BRIEF REPORT

Serum Neurofilament Light Chain in Replication Factor Complex Subunit 1 CANVAS and Disease Spectrum

Ilaria Quartesan, MD,[1,2] Elisa Vegezzi, MD,[1,2] Riccardo Currò, MD,[1,3] Amanda Heslegrave, BSc, PhD,[4,5] Chiara Pisciotta, MD, PhD,[6] Pablo Iruzubieta, MD,[7] Alessandro Salvalaggio, MD, PhD,[8] Gorka Fernande-Eulate, MD,[9] Natalia Dominik, MSc,[3] Bianca Rugginini, MD,[1] Arianna Manini, MD,[3,10] Elena Abati, MD,[3,11] Stefano Facchini, PhD,[2] Katarina Manso, MD,[12] Ines Albajar, MD,[7] Rhiannon Laban, MSc,[5] Alexander M. Rossor, MRCP, PhD,[3] Anna Pichiecchio, MD, PhD,[1,2] Giuseppe Cosentino, MD, PhD,[1,2] Paola Saveri, BSc,[6] Ettore Salsano, MD,[6] Francesca Andreetta, PhD,[6] Enza M. Valente, MD, PhD,[1,2] Henrik Zetterberg, MD, PhD,[4,5,12,13,14,15] Paola Giunti, MD, PhD,[16] Tanya Stojkovic, MD,[9] Chiara Briani, MD,[8] Adolfo López de Munain, MD, PhD,[7] Davide Pareyson, MD,[6] Mary M. Reilly, MD, FRCP, FRCPI,[3] Henry Houlden, MD, PhD, MRCP,[3] Cristina Tassorelli, MD, PhD,[1,2] and Andrea Cortese, MD, PhD[1,3*]

[1]*Department of Brain and Behavioral Sciences, University of Pavia, Pavia, Italy* [2]*IRCCS Mondino Foundation, Pavia, Italy* [3]*Department of Neuromuscular Diseases, UCL Queen Square Institute of Neurology, London, United Kingdom* [4]*Department of Neurodegenerative Disease, UCL Queen Square Institute of Neurology, London, United Kingdom* [5]*UK Dementia Research Institute at UCL, London, United Kingdom* [6]*Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy* [7]*Neurology Department, Donostia University Hospital, Osakidetza, and Biodonostia Health Research Institute-UPV-EHU, San Sebastián, Spain* [8]*Department of Neuroscience, University of Padova, Padova, Italy* [9]*Neuro-myology Department, Institut de Myologie, Pitié-Salpêtriére Hospital, APHP, Sorbonne University, Paris, France* [10]*Department of Neurology and Laboratory of Neuroscience, IRCCS Istituto Auxologico Italiano, Milan, Italy* [11]*Department of Pathophysiology and Transplantation (DEPT), University of Milan, Milan, Italy* [12]*Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden* [13]*Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden* [14]*Hong Kong Center for Neurodegenerative Diseases, Clear Water Bay, Hong Kong, China* [15]*Wisconsin Alzheimer's Disease Research Center, University of Wisconsin School of Medicine and Public Health, University of Wisconsin-Madison, Madison, Wisconsin, USA* [16]*Department of Clinical and Movement Neuroscience, UCL Queen Square Institute of Neurology, London, United Kingdom*

ABSTRACT: Background: Biallelic intronic AAGGG repeat expansions in the replication factor complex subunit 1 (*RFC1*) gene were identified as the leading cause of cerebellar ataxia, neuropathy, vestibular areflexia syndrome. Patients exhibit significant clinical heterogeneity and variable disease course, but no potential biomarker has been identified to date.

Objectives: In this multicenter cross-sectional study, we aimed to evaluate neurofilament light (NfL) chain serum levels in a cohort of *RFC1* disease patients and to correlate NfL serum concentrations with clinical phenotype and disease severity.

Methods: Sixty-one patients with genetically confirmed *RFC1* disease and 48 healthy controls (HCs) were enrolled from six neurological centers. Serum NfL concentration was measured using the single molecule array assay technique.

Results: Serum NfL concentration was significantly higher in patients with *RFC1* disease compared to age- and-sex-matched HCs ($P < 0.0001$). NfL level showed a moderate correlation with age in both HCs ($r = 0.4353$, $P = 0.0020$) and patients ($r = 0.4092$, $P = 0.0011$). Mean NfL concentration appeared to be significantly higher in patients with cerebellar involvement compared to patients without cerebellar dysfunction (27.88 vs. 21.84 pg/mL, $P = 0.0081$). The association between cerebellar involvement and NfL remained significant after controlling for age and sex ($\beta = 0.260$, $P = 0.034$).

Conclusions: Serum NfL levels are significantly higher in patients with *RFC1* disease compared to HCs and correlate with cerebellar involvement. Longitudinal studies are warranted to assess its change over time.

***Correspondence to:** Dr. Andrea Cortese, Department of Neuromuscular Diseases, UCL Queen Square Institute of Neurology, WC1N 3BG London, UK; E-mail: andrea.cortese@ucl.ac.uk; andrea.cortese@unipv.it

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Biallelic AAGGG repeat expansions in intron 2 of the gene encoding replication factor complex subunit 1 (*RFC1*) were identified as the cause of cerebellar ataxia with neuropathy and vestibular areflexia syndrome (CANVAS) and disease spectrum (here shortened as *RFC1* disease).[1]

Affected individuals exhibit significant clinical heterogeneity, starting with an isolated sensory neuropathy, with or without chronic cough, and progressing to a more complex ataxia with cerebellar dysfunction in later disease stages.[2,3] Bilateral vestibular areflexia is also often present but can be easily overlooked if not tested for. The clinical manifestations of *RFC1* disease have expanded beyond the classical CANVAS to encompass dysautonomia, parkinsonism, and cognitive impairment.[4-6]

Multiple studies have established that neurofilament light (NfL), a component of the axonal cytoskeleton released into the cerebrospinal fluid (CSF) and blood stream after neuronal damage,[7] is a promising fluid biomarker for neurodegenerative conditions, including Charcot–Marie–Tooth disease, hereditary transthyretin (ATTR) amyloidosis, and hereditary ataxias.[8-13] Increased CSF or serum NfL levels in patients carrying biallelic *RFC1* expansions have been observed in two single cases.[14,15] However, there is still scant evidence of its diagnostic role or its ability to reflect progression of the condition over time.

In this multicenter cross-sectional study, we aimed to assess whether serum NfL may represent a potential biomarker for *RFC1* disease using ultrasensitive single molecule array (Simoa) immunoassay technology. Furthermore, we sought to evaluate the correlation between NfL concentration and clinical phenotype and severity.

Patients and Methods

Serum samples (n = 50) were prospectively collected, after informed consent, from patients with a genetically confirmed diagnosis of *RFC1* disease[1] and consecutively attending neurology clinics in six neurological centers (London: 10; Pavia: 16; Milan: 1; Paris: 7; Padova: 7; San Sebastian: 9). In 11 patients, serum was retrieved from the biorepository (Milan).

Patients' neurological history and clinical signs, including the presence of sensory neuropathy, cerebellar dysfunction (defined by the presence of one or more of the following signs: broken pursuits, dysmetric saccades, gaze-evoked or downbeat nystagmus, dysarthria, and dysphagia), vestibular areflexia, dysautonomia, cognitive impairment, and parkinsonism, were collected based on a standard template. Patients with other neurologic diseases were excluded. In prospectively collected data, neurological examinations were performed at serum collection. In the 11 patients whose serum was retrieved from biorepositories, examination was performed at a mean of 15 months from serum sampling (ranging from 24 months before sampling to 12 months after sampling).

Based on the known progression of the disease from an isolated sensory neuropathy to a complex neuropathy with cerebellar dysfunction or full CANVAS,[3] patients were divided into two clinical subtypes as a proxy of disease severity: (1) *RFC1* disease without cerebellar involvement, which includes patients with isolated sensory neuropathy/neuropathy with bilateral vestibular areflexia, and (2) *RFC1* disease with cerebellar involvement, which includes patients with complex sensory and cerebellar ataxia or full CANVAS. Therefore, neuronal damage occurring within the cerebellum has been previously shown to be associated with higher elevation of NfL serum levels compared to neuropathies.[8-16] Loss of independent ambulation was also considered as a marker of advanced disease.

Serum samples from age-matched healthy controls (HC) were also collected from each participating center (London: 1 sample; Pavia: 13; Milan: 14; Paris: 4; Padova: 8; San Sebastian: 8). The assessment of HCs was based on medical history (through participants' interviews).

Blood sampling and storage were conducted following a standard operating procedure at each of the six different centers. In particular, blood was collected into serum separating tubes tubes and centrifuged at 20°C at 3500 rpm for 10 min. Serum was then aliquoted and stored at −20°C. Samples were anonymized and sent blinded for clinical details to the University College London (A.H., H.Z.) for analysis of NfL levels. Serum NfL concentration was measured using the Simoa NfL assay on an HD-X analyzer (Quanterix, Billerica, MA, USA) in one round of experiments with one batch of reagents. Four quality control samples were run in duplicate; the mean intra-assay coefficient of variation of duplicate determinations for concentration was 6.9%.

Statistical analysis was performed using SPSS, version 26.00 (IBM, Armonk, NY, USA) and GraphPad Prism 9.0.0 for Windows (GraphPad Software, San Diego, CA, USA).

Demographic and clinical data were described as mean (SD) or median (interquartile range) if normally or nonnormally distributed, respectively. Data normality was assessed using Q–Q plots and analytical tests (Kolmogorov–Smirnov test, Shapiro–Wilk test, and Anderson–Darling test), requiring consistent results from all tests to confirm normality.

Means between groups were compared using the *t* test for normally distributed data and the Mann-Whitney *U* test for nonnormally distributed data. Correlations were assessed using Spearman's or Pearson's coefficients, as appropriate for data distribution. We conducted a multiple linear regression analysis to examine the relationship between serum NfL (dependent variable), cerebellar involvement, age at time of blood collection, and sex (independent variables).

The study protocol was approved by the local institutional review boards and ethics committees. Written informed consent was obtained from all patients and HCs. The study adhered to all applicable ethical regulations.

Results

A total of 61 patients and 48 HCs were enrolled in the study. The demographics and clinical characteristics of participants are summarized in Table 1. There was no significant difference in the mean age at sample collection of the two groups ($P = 0.13$) or sex distribution ($P = 0.823$). Mean age at blood collection in patients with *RFC1* disease was 67.08 years (±10.70), with a mean age of onset of 55.16 years (±10.85) and a median disease duration of 11 years (6–15). Overall, 42 patients (69%) had signs and/or symptoms of cerebellar involvement. At the time of evaluation, 27 patients (44%) required walking aids. In 7 patients (11%) vestibular function was not assessed.

No significant difference in serum NfL levels was observed between stored samples and samples collected prospectively (23.18 vs. 26.62 pg/mL, $P = 0.22$).

Serum NfL concentration was significantly higher in patients with *RFC1* disease (24.34 pg/mL [IQR: 19.44–31.60]) compared to age-matched HCs (12.40 pg/mL ± 4.64) ($P < 0.0001$; Fig. 1A).

Receiver operating characteristic analysis showed that serum NfL levels could discriminate patients from controls with great accuracy (AUC of 0.9262, 95% CI [confidence interval]: 0.88–0.97) (Fig. 1B). A concentration of 15.86 pg/mL can effectively identify individuals with *RFC1* disease with a sensitivity of 92% and a specificity of 81%.

Serum NfL concentration showed a moderate correlation with age in both HCs ($r = 0.4353$, $P = 0.0020$) and patients ($r = 0.4092$, $P = 0.0011$) (Fig. 1C).

The significant difference in serum NfL concentration compared to controls was maintained for individual comparisons of controls versus patients without cerebellar involvement ($P < 0.0001$), and of controls versus patients with cerebellar involvement ($P < 0.0001$).

Also, mean NfL concentrations were significantly higher in patients with cerebellar involvement compared to patients without cerebellar dysfunction (27.88 vs. 21.84 pg/mL, $P = 0.0081$) (Fig. 1D), and the association remained significant after controlling for age and sex in a multiple linear regression model ($\beta = 0.260$, 95% CI: 0.411002, $P = 0.034$).

Conversely, there was no significant difference in NfL levels between patients without signs of vestibular dysfunction (n = 13) and patients with vestibular impairment (n = 41) (25.28 vs. 26.25 pg/mL, $P = 0.7061$). Serum NfL levels did not appear to correlate with

TABLE 1 *Demographic details of patients and healthy control cohorts*

Patient group	N	Age, y (min–max)	Sex, *F* (%)	NfL (pg/mL)
Controls	48	63.68 (37–79)	21 (44)	12.40 ± 4.64
RFC1 disease	61	67.08 (38–86)	28 (46)	24.34 ± 9.24
P-value		0.13	0.82	<0.0001
Without cerebellar involvement	19	64.11 (46–86)	6 (31)	21.84 ± 6.99
Isolated sensory neuropathy	11	66.91 (47–86)	4 (36)	24.48 ± 6.07
Sensory neuropathy with BVA	8	60.25 (46–77)	2 (25)	18.22 ± 6.84
With cerebellar involvement	42	68.42 (38–83)	22 (52)	27.88 ± 9.58
Complex sensory and cerebellar ataxia	9	65 (48–81)	6 (67)	26.69 ± 8.60
CANVAS	33	69.36 (38–83)	16 (48)	28.20 ± 9.94
P-value		0.14	0.14	0.0081

Abbreviations: NfL, neurofilament light; BVA, bilateral vestibular areflexia; CANVAS, cerebellar ataxia, neuropathy, vestibular areflexia syndrome.

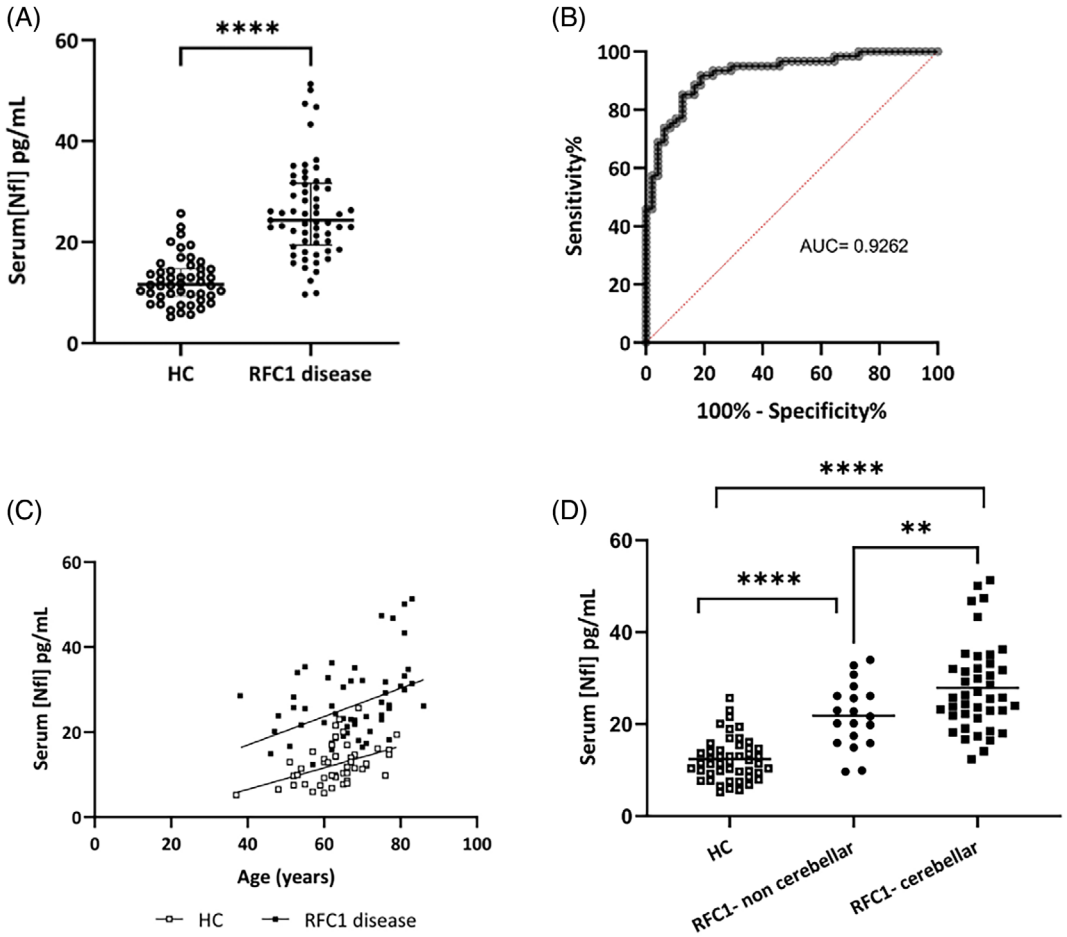

FIG. 1. (**A**) Increased serum NfL concentration in patients with *RFC1* disease compared to healthy controls (HC). (**B**) Receiver operator curve of serum NfL concentration for detecting patients with *RFC1* CANVAS and disease spectrum. (**C**) Correlation between serum NfL concentration and age in both patients carrying *RFC1* expansions and HCs. (**D**) Increased serum NfL concentration in patients with cerebellar involvement compared to patients without cerebellar dysfunction. Both subgroups show higher concentrations compared to HCs. Line is at mean. Error bars: standard deviation. *P*-value less than 0.05 is flagged with one star (*), *P*-value less than 0.01 is flagged with two stars (**), and *P*-value less than 0.001 is flagged with three stars (***). NfL: neurofilament light. CANVAS: cerebellar ataxia, neuropathy, vestibular areflexia syndrome. [Color figure can be viewed at wileyonlinelibrary.com]

disease duration ($r = 0.014$, $P = 0.917$) and did not differ between patients with independent walking (n = 42) and patients using walking aid (n = 27) (24.12 vs. 28.36 pg/mL, $P = 0.0820$).

One patient had clinically manifest parkinsonism, with an NfL value (31.78 pg/mL) above the 75th percentile, and 3 patients presented with clinical signs of dysautonomia (mean NfL: 28.82 ± 6.26 pg/mL). Cognitive impairment was not reported in any patients.

Discussion

This study is the first to investigate serum NfL levels as a biomarker in *RFC1* disease using ultrasensitive Simoa technology. We found significantly higher serum NfL levels in patients with biallelic *RFC1* expansions compared to HCs of the same age and sex. Elevated NfL levels were observed in various clinical phenotypes, including isolated sensory neuropathy, which is more common in early disease stages. NfL levels demonstrated excellent discriminatory power, supporting its potential as a reliable biomarker in various neurodegenerative ataxias,[12,13,17-19] as well as in hereditary neuropathies.[8,10]

NfL is an axonal cytoskeletal protein released after axonal damage. Abnormal NfL serum levels in our patients reflect the pathology and progression of *RFC1* disease. Postmortem examination revealed loss of sensory neurons in the dorsal root ganglia and Scarpa's ganglion, as well as marked loss of cerebellar Purkinje and granular cells.[14,20-22] A recent brain magnetic resonance imaging study also showed basal ganglia and brainstem volumetric reduction and involvement of the cerebral white matter in cases with advanced disease,[23] suggesting a widespread cerebral neurodegeneration.

We have also demonstrated that patients with cerebellar damage have significantly higher levels of serum NfL than those without cerebellar involvement.

This correlation persisted after correcting for age and gender. This may be explained by the high density of neurons in cerebellum, which would lead to a significantly increased release of NfL in the bloodstream when this structure is affected.

Conversely, serum NfL did not appear to correlate with disease duration and the need for walking aids. An explanation of this phenomenon is that NfL may increase rapidly in the initial stages of the disease and then reach a plateau above a certain degree of severity, as observed in other genetic ataxias.[12,13,18] Other possible explanations entail the difficulty in accurately defining the onset of the disease, because neuropathy symptoms may remain unnoticed for a long time, or the chronologically variable involvement of the cerebellum in early or late disease stages relative to onset, due to factors yet to be explored, including the repeat size and additional genetic modifiers. Also, no significant difference in serum NfL levels was found based on vestibular involvement. This could be due to the limited quantitative relevance of Scarpa's ganglion, which contains around 20,000 neurons, compared to the cerebellum's 50 billion neurons.

This study has some limitations. First, the small sample size limits the statistical power of the study. Second, the relatively wide range in the time interval between serum collection and neurological examination (within 24 months at the most) may have introduced variability. However, previous studies in patients with spinocerebellar ataxias and Charcot–Marie–Tooth disease showed no significant difference in serum NfL concentration after 1 or 2 years,[8,24] suggesting stability of NfL levels in the short time in patients with slowly progressive neurodegenerative diseases. Finally, the cross-sectional design of the study restricts causality assumptions and does not allow us to assess changes in serum NfL levels over time.

In conclusion, we have demonstrated that serum NfL holds promise as a reliable biomarker in *RFC1* disease, as NfL levels are elevated even in the early stages of the disease and exhibit a correlation with cerebellar involvement. Given the significant interindividual variability, NfL may prove to be a valuable tool for stratifying patients, and longitudinal studies are warranted to assess its ability to monitor disease progression.

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Data Availability Statement

Anonymized data from this study will be shared by request from any qualified investigator.

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Supporting Data

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Author Roles

I.Q.: drafting and revision of the manuscript; major role in the acquisition, analysis, and interpretation of data. E.V.: revision and critique of the manuscript; major role in the acquisition, analysis, and interpretation of data. R.C.: revision and critique of the manuscript; major role in the acquisition, analysis, and interpretation of data. A.H.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. C.P.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. P.I.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. A.S.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. G.F.-E.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. N.D.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. B.R.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. A.M.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. E.A.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. S.F.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. K.M.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. I.A.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. R.L.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. A.M.R.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. A.P.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. G.C.: revision and critique of the manuscript; acquisition, analysis, and interpretation of data. P.S.: revision and critique of the manuscript; acquisition and interpretation of data. E.S.: revision and critique of the manuscript; acquisition and interpretation of data. F.A.: revision and critique of the manuscript; acquisition and interpretation of data. E.M.V.: revision and critique of the manuscript; acquisition and interpretation of data. H.Z.: revision and critique of the manuscript; acquisition and interpretation of data. P.G.: revision and critique of the manuscript; acquisition and interpretation of data. T.S.: revision and critique of the manuscript; acquisition and interpretation of data. C.B.: revision and critique of the manuscript; acquisition and interpretation of data. A.L.M.: revision and critique of the manuscript; acquisition and interpretation of data. D.P.: revision and critique of the manuscript; acquisition and interpretation of data. M.M.R.: revision and critique of the manuscript; acquisition and interpretation of data. H.H.: revision and critique of the manuscript; acquisition and interpretation of data. C.T.: revision and critique of the manuscript; acquisition and interpretation of data. A.C.: study conceptualization, revision and critique of the manuscript, acquisition and interpretation of data. All authors have read and approved the final version of the manuscript.

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