ARTICLE

Serum neurofilament light

A biomarker of neuroaxonal injury after ischemic stroke

Steffen Tiedt, MD,* Marco Duering, MD,* Christian Barro, MD, Asli Gizem Kaya, MSc, Julia Boeck, Felix J. Bode, MD, Matthias Klein, MD, Franziska Dorn, MD, Benno Gesierich, PhD, Lars Kellert, MD, Birgit Ertl-Wagner, MD, Michael W. Goertler, MD, Gabor C. Petzold, MD, Jens Kuhle, MD, Frank A. Wollenweber, MD, Nils Peters, MD,‡ and Martin Dichgans, MD‡

Correspondence
Dr. Dichgans
martin.dichgans@med.uni-muenchen.de

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Abstract

Objective

To explore the utility of serum neurofilament light chain (NfL) as a biomarker for primary and secondary neuroaxonal injury after ischemic stroke (IS) and study its value for the prediction of clinical outcome.

Methods

We used an ultrasensitive single-molecule array assay to measure serum NfL levels in healthy controls (n = 30) and 2 independent cohorts of patients with IS: (1) with serial serum sampling at hospital arrival (n = 196), at days 2, 3, and 7 (n = 89), and up to 6 months post stroke; and (2) with standardized MRI at baseline and at 6 months post stroke, and with cross-sectional serum sampling at 6 months (n = 95). We determined the temporal profile of serum NfL levels, their association with imaging markers of neuroaxonal injury, and with clinical outcome.

Results

Patients with IS had higher serum NfL levels compared with healthy controls starting from admission until 6 months post stroke. Serum NfL levels peaked at day 7 (211.2 pg/mL [104.7–442.6], median [IQR]) and correlated with infarct volumes (day 7: partial $r = 0.736$, $p = 1.5 \times 10^{-15}$). Six months post stroke, patients with recurrent ischemic lesions on MRI (n = 19) had higher serum NfL levels compared to those without new lesions (n = 76, $p = 0.002$). Serum NfL levels 6 months post stroke further correlated with a quantitative measure of secondary neurodegeneration obtained from diffusion tensor imaging MRI ($r = 0.361$, $p = 0.001$). Serum NfL levels 7 days post stroke independently predicted modified Rankin Scale scores 3 months post stroke (cumulative odds ratio [95% confidence interval] = 2.35 [1.60–3.45]; $p = 1.24 \times 10^{-05}$).

Conclusion

Serum NfL holds promise as a biomarker for monitoring primary and secondary neuroaxonal injury after IS and for predicting functional outcome.

*These authors contributed equally to this work.

‡These authors contributed equally to this work.

From the Institute for Stroke and Dementia Research, University Hospital (S.T., M. Duering, A.G.K., J.B., B.G., F.A.W., M. Dichgans), and Graduate School of Systemic Neurosciences (S.T.), LMU Munich; Munich Cluster for Systems Neurology (SyNergy) (S.T., M. Dichgans), Munich, Germany; Neurologic Clinic and Policlinic (C.B., J.K.), Departments of Medicine, Biomedicine and Clinical Research, University Hospital Basel, University of Basel, Switzerland; German Center for Neurodegenerative Diseases (DZNE) (F.J.B., G.C.P.), Bonn; Department of Neurology (F.J.B., G.C.P.), University Hospital Bonn; Departments of Neurology (M.K., L.K.), Neuroradiology (F.D.), and Radiology (B.E.-W.), University Hospital, LMU Munich; Department of Neurology (M.W.G.), University of Magdeburg, University Hospital; German Center for Neurodegenerative Diseases (DZNE) (M.W.G.), Magdeburg, Germany; Stroke Center and Department of Neurology (N.P.), University Hospital Basel, Switzerland; and German Center for Neurodegenerative Diseases (DZNE) (M. Dichgans), Munich, Germany.

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Glossary

ANTs = Advanced Normalization Tools; **CIRCULAS** = Circulating Biomarkers in Acute Stroke; **DEMDAS** = German Center for Neurodegenerative Diseases—Mechanisms of Dementia After Stroke; **FLAIR** = fluid-attenuated inversion recovery; **FMRIB** = Oxford Centre for Functional Magnetic Resonance Imaging of the Brain; **FSL** = FMRIB's Software Library; **HC** = healthy control; **IS** = ischemic stroke; **LMU** = Ludwig-Maximilians University; **MD** = mean diffusivity; **mRS** = modified Rankin Scale; **NfL** = neurofilament light chain; **NIHSS** = NIH Stroke Scale.

Ischemic stroke (IS) presents with largely variable infarct sizes, outcomes, and recurrence rates. Infarct volumes predict stroke outcome.[1,2] However, determination of infarct volumes requires brain MRI or delayed CT scans as well as postprocessing and image segmentation, which are demanding in terms of logistics, personnel, and costs. Hence, there is great interest in circulating biomarkers that can easily be obtained from all patients and that correlate with the extent of neuronal injury.[3] Patients with IS show a high rate of recurrent ischemic lesions on MRI particularly within the first months after stroke.[4,5] Such lesions might also be reflected in circulating molecules.[6,7] Acute infarcts further induce secondary neurodegeneration outside the infarct area[8,9] including white matter tracts connected to the infarct.[10,11] Such secondary damage likely contributes to clinical outcome[8] and might also be reflected in molecules released from injured neurons. As such, circulating neuronal injury markers could be of interest for patient monitoring and for use in observational and interventional studies.[12]

Neurofilament light chain (NfL) is a neuronal scaffolding protein that is released into the extracellular space upon neuroaxonal damage.[13] Serum NfL levels increase during aging and are elevated in various neurologic conditions including traumatic brain injury,[14] multiple sclerosis,[15,16] and neurodegenerative diseases.[17,18]

We thus set out to explore the temporal evolution of serum NfL after IS and its association with infarct volumes, recurrent ischemic lesions, and an MRI measure of stroke-induced secondary neurodegeneration. We further determined its predictive value for functional outcome 3 months post stroke. To address these questions, we leveraged data from 2 prospective stroke cohorts with complementary study design.

Methods

Patient enrollment

CIRCULAS cohort

From February 2014 to March 2017, we prospectively recruited 277 patients with suspected IS within 24 hours of symptom onset through the Circulating Biomarkers in Acute Stroke (CIRCULAS) study. Patients were recruited through the emergency department of the university hospital at Ludwig-Maximilians University (LMU), a tertiary level hospital in Munich, Germany, as previously described.[19] All patients had a final diagnosis of IS as defined by an acute focal neurologic deficit in combination with a hyperintense lesion on diffusion-weighted MRI, or a new ischemic lesion on a delayed CT scan.[20] Patients with neurologic diseases within the last 3 months preceding the stroke were excluded (n = 81), leaving 196 patients for inclusion (figure 1A). We further included 30 healthy controls (HCs) (mostly spouses of patients) who were matched for age and sex and recruited through a single outpatient clinic at LMU.

DEMDAS cohort

From December 2013 to November 2016, we prospectively recruited 134 patients with IS through the DEMDAS study (German Center for Neurodegenerative Diseases—Mechanisms of Dementia After Stroke), a multicenter study conducted at 6 university hospitals in Germany.[21] For the current study, we used data from 3 centers. IS was defined as described for the CIRCULAS study. Patients were excluded if (1) MRIs did not meet prespecified quality criteria (n = 33), (2) they had neurologic diseases within the last 3 months preceding the stroke (n = 5), or (3) serum NfL levels were below detection limit (n = 1), leaving 95 patients for analyses (figure 1D).

Standard protocol approvals, registrations, and patient consents

All procedures were conducted in accordance with the Declaration of Helsinki and institutional guidelines. Written and informed consent was obtained from all participants in accordance with approval by the local ethics committee. The DEMDAS study was registered at clinicaltrials.gov (NCT01334749).

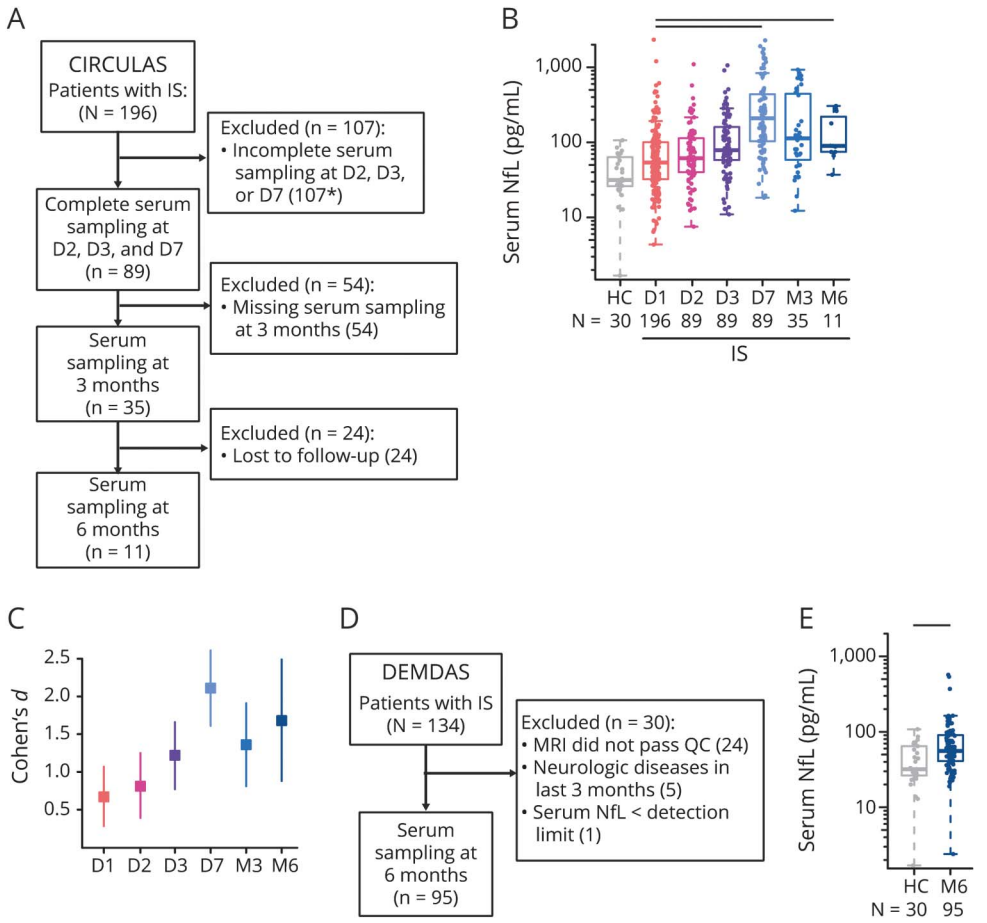
Serum sampling and processing

Blood samples from patients included in the CIRCULAS study were collected immediately on hospital arrival in the emergency department, at day 2 (D2), day 3 (D3), and day 7 (D7), as well as 3 months and 6 months post stroke (figure 1A). Blood samples from patients included in the DEMDAS study were collected at 6 months post stroke (n = 95). Samples were drawn using a tourniquet, 21-gauge needles, and uniform procedures. After 30 to 45 minutes at room temperature, separation of serum was achieved by differential centrifugation at 2,000*g* for 10 minutes at 15°C. Samples were aliquoted in screw cap vials and kept at −80°C.

NfL assay

Serum NfL levels were measured by a single-molecule array assay using the capture monoclonal antibody 47:3, and the

Figure 1 IS causes long-lasting elevations of serum NfL levels



(A) Study profile of the CIRCULAS study. (B) Temporal profile of serum NfL levels in patients with IS starting from admission (D1) up to 6 months post stroke and comparison with serum NfL levels in HCs. Shown are data from all patients with available NfL measurements (n = 196). (C) Effect sizes of serum NfL levels between HCs and different time intervals between stroke and blood sampling in patients with IS. (D) Study profile of the DEMDAS study. (E) Serum NfL levels in patients with IS from DEMDAS obtained 6 months post stroke compared to serum NfL levels in HCs. (B) Mann-Whitney test, Friedman test followed by the Conover test with Bonferroni correction; (C) Cohen *d*; and (E) Mann-Whitney test. The upper horizontal bar in panel B indicates a statistically significant difference between HCs and each time point in patients with IS covered by the bar. The lower horizontal bar in panel B and the horizontal bar in panel E indicate a statistically significant difference between all groups covered by the bar. *Includes 48 patients with serum sampling at day 7 and outcome data for 3 months post stroke. CIRCULAS = Circulating Biomarkers in Acute Stroke; D1 = day 1; D2 = day 2; D3 = day 3; D7 = day 7; DEMDAS = DZNE—Mechanisms of Dementia After Stroke; HC = healthy control; IS = ischemic stroke; M3 = month 3; M6 = month 6; NfL = neurofilament light chain; QC = quality control.

biotinylated detector monoclonal antibody 2:1 from Uman-Diagnostics (Umeå, Sweden) as previously described.[15] Interassay variabilities (coefficient of variation of concentrations) were 13%, 14%, and 5% for 3 control samples with mean concentrations of 8, 22, and 101 pg/mL, respectively. The mean intra-assay variability of duplicate determinations was 4.6%. Few samples showing intra-assay coefficients of variations above 20% were measured again.[15]

Neuroimaging

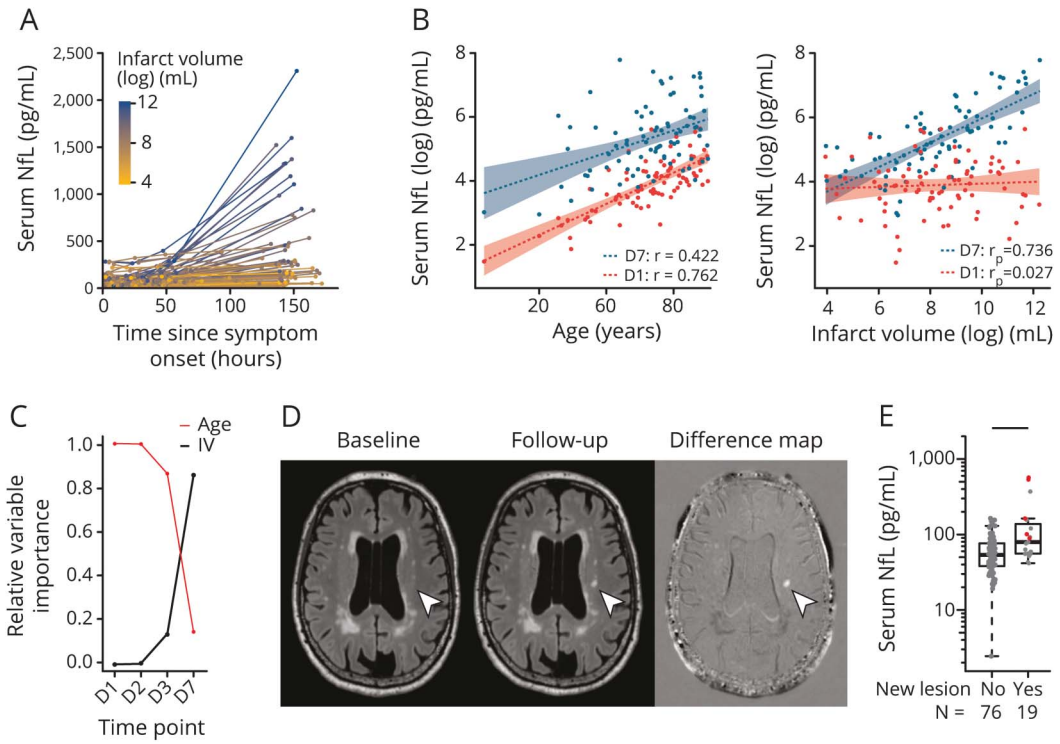
In CIRCULAS, infarct volumes were quantified on images from diagnostic scans (n = 83), either diffusion-weighted MRI (n = 69) or noncontrast CT (n = 14) obtained at least 24 hours after symptom onset. Infarcts were segmented manually slice-by-slice by trained raters.

In DEMDAS, patients were scanned using a standardized MRI protocol both at baseline and 6 months post stroke.[21] Infarct volumes and microstructural tissue integrity were quantified on diffusion-weighted scans (b = 1,000 s/mm^2, 2-mm isotropic resolution, 30 diffusion directions). Preprocessing of diffusion images included denoising and Gibbs ringing artifact removal as implemented in the mrtrix software package (version 3.0_RC2),[22–24] correction for motion and eddy current–induced distortions as implemented in the FMRIB Software Library (FSL,[25] version 5.0.10), and intensity bias correction as implemented in the N4 bias correction tool from the Advanced Normalization Tools (ANTs,[26] version 2.1.0).

For infarct volume calculation, preprocessed diffusion-weighted images were averaged and segmented into 2 tissue probability maps using FAST (FMRIB's Automated Segmentation Tool)[27] from FSL. Diffusion-restricted lesions were separated from CSF, which were in the same tissue probability map, by the Otsu histogram segmentation method. The segmentation maps were then manually edited and cleaned from misclassified artifacts using a custom, in-house, 3-dimensional (3D) editing tool and trained raters.

For the identification of incident fluid-attenuated inversion recovery (FLAIR) lesions between baseline and follow-up, we calculated difference maps. After N4 intensity bias correction and affine registration of baseline and follow-up images (3D FLAIR and diffusion tensor imaging, both 1-mm isotropic resolution) to halfway space using the ANTs toolbox,[28] difference maps were created by subtracting

Figure 2 Serum NfL levels correlate with infarct volumes and recurrent ischemic lesions



(A–C) Results obtained in the CIRCULAS study. (A) Serum NfL levels in individual patients with IS (n = 83) starting from admission until D7 and their association with infarct volumes. (B) Association of serum NfL levels with age and infarct volumes in patients with IS at hospital admission (D1) and at D7. (C) Relative variable importance of infarct volume compared to age for explaining serum NfL levels after IS over time. (D and E) Results obtained in the DEMDAS study. (D) Methodology used to identify recurrent ischemic lesions on brain MRI 6 months post stroke (for explanations see the methods section). (E) Serum NfL levels in patients with incident ischemic lesion at 6-month follow-up compared to patients without new lesion. Red points depict positive lesions on diffusion-weighted imaging. (B) Linear regression models; the linear fit (dashed line) and 95% confidence intervals are shown in color. (C) Random forest conditional variable importance analyses. (E) Mann-Whitney test. The black line indicates a statistically significant difference ($p < 0.05$). CIRCULAS = Circulating Biomarkers in Acute Stroke; D1 = day 1; D2 = day 2; D3 = day 3; D7 = day 3; DEMDAS = DZNE—Mechanisms of Dementia After Stroke; IS = ischemic stroke; IV = infarct volume; NfL = neurofilament light chain; r_p = partial correlation coefficient.

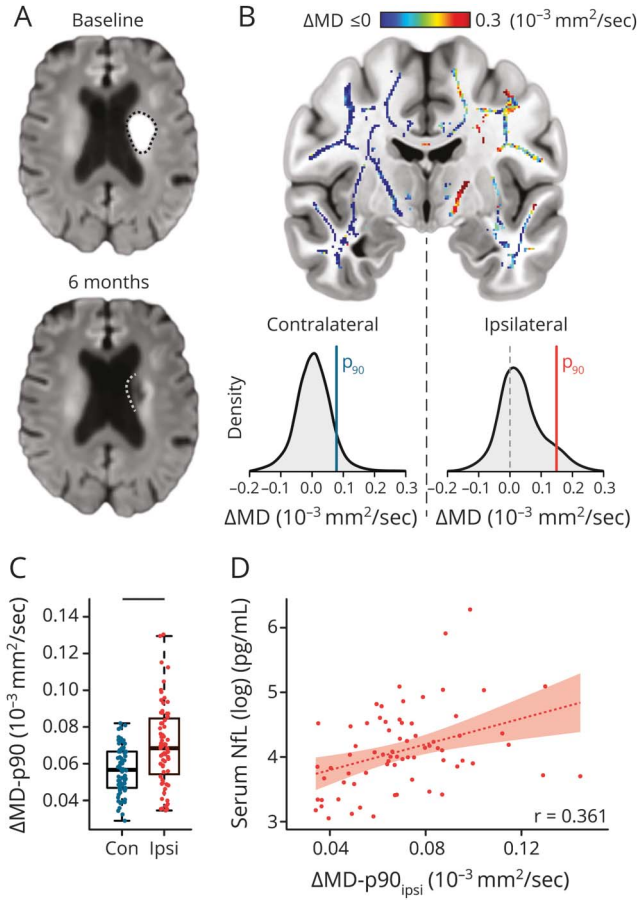
baseline images from follow-up images. An experienced rater visually checked for new lesions, which were easy to identify on the difference maps (figure 2D). The acute infarct segmentation was superimposed on top of the difference maps to identify recurrent lesions, i.e., FLAIR lesions not originating from the infarct visible on the baseline scan.

For the quantification of microstructural damage within major white matter tracts as a measure of secondary neurodegeneration, we first calculated fractional anisotropy and mean diffusivity (MD) maps for baseline and follow-up scans using "dtifit" from FSL. The maps were then reduced to the main fiber tract skeleton using the tract-based spatial statistics pipeline of FSL[29] with standard parameters. In addition, we applied a custom mask excluding areas close to CSF to minimize CSF contamination on MD values. The infarct area and a 2-mm spacer around the infarct were excluded from the analysis. We calculated the change in MD over time (ΔMD) for each voxel in the main tract skeleton. Next, we analyzed histograms derived from the ΔMD values for the hemisphere ipsilateral to the infarct and for the contralateral side (figure 3, A and B). We defined the 90th percentile of ΔMD values as a quantitative measure for microstructural changes in major white matter tracts between baseline and 6 months post stroke.[30]

Statistics

Statistical analyses were performed in "R," version 3.4.0.[31] To test for normality, we used the Shapiro-Wilk test. If significant, log transformation (base *e*) was used to achieve normal distribution. For group comparisons, the Mann-Whitney test was used. Paired data were analyzed using the Friedman test followed by the Conover test. All tests were performed 2-sided. A p value <0.05 was considered statistically significant. Where indicated, Bonferroni correction for multiple testing was applied. Effect sizes between HCs and patients with IS were calculated using Cohen *d*. We applied linear regression analysis to assess associations of serum NfL levels with infarct volume, age, and the change in MD (ΔMD-p90). Where indicated, adjustment was performed using analysis of residuals after regressing out confounders. To determine associations with the modified Rankin Scale (mRS) score (as dependent variable), we used simple ordinal logistic regression as implemented in the R package "ordinal."[32] Fulfillment of the proportional odds assumption was tested using the Brant test.[33] Random forest regression was used to assess conditional variable importance in a multivariable model. We used standard

Figure 3 Serum NfL levels correlate with secondary degeneration of major white matter tracts



(A) Coregistered DWI scans from a representative patient with ischemic stroke obtained at baseline (top) and 6 months post stroke (bottom). The acute infarct is visible as a DWI-positive lesion on the baseline scan. (B) Upper panel: coronary view showing the change in mean diffusivity (ΔMD) from baseline to 6 months post stroke within the main white matter tract skeleton (color-coded); lower panels: histograms of ΔMD quantified across the main white matter tract skeletons contralateral (left) and ipsilateral (right) to the infarct. Colored lines delineate the 90th percentile (p90) of the histogram. (C) ΔMD-p90 within white matter tracts ipsilateral to the infarct compared to white matter tracts contralateral to the infarct. (D) Association of serum NfL levels and ΔMD-p90 ipsilateral to the infarct. (C) Mann-Whitney test. The black line indicates a statistically significant difference ($p < 0.05$). (D) Simple linear regression analysis; the linear fit (dashed line) and 95% confidence intervals are shown in color. Con = contralateral; DWI = diffusion-weighted imaging; Ipsi = ipsilateral; MD = mean diffusivity; NfL = neurofilament light chain.

parameters, grew 1,501 trees per run, and performed 200 runs to calculate 95% confidence intervals ("party" package in R).[34]

Data availability

The raw data used in preparation of the figures and tables will be shared in anonymized format by request of a qualified investigator to the corresponding author for purposes of replicating procedures and results.

Results

Baseline characteristics of the study samples and HCs are presented in table 1 and data available from Dryad, table e-1, doi.org/10.5061/dryad.1s6s162.

IS causes long-lasting elevations of serum NfL

Compared to age- and sex-matched HCs, patients with acute IS had higher serum levels of NfL at admission (D1), throughout the first week (D2, D3, D7), and at 3 and 6 months after stroke (CIRCULAS study; figure 1, A and B). Serum NfL levels in patients increased up to D7 (all group comparisons between time points D1 to D7: $p < 2 \times 10^{-16}$, Friedman test with Bonferroni-corrected Conover post hoc tests) and remained elevated 3 and 6 months after stroke compared to admission (figure 1B). Similar results were obtained when restricting the analysis to patients with complete measurements from D1 to D7 (n = 89, data available from Dryad, figure e-1A, doi.org/10.5061/dryad.1s6s162), from D1 to 3 months (n = 35, data available from Dryad, figure e-1B), and from D1 to 6 months (n = 11, data available from Dryad, figure e-1, A–C). Accordingly, the effect size indicated the largest difference between HCs and patients on D7 (Cohen *d*: 2.11, 95% confidence interval: 1.61–2.61; figure 1C). Long-lasting elevations of serum NfL 6 months post stroke were further seen in a second independent cohort of patients with IS recruited through the DEMDAS study (55.6 vs 31.8 pg/mL [IS vs HC; n = 95/30; $p = 0.0005$]; figure 1, D and E, and table 1).

Serum NfL levels correlate with infarct volumes and recurrent ischemic lesions

Infarct volume

To assess the relationship between serum NfL levels and the extent of primary ischemic injury, we examined the relationship between infarct volumes and serum NfL levels during the acute phase (D1–D7, n = 83; figure 2A). Because of associations between serum NfL levels and both age and hypertension in patients at D1 (age: $r = 0.762$, $p < 2 \times 10^{-16}$; hypertension: $p = 0.006$) and D7 (age: $r = 0.422$, $p = 4.2 \times 10^{-5}$; hypertension: $p = 0.01$) (figure 2B; data available from Dryad, table e-2, doi.org/10.5061/dryad.1s6s162), all subsequent analyses were adjusted for age and hypertension. Infarct volumes correlated positively with NfL levels on D7 (partial correlation coefficient $r_p = 0.736$, $p = 1.5 \times 10^{-15}$) and D3 ($r_p = 0.370$, $p = 0.0003$) but not at earlier time points (figure 2B). Focusing on age and infarct volume, the relative variable importance of infarct volume to explain serum NfL levels increased from D1 to D7 (figure 2C).

Recurrent ischemic lesions on brain MRI during follow-up

We next investigated the relationship between recurrent ischemic lesions during follow-up and serum NfL levels at 6 months drawing on patients from the DEMDAS study (n = 95 patients). Nineteen patients (20%) developed recurrent ischemic lesions as defined by a new hyperintense lesion on the 6-month FLAIR (figure 2D) or diffusion-weighted imaging scans, 2 of which were symptomatic. Compared to patients without recurrent ischemic lesions, patients with recurrent ischemic lesions showed higher NfL levels at 6 months (78.7 vs 53.3 pg/mL, n = 19/76, $p = 0.002$; figure 2E). Recurrent

Table 1 Characteristics of healthy controls and patient cohorts

	Healthy controls (n = 30)	Patients with IS (CIRCULAS) (n = 196)	Patients with IS (DEMDAS) (n = 95)
Demographic characteristics			
Age, y, median (IQR)	78.0 (19.7)	74.9 (16.9)	65.0 (19.0)
Sex, female, n (%)	13 (43.3)	83 (42.3)	34 (35.8)
Vascular risk factors, n (%)			
Hypertension	14 (46.7)	154 (79.0)	45 (47.4)
Hypercholesterolemia	10 (33.3)	53 (27.3)	28 (29.5)
Diabetes	1 (3.3)	34 (17.5)	9 (9.5)
Current or past smoking	11 (36.7)	81 (43.3)	58 (61.1)
Family history	2 (6.7)	24 (13.0)	11 (11.6)
Prior stroke/TIA	0 (0.0)	40 (20.3)	7 (7.4)
Clinical scores, median (IQR)			
NIHSS score at D7[a]/baseline[b]	NA	2 (4)	2 (4)
mRS score at D7[a]/baseline[b]	NA	1 (2)	1 (1)
pmRS score	NA	0 (1)	0 (0)
Infarct volume, mL, mean (SD)	NA	27.7 (64.7)	9.1 (19.7)
Etiology of IS, n (%)			
Large artery atherosclerosis	NA	42 (21.4)	22 (23.2)
Cardioembolism	NA	62 (31.6)	21 (22.1)
Small vessel occlusion	NA	16 (8.2)	16 (16.8)
Arterial dissection	NA	5 (2.6)	3 (3.2)
Undetermined	NA	71 (36.2)	33 (34.7)

Abbreviations: CIRCULAS = Circulating Biomarkers in Acute Stroke; D7 = day 7; DEMDAS = DZNE—Mechanisms of Dementia After Stroke; IQR = interquartile range; IS = ischemic stroke; mRS = modified Rankin Scale; NA = not available; NIHSS = NIH Stroke Scale; pmRS = premorbid modified Rankin Scale.
Family history was considered positive if the patient had a parent and/or sibling with a history of stroke, myocardial infarction, treated angina, or of sudden cardiac death before age 55 years in men or age 65 years in women.
[a] In CIRCULAS patients.
[b] In DEMDAS patients (ranged from day 1 to day 3).

ischemic lesions remained an independent predictor of serum NfL levels at 6 months post stroke when adjusting for age, sex, hypertension, and infarct volume at baseline ($p = 0.014$).

Serum NfL levels correlate with secondary degeneration of major white matter tracts

Given previous evidence for secondary neurodegeneration after acute ischemic infarcts[8,9,11,35,36] and the observed long-lasting elevations of serum NfL levels 6 months post stroke, we next assessed the association between secondary neuroaxonal injury after stroke and serum NfL levels at 6 months in the DEMDAS cohort. As a quantitative measure for secondary neuroaxonal injury, we calculated the change in MD (ΔMD-p90) in major white matter tracts outside the infarct area between baseline and 6 months post stroke (figure 3, A and B). For methodologic reasons, we focused on patients with unilateral infarcts (n = 71). As expected, ΔMD-p90 in white matter tracts ipsilateral to the infarct (ΔMD-p90$_{ipsi}$) was higher than ΔMD-p90 in white matter tracts contralateral to the infarct (ΔMD-p90$_{contra}$) ($p = 0.0002$, figure 3C) and strongly correlated with infarct volume at baseline ($r = 0.624$, $p = 3.8 \times 10^{-9}$), consistent with secondary neuroaxonal injury. Of note, serum NfL levels at 6 months were associated with ΔMD-p90$_{ipsi}$ ($r = 0.361$, $p = 0.001$; figure 3D) but not with ΔMD-p90$_{contra}$ ($r = 0.141$, $p = 0.15$). Serum NfL levels at 6 months remained associated with ΔMD-p90$_{ipsi}$ after adjustment for age, sex, hypertension, and recurrent ischemic lesions ($p = 0.02$).

Serum NfL levels predict clinical outcome

As a final step, we explored the predictive value of serum NfL levels for clinical outcome. Because of the superior effect size of serum NfL levels on D7 post stroke, we performed the analyses in CIRCULAS patients with available sampling at D7

Table 2 Ordinal logistic regression analyses with mRS scores at 3 months as dependent variable

	p Value	Cumulative OR (95% CI)
Age	0.513	1.12 (0.79–1.60)
Sex, female	0.131	1.73 (0.85–3.52)
Infarct volume at baseline	0.002[a]	1.81 (1.25–2.61)
Serum NfL at D7	1.24e-05[a]	2.35 (1.60–3.45)
mRS score at D7	5.65e-08[a]	3.14 (2.08–4.75)
NIHSS score at D7	2.26e-06[a]	2.67 (1.78–4.01)
pmRS score	0.275	1.22 (0.85–1.75)

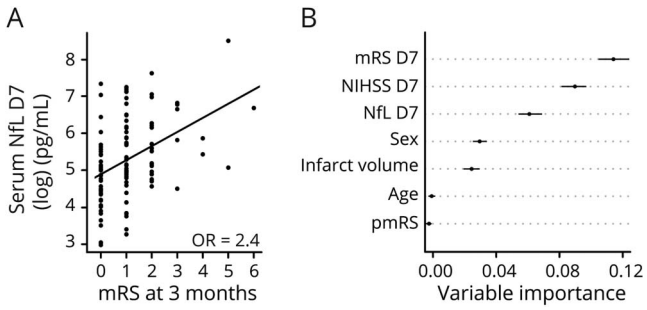
Abbreviations: CI = confidence interval; D7 = day 7; mRS = modified Rankin Scale; NfL = neurofilament light chain; NIHSS = NIH Stroke Scale; OR = odds ratio; pmRS = premorbid modified Rankin Scale.
Analyses were performed with data from patients from the CIRCULAS (Circulating Biomarkers in Acute Stroke) study (n = 110).
[a] Significant.

(n = 110). mRS scores at 3 months post stroke ranged from 0 to 6 (median = 1). In simple logistic regression analyses, serum NfL levels on D7, infarct volumes, mRS scores on D7, and NIH Stroke Scale (NIHSS) scores on D7 were associated with mRS scores 3 months post stroke (table 2 and figure 4A). To estimate the contribution of each variable, while accounting for high intercorrelation, we calculated the conditional variable importance from random forest regression. mRS scores on day 7, NIHSS scores on day 7, and serum NfL levels on day 7 were the most important predictors for mRS scores at 3 months post stroke (figure 4B).

Discussion

This study identifies serum NfL as a biomarker of both primary and secondary neuroaxonal injury after IS. Our main findings can be summarized as follows: (1) patients with IS had higher serum NfL levels compared to age-matched HCs starting from admission until 6 months post stroke; (2) NfL levels increased from D1 to D7 and correlated with infarct volumes; (3) NfL levels 6 months post stroke were associated both with recurrent ischemic lesions on brain MRI and secondary degeneration of major white matter tracts ipsilateral to the infarct; and (4) NfL levels 7 days post stroke independently predicted clinical outcome 3 months post stroke. Together, these findings highlight the potential utility of serum NfL as a biomarker for monitoring primary and secondary neuroaxonal injury in patients with IS.

Figure 4 Serum NfL levels predict clinical outcome



(A) Association of serum NfL levels at D7 with mRS at 3 months post stroke. (B) Variable importance of serum NfL levels at D7 compared to other variables to predict mRS scores 3 months post stroke. (A) Simple logistic regression analysis. (B) Random forest conditional variable importance analyses. Shown are point estimates and 95% confidence intervals obtained from 200 runs. D7 = day 7; mRS = modified Rankin Scale; NfL = neurofilament light chain; NIHSS = NIH Stroke Scale; OR = odds ratio; pmRS = premorbid modified Rankin Scale.

We found serum NfL levels to increase from hospital admission (D1) to D7. This finding broadly agrees with observations from a recent study in symptomatic patients with recent small subcortical infarcts that found increasingly higher NfL levels with increasing time from symptom onset to first blood sampling.[6] Of note, however, in the current study, we used serial blood sampling, thus controlling for potential bias influencing the timing of sampling. Also, we did not exclude patients with cortical infarcts, larger infarct sizes, or specific stroke etiologies. Hence, our findings can be considered representative of the spectrum of IS. The observed association between serum NfL levels and age in patients with stroke on admission (D1) mirrors the reported correlation between serum NfL and age in other cohorts.[15,17,37] Of note, however, the importance of age for explaining NfL levels in patients decreased from D1 to D7, whereas the importance of infarct volume greatly increased from D1 to D7, evidencing infarcted tissue as the primary source of increasing serum NfL levels within the first week after stroke. The effects of vessel recanalization on serum NfL levels should be further explored in future studies. Given the observed association between NfL levels at D7 and both infarct size and clinical outcome (see below), serum NfL might be of great interest as an adjunct outcome marker for future therapeutic trials, although this would need to be determined in the setting of a prospective trial.[38]

We further found serum NfL levels 6 months post stroke to be associated with recurrent ischemic lesions on brain MRI 6 months post stroke. The majority of these lesions were negative on diffusion-weighted imaging (figure 2E), suggesting that recurrent lesions of variable age[39–41] contributed to elevations of serum NfL levels at follow-up. The range of serum NfL levels in patients with recurrent lesions differed markedly from those in patients without recurrent lesions. Hence, serum NfL might assist in monitoring patients with elevated vascular risk and selecting patients for further diagnostic workup, although such an approach would need to be validated in a separate study. Again, our findings broadly agree with those from a recent study in patients with small subcortical infarcts, which found higher serum NfL levels in individuals with new vascular lesions at follow-up.[6] However, that study did not control for potential confounders, whereas we found recurrent ischemic lesions to influence serum NfL

levels independent of demographic factors and baseline infarct volume.

Among the most notable findings is the observed association between serum NfL levels 6 months post stroke and an MRI marker of secondary neurodegeneration. Specifically, we found higher serum NfL levels to moderately associate with a shift of MD (ΔMD-p90) toward higher values in major white matter tracts ipsilateral to the infarct. The validity of ΔMD-p90 as a measure for secondary neurodegeneration is supported by several observations: (1) we previously showed that ischemic infarcts cause focal degeneration in remote cortex via degeneration of connecting fiber tracts quantified using ΔMD[8,9,11]; (2) we now found ΔMD-p90 in white matter tracts ipsilateral to the infarct (ΔMD-p90$_{ipsi}$) to be significantly higher than ΔMD-p90 in white matter tracts contralateral to the infarct (ΔMD-p90$_{contra}$) as would be expected in the presence of secondary degeneration; and (3) ΔMD-p90$_{ipsi}$ strongly correlated with infarct volume as would also be expected in the presence of secondary neurodegeneration. Of note, we excluded infarct voxels and voxels surrounding the infarct from the MD measurements thus eliminating signal contamination by the infarct itself and by signal alterations in close vicinity to the infarct. Moreover, visual inspection of the ΔMD maps revealed the highest values in white matter tracts connected to the infarct as evidenced, e.g., by the pyramidal tract in figure 3B. NfL is predominantly expressed in large myelinated axons such as motor neurons[13] but is likewise present in neuronal bodies and dendrites with a presumed role in dendritic growth.[42] Collectively, these findings suggest that elevated serum NfL levels post stroke in part reflect secondary neurodegeneration of connecting white matter tracts.

Given the association between serum NfL levels at 6 months and recurrent ischemic lesions as well as secondary neurodegeneration during follow-up, serum NfL might have clinical utility for patient monitoring. Specifically, serum NfL levels might help in identifying patients with stroke who would benefit from repeated diagnostic workup and potentially more intensive secondary prevention, although this would need to be examined in the context of a clinical trial. Future studies may further determine the half-life of serum NfL, which cannot be estimated from our study but would help when interpreting repeat measurements.

We further found serum NfL levels on D7 to predict clinical outcome 3 months post stroke independent of other variables including age, infarct volume, stroke severity (D7), and functional status (D7). In fact, serum NfL levels on D7 showed higher predictive value compared to earlier[43] and later, clinically less meaningful, time points[44,45] and were the most important predictor of mRS scores 3 months post stroke following clinical scores at baseline. This might relate to serum NfL capturing both primary and secondary neuroaxonal injury, therefore also reflecting vascular injury preceding the stroke as evidenced by our findings in DEMDAS. Along the same lines, serum NfL levels in our patients might have been influenced by unrecognized neurodegenerative disease that might also have contributed to disability. Also, it might be that NfL captures aspects of stroke-related tissue injury that are particularly relevant for functional outcome. Conceivably, its specificity for neuroaxonal damage might distinguish serum NfL from other blood-based biomarkers with predictive value for stroke outcome reflecting other biological processes, such as copeptin,[46] brain natriuretic peptide,[47] and C-reactive protein.[48] Whether patients with elevated serum NfL levels on D7 benefit from more intensive rehabilitative treatment would need to be investigated in a prospective study.

In contrast, the utility of serum NfL for diagnosing IS on admission seems limited. First, the difference in serum NfL levels between patients with IS and HCs on D1 was low and of less utility compared to neuroimaging and other biomarkers,[19,49] in accord with a recent study in patients with acute cerebrovascular events.[43] Second, elevations of serum NfL are seen in various conditions including neurodegenerative diseases,[17,18] multiple sclerosis,[15,16] and brain trauma.[14] Third, we found serum NfL levels to be influenced by ischemic lesions occurring weeks or months before blood sampling as evidenced by our findings on recurrent ischemic lesions in DEMDAS. Still, the combination of 2 measurements, one at admission and one on D7, would be instructive and might indeed provide valuable information in patients without infarcts on admission scans. Additional data involving serial sampling in different patient groups, including patients with TIAs, are needed to define the utility of such an approach.

Our study has several methodologic strengths. First, we used data from 2 prospective cohorts with systematic follow-up and complementary study design enabling us to study multiple aspects of stroke and vascular brain injury. Second, our sampling strategy in CIRCULAS involved serial blood sampling starting immediately upon hospital admission before any medical interventions and extended up to 6 months post stroke thus enabling us to study the dynamics of serum NfL levels both in the overall sample and in individual patients. Third, quantification of serum NfL levels was performed using the latest technology (single-molecule array [Simoa]) and an extensively validated ultrasensitive single molecule assay.[50] Also, neuroimaging in the DEMDAS cohort involved state-of-the-art longitudinal MRI at 3-tesla and postprocessing, including isotropic 3D T1 and FLAIR sequences, difference imaging, and a high-quality diffusion tensor imaging analysis, enabling the quantification of changes in fiber tract integrity over time as a measure for secondary neurodegeneration. Finally, recurrent ischemic lesions were identified on difference images, which is largely observer-independent.

Our study also has limitations. Because of the study-related requirements with multiple assessments and follow-up, we might have selected patients with minor stroke as reflected by the low admission NIHSS scores. Correspondingly, infarct sizes were in the smaller range and the median mRS scores at

3 and 6 months were low. We cannot exclude a differential behavior of serum NfL levels in patients with severe stroke and large infarct sizes. However, we would argue, if at all, the preferential inclusion of patients with minor stroke would have resulted in an underestimation of strength of associations between serum NfL levels, MRI measures, and clinical outcome. Also, the clinical utility of serum NfL levels to monitor disease activity is likely to be highest in patients with minor stroke, who have the largest expected gain in prevention. Another limitation is the high proportion of patients from CIRCULAS with incomplete blood sampling at D2 or later time points who were therefore excluded from the analyses. Of note, however, sensitivity analyses showed that serum NfL levels at earlier time points were similar across participants with missing values at later time points. Also, we were not able to determine the exact time point of peak serum NfL levels because our sampling strategy did not cover the time interval between D7 and 3 months. Furthermore, DEMDAS had no standardized blood sampling at baseline, thus precluding analyses involving both baseline NfL and follow-up MRI. Finally, the number of patients with both serum NfL levels at D7 and outcome data was relatively small, thus requiring validation in additional patient cohorts.

Herein, we identify serum NfL as a promising biomarker of primary and secondary neuroaxonal injury after IS. Serum NfL levels in part reflect neuroaxonal degeneration of white matter tracts after acute ischemic lesions. As such, serum NfL levels seem suited to monitor multiple aspects of disease activity in patients with vascular brain lesions. Our findings further show that serum NfL levels obtained 7 days after stroke might have clinical utility for the prediction of clinical outcome and as an adjunct readout for future therapeutic trials.

Author contributions

Steffen Tiedt, MD: conception and design of the study, acquisition, analysis, and interpretation of data, drafting a significant portion of the manuscript or figures. Marco Duering, MD: conception and design of the study, acquisition, analysis, and interpretation of data, drafting a significant portion of the manuscript or figures. Christian Barro, MD: acquisition, analysis, and interpretation of data. Asli Gizem Kaya: acquisition, analysis, and interpretation of data. Julia Boeck: acquisition, analysis, and interpretation of data. Felix J. Bode, MD: acquisition, analysis, and interpretation of data. Matthias Klein, MD: acquisition, analysis, and interpretation of data. Franziska Dorn, MD: acquisition, analysis, and interpretation of data. Benno Gesierich, PhD: acquisition, analysis, and interpretation of data. Lars Kellert, MD: acquisition, analysis, and interpretation of data. Birgit Ertl-Wagner, MD: acquisition, analysis, and interpretation of data. Michael W. Goertler, MD: acquisition, analysis, and interpretation of data. Gabor C. Petzold, MD: acquisition, analysis, and interpretation of data. Jens Kuhle, MD: acquisition, analysis, and interpretation of data. Frank A. Wollenweber, MD: conception and design of the study, acquisition, analysis, and interpretation of data. Nils Peters, MD: conception and design of the study, acquisition, analysis, and interpretation of data. Martin Dichgans, MD: conception and design of the study, drafting a significant portion of the manuscript or figures.

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Disclosure

The authors report no disclosures relevant to the manuscript. Go to Neurology.org/N for full disclosures.

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