Supplementary Material

Immunophenotyping Tracks Motor Progression in Parkinson's Disease Associated with a TH Mutation

Supplementary Table 1. Antibodies

Specificity	Clone/Species	Conjugate	Vendor	Catalog Number	Purpose	Dilution	Concentration
TH	Polyclonal/Rabbit	N/A	Sigma	AB152	FC	1:100	0.01 mg/mL
DAT	MAB369/Rat	N/A	Sigma	MAB369	FC	1:100	0.01 mg/mL
Rabbit	Polyclonal/Goat	BV421	BD	565014	FC	1:40	0.005 mg/mL
Rat	Polyclonal/Goat	APC	BD	551019	FC	1:40	0.005 mg/mL

Supplementary Table 2. Reagents and Materials

Reagent	Supplier	Catalog Number	Purpose	Concentration
Ficoll-Paque Plus	GE	45-001-750	PBMC isolation	N/A
PBS	In house	N/A	PBMC isolation, FC	1x
K2EDTA Vacutainer	BD	366643	Blood collection	N/A
Butterfly blood collection device	BD	367342	Blood collection	N/A
Leucosep Tubes	Greiner BioOne	227290P	PBMC isolation	N/A
Trypan Blue	MP Biomedicals	1691049	Cell counting	Stock
Fix/Perm Kit	eBioscience	88-8824-00	FC	Stock

Supplementary Table 3. Equipment

Equipment	Supplier	Part Number	Purpose
Centrifuge	Sorvall	ST8	PBMC isolation
Cytometer	BD	Canto II	FC
Spectral Analyzer	Cytek	Aurora	FC
Microcentrifuge	Fisher	59A	FC

MATERIALS AND METHODS

Human subjects

Blood samples from age-matched healthy subjects were obtained from two sources: the Movement Disorder Clinic at the University of Florida *via* an approved IRB protocol with written informed consent (IRB201701195), or the Lifesouth Community Blood Center, Gainesville, FL where deidentified samples exempt from informed consent (IRB201700339) were purchased. According to Lifesouth regulations, healthy donors were individuals aged 40-80 years old of any gender, who were not known to have any blood borne pathogens (both self-reported and independently verified), and were never diagnosed with a blood disease, such as leukemia or bleeding disorders. In addition, none of the donors were using blood thinners or antibiotics, or were exhibiting signs/symptoms of infectious disease, or had a positive test for viral infection in the previous 21 days.

Inclusion/exclusion criteria for human subjects

Parkinson's disease patients: Potential study participants were evaluated by a board-certified neurologist specializing in movement disorders. Patients were eligible to participate if 1) they had a confirmed PD diagnosis, 2) there was absence of comorbid movement disorder (i.e. essential tremor), 3) there was absence of any psychiatric diagnoses, 4) they were not prescribed psychotropic medications (i.e. neuroleptics), 5) they had no current or recent diagnosis of cancer (within 18 months) and were not on current or recent (within 18 months) treatment for the same, 6) had not been diagnosed with viral, bacterial or other infections within the preceding 21 days and were currently not being treated for the same.

Healthy control subjects: While not evaluated explicitly by a movement disorder specialist, healthy control subjects were present at the time of blood draw for PD patients and most frequently included the patient's spouse, allowing for control of environmental factors that may influence immune factors being studied. Participants were eligible to participate if 1) they report no current or past diagnosis of motor disorder (PD, ET, dystonia), 2) they were currently not taking medication for the same (self-reported), and 3) were not exhibiting overt symptoms of movement disorder.

Demographic information: Age, disease duration, sex distribution and motor scales for groups used for analysis are given in Supplementary Table 4.

PBMC isolation and flow cytometry

Materials and equipment are listed in Tables 1, 2, and 3. As previously published [1-3], whole blood was collected in K2EDTA vacutainer blood collection tubes (BD, 366643) and kept at room temperature for up to 2 h prior to PBMC isolation. Briefly, blood from healthy volunteers and PD patients was overlaid in Leucosep tubes for PBMC isolation, centrifuged for 20 min at 400 g with brakes turned off and acceleration set to minimum. PBMCs were collected from the interphase of Ficoll and PBS, transferred to a fresh 15 mL conical tube, resuspended in 8 mL sterile PBS and centrifuged for 10 min at 100 x *g*, and repeated twice more. Cells were counted with a hemacytometer using trypan blue exclusion of dead cells, and density-adjusted with PBS for flow cytometry staining.

Patient and healthy control subject PBMCs were stained for flow cytometry analysis in 100 µL staining volume containing 1 million cells per condition. Staining for intracellular epitopes of DAT and TH was performed at room temperature in permeabilization buffer (eBioscience, 88-842-00), followed by species-specific secondary antibodies. We note that the flow cytometry method used to detect DAT and TH expressing cells does not allow for assessment of protein levels of these markers. Samples were resuspended in 500 µL PBS after the final wash. Data were collected within 2 h on BD Canto II or Cytek Aurora Spectral Cytometer. Each experiment included single color compensation, followed by automatic compensation calculation. Compensation matrices were not altered thereafter. Data were analyzed using Flowjo Software (BD Biosciences). All gates were set via fluorescence-minus-one (FMO) analysis, arriving at the final gates used for analysis. When assessing the percentage DAT+ or TH+ PBMCs, the entire gated region shown in the histogram of Supplementary Figure 2 are reported as the percentage of DAT+ or TH+ PBMCs. Gating strategy is shown in Supplementary Figure 2.

Genetics: Samples and data is processed and stored according to HIPAA compliance requirements following CAP guidelines [4] and CLIA standards for quality and competence [5] at UF Health Medical Laboratories Whole genome sequencing (WGS) is benchmarked using the National Institute of Standards and Technology 'Genome in a Bottle' Consortium standards (HG002 son, HG003 & HG004 parental genomes [6]).WGS precision metrics have been explored from 5-30× depth for the entire genome and results are concordant with the NIST/PrecisionFDA data "truth sets" [7-9] with > 95.2% analytical sensitivity and > 97.3% precision. DNA is extracted from a buccal swab using the Qiagen QIAmp DNA Mini Kit. DNA is quantified by fluorescence on an Invitrogen Qubit Fluorometer. Individually-indexed genomic libraries are prepared using

dual unique indexes from ~200ng DNA/individual (New England Biolabs NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®). Genome library quality and quantity are confirmed by automated electrophoresis on an Agilent 2100 bioanalyzer and by qPCR. Individual libraries are normalized and pooled in equimolar ratios for 2× 150 bp paired-end sequencing at 35× depth on an Illumina NovaSeq 6000. To expedite and enable innovation, speed, data and code sharing, and to maintain security and PHI/HIPAA compliance, versioned bioinformatic pipelines for clinical genome variant calling have been developed in AWS in a containerized compute environment. Bioinformatic analyses include index deconvolution of pooled samples, read trimming, alignment, QC analyses, variant calling and annotation, with gene set panel filtering followed by variant prioritization by expert review [10-12] In brief, .fastq reads are aligned to the human genome reference (GRCh37/hg19) and variants are called and annotated using 'versioned' open source softwares including: TrimmomaticPE 0.39 [13], FastQC 0.11.9, MultiQC 1.9 [14], BWA MEM 0.7.17-r1188 [15], samtools 1.10 [16], Picard 2.23.8 [17], Strelka2 2.9.10 [18], bcftools 1.10.2 [16], snpEff 5.0c [19], ExpansionHunter 4.0.2 [20], Manta [21], and cn.mops 1.8.0 [22]. Computation is optimized by Nextflow [23]. Quality control reports are generated and examined for all individual samples and batched runs, including general statistics on WGS coverage per sample, on mapping quality and on the proportion of reads surviving that process. These data quantify sequence read counts (unique, duplicate, and overrepresented %), quality (Phred scores across reads, per sequence quality scores, length distributions, GC content and 'N' scores). Aligned files (.bam, .bai) and annotated variant files include quality scores, read orientations and depths. Per sample variability is documented as a composite variant call file (VCF) that includes all intergenic regions, intronic variants, downstream and upstream gene variants, non-coding, missense, nonsense and silent/synonymous variants, frameshift, stop gain, splice, disruptive in-frame deletions and duplications, start loss, stop loss and gene fusions. Our annotation approach is exact, comprehensive [24, 25] and includes CADD [26] and Revel scores [27], gnomAD frequencies [28], ClinVar [29], and OMIM entries [30]. All ~22,000 genes that make up the human genome are sequenced. Reporting is restricted to exonic nonsynonymous and splicing (± 20 bp) substitutions. Only variants with > 10× coverage are reported. The clinical significance of the filtered variants is assessed according to ACMG recommendations [11]. This sequence reported here is performed as research using de-identified swabs/DNA samples provided by collaborating investigators. However, this variant had been previously identified in this patient in a commercial CAP-accredited clinical lab.

DaTScan: DaTscan imaging was performed as published in Catafau 2004 [31]. SPECT imaging was obtained 3 to 6 h following intravenous injection of ^{123}I-Ioflupane (111-185 MBq; DaTscan GE Healthcare, Amersham, UK). A dose of 4.36 mCi of ^{123}I-Ioflupane were utilized. Images were acquired using a gamma camera fitted with high-resolution collimators and set to a photopeak of 159 keV with a ± 10% energy window. Subject was supine with the head on an off-the-table headrest, a flexible head restraint such as a strip of tape across the chin or forehead may be used to help avoid movement and set a circular orbit for the detector heads with the radius as small as possible. Interpretation of results indicated asymmetric uptake and activity (e.g., activity in the region of the putamen of one hemisphere is absent or greatly reduced with respect to the other) and reported as abnormal results. There was reduced activity in both right and left putamen alone or also in the caudate nuclei.

Statistics

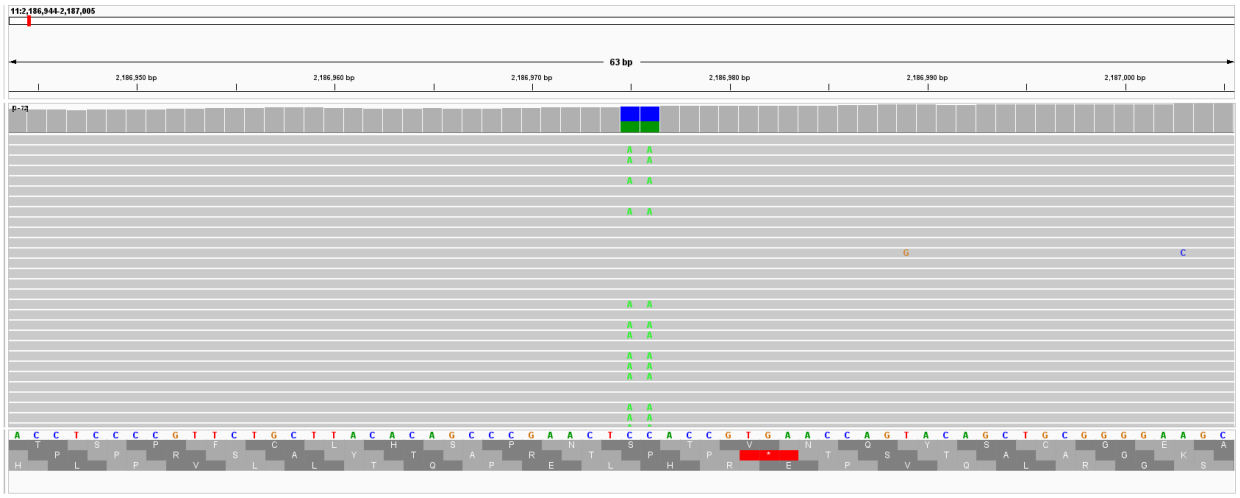
Unpaired Student's t-test (two-tailed) was used when comparing two groups with Tukey's correction for multiple comparisons. $p<0.05$ was considered statistically significant. All statistical analyses were performed in GraphPad Prism 10.

Supplementary Table 4. Patient demographics

# Female	# Male		Disease Duration (y)	Age (y)	HY Score*	UPDRS-III (Off)*	UPDRS-III (On)*	LED*
47	83	**Average**	10.66	67.72	2.51	31.03	24.46	1111.81
		Range	0-27	41-81	1.5-5	13-67	2-51	0-25000
		Standard Deviation	*7.17*	*7.99*	*0.75*	*10.95*	*9.83*	*2252.61*

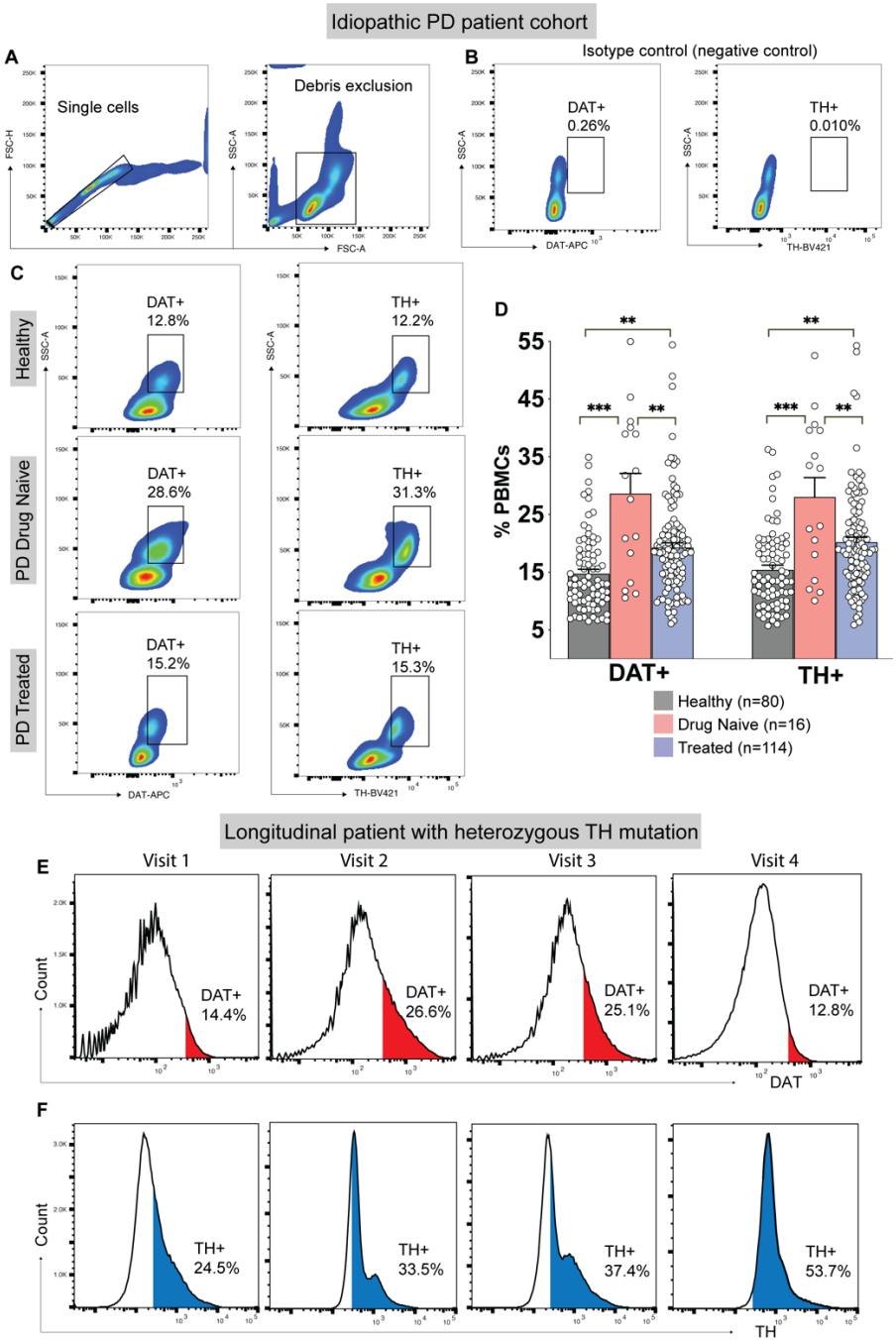
*HY, Hoehn and Yahr score; UPDRS-III, Unified Parkinson's Disease Rating Scale part 3, motor subscale; On and Off refer to on drug or off drug status; LED, Levodopa Equivalence Dose

Supplementary Figure 1. Sequencing results.



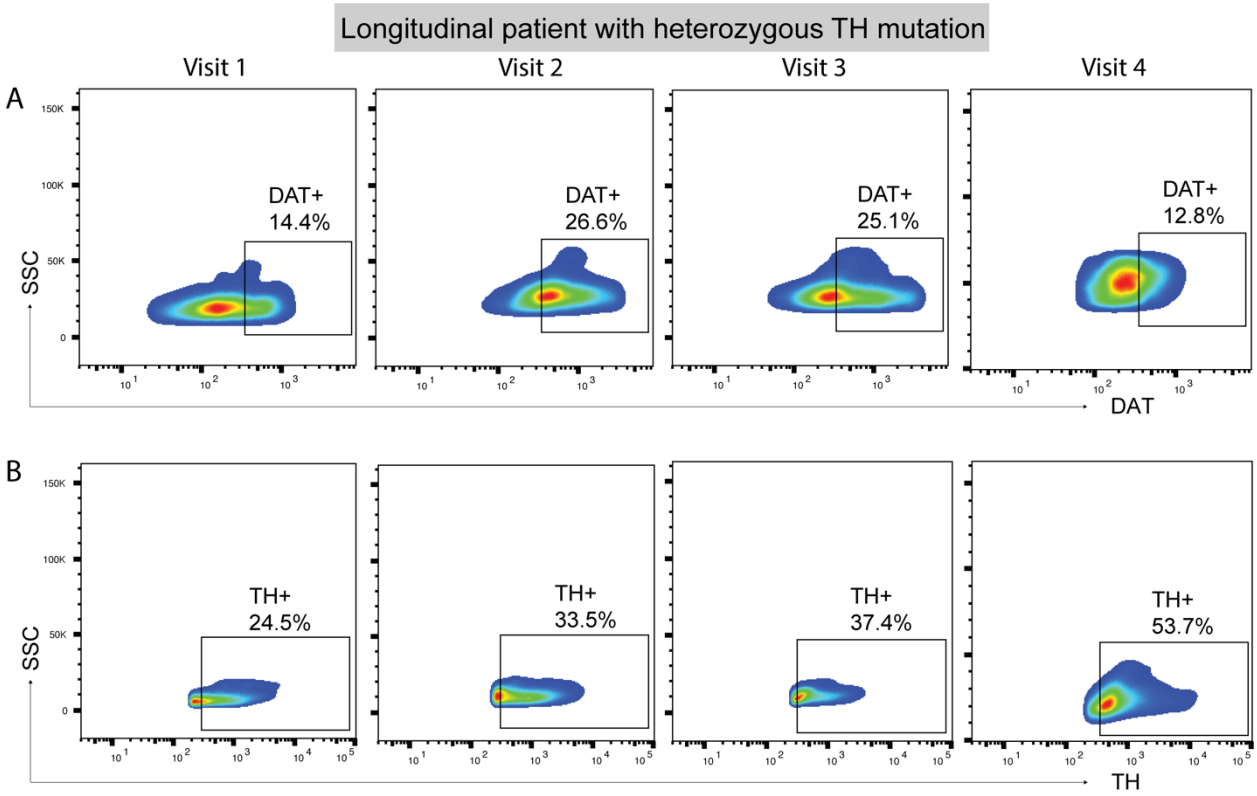
Integrated genome view of chromosome 11: 2,186,944-2,187,005 interval spanning the TH locus, exon 12, and the position of the g.2,186,975-6 CC>AA (c.1215_1216delCCinsTT; p. Glu406*) pathogenic variant. Reads and their orientation are illustrated in gray, the consensus nucleotide sequence is shown beneath, along with possible open reading frames and the translated protein normally encoded (blue bar). 'CTC' encoding "E", the single letter amino acid for glutamine (Glu) is mutated to 'ATC' encoding the amber stop codon.

Supplementary Figure 2. Gating strategy and representative DAT+ and TH+ PBMCs in a cohort of idiopathic PD patients, healthy control subjects, and a PD patient carrying a TH mutation.



Isolated PBMCs were stained and analyzed via flow cytometry after A) gating single cells and excluding debris. B) Non-immune isotype control was used to assess specificity of DAT and TH signals. C, D) Relative to healthy control subjects, drug naïve idiopathic PD patients show a significantly increased percentage of DAT+ and TH+ PBMCs, which are significantly reduced in treated patients, but remain higher than healthy control levels (**p<0.01, ***p<0.001; One-way ANOVA, with Tukey's correction for multiple comparisons; alpha=0.05; f(2,3)=305.7). E) In a patient carrying a heterozygous TH mutation, DAT+ PBMCs follow the trend established in idiopathic PD, where DAT+ PBMCs are increased prior to treatment, and subsequently decrease towards healthy control levels (Fig. 3) following treatment. In contrast to idiopathic PD patients, F) the subject carrying a TH mutation shows TH+ PBMCs continuing to increase despite treatment for PD, indicating TH+ PBMCs are uncoupled to treatment response.

Supplementary Figure 3. Dot plot representation of longitudinal changes in DAT+ and TH+ PBMCs over four visits, in a patient carrying a TH mutation.



A) In a patient carrying a heterozygous TH mutation, DAT+ PBMCs follow the trend established in idiopathic PD, where DAT+ PBMCs are increased prior to treatment, and subsequently decrease towards healthy control levels (Fig. 3, Supplementary Figure 2) following treatment. In contrast to idiopathic PD patients, B) the subject carrying a TH mutation shows TH+ PBMCs continuing to increase despite treatment for PD, indicating TH+ PBMCs are uncoupled to treatment response.

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