Prostatic hyperplasia	5	0	1
Ankylosing spondylarthritis	5	0	1
Migraine	10	5	1
Bipolar disorder	0	10	0.487
Hypothyroidism (controlled)	0	10	0.487
Congenital cardiomyopathy	0	5	1
Autoimmune disease	5	20	0.342
Diabetes	0	5	1
Medication			
Levodopa	80	0	**<0.001**
Dopamine agonist	60	0	**<0.001**
COMT inhibitor	20	0	0.106
MAO inhibitor	30	0	**0.020**
Amantadine	15	0	0.231
Anticholinergic	15	0	0.231
Beta-adrenergic receptor agonist	5	0	1
Selective serotonine reuptake inhibitor	10	5	1
Benzodiazepine	25	5	0.182
GLP-1R agonist	5	0	1
Statin	20	10	0.661
Acetylsalicylic acid, clopidogrel	15	5	0.605
Anticoagulant therapy	20	0	0.106
Tadalafil	5	0	1
Tamsulosin	5	0	1
Beta-blocker	20	10	0.661
Antihypertensive treatment	20	15	1
Laxatives	5	10	1
Bisphosphonates, calcium, vitamin D	15	20	1
Proton pump inhibitor	5	0	1
Antiepileptic drugs	0	5	1
Thyroxine	0	15	0.231
Antiinflammatory	0	5	1
Raloxifene	0	5	1
Anti-histaminic drug	0	5	1
Anti-diabetes drug	0	5	1
Neuroleptic	0	5	1
Lithium	0	5	1

* Obtained using Fisher's exact test

Supplementary Material C

16SrRNA gene amplicon dataset

Sequencing of the 16S rRNA gene amplicons from patients and controls' saliva and plaque samples generated 16,365,221 row reads. After quality filtering steps, 12,889,410 reads remained, with a median of 163,134 reads (range 35,030–282,491) per sample.

Supplementary Material D

Comparisons between saliva and plaque samples composition

PERMANOVA test showed that differences in community profiles between the saliva versus dental plaque samples were statistically significant (p <0.0001). The 20 zOTUs with an average relative abundance >2% in at least one of the two sample types were significantly differentially abundant (Supplementary Table 1).

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Supplementary Figure 1. Bacterial community composition according to the type of sample (saliva vs. dental plaque) using a Principal Coordinates Analysis of Bray-Curtis similarity matrices. Saliva and plaque samples were clearly separated in the first two PCo axes. The visualization within the first two PCo axes explained 27.1% of the difference in bacterial composition.