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Characterization of molecular biomarkers in cerebrospinal fluid and serum of E46K-*SNCA* mutation carriers

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ABSTRACT

Introduction: Blood and cerebrospinal fluid represent emerging candidate fluids for biomarker identification in Parkinson's disease (PD).

Methods: We studied 8 individuals carrying the E46K-*SNCA* mutation (3 PD dementia (PDD), 1 tremor-dominant PD, 2 young rigid-akinetic PD and 2 asymptomatic) and 8 age- and sex-matched healthy controls. We quantified the levels of total alpha-synuclein (a-syn), neurofilament light chain (NfL), glial fibrillary acidic protein (GFAP), Tau and ubiquitin carboxy-terminal hydrolase L1 (UCHL1) with SiMoA (Quanterix) in cerebrospinal fluid (CSF) of mutation carriers and in serum of all participants. The correlation between the concentration of biofluid markers and clinical outcomes was evaluated.

Results: Although based on a small number of cases, CSF a-syn was decreased in symptomatic E46K-*SNCA* carriers compared to the asymptomatic ones. Asymptomatic carriers exhibited similar serum biomarker levels as compared to matched controls, except for serum a-syn, which was higher in asymptomatic individuals. Carriers with PDD diagnosis displayed increased levels of serum NfL and GFAP compared to matched controls. These findings highly correlated with cognitive and motor status of E46K-*SNCA* carriers, but not with disease duration.

Conclusions: Patients with familial forms of neurodegenerative disease exhibit variable penetrance of the phenotype and are exceptionally valuable for delineating biomarkers. Serum and CSF molecular biomarkers in E46K-*SNCA* mutation carriers show that a-syn might be suitable to track the conversion from asymptomatic to PD, whereas NfL and GFAP might serve to foresee the progression to PD dementia. These findings should be interpreted with caution and need to be replicated in other genetic synucleinopathy cohorts.

1. Introduction

Parkinson's disease (PD) is the most common motor neurodegenerative disease that is neuropathologically characterized by the loss of dopaminergic neurons in substantia nigra. Although the precise mechanisms promoting neurodegeneration are unknown, aggregates of misfolded alpha-synuclein (a-syn) in the nervous system forming Lewy bodies and Lewy neurites are believed to be central in the pathogenesis

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of PD [1]. The clinical diagnosis of PD is based on its canonical motor symptoms, but there is a wide heterogeneity of clinical presentations with respect to the motor phenotype, development of cognitive impairment and the rate of disease progression [2], which highlights the need to develop reliable biomarkers to unequivocally diagnose the disease or monitor disease progression. Moreover, even when causative PD mutations are identified in a patient, penetrance of those mutations is highly variable and often far from complete. Predictive biomarkers in such cases would be invaluable in identifying those patients most at-risk of developing the disease.

The use of fluid-based biomarkers is an urgent need for disease diagnosis and monitoring in PD. Testing blood or cerebrospinal fluid (CSF) over nuclear medicine neuroimaging techniques offers some advantages, such as ease of accessibility for screening and follow-up in a wide range of medical facilities. Although blood and CSF-based biomarkers are currently in their early phase of development for PD diagnosis and monitoring, they provide access to understand the molecular mechanisms that may underlie PD [3]. Most studies conducted so far have focused on the measurements of a-syn or neurofilament light chain (NfL) in CSF and peripheral blood in different cohorts of idiopathic PD patients. These studies have shown consistent results in CSF, observing decreased a-syn and increased NfL levels in sporadic PD patients [4,5]. Regarding blood plasma and serum findings, results are more variable and increased [6,7], decreased [8,9], or no changes [10,11] of blood a-syn and NfL have been described in PD compared to controls. However, few biomarker studies have been performed in genetic PD cohorts. These cohorts offer some exceptional opportunities for correlating biomarkers with clinical indices and phenotypic penetrance.

SNCA-linked dominant mutations are rare and limited to few families worldwide. In 2004, our group described a novel point mutation in the a-syn gene (G188A), which results in glutamic acid substitution by lysine in position 46 of the *SNCA* gene (E46K-*SNCA*) [12]. This mutation causes a dominant autosomal inherited PD and induces PD with clinical and pathological features reminiscent of dementia with Lewy bodies [12]. Their clinical phenotype is characterized by rapid progressing parkinsonism with sleep disturbances preceding the parkinsonian signs [13], dysautonomia, and cognitive impairment with prominent posterior cortical dysfunction from early stages of the disease [14,15]. So far, 8 subjects of the same family with E46K-*SNCA* mutation with different degrees of disease severity have been longitudinally followed. Symptomatic carriers of the E46K-*SNCA* mutation represent a clinically aggressive genetic model of pure Lewy body disease (LBD) with accelerated neurodegeneration. Nonetheless, the fact that few individuals are asymptomatic, this family offers a unique opportunity to study biological samples and their correlation with clinical outcomes in the whole spectrum of PD.

In this work, we analyzed the levels of total a-syn, NfL, glial fibrillary acidic protein (GFAP), ubiquitin carboxy-terminal hydrolase L1 (UCHL1) and Tau in serum and CSF of E46K-*SNCA* mutations carriers to better understand the molecular changes associated with this mutation, and to test whether any biofluid marker could be used for disease monitoring and phenotypic conversion.

2. Methods

2.1. Study population

Eight carriers of E46K-*SNCA* mutation and 8 age- and sex-matched healthy controls were recruited for the present study between 2019 and 2020. From the 8 mutation carriers, 2 were asymptomatic, 1 was recently diagnosed with PD, 2 were young carriers with PD diagnosis and 3 presented PD dementia (PDD) (Table 1). All participants were recruited through the Department of Neurology at Cruces University Hospital. Patients with PD fulfilled Parkinson's UK Brain Bank criteria for the diagnosis of PD.

The study protocol was approved by the regional Basque Clinical Research Ethics Committee. All participants gave written informed consent prior to their participation in the study, in accordance with the tenets of the Declaration of Helsinki.

2.2. Clinical evaluations

One experienced neurologist in the field of movement disorders recorded disease duration, Hoehn & Yahr Scale score, Unified PD Rating Scale (UPDRS) score, and Levodopa Equivalent Daily Dose (LEDD). In E46K-*SNCA* carriers, general cognition was assessed with Montreal Cognitive Assessment (MoCA), A Single-Question Screen for REM Sleep Behavior Disorder (RBDQ1) was administered, and olfaction was evaluated with Brief Smell Identification Test (BSIT). Symptomatic mutation carriers were studied in an on-medication condition to complete all study assessments.

2.3. Sample collection and processing

Blood samples were collected by venipuncture in the morning using Vacutainer tubes (BD Vacutainer SST II Advance, Ref.: 367955) for serum separation. Lumbar puncture was only performed in E46K-*SNCA* mutation carriers and not in controls, following Ethics Committee requirements. CSF and serum were collected on the same day in low adhesion polypropylene tubes. Serum samples were centrifuged at 2500 rpm for 20 min, while CSF was centrifuged at 2000 rpm for 10 min at room temperature. Following centrifugation, serum was transferred to polypropylene tubes and CSF to Micronic tubes in 500 μl aliquots. Biospecimens were stored at −80 °C within 60 min after collection.

2.4. Immunoassay (SiMoA)

Serum and CSF protein levels were quantified by commercially available single molecule array (SiMoA) kits and analyzed on a fully automated SiMoA HD-1 Analyzer (Quanterix). NfL, Tau, GFAP and UCHL1 biomarkers were measured with Neurology 4-Plex A assay (Cat. No. 102153) following manufactures instructions [16]. For total a-syn

Table 1
Demographic and clinical characteristics of study participants.

E46K-SNCA mutation carriers												Matched Control Group		
ID	Sex	Age	Diagnosis	Disease duration (years)	HY	UPDRS				LEDD (mg)	MoCA	ID	Sex	Age
						I	II	III	IV					
A07	Male	68	PDD	17	5	11	34	66	12	852	3	CA02	Male	67
A05	Female	62	PDD	19	3	10	30	46	10	750	11	CA09	Female	62
A03	Female	56	PDD	13	3	7	19	35	8	750	9	A08	Female	55
A06	Male	63	PD	0.5	1	0	3	9	2	100	27	CA11	Male	62
A01	Male	37	PD	8.2	2	1	9	25	3	310	29	CA07	Male	35
A09	Male	39	PD	4.6	2	1	8	22	4	100	28	CA06	Male	39
A04	Female	60	Asymptomatic		–	0	0	0	0	0	29	CA03	Female	59
A02	Male	39	Asymptomatic		–	0	0	0	0	0	28	CA05	Male	39

quantification, the Human Alpha-Synuclein assay (Cat. No. 102233) was used, and manufactures instructions were followed for CSF [17]; serum samples were diluted to a final dilution range of 40x to 60x to fit within the dynamic range of the standard curve (0–10,000 pg/mL). The concentration of 3 samples was slightly above the range of the standard curve. In those cases, the maximum value of the standard curve was assigned for quantitative analyses. All samples were run in duplicates, and the average concentrations calculated. Investigators performing the analysis were blinded to the diagnosis.

2.5. Statistical analysis

All analyses were performed using RStudio software (v 1.4.1717). Due to the heterogeneity of clinical phenotype of E46K-*SNCA* mutation carriers, E46K-*SNCA* carriers were stratified into young and older patients for descriptive statistics. Within each subgroup, we compared the levels of blood and CSF markers between asymptomatic vs. the symptomatic patients. No statistical tests were applied due to the small number of subjects within subgroups. When comparisons were performed between E46K-*SNCA* carriers and matched healthy controls, a two-sample T-test was applied. Non-normally distributed continuous variables were log-transformed for statistical hypothesis testing. Pearson's correlation coefficient was calculated for continuous variables, and Kendall's correlation coefficient for ordinal variables. P-values < 0.05 were considered statistically significant.

3. Results

3.1. Clinical characteristics

The demographical and clinical features of E46K-*SNCA* mutation carriers and age and sex-matched healthy controls are displayed in

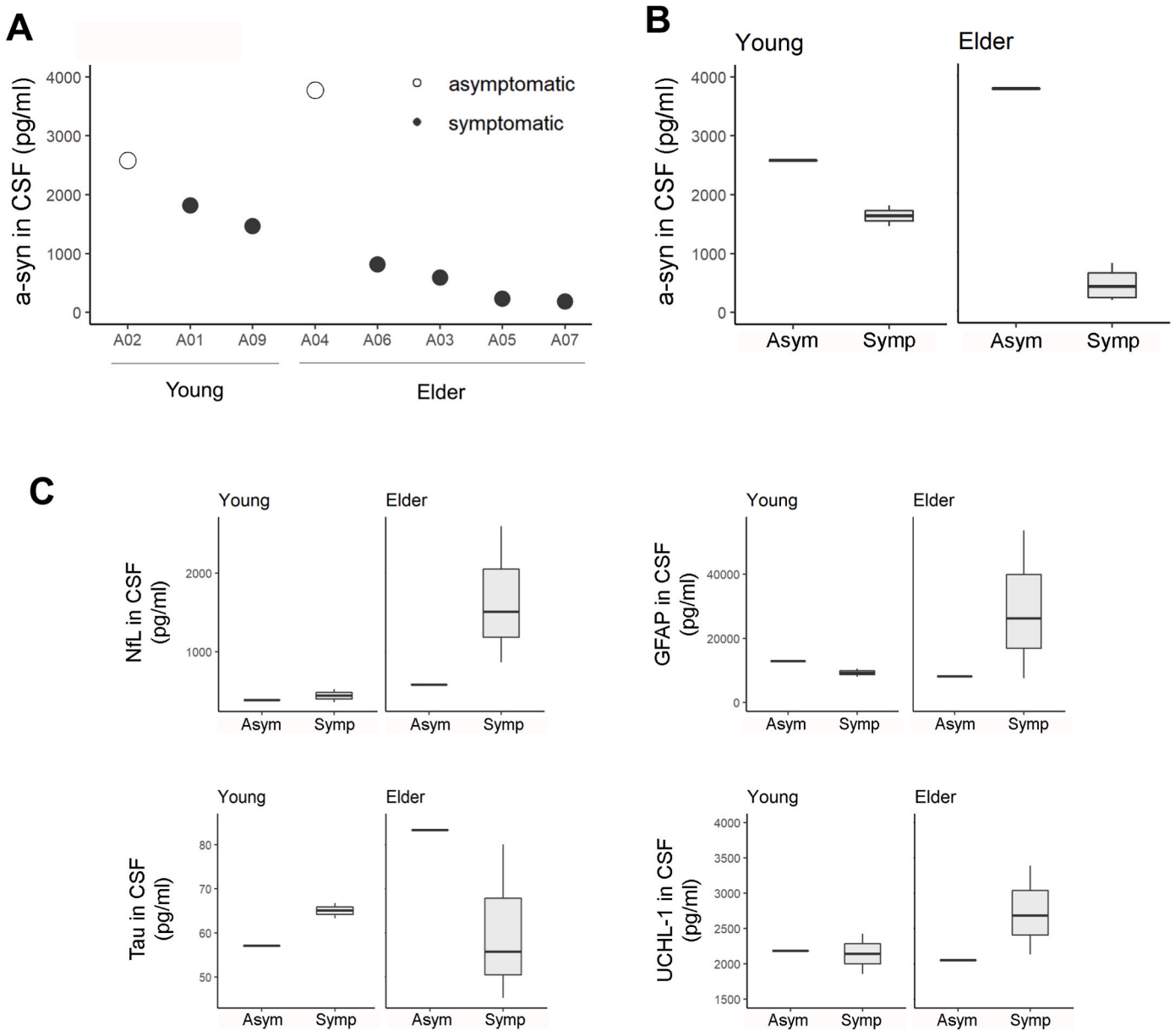

Fig. 1. Cerebrospinal fluid levels of alpha-synuclein (A), NfL, GFAP, Tau and UCHL1 (B) in E46K-*SNCA* mutation carriers. The concentration of each protein in asymptomatic vs. symptomatic carriers is represented in young (n = 3) and elder subjects (n = 5) separately. No statistical tests were performed due to the small sample size of subgroups after stratifying individuals by age. *Abbreviations*: Asymp: asymptomatic E46K-SNCA carrier; GFAP, glial fibrillary acid protein; NfL, neurofilament light chain; Symp: symptomatic E46K-*SNCA* mutation carrier; UCHL1, ubiquitin carboxy-terminal hydrolase L.

Table 1. Asymptomatic E46K-*SNCA* carriers underwent SPECT DatScan in 2021 to ascertain that they did not present presynaptic dopaminergic deficiency in basal ganglia and were free of PD-disease. Moreover, none of the asymptomatic carriers referred symptoms compatible with RBD and presented normal olfaction at study inclusion (BSIT ≥11). In contrast, all symptomatic carriers had polysomnography-confirmed RBD and hyposmia (BSIT ≤6). Additional clinical information of E46K-*SNCA* mutation carriers can be found in Supplementary Material.

3.2. CSF protein levels in E46K-SNCA mutation carriers

We first analyzed the levels of a-syn, NfL, GFAP, UCHL1 and Tau in CSF, as this biofluid has shown the most consistent results in PD. Asymptomatic carriers of E46K-*SNCA* mutation exhibited higher concentrations of CSF a-syn compared to symptomatic carriers (Fig. 1A). Stratified comparisons revealed that CSF a-syn levels were lower in older symptomatic (n = 3, 456 ± 300 pg/ml) compared to the older asymptomatic individual (n = 1, 3772 pg/ml), Fig. 1B). Similarly, young symptomatic individuals exhibited lower concentrations of CSF a-syn compared to young asymptomatic carriers, with a mean difference of 941.85 pg/ml (35% change). Moreover, CSF a-syn levels tended to be higher in young symptomatic carriers (n = 2, 1639 ± 248 pg/ml) compared to older symptomatic ones.

Further analysis revealed that, in young carriers, CSF concentrations of NfL, GFAP, UCHL1 and Tau were similar between asymptomatic and symptomatic carriers. However, older carriers showed a trend towards increased NfL, GFAP and UCHL1 concentrations compared to the asymptomatic elder carrier (A04), whereas CSF levels of Tau showed the opposite trend (Fig. 1C). It is worth mentioning that CSF levels of the latter proteins in the elder symptomatic carrier with TD-PD diagnosis were very similar to the levels of the asymptomatic carrier, except for Tau, which was decreased by 54% in TD-PD carrier compared to the asymptomatic elder relative.

3.3. Correlation between CSF and serum markers

Within the E46K-*SNCA* group, we found positive and significant correlations between levels in serum and CSF of NfL (r = 0.87, p = 0.005) and GFAP (r = 0.74, p = 0.03), but not in the concentrations of a-syn (r = 0–21, p = 0.60), Tau (r = −0.31, p = 0.45) or UCHL1 (r = 0.66, p = 0.07).

3.4. Serum markers in comparison with the control group

Next, we assessed the differences in serum concentration of the proposed biomarkers in E46K-*SNCA* mutations carriers (n = 8) in comparison with age- and sex-matched control group (n = 8). Measurements of serum NfL revealed that the concentration was similar between age and sex-matched healthy controls and young asymptomatic, young symptomatic, elder asymptomatic and elder TD-PD carriers (Fig. 2A). However, E46K-*SNCA* carriers with PDD showed increased serum NfL levels vs. matched healthy controls (PDD E46K-*SNCA* (n = 3), 30.6 ± 15.1 pg/ml vs. control (n = 3) 11.4 ± 4.0 pg/ml, t = 2.88, p = 0.048). A similar pattern was observed for serum GFAP levels (Fig. 2B), older carriers with PDD showing increased serum GFAP levels compared to controls (E46K-*SNCA*, 361.9 ± 160 pg/ml vs. control 148.7 ± 53 pg/ml), but differences were non-significant (t = 2.28, p = 0.091). Tau and UCHL1 levels in serum were comparable between E46K-*SNCA* carriers and matched healthy controls, UCHL1 levels showing large coefficients of variation (>20% in about 35% of the samples). In E46K-*SNCA* carriers, a-syn showed great variability and, although an overall trend towards increased levels was observed in symptomatic carriers compared

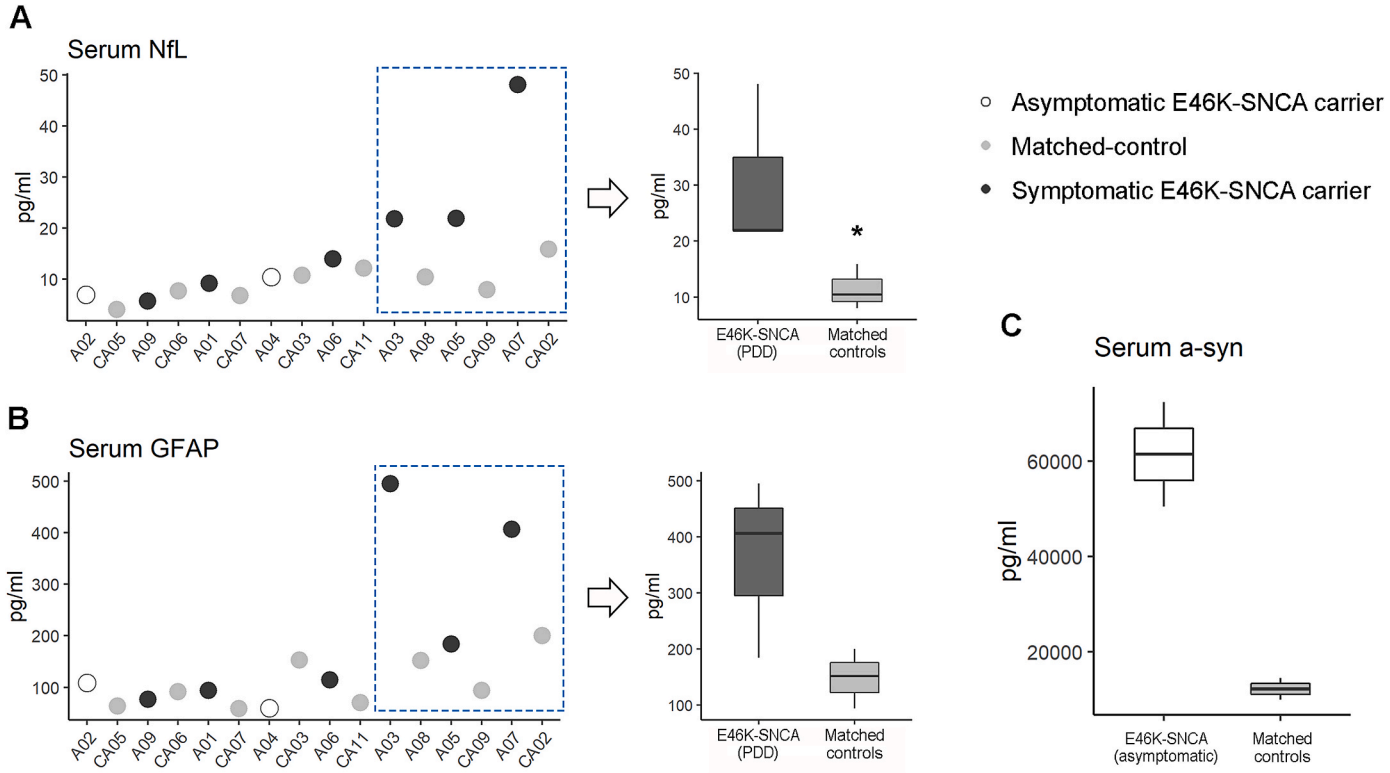

Fig. 2. Serum levels of NfL (A) and GFAP (B) in E46K-*SNCA* mutation carriers and age- and sex-matched controls. Dots represents the mean value of duplicated in SiMoA testing in each subject. Boxplots in (A) and (B) show the serum concentrations in E46K-*SNCA* carriers with PDD diagnosis and their matched-control counterparts. (C) Boxplot showing serum a-syn levels in asymptomatic E46K-*SNCA* mutation carriers compared to age- and sex-matched controls. No statistical test was applied due to the small sample size in each group (n = 2). *Abbreviations*: a-syn, alpha-synuclein; GFAP, glial fibrillary acidic protein; NfL, neurofilament light chain; PDD, Parkinson's disease dementia. *p < 0.05.

to asymptomatic carriers, the TD-PD (A06) and eldest PDD (A07) presented lower levels than their matched healthy controls. Intriguingly, both asymptomatic carriers showed increased serum a-syn levels compared to controls (asymptomatic E46K-*SNCA* (n = 2), 61.5 ± 15.4 ng/ml vs. matched healthy controls (n = 2) 12.2 ± 0.3 ng/ml) (Fig. 2C).

4. Correlation between biological fluid-derived biomarkers and clinical outcomes

Finally, we explored whether CSF or serum protein concentrations were correlated with clinical variables in E46K-*SNCA* mutation carriers, including motor impairment measured with UPDRS III and HY, general cognition, and disease duration. We found that serum levels of NfL (r = 0.88, p = 0.021), GFAP (r = 0.81, p = 0.047), and Tau (r = 0.83, p = 0.04) were significantly correlated with motor impairment. Serum measurements of NfL and GFAP were also significantly correlated with cognitive decline (r = 0.89, p = 0.003 and r = 0.88, p = 0.004, respectively). Serum a-syn levels did not correlate with disease duration, MoCA score or UPDRS III score.

Contrarily, CSF NfL measurements revealed that, although the correlation coefficients for MoCA and UPDRS III were high (r = −0.67, r = 0.58, respectively), results were not statistically significant. On the other hand, CSF a-syn levels were negatively correlated with HY stage (tau = −0.69, p = 0.021) and positively with MoCA score (r = 0.74, p = 0.035) (Fig. 3).

None of the serum or CSF biomarker measurements correlated with disease duration.

5. Discussion

The results of our study reveal that CSF a-syn levels were decreased in symptomatic E46K-*SNCA* mutation carriers compared to asymptomatic carriers, but this difference was not reproduced in serum samples. Interestingly, asymptomatic carriers showed higher serum a-syn concentration than their matched healthy control counterparts. On the other hand, serum NfL and GFAP levels highly correlated with CSF concentrations, rendering these blood-derived markers as good surrogate measures of CSF proteins. We observed that NfL and GFAP serum levels were significantly increased in E46K-*SNCA* carriers with PDD diagnosis compared to matched healthy controls. Finally, CSF a-syn and serum NfL highly correlated with disease severity of E46K-*SNCA* carriers, but not with disease duration. Taken together, our results suggest that increased a-syn levels may indicate presymptomatic or early stages of PD. As the disease progresses, we speculate that CSF a-syn decreases because of the deposition of a-syn in the nervous system, and the subsequent neurodegeneration contributes to a gradual increase in serum NfL and GFAP that is related to the severity of the disease.

A-syn is one of the leading biomarkers for PD due to its central role in the pathogenesis of the disease. Several studies have quantified total a-syn levels in CSF to discriminate PD patients from healthy subjects. These studies have concluded that PD patients consistently present decreased CSF a-syn levels [18–20]. Although few studies have been conducted in genetic variants, recent evidence shows that the prior statement is also true for PD patients with LRRK2 [21] and GBA1 mutations [22]. In line with previous findings, our results also show that symptomatic E46K-*SNCA* carriers displayed lower levels of CSF a-syn compared to disease-free asymptomatic mutation carriers that nicely correlated with the clinical status of patients in terms of HY stage and cognitive decline.

On the other hand, peripheral blood has been suggested to be an excellent candidate for biomarker detection due to the ease of accessibility and lesser invasiveness over CSF collection for screening and patient follow-up. Nowadays, ultrasensitive assays such as SiMoA has paved the way to investigate different protein levels in peripheral blood with minimal concentration improving its clinical applicability. Previous studies have shown variable results regarding peripheral blood a-syn levels in PD patients, although a recent meta-analysis performed by Bougea et al. [23] suggests that blood total a-syn is increased in PD patients compared to controls. The analytical method of choice were ELISAs in most of the previous studies. In this work, we used a more sensitive SiMoA and obtained variable results from serum samples in E46K-*SNCA* mutation carriers. Although an overall increase in serum a-syn was observed in E46K-*SNCA* carriers, both in symptomatic and asymptomatic, compared to matched healthy controls, some patients

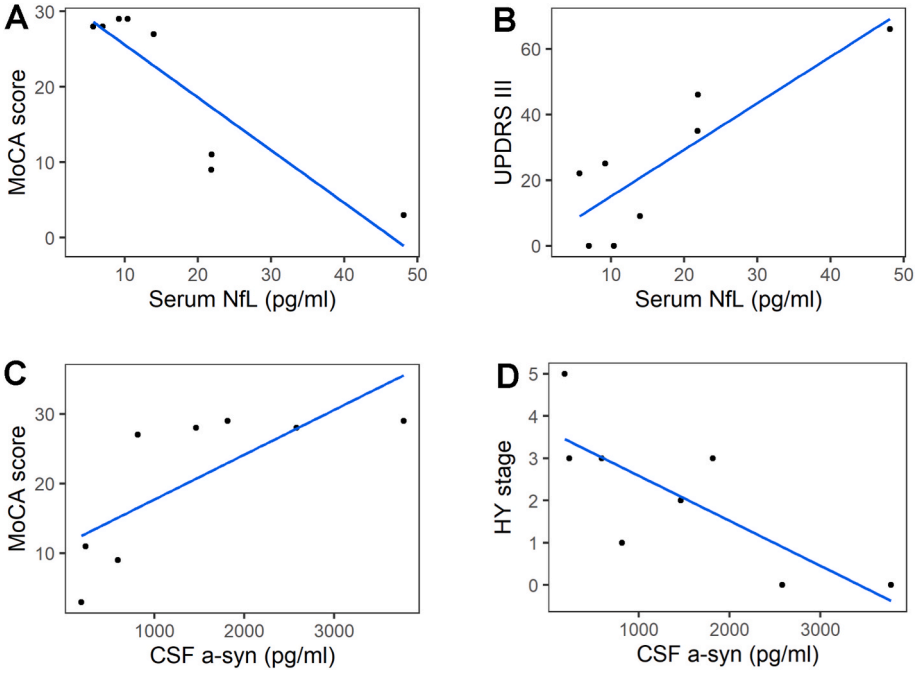

Fig. 3. Scatterplots showing the correlations between biofluid markers and cognitive status (A) and (C) or motor impairment (B) and (D). (A) and (B) show the correlation with serum NfL, whereas (C) and (D) show the correlation with CSF a-syn levels. *Abbreviations*: a-syn, alpha-synuclein; CSF, cerebrospinal fluid; HY, Hoenh & Yahr Scale; MoCA, Montreal Cognitive Assessment; NfL, neurofilament light chain; UPDRS III, Unified Parkinson's disease Rating Scale, part III.

displayed decreased levels. The mechanisms mediating these discrepant outcomes is uncertain. It might be that concomitant diseases of some carriers, like anemia or diabetes mellitus, could alter peripheral blood a-syn levels, as associations between a-syn with hematopoiesis [24] and insulin-resistance [25] have already been described. Another plausible explanation could be that synuclein homeostasis differs between blood and brain compartments. It is interesting to note that carriers of A53T mutation in *SNCA* gene, who have a similar clinical phenotype to E46K-*SNCA* carriers, also exhibit lower a-syn levels in blood than controls but asymptomatic A53T carriers show slightly higher concentrations than controls [26]. Similarly, in a previous study of our group, we detected phosphorylated a-syn levels in the skin of E46K-*SNCA* mutation carriers by immunohistochemistry [27], where the degree of deposition was particularly high in asymptomatic and presymptomatic patients. These findings suggest that peripheral a-syn levels might fluctuate during the natural course of the disease, observing increased levels during presymptomatic and early stages as compared to controls, and decreased levels as the severity of the disease progresses.

Regarding NfL levels measured in serum, higher concentrations were measured in E46K-*SNCA* mutations carriers with PDD diagnosis compared to age- and sex-matched controls. However, this was not observed in young symptomatic mutation carriers, suggesting that NfL serum levels might reflect a generalized neurodegeneration that usually coincides with cognitive disability. This idea is supported by neuroimaging studies, in which serum NfL levels have been associated with cognitive deterioration and posterior cortical degeneration measured with MRI [28]. Posterior cortical deterioration delineates the progression to PDD and is one of the earliest features of E46K-*SNCA* mutation carriers, as suggested by their visuospatial and language compromise [14]. This fact, together with the observation that serum NfL highly correlated with MoCA score in E46K-*SNCA* carriers, may indicate that NfL blood measure could be a useful biomarker to track PD dementia.

Few studies have explored GFAP blood levels in PD patients. GFAP is an intermediate filament protein that is expressed in the astroglial cytoskeleton and is released upon cell death or injury. According to the results of Su et al. [29], a cohort of sporadic PD patients showed increased blood GFAP, which coincides with the current results. However, GFAP increase was mainly due to the high levels observed in E46K-*SNCA* carriers with PDD diagnosis. Release of GFAP into the blood stream could be attributed to structural disintegration of astroglial cells during neurodegeneration or, more plausibly, GFAP overexpression could be a homeostatic response to neuronal damage, given that similar patterns were observed in NfL and GFAP blood levels for E46K-*SNCA* mutation carriers, and both correlated with motor and cognitive impairment.

A major limitation of the current study is the small sample size and therefore *p*-values should be interpreted with caution. However, *SNCA* mutations are rare and E46K point mutation is an exceptionally interesting multi-generation kindred that exhibits striking variable penetrance of a dominant a-syn mutation. Therefore, it is especially relevant to perform biomarker studies in these subjects, as their clinical profile might not completely coincide with idiopathic PD (iPD) patients, and future studies including a subgroup of iPD patients are required. Related to this, it should be stressed that blood biomarker differences described in the literature so far between iPD and controls are small and highly variable. In our study, young carriers and control subjects presented serum NfL and CSF a-syn levels similar to those described before [11, 30], whereas E46K-*SNCA* carriers with PDD diagnosis presented more pronounced differences compared to previous reports in iPD. Thus, we recommend relying on fluid biomarker levels in each group as measures of evidence rather than extracting conclusion based on *p*-values.

In conclusion, this work shows that CSF a-syn levels are decreased in symptomatic E46K-*SNCA* mutation carriers compared to asymptomatic carriers, and that serum levels of NfL and GFAP are increased, especially in carriers with PDD. Longitudinal studies in biofluid markers within E46K-*SNCA* carriers will determine whether declining CSF and serum a-syn mark the conversion from asymptomatic to symptomatic phase and if increasing NfL and GFAP serum levels may forecast conversion to dementia.

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Author contribution statement

A.M-G. and JC.G-E. contributed to the conception and design of the study. A.M-G., R.P., N.A., M.A., M.C-A., T.F., B.T. and I.G. contributed to the acquisition of clinical data and collection of biospecimens. E.C.-Z., F. Z., R.C., C.M., and F.C. designed and performed the SiMoA immunoassays. A.M-G. performed data analysis and drafted the initial manuscript. R.S.-P. and V.K. contributed to data interpretation. A.M-G., M.A., and JC.G-E. contributed to preparing the figures. All authors contributed to the revision of the final manuscript.

Declaration of competing interest

No potential conflict of interest to report.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2022.01.024.

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