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ARTICLE

A leucine-rich repeat kinase 2 (LRRK2) pathway biomarker characterization study in patients with Parkinson's disease with and without LRRK2 mutations and healthy controls

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Abstract

Increased leucine-rich repeat kinase 2 (LRRK2) kinase activity is an established risk factor for Parkinson's disease (PD), and several LRRK2 kinase inhibitors are in clinical development as potential novel disease-modifying therapeutics. This biomarker characterization study explored within- and between-subject variability of multiple LRRK2 pathway biomarkers (total LRRK2 [tLRRK2], phosphorylation of the serine 935 (Ser935) residue on LRRK2 [pS935], phosphorylation of Rab10 [pRab10], and total Rab10 [tRab10]) in different biological sources (whole blood, peripheral blood mononuclear cells [PBMCs], neutrophils) as candidate human target engagement and pharmacodynamic biomarkers for implementation in phase I/II pharmacological studies of LRRK2 inhibitors. PD patients with a LRRK2 mutation ($n=6$), idiopathic PD patients ($n=6$), and healthy matched control subjects ($n=10$) were recruited for repeated blood and cerebrospinal fluid (CSF) sampling split over 2 days. Within-subject variability (geometric coefficient of variation [CV], %) of these biomarkers was lowest in whole blood and neutrophils (range: 12.64%–51.32%) and considerably higher in PBMCs (range: 34.81%–273.88%). Between-subject variability displayed a similar pattern, with relatively lower variability in neutrophils (range: 61.30%–66.26%) and whole blood (range: 44.94%–123.11%), and considerably higher variability in PBMCs (range: 189.60%–415.19%). Group-level differences were observed with elevated mean pRab10 levels in neutrophils and a reduced mean pS935/tLRRK2 ratio in PBMCs in PD LRRK2-mutation carriers compared to healthy controls. These findings suggest that the evaluated biomarkers and assays could be used to verify pharmacological mechanisms of action and help explore the dose–response of LRRK2 inhibitors in early-phase clinical studies. In addition, comparable α-synuclein aggregation in CSF was observed in LRRK2-mutation carriers compared to idiopathic PD patients.

[Correction added on 5 August 2023, after first online publication: A new co-author, 'Diana R. Pereira' has been added to the author list in this version.]

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Total leucine-rich repeat kinase 2 protein (tLRRK2), phosphorylation of the serine 935 residue on LRRK2 (pS935), phosphorylation of the threonine 73 (Thr73) residue on the direct LRRK2 substrate Rab10 (pRab10), and total Rab10 (tRab10) have been identified as LRRK2 pathway and inhibition biomarkers.

WHAT QUESTION DID THIS STUDY ADDRESS?

This study explored within- and between-subject variability in tLRRK2, pS935, pRab10, and tRab10 in whole blood, peripheral blood mononuclear cells (PBMCs), and neutrophils as candidate human safety, target engagement, and pharmacodynamic biomarkers for implementation in phase I/II pharmacological studies of novel LRRK2 inhibitors. It also looked at α-synuclein (αSyn) aggregation in cerebrospinal fluid (CSF) in PD patients with a LRRK2 mutation.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

Total LRRK2 and pS935 can be evaluated in whole blood as LRRK2-inhibitor target engagement markers in a clinical study setting with low variability. pRab10 and tRab10 display significantly larger variability in PBMCs and therefore in clinical studies these biomarker levels should be analyzed relative to an individual's baseline value. αSyn aggregation in CSF seems present in at least a similar extent in LRRK2-mutation carriers compared to idiopathic PD patients.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Together with CSF drug concentrations, measuring tLRRK2, pS935, tRab10, and pRab10 peripherally can verify pharmacological mechanisms of action and help explore the dose–response of novel LRRK2 inhibitors in early-phase clinical studies in healthy subjects and patients.

INTRODUCTION

Gain-of-kinase-function mutations in the gene encoding the leucine-rich repeat kinase 2 (LRRK2) protein confer the highest population-attributable risk to Parkinson's disease (PD), accounting for approximately 4%–5% of familial PD and 1%–2% of sporadic PD.[1–3] Moreover, emerging evidence suggests that LRRK2 activity is also increased in a proportion of idiopathic PD patients,[4,5] which has sparked an interest in the development of LRRK2 kinase inhibitors as potential disease-modifying therapeutics.[6–10]

Pathogenic LRRK2 mutations associated with PD reside in the guanosine triphosphatase (GTPase) and kinase domains of the protein (Figure 1). The most common LRRK2 mutation, G2019S, is located in the kinase domain and increases LRRK2 kinase function approximately 2–3-fold.[6,7,11–14] Surprisingly, mutations in the neighboring GTPase domain, including the R1441C/G/H and Y1699C mutations, seem to have substantially larger (indirect) effects on LRRK2 kinase activation of up to 2–15-fold in model systems.[6,7] This effect appears to be mediated through intramolecular regulation of the kinase activity by the GTPase.[7,12,15,16] Increased LRRK2 kinase activity results in excessive phosphorylation of substrates, including a subset of Rab GTPases. These Rab GTPases are a family of key players in intracellular trafficking events and lysosomal homeostasis.[6] Together, the LRRK2-Rab GTPase pathway is believed to play a role in regulating endo-lysosomal biology via multiple mechanisms, including endocytosis, autophagy, and lysosomal functioning.[15,17–20] In PD, lysosomal damage and dysfunctioning (resulting from increased LRRK2 kinase activity) may be a central mechanism impairing degradation of proteins, ultimately resulting in the accumulation of α-synuclein (αSyn), a cardinal pathological feature of PD.[8,21–23]

Several LRRK2 pathway and inhibition biomarkers have been described in recent years including total LRRK2 (tLRRK2) protein for safety evaluation, potential pathway engagement, and normalization purposes,[7,8,10,24–29] phosphorylation of the serine 935 (pS935) residue on LRRK2 as an indirect LRRK2-inhibitor target engagement marker,[7,8,13,30,31] phosphorylation of the threonine 73 (Thr73) residue on the direct LRRK2 substrate Rab10 (pRab10) as a direct pharmacodynamic marker,

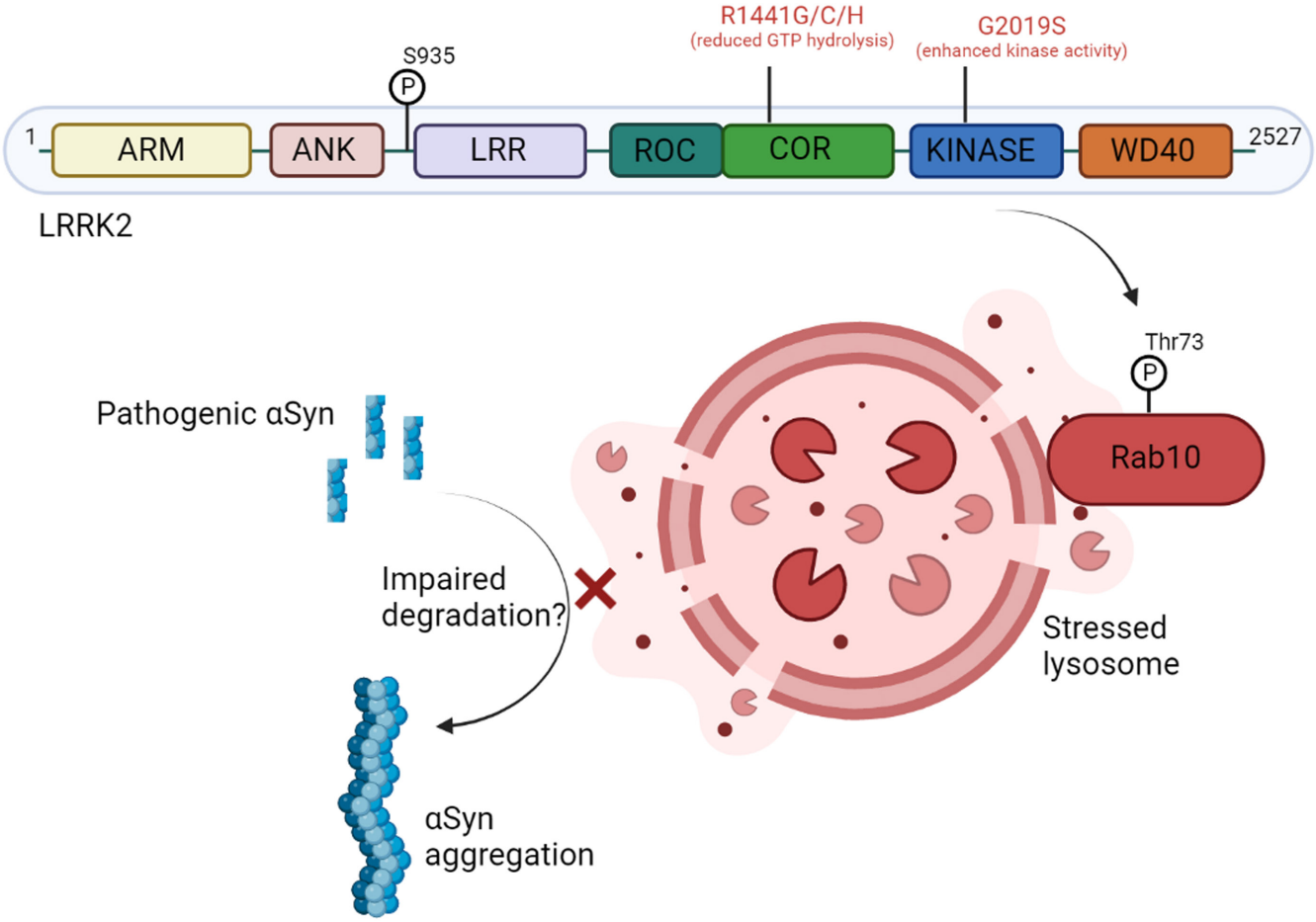

FIGURE 1 Leucine-rich repeat kinase 2 (LRRK2) protein schematic, downstream substrate, phosphorylation sites, and common mutations. LRRK2 is a 2527 amino acid, multidomain protein consisting of a guanosine triphosphatase (GTPase) domain – comprised of the Ras of complex protein (ROC) terminating with a spacer domain called the C-terminal of the Roc-domain (COR) – immediately followed by a serine/threonine kinase domain and surrounded by several protein–protein interaction domains including the leucine-rich repeat (LRR) domains towards the N-terminus and WD40 domain at the C-terminus.[12] LRRK2 has a phosphorylation site at serine 935 (involved with inactive LRRK2 binding to the 14-3-3 family of proteins), that has been demonstrated to dephosphorylate upon LRRK2-inhibitor binding. The Rab10 GTPase is a direct substrate for the LRRK2 kinase domain with phosphorylation at the threonine 73 residue. Phosphorylated Rab10 is recruited onto stressed lysosomes in Parkinson's disease (PD) and may impact lysosomal vesicle formation/budding and exocytosis, which in turn may impair protein degradation (red cross in figure) and aggregation of misfolded proteins including pathogenic α-synuclein (αSyn). Common pathogenic mutations associated with PD include R1441C/G/H in the GTPase domain and G2019S in the kinase domain, increasing LRRK2 kinase activity 2–3- and 2–15-fold, respectively. Image created with BioRender.com.

and total Rab10 (tRab10) for pRab10 normalization purposes (Figure 1).[6,8,9,13,31–33] In addition, LRRK2 autophosphorylation of serine 1292 and phosphorylation of several other Rab family substrates (e.g. Rab1B, Rab7A, Rab8A, and Rab12) have been investigated as potential LRRK2 pathway biomarkers, but these carry less ideal properties for use in a clinical setting.[8,31–34] More downstream pathophysiological biomarkers that could be of interest in clinical studies with LRRK2 inhibitors include bis(monoacylglycerol)phosphate (BMP) isoforms as a readout of lysosomal functioning[9,35] and/or potentially direct measurement of pathologic αSyn aggregation potency.[36,37]

To establish a potential treatment effect in PD, LRRK2 inhibition would need to reach the central nervous system (CNS). CNS tissue, however, cannot be used to assess biomarker levels of target and pathway engagement in a clinical setting. Fortunately, LRRK2 is present throughout the brain and body with the highest expression in circulating immune cells, the lungs, and the kidney, which offers an opportunity to investigate blood-based biomarkers as surrogates for CNS LRRK2 activity.[7,38]

In addition, it has recently been demonstrated that total LRRK2 can be evaluated in cerebrospinal fluid (CSF).[29] In vitro and in vivo experiments have demonstrated that LRRK2 inhibition shows a similar dose–response pattern in peripheral tissues and cells compared to the CNS.[9,13] This provides an opportunity for clinical studies with LRRK2 inhibitors to use blood (and CSF)

-based pathway biomarkers, combined with drug concentration measurements in blood and CSF, to predict CNS pharmacodynamic effects.[7,9,13,38] Moreover, recently new assay techniques have become available that make it possible to detect small quantities of αSyn aggregates circulating in CSF.[36] αSyn aggregation potency might provide an interesting pathophysiological response biomarker to LRRK2-inhibtion, and more relevant than total αSyn levels that can display little difference between PD patients and healthy controls.[37]

Before any anticipated pharmacological effect biomarker can be implemented in a clinical study, it is essential to understand the within- and between-subject variability as this influences the minimal detectable effect sizes as well as the overall biomarker sampling and analysis strategy. Therefore, the purpose of this biomarker characterization study was to explore within- and between-subject variability in tLRRK2, pS935, pRab10, and tRab10 in different biological sources (whole blood, peripheral blood mononuclear cells [PBMCs], neutrophils) as candidate human target engagement, pharmacodynamic, and potential patient stratification biomarkers for further formal validation studies.[13] Additionally, this study aimed to explore group-level differences in LRRK2 pathway activity and αSyn aggregation in CSF between PD patients with and without a LRRK2 mutation and healthy control subjects, all with the aim to develop a robust biomarker strategy for implementation in phase I/II pharmacological studies of novel LRRK2 inhibitors.[9,10]

METHODS

Study design and population

This single-center, non-interventional study used a design with repeated blood sampling split over 2 days to investigate both day-to-day within-subject (intraindividual) and between subject (interindividual) variability in LRRK2 pathway biomarkers. In addition, group-level differences in biomarker levels between PD patients with a LRRK2 mutation (LRRK2+PD), idiopathic PD patients (iPD) and healthy matched control subjects (HC) without PD were assessed. In each group six subjects were planned to complete two visits to the clinic at least 10 days and up to 4 weeks apart, for blood sample and CSF collection after a low-fat breakfast followed by 4 h of fasting. Four additional HC subjects were planned to complete only one clinic visit for blood sample and CSF collection in a fasted state. All biomarker samples were collected during the same part of the day (morning) at approximately the same time. Subjects had a safety follow-up visit or telephone call approximately 1 week after completing the last clinic visit.

The LRRK2+PD and iPD patients were recruited from a database of 3402 genotyped PD patients (Centre for Human Drug Research [CHDR], Leiden, the Netherlands) in 2018.[39] LRRK2+PD patients had to have completed genetic screening showing one of the following LRRK2 mutations: G2019S, I2020T, R1441G, R1441C, R1441H, N1437H, or Y1699C, absence of PD-associated glucocerebrosidase gene (GBA) mutations, and a clinical diagnosis of PD (Hoehn & Yahr [H&Y] stage I-IV). The iPD patients had to have completed genetic screening showing absence of PD-associated LRRK2 and GBA mutations. Healthy control subjects were matched to a LRRK2+PD or iPD patient for gender, age (±5 years) and body mass index (BMI) (±3.5 kg/m^2), had to have no clinical history or signs/symptoms of PD, and no first-order relatives diagnosed with PD. Subjects were allowed to maintain stable doses and regimens for concomitant medication, herbal treatments, medical marijuana, and dietary supplements during the study. Only non-smokers were included.

The sample size was based on practical considerations based on the estimated prevalence of LRRK2+PD in the Netherlands and is considered sufficient to provide descriptive information on LRRK2 pathway biomarkers.

Biomarker assessments

Neutrophil and PBMC lysates were analyzed for pS935, tLRRK2, pRab10, and tRab10. Whole blood was analyzed for pS935 and tLRRK2 only, because Rab10 was not detectable in whole blood with available assays.

For neutrophil isolation, whole blood was collected in a K2EDTA tube and neutrophil isolation was performed within 1 h using a Direct Human Neutrophil Isolation Kit and RoboSep device (StemCell) following the manufacturer's protocol. Frequency of neutrophils in whole blood, yield, and purity (CD16+, CD66b+, CD45+) of the negative fraction containing the neutrophils were assessed by flow cytometry using a MACSQuant 10 analyzer (Miltenyi Biotec). Neutrophils were pelleted by centrifugation and then resuspended in lysis buffer. The lysates were incubated on ice for 20 min, followed by centrifugation. Supernatants were aliquoted and stored at −80°C for later immunoassay analysis.

For PBMC analysis, blood was collected into CPT-sodium heparin tubes and PBMCs were isolated following the manufacturer's protocol. Cells were counted on a MACSQuant 10 analyzer (Miltenyi Biotec). PBMCs were pelleted by centrifugation and resuspended in PBMC lysis buffer.[13] The lysates were incubated on ice for 20 min, followed by centrifugation. Supernatants were

aliquoted and stored at −80°C for later immunoassay analysis.

pS935, tLRRK2, pRab10, and tRab10 in all samples were quantified using Meso Scale Discovery (MSD)-based assays according to methods described elsewhere,[13] with the exception that at the time of this study these assays were still in a developmental stage and not yet validated. Results were plotted with MSD arbitrary units (A.U.).

CSF was collected in polypropylene tubes following lumbar puncture with an atraumatic 22G needle, centrifuged, and the supernatant was aliquoted and stored at −80°C. Samples were analyzed using an αSyn Seed Amplification Assay (αS-SAA) that uses amplification cycles and an excess of recombinant αSyn to elongate and detect misfolded αSyn aggregates (αSyn seeds) in CSF. The assay was performed by Amprion using previously published methods.[40–42] Briefly, CSF samples were blindly analyzed in triplicate (40 µL each) in a reaction mixture comprising 0.3 mg/mL recombinant αSyn (Amprion, cat# S2020), 100 mM PIPES pH 6.50, 500 mM NaCl, 10 µM Thioflavin T (ThT), and one 3/32″ Si3N4 bead blocked with 1% BSA. Reaction mixtures were analyzed in 96-well plates. Plates were orbitally shaken at 800 rpm for 1 min every 29 min at 37°C for 150 h. Fluorescence readings (relative fluorescence units [RFU], 440–10/490–10) were collected every 30 min. The results from each triplicate were combined to determine the samples' result (positive/negative for αSyn seeds) using a probabilistic algorithm already described.[40]

Statistical analysis

All statistical analysis was performed using SAS statistical software (version 9.4). Within-subject variability between two visits and between-subject variability are expressed as geometric coefficient of variation (CV, %). Geometric CV was estimated within a repeated measures mixed effects model with group, visit, and group by visit as fixed factors, and visit as repeated factor within subject, and a compound symmetry variance/covariance structure within group, if possible. Where possible, pS935 was normalized to tLRRK2 (pS935/tLRRK2) and pRab10 to tRab10 (pRab10/tRab10) and to tLRRK2 (pRab10/tLRRK2) to explore if this would reduce variability. Neutrophil and PBMC biomarker values were also normalized to cell number and glyceraldehyde 3-phosphate dehydrogenase (GADPH; PBMCs only) as an exploratory analysis to determine whether this would reduce within- and between-subject variability. Within the model, mean (95% confidence intervals [CI]) group-level differences in pS935, tLRRK2, pS935/tLRRK2, pRab10, tRab10, pRab10/tRab10, and pRab10/tLRRK2 levels were explored on log-transformed data. Inconclusive test results in the αS-SAA were treated as false-negatives for calculation of the assay sensitivity and specificity, with their 95% CI (Wilson Score Intervals). The αS-SAA ThT fluorescence signal between the LRRK2+PD, iPD, and HC groups was analyzed with a repeated measures mixed effects model, with time as repeated factor within subject by visit, and group, visit, time, and group by time as fixed factor. The contrasts between the three groups were estimated within the model, with their 95% CI. Estimated means per group and timepoint were generated and graphically presented. The level of significance was set at $p < 0.05$.

Ethics statement

This study was conducted in accordance with the International Conference for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), Good Clinical Practice, and the principles of the Declaration of Helsinki. The study was registered in the Netherlands Trial Register (NTR7647), approved by an independent ethics committee (Stichting Beoordering Ethiek Biomedisch Onderzoek, Assen, the Netherlands), and all subjects provided their written informed consent before participation.

RESULTS

Demographics and baseline characteristics

Six LRRK2+PD, six iPD, and 10 matched HC subjects were enrolled into the study, with an age of between 47 and 81 years and a BMI between 21.0 and 32.9 kg/m^2. Most subjects were male (86%). Baseline characteristics (except for the PD diagnosis) were generally comparable between the three groups (Table 1). The six LRRK2+PD patients had a shorter mean time since diagnosis (6.7 ± 4.0 vs. 9.8 ± 3.8 years), a slightly lower mean H&Y score (1.8 ± 1.2 vs. 2.0 ± 0.8), and included more subjects of North African descent (33% vs. 0%) compared to the six iPD patients. Five (83%) LRRK2+PD patients carried a G2019S LRRK2 mutation and one (17%) patient carried a R1441C mutation. None of the iPD subjects had mutations in the LRRK2 and GBA genes. All patients with PD and six healthy control subjects completed two visits to the clinic, and four healthy control subjects only completed one clinic visit (Table 1). Two LRRK2+PD patients only completed one of the two planned lumbar punctures.

TABLE 1 Subject baseline characteristics at screening.

Characteristic	PD patients with LRRK2 mutation (LRRK2+PD)	Idiopathic PD patients (iPD)	Healthy controls (HC)	
	Subjects with 2 visits	Subjects with 2 visits	Subjects with 2 visits	Subjects with 1 visit[a]
Total subjects (*N*)	6	6	6	4
Age, years, mean (SD)	62.3 (11.8)	62.7 (10.7)	64.0 (10.2)	66.0 (3.1)
Gender, male, *n* (%)	5 (83)	5 (83)	5 (83)	4 (100)
BMI, kg/m^2, mean (SD)	27.8 (3.7)	28.5 (2.8)	27.0 (3.3)	26.8 (2.6)
Race, *n* (%)				
White	4 (67)	6 (100)	6 (100)	3 (75)
North African	2 (33)	–	–	–
Mixed (White/Asian)	–	–	–	1 (25)
Years since PD diagnosis at screening, mean (SD)	6.7 (4.0)	9.8 (3.8)	N/A	N/A
Baseline H&Y stage, *n* (%)				
1	4 (66)	2 (33)	N/A	N/A
2	–	2 (33)		
3	1 (17)	2 (33)		
4	1 (17)	–		
LRRK2 mutation status, *n* (%)				
G2019S	5 (83)	N/A	N/A	N/A
R1441C	1 (17)			

Abbreviations: BMI, body mass index; H&Y, Hoehn & Yahr; LRRK2, leucine-rich repeat kinase 2; N/A, not available; PD, Parkinson's disease; SD, standard deviation.

[a]The four subjects that had only one clinic visit are not included in the analyses of within-subject variability.

Within-subject variability per biomarker and biological source

Within-subject variability (geometric CV, %) between visit 1 and 2 for each biomarker (pS935, tLRRK2, pS935/tLRRK2, pRab10, tRab10, pRab10/tRab10, and pRab10/tLRRK2) in each biological source (whole blood, PBMCs, and neutrophils) is depicted in Figure 2a. Within-subject variability was lowest in whole blood (ranging from 12.64% to 30.37% [pS935/tLRRK2 and pS935, respectively]) and neutrophils (ranging from 43.55% to 51.32% [tRab10 and pRab10, respectively]). Both pS935 and tLRRK2 were below quantification limits in all neutrophil lysate samples. In PBMCs within-subject variability was considerably higher for all biomarkers (ranging from 121.49% to 273.88% [pS935/tLRRK2 and tLRRK2, respectively]), except for tRab10 (34.81%). When normalizing pS935 to tLRRK2 (pS935/tLRRK2) and pRab10 to tRab10 (pRab10/tRab10) within-subject variability decreased in each biological source, though only marginally (Figure 2a). Normalizing pRab10 to tLRRK2 (pRab10/tLRRK2) in PBMCs resulted in the highest within-subject variability with a geometric CV of 789.46%. Normalizing to total cell number or GADPH (PBMCs only) did not decrease variability observed in PBMCs (Figure S1A) or neutrophils (data not shown).

Between-subject variability within each subject group

Between-subject variability (geometric CV, %) for each biomarker in each biological source is depicted in Figure 2b. Between-subject variability was lowest in neutrophils (ranging from 61.30% to 66.26% [pRab10/tRab10 and pRab10, respectively]), followed by whole blood (ranging from 44.94% to 123.11% [pS935 and tLRRK2, respectively]). In PBMCs between-subject variability was again considerably higher (ranging from 189.60% to 415.19% [pS935 and pRab10/tRab10, respectively]), except for tRab10 (74.32%) and pS935/tLRRK2 (96.27%). Normalizing pS935 to tLRRK2 (pS935/tLRRK2) approximately halved between-subject variability of pS935 in PBMCs, but more than doubled it in whole blood. Normalizing pRab10 to tRab10 (pRab10/tRab10) did not significantly decrease between-subject

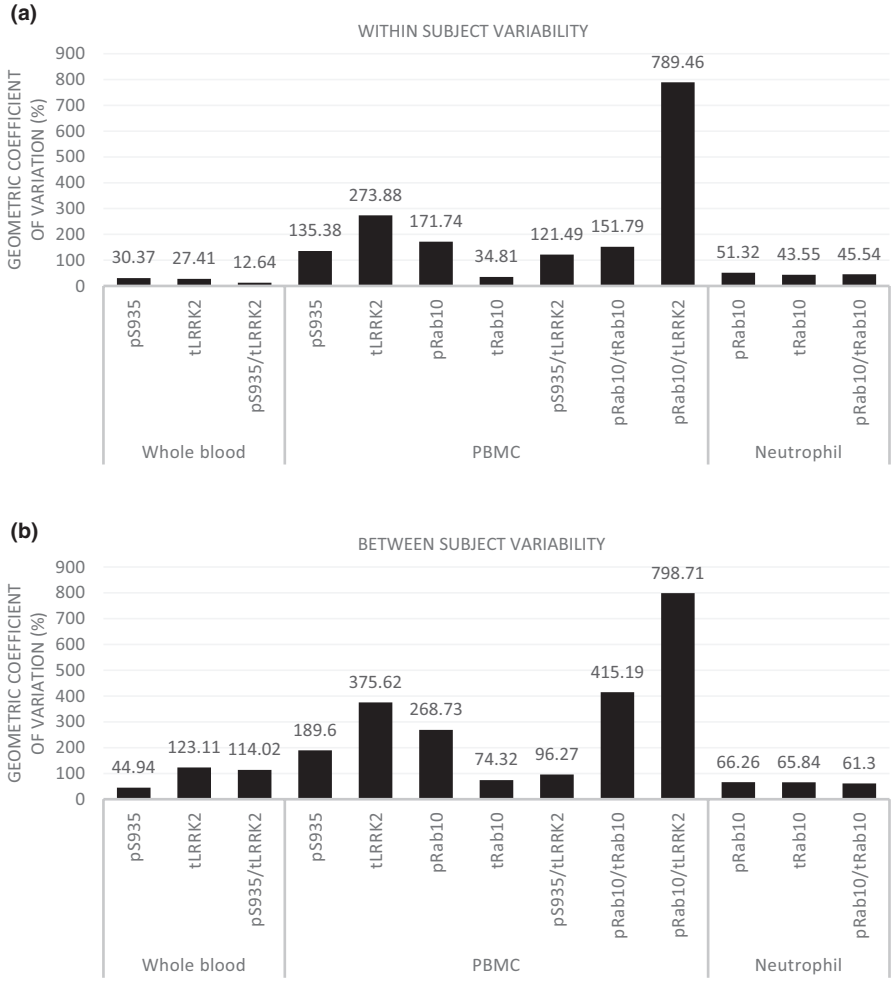

FIGURE 2 Variability of each biomarker in each biological source. (a) Within-subject (intrasubject) variability between visits 1 and 2, and (b) between-subject (intersubject) variability of pS935, tLRRK2, pS935/tLRRK2, pRab10, tRab10, pRab10/tRab10, and pRab10/tLRRK2 in whole blood, peripheral blood mononuclear cells (PBMCs), and neutrophils expressed as geometric coefficient of variation (%). Data from all three subpopulations (leucine-rich repeat kinase 2 [LRRK2] + Parkinson's disease [PD], idiopathic Parkinson's disease [iPD], and healthy controls [HC]) was pooled for this analysis. Within-subject and between-subject variability were low in whole blood and neutrophils, and substantially higher in PBMCs. See Figure S2A-F for a breakdown per subpopulation.

variability in neutrophils and increased variability in PBMCs (Figure 2b). Controlling pRab10 for tLRRK2 (pRab10/tLRRK2) in PBMCs resulted in the highest between-subject variability with a geometric CV of 798.71%. Normalizing to total cell number or GADPH (PBMCs only) did not decrease variability observed in PBMCs (Figure S1B) or neutrophils (data not shown). There were no apparent differences in between-subject variability between the three populations, except for substantially higher between-subject variability in tLRRK2, pRab10, and pRab10/tRab10 in the HC group, and for pRab10/tLRRK2 in the HC and LRRK2+PD groups (Figure S2E).

Group-level differences for each biomarker

No group-level differences were observed for pS935, tLRRK2, and tRab10 between the LRRK2+PD, iPD, and HC groups in either whole blood, PBMCs, or neutrophils. Furthermore, no group-level differences were observed for pRab10 in PBMCs (Table S1). However, the mean phosphorylation of Rab10 (pRab10) was significantly elevated in the LRRK2+PD (4795 A.U.) compared to the HC (2595 A.U.) group in neutrophils ($p = 0.0404$). Though numerically higher, the mean pRab10 level in neutrophils in the iPD group (3309 A.U.) did not significantly differ from the HC group (Figure 3a). After correcting for tRab10 in neutrophils, the mean pRab10/tRab10 ratio was approximately two-fold higher in the LRRK2+PD (1.40) compared to the HC (0.67) group ($p = 0.0062$). The mean pRab10/tRab10 ratio in the iPD group (1.26) was also elevated compared to the HC group, but this difference was not significant ($p = 0.0698$), nor was there a significant difference between the LRRK2+PD and iPD groups. The single subject with a $LRRK2^{R1441C}$ mutation had the highest pRab10 level in neutrophils of all participants (pink dot, Figure 3a). No group-level differences were observed for pRab10/tRab10 or pRab10/tLRRK2 in PBMCs.

When pS935 was corrected for tLRRK2, the mean pS935/tLRRK2 ratio was significantly lower in LRRK2+PD versus HCs (1.53 vs. 3.12, $p = 0.0327$) and in LRRK2+PD versus iPD (1.53 vs. 4.46, $p = 0.0006$) in PBMCs (Figure 3b), but not in whole blood.

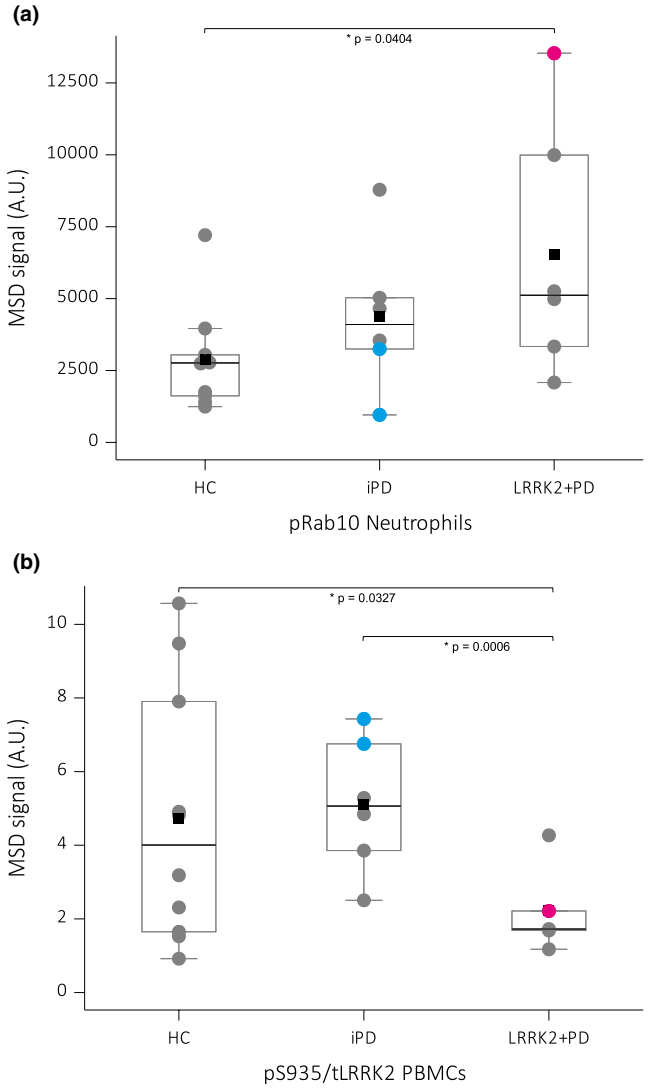

FIGURE 3 Group-level differences in biomarker expression levels in neutrophils and peripheral blood mononuclear cells (PBMCs). (a) Differences in phosphorylation of Rab10 in neutrophils between leucine-rich repeat kinase 2 (LRRK2)+Parkinson's disease (PD), idiopathic Parkinson's disease (iPD) and healthy control (HC) subjects. The mean pRab10 level was significantly higher in neutrophils in the LRRK2+PD group compared to the HC group ($p=0.0404$). The mean pRab10 levels did not significantly differ between the LRRK2+PD and iPD, and the iPD and HC groups. For subjects with two visits the values are averaged for each timepoint. (b) Differences in phosphorylation of serine residue 935 (Ser935), corrected for total LRRK2 (pS935/tLRRK2) in PBMCs between LRRK2+PD, iPD, and HC groups. The mean pS935/tLRRK2 level was significantly lower in PBMCs in the LRRK2+PD group compared to both the iPD ($p=0.0006$) and the HC group ($p=0.0327$). There was no difference between the iPD and HC groups. Values expressed with interquartile range and group-level mean (black square). Each dot represents an individual subject (grey dots). The pink dot marks the subject with a $LRRK2^{R1441C}$ mutation. The blue dots mark two iPD subjects that tested negative for α-synuclein (αSyn) seeds in cerebrospinal fluid with the αS-SAA at both visits. The strength of the pRab10 signal in the plate-based immunoassays is expressed in arbitrary units (A.U.). One timepoint for one subject in the iPD group was confirmed to be an outlier and not included in the final analysis. MSD, Meso Scale Discovery.

Presence of pathogenic αSyn in CSF

Five of six (83%) LRRK2+PD patients tested positive for αSyn seeds in all three replicates of all CSF samples, and one (17%, G2019S mutation) subject only tested positive in two of the three replicates in both CSF samples (result considered inconclusive). For the six iPD patients, four (67%) tested positive in all three replicates of both visits' samples, one patient (17%; 70 years; H&Y stage 2) only tested positive in one of the three replicates for both visits (result considered negative), and one patient (17%; 47 years; H&Y stage 3, dopamine transporter single-photon emission computed tomography [DAT SPECT] confirmed) did not test positive in any of the three replicates for both visits. These two αSyn seeds-negative iPD patients had the lowest mean pRab10 and highest pS935/tLRRK2 levels within their group (blue dots, Figure 3). Eight of the 10 (80%) HC subjects tested negative in all replicates. One HC subject (10%) tested negative in all replicates during his first visit, but positive in all three replicates on his second visit, and one HC subject (10%) tested positive in all three replicates during his first and only study visit. Assuming diagnosis of the participants is correct, the calculated overall sensitivity (%, 95% CI) of the αS-SAA was 80.0% (49.0%–94.3%) for the LRRK2+PD and 58.3% (32.0%–80.7%) for the iPD population. Specificity was 87.5% (64.0%–96.5%) in the HC. The kinetics of αS-SAA aggregation (mean ± 95% CI) for all three groups are shown in Figure 4. The mean αS-SAA signal (RFU) was highest in the LRRK2+PD group and lowest in the HC group. Though the mean time to reach 50% aggregation (T50) was not significantly different between the LRRK2+PD and iPD groups, the total mean αS-SAA signal at the last recorded timepoint was approximately 1.3-fold higher in the LRRK2+PD group.

DISCUSSION

Variability

Reproducible, blood-based, biomarker assays are essential to allow for simple and reliable evaluation of LRRK2 pathway and inhibition levels in clinical studies with LRRK2 inhibitors. Such biomarker assays are especially helpful in early-phase studies to explore the safety profile of novel compounds at different levels of LRRK2 inhibition and to help select dose levels within the anticipated therapeutic window for follow-on larger late-stage patient trials.[38]

The within-subject variability observed between two visits for pS935 and tLRRK2 (CV = 30.37% and 27.41%) in

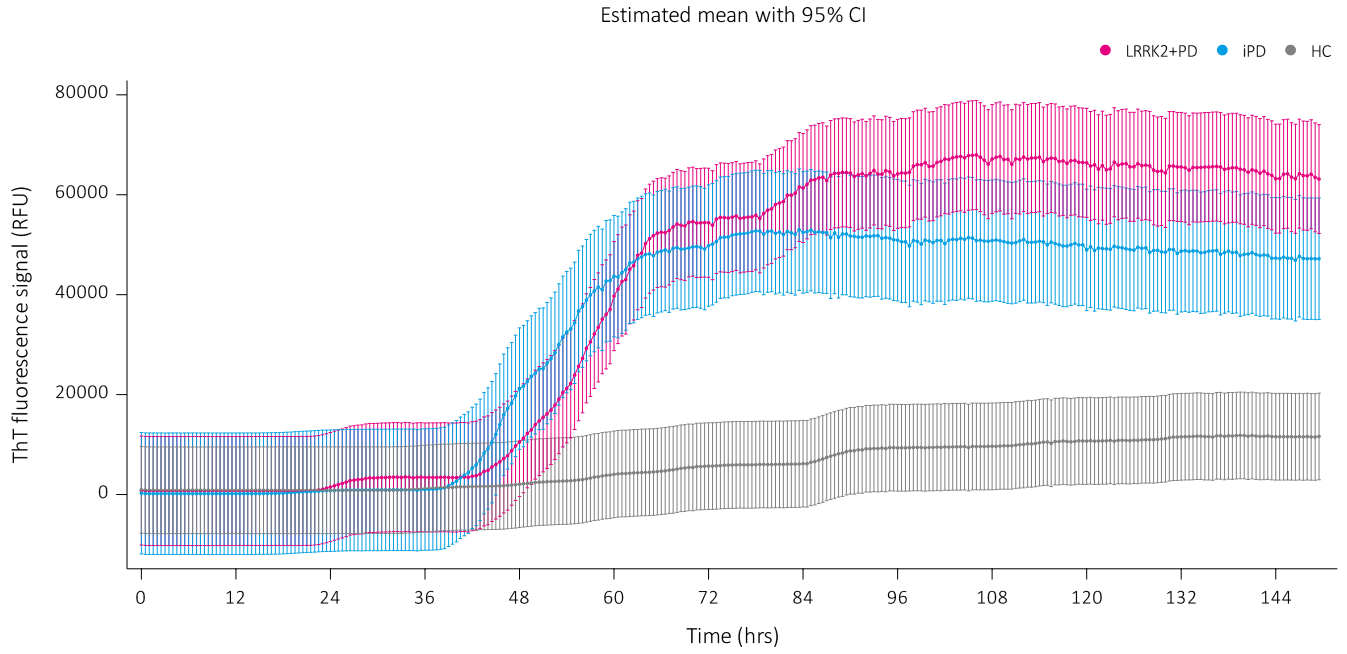

FIGURE 4 α-Synuclein (αSyn) Seed Amplification Assay (αS-SAA) in cerebrospinal fluid (CSF) samples from leucine-rich repeat kinase 2 (LRRK2) + Parkinson's disease (PD), idiopathic Parkinson's disease (iPD), and healthy control (HC) subjects. Values correspond to the mean (±95% confidence interval [CI]) for each subject's group, with each individual sample analyzed in triplicate. All but two LRRK2+PD and four HC subjects had CSF collected during two visits. Curves of the LRRK2+PD and iPD groups were not significantly different as evaluated by a mixed effects model ($p = 0.2882$), but both PD groups did differ from the HC subjects ($p < 0.0001$). Two iPD patients that tested negative for αSyn with the αS-SAA are excluded from this analysis. RFU, relative fluorescence units; ThT, Thioflavin T.

whole blood suggests that the investigated assays would be fit-for-purpose to evaluate tLRRK2 levels and LRRK2 inhibition via pS935 in whole blood in a clinical study setting. During further validation of the assays described in this article, even lower within-subject variability was observed over the course of 24 h with a CV of 16% and 7% for pS935 and tLRRK2, respectively,[13] and these assay have now successfully been used during the early-stage clinical evaluation of two novel LRRK2-inhibitors.[9,10] Much higher within-subject variability with CVs > 100% was observed for the investigated LRRK2 and downstream Rab10 biomarkers in PBMCs, which could make it difficult to quantitatively differentiate the lower end of the LRRK2 inhibition dose–response curve in PBMCs in a clinical setting. However, it was anticipated that in a clinical study a level of LRRK2 inhibition would be achieved that would enable characterization of the pRab10 pharmacodynamic response to LRRK2 inhibition in PBMCs. Measurement of pRab10 reduction in PBMCs was subsequently implemented in phase I and Phase Ib studies of two LRRK2 inhibitors which showed a clear pharmacodynamic response alongside pS935 reduction, demonstrating that pathways downstream of LRRK2 were inhibited in those studies.[9,10] Total LRRK2 in human PBMCs can vary widely among individuals;[43] however, correcting for tLRRK2 or tRab10 for that matter did not significantly reduce the pS935 or pRab10 variability observed in PBMCs in this small-size exploratory study. Correcting for cell number or GAPDH also did not meaningfully reduce variability in PBMCs. An observed higher biomarker variability in PBMCs can be explained by the fact that PBMCs consist of a heterogeneous cell population and only a minority of these cells (monocytes, which make up 5%–20% of PBMCs) express LRRK2 and Rab10.[31] LRRK2 and Rab10 are expressed roughly two-fold higher in neutrophils compared to monocytes, which likely translates to the considerably lower within-subject variability observed for pRab10 and tRab10 in the neutrophils (CV = 51.32% and 43.55%, respectively). The downside of working with neutrophils in a clinical setting, however, is a more complicated isolation procedure and the fact that LRRK2 in neutrophil extracts may undergo considerable proteolytic degradation, which could explain why pS935 and tLRRK2 in this study's neutrophil assays were below the limit of detection.[31] The source of variability of pS935, total LRRK2, pRab10, and tRab10 in this study is likely to be due mainly to biological variability, as a subsequent characterization of the assay technical variabilities in whole blood and PBMCs showed CVs < 20% between technical replicates.[13] In addition, only two observations were used to calculate the within-subject variability which introduces a risk for overestimating the variability.

Between-subject variability was higher than within-subject variability for all investigated biomarkers in all biological sources, which is consistent with observations by others.[13,31] There are a few (counterintuitive) exceptions to this observation when the within- and between-subject variability is broken down per subgroup (Figure S2B,C,E,F), which likely results from the small sample size per subgroup and the fact that only two observations were used to calculate the within-subject variability. The considerable between-subject variability indicates that when assessing LRRK2 levels and inhibition in clinical studies, values should be analyzed relative to an individual's baseline value, rather than looking at absolute group-level differences between active treatment and placebo, as has been done in published clinical studies with LRRK2 inhibitors.[9,10]

One disadvantage of monitoring LRRK2 biomarkers in the peripheral circulation only is that it will always leave some uncertainty about how well this correlates to biomarker levels in the brain and peripheral organs including the lungs and kidney.[27] This caveat has been partially addressed in a recent report showing similar LRRK2 inhibition in the periphery (PBMC) and brain in cynomolgus macaques treated with DNL201, as assessed by pS935.[9] Currently, there are no clinically translatable methods to monitor LRRK2 inhibition using CSF-based methods or via imaging directly in the brain.

In addition, one caveat in using pRab10 as a biomarker for LRRK2 kinase inhibition is that although in peripheral tissues and cells such as PBMCs, kidney, and lung LRRK2 knockout or kinase inhibition as measured by pS935 is accompanied by a reduction in pRab10 similar in magnitude to pS935, in brain phosphorylation of Rab10 is only partially reduced with LRRK2 genetic ablation or kinase inhibition.[13,44] This may indicate that other kinases besides LRRK2 are also able to phosphorylate Rab10, especially in the brain where LRRK2 expression is restricted to specific cell types including microglia and oligodendrocyte precursor cells.[45] This seems to be confirmed by the recent discovery that PPM1H can act as a modulator of LRRK2 signaling via controlling dephosphorylation of Rab proteins.[46] In the absence of a clinically translatable biomarker of LRRK2 activity in CSF, peripheral pRab10 may serve as a useful surrogate likely to be indicative of inhibition of LRRK2 in the subset of brain cells that do express LRRK2, on the condition that the investigational compound is also demonstrated to be highly CNS-penetrant with comparable peripheral and central (unbound) drug exposure levels.

Group-level differences

LRRK2 kinase function has been reported to be elevated in the range 2–15-fold in LRRK2-mutation carriers, while total LRRK2 levels were comparable between wildtype and LRRK2^{G2019S} mutation carriers.[6,7,11–13] These previous observations are replicated in this study, despite its small sample size, with no observed group-level difference in total LRRK2 and an approximately two-fold elevation in LRRK2's phosphorylation of Rab10 in neutrophils in the LRRK2+PD group, suggesting ≥50% LRRK2-inhibition as target for therapeutic efficacy in clinical studies.[9] Although not significant, potentially due to the small sample size, pRab10 levels were also numerically higher in the iPD group compared to HCs, which is in line with some previous reports[4,47] but has not been universally reported in studies of PBMC LRRK2 biochemical or downstream pathway activity in iPD patients.[9,13,31,32,48,49] The elevation in pRab10 in iPD patients could potentially be explained by an increase in LRRK2 activity in response to lysosomal stress and inflammatory stimuli in idiopathic Parkinson's disease.[13] Interestingly, the single LRRK2^{R1441C} mutation carrier had the highest observed pRab10 levels in neutrophils, which matches previous findings where an R1441C mutation displayed an approximate four-fold and a G2019S mutation an approximate two-fold increase in Rab10 phosphorylation compared to LRRK2 wildtype.[6,8,33] As LRRK2 kinase is only known to affect the phosphorylation and not production of Rab10,[6] no group-level differences in tRab10 were expected or observed.

When corrected for tLRRK2, phosphorylation of LRRK2-Ser935 (pS935/tLRRK2) was found to be reduced in the LRRK2 mutation carriers compared to the iPD and HC groups in PBMCs, but not in whole blood, similar to previous findings in PBMCs.[13,43] In PD, pS935 plays a role in LRRK2 binding to the 14-3-3 protein family that can regulate LRRK2 kinase activity, drive translocation of LRRK2 into exosomes followed by secretion into the urine, and protect LRRK2 from proteasomal degradation by inhibiting ubiquitylation.[50] Observations of reduced Ser935 phosphorylation have also been reported in G2019S knockin mouse astrocytes, in R1441C, R1441G, R1441H, Y1699C, and I2020T knockin HEK-293 cells, and in the substantia nigra of iPD patients.[13,16,51]

Presence of pathogenic αSyn in CSF

The percentage of αSyn seeds-positive subjects in the LRRK2+PD group (83%) was higher compared to previous reports using similar assays (40%; $n=15$ and 78%; $n=9$), which could result from assay differences and/or the low sample size in these studies, but does seem to contradict speculated αSyn structural, self-aggregation potency, and/or αSyn burden diversity between LRRK2+PD and iPD patients.[37,52]

In the αS-SAA both T50 and the top fluorescence value appear to be related to the concentration of αSyn seeds in the original CSF sample.[36] The T50 and the top fluorescence value were comparable between the LRRK2+PD and iPD groups, despite on average a shorter time since PD diagnosis in the LRRK2+PD group. This could suggest that αSyn aggregation is present at least to a similar extent in LRRK2-mutation carriers compared to iPD patients, which is further supported by previous reports of elevated αSyn levels in CSF for LRRK2-mutation carriers.[37,53,54] However, at this time the αS-SAA is not validated to detect and/or quantify potential αSyn aggregation-level differences and therefore these observations should be interpreted with care. However, this does open up the possibility to investigate αS-SAA as a potential pharmacodynamic biomarker in the future.

Two iPD patients tested negative for αSyn with the αS-SAA, resulting in a low assay sensitivity for this population (58.3%), which is surprising considering the αS-SAA's previously reported high sensitivity of 88.5%,[36] and these patients' confirmed PD diagnosis. Interestingly, these two patients also had the lowest pRab10 levels within the iPD group. This could support a correlation between LRRK2-activity and αSyn aggregation, which could make αS-SAA an interesting pathophysiological biomarker in future LRRK2-inhibitor studies, although this would first require confirmation in a larger population. The two HC subjects that tested positive via the αS-SAA do not have a clinical PD diagnosis to date. The high sensitivity and specificity of the αS-SAA in larger cohorts supports its continued use and investigation as a diagnostic assay in PD.[41]

To conclude, LRRK2 inhibition offers a promising therapeutic strategy for the treatment of PD patients with LRRK2 mutations and potentially also for idiopathic Parkinson's disease. While it has proved challenging to robustly demonstrate target engagement and pharmacodynamic effects of LRRK2 inhibition in the central compartment, several reliable peripheral biomarkers and assays have been identified over the past few years. Together with CSF concentrations of an investigational compound, measuring tLRRK2, pS935, tRab10, and pRab10 peripherally could verify pharmacological mechanisms of action and help explore the dose–response of novel LRRK2 inhibitors in early-phase clinical studies. The large variability observed for pRab10 in PBMCs indicates that this true LRRK2 pharmacodynamic marker would be useful only in situations where there is a very large effect size (e.g., as pRab10 approaches depletion due to LRRK2 inhibition[9,10]) and is unlikely in this assay to be very useful as a method to distinguish very small changes in pRab10 (e.g., as a patient stratification marker). pS935 and tLRRK2 in whole blood, on the other hand, seem particularly suitable to explore the full dose–response curve in early-phase LRRK2-inhibitor trials.

AUTHOR CONTRIBUTIONS

All listed authors meet authorship criteria and no others meeting the criteria have been omitted. M.F.J.M.V. wrote the manuscript. M.D.T., J.A.A.C.H., G.J.G., D.R.P., and S.H.R. designed the research. E.T., J.A.A.C.H., D.R.P., and G.J.G. performed the research. M.F.J.M.V., M.D.T., J.A.A.C.H., G.J.G., D.R.P., and S.H.R. analyzed the data. S.H.R. contributed new reagents/analytical tools.

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CONFLICT OF INTEREST STATEMENT

S.H.R. and M.D.T. are employees of and may hold stocks in Denali Therapeutics Inc. All other authors declared no competing interests for this work.

INFORMED CONSENT

Written informed consent was signed by participants prior to performing any study procedure including screening.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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