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TITLE: Interactions of Gut Microbiome, Genetic Susceptibility, and Environmental Factors in Parkinson's Disease

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14. ABSTRACT Genetic and environmental factors explain a fraction of Parkinson's disease risk, prompting the question if the microorganisms in the gut hold the trigger. Attempts to identify PD-associated microorganisms have produced inconsistent results. It is unknown if low reproducibility can be overcome by rigorous study design or its unsurmountable due to dynamic nature of microbiome. Our aim was to determine if it is possible to attain robust and reproducible association signal with gut microorganisms. If so, to identify the microorganisms responsible for the dysbiosis in Parkinson's. We adopted standards of rigor from GWAS, used two datasets for validation, sequenced 16S rRNA V4-region using DNA from stool, investigated 46 potential confounders, conducted microbiome-wide association study with two methods (ANCOM, Kruskal-Wallis), followed by co-occurrence network analysis to infer interactions. 15 genera were associated with Parkinson's at microbiome-wide significance level, in both datasets, with both methods, with or without covariate adjustment. The associations were not independent, rather represented 3 polymicrobial clusters. Cluster 1 was composed of opportunistic pathogens; all were elevated in PD. Cluster 2 were short-chain-fatty-acid producing bacteria; all were reduced in PD. Cluster 3 were carbohydrate-metabolizer probiotics; elevated in PD. In conclusion, robust, reproducible results are attainable. Overabundance of pathogens in PD gut is a novel finding and their identity provides the lead to experimentally test their role in triggering disease.

15. SUBJECT TERMS
Parkinson disease, gut microbiome, opportunistic pathogens, short-chain fatty acids, probiotics.

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- **INTRODUCTION:**

Parkinson's disease (PD) is a common, progressive and debilitating disease which currently cannot be prevented or cured. Numerous genetic and environmental risk factors have been identified but none is sufficient to cause disease, not individually, not in combination, and not in interaction. There must be more to gene-environment interaction in PD than the human genome and the environmental factors that we know of. This proposal is based on new evidence that has revealed the gut microbiome is a key player in pathogenesis of PD. The project will identify the microbial pathways that contribute to PD pathogenesis, and identify the specific microorganism that interact with genetic and environmental risk factors.

- **KEYWORDS:**

Parkinson's disease, gut microbiome, genetics, environmental factors

- **ACCOMPLISHMENTS:**
  - **What were the major goals of the project?**

See next page.

Major Goals	Timeline in months	% Complete
Aim 1: Delineate the dysbiosis of microbiome in PD		
Major Task 1: Enrollment of 500 PD cases and 300 controls		
Subtask 1: Identifying potential subjects in the clinic. 500 PD cases and 300 controls	1-20	79%
Subtask 2: Enrolling subjects. 500 PD cases and 300 controls • Consent • Blood draw in clinic, • Collecting Environmental Exposure Questionnaire, • Give subjects stool collection kit, go over the instructions on collecting stool and filling out Gut Microbiome Questionnaire and mailing them back.	1-20	• Consent 79% • Blood, saliva 63% • Returned EEQ 54% • Returned stool & GMQ 54%
Major Task 2: Specimen/Data processing 500 PD cases and 300 controls		
Subtask 1: Extracting DNA at UAB Core. 500 PD cases and 300 controls • Human DNA and plasma from blood. 500 PD cases and 300 controls • Microbiome DNA from stool. 500 PD cases and 300 controls	1-20	11% Human DNA
Subtask 2: Banking serum, PAXgene, and DNA in -80 freezers. 500 PD cases and 300 controls	1-20	79%
Subtask 3: Data entry 500 PD cases and 300 controls • Environmental Exposure Questionnaire. 500 PD cases and 300 controls • Gut Microbiome Questionnaire. • 500 PD cases and 300 controls • Data extracted from medical records. 500 PD cases.	1-20	
Major Task 3: Data generation. 500 PD cases and 300 controls		
Subtask 1: Genotyping will be done in two batches at the end of year 1 and year 2 at Genotyping Core of HudsonAlpha Inst. For Biotechnology. 250 PD cases, 150 controls in batch 1 (month 11-13), and 250 PD cases, 150 controls in batch 2 (month 21-23)	11-13 and 21-23	
Subtask 2: Sequencing microbiome will be done in two batches at the end of year 1 and year 2 at Sequencing Core of HudsonAlpha Inst. for Biotechnology. 250 PD cases, 150 controls in batch 1 (month 11-13), and 250 PD cases, 150 controls in batch 2 (month 21-23)	11-13 and 21-23	
Subtask 3: QC Approximately 250 cases, 150 controls in batch 1 (month 11-13), and 250 cases, 150 controls in batch 2 (month 21-23), and all data combined 500 cases and 300 controls. • Genotypes • Sequences	14-16 and 24-26	
Major Task 4: Data analysis Dataset 1= 539 PD cases, 307 controls Dataset 2= 500 PD cases, 300 controls Meta analysis =1039 PD cases, 607 controls.		
Assemble exposure, genotype and microbiome data, assemble covariate and confounder data, conduct bioinformatics, and statistics analysis on dataset 1 (enrolled prior to this study, data will be analyzed here), dataset 2 in two batches genotyped in year 1 and 2, meta-analysis of dataset 1 and 2. Dataset 1= 539 PD cases, 307 controls Dataset 2= 500 PD cases, 300 controls Meta analysis=1039 PD cases, 607 controls.	1-24	Dataset 1 completed.
Milestone #1: Co-author manuscript on in-depth characterization of dysbiosis of microbiome in PD	24	

Major Goals	Timeline in months	% Complete
Aim 2: Investigate the interaction of the microbiome with the host genome Dataset 1= 539 PD cases, 307 controls Dataset 2= 500 PD cases, 300 controls		
Major Task 1: Hypothesis testing (data were generated, cleaned, and assembled in Aim 1) Dataset 1= 539 PD cases, 307 controls Dataset 2= 500 PD cases, 300 controls		
Subtask 1: Metagenome-wide interaction test with each of 28 PD susceptibility loci, starting with the highest GWAS peak (SNCA) and working down the list sequentially, in dataset 1. Dataset 1= 539 PD cases, 307 controls Subtask 2: Replicate in dataset 2 Dataset 2= 500 PD cases, 300 controls Subtask 3: delineate the replicated findings at species/strain level Dataset 2= 500 PD cases, 300 controls Subtask 4: discern gene function and pathway Dataset 2= 500 PD cases, 300 controls Subtask 5: use two datasets explore microbiome x host genome interaction both genome-wide Datasets 1 and 2= 1039 PD cases, 607 controls.	25-36	
Milestone #2: Co-author (several) manuscripts on the interaction of microbiome and host genome on PD	36	
Aim 3: Investigate the interaction of the microbiome with the environmental risk factors Dataset 1= 539 PD cases, 307 controls Dataset 2= 500 PD cases, 300 controls		
Major Task 1: Hypothesis testing with smoking and caffeine (data were generated, cleaned, and assembled in Aim 1) Dataset 1= 539 PD cases, 307 controls Dataset 2= 500 PD cases, 300 controls		
Subtask 1: test effects of smoking/caffeine on microbiome in dataset 1 Dataset 1= 539 PD cases, 307 controls Subtask 2: conduct metagenome-wide interaction test with smoking/caffeine in dataset 1, Dataset 1= 539 PD cases, 307 controls Subtask 3: Replicate in dataset 2 Dataset 2= 500 PD cases, 300 controls Subtask 4: delineate the replicated findings at species/strain level Dataset 2= 500 PD cases, 300 controls Subtask 5: discern gene function and pathway Dataset 2= 500 PD cases, 300 controls	37-42	
Milestone #3: Co-author manuscripts on the interaction of smoking, microbiome and PD	36	
Milestone #4: Co-author manuscripts on the interaction of caffeine, microbiome and PD	36	

Major Goals	Timeline in months	% Complete
Aim 4. Develop a microbiome-based predictive biomarker 1039 PD cases, 607 controls, 100 RBD cases		
Major Task 1: Enrollment of 100 RBD cases		
Subtask 1: Identifying and enrolling subjects with RBD without PD or neurological symptoms 100 RBD cases • Consent • Blood draw in clinic, • Collecting Environmental Exposure Questionnaire, • Give subjects stool kit, go over instructions on collecting stool and filling out Gut Microbiome Questionnaire and mailing them back to the attending physician • Send questionnaires and specimen to Payami lab Send data on conversion to PD to Payami lab	1-48	10%
Major task 2: Specimen/Data processing (as in aim 1) 100 RBD cases	1-48	10%
Major task 3: Data generation (as in aim 1) 100 RBD cases		
Subtask 1: genotyping (as aim 1) 100 RBD cases Subtask 2: sequencing (as in aim 1) 100 RBD cases Subtask 3: QC (as in aim 1) 100 RBD cases	42-45	
Major task 4: Data analysis 100 RBD cases – using results from 1039 PD cases, 607 controls	45-48	
Milestone #5: Co-author manuscript on microbiome/RBD/PD	36	

- **What was accomplished under these goals?**

Enrollment. We have enrolled 639 new subjects (481 PD cases+148 controls+ 10 RBD) under DoD protocol that will constitute Dataset 2 when completed. We are ahead of targeted enrolment for PD. We are behind on the targeted enrollment for controls, because many patients are not bringing a spouse to their clinic visit. To expand our reach for controls beyond spouses in clinic, we obtained IRB approval to recruit from outside clinic. We have posted fliers at the University and have begun to get volunteers. We are behind in RBD enrolment because the Montreal site is still awaiting for HRPO approval to begin enrolment. 30% of subjects who consent to study and take the packet home do not return the stool sample, despite emphasis at clinic and follow-up calls. Hence we need to enroll larger numbers to meet the targeted goal with complete data.
Data analysis The raw data for Dataset 1 was in hand at start of this grant and was planned to get cleaned and analyzed as part of this grant. 16S rRNA sequences and metadata for dataset 1 was interrogated for quality control. DATA2 was used for bioinformatic pipeline, and SILVA for taxonomic assignment. Global composition of microbiome was tested using PERMANOVA . Thirteen methods for differential abundance testing were assessed and two were chosen for

analysis. Population structure was detected using principal component analysis. Covariates were interrogated as potential confounders by (a) inclusion as covariate in model and adjusting (b) stratified analysis.

Major findings. Within dataset 1, unexpected difference in gut microbiome was found between data collected in Alabama vs. data collected in Seattle, New York and Atlanta. Since this so called "population structure" is a well-known confounder for association studies, the geographic sites were kept separate and analyzed in parallel. Dysbiosis of gut microbiome in PD was evident and highly significant irrespective of geography and confounders. We detected overabundance of opportunistic pathogens and reduced levels of short-chain fatty acid producing organisms in the gut microbiome of persons with PD.

- **What opportunities for training and professional development has the project provided?**

Zachary Wallen is a graduate student working full time on this project. He is being personally trained by the PI. He has conducted bioinformatic and statistical analyses of dataset 1 outlined above. He attended and presented a poster at the 5th World Parkinson Congress, June 2019, Kyoto, Japan. The received a travel Award from the Congress; and his Abstract was in top 25% and chosen for additional evening poster tour.

- **How were the results disseminated to communities of interest?**

Nothing to report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Aim 1.

Major task 1:

- continue and complete enrolment of PD cases and controls

Major task 2:

- extract DNA from blood, saliva and stool
- Bank DNA
- Begin data entry of dataset 2

Major task 3:

- GWAS genotyping the first batch of dataset 2 (250 PD, 150 controls)
- Sequence metagenome the first batch dataset 2 (250 PD, 150 controls)

Major Task 4

- Data analysis

Reach Milestone 1:

- Publish a paper on characterization of the dysbiosis of gut microbiome in PD

Aim 4

Major Task 1

- Continue to enroll RBD subjects at UAB
- Begin enrolling RBD at Montreal site.

- **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

Nothing to report

- **What was the impact on other disciplines?**

Nothing to report.

- **What was the impact on technology transfer?**

Nothing to report

- **What was the impact on society beyond science and technology?**

Nothing to report

- **CHANGES/PROBLEMS:**
  - **Changes in approach and reasons for change**

Nothing to report

- **Actual or anticipated problems or delays and actions or plans to resolve them**

- Problem: We are behind in enrollment of controls. We had relied on generosity of spouses of patients, but many patients come to clinic alone, and many spouses are not willing to participate
- Corrective action: We have obtained IRB approval to post fliers at the University and the Hospital for healthy volunteers. We also obtained IRB approval and cooperation of memory disorder clinic clinicians to recruit spouses and caregiver.

- Problem: Up to 30% of individuals who agree to the study do not return the take home part which includes stool and questionnaires.
- Corrective action: We tried different strategies to increase compliance (see below) but we are stuck with 70% compliance, and the only way we can make up for non-compliance is to enroll more subjects than we projected to reach the projected numbers with complete data.

(1) We made an effort to emphasize in clinic during recruitment that only if they are willing and able to complete the take home part, especially the stool, they should enroll in study. They agree, yet 30% do not comply.

(2) For a period, we made follow-up calls with reminders, some say they forgot, promise to send it, but they don't. Some say they lost it, ask for another kit, we send them a replacement, and still they don't return. We have learned if they do not send the kit back in a week or two after enrollment, they are not going to.

- Problem: We have not started enrolment at the Montreal site. There was a delay in HRPO review due to personnel change, but they are now back on track working with HRPO and their IRB to get HRPO approval.
- Correction action: We contacted Dr. Stephen Grate and he helped identify the source of initial delay and got the paperwork back on track for HRPO review.

We do not anticipate any major problems, because we are identifying problems and taking immediate action to stay on course. The only concern for which we have no control is getting HRPO approval so Montreal can begin enrollment.

- **Changes that had a significant impact on expenditures**

No lasting changes. We would like to explain why our actual expenditure ($287,561 including indirect) is less than the projected budget ($594,834 including indirect) for year 1, noting that it reflects only a delay, and we are confident we can catch up and carry out the projects as proposed, albeit with a few months adjustment.

- The largest unspent item is DNA extraction, sequencing and genotyping. We had proposed to process the specimen in two batches of 250 cases and 150 controls staring in year 1 (DNA extraction from blood and stool, sequencing metagenome and GWAS genotyping, $194,000 (direct) x1.48 (indirect) = $288,000 in year 1). However, we have exceeded enrolment of cases and but are behind on controls. If we proceed now, we will have an imbalance (i.e., mostly cases in year 1 and mostly controls in year 2) that will cause a batch effect, a well-known confounder that can skew results. Hence we are waiting until we reach a scientifically balanced number of cases and controls before processing them, which we anticipate will happen in year 2.
- Another unspent item is the subaward with Montreal ($23,000 year 1). They are still waiting for HRPO approval. Montreal site is confident they can catch up, hence we will carry forward their subaward from year 1.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report

- **Significant changes in use or care of human subjects**

Nothing to report

- **Significant changes in use or care of vertebrate animals.**

Nothing to report

- **Significant changes in use of biohazards and/or select agents**

- **PRODUCTS:**

Nothing to report

- **Publications, conference papers, and presentations**

Journal publications. Nothing to report
Books or other non-periodical, one-time publications. Nothing to report
Other publications, conference papers, and presentations. Nothing to report
Website(s) or other Internet site(s) Nothing to report
Technologies or techniques Nothing to report
Inventions, patent applications, and/or licenses Nothing to report

- **Other Products**
  - data or databases: We have demographic, clinical and exposure data on subjects enrolled in the study. Currently, they are on paper. An electronic database is being built to enter the data.
  - biospecimen collections: We have blood or saliva on all subjects enrolled in the study. We have received stool sample from ~70% of subjects (we aimed for 100%, 30% are non-compliant)

- **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
  - **What individuals have worked on the project?**

Name: Haydeh Payami, PhD
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID): 0000-0001-9084-5338
Nearest person month worked: 3.9 CM
Contribution to Project: Responsible for the study. Implemented the study, manages the daily activities, ensuring standardized and rigorous study material and methods of subject selection. Tracks and logs and coordinates subjects selection, enrollment, data processing, and banking.(b) enrolls RBD subjects at UAB.

Name: Mary Appah
Project Role: Data Analyst
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 3.3 CM
Contribution to Project: Data manager. Assists with flow of data collection, error control, and manages database.

Name: Wanda Hall
Project Role: Subject Recruitment, Staff of UAB CCTS Clinical Research Support Program
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 10-20 hrs per week, charged as hourly service to CCTS
Contribution to Project: Subject enrollment

Name: Jake Orr
Project Role: Subject Recruitment
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: I CM
Contribution to Project: Subject enrollment

Name: Marissa Dean, MD
Project Role: Movement Disorder Specialist Neurologist
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 0.57 CM
Contribution to Project: Identification of qualified subjects for enrollment

Name: Zachary Wallen, MS*
Project Role: Graduate student
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 12 CM
Contribution to Project: Bioinformatics and statistical analysis, Biospecimen processing

* Zachary Wallen is a graduate students working full time on this grant. He is not paid by this DoD grant because he is on an NIH training grant. He has been working on this grant for the entire past year, since the start of the grant. We did not include him in this list for quarterly reports because we assumed it included only people who were paid by the grant. We noted "*regardless of the source of compensation*" in the instructions of this report.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to report
What other organizations were involved as partners? Nothing to report

- **SPECIAL REPORTING REQUIREMENTS**
  - **COLLABORATIVE AWARDS: Nothing to report**
  - **QUAD CHARTS: Attached**
- **APPENDICES: None.**

Interactions of gut microbiome, genetic susceptibility and environmental factors in Parkinson's disease

PD170080
W81XWH1810508

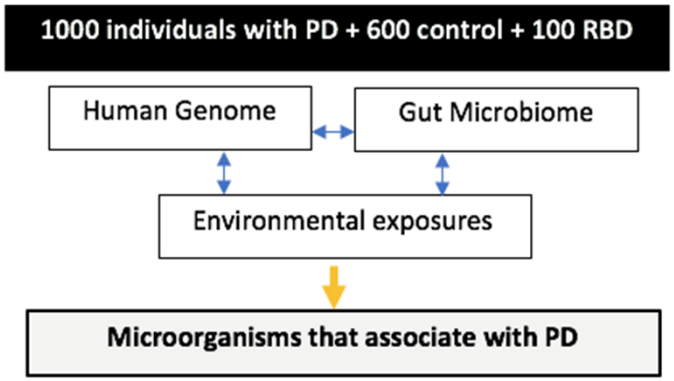
PI: Haydeh Payami, PhD **Org:** University of Alabama at Birmingham **Award Amount:** $1,861,029

Study/Product Aim(s)

- Hypothesis 1. Specific microorganisms in the gut contribute to the pathogenesis of PD.
- Hypothesis 2. Specific microorganisms determine if a genetically susceptible individual will develop PD.
- Hypothesis 3. The association of smoking and caffeine with PD is mediated by gut microbiome.
- Hypothesis 4. There exists a gut microbiome signature for prodromal PD.

Approach

We have begun enrolling the projected 500 persons with PD, 300 controls and 100 with RBD; we are collecting blood or saliva, stool, and environmental and clinical data for each subject. Later during the study we will generate genotypes, sequence the microbiome, and then analyze the data to test the 4 hypotheses.



Created standardized tools and methods, obtained regulatory approvals, launched the study and have enrolled 508 subjects. Analyzed dataset 1 (existing), demonstrated dysbiosis in gut microbiome in PD and identified candidate microorganisms.

Timeline and Cost

Activities / CY	18	19	20	21	22
Enrollment and data collection					
Genotyping human genome, sequencing gut microbiome					
Data analysis					
Publishing results					
Estimated Budget ($K)	**$198**	**$590**	**$489**	**$330**	**$254**

Updated: 9/10/19

Goals/Milestones

CY18 Goals – Launch study to enroll PD, control, and RBD subjects.
✓ Create study documents and obtain IRB and HRPO approval
✓ Begin enrollment, collect blood, stool and environmental data

CY19 Goal – Continue data collection
✓ Enrollment and data collection on PD, controls, RBD
☐ Generate genome-wide genotype data
☐ Generate microbiome sequence data

CY20, CY21, CY22 Goal
☐ Continue and complete data collection, genotyping and sequencing
☐ Data analysis
☐ Manuscript publication

Comments/Challenges/Issues/Concerns
- If timelines change, comment here.
- If off by more than one quarter in spending, comment here.

Budget Expenditure to Date (9/1/18 – 8/31/19)
Projected Expenditure: $594,834
Actual Expenditure: $287,561