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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Polyglutamine-Expanded Ataxin-3: A Target Engagement Marker for Spinocerebellar Ataxia Type 3 in Peripheral Blood

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ABSTRACT: Background: Spinocerebellar ataxia type 3 is a rare neurodegenerative disease caused by a CAG repeat expansion in the *ataxin-3* gene. Although no curative therapy is yet available, preclinical gene-silencing approaches to reduce polyglutamine (polyQ) toxicity demonstrate promising results. In view of upcoming clinical trials, quantitative and easily accessible molecular markers are of critical importance as pharmacodynamic and particularly as target engagement markers.

Objective: We aimed at developing an ultrasensitive immunoassay to measure specifically polyQ-expanded ataxin-3 in plasma and cerebrospinal fluid (CSF).

Methods: Using the novel single molecule counting ataxin-3 immunoassay, we analyzed cross-sectional and longitudinal patient biomaterials.

Results: Statistical analyses revealed a correlation with clinical parameters and a stability of polyQ-expanded ataxin-3 during conversion from the pre-ataxic to the ataxic phases.

Conclusions: The novel immunoassay is able to quantify polyQ-expanded ataxin-3 in plasma and CSF, whereas ataxin-3 levels in plasma correlate with disease severity. Longitudinal analyses demonstrated a high stability of polyQ-expanded ataxin-3 over a short period.
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Key Words: ataxin-3; Machado-Joseph disease; spinocerebellar ataxia type 3; singulex technology; target engagement biomarker

Spinocerebellar ataxias are a heterogeneous group of dominantly inherited, progressive diseases. The most common among them globally is spinocerebellar ataxia type 3 (SCA3), also known as Machado–Joseph disease, a multisystem disorder characterized by the degeneration of spinocerebellar tracts, dentate nucleus, brainstem nuclei, and basal ganglia. It is caused by an unstable expansion of a polyglutamine (polyQ)-encoding CAG repeat in the *ATXN3* gene resulting in the expression of an abnormally elongated ataxin-3 protein that is considered to be the major cause of neurodegeneration in SCA3. Currently, there is no treatment, but new approaches aiming to silence the disease gene are close to clinical trials.[1]

To demonstrate target engagement in such trials, the availability of ultrasensitive quantitative immunoassays to measure the concentration of polyQ-expanded ataxin-3 in body fluids is mandatory. Recently, an immunoassay that detected polyQ-expanded ataxin-3 in body fluids, including cerebrospinal fluid (CSF), and discriminated between SCA3 patients and healthy controls was developed.[2] In addition, an assay based on time-resolved fluorescence energy transfer (TR-FRET) was reported, which was capable of measuring ataxin-3 concentrations in peripheral blood mononuclear cells (PBMCs) but failed to detect ataxin-3 in body fluids.[3]

Here, we report a novel single molecule counting (SMC) ataxin-3 immunoassay to specifically measure polyQ-expanded ataxin-3 in plasma and CSF. Using this assay, we found strong correlations between plasma polyQ-expanded ataxin-3 concentrations and clinical parameters. In a longitudinal study, we observe a high stability of polyQ-expanded ataxin-3 in pre-ataxic and ataxic mutation carriers.

Patients and Methods

Ethics and Consent to Participate

The study was approved by the local committees of all participating centers. Informed and written consent was obtained from all study participants at enrollment.

Study Participants

Blood and CSF samples were obtained from participants of the European Spinocerebellar Ataxia Type-3/Machado–Joseph Disease Initiative (ESMI) cohort. Biosamples were collected under highly standardized protocols at all participating centers. Details are provided in Table 1 and Appendix S1.

Age at ataxia onset (AAO) in ataxic mutation carriers was defined as the reported age at onset of gait difficulties. In the pre-ataxic mutation carriers, predicted AAO was calculated based on age at recruitment and CAG repeat length.[4] The Scale for the Assessment and Rating of Ataxia (SARA) was used to assess the severity of ataxia.[5] Mutation carriers were classified as either pre-ataxic (SARA < 3 points) or ataxic (SARA ≥ 3). The CAG repeat length of the expanded allele was determined using PCR-based fragment-length analysis.

Ataxin-3 SMC Assay

The assay employs SMC technology that provides ultrasensitivity and a wide linear detection.[6] Specific detection of polyQ-expanded ataxin-3 occurs by bead-based immunoreaction with antibody combination 1H9 and MW1.[7] Epitope-binding sites are shown in Figure 1A. Biomaterials were subsequently measured using the SMCxPro platform. All assays were performed by operators blinded to the genotype and

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TABLE 1 *Demographic and clinical characteristics of participants in the analyzed ESMI cohort and comparison of ultrasensitive immunoassays to measure polyQ-expanded ataxin-3 in human biofluids*

Demographic information	Controls	Pre-ataxic SCA3	Ataxic SCA3
Exploratory cohort			
Sample size (female)	9 (44%)	ND	10 (50%)
Age (y)	45 (39.5–58)	ND	53 (36.7–56.5)
Reported age at onset, AAO (y)	NA	NA	47 (27.5–49.5)
SARA score	0.5 (0–1)	ND	10.5 (7.5–15.5)
Repeat count (long allele)	NA	ND	67.5 (64.7–69)
Validation cohort			
Sample size (female)	15 (46.6%)	11 (54.5%)	45 (51.1%)
Age (y)	43.5 (21.1–68.2)	35 (21–42)	49.8 (40.2–61)
Predicted/reported AAO (y)	NA	44 (36.5–49.5)	39.1 (13–68)
SARA score	0.2 (0–2)	0.7 (0–1.5)	15.3 (4–34.5)
Repeat count (long allele)	NA	68 (62–71)	69 (58–73)
CSF cohort			
Sample size (female)	18 (55.5%)	5 (80%)	12 (66.6%)
Age (y)	45 (34–52)	37 (35.5–41)	47.2 (39.2–57.7)
Reported AAO (y)	NA	NA	42 (29–45)
SARA score	0 (0–1.2)	1 (1–1.5)	10.2 (8–17.5)
Repeat count (long allele)	NA	69.5 (69–70.7)	69.5 (66.5–70.7)
Longitudinal cohort			
Sample size (female)	4 (50%)	5 (80%)	28 (53.5%)
Participants for whom disease status changed	0	3	2
Age (y) at baseline	44.5 (23.5–65.9)	36 (26–38.5)	51.5 (43.7–60.2)
Reported AAO (y): BL and FUP	NA	BL: NA FUP: 31 (26–37)	41 (33–47.5)
SARA score: BL and FUP	BL: 0.5 (0–1.7) FUP: 0 (0–1.5)	BL: 1 (0.5–1) FUP: 3.5 (1–6.2)	BL: 12.5 (6.5–17.5) FUP: 11.7 (9–19.5)
Repeat count (long allele)	NA	69 (65.5–70.5)	70 (65.5–71.7)
Time span between BL and FUP	14.1 (11.5–15.2)	13.2 (12.0–14.4)	13.0 (11.3–17.7)

Assay comparison	Meso Scale (Prudencio et al 2020)	SMC (described here)
Antibodies used	Atxn3 clone 1H9 and polyQ 3B5H10	Atxn3 clone 1H9 and polyQ MW1
Applicable in	Plasma and CSF	Plasma and CSF
Dynamic range	pg/mL	pg/mL
LoD, LLoQ, and ULoQ	Not provided in the publication	LOD, 0.07 pg/mL; LLoQ, 0.253 pg/mL; ULoQ, 427.69 pg/mL
AUC plasma		
cntrs vs. mut carrier	1.0	1.0
Pre- vs. ataxic	0.70	0.78

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TABLE 1 *Continued*

Assay comparison	Meso Scale (Prudencio et al 2020)	SMC (described here)
AUC CSF		
cntrs vs. mut carrier	1.0	1.0
Pre- vs. ataxic	0.89	0.58
Correlation with clinical data: CSF	None	None
Plasma	AAO ($P = 0.020$)	AAO ($P = 0.0003$)
	Gait ($P = 0.030$)	SARA ($P = 0.0202$)
	Not corrected for age or polyQ lengths	Corrected for age and polyQ lengths
Correlation between CSF and plasma polyQ-atxn3	None ($P = 0.82$)	None ($P = 0.45$)
Longitudinal data	ND	High stability in 1-year follow-up

Data are reported as median and interquartile range.
Abbreviations: SMC, single molecule counting; SCA3, spinocerebellar ataxia type 3; ND, not determined; AAO, age at ataxia onset; NA, not applicable; SARA, Scale for the Assessment and Rating of Ataxia; CSF, cerebrospinal fluid; BL, baseline; FUP, follow-up visit; LOD, limit of detection; LLoQ, lower limit of quantification; ULoQ, upper limit of quantification; AUC, area under the curve (determination of discrimination efficiency between different genotypes); cntrs, healthy controls; mut carrier, mutation carriers including pre-ataxic and ataxic SCA3 mutation carriers; pre-, pre-ataxic; polyQ, polyglutamine.

clinical state of the participant. Detailed assay description is presented in Appendix S1.

Statistical Analysis

Analyte distribution was tested for normality using Shapiro–Wilk test. Nonparametric group analyses were performed using two-sided Mann-Whitney *U* test, with Bonferroni correction for multiple comparison. For linear correlation we used partial Spearman correlation. Data were adjusted for age and CAG repeat length. CAG repeat adjustment was included as MW1 antibody can bind 16 polyQ repeats with increasing intensity for longer repeats.[7] Both age and expanded CAG repeat length were identified as covariable in our data set and as an independent modifier of SCA3 disease severity.[8,9] Multivariate analyses revealed that age at onset, disease duration, and sex did not correlate with our data sets. Therefore, all statistical analyses were corrected only for age and expanded CAG repeat length. Correlation analyses of plasma and CSF ataxin-3 levels were performed on *z*-transformed data sets. Effect sizes (r) were calculated as Cohen's d. Intraclass variation (ICC) was performed to analyze the stability of the analyte ataxin-3 at the longitudinal study design. To test the quality of classification of the cohort into healthy controls and mutation carriers, we calculated receiver operating characteristic (ROC) curves and determined the area under the curve (AUC).

Data are presented as median and interquartile range. Statistical significance is demonstrated by *P*-values (≤0.01 [**], ≤0.001 [***]).

All statistical and graphical evaluations were performed with GraphPad prism 8.0. For linear regression, we used IBM SPSS Statistics version 27.

Results

SMC Immunoassay Quantifies PolyQ-Expanded Ataxin-3 with High Specificity and Sensitivity

SMC immunoassay validation using human recombinant ataxin-3 with normal (15Q) or elongated (62Q) polyQ length demonstrated a high specificity for polyQ-expanded ataxin-3 over normal ataxin-3 (Fig. 1B). Determination of LOD (limit of detection, 0.07 pg/mL), lower limit of quantification (LLoQ, 0.252 pg/mL), and upper limit of quantification (ULoQ, 427.695 pg/mL) showed a low picomolar detection threshold and broad dynamic range (Fig. 1C). Spiking in recombinant polyQ-expanded ataxin-3 protein in human CSF or plasma from control subjects revealed a signal recovering rate of more than 99.5% and demonstrated the capacity of the assay to measure reference analytes in real human biomaterials. In the exploratory cohort, polyQ-expanded ataxin-3 plasma concentrations in SCA3 mutation carriers ranged from 18 to 87 pg/mL (59.63 pg/mL [48.93–74.37]), whereas polyQ-expanded ataxin-3 was not detectable in healthy controls (1.15 pg/mL [0.27–6.0]) (Fig. 1D).

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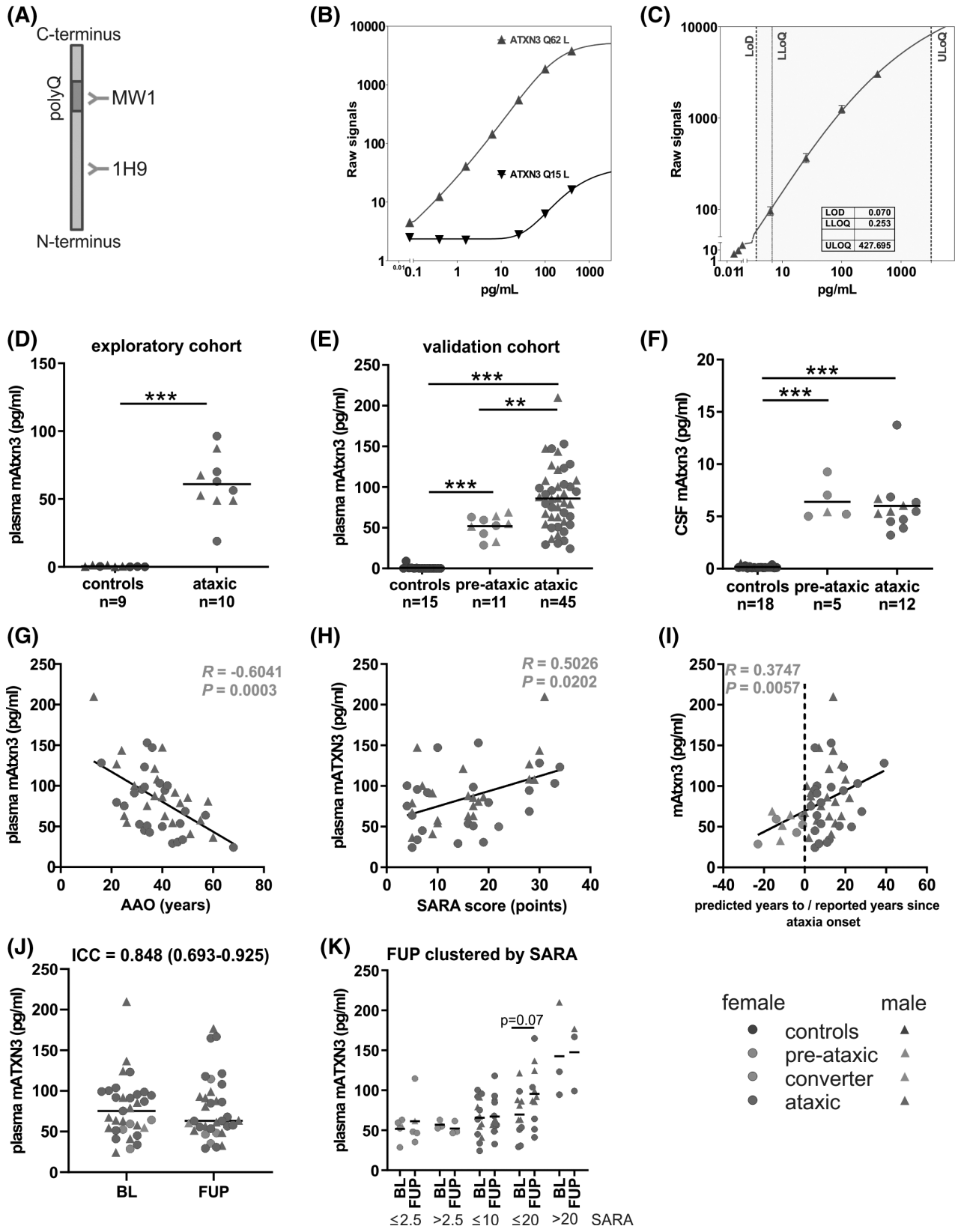

FIG. 1. Legend on next page.

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PolyQ-Expanded Ataxin-3 Is Quantifiable in Mutation Carriers

In plasma and CSF samples from the validation cohort, polyQ-expanded ataxin-3 was quantifiable (plasma: 72.25 pg/mL [52.34–100.3], $P < 0.0001$, $r = 0.84$; CSF: 5.48 pg/mL [4.85–6.77], $P < 0.0001$, $r = 0.869$) in SCA3 mutation carriers, whereas concentrations were below the detection threshold in healthy controls (plasma: 0.14 pg/mL [0.1–0.4]; CSF: 0.11 pg/mL [0.08–0.15]; Fig. 1E,F). PolyQ-expanded ataxin-3 concentrations were higher in plasma samples of ataxic than those of pre-ataxic mutation carriers (83.30 pg/mL [55.38–106.6] vs. 53.80 pg/mL [40.28–63.37]; $P = 0.009$, $r = 0.50$) (Fig. 1E). In particular, patients with a more severe disease presented with higher ataxin-3 levels (SARA < 10: 75.80 pg/mL [41.03–95.65], SARA ≥ 10: 86.34 pg/mL [60.45–122.2]). CSF concentrations of polyQ-expanded ataxin-3 did not differ between ataxic and pre-ataxic mutation carriers. Correlation analysis failed to reveal an association between CSF and plasma ataxin-3 levels ($R = 0.210$, $P = 0.45$). No sex-specific differences of polyQ-expanded ataxin-3 protein levels were detected (Fig. 1D,F). The level of polyQ-expanded ataxin-3 in plasma and CSF perfectly discriminated between mutation carriers and healthy controls with AUC values of 1.00 in the ROC analysis. Plasma polyQ-expanded ataxin-3 showed a good discrimination ability comparing pre-ataxic and ataxic mutation carriers (AUC = 0.78) but failed for CSF (AUC = 0.58) (Fig. S1A–D, Appendix S1).

Plasma PolyQ-Expanded Ataxin-3 Level Correlates with Clinical Parameters and Remain Stable over a 1-Year Period

Plasma polyQ-expanded ataxin-3 were positively correlated with SARA ($R = 0.5026$, $P = 0.020$) (Fig. 1H), whereas it was negatively correlated with AAO ($R = -0.6041$, $P < 0.001$) (Fig. 1G): these results, however, were not replicable in CSF samples. Cross-sectional analyses of plasma polyQ-expanded ataxin-3 protein level relative to time to predicted/reported years from ataxia onset revealed a positive linear correlation ($R = 0.3747$, $P = 0.005$), demonstrating that the polyQ-expanded ataxin-3 protein levels are higher at a later stage of the disease (Fig. 1I). Longitudinal measurements of 33 mutation carriers over a 1-year period revealed a high stability of polyQ-expanded ataxin-3 in SCA3 mutation carriers, including three mutation carriers that converted from the pre-ataxic to the ataxic stage (ICC = 0.848 [0.693–0.925]) (Fig. 1J). Only at later disease stages were higher protein levels observed after a 1-year period (Fig. 1K).

Discussion

To demonstrate target engagement in future trials that aim at silencing the SCA3 disease gene, the availability of an ultrasensitive, quantitative immunoassay to measure the concentration of polyQ-expanded ataxin-3 in body fluids is mandatory.[1]

Here, we report on the successful generation and validation of a new ultrasensitive and quantitative immunoassay to specifically measure low concentrations (pg/mL) of polyQ-expanded ataxin-3 in human biofluids like blood plasma and CSF. Our SMC immunoassay perfectly discriminated between healthy controls and SCA3 mutation carriers, yielding discrimination values like a recent published ataxin-3-specific mesoscale assay.[2] In addition, our assay allowed for a discrimination between pre-ataxic and ataxic mutation carriers in plasma. Moreover, polyQ-expanded ataxin-3 plasma levels correlated with the clinical features of the disease, namely SARA, suggesting that our assay might indeed quantify polyQ-expanded ataxin-3 in a way that reflects the severity of ataxia of SCA3. These findings extend our pilot study where we used a TR-FRET-based technique to quantify ataxin-3 in PBMCs,[3] by demonstrating that polyQ-expanded ataxin-3 protein serves as a biomarker even in plasma and possibly CSF.

We did not find an association of polyQ-expanded ataxin-3 levels in plasma and CSF. Therefore, the pool of polyQ-expanded ataxin-3 in CSF differs from that in peripheral blood and blood cells, as reported earlier.[2] This notion is further supported by the observation that

FIG. 1. PolyQ (polyglutamine)-expanded ataxin-3 is detectable across different fluids from SCA3 (spinocerebellar ataxia type 3) mutation carriers, including strong effect sizes in plasma. (**A**) Epitope binding sites of MW1 and 1H9 antibody within the ataxin-3 protein. (**B**) Detection of purified recombinant ataxin-3 proteins with the SMC (single molecule counting) immunoassay showed specificity for polyQ-expanded ataxin-3 (ATXN3 Q62) over normal human ataxin-3 protein (ATXN3 Q15). (**C**) Determination of LOD (limit of detection, 0.07 pg/mL), LLoQ (lower limit of quantification, 0.253 pg/mL), and ULoQ (upper limit of quantification, 427.695 pg/mL) revealed a low picomolar detection threshold and an acceptable dynamic range. (**D**) In a small exploratory cohort, a discrimination between healthy controls and ataxic mutation carrier was possible in plasma samples ($P < 0.001$). (**E**, **F**) In a larger validation cohort, polyQ-expanded ataxin-3 protein levels were measured in plasma and CSF and demonstrated a significant discrimination between healthy controls and mutation carriers (each $P < 0.001$). Only in plasma was a separation between pre-ataxic and ataxic mutation carriers possible ($P = 0.009$). (**G**, **H**) Plasma polyQ-expanded ataxin-3 level correlated negatively with (**G**) age at onset (AAO, $P = 0.0003$) and positively with the (**H**) clinical scores SARA (Scale for the Assessment and RATING OF Ataxia) ($P = 0.02$). (**I**) Cross-sectional correlation of predicted years or reported years since ataxia onset (SARA ≥ 3) to polyQ-expanded ataxin-3 levels revealed a significant linear regression ($P = 0.005$). (**J**) Longitudinal analyses of 33 mutation carriers with 1-year follow-up revealed a high stability of polyQ-expanded ataxin-3 during disease progression. (**K**) Only at later disease stages (SARA > 10) increased polyQ-expanded ataxin-3 levels were observed after a 1-year follow-up. Scatter plots show unadjusted values. Indicated R and P values are age- and CAG-repeat-adjusted. Stability analyses of ataxin-3 values over a 1 year period were analyzed by intraclass variation (ICC). Data were calculated using the 2-tailed Mann-Whitney U tests and Bonferroni-corrected (**D**–**F**, **K**). The number of analyzed patients per study group is included below the graphs. [Color figure can be viewed at wileyonlinelibrary.com]

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the levels of polyQ-expanded ataxin-3 were >10 times higher in plasma as in CSF.

So far, neither our SMC™ nor the mesoscale-based immunoassay (comparison of both immunoassays in Table 1) showed any association between CSF polyQ-expanded ataxin-3 protein levels and clinical features of the disease. This could be explained by the assumption that CSF polyQ-expanded ataxin-3 represents a—rather disease-stage-independent—trait biomarker of the disease, as demonstrated for the respective key proteins of other neurodegenerative diseases, for example, C9orf72 dipeptides in ALS/FTD.[10] Alternatively, it might be due to the sample size and composition of our SCA3 subject group. The total number of CSF samples, in general, was low. This applied even more to CSF from patients in the later stages of the disease, in whom we found higher plasma concentrations of polyQ-expanded ataxin-3. The lack of correlation of CSF polyQ-expanded ataxin-3 concentrations and disease severity, however, does not call into question the potential usefulness of CSF polyQ-expanded ataxin-3 that might serve as a target engagement biomarker in future interventional trials that investigate gene-silencing approaches.

Our longitudinal analyses revealed a high stability of plasma-derived polyQ-expanded ataxin-3 protein levels over a period of 1 year. If confirmed in a larger cohort and longer time period, the high degrees of stability of this biomarker would allow to reduce sample sizes in trials that include polyQ-expanded ataxin-3 as one of the endpoints.

In conclusion, our novel SMC immunoassay is able to quantify polyQ-expanded ataxin-3 in plasma and CSF while ataxin-3 levels in plasma correlating with disease severity. First longitudinal analyses demonstrated a high stability of polyQ-expanded ataxin-3 over a period of 1 year. Therefore, this immunoassay has the potential to support the clinical development of therapeutic drugs in SCA3, allowing to determine the levels of polyQ-expanded ataxin-3 as a target engagement biomarker in human biofluids, especially peripheral blood. ■

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Data Availability Statement

All data are available within the manuscript and the supplementary material

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Neuropathological Findings in a Case of Parkinsonism and Developmental Delay Associated with a Monoallelic Variant in *PLXNA1*

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