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### Title and Subtitle

The Parkinson Transcriptome Project: Roadmap to Personalized Care

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### Abstract

This study provides a comprehensive catalog of gene expression - both protein-coding and non-coding - specifically in dopamine neurons *in situ* in human brain. Going beyond traditional messenger RNA sequencing, all polyadenylated and non-polyadenylated transcripts over 50 bases were deeply sequenced using ribo-depleted RNA from dopamine neurons laser-captured from human post-mortem brains of patients with Parkinson’s disease using laser-capture RNA sequencing. We show unequivocally that non-coding RNAs are abundantly transcribed in human dopamine neurons and highlight significant changes in the expression of both protein-coding and non-coding transcripts in patients with clinical and neuropathological Parkinson’s disease. Expression Quantitative Trait Locus analysis indicates that the expression of many non-coding and protein-coding transcripts in nigral dopamine neurons is associated with genetic variants. Finally, these pilot phase data support the feasibility of measuring select RNAs linked to Parkinson’s in vulnerable dopamine neurons as possible biomarkers in blood.

### Subject Terms

Parkinson’s disease, non-coding RNA, RNA sequencing, expression Quantitative Trait Locus, biomarkers
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1. Introduction

The recent discovery of a hidden universe of tens of thousands of non-protein-coding RNAs is revolutionizing our thinking about the flow of genetic information that shapes the complexity of the human brain and underlies its diseases. A revised view suggests parallel processing from DNA to protein-coding mRNAs as well as non-coding RNAs that are themselves active (without translation into proteins) --- with important biologic functions as regulators and biosensors.

We hypothesize that in complex neurodegenerative diseases such as Parkinson’s genetic and epigenetic risks (from aging and environmental exposures) exert their disease-causing effects in part through a concerted modulation of transcriptional programs that include non-coding and protein-coding RNA functions. The aims of this pilot study were: 1, Establishing a catalog of tens of thousands previously unexplored, non-coding RNAs and assess their association with Parkinson’s precisely in the preferentially vulnerable cell-type --- dopaminergic neurons laser-captured from human substantia nigra --- using massively parallel sequencing and unlimited ab initio reconstruction of the whole transcriptome 2, Beginning to delineate the genetic variability that underlies differences in non-coding RNA abundance and processing in human nigral dopamine neurons using transcriptome genetics. 3, Evaluating PD-relevant transcripts as potential biomarkers in a pilot study of blood samples from patients with PD and controls enrolled in the Harvard Biomarker Study.

In this study we were able to provide a first look at the “dark universe” of thousands of short and long non-coding RNAs expressed in the dopamine neuron of normal brains and in Parkinson’s disease. Moreover, the study begins to clarify the role of genetic variants in dopamine neurons and to translate transcripts associated with PD-neuropathology. Finally, we provide proof of the concept that select RNAs associated with Parkinson’s in vulnerable dopamine neurons can be translated into personalized, dual-use, peripheral biomarkers. Validated biomarkers will be clinically relevant for identifying PD-vulnerable warfigthers and civilians, and for monitoring neurotoxin exposure and therapeutic response.

2. Keywords

Parkinson’s disease, non-coding RNA, RNA sequencing, expression Quantitative Trait Locus, biomarkers
3. Overall project summary

Aim 1. Delineating non-coding RNAs associated with Parkinson’s in nigral dopamine neurons (Fig. 1)

33% of all unique RNAs robustly detected in nigral dopamine neurons are long or short non-protein-coding RNAs (ncRNAs) (Fig. 1a). In control brains, 8,493 ncRNAs many of which had never before been described in human brain were identified by ab initio transcriptome reconstruction (Fig. 1b, stacked colored bar). These include short ncRNAs (e.g. 17 snoRNAs and 11 snRNAs) as well as...
thousands of long, regulatory, ncRNAs (e.g. 2,846 pseudogenes, 2,806 lincRNAs, and 2,454 natural antisense RNAs.

121 non-coding RNAs (Fig. 1c, d) were statistically significantly differentially expressed in dopamine neurons laser-captured from substantia nigra pars compacta of 19 patients with PD compared to 62 controls without PD with genome-wide significance (adjusted for multiple testing using the false discovery rate method) and adjusting for the pertinent covariates of age, gender, RIN number, postmortem interval, and batch). These PD-associated non-coding RNAs included 77 expressed pseudogenes, 22 lincRNAs, 17 antisense transcripts, as well as sense-intronic, sense-overlapping, and other non-coding RNAs. Moreover, we identified 1,178 protein-coding mRNAs significantly over- or under-expressed in dopamine neurons captured from brains of patients with PD (Fig. 1c, d). The scatter plot in Fig. 1d visualizes non-coding and coding RNAs more than three-fold differentially expressed in dopamine neurons of patients with PD compared to controls. Non-coding RNAs are shown in magenta. The lincRNA, LINC00674 and the expressed pseudogene UQRBP1 are highlighted in Fig. 1d and in the box plots in Fig. 1e. Moreover, expression changes in two genes, which when mutated cause familial PD, LRRK2 and DNAJC61,2 (Fig. 1d, e) were significant in dopamine neurons of patients with PD compared to controls.

Expression of the dopamine neuron-specific ALDH1A1 gene is lost in neurons from patients with Parkinson’s (Fig. 2).

We compared the RNA content of dopamine neurons and pyramidal neurons laser-captured from control brains. Variance- and sex- adjusted differential expression analysis was performed. 115 genes were exclusively transcribed in dopamine neurons and not in pyramidal neurons with $P < 10^{-6}$. Fig. 2a shows the top-ranked 22 dopamine neuron-specific genes (black) and the top 5 pyramidal neuron-specific genes (magenta). Confirming the cell-type precision of our method, these dopamine-neuron specific transcripts included the established parts of the dopamine metabolism machinery, the vesicular monoamine transporter 2 (VMAT2), dopamine receptor D2 (DRD2), tyrosine hydroxylase (TH), and dopamine decarboxylase (DDC). Surprisingly, the by far most abundant dopamine neuron-specific transcript however was Aldehyde Dehydrogenase 1A1 (ALDH1A1). ALDH1A1 was significantly underexpressed in dopamine neurons of cases with PD compared to dopamine neurons of controls ($P = 10^{-5}$; Fig. 2b). ALDH1A1 mediates the oxidation of toxic dopamine-3,4-dihydroxyphenylacetaldehyde (DOPAL) into non-toxic 3,4-dihydroxyphenylacetic acid (DOPAC). In mice ALDH1A1 overexpression suppresses A53T-α-synuclein-induced dopaminergic neurodegeneration and ALDH1A1 knockout enhances dopaminergic neurodegeneration in model mice 3. ALDH1A1 expression might also be reduced in blood of patients with PD 4.

Aim 2. Delineating the genetic variants regulating non-coding RNAs in nigral dopamine neurons. In order to elucidate the relationship between genetic variation and gene expression, we have developed a strategy by combining PEER5 and Matrix-eQTL6 to conduct expression quantitative trait loci (eQTL) analysis. In brief, we normalized the gene expression by adjusting both known and
hidden factors learnt from the Bayesian framework PEER, then used Matrix-eQTL to call significant cis-eQTL on the residuals of all genes for both genotyped and imputed SNPs.

Expression of several genes was associated with multiple cis-acting SNPs (eSNPs). The expression of 118 genes was linked to 5,024 genetic variants with a false discovery rate (FDR) < 0.05. These “e-genes” included 47 protein-coding genes and a surprising number of non-coding genes, including 30 expressed pseudogenes, 18 antisense genes, and 15 lincRNAs. 2,602 eSNPs were associated with RP11-707O23.5 expression, a pseudogene of MAPK8IP1 (mitogen-activated protein kinase 8 interacting protein 1). 51 eSNPs overlapped with PD GWAS risk SNPs in the MAPT susceptibility locus. Importantly, eQTL analysis suggests that these variants (although physically located in the proximity of the MAPT locus) do not regulate expression of the MAPT gene, but instead of three other genes physically located nearby (i.e. RP11-707O23.5 with FDRs as low as 1.08^{-4}, KANSL1-AS1 with FDR of 0.013, and LRRC37A2 with FDR of 0.0014).

When looking at the regression of gene expression on genotype, we observed distinct patterns relating minor allele dosage to expression. For example, expression levels of the lincRNA AP000350.5 negatively correlated with minor allele dosage of eSNP rs5760099 (Fig. 3a), suggesting a repressive effect of the minor allele. By contrast, minor allele dosage of the genetic variant rs7955007 was associated with increased expression of RP11-726G1.1 (a processed transcript that overlaps with a piRNA; Fig. 3b), suggesting an activating, additive effect. We also observed eSNP-gene expression pairs with an apparently dominant, dosage-independent relation (Fig. 3c). For example, expression levels of the antisense transcript TMLHE-AS1 showed a binary increase associated with the presence (vs. absence) of the minor allele for rs73569697, but not with its dosage.

Aim 3. Evaluating PD neuropathology-linked RNAs as personalized blood biomarkers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mean mRNA counts in blood of patients with PD (SD)</th>
<th>Mean mRNA counts in blood of healthy controls (SD)</th>
<th>Difference of means in blood (%)</th>
<th>P-value* (in blood)</th>
<th>P-value* (in dopamine neurons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNCA</td>
<td>2,117 (1,732)</td>
<td>3,042 (2,030)</td>
<td>- 30.4%</td>
<td>3.8x10^{-15}</td>
<td>0.02</td>
</tr>
<tr>
<td>LRRK2</td>
<td>10,531 (3,451)</td>
<td>9,352 (3,159)</td>
<td>12.6%</td>
<td>4.7x10^{-08}</td>
<td>0.001</td>
</tr>
<tr>
<td>CHMP3</td>
<td>2,259 (495)</td>
<td>2,135 (442)</td>
<td>5.8%</td>
<td>4.5x10^{-05}</td>
<td>0.01</td>
</tr>
<tr>
<td>COMT</td>
<td>390 (81)</td>
<td>405 (97)</td>
<td>- 3.7%</td>
<td>0.02</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* P value derived from generalized linear models analysis adjusting for age at baseline, gender, RNA Integrity Number, white blood cell counts, red blood cell counts, and platelet counts. * P value comparing transcript counts in dopamine neurons of cases vs. controls adjusting for age, gender, RNA Integrity Number, and batch number.

We evaluated the feasibility of translating transcripts associated with PD neuropathology in dopamine neurons of human brain (from Aim 1) into potentially clinically useful biological markers detectable in peripheral blood.

We performed a cross-sectional study using blood samples from cases representing untreated de novo PD as well as treated early-stage PD and age- and sex-matched healthy controls enrolled in...
the Harvard Biomarker Study (HBS)\textsuperscript{7-12} --- a study of >2,500 participants with detailed clinical phenotypes and longitudinal biospecimens collection co-founded and co-directed by Dr. Scherzer. PD patients met stringent UK PD Brain Bank Society's (UKPDBBS) diagnostic criteria and were at an early-stage of the disease as defined by a Modified Hoehn & Yahr stage \( \leq 3 \) assessed by a movement disorders specialist. This single-center study followed stringent rules of evidence for molecular marker discovery and validation and was made possible by our existing collection of high-quality RNA already extracted from blood samples of >2,500 individuals enrolled in HBS.

Excitingly, in this proof-of-concept pilot we were already able to translate four transcripts that are pathologically expressed in laser-captured dopamine neurons of patients with PD into peripheral, easily accessible possible blood biomarkers. These transcripts are \( \alpha \)-synuclein (\( SNCA \)) and \( LRRK2, \ COMT, \) and \( CHMP3 \) (Table 1 and Fig. 4). These four transcripts were statistically significantly differentially expressed in dopamine neurons of patients with PD compared to controls based on our laser-capture, total RNAseq method (from Aim 1; Table 1) as well as in peripheral blood samples from the Harvard Biomarker Study (HBS) on the precise Nanostring platform (Fig. 4). The \( P \) values for associations in blood as well as in dopamine neurons are shown in Table 1. Importantly, two of these four transcripts are also genetically linked to familial and sporadic PD: \( \alpha \)-synuclein (\( SNCA \)) and \( LRRK2 \).

The \( SNCA \) transcript was further evaluated in neuroimaging-supported, early stage PD on the qPCR, microarray, and NanoString platform in the PROBE and PPMI study, in addition to HBS. We found that \( SNCA \) transcripts in circulating blood cells were reduced in early stage, untreated, and dopamine transporter neuroimaging-supported Parkinson's disease (PD) in three independent regional, national, and international populations representing 500 cases and 363 controls and on three analog and digital platforms with \( P = 0.0001 \) in meta-analysis. Individuals with \( SNCA \) transcripts in the lowest quartile of counts had an odds ratio for PD of 2.45 compared to individuals in the highest quartile. Disease-relevant transcript isoforms were low even near disease onset. Importantly, low \( SNCA \) transcript abundance predicted cognitive decline in patients with Parkinson's during up to five years of longitudinal follow-up. These important findings on \( SNCA \) were published in the prestigious journal Brain in August of 2015 (Locascio et al., Brain, 2015; the reprint is attached in the Appendix).

The other three transcripts - \( LRRK2, \ COMT, \) and \( CHMP3 \) - significantly associated with early stage PD in blood (Fig. 4) are also directly relevant to the neuropathology with significant expression changes in dopamine neurons (Table 1). \( LRRK2 \) is genetically strongly implicated in both familial and sporadic PD. \( COMT \) transcripts encode catechol-O-methyl-transferase, an essential gene in the metabolism of L-dopa. Finally, the vacuolar Protein Sorting 24 Homolog (\( CHMP3 \)) was pathologically expressed in our RNAseq analysis of dopamine neurons from patients with PD. It is a novel PD-associated transcript, to our knowledge previously not linked to Parkinson’s. Interestingly, the gene \( CHMP3 \) encodes a protein that sorts transmembrane proteins into lysosomes/vacuoles via the multivesicular body pathway. It is part of endosomal sorting complexes required for transport (ESCRT). This pathway is implicated in the pathogenesis of sporadic PD\textsuperscript{13}.

This study provides proof of the concept that select RNAs associated with Parkinson’s in vulnerable dopamine neurons can be translated into personalized, dual-use, peripheral biomarkers.
4. Key research accomplishments

- This study provides the first comprehensive catalog of gene expression - both protein-coding and non-coding - in a specific cell type, not in cultured cells or mice, but in situ in human brain.

- Going beyond traditional mRNA sequencing, all polyadenylated and non-polyadenylated transcripts over 50 bases were ultra deeply sequenced using ribo-depleted RNA from dopamine neurons laser-captured from human post-mortem brains of patients with PD and controls using a new method we developed, termed laserRNaseq.

- A vast universe of annotated as well as novel non-coding RNAs expressed in dopamine neurons was discovered suggesting a more diverse and much more complex transcriptional architecture than previously imagined.

- The expression of 121 non-coding transcripts and more than a thousand protein-coding transcripts in nigral dopamine neurons was significantly associated with Parkinson’s Lewy body neuropathology.

- Expression of the gene ALDH1A1 that plays a critical role in scavenging toxic dopamine metabolites is lost in dopaminergic neurons laser-captured from patients with PD.

- This study provides proof of the concept that select RNAs associated with Parkinson’s in vulnerable dopamine neurons can be translated into personalized, dual-use, peripheral biomarkers. Specifically, we were able to translate four transcripts (SNCA, LRRK2, COMT and CHMP3) that were abnormally transcribed in laser-captured dopamine neurons of PD brains into potential blood biomarkers for patients with Parkinson’s.

5. Conclusion

This study provides a first look at the “dark universe” of tens of thousands of short and long non-coding RNAs expressed in the human dopamine neuron of controls and in Parkinson’s disease. We show unequivocally that non-coding RNAs are abundantly transcribed in dopamine neurons of human brain. We identify highly significant changes in the expression of both protein-coding and non-coding transcripts in Parkinson’s disease. These changes indicate key roles for non-coding RNAs and for the protein-coding familial PD gene DNAJC6 and ALDH1A1 in the survival of dopamine neurons in Parkinson’s. ALDH1A1 mediates the oxidation of toxic dopamine-3,4-dihydroxyphenylacetaldehyde (DOPAL) into non-toxic 3,4-dihydroxyphenylacetic acid (DOPAC). In mice ALDH1A1 overexpression suppresses A53T-α-synuclein-induced dopaminergic neurodegeneration and ALDH1A1 knockout enhances dopaminergic neurodegeneration.

By translating four transcripts (that were found to be pathologically expressed in dopamine neurons of patients with PD) into potential biomarkers accessible in blood of patients and controls, we provide proof of concept for the idea that the activity of select PD-relevant genes can be precisely monitored in blood tests. In support of this view we show robust evidence indicating significant associations between SNCA, LRRK2, COMT, and CHMP3 mRNA counts in blood and Parkinson’s using precise digital gene expression analysis with molecular barcodes on the NanoString platform. Additionally, previous reports indicate that ALDH1A1 expression might also be reduced in blood of patients with PD.

In future steps, we will continue to delineate all regions of the human genome actively transcribed in dopamine neurons. We will fully decode all non-coding RNAs active in dopamine neurons. We will extend our investigation from known non-coding RNAs annotated in the GENECODE database to the characterization of thousand of novel, previously not described non-coding RNAs emerging from these analyses (such as novel transcribed enhancers or eRNAs and new classes of...
RNA biotypes). We will increase statistical power and scientific knowledge by extending the current observations to larger numbers of high-quality brains, to pre-clinical Lewy body neuropathology, and to additional brain cell-types (e.g. pyramidal cells). Genetically, as our sample size becomes larger we will be increasingly powered to uncover how genetic risk variants causally contribute to the degeneration of dopamine neurons via dysregulation of cell-type-specific transcription. Clinically, we will continue to translate PD-associated transcripts into peripheral tests in increasingly large\textsuperscript{6} and definitive studies. Moreover, we aim to assemble validated transcriptional and genetic markers into polygenic test panels with improved sensitivity and specificity. Validated biomarkers will be clinically relevant for identifying PD-vulnerable warfigthers and civilians, and for monitoring neurotoxin exposure and therapeutic response in clinical trials and eventually, clinical practice.
6. Publications since submission of the original application (chronologic order; *, denotes corresponding or co-corresponding author)


Presentations

2013 Can BIG DATA solve big questions?
Movement Disorders Conference, Department of Neurology
Massachusetts General Hospital
Boston, MA

2014 Can BIG data help neurological disease?
Massachusetts General Hospital, J. Philip Kistler Stroke Center Meeting
Massachusetts General Hospital, Boston, MA

2014 From BIG data to personalized medicine for Parkinson’s
Icahn School of Medicine at Mount Sinai
New York, NY

2014 Mapping transcriptome space in Parkinson's: from the known world to an expanding universe
Molecular Pathology Seminar
Johns Hopkins University, Baltimore, MD

2014 The PD Brain Map: How does the human genome function in dopamine neurons?
NIH/NINDS Parkinson’s Disease Genetics and Systems Biology Workshop
Bethesda, MD

2014 Human genome function in Parkinson’s
Neurology Department, Invited Lecture
Goethe University, Frankfurt, Germany

2015 Parkinson’s disease: How Big Data will enable a future personal medicine
Genzyme
Framingham, MA

Abstracts

2013 The PD Brain Map Project: Mapping the Transcriptional Architecture of Dopamine Neurons in Human Brain
Annual Meeting of the American Society for Human Genetics
Boston, MA

2015 Functional characterization of the human brain transcriptome in Parkinson's disease
European Molecular Biology Laboratory Meeting
Heidelberg, Germany

2015 BRAINCODE: How Does the Human Genome Function in Specific Brain Neurons?
Annual Meeting of the American Society for Human Genetics
Baltimore, MD

7. Inventions, Patents and Licenses - Nothing to report

8. Reportable Outcomes - Nothing to report
9. Other Achievements

2015 - Scientific Advisory Board, American Parkinson Disease Association


10. References


11. Appendix

Association between α-synuclein blood transcripts and early, neuroimaging-supported Parkinson's disease


Association between α-synuclein blood transcripts and early, neuroimaging-supported Parkinson’s disease

Joseph J. Locascio,1,2,* Shirley Eberly,3,* Zhixiang Liao,1,4,* Ganqiang Liu,1,4 Ashley N. Hoesing,1,4,5 Karen Duong,1,4,5 Ana Trisini-Lipsanopoulous,1,4,5 Kaltra Dhima,1,4,5 Albert Y. Hung,2 Alice W. Flaherty,2,6 Michael A. Schwarzschild,2 Michael T. Hayes,7 Anne-Marie Wills,2,5 U. Shivraj Sohur,2 Nite I. Mejia,2 Dennis J. Selkoe,4,7 David Oakes,3 Ira Shoulson,8 Xianjun Dong,1,4 Ken Marek,9 Bin Zheng,1,4 Adrian Ivinson,4,5 Bradley T. Hyman,2,5 John H. Growdon,2 Lewis R. Sudarsky,7 Michael G. Schlossmacher,10 Bernard Ravina11 and Clemens R. Scherzer1,2,4,5,7

*These authors contributed equally to this work.

There are no cures for neurodegenerative diseases and this is partially due to the difficulty of monitoring pathogenic molecules in patients during life. The Parkinson’s disease gene α-synuclein (SNCA) is selectively expressed in blood cells and neurons. Here we show that SNCA transcripts in circulating blood cells are paradoxically reduced in early stage, untreated and dopamine transporter neuroimaging-supported Parkinson’s disease in three independent regional, national, and international populations representing 500 cases and 363 controls and on three analogue and digital platforms with \( P < 0.0001 \) in meta-analysis. Individuals with SNCA transcripts in the lowest quartile of counts had an odds ratio for Parkinson’s disease of 2.45 compared to individuals in the highest quartile. Disease-relevant transcript isoforms were low even near disease onset. Importantly, low SNCA transcript abundance predicted cognitive decline in patients with Parkinson’s disease during up to 5 years of longitudinal follow-up. This study reveals a consistent association of reduced SNCA transcripts in accessible peripheral blood and early-stage Parkinson’s disease in 863 participants and suggests a clinical role as potential predictor of cognitive decline. Moreover, the three independent biobank cohorts provide a generally useful platform for rapidly validating any biological marker of this common disease.

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Introduction

Nearly 5 million people have Parkinson’s disease, a sporadic, progressive neurodegenerative disorder linked to a complex genetic architecture and environmental exposures. Over the next 15 years, this number will almost double to 9 million patients worldwide (Dorsey et al., 2007). Already today, the costs to public health are enormous—estimated at $10.8 billion annually in the USA alone (O’Brien et al., 2009). There are no cures or disease-modifying therapies for Parkinson’s disease, and this may be due in part to our inability to monitor biological markers of early disease. Currently, patients with Parkinson’s disease are diagnosed, cared for, and assigned to clinical trials based on medical history and physical exam.

To translate research progress into the clinic, simple, dynamic biomarkers are needed that are rooted in genetic discoveries. Traditionally, Parkinson’s disease has been approached from a clinical perspective, as a homogeneous movement disorder typified by the triad of resting tremor, bradykinesia and rigidity. Based on this clinical view of the disease, it was postulated that a single laboratory or imaging biomarker for Parkinson’s disease would be developed to serve as a universal diagnostic. This goal has been elusive. More recently, a human genome-inspired view of Parkinson’s disease has provided unequivocal evidence for a diversity of genetic variants, each exacting a small, but definitive effect, underlying the clinical phenotype (Nalls et al., 2014). It is becoming clearer that Parkinson’s disease is aetiologically, genetically, pathologically and even clinically more heterogeneous than previously implied (Scherzer and Feany, 2004; Scherzer et al., 2004; Forman et al., 2005). In light of this diversity the traditional expectation of a ‘one size fits all’ laboratory diagnostic that can detect all patients and answer all clinical questions may need to be revised. Personalized biomarkers, tailored to patient endophenotypes and specific clinical applications, are likely to emerge. They may more appropriately describe the molecular architecture of the disease.

What are the practical applications that such biomarkers may serve, if they cannot be used as a universal diagnostic? Prognostic and mechanism-directed biomarkers will help to stratify patients for clinical trials. Risk markers are required for identifying high-risk individuals prior to the onset of overt clinical symptoms, when disease-modifying interventions will be most effective. Pharmacodynamic markers are required to determine whether an investigational drug engages the intended target. In cancer drug development, biomarker use has increased average phase III trial success by 34% (Hayashi et al., 2013) and shortened total drug development timelines (Chin et al., 2011). Emerging CSF tests for Parkinson’s disease (Mollenhauer et al., 2011) and dopamine transporter imaging are relatively invasive or time-consuming and expensive. Blood-based tests are attractive, but no disease-causing molecule has been rigorously validated.

Accumulation of α-synuclein protein (encoded by the SNCA gene) in the brain is a hallmark of Parkinson’s disease (Braak et al., 2002). For nearly two decades since its characterization as an electric ray protein, the Parkinson’s disease gene α-synuclein was thought of as ‘neuron-specific’ (Maroteaux et al., 1988), but this view was disproven with the discovery of high levels of α-synuclein expression in blood cells, particularly those of erythroid lineage (Nakai et al., 2007; Scherzer et al., 2008; Maitta et al., 2011). Here we tested whether levels of SNCA transcripts (mRNAs) in circulating blood cells might serve to identify individuals with increased risk of Parkinson’s disease. We evaluated whether levels of α-synuclein gene (SNCA) transcripts are associated with the presence of early-stage, clinically and neuroradiologically confirmed Parkinson’s disease in a platform of three independent biomarker study cohorts.

Materials and methods

Brief methods are summarized below. See the online Supplementary material for detailed clinical, laboratory and statistical experimental methods for the Harvard Biomarker Study (HBS), Prognostic Biomarkers in Parkinson’s Disease Study (PROBE) and Parkinson’s Progression Markers Initiative (PPMI), respectively, and for the meta-analysis across three studies.

Biospecimen collections

Briefly, the studies were designed to minimize bias from sample processing by collecting, handling and analysing specimens of cases and controls in a standardized manner following defined operating procedures and according to rules of evidence (Ransohoff, 2004, 2005; Hennecke and Scherzer, 2008). Cases and controls were processed in parallel by technicians blinded to diagnosis to avoid bias due to ‘run order’ of samples. Venous whole blood was collected in PAXgene™ tubes.
Blood and PAXgene™ reagents were mixed by gently inverting 8–10 times.

**RNA isolation and quality control**

RNA was extracted according to the PAXgene™ Blood RNA kit manual extraction protocol including DNase treatment. RNA quality was determined with the RNA 6000 Nano Chip on an Agilent 2100 Bioanalyzer (Agilent Technologies) and the RNA Integrity Number package (Imbeaud et al., 2005).

**Quantitative PCR and microarray expression analyses**

Quantitative PCR was performed similar to Scherzer et al. (2008). Microarray procedures were performed as described (Zheng et al., 2010) using Illumina HumanHT-12v3 Expression BeadChips. The PROBE microarray data set has been submitted to the GEO database (accession number GSE57475).

**NanoString assay performance**

Probes were designed according to the manufacturer’s design principles (Geiss et al., 2008), including screening for inter- and intra-reporter and capture probe interactions, and selection for probes with optimal melting temperatures (Geiss et al., 2008). Direct counts of the target RNAs were measured in 12.5 ng of RNA by digital expression analysis based on NanoString technology (without reverse transcription into cDNA). Probes for the target and control RNAs were multiplexed and assayed on the nCounter Digital Analyzer. The laboratory running the assay was blinded to the diagnosis. No-template (negative) controls containing water substituted for template were run and no signal was detected. To avoid run-order bias, samples of cases or controls were randomly assigned to plates. To control for plate-to-plate variation and drift, equal amounts of Human Universal Reference RNA were included at the beginning, within, and at the end of the entire experiment. Transcript counts (excluding SNCA-007) measured in reference RNA were highly correlated with R² > 0.999 both within one plate and in-between different plates, thus excluding drift as a potential source of bias in the experiment. Furthermore, 5% of participants’ samples were randomly resampled to verify the retest reliability (technical precision). The average R² was 0.98 for these correlations. The NanoString data are accessible through the PPMI website (http://www.ppmi-info.org).

**Results**

**Harvard Biomarker Study: SNCA mRNA abundance in early-stage Parkinson’s disease**

We first evaluated relative SNCA mRNA abundance in a case-control study nested in the HBS using precise, kinetic, quantitative PCR based on fluorogenic 5’ nuclease chemistry (quantitative PCR). We specifically designed the HBS as a clinical biomarker study with rigorous, predefined collection and processing protocols. Cases and controls had similar ages, but patients with Parkinson’s disease were more likely to be males (Table 1). Cases were at an early stage of the disease, with a mean modified Hoehn and Yahr stage of 2.1. A large majority of cases (201 of 222, 90.5%) were on medications that ameliorate the dopamine deficiency caused by the degeneration of neurons in the substantia nigra, while 9.5% (21 of 222) were untreated, de novo patients. The controls were recruited from the same source population. Case and control samples were collected, processed, and analysed in parallel. Samples were required to meet stringent quality control criteria in order to enter the study including a RNA Integrity Number (RIN) (Auer et al., 2003) threshold of ≥7.5 indicating high RNA quality. The two groups had excellent RNA quality with mean RINs of 8 ± 0.4 versus 8.05 ± 0.4 [mean ± standard deviation (SD), respectively.

Relative SNCA transcript abundance in venous blood was significantly lower for patients with Parkinson’s disease than for controls (difference of 20%; P-value = 0.0004;
Table 2: Blood SNCA expression is associated with early-stage clinical and DAT-neuroimaging supported Parkinson’s disease in three independent populations and on three independent assay platforms

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
<th>Difference of means</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantitative PCR platform</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (total n = 405)</td>
<td>222</td>
<td>183</td>
<td>−20</td>
<td>0.0004</td>
</tr>
<tr>
<td>Unadjusted relative SNCA expression (mean, SD)</td>
<td>51.5 (1.97)</td>
<td>64.2 (1.8)</td>
<td>−20</td>
<td>0.0004</td>
</tr>
<tr>
<td>Adjusted relative SNCA expression (mean, 95% CI)</td>
<td>49 (45–54)</td>
<td>61 (55–68)</td>
<td>−17</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>PROBE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantitative PCR platform</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>n (total n = 118)</td>
<td>76</td>
<td>42</td>
<td>−22</td>
<td>0.025</td>
</tr>
<tr>
<td>Unadjusted relative SNCA expression (mean, SD)</td>
<td>75.6 (2.27)</td>
<td>96.3 (1.7)</td>
<td>−22</td>
<td>0.025</td>
</tr>
<tr>
<td>Adjusted relative SNCA expression (mean, 95% CI)</td>
<td>72 (61–85)</td>
<td>96 (76–122)</td>
<td>−25</td>
<td>0.046</td>
</tr>
<tr>
<td>Microarray platform</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n (total n = 142)</td>
<td>93</td>
<td>49</td>
<td>−18</td>
<td>0.01</td>
</tr>
<tr>
<td>Unadjusted relative SNCA expression (mean, SD)</td>
<td>2846 (1962)</td>
<td>3469 (1622)</td>
<td>−18</td>
<td>0.01</td>
</tr>
<tr>
<td>Adjusted relative SNCA expression (mean, 95% CI)</td>
<td>2452 (2167–2775)</td>
<td>3098 (2635–3644)</td>
<td>−21</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>PPMI</strong></td>
<td></td>
<td></td>
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<tr>
<td>Digital gene expression platform</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>n (total n = 340)</td>
<td>202</td>
<td>138</td>
<td>−21</td>
<td>0.03</td>
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<td>Unadjusted analyses</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3E4-SNCA transcript counts (mean, SD)</td>
<td>13 458 (7812)</td>
<td>15 959 (12 576)</td>
<td>−16</td>
<td>0.08</td>
</tr>
<tr>
<td>E4E6-SNCA transcript counts (mean, SD)</td>
<td>2826 (1860)</td>
<td>3496 (3,119)</td>
<td>−19</td>
<td>0.04</td>
</tr>
<tr>
<td>Long 3’UTR-SNCA transcript counts (mean, SD)</td>
<td>573 (387)</td>
<td>781 (1013)</td>
<td>−27</td>
<td>0.007</td>
</tr>
<tr>
<td>SNCA-007 transcript counts (mean, SD)</td>
<td>23 (16)</td>
<td>25 (21)</td>
<td>−8</td>
<td>0.6</td>
</tr>
<tr>
<td>Adjusted models</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjusted E3E4-SNCA transcript counts (mean, 95% CI)</td>
<td>2409 (2196–2643)</td>
<td>2784 (2495–3107)</td>
<td>−13</td>
<td>0.046</td>
</tr>
<tr>
<td>Adjusted long 3’UTR-SNCA transcript counts (mean, 95% CI)</td>
<td>485 (440–534)</td>
<td>578 (515–647)</td>
<td>−16</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*ILMN-PROBE1 indicates Illumina probe ILMN1701933 that targets the 3’UTR of SNCA.
+ILMN-PROBE2 indicates Illumina probe ILMN1766165 that targets exon 5 of SNCA.

All statistical analyses for quantitative PCR assays were conducted using the natural logarithm of ΔCT values. For presentation purposes in Table 2 and Figs 1 and 2, a linearizing transformation of mean ΔCTs was performed. Values shown are in arbitrary units.

Indicates per cent reduction in expression in cases compared to controls determined using the comparative CT method.

Indicates significance analysis for all measurement values was performed on log-transformed data.

Table 2 and Fig. 1A). Post hoc analysis of the subgroup of untreated, de novo cases showed consistent results with a >20% lower mean relative SNCA transcript abundance in the de novo cases compared to the controls (P-value = 0.01). Counts of the four endogenous reference genes (Fig. 1B) and their geometric mean (that was used to normalize for input RNA) were virtually identical in cases and controls (Fig. 1C). Importantly, complete blood counts in cases and controls could not account for the reduction in relative SNCA mRNA abundance in patients consistent with previous reports by others (Kasten et al., 2010). Specifically, white blood cell counts (Fig. 1D) and platelet counts (Fig. 1F) were unchanged between cases and controls, while red blood cell counts were marginally higher in cases than in controls (P = 0.03; Fig. 1E).

General linear model analysis was performed adjusting for the covariates of counts of white and red blood cells, and gender. In this covariate-adjusted analysis the mean relative abundance of SNCA expression was 17% lower in cases than in controls with P = 0.003.

Odds ratios (OR) for the lowest (first) quartile relative to the highest (fourth) quartile for blood SNCA transcript abundance were associated with Parkinson’s disease status in the unadjusted analysis and remained associated following adjustments for the covariates of age, hours at 4°C, red and white blood cells, and platelets (Model 1) or for gender (Model 2) with odds ratios ranging from 2.14 (95% CI, 1.10–4.14) to 2.15 (95% CI, 1.20–3.82) (Table 3).

**PROBE study**

**Replication of low SNCA mRNA abundance in dopamine transporter imaging-supported Parkinson’s disease**

To be of use in clinical trials, it must be feasible to measure a biomarker in a multicentre study design. We thus further evaluated SNCA mRNA levels in the USA-wide, multicentre PROBE study (Supplementary Table 1). There were no significant baseline inequalities for age, gender, and white and red blood cell counts. Cases had a mean
Hoehn and Yahr stage of 2.0, a mean total UPDRS score of 30.6, and mean disease duration of 5.5 years. The clinical diagnosis was supported by dopamine transporter (DAT) imaging with $^{123}$I-$\beta$-CIT. A subset of 76 cases with Parkinson’s disease and 42 controls with available high-quality RNA were analysed by quantitative PCR. The mean relative abundance of $\alpha$-synuclein ($\alpha$-Syn) expression was 22% lower in cases than in controls with $P = 0.025$ in the unadjusted analysis (Table 2 and Fig. 2A). This remained significant after adjusting for the covariates of white and red blood cells, and gender with $P = 0.046$. Quantitative PCR allows for precise relative, but not absolute quantification. Thus, the difference in $\alpha$-Syn mRNA abundance in cases relative to controls found in PROBE was comparable to the 20% difference seen in HBS (i.e. in Figs 1A and 2A), while the absolute expression values (in arbitrary units) were not directly comparable due to different calibrators.

We confirmed these expression changes using two $\alpha$-Syn probes represented on a secondary, microarray expression platform. Results for both probes were available for 93 cases and 49 controls enrolled in PROBE and confirmed that relative blood $\alpha$-Syn expression was lower in cases compared to controls (Table 2 and Fig. 2B and C). Results for ILMN-PROBE1 confirmed an association between $\alpha$-Syn expression and Parkinson’s disease adjusting for the covariates of white and red blood cell counts, and gender with $P = 0.02$. The association was further confirmed by ILMN-PROBE2 with $P = 0.03$, again adjusting for the same covariates.

Individuals with $\alpha$-Syn transcript abundance in the lowest quartile of values had an OR for Parkinson’s disease of 4.5 (95% CI, 1.3–15.0) compared to individuals in the highest quartile of values (Table 3). The corresponding odds ratios for the two microarray probes were 4.4 (95% CI, 1.4–14.5) and 4.6 (95% CI, 1.5–13.7), respectively. Thus, measuring blood $\alpha$-Syn expression was feasible in a multicentre study and reduced blood $\alpha$-Syn expression in Parkinson’s disease was confirmed in this independent population on two distinct analysis platforms.
Reduced abundance of disease-relevant SNCA isoforms already near disease onset

These associations prompted us to further extend and verify our findings in patients near disease onset, when individual motor symptoms are just beginning to manifest. Two hundred and two cases with de novo motor Parkinson’s disease, with less than 2 years of disease and with confirmed loss of dopaminergic substantia nigra terminals by DAT imaging and 138 age- and sex-matched controls without neurologic disease and with normal DAT imaging, who met stringent RNA quality-control criteria (RIN ≥ 7.3) were included. Key eligibility criteria for PPMI require the presence of asymmetric resting tremor or asymmetric bradykinesia, a supportive DAT scan, and a diagnosis of Parkinson’s disease for 2 years or less, without current dopamine replacement medication (Parkinson Progression Marker Initiative, 2011). Patients therefore were clinically at a very early stage and did not yet necessarily fulfill the standard, full clinical diagnostic criteria of the UK Parkinson’s Disease Society Brain Bank that require bradykinesia and a second mandatory clinical cardinal sign and three supportive clinical criteria to be present for diagnosis (Hughes et al., 1992). Cases and controls were frequency matched for age and gender (Supplementary Table 2). The PPMI population was remarkable for the virtual absence of Parkinson’s disease medications, and the equal distribution of potentially confounding variables of age, gender, and blood cell counts among cases and controls (Supplementary Table 2 and Fig. 3G–I).

The advent of digital expression analysis with molecular barcodes (Geiss et al., 2008; Malkov et al., 2009) allowed us to count the abundance of SNCA transcripts directly in RNA from blood cells, without the need for reverse transcription or PCR amplification. No signal was detected in controls lacking template. To avoid run-order bias, samples of cases or controls were randomly assigned to plates.

### Table 3

The odds ratio for Parkinson’s disease prevalence is increased in individuals with blood SNCA expression in the lowest quartile of values compared to individuals with SNCA expression in the highest quartile of values in three study populations

<table>
<thead>
<tr>
<th></th>
<th>Quartile</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HBS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative SNCA mRNA abundance</td>
<td>5–37a</td>
<td>37–60a</td>
<td>60–91a</td>
<td>91–294a</td>
<td></td>
</tr>
<tr>
<td>Parkinson’s disease (n = 222), n/total (%)</td>
<td>67/222 (30)</td>
<td>58/222 (26)</td>
<td>50/222 (23)</td>
<td>47/222 (21)</td>
<td></td>
</tr>
<tr>
<td>Healthy control (n = 183), n/total (%)</td>
<td>34/183 (18.6)</td>
<td>43/183 (23.5)</td>
<td>51/183 (27.9)</td>
<td>55/183 (30)</td>
<td></td>
</tr>
<tr>
<td>Unadjusted OR, (95% CI)b</td>
<td>2.31 (1.31–4.1)</td>
<td>1.58 (0.91–2.75)</td>
<td>1.15 (0.66–1.99)</td>
<td>1 (         )</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.0036</td>
<td>0.11</td>
<td>0.63</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Model 1, OR (95% CI)c</td>
<td>2.14 (1.10–4.14)</td>
<td>1.86 (1.01–3.42)</td>
<td>1.07 (0.6–1.92)</td>
<td>1 (         )</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.024</td>
<td>0.046</td>
<td>0.82</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Model 2, OR (95% CI)d</td>
<td>2.15 (1.2–3.82)</td>
<td>1.61 (0.92–2.83)</td>
<td>1.12 (0.64–1.96)</td>
<td>1 (         )</td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>0.0096</td>
<td>0.098</td>
<td>0.69</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**PROBE**

|                  |          |            |            |            |            |
| Relative SNCA mRNA abundance | 16–52a   | 56–79a     | 84–119a     | 119–2702a  |
| Parkinson’s disease (n = 76), n/total (%) | 24/76 (32) | 20/76 (26)  | 17/76 (22)  | 15/76 (20) |
| Healthy control (n = 42), n/total (%) | 5/42 (12)  | 10/42 (24)  | 13/42 (31)  | 14/42 (33) |
| OR (95% CI)f | 4.5 (1.3–15.0) | 1.9 (0.7–5.3) | 1.2 (0.4–3.4) | 1 (         ) |
| P-value           | 0.02     | 0.90       | 0.25       | 1          |

**PPMI**

|                  |          |            |            |            |            |
| Long-3’UTR-SNCA transcript counts | 59–328   | 335–497    | 505–786    | 796–10861  |
| Parkinson’s disease (n = 202), n/total (%) | 58/202 (29) | 48/202 (24) | 55/202 (27) | 41/202 (20) |
| Healthy control (n = 138), n/total (%) | 27/138 (19.6) | 37/138 (26.8) | 30/138 (21.7) | 44/138 (31.9) |
| Unadjusted OR, (95% CI)g | 2.3 (1.2–4.3) | 1.4 (0.8–2.5) | 1.97 (1.1–3.6) | 1 (         ) |
| P-value | 0.009    | 0.28       | 0.03       | 1          |
| Adjusted OR (95% CI)h | 2.1 (1.1–4.0) | 1.4 (0.8–2.6) | 2.1 (1.1–3.9) | 1 (         ) |
| P-value | 0.02     | 0.25       | 0.02       | 1          |

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a All statistical analyses for quantitative PCR assays were conducted using ΔCT values. For presentation purposes the ranges of each quartile of values shown were linearly transformed, e.g. 2(lowest)ΔCT and 2(highest)ΔCT.
b Unadjusted odds ratio (OR) and confidence intervals (CI) were calculated using logistic regression models comparing the risk of each of quartiles 1 to 3 to the reference quartile 4 (highest relative SNCA transcript abundance).
c Model 1 consists of covariates age, hours in 4°C, red blood cells, platelets, and white blood cells in addition to the SNCA quartiles.
d Model 2 consists of covariate sex in addition to the SNCA quartiles.
e PROBE results on quantitative PCR platform are shown, similar results were obtained on the microarray platform.
f No adjustments were indicated.
g Model consists of the covariate white blood cells in addition to the quartiles of long 3’UTR-SNCA counts.
Transcript counts (excluding SNCA-007) measured in reference RNA were highly correlated with $R^2 > 0.999$ both within one plate and in-between different plates, thus excluding drift as a potential source of bias. Furthermore, 5% of participants’ samples were randomly resampled to verify the retest reliability (technical precision). The average $R^2$ was 0.98 for these correlations.

Alternative SNCA transcript isoform usage has been suggested as a potential convergent mechanism in Parkinson’s disease pathology (Rhinn et al., 2012). We therefore designed multiple probes (Supplementary Fig. 1) to target the boundaries of exon 3 and exon 4 (henceforth termed E3E4-SNCA transcript isoform), transcripts specifically with a long 3’UTR region (which may redirect α-synuclein towards mitochondria; Rhinn et al., 2012), transcripts that skip exon 5 (termed E4E6-SNCA transcript isoform) and are translated into a truncated, aggregation-prone 112 amino acid protein (Murray et al., 2003), or the rare short SNCA-007 transcript isoform that comprises exons 1–4. Relative counts of long 3’UTR-SNCA transcripts were reduced by 27% in cases with Parkinson’s disease compared to controls in the unadjusted analysis with $P = 0.007$ (Table 2 and Fig. 3A). This was confirmed with a second digital molecular probe directed at a different target sequence in long-3’UTR-SNCA transcripts. The relation between long-3’UTR-SNCA transcripts and Parkinson’s disease remained significant with a $P$-value of 0.02 after adjusting for the covariates of white blood cell, red blood cell, and gender (Table 2). Counts of exon 5-skipping transcripts were also associated with Parkinson’s disease in unadjusted and adjusted analyses with $P$-values of 0.04 and 0.046, respectively (Table 2 and Fig. 3B). Counts for E3E4-SNCA transcripts were 16% lower in cases compared to controls but did not reach significance. Counts of the rare SNCA-007 transcript did not significantly differ between cases and controls. To estimate whether SNCA expression is specially associated with Parkinson’s disease or whether Parkinson’s disease-linked transcripts are generally perturbed in circulating blood cells of patients with Parkinson’s disease, we examined the expression of loci linked to familial Parkinson’s disease [PARK7 (also known as DJ-1) and PARK15 (also known as FBX07)] and ZNF746 (implicated in mediating the effects of mutant PARK2; Shin et al., 2011). Furthermore, the QDPR gene produces an essential cofactor for dopamine biosynthesis and was also evaluated. These Parkinson’s disease-linked transcripts were similarly expressed in cases and controls with no or minimal fold-changes of <5% and non-significant $P$-values [although one of five PARK15/FBX07 isoforms tested (FBX07-001) showed a marginal trend with $P = 0.06$]. For example, mean counts for PARK7 mRNAs were 1144 in de novo cases and 1160 in controls (1.5% change, $P = 0.4$; Fig. 3C). Counts (Fig. 3D and E) and geometric mean (Fig. 3F) of the six endogenous reference genes (used to normalize for input RNA) were nearly identical in cases and controls. Cases and controls did not significantly differ in white cells, red cells, and platelets counts (Fig. 3G–I).

Individuals with long-3’UTR-SNCA transcript counts in the lowest quartile of values had an OR $> 2$ for Parkinson’s disease compared to individuals in the highest quartile of values in both the unadjusted analysis and analyses adjusted for white blood cell count, the only covariate retained in a step-wise backward elimination procedure (Table 3).

**Risk of Parkinson’s disease associated with relative SNCA transcript abundance**

Clinically useful biological markers of complex diseases can have modest areas under the curve (AUC) and a wide range
of values that overlap in subjects with a clinical disease and in those without it (Manolio, 2003). Consistent with this notion, SNCA transcripts showed AUCs for Parkinson’s disease in the range of a possible risk marker. The AUC for SNCA transcripts as a predictor of Parkinson’s disease was 0.6 in HBS, ranged from 0.63 to 0.66 in PROBE (for quantitative PCR and microarray probes, respectively), and was 0.58 in PPMI.

A meta-analysis was made possible by the similar collection protocols between the cohorts and allowed for powerfully combining the three studies to estimate the association between SNCA transcript abundance and risk of early-stage Parkinson’s disease. It increased the sample size to 500 cases and 363 controls (total of 863). SNCA variables estimated on quantitative PCR and digital expression platforms were standardized and ranked. The covariates of

Figure 3  PPMI: disease-relevant SNCA isoforms are already reduced near disease onset. (A and B) SNCA isoforms were probed on a highly robust digital expression platform in 202 cases with de novo motor Parkinson’s disease (PD), with less than 2 years of disease and with confirmed loss of dopaminergic substantia nigra terminals by DAT imaging and 138 age- and sex-matched controls (HC) without neurological disease and normal DAT imaging participating in PPMI. Unadjusted counts of disease-relevant SNCA transcript isoforms with long 3′ UTR (A) or skipping exon 5 (B), were reduced by 27% and 19%, respectively, in de novo cases compared to controls (P = 0.007 and 0.04, respectively). (C) By contrast mean counts for the gene PARK7 (D-J) mutated in rare autosomal recessive Parkinson’s disease were not significantly different in de novo cases with sporadic Parkinson’s disease compared to controls (difference of 1.5%). Box plots visualize first, third quartiles and means; the ends of the whiskers represent the lowest (or highest) value still within 1.5-times the interquartile range. (D–F) Reference genes were stably expressed in blood cells of cases compared to controls. Counts of the six endogenous reference genes used in PPMI (D and E) were virtually identical in the 202 cases and 138 controls. Mean counts (standard error) are shown for cases (orange bars) and controls (grey bars). (F) The geometric mean of these six endogenous reference genes was used to normalize for input RNA. It was nearly identical in cases and controls. (G–I) White (WBC) and red (RBC) blood cell, and platelet counts did not differ significantly between cases and controls. Bar graphs show means and standard errors.
white and red blood cell count, gender, and age were adjusted for in the meta-analysis. For PPMI we first conservatively included expression measures for the generic probe (E3E4-SNCA) that targets most SNCA transcripts. In a second iteration, we included the specific probe targeting long 3’UTR-SNCA. In both scenarios the association between SNCA blood transcripts and early Parkinson’s disease was highly significant with \( P = 0.0001 \) and \( P < 0.0001 \), respectively. Overall, individuals with SNCA transcript levels in the lowest quartile had a highly significant OR for Parkinson’s disease of 2.18 (96% CI, 1.47–3.22) and 2.45 (96% CI, 1.65–3.64; Fig. 4) for each of the two scenarios, respectively. Cochran’s Q statistic equalled 2.67 (\( P = 0.26 \)), giving no evidence of heterogeneity among the three studies.

Taken together these results indicate that reduced SNCA mRNA levels in circulating cells are associated with Parkinson’s disease, even near disease onset and in patients with DAT-imaging supported loss of dopaminergic nigral projections. Exploration of specific transcript isoforms using digital transcript counting indicated that SNCA transcripts containing a long 3’UTR may be particularly relevant for this association.

**SNCA transcript abundance predicts cognitive decline**

Predictors of cognitive decline would be valuable for stratifying clinical trials and for advancing clinical care (Chen-Pplotkin, 2014). We have tracked Mini-Mental State Examination (MMSE) scores in the HBS and the PROBE populations during multiple longitudinal in-person follow-up visits for ~5 years. To evaluate whether SNCA transcript levels measured at enrolment can longitudinally predict cognitive prognosis in Parkinson’s disease, we analysed the 222 patients with Parkinson’s disease that were longitudinally followed in the HBS. In HBS, MMSE scores were obtained during in-person study visits at enrolment, after 12, 24, and 60 months in the study. There was overall a significant relation between SNCA transcript abundance at enrolment and MMSE scores during the longitudinal follow-up period with \( P = 0.0007 \). We then tested whether low versus high SNCA expression status measured at enrolment could predict longitudinal cognitive decline in these patients. The 25% of patients with Parkinson’s disease with the highest SNCA transcript levels at enrolment (\( n = 55 \); termed henceforth ‘SNCA high expressors’) and the 25% of patients with the lowest SNCA transcript levels at enrolment (\( n = 55 \); termed ‘SNCA low expressors’) were compared (Fig. 5). Notably, at enrolment high and low SNCA expressors had nearly identical mean (SD) MMSE scores of 28.37 (1.95) versus 28.53 (2.28) (\( P = 0.3 \)). Moreover, the two groups showed no statistically significant differences in other clinical characteristics at enrolment, including gender, age, disease duration, total Unified Parkinson’s Disease Rating Scale (UPDRS) score, Hoehn and Yahr stage, medications, and years of education (Supplementary Table 3). Notably, low SNCA expressors showed accelerated longitudinal cognitive decline compared to high expressors with \( P = 0.01 \) in the longitudinal mixed random and fixed effects model analysis (Locascio and Atri, 2011) adjusting for age, gender, disease duration upon enrolment, and years of education (Fig. 5A).

We then asked whether low and high expressor status can also predict cognitive decline in PROBE. Prospective longitudinal clinical data were available for 65 of the 76 patients in PROBE with relative SNCA abundance measured by quantitative PCR at enrolment. Again, the 25% of patients with Parkinson’s disease with the highest SNCA transcript levels at enrolment into PROBE (\( n = 16 \) in PROBE) and the 25% of patients with the lowest SNCA transcript levels at enrolment (\( n = 16 \) in PROBE) were tested. High and low SNCA expressors had similar MMSE scores of 29.19 (0.98) versus 28.44 (2.78) at enrolment (\( P = 0.95 \)) (Supplementary Table 2). Mixed effects analysis adjusting for the covariates of age, gender, disease duration upon enrolment, and years of education indicated a similar trend as observed in the larger HBS population, but this did not reach statistical significance, likely because of insufficient power (Fig. 5B). PPMI was not included in the longitudinal analysis because the multi-year longitudinal follow-up data needed are not yet available. Finally, to increase power, we performed a mixed random and fixed effects meta-analysis across the low and high expressors from each of the HBS and PROBE populations. The combined analysis robustly confirmed SNCA expression status as predictor of longitudinal cognitive decline in Parkinson’s disease with \( P = 0.005 \) after adjusting for pertinent covariates and for study (Fig. 5C). Similar analyses were performed to evaluate whether SNCA expression can also predict decline in longitudinal total UPDRS scores.
These analyses were inconclusive and will require further evaluation in larger, longitudinally followed patient populations.

Discussion

We show here that SNCA transcript counts in circulating blood cells are reduced in early Parkinson’s disease—even in the landmark PPMI study that enrolled patients before they met standard clinical diagnostic criteria. The association between SNCA expression and Parkinson’s disease was generalizable across platforms and across populations (Table 2 and Figs 1–3). It was observed in studies from a single health care system (HBS), from 22 USA sites (PROBE), and from 24 USA and international sites (PPMI). This association remained significant when adjusted for confounding variables such as age, gender, white and red blood cell counts, Parkinson’s disease medications, or sample handling.

Biological markers of processes that lead to complex diseases such as Parkinson’s disease are not simply present or absent, but have a wide range of values that overlap in subjects with a clinical disease and in those without it (Manolio, 2003). For example, total cholesterol has modest specificity and sensitivity for predicting cardiovascular disease (with an area under the receiver operating curve of 0.59 in the Women’s Health Study; Ridker et al., 2000)—well short of the >90% specificity required for a bona fide diagnostic test. Nonetheless, biological markers of complex diseases can have powerful clinical applications. Cholesterol metabolism-related biomarkers have transformed cardiology. They enable early intervention with cholesterol-lowering medications because of the favourable risk/benefit ratio (low side-effect profile combined with clear benefits for reducing debilitating cardiovascular events). Levels of these commonly used biomarkers are also valuable in tracking therapeutic response and patient stratification. Thus, if confirmed in prospective studies, SNCA blood transcripts could be developed into future markers of risk, of patient stratification for clinical trials, and of response to novel therapeutics (particularly those targeting α-synuclein production or clearance).

We have begun to explore a clinical application for this biological marker as a predictor of cognitive decline. Cognitive decline is one of the most debilitating manifestations of disease progression in Parkinson’s and is closely correlated with the presence of α-synuclein protein-positive Lewy body burden throughout the neocortex (Braak et al., 2002). Predictors of cognitive decline would be valuable for stratifying clinical trials and for advancing clinical care (Chen-Plotkin, 2014). Several previous studies have examined the association between cognitive decline and biological markers in cross-sectional studies (Mollenhauer et al., 2014).
2014) or with a single longitudinal follow-up after 1–2 years (Siderowf et al., 2010; Chen-Plotkin et al., 2011). Longitudinal studies with repeated measurements provide more independent information than a single measurement (Hedeker and Gibbons, 2006; Locascio and Atri, 2011). In our study low SNCA transcript abundance at enrolment predicted cognitive decline in patients with Parkinson’s disease that were cognitively reassessed during multiple longitudinal visits over up to 5 years of follow-up with a P-value of 0.005 in the combined analysis of HBS and PROBE studies.

Beyond these translational implications, the association here observed offers an immediate clue towards unravelling the biological function of α-synuclein in health and disease. α-Synuclein had been thought of as a neuron-specific gene for decades, but this view was turned on its head with the discovery of high levels of α-synuclein present in blood cells, particularly in reticulocytes (circulating immature red blood cells) and during retinal erythroid differentiation and in megakaryocytes (Scherzer et al., 2008; Maitta et al., 2011), with low or no expression in other tissues (Maroteaux et al., 1988). In erythroid cells SNCA expression is regulated by the GATA-1 transcription factor in synchrony with machinery essential for incorporating iron into heme within mitochondria (Scherzer et al., 2008) thereby pointing at a biological link between three nodes of Parkinson’s disease: α-synuclein, mitochondria (Zheng et al., 2010), and iron (Rhodes and Ritz, 2008). Our study suggests that this link might be perturbed in early disease stages. Alternative α-synuclein transcript isoform usage has been suggested as a potential convergent mechanism in Parkinson’s disease pathology (Rhinn et al., 2012). 3’UTR ends of mRNAs are critical for targeting transcripts to specific subcellular compartments and for translational control (Andreassi and Riccio, 2009). Specific transcript isoforms of SNCA with an extended 3’UTR impact accumulation of α-synuclein protein, which appears redirected away from synaptic terminals and towards mitochondria (Rhinn et al., 2012). This might be relevant to disease processes as mitochondrial dysfunction is implicated in the onset of Parkinson’s disease neuropathology (Zheng et al., 2010). By contrast, deletion of exon 5 of the SNCA gene results in the translation of a truncated 112 amino acid protein that is prone to aggregation and increases seeding efficiency (Murray et al., 2003).

In sporadic Parkinson’s disease, α-synuclein protein (and iron) accumulates in the brain and peripherally α-synuclein aggregates are found in skin, glands, intestines and epicardium (reviewed in Jellinger, 2011). In a few dozen patients a triplication of the SNCA locus causes monogenic familial Parkinson’s disease. The direction of the change observed here was therefore surprising, even paradoxical, at first glance. How can it be that SNCA blood transcript levels are consistently reduced in each of the three studies? Ample precedent exists for paradoxical changes in peripheral markers of neurological diseases. In Parkinson’s disease, extracellular α-synuclein protein levels are reduced in CSF for reasons unknown (Mollenhauer et al., 2011). In Wilson’s disease, copper accumulates in the basal ganglia, but serum levels are paradoxically low. In Alzheimer’s disease, amyloid-β_{42} levels are reduced in CSF (Hansson et al., 2006). We hypothesize that intracellular SNCA transcript levels in clinically manifest, sporadic Parkinson’s disease may be reduced as a consequence of intracellular α-synuclein protein accumulation (due to impaired clearance or other mechanisms) that leads to a feedback repression of transcription—in a homeostatic effort to ameliorate the ‘log jam’ of excessive α-synuclein protein products. In an alternate view, because SNCA is so highly expressed in reticulocytes, reduced SNCA expression could be a marker of generally abnormal reticulocyte biogenesis or function in early-stage Parkinson’s disease. We performed association studies that do not imply causality and that were not designed to distinguish between these alternative hypotheses. However, neither ours nor previous studies (Kasten et al., 2010) found evidence for an association between anaemia and Parkinson’s disease. By contrast, anaemia during early life, preceding disease onset by up to 20–29 years (Savica et al., 2009), and multiple recent blood donations have been correlated to increased risk of Parkinson’s disease (Logroscino et al., 2006).

The ease and non-invasiveness of an α-synuclein blood test is appealing. Previous attempts to search for α-synuclein blood tests centred on α-synuclein plasma protein, involved small cohorts, and were inconclusive (for review see Chahine et al., 2014). That approach poses considerable technical challenges as plasma can be easily contaminated with α-synuclein released from erythrocytes ruptured during sample transport and centrifugation. Our studies leapfrogged this technical challenge. Patient blood was directly collected into an FDA-approved system for preserving ‘intracellular RNA from whole blood for RT-PCR used in molecular diagnostic testing’ (without a need for plasma separation) where chemicals immediately stabilize the in vivo gene transcription profile by reducing in vitro RNA degradation and minimizing gene induction (Pahl and Brune, 2002a, b; Rainen et al., 2002; Kim et al., 2014). The HBS, PROBE and PPMI studies were purposefully designed to minimize the threats of bias from sample collection, processing and storage (Ransohoff, 2004). Case and control samples for HBS and PPMI were collected, processed and analysed in parallel using standardized procedures for each step. In contrast to other studies, RNA integrity, complete blood counts, and several processing and quality indicators were carefully measured for all three studies.

Although each of the three biomarker studies here presented has limitations when taken on its own, these individual constraints are compensated for by the collective strengths. For example, while the PROBE and HBS study could not completely rule out an undue influence of Parkinson’s disease medications on SNCA expression, this question was fully addressed by exclusively recruiting de novo cases in PPMI. We clearly show that this is not simply a marker of sick patients; instead, the association...
is already detectable in individuals who are very early in the disease course and highly functional. Clinical diagnostic certainty was enhanced through DAT neuroimaging.

Beyond revealing an association between lowered SNCA expression and Parkinson’s disease, this study presents a generally useful resource of three purpose-designed biobanks and linked clinical data. This platform of biobanks can now be used for accelerating the exploration of nearly any biofluid marker for Parkinson’s disease by decreasing the time from discovery to replication from multiple years down to the assay run-time.

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PROBE Study. PROBE Steering Committee: Voyager Therapeutics: Bernard Ravina; Brigham and Women’s Hospital: Clemens Scherzer, University of Ottawa: Michael Schlossmacher, Avid Radiopharmaceuticals: Andrew Siderowf; University of Rochester: David Oakes; Institute for Neurodegenerative Disorders: Kenneth Marek; Georgetown University: Ira Shoulson. The investigators who contributed to the PROBE cohort and collection of clinical data and blood samples are listed in the Appendix of Ravina et al., A Longitudinal Program for Biomarker Development in Parkinson’s Disease: A Feasibility Study, Movement Disorders, Vol. 24, No. 14, 2009, pp. 2081–2090.

PPMI Study. The PPMI data used in this study were obtained from the Parkinson’s Progression Markers Initiative (PPMI) database (www.ppmi-info.org/data). For up-to-date information on the study, visit www.ppmi-info.org.

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Supplementary material

Supplementary material is available at Brain online.

References

α-Synuclein transcripts in early Parkinson's disease.