Whole-exome sequencing of 228 patients with sporadic Parkinson’s disease

Cynthia Sandor1,2,*, Frantisek Honti1,†, Wilfried Haerty2,‡, Konrad Szewczyk-Krolikowski1,3, Paul Tomlinson1,3, Sam Evetts1,3, Stephanie Millin1,2, Thomas Keane*, Shane A. McCarthy4, Richard Durbin4, Kevin Talbot1,3, Michele Hu1,3, Caleb Webber1,2, Chris P. Ponting1,2,4,§ & Richard Wade-Martins1,5

Parkinson’s disease (PD) is the most common neurodegenerative movement disorder, affecting 1% of the population over 65 years characterized clinically by both motor and non-motor symptoms accompanied by the preferential loss of dopamine neurons in the substantia nigra pars compacta. Here, we sequenced the exomes of 244 Parkinson’s patients selected from the Oxford Parkinson’s Disease Centre Discovery Cohort and, after quality control, 228 exomes were available for analyses. The PD patient exomes were compared to 884 control exomes selected from the UK10K datasets. No single non-synonymous (NS) single nucleotide variant (SNV) nor any gene carrying a higher burden of NS SNVs was significantly associated with PD status after multiple-testing correction. However, significant enrichments of genes whose proteins have roles in the extracellular matrix were amongst the top 300 genes with the most significantly associated NS SNVs, while regions associated with PD by a recent Genome Wide Association (GWA) study were enriched in genes containing PD-associated NS SNVs. By examining genes within GWA regions possessing rare PD-associated SNVs, we identified RAD51B. The protein-product of RAD51B interacts with that of its paralogue RAD51, which is associated with congenital mirror movements phenotypes, a phenotype also comorbid with PD.

Parkinson’s disease (PD) is a common, multifactorial and genetically heterogeneous neurodegenerative disorder, primarily of old age. It is characterized by progressive loss of dopamine neurons in the substantia nigra pars compacta together with the accumulation of intracellular protein inclusions termed Lewy bodies1. Highly penetrant Mendelian variants, such as in SNCA, LRRK2, PARKIN, PINK1, and PARK7 genes, explain less than 10% of familial PD2. Twenty-eight DNA variants across 24 loci that predict risk, albeit to a minor degree, for sporadic PD have been identified using genome-wide association studies (GWAS) involving over ten thousand individuals with PD3.

Despite this progress, most sporadic PD cases remain unexplained genetically. With larger studies and the increased application of genome sequencing technologies, it is anticipated that there will be a better understanding of the genetic contributions to PD risk that would directly inform on the molecular and cellular etiology of the disease. Exonic sequence is highly enriched in variants that explain the heritability of complex traits4. By sequencing the exons of protein-coding genes (in other words, the exome) of PD subjects, and identifying their variants, which are rare in a control cohort, there is the prospect of identifying further PD disease genes. Nevertheless, only

1Oxford Parkinson’s Disease Centre, University of Oxford, Oxford, United Kingdom. 2MRC Functional Genomics Unit, Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, OX1 3PT, United Kingdom. 3Nuffield Department of Clinical Neurosciences, John Radcliffe Hospital, West Wing Level 6, Headley Way, Oxford OX3 9DU, United Kingdom. 4Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1SA, United Kingdom. 5Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, OX1 3QX, United Kingdom. *Present address: Royal Brompton & Harefield NHS Foundation Trust, Sydney Street, London SW3 6NP, United Kingdom. †Present address: Joule Institute Norwich Research Park Innovation Centre, Colney Lane, Norwich NR4 7UH, United Kingdom. ‡Present address: MRC Human Genetics Unit, MRC IGM, University of Edinburgh, Edinburgh, United Kingdom. §Present address: MRC Human Genetics Unit, MRC IGM, University of Edinburgh, Edinburgh, United Kingdom. ¶These authors contributed equally to this work. Correspondence and requests for materials should be addressed to C.W. (email: caleb.webber@dpag.ox.ac.uk) or C.P.P. (email: chris.ponting@igm.ed.ac.uk) or R.W.-M. (email: richard.wade-martins@dpag.ox.ac.uk)
when very large cohorts (>10,000 samples) are investigated will there likely be sufficient power for such individual rare disease-associated variants to be revealed5.

Variants that occur more frequently in the patient cohort than in controls could still be of interest for predicting PD susceptibility despite individually not being genome-wide significant. For example, individual genes, or else multiple genes encoding functionally interacting proteins, might be found that harbour an unusually large number of such sub-genome-wide significant variants. These genes would then add to our understanding of the molecular deficiencies contributing to neurodegeneration in PD.

Here, we analyse exonic variants found in 228 individuals with sporadic PD, all recruited from a relatively homogeneous UK population. We apply multiple approaches at the level of the variant, the gene and the pathway to analyse whether – considered individually or together – these variants can contribute to PD diagnosis.

Results
Exomes of 244 Parkinson's disease (PD) patients selected from the Oxford Parkinson's Disease Centre Discovery Cohort were sequenced. After quality control, and after one individual with relatively strong identity-by-descent (>0.1875) was discarded, 228 PD patient exomes were considered for analysis and compared with 884 control exomes from UK10K cohort and matching our control in terms of ancestry (Method). 94,369 sites with base calls in at least 99% of samples were retained for analysis. Eight individuals known to carry variants in GBA (N370S and L444P) or LRRK2 (R1441C and G2019S) were included as positive controls for mutation detection through exon resequencing. All known variants were detected, although the single GBA L444P variant call failed to exceed the VQSLOD threshold. We called a total of 94,369 single nucleotide variants (SNVs), of which 39,569 had a minor allele frequency of less than 1% (in controls), affecting 10,252 genes. Of 94,369 SNVs, 21,235 were non-synonymous (NS) affecting 9444 protein coding genes, including 313 that caused the gain or loss of a stop codon and 6,541 that were considered by Polyphen-26 as being damaging (Supplementary Fig. 1).

No SNV/gene reached genome-wide significance for PD association. Fisher's exact test and logistic regression using between 1 and 10 covariates were performed using PLINK7 (http://pngu.mgh.harvard.edu/purcell/plink/). The best-fitting quantile-quantile (Q–Q) plot was obtained with the use of two covariates in logistic regression and the control sets listed in the Methods (Figs 1A and 2). As expected, given the low number of cases and the multiple testing correction required, no substitution achieved significance of association with PD status (p-value upper threshold $2.4 \times 10^{-6}$; Fig. 2). We next used SKAT8 to perform a burden test which found no single gene showing a significant association of multiple SNVs with PD risk. (Fig. 1B and Supplementary Fig. 2). After performing burden analyses, we again failed to find a significant enrichment in the number of NS per individual between cases and controls (NScases $= 6639$ vs NScontrols $= 6650$, p-value $= 0.34$).

PD association in previous reported PD candidate genes. We then examined globally whether known genes involved in the familial or sporadic forms of PD were enriched in PD associated NS SNV (Method). For 15 genes involved in familial PD$^	ext{a}$ (Supplementary Table 1), we did not find a significant enrichment of PD-associated NS SNVs (10/15 genes had at least one NS SNV; p-value enrichment based on a logistic regression test $= 0.054$ and p-value enrichment based on the SKAT test $= 1$). However, for 329 genes harboured within 26 GWA intervals (Methods), a significant 1.2-fold enrichment in PD-associated NS SNVs was achieved (163/329 genes had at least one NS SNV, p-value enrichment based on logistic regression test $= 0.036$).

Next, we looked amongst 15 genes involved in familial and 329 genes harboured in 26 GWA intervals (Fig. 1, Tables 1 and 2) for NS SNVs with nominal p-value associations of $<0.05$ with PD. We identified two PD-associated NS SNVs with MAF $<1\%$ within genes harboured in PD risk GWA intervals, each with an odds ratio $>3.5$. Specifically, these were (1) rs34094401, a missense mutation (L172W) in the RAD51B gene encoding a protein essential for DNA repair (18 cases versus 20 controls are heterozygous for this variant) lying in the PD GWA interval defined by the lead SNP rs1555399, and (2) rs41309351, a missense mutation (R480W) in the
CPXM1 gene encoding for a carboxypeptidase (16 cases versus 12 controls are heterozygous for the variant) lying in the PD GW A interval defined by the lead SNP rs55785911. Truncating and missense variants in the RAD51B-interacting paralog RAD51 have been reported to cause congenital mirror movement syndrome10,11. By examining the frequencies for rs34094401 in an independent cohort including 536 PD patients and 260 controls matching PD patients for age and genotyped on HumanCoreExome chip from Illumina12, we observed a similar but non-significant effect at the RAD51B locus (p-value = 0.14): MAF cases = 0.01306 (14 cases heterozygous) vs MAF controls = 0.005769 (3 controls heterozygous). We noted that the control minor allele frequency of these two SNPS were consistent with those reported by the Exome Aggregation Consortium (ExAC) in the European (Non-Finnish) population 13: rs34094401 MAF ctr = 0.010180995 & MAF ExAC = 0.011834053 while rs41309351 MAFctr = 0.00678733 & MAFExAC = 0.003578.

Functional analyses of PD-associated SNVs. Although we did not find genome-wide significant results for PD association, genes whose proteins contribute to a common cellular process or pathway could contain an enrichment of SNVs that are sub-genome-wide significant for PD association. For this analysis, we examined whether the top N genes with most PD-associated NS SNVs (N = 100, 200, 300) were enriched in particular Gene Ontology terms14. Only when considering such a large number of genes, the genes annotated with a role in extracellular matrix disassembly include those encoding the metalloproteases MMP-7 and MMP-8, and the reduced expression of metalloproteases has been noted in PD post-mortem brain tissue15 while a polymorphism in MMP-9 has been associated with PD and amyotrophic lateral sclerosis16.

Discussion
In this study, we compared the exomes of 228 PD cases with 884 controls exomes drawn from the UK10K17 study. We performed association tests both at the level of single-nucleotide variants and at the gene level but found that no variant was significantly associated with PD after applying a multiple-testing correction. While power for discovery is clearly the major issue, there may be additional considerations affecting our study. As the heritability of PD is not high (34–40%), the identification of causative variants or risk alleles for PD may be more difficult than for some other, common neurological disorders18,19. Furthermore, as our controls are not aged-matched, we do not know whether control individuals will go on to develop PD. Indeed, for example the LRRK2-G2019S

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Table 1. Suggestive PD risk non-synonymous variant (p-value < 0.05) within genes known to be involved in familial form of PD.

| SNV          | Chromosome | Position | Gene     | AA substitution | Allele | MAF (Cases) | MAF (Controls) | Odds Ratio | Pval |
|--------------|------------|----------|----------|----------------|--------|-------------|----------------|------------|------|
| rs2302464    | 4          | 15709252 | BST1     | R/Q            | A/G    | 0.018       | 0.039          | 0.44       | 0.042|
| rs1801334    | 6          | 161781225| PARK2    | D/N            | T/C    | 0.026       | 0.046          | 0.56       | 0.038|
| rs33995883   | 12         | 40740686 | LRRK2    | N/D            | G/A    | 0.020       | 0.010          | 1.96       | 0.044|
| rs2279068    | 3          | 182755209| MCCC1    | H/P            | Y/G    | 0.285       | 0.230          | 1.26       | 0.014|

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Figure 2. Manhattan plot showing the significance (−log10(p-value)) of association of non-synonymous variants with PD status. The horizontal line indicates the Bonferroni multiple testing correction threshold at p = 2.4 × 10−6. Amongst the black points, the blue and green points represent the significance of association for non-synonymous variants within genes involved in the familial form of PD (blue) or genes harboured in one of 26 published PD GW A intervals (green), respectively. The names of genes are given in the top part of plot in blue and green for mono and GW A genes respectively. For 26 GW A intervals, only the name of the gene(s) that possesses the most significant association from the exome non-synonymous variants is showed.
Table 2. Suggestive PD risk non-synonymous (p-value < 0.05) within genes harbored in PD GWAS intervals.

mutation has a penetrance of 28% at age 59 years, 51% at age 69 years, and 74% at age 79 years20. Burden tests or collapsing methods, such as SKAT, might be expected to improve power over single-marker tests, but they still require thousands of samples to reach acceptable statistical power8,21. We considered different subsets of the UK10K controls for this study and the best-fitting Q–Q plot was obtained using a set of six UK10K data sets 17 (see Methods). Notably for other studies seeking exome controls, the use of the two largest UK10K cohorts, COHORT_TWINS (~1,700 samples) and COHORT_ALSPAC (740 samples), produced inflated p-values that could not be controlled by filtering or covariates. Some of the minor allele frequencies from these two cohorts that were responsible for this effect were very different from the allele frequencies in other controls, suggesting a population stratification problem. While DNA sequencing enables the direct detection of causal mutations and may supersede microarray-based genotyping methods, some of the limitations of GWAS still apply to these studies. Thousands of cases and controls are required to robustly associate rare variants with a common disease and population stratification has to be controlled for5,22.

NS SNVs most associated with PD were enriched both in genes whose proteins have roles associated with the extracellular matrix and in genes lying within PD-associated GWA intervals. Furthermore, we identified a nominally-associated SNV in the RAD51B gene which lies within a region of the genome associated with PD through GWA. Variants in the paralog RAD51, whose protein product interacts with that of RAD51B, have been associated with congenital mirror movement disorders (OMIM 614508) and mirror movement phenotypes are also associated with PD23. Thus, RAD51B represents an interesting candidate gene for further examination within this GWA locus.
Methods
Clinical characteristics of PD subjects. Established in September 2010, the Oxford Discovery Cohort (www.opdc.ox.ac.uk) comprises patients with idiopathic PD diagnosed in the previous 3.5 years according to UK PD Society Brain Bank diagnostic criteria recruited from a 2.4 million Thames Valley population with the aim of following up the cohort over the natural history of their disease. PD patients were prospectively recruited over two years from October 2010 to September 2012 and underwent a 2-hour standardised assessment with a movement disorders neurologist and research nurse, following which patients were diagnosed as having PD, with an estimated clinical probability for this diagnosis being made by the research neurologist. Atypical parkinsonian features were systematically screened for using the NINDS Parkinson’s tool, and patients with secondary parkinsonism due to head trauma or medication use, or features of atypical parkinsonism syndromes, were excluded from the study. Subjects were also excluded from subsequent analysis if they had a <80% baseline probability of manifesting idiopathic PD. Clinical assessments were performed while the subject was taking their normal medications, and in the ‘on’ state. The study was undertaken with the understanding and written consent of each subject, with the approval of the NHS South Central Berkshire Research Ethics Committee (study reference 10/H0505/71), and in compliance with national legislation and the Declaration of Helsinki.

244 consecutive PD subjects from the Discovery cohort were recruited to this exome study, of whom 224 (90%) were selected on the following basis: (i) possessing a LRRK2 R1441C or G2019S variant (n = 4), (ii) possessing a GBA N370S or L444P variant (n = 4), (iii) subject had undergone MRI brain scan (n = 20), (iv) subject had undergone Cerebrospinal Fluid (CSF) examination (n = 39), (v) subject had undergone skin biopsy (n = 16), (vi) subject had at least 1 first degree PD affected relative (n = 52), (vii) subject had at least 1 second degree PD affected relative (n = 21) and (viii) subject had a Montreal Cognitive Assessment (MoCA) score ≤22 indicating significant cognitive impairment (n = 68). Of the 244 PD subjects, (i) no female ratio was 0.60:0.40, mean (SD) age at diagnosis was 66.3 (9.4) years (age range 31.6–87.4 years, mean (SD) Hoehn and Yahr scale was 1.90 (0.5) range 1.0–4.0, median = 2, interquartile range = 2, 2) and mean (SD) disease duration from diagnosis was 1.7 (1.1) years. Ethnicity of the PD subjects comprised 92% white British, 2.6% white Irish, 2.9% white other, 0.4% Indian, and 2.1% other.

Control Exome samples. As our PD exome patients have ben recruited in UK, we used public whole-exome sequencing data or individuals matching our PD cohort in term of ancestry and thus with UK origin. The following exome sample datasets were used from the UK 10K dataset: NEURO_IOP_COLLIER (152 samples), NEURO_ABERDEEN (317 samples), NEURO_EDINBURGH (213 samples), RARE_NEUROMUSCULAR (72 samples), RARE_THYROID (18 samples) and NEURO_IMGSAC (112 samples), making a total of 884 control exomes. The two largest UK10K cohorts, COHORT_TWINS and COHORT_ALSPAC, were not used as they contained minor allele frequencies that differed from those of other control cohorts, indicating a possible population stratification problem. Furthermore, to avoid inflation of low p-values due a stratification issue with particular subsets of the UK10K controls, best-fitting Q–Q plot was obtained using the set of six control data sets.

Exome sequencing. 120 μl of 25 ng/μl of DNA was fragmented to an average size of 150 bp (75–300 bp), and subjected to Illumina DNA sequencing library creation using Bravo automated liquid handling. Adapter ligated libraries were amplified via 6 cycles of PCR. 500 ng/μl of each amplified library was hybridized to RNA baits (SureSelect Human All Exon 50 Mb) and post sequence capture processed following the Agilent SureSelect protocol. Enriched libraries were post capture indexed using a unique DNA barcode via 12 cycles of PCR. Equimolar pools of 8 libraries were sequenced using the Illumina HiSeq machine using the 75 bp paired end protocol.

Data processing. Sequencing reads from each lane were aligned to the human reference genome used in the 1000 Genomes phase 2 (NCBI build 37 + decoy v5) using BWA version 0.5.9-r16 and the parameters ‘-q 15 –t 6’. The GATK27 ‘IndelRealigner’ was used to realign reads near known indels [ftp://ftp.1000genomes.ebi.ac.uk/ftp/1000genomes/phase2_mapping_resources/]. The BAM files were then re-sorted and quality scores were recalibrated using GATK ‘TableRecalibration’ ignoring all SNP positions in dbSNP v135 28. Finally, SAMtools ‘calmd’ was used to recalculate MD/NM tags in the BAM files. All lanes from the same library were then merged into a single BAM file using Picard tools29 and PCR duplicates were marked using Picard ‘MarkDuplicates’. Enriched libraries were then capture indexed using a unique DNA barcode via 12 cycles of PCR. Equimolar pools of 8 libraries were sequenced using the Illumina HiSeq machine using the 75 base paired end protocol.

Variant calling. SNP and indel calls were made using samtools30 by pooling the alignments from all 246 individual exome BAM files. A genotype likelihood (bcf) file was created with samtools (v0.1.18-r572) mpileup, for every site in the exome bait regions (+/– 100bp) [samtools mpileup –EDVSP –C50 –L500 –m3 –F0.2 –d 160000 –P ILLUMINA –l baits.w100.bed]. Variants were then called using bcftools (v0.1.18-r572) [bcftools view –m.9 –vegN].

SNP filtering. Variant Quality Score Recalibration (VQSR)32 was used to filter SNPs. The GATK (v1.6-13-g91f02df2) UnifiedGenotyper was used to recall the sites/alleles discovered by samtools in order to generate annotations to be used for recall. The GATK VariantRecalibrator was then used to model the variants, followed by GATK ApplyRecalibration which assigns VQSLOD (variant quality score log odds ratio) values to the variants. Annotations used for training were QD, HaploTyperScore, MQRankSum, ReadPosRankSum, FS, MQ, and InbreedingCoeff. The training sets were HapMap3.3 and Omnii2.5M sites. The truth set used was HapMap3.3 sites while the known set was dbSNP 132 sites. A VQSLOD cut off was chosen and the following filters applied to SNP sites:

LowQual,Description = “Low quality variant according to GATK (GATK)”
MinVQSLOD,Description = “Minimum VQSLOD score [SNPs=−1.8356, truth sensitivity 99.5%]”. 
**Indel filtering.** Indels were left-aligned using GATK (v1.6-13-g9f102df) LeftAlignVariants. The following filters were then applied to indel sites:

- **StrandBias,** Description = "Min P-value for strand bias (INFO/PV4) [0.0001]"
- **BaseQualBias,** Description = "Min P-value for baseQ bias (INFO/PV4) [1e-100]"
- **EndDistBias,** Description = "Min P-value for end distance bias (INFO/PV4) [0.0001]"
- **GapWin,** Description = "Window size for filtering adjacent gaps [3]"
- **MaxDP,** Description = "Maximum read depth (INFO/DP or INFO/DP4) [8000000]"
- **MinAB,** Description = "Minimum number of alternate bases (INFO/DP4) [2]" > **MinDP,** Description = "Minimum read depth (INFO/DP or INFO/DP4) [16000]
- **MinMQ,** Description = "Minimum RMS mapping quality for SNPs (INFO/MQ) [10]"
- **Qual,** Description = "Minimum value of the QUAL field [10]"
- **RefN,** Description = "Reference base is N []".

**Additional filtering.** Additionally, the following filters were applied to individual genotype calls based on their depth or genotype quality:

- **IndelsMinSampleDP,** Description = "Genotypes set to. for samples with DP < 4 (FORMAT/DP)"
- **IndelsMaxSampleDP,** Description = "Genotypes set to. for samples with DP > 2000 (FORMAT/DP)"
- **SNPsMinSampleGQ,** Description = "Genotypes set to. for samples with GQ < 20 (FORMAT/GQ)"
- **IndelsMinSampleGQ,** Description = "Genotypes set to. for samples with GQ < 60 (FORMAT/GQ)".

**Annotation.** The calls were annotated with dbSNP 137 rsIDs and population allele frequencies from the 1000 Genomes Phase 1 26 integrated (v3) callset [ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/phase1/analysis_results/integrated_call_sets]. Variant consequence annotations were added using the Ensembl Variant Effect Predictor2 12 (v2.4) against Ensembl 66. This provides coding consequence predictions and SIFT, PolyPhen-2 and Condel annotations.

**Association analysis.** VCF files were converted to PED format (format of file used by PLINK (Purcell et al. 2007)) with VCFTools 8733 [—remove-indels —remove-filtered-all]. We performed single variant association analyses by using logistic regression test incorporating two first ancestry covariate. We evaluated evidence for association at the genetic level by using the sequence kernel association test (SKAT)8 that allows for risk and protective effects in the same gene. We used asymptotic p-values and incorporated ancestry covariate (two first Principal Components). SKAT assumes that rare variants are more likely to be causal variants with large effect sizes and assigns weight to the variant according to minor allele frequency (we used the default frequency weight (1 and 25 as the two beta distribution shape parameters, to up-weight lower frequency mutations)). As power was clearly an issue in this study, we did not repeat these analyses by functional category of variants.

**PD GWA intervals.** The 26 PD GWA intervals were defined: (i) by identifying the most distant pair of SNPs with either (i) an \( r^2 > 0.5 \) with the lead SNP reported in http://pdgene.org3,34 using the haplotypes of 1000 Genome Project or (ii) an \( r^2 > 0.5 \) computed by considering the European haplotypes only, and then adding an additional 250 kb on either side of the interval to include genes that may be regulated by GWA-associated regulatory variants.

**Gene Sets enriched in genes with PD-associated Non-synonymous SNVs.** To evaluate if a set of genes was enriched in PD associated NS SNVs, we computed the sum of \(-\log_10\) of p-value association of logistic regression test of NS SNVs within these genes and compared this measure with this got with random set genes matching each gene of test gene set for the number of NS SNVs. We performed an analogue test by replacing the p-value association of logistic regression by p-value associated with SKAT test.

**Tissue enrichment analysis.** FPKM => Feature Scaling => Euclidean Normalization (such that the square root of the sum of the square of expression levels is one). Compare the sum of expression value for PD risk gene with a set of random gene (match for CDS length).

**Overlap with PD GWAS intervals.** 26 genome-wide significant PD intervals were acquired from http://pdgene.org3,34. Intervals were extended at each end by 500kb. The number of the top N genes (10 \( \leq N \leq 100 \)) most significantly associated by SKAT with PD located within either set of intervals was compared with the number of randomly sampled length-matched genes in these intervals.

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Author Contributions
R.W.-M., C.P.P. and K.T. conceived and co-ordinated the project. M.H. designed the cohort study and selected patients for sequencing. K.K., St.Mi. and C.P.P. undertook bioinformatics genome analysis. C.W., C.S., M.H., C.P.P. and R.W.-M. wrote the manuscript.

Additional Information
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