*Main title*

**Serum neurofilament light chain protein is a measure of disease intensity in frontotemporal dementia**

*Running title*

**Serum neurofilament light in FTD**

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**Contributors**

JR, KD, EB, SM, JS, NF and JW were involved in patient recruitment and collection of data. JT, RD, NN, UF, KB and HZ were involved in assay development, sample processing and analysis. JR, IW, KD, EB, EG, AF, MJC and SO were involved in analysis of the psychometric and imaging data. JR and IW drafted the initial version and figures. JR, IW and JN performed the statistical analysis. All authors contributed to reviewing and editing the manuscript.

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**ABSTRACT**

**Objective:** The objective of the study was to investigate serum neurofilament light chain (NfL) concentrations in frontotemporal dementia (FTD), and to see whether they are associated with the severity of disease.

**Methods:** Serum samples were collected from 74 participants (34 with behavioural variant FTD (bvFTD), three with FTD and motor neurone disease and 37 with primary progressive aphasia (PPA)) as well as 28 healthy controls. Twenty-four of the FTD participants carried a pathogenic mutation in *C9orf72* (9), microtubule-associated protein tau, *MAPT* (11) or progranulin, *GRN* (4). Serum NfL concentrations were determined using the NF-Light kit transferred onto the Simoa platform, and compared between FTD and healthy controls, as well as between the FTD clinical and genetic subtypes. We also assessed the relationship between NfL concentrations and measures of cognition and brain volume.

**Results:** Serum NfL concentrations were higher in FTD patients overall (mean 77.9 (standard deviation 51.3) pg/ml) than controls (19.6 (8.2) pg/ml; p<0.001). Concentrations were also significantly higher in bvFTD (57.8 (33.1) pg/ml) and both the semantic and nonfluent variants of PPA (95.9 (33.0) pg/ml and 82.5 (33.8) pg/ml respectively) compared with controls, and in semantic variant PPA compared with logopenic variant PPA. Concentrations were significantly higher than controls in both the *C9orf72* and *MAPT* subgroups (79.2 (48.2) pg/ml and 40.5 (20.9) pg/ml respectively) with a trend to a higher level in the *GRN* subgroup (138.5 (103.3) pg/ml). However there was variability within all groups. Serum concentrations correlated particularly with frontal lobe atrophy rate (r=0.53, p=0.003).

**Conclusion:** Increased serum NfL concentrations are seen in FTD but show wide variability within each clinical and genetic group. Higher concentrations may reflect the intensity of the disease in FTD and are associated with more rapid atrophy of the frontal lobes.

**INTRODUCTION**

Frontotemporal dementia (FTD) is a common cause of early onset dementia [1]. Clinically, patients present with either changes in personality (behavioural variant FTD, bvFTD) or impaired language (primary progressive aphasia, PPA) although overlap with motor neurone disease (FTD-MND) is not uncommon [1]. FTD has an autosomal dominant genetic cause in around a quarter of people, with mutations in the progranulin (*GRN)*, chromosome 9 open reading frame 72 (*C9orf72*) and microtubule-associated protein tau (*MAPT*) genes being commonest [2].

Few fluid biomarkers have been investigated in FTD, although there have now been a number of studies of neurofilament concentration in the cerebrospinal fluid (CSF) [3-11]. Higher neurofilament light chain (NfL) levels are believed to represent axonal degeneration [12,13], and whilst early studies showed variability in CSF concentrations in FTD [4-10], a more recent study has suggested that CSF NfL levels correlate with disease severity [11].

There is considerable interest in developing blood-based biomarkers because of their convenience and higher acceptability relative to CSF. NfL can be measured in serum using standard immunoassay formats [14], but those based on ELISA or electrochemiluminescence methods lack the analytical sensitivity to measure low levels. For this reason, we developed a novel immunoassay based on the Simoa technique [15] that allows quantification down to subfemtomolar concentrations (below 1 pg/ml) of the analyte, and is 25-fold as sensitive as the previous electrochemiluminescence-based method [16]. Using this assay, we aimed to investigate serum NfL concentrations in FTD. Our hypotheses were that, firstly, serum NfL concentration would be elevated in FTD compared with healthy controls, secondly, that concentrations would vary between FTD subgroups, and thirdly, that increased serum NfL levels would reflect the disease intensity or rate of progression.

**METHODS**

74 participants were consecutively recruited from the University College London FTD study: 34 participants with bvFTD according to Rascovsky criteria [17], three participants with FTD-MND [18] and 37 participants with PPA according to the Gorno-Tempini criteria [19]. Of the 37 PPA participants, 13 had the nonfluent variant (nfvPPA), 10 had the semantic variant (svPPA), seven had the logopenic variant (lvPPA) and seven did not fit criteria for any of the three variants (here called PPA-NOS, not otherwise specified). We did not include patients fulfilling criteria for lvPPA in the overall FTD analysis as they are likely to have underlying Alzheimer’s disease pathologically [1,2]. Data was compared with 28 healthy control participants matched for age and gender who had been collected as part of a study into neurodegenerative disease (Table 1). Twenty-four of the FTD participants carried a pathogenic mutation: nine with an expansion in *C9orf72* (8 with bvFTD, 1 with nfvPPA), 11 with a *MAPT* mutation (all with bvFTD) and four with a *GRN* mutation (1 with bvFTD, 1 with nfvPPA and 2 with PPA-NOS). No mutations were found in the other participants. No significant differences were noted in age or gender between any of the groups, and no significant difference in disease duration was seen between the clinical or genetic FTD subgroups.

*Standard Protocol Approvals, Registrations, and Patient Consents*

Approval for the study was obtained from the local ethics committee and all participants provided written consent to take part.

*Measurement of NfL concentrations*

Serum samples were collected from each of the participants and then processed, aliquoted and frozen at -80°C according to standardized procedures. Serum NfL concentrations were measured using the NF-Light assay from Uman Diagnostics (UmanDiagnostics, Umeå, Sweden), transferred onto the Simoa platform employing a homebrew kit (Quanterix Corp, Boston, MA, USA) and detailed instructions can be found in the Simoa Homebrew Assay Development Guide (Quanterix). In short, paramagnetic carboxylated beads (Cat#: 100451, Quanterix) were activated by adding 5% (v/v) 10 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, Cat#: 100022, Quanterix) to a magnetic beads solution with 1.4·106 beads/µl. Following a 30 min incubation at room temperature (RT) the beads were washed using a magnetic separator and an initial volume, i.e., EDAC + bead solution volumes in the previous step, of 0.3 mg/mL ice cold solution of the capture antibody (UD1, UmanDiagnostics) was added. After 2 h incubation on a mixer (2000 rpm, Multi-Tube Vortexer, Allsheng, China) at RT the beads were washed and an initial reaction volume of blocking solution was added. After three washes the conjugated beads were suspended and stored at 4°C pending analysis. Prior to analysis the beads were diluted to 2500 beads/µl in bead diluent. The detection antibody (1 mg/mL, UD2, UmanDiagnostics) was biotinylated by adding 3% (v/v) 3.4 mM EZ‐Link™ NHS‐PEG4‐Biotin (Quanterix) followed by 30 min incubation at RT. Free biotin was removed using spin filtration (Amicon® Ultra-2, 50 kD, Sigma) and the biotinylated antibody was stored at 4°C pending analysis. The serum samples were assayed in duplicate on a Simoa HD-1 instrument (Quanterix) using a 2-step Assay Dilution protocol that starts with an aspiration of the bead diluent from 100 L conjugated beads (2500 beads/µl) followed by addition of 20 L biotinylated antibody (0.1 μg/ml) and 100 l 4-fold diluted sample (or undiluted calibrator) to the bead pellet. For both samples and calibrator the same diluent was used [PBS; 0.1% Tween-20; 2% BSA; 10 μg/ml TRU Block (Meridian Life Science, Inc., Memphis, TN, USA)]. After a 47 cadances incubation (1 cadance = 45 s) the beads were washed followed by addition of 100 L of the streptavidin-conjugated -galactosidase (150 pM, Cat#: 100439, Quanterix). This was followed by a 7 cadences incubation and a wash. Prior to reading, 25 L resorufin D-galactopyranoside (Cat#: 100017, Quanterix) was added. The calibrator curve was constructed using the standard from the NfL ELISA (NF-light®, UmanDiagnostics) in triplicate. The lower limits of detection and quantification, as defined by the concentration derived from the signal of blank samples (sample diluent) + 3 and 10 standard deviations, were 0.97 pg/ml and 2.93 pg/ml, respectively. To evaluate the linearity of the assay six different samples were analyzed at 4 (default), 8, and 16-fold dilution and the average coefficient of variation (CV) for the concentration measured at the different dilutions was 11.5%. All samples were measured as duplicates. The mean CV of duplicate concentrations was 4.3%. In addition, a quality control (QC) sample was measured in duplicate on each of the seven runs used to complete the study. The intra-assay CV for this sample was below 10%. All measurements were performed by board-certified laboratory technicians in one round of experiments using one batch of reagents.

*Psychometric assessment*

Forty-seven participants had psychometric testing at baseline usually on the same day as serum sampling but at a maximum of six months from the time of sample collection (mean (standard deviation) interval 0.0 (0.2)) years: 22 with bvFTD, 2 with FTD-MND and 23 with PPA (9 with nfvPPA, 9 with svPPA and 5 with PPA-NOS). Twenty-nine participants had follow up psychometric testing at an interval of 1.1 (0.2) years: 11 with bvFTD, 2 with FTD-MND and 16 with PPA (5 with nfvPPA, 7 with svPPA and 4 with PPA-NOS). Testing included the Weschler Abbreviated Scale of Intelligence (WASI) Vocabulary, Block Design, Similarities and Matrices subtests [20], the Recognition Memory Tests for Faces and Words [21], the Graded Naming Test [22], the Graded Difficulty Calculation Test [23] and the D-KEFS Color-Word Interference Test [24], as well as the Mini-Mental State Examination [25].

*Neuroimaging analysis*

Forty-six of the FTD participants had had volumetric T1 brain magnetic resonance imaging on a 3T Siemens Trio scanner performed usually on the same day as serum sampling but at a maximum of six months from the time of sample collection (mean (standard deviation) interval 0.0 (0.2)): 24 with bvFTD, 2 with FTD-MND, and 20 with PPA (8 with nfvPPA, 8 with svPPA, 4 with PPA-NOS). Twenty-nine participants had a follow up scan at 1.1 (0.4) years after the baseline scan: 13 with bvFTD, 2 with FTD-MND and 14 with PPA (5 with nfvPPA, 6 with svPPA and 3 with PPA-NOS). Whole brain volumes were measured using a semi-automated segmentation method [26] with annualized whole brain atrophy rates calculated using the boundary shift integral (BSI) [27]. Individual lobar cortical volumes were measured using a multi-atlas segmentation propagation approach following the brainCOLOR protocol (www.braincolor.org), combining regions of interest to calculate grey matter volumes for each lobe [28,29]. Annualized lobar atrophy rates were calculated using the differences in volumes between baseline and follow up scans, and dividing by the interval between scans.

Serum NfL concentrations were initially compared between the control group and the total FTD group. Levene’s test for homogeneity demonstrated unequal variances between these two groups (Levene’s statistic = 22.8; p<0.001), and therefore Welch’s t test (without assumptions for equal variance) was used to compare the groups. Serum NfL data was normally distributed (Kolmogronov-Smirnov test) and so an ANOVA was used to compare mean serum NfL concentrations across each of the clinical subgroups (bvFTD, FTD-MND, nfvPPA, svPPA, lvPPA and PPA-NOS) and across the genetic FTD subgroups (*MAPT, GRN,* and *C9orf72*), and to compare each of these subgroups with the control group. To allow for unequal variance, the Games-Howell correction was used for post-hoc pairwise comparisons between groups. The same statistical methods were also used to compare NfL levels between the genetic subgroups, and between each of these groups and the control group. Pearson’s correlation coefficient was used to examine the association between serum NfL concentrations and each of the cognitive and imaging measures (with a Bonferroni correction for multiple comparisons also assessed i.e. a p value of <0.005 for the cognitive measures, and <0.007 for the imaging measures).

**RESULTS**

Serum NfL concentrations in the control and total FTD groups as well as each clinical subgroup are shown in Table 1. The lowest serum NfL concentration in the study (7.2 pg/mL) was well above the lower limits of detection and quantification of the assay. Serum NfL concentrations were significantly higher in the total FTD group versus controls (mean (standard deviation) = 77.9 (51.3) pg/ml and 19.6 (8.2) pg/ml respectively; mean difference = 58.3 pg/ml, 95% confidence intervals 45.4, 71.1; p<0.001). In distinguishing FTD from controls, a cutoff of 33 pg/ml gave a sensitivity of 84% and specificity of 96%. Serum NfL concentrations were also significantly higher in the majority of the clinical FTD subgroups compared with controls (Welch statistic = 25.1, df 5, 13.2; p<0.001) (Figure 1A, Table 2A). Compared with controls, serum NfL concentrations were higher in patients with bvFTD, nfvPPA and svPPA. Although patients with FTD-MND had higher mean serum NfL concentrations than controls (and all of the other groups) this difference did not reach statistical significance, likely due to the small sample size of the FTD-MND group. Serum NfL concentrations did not significantly differ between any of the clinical FTD subgroups, although there was a (non-significant) trend towards a higher level in patients with svPPA compared with bvFTD patients (mean difference = 38.1, p=0.070). There was a significantly higher level in patients with svPPA compared with lvPPA (mean difference = 46.3, p = 0.032).

Mean NfL concentrations were higher than controls in each of the genetic subgroups (Figure 1B, Table 2B): mean (SD) levels 138.5 (103.3) pg/ml in *GRN*, 79.2 (48.2) pg/ml in *C9orf72* and 40.5 (20.9) pg/ml in *MAPT* mutations. However, only the *MAPT* subgroup (mean difference from controls = 20.8, 95% CI = 1.4, 40.3; p=0.035) and the *C9orf72* subgroup (mean difference from controls = 59.5, 95% CI = 8.0, 111.0; p=0.025) were significantly different, with the lack of difference in the *GRN* subgroup likely due to small sample size (Table 2B). Despite the apparent larger mean NfL levels in *GRN* and *C9orf72* compared with MAPT mutations, there was no significant difference in levels between the genetic subgroups (Table 2B).

Baseline and longitudinal cognitive and imaging measures are shown in Table 3. Serum NfL concentrations correlated with baseline measures of executive dysfunction [WASI similarities (r = -0.32, p = 0.03) and D-KEFS Color-Word Interference ink colour naming task (r = -0.35, p = 0.03)] but not with other baseline psychometric tests, nor with longitudinal changes in psychometric measures. However no cognitive measures survived correction for multiple comparisons. There were also no significant correlations with baseline brain volumes. However, serum NfL levels were correlated with rates of whole brain (r = 0.46, p = 0.01), frontal lobe (r = 0.53, p = 0.003) (Figure 2) and parietal lobe atrophy (r = 0.38, p = 0.04), although not with other lobar atrophy rates. Only the correlation with frontal lobe atrophy rate survived correction for multiple comparisons.

**DISCUSSION**

Using a novel ultrasensitive immunoassay, we show that serum NfL concentrations are raised in FTD and that higher concentrations are associated with faster rates of brain atrophy. This suggests that serum NfL concentrations reflect the intensity of the disease in FTD and higher concentrations are associated with a more rapid disease progression. Within the FTD subtypes, there was a tendency for groups with probable TDP-43 pathology (svPPA and FTD-MND clinically, *GRN* and *C9orf72* mutations genetically) to have raised levels compared with those associated with tau pathology (*MAPT* mutations) although within all groups there is substantial variability. With a lower limit of quantification of 0.26 pg/mL, all samples, including those from normal controls, could be reliably quantified, which is an advantage over earlier studies on serum NfL in other conditions [14, 30-32].

The results in this study are consistent with those found in previous CSF studies of NfL concentrations in FTD: levels are consistently higher in cases with FTD [4-11] and tend to be increased in those with probable TDP-43 pathology [10,11]. Certainly for genetic FTD, where *GRN* and *C9orf72* mutations are associated with TDP-43 pathology, this is consistent with the more rapid progression (and shorter disease duration) seen in many patients within these two mutation groups (independent of clinical syndrome) compared with the relatively slower progression of patients with *MAPT* mutations (which is associated with tau pathology) [33]. One previous study also suggested a correlation of CSF NfL with measures of disease severity, and consistent with our study showed an association of levels with frontal lobe atrophy [11].

We found that serum NfL levels were correlated with the rate of subsequent brain atrophy but not with the baseline brain volumes. Measures of brain atrophy are likely to be better measures of the disease intensity than just a single cross-sectional measure of the whole brain or lobar volumes which reflect disease duration and normal variation as well as disease activity. Serum NfL levels correlated with baseline measures of executive function but not with longitudinal measures. A number of the patients had scored at near floor on executive tasks at baseline and therefore there is less ability to measure progression with such measures when assessed longitudinally.

It will be important to investigate patients at different stages of the disease as this may influence the association between NfL and rates of atrophy. The GENFI study ([www.genfi.org.uk](http://www.genfi.org.uk)) has recently shown that pathological rates of brain atrophy appear to start up to ten years before symptom onset but are variable between different genetic mutations [29]. If serum NfL concentrations represent measures of disease intensity then we would predict that levels would start to increase around ten years before onset and may provide a useful non-invasive marker of proximity to symptom onset.

There are a number of limitations to this study. In particular the majority of the patients did not have pathological confirmation of the cause of their illness, and future studies should investigate serum NfL levels in different FTD pathologies. Although there are a relatively large number of cases for a study of a rare disorder like FTD, the individual numbers are small in each subgroup (particularly the FTD-MND group) and it would be useful for future studies to investigate larger groups of the individual clinical and genetic subtypes. Clinical measures of disease staging in FTD have only recently been designed (such as the FTLD-CDR [34] and Frontotemporal dementia Rating Scale (FRS) [35]) and were not available in this cohort; it will be important for future studies to compare such measures with serum NfL levels.

Higher serum NfL concentrations are associated with more rapid brain atrophy and may therefore reflect disease intensity in FTD. As blood sampling is less invasive and has better patient acceptability than lumbar puncture, serum NfL may provide important prognostic information, and prove to be a useful outcome measure for clinical trials in FTD. However, further studies will be required in order to understand the factors affecting the variability in NfL concentration and to determine whether it can be a useful measure within individual patients.

**Table 1 Demographic characteristics of the study participants. *FTD = frontotemporal dementia (total FTD cases do not include lvPPA), bvFTD = behavioural variant FTD, FTD-MND = FTD with motor neurone disease, PPA = primary progressive aphasia, nfvPPA = nonfluent variant PPA, svPPA = semantic variant PPA, lvPPA = logopenic variant PPA, PPA-NOS = PPA not otherwise specified.***

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Disease group** | **Controls** | **Total FTD** | ***bvFTD*** | ***FTD-MND*** | ***nfvPPA*** | ***svPPA*** | ***lvPPA*** | ***PPA-NOS*** |
| **Number** | 28 | 67 | *34* | *3* | *13* | *10* | *7* | *7* |
| **Age,**  **years [mean (SD)]** | 63.9 (7.2) | 64.5 (7.9) | *63.0 (8.3)* | *65.0 (0.3)* | *67.5 (9.7)* | *65.2 (6.4)* | *65.6 (5.9)* | *63.9 (5.2)* |
| **Male gender [%]** | 46.4 | 61.2 | *73.5* | *66.7* | *23.1* | *60.0* | *71.4* | *71.4* |
| **Disease duration,**  **years [mean (SD)]** | N/A | 5.5 (3.7) | *6.2 (4.6)* | *6.0 (4.6)* | *3.8 (1.5)* | *6.0 (2.1)* | *6.4 (2.9)* | *4.5 (2.5)* |
| **Serum neurofilament light chain,**  **pg/ml [mean (SD)]** | 19.6 (8.2) | 77.9 (51.3) | *57.8 (33.1)* | *195.0 (69.9)* | *82.5 (33.8)* | *95.9 (33.0)* | *49.5 (19.4)* | *91.2 (86.6)* |

**Table 2 Comparison of serum NfL concentrations between A) the disease subgroups and controls, and B) the genetic subgroups and controls. *Values are displayed as mean difference in serum neurofilament concentration between groups [SEM, p value] and refer to comparison of rows versus columns.***

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **bvFTD** | **FTD-MND** | **nfvPPA** | **svPPA** | **lvPPA** | **PPA-NOS** |
| **Controls** | **-38.2 [5.9, <0.001]** | -175.3 [40.4, 0.185] | **-62.9 [9.5, <0.001]** | **-76.2 [10.5, 0.001]** | -29.9 [7.5, 0.053] | -71.6 [32.8, 0.413] |
| **bvFTD** |  | -137.2 [40.8, 0.276] | -24.7 [10.9, 0.308] | -38.1 [11.9, 0.070] | 8.3 [9.3, 0.968] | -71.6 [32.8, 0.413] |
| **FTD-MND** |  |  | 112.4 [41.5, 0.374] | 99.1 [41.7, 0.451] | 145.4 [41.0, 0.248] | 103.8 [51.9, 0.509] |
| **nfvPPA** |  |  |  | -13.3 [14.0, 0.959] | 33.0 [11.9, 0.136] | -8.7 [34.0, 1.000] |
| **svPPA** |  |  |  |  | **46.3 [12.7, 0.032]** | 4.7 [34.4, 1.000] |
| **lvPPA** |  |  |  |  |  | -41.7 [33.5, 0.857] |

|  |  |  |  |
| --- | --- | --- | --- |
|  | ***MAPT*** | ***GRN*** | ***C9orf72*** |
| ***Control*** | **-20.8 [6.5, 0.035]** | -118.8 [51.7, 0.277] | -**59.5 [16.2, 0.025\*** |
| ***MAPT*** |  | -98.0 [52.0, 0.386] | -38.7 [17.3, 0.175] |
| ***GRN*** |  |  | 59.3 [54.1, 0.712] |

**Table 3 Cognitive and imaging characteristics of the FTD study participants. *Baseline cognitive measures are standard scores apart from the MMSE (out of 30). Longitudinal cognitive scores are annualized change in standard score (or change in MMSE score: a negative score is a decrease in score. Baseline brain volumes are expressed as a percentage of total intracranial volume (measured in SPM12). Longitudinal imaging measures are annualized rates of atrophy (%).***

|  |  |  |
| --- | --- | --- |
|  | **Baseline**  **Mean (SD)** | **Longitudinal**  **Mean (SD)** |
| ***Cognitive measures*** |  |  |
| **Number of participants** | 47 | 29 |
| **MMSE** | 23.8 (5.7) | -1.7 (5.0) |
| **WASI Vocabulary** | 4.4 (4.4) | -0.9 (2.9) |
| **WASI Block Design** | 8.7 (4.3) | -0.4 (2.6) |
| **WASI Similarities** | 5.9 (4.3) | -1.5 (3.1) |
| **WASI Matrices** | 9.4 (4.3) | 0.1(2.6) |
| **RMT Faces** | 5.2 (4.1) | -0.9 (3.7) |
| **RMT Words** | 6.1 (4.5) | -1.5 (4.1) |
| **Graded Naming Test** | 4.2 (4.4) | -1.7 (3.0) |
| **Graded Difficulty Calculation Test** | 7.8 (5.0) | -0.9 (2.4) |
| **D-KEFS Color-Word Interference Test** | 6.1 (5.1) | -1.7 (2.5) |
| ***Imaging measures*** |  |  |
| **Number of participants** | 46 | 29 |
| **Whole brain** | 72.6 (5.0) | 1.9 (1.5) |
| **Frontal** | 10.4 (1.0) | 2.2 (2.7) |
| **Temporal** | 7.0 (0.9) | 2.7 (2.4) |
| **Parietal** | 6.0 (0.5) | 1.2 (2.9) |
| **Occipital** | 4.9 (0.4) | 0.7 (2.5) |
| **Insula** | 0.8 (0.1) | 2.6 (2.6) |
| **Cingulate** | 1.6 (0.1) | 1.2 (2.0) |

**FIGURES**

**Figure 1**

**Title: Serum NfL concentrations in participants by A) clinical diagnosis and B) genetic status.**

***Legend: All patients have bvFTD except for \*with nfvPPA and #with PPA-NOS.***

**Figure 2**

**Title: Relationship of serum NfL concentrations to frontal lobe atrophy rate.**

***Legend:* *Serum neurofilament light chain concentrations are correlated with frontal lobe atrophy rates [r=0.53, p=0.003]. Points indicate individual patient values and the straight line indicates the line of best fit from a linear regression model of serum NfL on annualized frontal lobe atrophy rate.***

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