Increased Phospho-AKT in Blood Cells from *LRRK2* G2019S Mutation Carriers

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The purpose of this study was to investigate whether differential phosphorylation states of blood markers can identify patients with LRRK2 Parkinson's disease (PD). We assessed phospho(P)-Ser-935-LRRK2 and P-Ser-473-AKT levels in peripheral blood cells from patients with G2019S *LRRK2*-associated PD (L2PD, n = 31), G2019S *LRRK2* non-manifesting carriers (L2NMC, n = 26), idiopathic PD (iPD, n = 25), and controls (n = 40, total n = 122). We found no differences at P-Ser-935-LRRK2 between groups but detected a specific increase of P-Ser-473-AKT levels in all G2019S carriers, either L2PD or L2NMC, absent in iPD. Although insensitive to LRRK2 inhibition, our study identifies P-Ser-473-AKT as an endogenous candidate biomarker for peripheral inflammation in G2019S carriers using accessible blood cells.

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L eucine-rich repeat kinase 2 (*LRRK2*) mutations cause *LRRK2*-associated Parkinson's disease (L2PD), the most frequent form of monogenic Parkinson's disease (PD), which has a clinicopathological phenotype similar to idiopathic PD (iPD).[1] Non-manifesting *LRRK2* mutation carriers (L2NMCs) are at high risk of PD but predicting disease onset is challenging given the low penetrance and the lack of progression biomarkers.[2,3] In vitro, *LRRK2* pathogenic mutations, including G2019S at the Serine (Ser) / Threonine (Thr) kinase, or R1441G at the GTPase ROC domains, increase LRRK2 kinase activity in neurons by a toxic gain-of-function.[4] Thus, the P-Ser-1292-LRRK2 and P-Ser-935-LRRK2 phospho-sites reflecting auto-phosphorylation or phosphorylation by upstream kinases, respectively,[5] represent candidate biomarkers for target engagement in LRRK2 inhibitor clinical trials.[6] Decreased blood P-Ser-935-LRRK2 levels have been described in patients' with L2PD compared to L2NMC only but not to controls in North Americans,[7] but not validated in another study using the same cohort.[8] Increased P-Thr-73-RAB10 levels, the only known endogenous substrate of LRRK2,[9] has been shown in a large cohort of patients with L2PD carrying the R1441G mutation, but not for G2019S.[10] Therefore, biomarker research in L2PD needs to be evaluated and expanded in additional cohorts.

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The AKT Ser/Thr kinase 1 (AKT) is a central hub in the key PI3K/AKT/mTOR pathway. AKT is ubiquitously expressed and has a critical role in regulating diverse cellular signals, managing the balance between catabolism and anabolism.[11] Moreover, AKT is related to broad physiological functions across cells, tissues, and pathologies, including cancer, autoimmune diseases, and neurodegenerative disorders.[11] P-Ser-473-AKT has been proposed as an LRRK2 phosphorylation substrate in PD animal models[12] and L2PD induced pluripotent stem cell (iPSC)-derived neurons.[13] In addition, postmortem iPD nigral neurons had diminished P-Ser-473-AKT levels,[14,15] whereas fibroblasts from patients with L2PD showed increased AKT signaling.[16] Overall, it is expected that phospho-protein differences related to common *LRRK2* mutations, such as G2019S, could act as progression or drug response biomarkers for L2NMC and L2PD. Here, we expanded biomarker research in G2019S mutation carriers from PD by analyzing the endogenous levels of P-Ser-935-LRRK2, P-Ser-1292-LRRK2, and P-Ser-473-AKT in peripheral blood mononuclear cells (PBMCs) from a large Spanish *LRRK2* cohort (n = 111) encompassing patients with G2019S L2PD, PD at-risk G2019S L2NMC, patients with iPD, and controls. To further assess the potential direct effects of G2019S on P-Ser-935-LRRK2 and P-Ser-473-AKT, we collected fresh PBMC lysates from additional subjects (n = 11), including patients with heterozygous and homozygous G2019S L2PD, heterozygous R1441G L2PD, and controls. We treated these samples with the 3-(4-Pyrimidinyl) Indazole (MLi-2),[17] a potent and selective LRRK2 inhibitor that reduces its kinase activity.

Methods

Subjects

Blood samples were obtained at the Movement Disorders Unit from the Hospital Clínic de Barcelona as part of the Barcelona LRRK2 biorepository cohort supported by the Michael J. Fox Foundation for Parkinson's research (MJFF) at our center. The ethics committee of the Hospital Clínic de Barcelona approved the study, and all subjects gave written informed consent. PD diagnosis was made according to the UK PD Society Brain Bank criteria, except that more than one affected relative with PD was not an exclusion criterion.[18] The studied cohort included 111 subjects consisting of heterozygous G2019S L2PD (n = 25) and G2019S L2NMC (n = 25), patients with iPD (n = 25), and healthy controls (n = 36; Table). The patients with iPD and controls were age-matched with patients with L2PD, and the L2NMC were recruited as available. We selected the healthy control group from among the spouses from the patients with PD visiting our outpatient clinic and other genetically unrelated relatives, caregivers, or friends. To further assess the effects of the MLi-2 LRRK2 kinase inhibitor on P-Ser-935-LRRK2, P-Ser-1292-LRRK2, and P-Ser-473-AKT, we additionally collected fresh PBMC samples from 11 subjects, including heterozygous G2019S L2PD (n = 5), homozygous G2019S L2PD (n = 1), heterozygous R1441G L2PD (n = 1), and healthy controls (n = 4; Table S1). Except for one homozygous G2019S carrier with North-Arab Berber origin, all participants were White race of Spanish descent.

Genotyping

We used commercial Taqman SNP assays on-demand on a Step One Plus Real-time PCR System (Life Tech) to genotype the *LRRK2* G2019S mutation (Thermo Fisher Scientific; #C-63498123-10) and R1441G/C/H, as previously described.[19] The studied *LRRK2* mutations were absent in the patients with iPD and healthy controls. No systematic sequencing was done to exclude additional mutations causing monogenic PD in iPD cases.

PBMC Isolation

A total of 8ml of blood was drawn by peripheral vein puncture in fasting. PBMCs were isolated by density gradient using sodium-citrate collector tubes according to

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TABLE: Clinicodemographic Features from Patients With G2019S L2PD, L2NMC, iPD, and Healthy Controls

Subjects (n = 111)	Mean age (yr) ± SD	Sex (male %)	Disease duration	Mean MDS UPDRS-III ± SD	Mean H&Y ± SD	Mean S&E (minimum - maximum)
L2PD (n = 25)	65.28 ± 11.16	32%	10.84 ± 9.92	24.08 ± 12.83	1.96 ± 0.78	89% (70–100)
L2NMC (n = 25)	51.13 ± 13.34	48%	0	1.96 ± 2.72	0	100%
iPD (n = 25)	69.12 ± 9.88	60%	7.64 ± 7.23	21.32 ± 11.10	1.72 ± 0. 54	96% (70–100)
C (n = 36)	66.33 ± 11.54	55%	0	3.56 ± 3.30	0	100%

Abbreviations: C = healthy controls; H&Y = Hoehn and Yahr motor disability scoring; iPD = idiopathic Parkinson's disease; L2NMC = non-manifesting LRRK2 G2019S carriers; L2PD = LRRK2-associated Parkinson's disease; MDS UPDRS III, Unified Parkinson's Disease Rating Scale scoring from the Movement Disorders Society; S&E = Schwab and England activities of daily living scoring; SD, standard deviation.

the manufacturer's instructions (BD Vacutainer CPT, #EAN30382903627821). Dry pellets were flash-frozen in liquid N_2 and stored at $-80°C$ until use.

Supplementary Methods

Please see Supplementary Methods for Western blotting analysis and LRRK2 inhibition by MLi-2 treatment.

Results

Analysis by Western blot of PBMCs from patients with G2019S L2PD, G2019S L2NMC, iPD, and controls showed similar levels of total LRRK2 and P-Ser-935-LRRK2 across groups (Fig 1B, C). On the contrary, we observed statistically significant phospho-protein differences for P-Ser-473-AKT (Kruskal-Wallis test; $p = 0.0031$). More specifically, all G2019S mutation carriers as a group (ie, L2PD and L2NMC), showed increased P-Ser-473-AKT levels compared to controls (Fig 1E; Dunn test adjusted $p = 0.0022$) or iPD (see Fig 1E; Dunn test adjusted $p = 0.0260$). Segregating by disease status, we also found increased P-Ser-473-AKT in G2019S L2PD (Fig 1D; Dunn test adjusted $p = 0.0353$) and G2019S L2NMC independently compared to controls (Fig 1D; Dunn test adjusted $p = 0.0170$), but no changes in
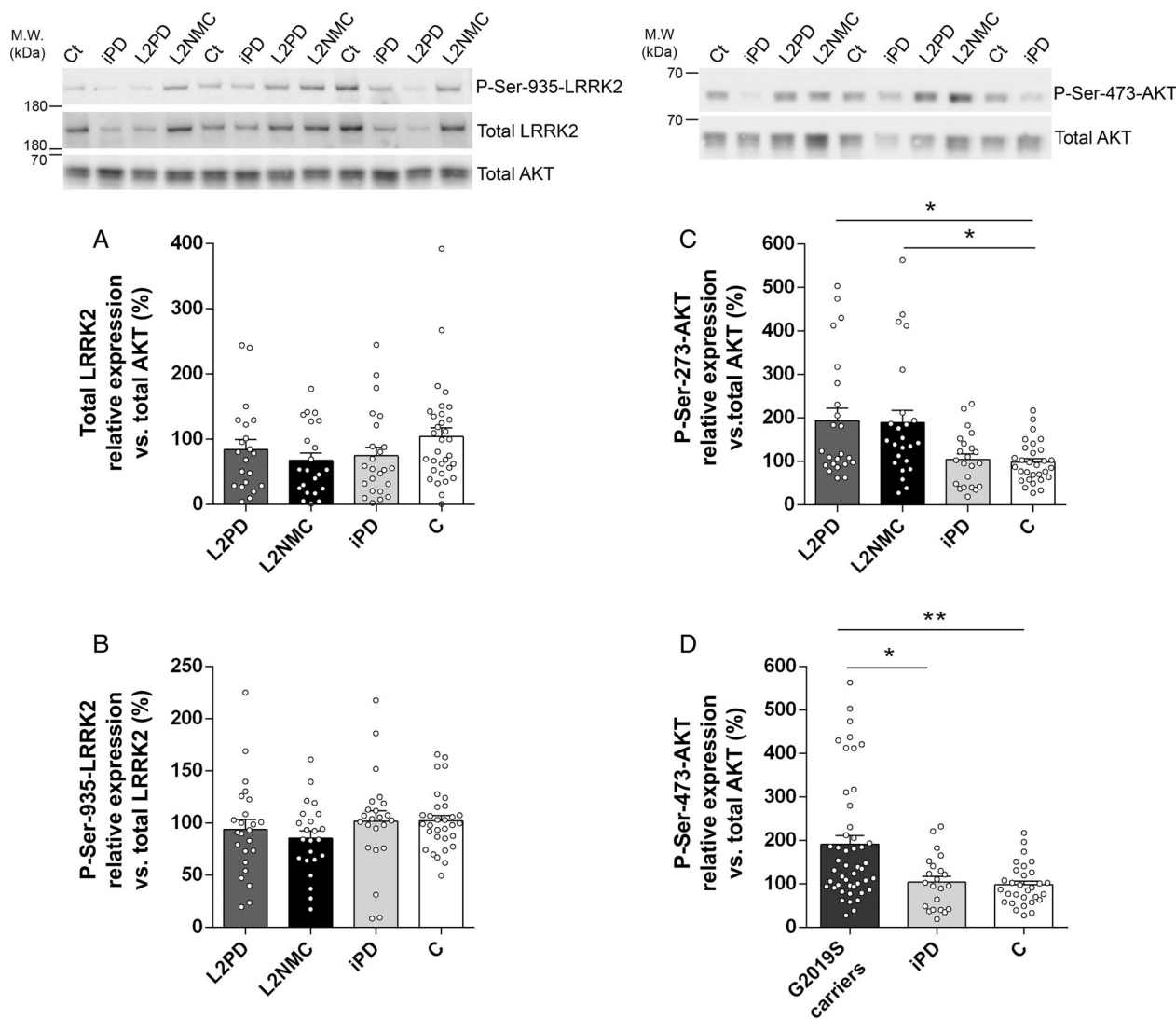

FIGURE 1: Analysis of LRRK2 and AKT phosphorylation levels in PBMC lysates from G2019S L2PD, G2019S L2NMC, iPD, and healthy controls. (A) Western blotting analysis of representative samples (MW = molecular weight in KDa). (B) Total LRRK2 protein levels relative to total Akt. (C) P-Ser-935-LRRK2 levels relative to total LRRK2. (D) P-Ser-473-AKT levels relative to total AKT. (E) P-Ser-473 AKT levels in all G2019S mutation carriers (L2PD and L2NMC). Bar plot data is represented as mean ± standard error of the mean (SEM; samples included in the densitometry analyses in A: 21 L2PD, 22 L2NMC, 24 iPD, and 32 C; B: 24 L2PD, 23 L2NMC, 23 iPD, and 31 C; C: 23 L2PD, 24 L2NMC, 22 iPD, and 30 C; D: 47 G2019S carriers, 22 iPD, and 30 C). Group comparisons were made using Kruskal-Wallis analysis followed by Dunn post hoc test. Statistical significance is referred as (*) *p* below 0.05; (**) *p* below 0.01. iPD = idiopathic Parkinson's disease; L2NMC = non-manifesting LRRK2 G2019S carriers; L2PD = LRRK2-associated Parkinson's disease; PBMC = peripheral blood mononuclear cell.
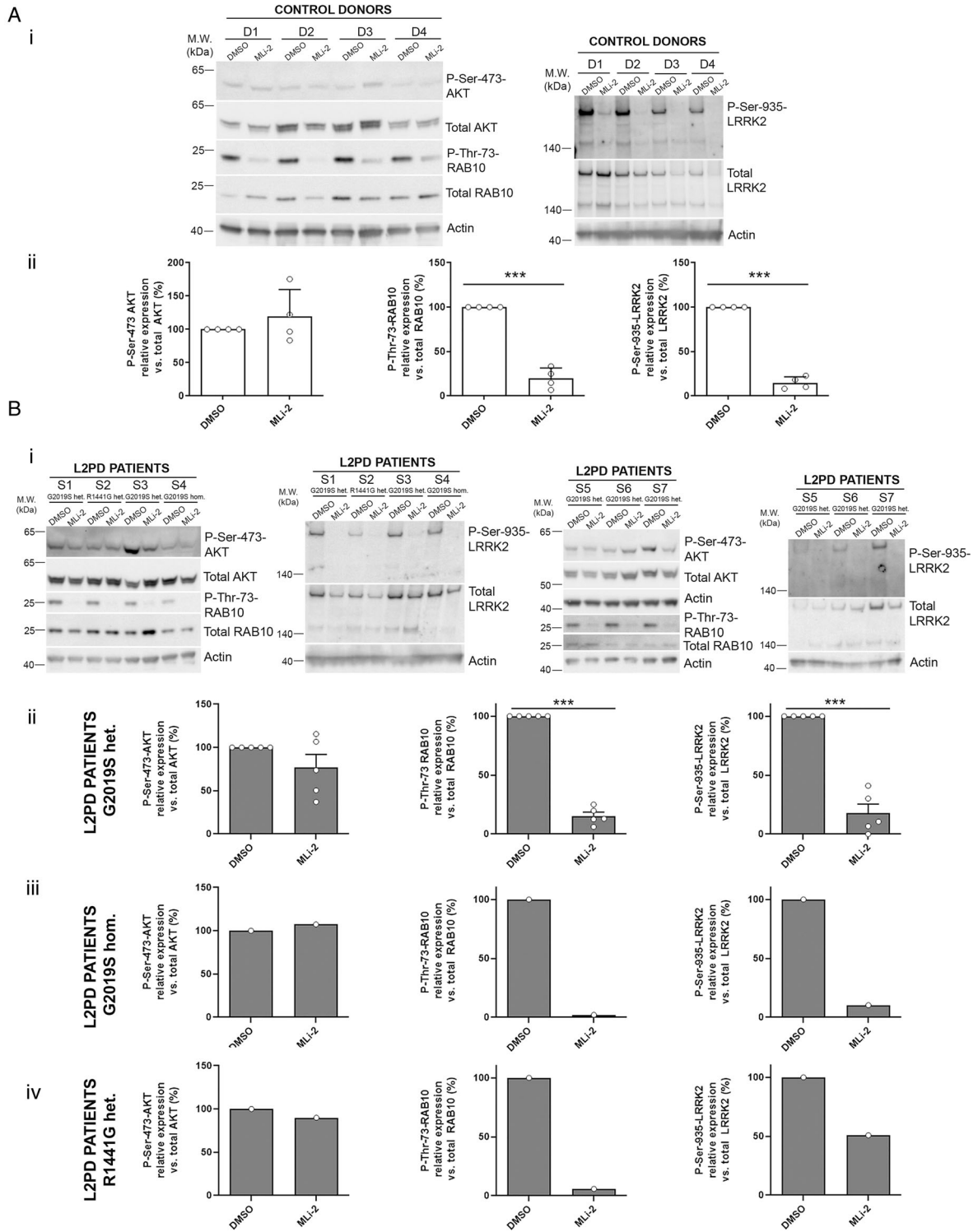
FIGURE 2: Analysis of AKT phosphorylation in PBMC lysates from (A) healthy donors and (B) patients with L2PD treated with small-molecule LRRK2 inhibitors. LRRK2 kinase activity was inhibited with 1 hour treatment of 100 nM 3-(4-Pyrimidinyl) Indazole (MLi-2) using DMSO as a vehicle. Inhibition of LRRK2 is proved by the decrease in RAB10 and LRRK2 phosphorylation levels (P-Thr-73-RAB10 and P-Ser-935-LRRK2). (A) (i) Western blotting panels of PBMCs protein extracts from healthy donors D1 to D4 (n = 4). (ii) Densitometric analysis of P-Ser-473-AKT protein levels relative to total AKT, P-Thr-73-RAB10 relative to total RAB10, and P-Ser-935-LRRK2 relative to total LRRK2. (B) (i) Western blotting panels of PBMCs from study subjects S1 to S7 (n = 7), including patients with L2PD carrying the mutations G2019S or R1441G in heterozygosis (het.) or homozygosis (hom.). Densitometric analysis of P-Ser-473-AKT levels relative to total AKT, P-Thr-73-RAB10 relative to total RAB10 and P-Ser-935-LRRK2 relative to total LRRK2 in (ii) L2PD G2010 heterozygous patients (n = 5), (iii) a patient with L2PD G2019S homozygous (n = 1) and (iv) a patient with L2PD R1441G homozygous (n = 1). In every patient, the densitometric value is normalized to its respective DMSO control as percentage of change after MLi-2 treatment. Data were analyzed by Student's *t* test (***p < 0.001 vs. DMSO control). L2PD = LRRK2-associated Parkinson's disease; PBMC = peripheral blood mononuclear cell.

iPD. These results indicate that increased P-Ser-473-AKT levels are associated with the G2019S mutation suggesting augmented P-Ser-473-AKT phosphorylation as a state marker of G2019S carriers, either patients with L2PD or patients with PD with at-risk L2NMC.

To further investigate whether P-Ser-473-AKT upregulation may be related to enhanced kinase activity of G2019S mutant LRRK2, we subsequently collected fresh PBMC samples from 11 additional subjects, including 5 patients with heterozygous G2019S L2PD, one patient with homozygous G2019S L2PD, one patient with heterozygous R1441G L2PD, and 4 healthy controls (Fig 2). In parallel, we treated these PBMC samples either with the LRRK2 kinase inhibitor MLi-2 (100 nM, 1 hour) or the DMSO vehicle alone. Whereas the MLi-2 treatment significantly reduced P-Ser-935-LRRK2 and P-Thr-73-RAB10 levels in G2019S carriers and controls, we observed no significant effect of MLi-2 on P-Ser-473-AKT levels. Interestingly, patients with G2019S L2PD under LRRK2 inhibition showed a trend toward lower levels of P-Ser-473-AKT, which warrants further investigation.

Discussion

Ours is the first study reporting increased P-Ser-473-AKT levels in blood cells from G2019S carriers, either patients with L2PD with a manifest disease or L2NMC at risk of PD, but not in iPD indicating an association with the G2019S mutation. Different groups have reported that in the central nervous system, AKT activation by phosphorylation at Ser-473 promotes neuroprotection in PD and other neurodegenerative disorders, such as Alzheimer's disease (AD)[14,20,21] or Huntington's disease.[22,23] In neural tissue from Drosophila PD models, P-Ser-473-AKT was proposed as a downstream target of LRRK2, although these findings have not been further replicated yet.[12] In addition, human *LRRK2* G2019S and R1441C dopaminergic neurons showed enhanced apoptosis mediated by decreased P-Ser-473-AKT levels.[24] Moreover, neurodegeneration mediated by low P-Ser-473-AKT was described in iPSC-derived neurons of patients with PD carrying the *LRRK2* I2020T pathogenic mutation from the Japanese family from Sagamihara, in which the *LRRK2* locus PARK8 was first reported.[12]

However, in peripheral non-neural tissues from various disease models, P-Ser-473-AKT activation has been linked to inflammation, indicating different tissue-specific effects of AKT deregulation (eg, promoting neutrophil[25] and lymphocyte survival),[26] or platelet activation.[27] Similarly, autoimmune disorders with inflammation, such as systemic lupus erythematosus, exhibited increased P-Ser-473-AKT.[28–30] In neurological diseases with a recognized immune component, such as AD, enhanced AKT signaling led to neuroinflammation

by activating microglia, consistent with its peripheral immune origin.[31,32] In PD, a-synuclein fibrils treatment of microglia showed increased AKT phosphorylation.[30] Another study in G2019S fibroblasts from patients with L2PD reported peripheral AKT activation at Thr-308.[16] Indeed, *LRRK2* mutations have been associated with inflammatory disease in G2019S L2PD and L2NMC.[29,33–35] Our findings of high P-Ser-473-AKT levels in blood cells from G2019S carriers support the recently recognized immune component of PD at multiple levels,[36–39] and may hold potential therapeutic implications for peripheral modulation of AKT.

We also observed that elevated P-Ser-473-AKT levels were insensitive to LRRK2 inhibition by Mli-2,[17] suggesting that AKT is not a direct substrate of LRRK2, at least in blood cells. Nevertheless, crosstalk between LRRK2 and other kinases with a preference for P-Ser-473-AKT cannot be ruled out. Moreover, increased P-Ser-473-AKT levels directly related to enhanced LRRK2 activity (ie, reversible by MLi-2),[17] were shown in peripheral phagocytes only in the physiological context of active macropinocytosis,[40] suggesting potentially different effects in vivo and in cell pellets. In our study, the differential P-Ser-473-AKT levels in G2019S carriers identify phospho-AKT as a candidate biomarker of chronic inflammation associated with the G2019S mutation[33,34] in L2PD and L2NMC. P-Ser-473-AKT could serve as a pharmacodynamic readout of AKT modulation in L2PD clinical trials of AKT inhibitors reducing inflammation in blood cells or microglia. This point is relevant given that several AKT modulation strategies are being explored in neurodegenerative diseases, including AD or PD.[41,42] If valid, similarly as proposed for LRRK2 inhibitors,[3,43] initiation of AKT modulators in L2NMC would be an option to investigate in future clinical trials.

Our study also found that P-Ser-935-LRRK2 levels were similar in PBMCs from patients with G2019S L2PD, G2019S L2NMC, iPD, and controls. P-Ser-935-LRRK2 phosphorylation is catalyzed by other kinases upstream of LRRK2.[5] Results in previous studies have been slightly contradictory, with one study showing decreased P-Ser-935-LRRK2 in G2019S L2PD PBMCs compared to iPD only but not with controls,[7] and another study using PBMCs from the same LRRK2 cohort[7] reporting no P-Ser-935-LRRK2 changes in G2019S L2PD or G2019S L2NMC versus controls but increased levels in iPD.[8] Although these studies[7,8] used sensitive enzyme-linked immunosorbent assay (ELISA)-based methods with broader detection dynamic ranges than Western blotting, the sample size and antibodies were similar to our study (Table S2). In addition, one Western blotting analysis of LRRK2 neutrophils showed no P-Ser-935-LRRK2 differences in G2019S L2PD or iPD versus controls.[10] Altogether, these works agree in reporting no P-Ser-935-LRRK2 differences between G2019S

carriers and controls, indicating that P-Ser-935-LRRK2 is not an endogenous biomarker of G2019S carriers. Yet, P-Ser-935-LRRK2 represents an appealing pharmacodynamic readout for LRRK2 target engagement,[44,45] as we also observed by treating fresh PBMCs with MLi-2.

There are 2 unmet needs in the LRRK2 field. First, it is unknown why some LRRK2 carriers develop PD and some do not, which our study does not resolve, given that P-Ser-473-AKT levels were equally increased in all G2019S carriers regardless of their disease status. Second, there is a limited target engagement in L2PD clinical trials, especially for early neuroprotective measures before the motor onset. Our study explicitly tackles the former need by identifying P-Ser-473-AKT as a novel candidate biomarker of peripheral inflammation in G2019S carriers. However, future phospho-AKT studies in longitudinal LRRK2 cohorts are needed. In summary, we have identified P-Ser-473-AKT as an endogenous candidate biomarker of peripheral inflammation in LRRK2 G2019S carriers using accessible blood cells. Our findings warrant further assessment in additional G2019S cohorts and, if validated, may hold implications for clinical trials investigating AKT modulators in L2PD but also in other neurological diseases cursing with peripheral inflammation.

Acknowledgments

Author Contributions

R.F-S., C.M., M.E., and M.J.M. contributed to conception and design of the study. All authors contributed to the acquisition and analysis of data. R.F-S., M.E., C.M., A.G., L.P-S., G.C-C., J.S-B., and N.M-F. contributed to drafting the text or preparing the figures.

Potential Conflicts of Interest

The authors declared no conflict of interest.

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