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Total number of authors:
14

Published in:
Neurology: Neuroimmunology and NeuroInflammation

Link to article, DOI:
10.1212/NXI.0000000000200335

Publication date:
2025

Document Version
Publisher's PDF, also known as Version of record

RESEARCH ARTICLE OPEN ACCESS

Temporal Dynamics of Plasma Neurofilament Light in Blood Donors With Preclinical Multiple Sclerosis

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Neurol Neuroimmunol Neuroinflamm 2025;12:e200335. doi:10.1212/NXI.0000000000200335

Abstract

Background and Objectives

Multiple sclerosis (MS) is a CNS disease, characterized by demyelination, inflammation, and neurodegeneration. Recent advances in technology allow measurement of the axonal damage marker neurofilament light chain in peripheral blood. Two studies have shown that patients with MS have elevated neurofilament light levels before their first symptom, but longitudinal studies are lacking. We aimed to investigate the intraindividual neurofilament light dynamics during the presymptomatic phase of MS.

Methods

The Danish Blood Donor Study (DBDS) has stored plasma samples from blood donors for more than 10 years. We identified DBDS participants, who had subsequently been diagnosed with MS, and included all samples donated before their first demyelinating symptom (median 5.00 samples per case). As controls, we included 2 healthy donors per case. Plasma levels of neurofilament light were measured and compared with quality-of-life data. We used a Bayesian approach to derive estimates for the percentage of cases with presymptomatic increased neurofilament light levels.

Results

We observed that 12 (17%, 95% CI 9%–28%) of 69 presymptomatic MS donors had intermittently increased neurofilament light levels preclinically. Increased levels were present up to 9 years before clinical onset, also in primary progressive MS. Healthy donors and presymptomatic MS donors with and without increased neurofilament light levels reported equally high physical and mental well-being. Model-based estimates suggested that 55% of cases (95% credible interval [28%–87%]) had experienced increased presymptomatic neurofilament light levels.

Discussion

Patients with MS periodically sustain axonal injury up to 9 years before clinical onset, even in primary progressive disease. This most likely represents asymptomatic disease activity. Some or even all patients are affected by this intermittent axonal injury, prompting the need for further studies of the presymptomatic phase in relation to prognosis and as a therapeutic window of opportunity.

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Go to Neurology.org/NN for full disclosures. Funding information is provided at the end of the article.

The Article Processing Charge was funded by the authors.

Glossary

ARMSS = age-related multiple sclerosis severity score; **BMI** = body mass index; **CIS** = clinically isolated syndrome; **DBDS** = Danish Blood Donor Study; **DMSR** = Danish Multiple Sclerosis Registry; **IQR** = interquartile range; **MS** = multiple sclerosis; **NfL** = neurofilament light chain; **NPR** = National Patient Registry; **RIS** = radiologically isolated syndrome.

Introduction

The exact mechanism and time of onset in multiple sclerosis (MS) remain unclear. Most patients are diagnosed with MS when they experience their first or second demyelinating symptom, but MS disease activity most likely commences before this.[1] Indeed, most patients with MS have more than 1 white matter lesion on the MRI performed after their first demyelinating symptoms,[2] indicating that some inflammatory activity takes place before clinical onset. Furthermore, individuals who have white matter lesions on brain MRI, but no previous symptoms of demyelinating disease (radiologically isolated syndrome [RIS]), have a 51% risk of developing MS.[3] Studies have shown an increased prevalence of fatigue,[4] cognitive impairment,[5,6] depression,[7] and utilization of health care services[8] among individuals who later develop MS, symptoms collectively described as a prodromal phase.[9] These data support the notion that subtle MS-induced changes take place before the first demyelinating event. MS disease activity can be monitored using neurofilament light chain (NfL), a component of the neuroaxonal skeleton. When axons are damaged, NfL is released to the CSF and the blood stream. Several studies have shown a robust association between MS disease activity and NfL levels in CSF[10] and blood.[11] This makes NfL a good biomarker of neuroaxonal injury. Furthermore, NfL dynamics reflect bouts of disease activity, as serum NfL levels are increased 0–90 days before and 0–90 days after the development of gadolinium-enhancing lesions on MRI brain scans.[12]

In healthy individuals, serum NfL increases by approximately 3%[13,14] with every year of age. The body mass index (BMI) may also affect NfL levels.[15,16] NfL seems to show minor day-to-day intrapersonal variation, but some degree of interindividual variation.[17] In patients with MS, a doubling of individual serum NfL levels has been associated with an increased risk of a subsequent MS relapse.[18]

Two studies have investigated serum NfL levels preclinically in MS.[19,20] Both found that serum NfL levels were increased in patients with MS compared with healthy controls, up to 6 and 10 years before first symptom, respectively. Furthermore, one of them found that an intrapersonal increase in serum NfL was associated with an increased risk of MS, based on 2 samples per person.[19] This is consistent with a study that showed that high CSF NfL levels in individuals with RIS were associated with a shorter time to MS development.[21] Taken together, these studies indicate that MS pathology starts up to 10 years before the first demyelinating symptom and that the disease process could therefore already be advanced at the time of diagnosis. However, they do not clarify whether presymptomatic NfL levels fluctuate as a reflection of intermittent, clinically silent disease activity or if they display a stable, modest elevation. Thus, longitudinal studies of NfL dynamics in presymptomatic MS are needed.

In this study, we aim to investigate the intraindividual NfL dynamics during the presymptomatic phase of MS.

Methods

The Danish Blood Donor Study

The Danish Blood Donor Study (DBDS) is a longitudinal, prospective, and ongoing national multicenter public health study and biobank. At the time of this study, more than 100,000 donors (aged 18–67 year old) had been enrolled in the study since 2010, donating plasma samples in connection with every blood donation. The DBDS cohort has been described in detail elsewhere.[22] Blood donors represent a highly selected group, who are generally healthier than the average population.[23] However, despite being initially healthy, many blood donors eventually develop diseases over time, including MS.

On entry into the DBDS and during the study, the blood donors fill out questionnaires about lifestyle and health. The DBDS questionnaire contains the validated Short Form-12 (SF12) questionnaire,[24] which determines a score for physical and mental health based on 12 questions.

Administrative Registry Data

The Danish Multiple Sclerosis Registry (DMSR) is a nationwide population-based patient registry that has collected detailed information about all patients with MS or related disorders in Denmark since 1948.[25] From the DMSR, we gathered information on date of birth, sex, age at MS clinical onset, current clinical phenotype, Expanded Disability Status Scale scores, and MRIs.

The Danish National Patient Registry (NPR) includes every diagnosis requiring hospitalization of every individual in Denmark since 1977.[26] Furthermore, every individual in Denmark is identified by the same personal identification number in these registries, which enables linking data from all national registries. We used the NPR data on MS and other demyelinating diagnoses to exclude demyelinating disease among the healthy donors. We also used the NPR data post hoc to investigate other potential sources of raised NfL levels in individuals with identified increases in NfL levels, among both the healthy donors and individuals with MS (including all types of neurologic conditions,

head trauma, neuropathies as part of other diagnoses, and diseases in the optic nerve and visual system [ICD10 DG00-DG99, DH46-48, DS04, DS06, DS07, DS09, DM472B, DM494, DE851A, DM146, DH940, DH933A, DT983DD]).

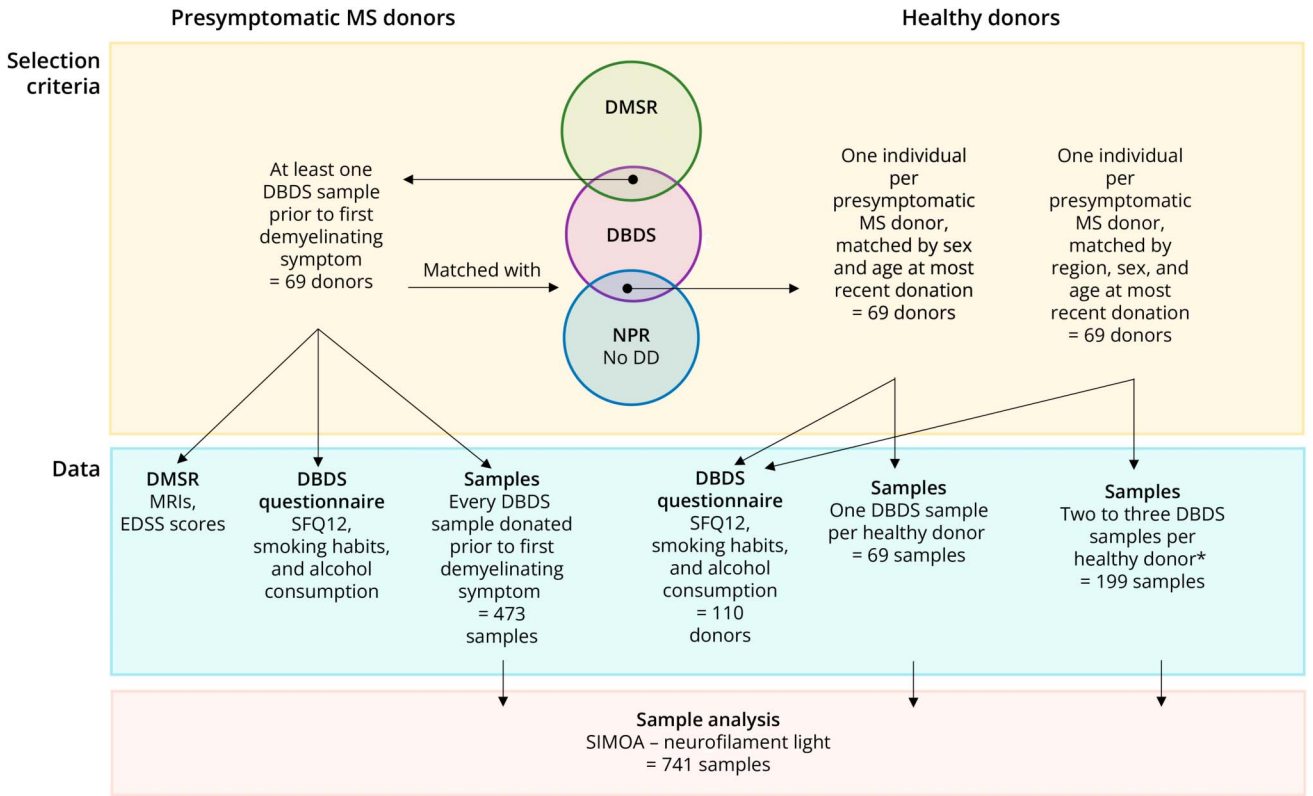
Study Design

We defined cases as DBDS participants who had developed MS or clinically isolated syndrome (CIS) after donating at least 1 blood sample to the DBDS. To identify these case-patients, we merged data from The Danish DMSR and the DBDS registry (Figure 1). We included every available plasma sample from blood donations before the first demyelinating symptom, as determined in the DMSR, in the study. Subsequently, we merged the DBDS, including only donors who were not present in the DMSR, with the NPR to identify healthy donors, who had not developed MS or any other demyelinating diseases, based on both registries. From this group, we identified 2 cohorts of healthy donors. In the first cohort, we matched each MS and CIS case with 1 DBDS donor of the same sex, region, and age at the time of the most recent donation and included 1 biobank sample from each healthy donor. For the second cohort, we matched each MS and CIS case with 1 DBDS donor of the same sex and included 2 to 3 biobank samples from each healthy donor—one matched to the age of the cases at the time of the most recent donation and the other 2 approximately 3 months and 1 year apart, respectively. We extracted information about alcohol consumption, tobacco use, height, weight, and the SF12 from the DBDS questionnaire for all donors. We adhered to the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) statement case-control checklist for this study.

Sample Processing and Laboratory Assessment of Neurofilament Light

All DBDS blood samples were collected in 2 mL plasma EDTA tubes with a gel separator (K2EDTA/gel), which were centrifuged and then frozen at −20°C within 6–12 hours. For this study, all included samples were thawed at room temperature for maximum 90 minutes and separated into 150 µL vials, using the Hamilton Star liquid handling system. The aliquots were stored at −80°C until analysis. NfL concentrations were analyzed in duplicate with the SiMoA NF-light Advantage Kit (cat no. 103186) using a SiMoA SR-X system (Quanterix, MA). Samples with a coefficient of variation above 20% between duplicates were reanalyzed. Each plate contained a mix of presymptomatic MS donors and healthy donors. The laboratory personnel who performed the

Figure 1 Study Design



*One sample matched to the age of the cases at the time of the most recent donation, the other 2 approximately 3 months and 1 year apart, respectively.
DBDS = The Danish Blood Donor Study; DMSR = The Danish Multiple Sclerosis Registry; MS = Multiple sclerosis; NPR = The Danish National Patient Registry; NPR No DD = Selection of individuals in NPR without demyelinating diagnoses; SFQ12 = The Short Form-12 Questionnaire; The MS Registry = The Danish Multiple Sclerosis Registry.

analyses was blinded to the clinical status of the samples. Interassay and intra-assay mean coefficient of variation was 7.4% and 6.8%, respectively.

Statistical Analysis

NfL values were log-transformed using the natural logarithm to approximate normality and to stabilize the variance.

We fitted a multivariable linear regression model to the healthy donor data (one sample per healthy donor) with log(NfL) as the outcome variable, and age, sex, and storage time as predictor variables. These variables were chosen based on previously shown associations.[14,15] BMI was not included as a variable because the available BMI data were incomplete. We determined the upper 97.5% NfL reference level of the healthy donors by adding 2 residual standard deviations to the predictions from the linear model. The resulting upper limit of prediction was then transformed back to the original NfL scale. This model was used to predict the 97.5 percentile values expected in healthy donors for each case sample. For each presymptomatic donor sample, we calculated an NfL ratio by dividing the actual NfL measurement by the predicted upper 97.5% value for a healthy donor with the same covariates (age, sex, and storage time).[27] To be conservative, every sample with a ratio more than 10% higher than the predicted 97.5% level was defined as increased. The same procedure was followed when examining for increased NfL levels in the 130 of 199 consecutive samples from healthy donors, which were not used to generate the reference interval.

The presymptomatic MS donors were divided into 2 groups, based on whether they had increased NfL samples, according to this criterion. The number of samples per person per year of observation was calculated by dividing the number of samples by time from first to last sample for each individual. For those with only 1 sample, time was set to 3 months, because this is the recommended interval between blood donations. The number of samples per person per year of observation, age at onset, and the global age-related MS severity score (ARMSS)[28] were compared using the Wilcoxon rank-sum test. The end of follow-up was defined as the most recent visit recorded in the DMSR.

The DMSR MRI data were incomplete. Therefore, for each presymptomatic MS donor, we included the earliest MRI scan performed <2 years of onset, with information about the number of cerebral T2 lesions. We dichotomized the MRI scan results (>/≤10 lesions). The intraindividual geometric mean of NfL was used when calculating interindividual medians of values based on more than 1 sample per person.

Kaplan-Meier analysis was used to determine the median time from donation of the first sample to clinical onset, time from clinical onset to the first MRI scan with T2 lesion information, and the years of follow-up after clinical onset. The end of follow-up was defined as the most recent visit recorded in the DMSR data. The log-rank test was used to compare differences between 2 groups. A Cox regression model was used to investigate the difference in time from the MS diagnosis to the first relapse among the group with and the group without increased NfL levels.

The Fishers exact test was used to compare 2 groups of categorical values. The Wilcoxon rank-sum test was used to compare 2 groups of continuous data with a nonnormal distribution.

When comparing 3 groups, Welch analysis of variance (ANOVA) was used for continuous data with a normal distribution but unequal variances. The Kruskal-Wallis test was used if the variances were unequal. Differences between groups of categorical values were tested through the Pearson χ^2 test.

We used the means and standard deviations derived from a study by Piehl et al.,[29] a power level of 90% and an α level of 0.05 to estimate the required minimum sample size, which was 41 cases.

p Values < 0.05 were considered statistically significant. We additionally analyzed the data using Bayesian statistical modeling. Bayesian methods are well suited for addressing challenges such as varying sampling frequencies between donors and also the hierarchical data structure (There are multiple measurements from some individuals, and these data points are therefore not independent.) The Bayesian approach further allowed us to compute posterior probabilities for questions of interest.

The Bayesian analysis methods are described in detail in the eMethods section. In brief, we used 2 different approaches to analyze the data under the Bayesian paradigm: first, similarly to the frequentist approach, we fitted a regression model to only data from the healthy controls and then used the fitted model to assess whether case-samples had higher NfL levels than expected. Second, we fitted a model that also included the case data, which we could use to directly estimate the fraction of cases with presymptomatic peaks, given the interpatient variation in sampling frequency, and also the probability that any given sample corresponded to a peak.

R (version 4.0.0)[30] was used to perform all statistical analysis and plots.

Standard Protocol Approvals, Registrations, and Patient Consents

The research protocol was approved by the Capital Region Ethical Committee (H-18053295). The DBDS was approved by the Ethical Committees (H-22021178; 1700407; SJ-740) and Danish Data Protection Agency (P-2019-99). Written informed consent was obtained in accordance with the Declaration of Helsinki from all participants at enrollment into the DBDS.

Data Availability

The data that support the findings of this study are available on reasonable request from the corresponding author. The

data are not publicly available due to restrictions that they contain information that could compromise the privacy of research participants.

Results

Presymptomatic MS Cohort

Of the approximately 100,000 blood donors in the DBDS at the time of the study, we identified 64 donors who had developed MS and 5 who had developed CIS, all of whom had donated at least 1 sample before experiencing their first demyelinating symptom (Figure 1). The median age of the donors was 36.4 year old (interquartile range [IQR] 28.6–44.1) at their most recent donation, and 71% were female. Collectively, they donated 473 samples in this period, corresponding to a sampling frequency of 2.0 samples per observed year per person (IQR = 1.5–2.6, range = 0.4–6.0). The presymptomatic MS donors and the healthy donors were comparable regarding sex, age, and BMI (Table 1).

Neurofilament Light Levels in Healthy Donors

We included 138 healthy donors with no record of demyelinating diseases in the study. The multivariable regression analysis revealed a highly significant, positive linear association between log(NfL) and age (p = 0.0001), indicating that NfL increased by 1.4% (95% CI 1.0%–2.1%) with each additional year of age, whereas it showed a weak, inverse association with storage time (p = 0.02), suggesting a 0.3% (95% CI 0.1%–0.5%) decrease with each additional year of storage; additionally, NfL was 17.0% (95% CI 0.0%–37.9%) higher in male than in female patients, although the association between NfL and sex was not statistically significant (p = 0.054). As such, the 97.5% prediction limit was NfL = $12.4 * 1.014^{age} * 0.997^{storage\ time} * 1.17^{sex}$. A model including only donors with BMI data (n = 86) showed no statistically significant association between log(NfL) and BMI (data not shown).

To study the intraindividual NfL variation among the healthy donors, we examined the 130 of the 199 consecutive samples from healthy donors that were not used to generate the reference interval (sample overview in Figure 1). Only 1 of these 130 samples from healthy donors displayed an increased NfL level (Figure 2). The NPR did not contain any diagnoses which could explain this NfL level.

Neurofilament Light Levels Are Periodically Increased in Presymptomatic MS

Based on the regression model, there were 22 samples with increased NfL levels (Figure 3A), which were donated by 12 (17%) different presymptomatic MS donors. The median NfL level of the samples with increased levels was 24.1 ng/L (IQR 20.1–29.6 ng/L, range 14.2–40.2), and the donors were 35.4 year old (median, IQR 31.2–50.2 year old) when they donated the samples.

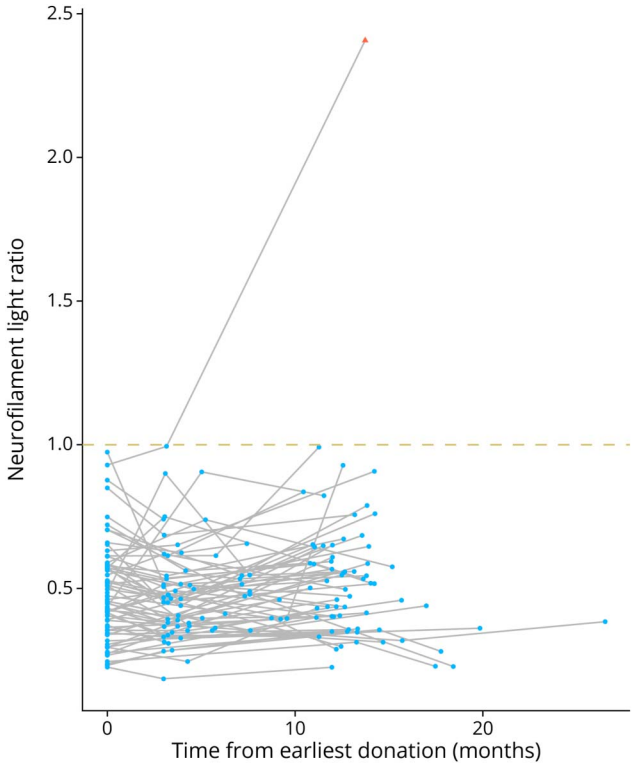
Most of the presymptomatic MS donors exhibited low baseline levels of NfL, which were comparable with those of the healthy donors, interspersed with intermittently increased NfL levels (NfL peaks) (Figure 3B). Some donors, such as donor B and F, had several samples with increased NfL levels, whereas others, such as donors A and K (Figure 3B), only had 1 or 2 samples with increased NfL levels during the observation period. For 2 donors (K and L), the NfL levels seemed to rise in the time leading up to onset. We detected increased NfL levels up to 9 years before the initial demyelinating symptom. Our cohort included 4 donors who had been diagnosed with primary progressive MS. Of interest, 2 of the donors who had developed primary progressive MS (Figure 3B, no. 56 and no. 65), had samples with NfL peaks preclinically. None of the donors with CIS had preclinically increased NfL levels. A post hoc analysis of NPR data in the period before MS onset in donors with NfL peaks did not contain any alternative diagnoses which could explain the increased NfL levels.

Table 1 Demographic Characteristics of the Study Population

	Healthy donors (n = 138)	Presymptomatic multiple sclerosis donors (n = 69)	Group comparison
No. of samples per person (median [IQR])	1.0 (1.0–1.0)	5.0 (3.0–10.0)	$p < 2.2 \times 10^{-16}$,[b]
Sex, n (%)			
Female	98 (71)	49 (71)	$p = 1.0$[c]
Male	40 (29)	20 (29)	
Age at donation (median [IQR], y)	36.7 (29.1–44.5)	36.4 (28.6–44.1)	$p = 0.8$[b]
BMI (median [IQR])[a]	24.9 (22.6–26.8)	25.1 (22.9–27.1)	$p = 0.5$[b]
Storage time (median [IQR], y)	7.7 (6.6–8.6)	9.4 (7.7–10.6)	$p = 1.3 \times 10^{-6}$[b]
Geometric mean NfL (median [IQR], ng/L)	7.1 (5.6–10.3)	7.8 (6.4–10.4)	$p = 0.2$[b]

Abbreviations: BMI = body mass index; IQR = interquartile range; NfL = neurofilament light chain.
[a] n = 63 for the presymptomatic multiple sclerosis donors, n = 129 for the healthy donors.
[b] Wilcoxon rank-sum test.
[c] Fisher exact test.

Figure 2 Neurofilament Light Levels Dynamics in Consecutive Healthy Donor samples



Neurofilament light ratio in relation to No. of months since earliest donation in healthy donors. The ratio of actual neurofilament light values to the predicted 97.5% upper reference limit is displayed on the y-axis, and the upper reference limit is denoted by the dashed line. Red triangle: increased neurofilament light level. Blue circle: neurofilament light level within the normal range.

The Bayesian analysis corroborated these findings (eFigure 1A and 1B, eMethods), showing a much higher proportion of extreme NfL values in the presymptomatic MS donor group than expected based on the covariates (age and storage time).

Clinical Characteristics of Presymptomatic MS Donors

The clinical characteristics of the presymptomatic MS donors with and without observed increased NfL levels are summarized in Table 2. The number of samples per year of observation was comparable between the presymptomatic donors with and without NfL peaks (Table 2, $p = 0.4$). Furthermore, these groups were comparable about the number of years from the first sample to the first demyelinating symptom ($p = 0.3$).

All presymptomatic donors with NfL peaks and an available MRI with T2 lesion data had at least 1 T2 cerebral or spinal cord T2 lesion on the first MRI performed within 2 years after onset. The majority (85.7%) of the presymptomatic MS donors who had NfL peaks had 10 or more white matter lesions on their first MRI after onset.

There was no statistically significant difference in the time to the first clinical relapse after the MS diagnosis (hazard ratio = 0.81 for donors with presymptomatic increased NfL levels, 95% CI 0.24–2.72, $p = 0.73$) and the postonset global ARMSS ($p = 0.3$, Table 2).

Axonal Injury Is Preclinical and Asymptomatic

The DBDS questionnaire was filled out by 82% of the donors during the observation time. On average, the donors were 35 years (IQR 28–43) old when they answered the questionnaire. Eight of the donors with NfL peaks had already experienced an NfL peak when they filled out the questionnaire, and 2 of these experienced NfL peaks within 6 months before (n = 1) or after (n = 1) filling out the questionnaire.

The average physical and mental composite SF12 scores of the presymptomatic MS donors with NfL peaks were similar to the average scores of both the presymptomatic MS donors without NfL peaks, and the healthy donors (Table 3). Furthermore, the presymptomatic MS donors with and without NfL peaks and the healthy donors were comparable about lifestyle factors, such as BMI, alcohol consumption, and smoking habits (Table 3).

Estimating the Frequency of Presymptomatic Disease Activity

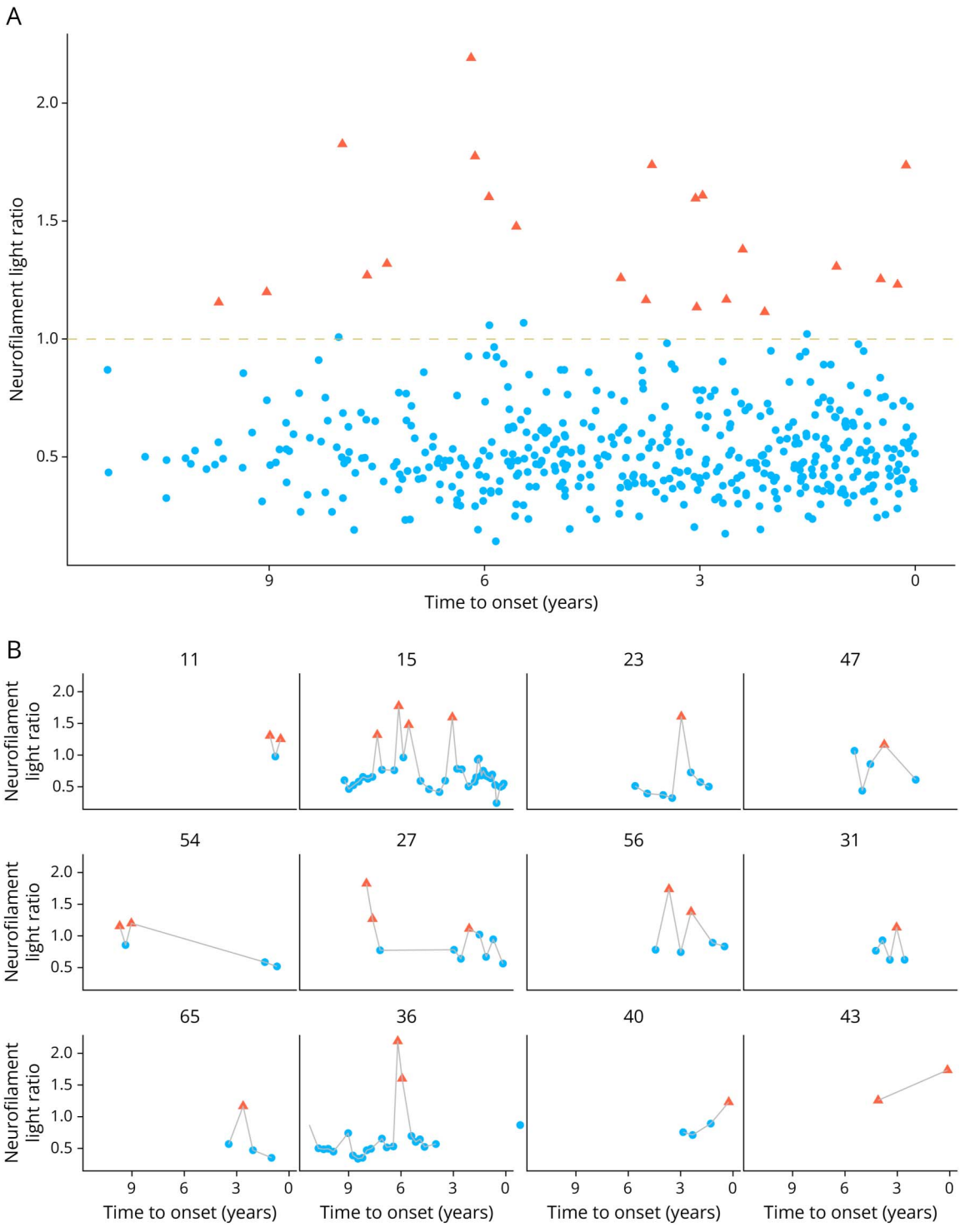
Using a Bayesian approach accounting for the sampling frequency variation between donors and for the hierarchical structure of the data, we estimated that 55% (posterior mean; 95% credible interval [28%–83%]) of presymptomatic MS donors had experienced increased NfL levels before their first clinical symptom, although not observed in our measurements. We are 99.98% certain that this proportion is at least 10% of presymptomatic donors. The estimated proportion of the samples donated by these individuals, corresponding to peak levels, was 19% (posterior mean; 95% credible interval [8%–31%]). A model which assumed that all the donors experienced increased NfL levels also fitted the data well, suggesting that based on these data, we cannot exclude the possibility that all patients with MS experience presymptomatic NfL peaks.

For each case, we furthermore computed the probability that any individual case belonged to the class experiencing presymptomatic NfL peaks, as well as the probability that any individual measurement was such a peak. eFigure 2 shows the NfL measurement time series for all cases, with likely peaks highlighted in color (shades indicating certainty of a peak). For each case, the certainty of this subject belonging to the peak class is furthermore indicated. Note that participants with very few, nonpeak measurements displayed a certainty close to 50%, in contrast to participants with a large number of nonpeak measurements displaying a certainty below 40% (eFigure 2).

Discussion

In this study, we have for the first time shown how NfL levels fluctuate within an individual in the years preceding the first clinical, demyelinating symptom. Our data strongly indicate

Figure 3 Neurofilament Light Levels in Preclinical Multiple Sclerosis Donor Samples



(A) Neurofilament light ratio in relation to number of years to the first demyelinating symptom in all samples from presymptomatic MS donors. The ratio of actual neurofilament light values to the predicted 97.5% upper reference limit is displayed on the y-axis. The upper reference limit is denoted by the blue dashed line. Samples with levels more than 10% above the upper reference limit are highlighted in red; (B) neurofilament light ratio in relation to years to the first demyelinating symptom in presymptomatic MS donors with increased neurofilament light levels. Red: increased neurofilament light level. Blue: neurofilament light level within the normal range.

that a substantial proportion of presymptomatic MS donors have intermittently increased NfL levels. Of interest, a recent study of patients with MS found that intraindividual increases in serum NfL were predictive of future clinical or MRI-based disease activity.[16] Furthermore, a study of patients with MS found that that serum NfL levels were higher than remission

Table 2 Comparison of the Multiple Sclerosis Characteristics of the Presymptomatic Multiple Sclerosis Cases With and Without Increased Neurofilament Light Levels

	Presymptomatic multiple sclerosis donors		
	With increased NfL levels (n = 12)	Without observed increased NfL levels (n = 57)	Group comparison
No. of samples per y of observation (median [IQR])	1.6 (1.4–2.3)	2.0 (1.4–3.0)	$p = 0.4$[b]
Time (y) from first sample to first symptom (median [25%–75% quantile])	5.0 (3.8–8.6)	5.6 (2.2–7.2)	$p = 0.3$[c]
Age at onset (median [IQR], y)	38.6 (32.2–50.5)	38.1 (30.1–45.0)	$p = 0.8$[b]
No. of participants with an MRI with T2 cerebral lesion data within 2 years of onset, n (%)			
Yes	58	42	$p = 0.091$[d]
No	58	42	
No. of T2 brain lesions on first MRI, n (%)			
0–9	14.3	57.6	
10+	85.7	42.4	
Time (mo) from clinical onset to MRI (median [25%–75% quantile], [range])	9.4 (1.2–11.0) (0.1–22.9)	4.1 (1.3–10.0) (0.5–21.3)	$p = 0.5$[c]
Global ARMSS score (median [IQR])[a]	2.1 (2.6)	2.4 (2.7)	$p = 0.3$[b]
Follow-up time (y) from diagnosis (median [25%–75% quantile])	5.3 (4.0–6.9)	6.2 (4.3–7.9)	$p = 0.2$[c]

Abbreviations: ARMSS = age-related multiple sclerosis severity; IQR = interquartile range; NfL = neurofilament light chain.
[a] Range: 0.17–7.06.
[b] Wilcoxon rank-sum.
[c] Log-rank test.
[d] Fisher exact test.

levels 0–90 days before and after the formation of a gadolinium-enhancing lesion.[12] The presymptomatic MS donors who experienced NfL peaks all had T2 brain lesions on MRI scans performed within the first 2 years after the first demyelinating symptom and the majority had >10 lesions. This supports the notion that these NfL peaks represent presymptomatic MS disease activity with axonal injury, in a similar manner to patients with symptomatic MS.

Two studies detected higher NfL levels in presymptomatic MS cases compared with healthy controls up to 6 and 10 years, respectively, before MS onset.[19,20] Similarly, we found that NfL peaks occurred up to 9 years before the first demyelinating symptom. However, our results emphasize the complexity of NfL dynamics and highlight that sequential measurements are needed to adequately identify individuals with preclinical MS activity at risk of developing symptomatic MS. In accordance with this, a very recent study detected a common signature of autoantibody proteins preclinically in a subset of patients with MS, highlighting this phase of the disease as crucial, prompting further immunologic studies.[31] The identified patient subset also displayed higher levels of peripheral blood NfL preclinically, based on 1 preclinical sample per patient. We have shown that individual NfL levels vary greatly preclinically, just as they do after onset, suggesting that caution is needed when interpreting the significance of singular peripheral blood NfL measurements.

We found that, despite detectable NfL peaks, these donors reported similar mental and physical well-being as donors without NfL peaks and healthy donors. This may seem to be in contrast to reports of prodromal symptoms in the preclinical MS population.[1] However, we suspect that part of the reason for this apparent disparity lies in the selective nature of blood donation; to be permitted to donate, the donors have to feel healthy. This screening may have removed preclinical MS donors who did experience prodromal symptoms. As such, our study design is not suited to investigate prodromal symptoms in patients with preclinical MS. However, it is well suited to study whether it is possible for preclinical MS donors to have raised NfL levels without notable symptoms. Our results suggest that some patients with MS may have clinically silent presymptomatic axonal injury. Thus, the therapeutic window of opportunity could be much earlier than the time of treatment initiation today.

We detected NfL peaks in 2 donors who developed primary progressive MS. This corroborates that some patients with primary progressive MS may have episodic disease activity in the early stages of the disease.[32] This could provide interesting perspectives regarding early identification and treatment of this MS subgroup with an overall poor prognosis. However, the number of observations was limited, and this finding needs to be corroborated in other populations.

Table 3 Danish Blood Donors Study Questionnaire Data for Healthy Donors and Presymptomatic Multiple Sclerosis Donors With and Without Increased Neurofilament Light Levels

		Presymptomatic multiple sclerosis donors		
	Healthy donors (n = 110)	With increased NfL levels (n = 10)	Without observed increased NfL levels (n = 49)	Group comparison
Respondent age (median [IQR])	35.0 (27.8–43.1)	35.0 (29.4–48.6)	35.3 (28.0–42.9)	$p = 0.9$[g]
Sex, n (%)				
Female	76 (69)	5 (50)	37 (76)	$p = 0.3$[h]
Male	34 (31)	5 (50)	12 (24)	
BMI (median [IQR])[a]	25.0 (22.5–26.7)	24.9 (23.3–26.3)	25.3 (22.9–27.8)	$p = 0.7$[g]
Physical component summary (median (IQR)[b]	55.5 (53.5–56.7)	56.0 (51.0–58.6)	55.4 (53.0–55.9)	$p = 0.7$[g]
Mental component summary (median [IQR])[c]	55.9 (51.5–57.8)	55.1 (51.0–58.6)	54.9 (53.0–55.9)	$p = 0.5$[g]
Current smoking status, (n (%)[d]				
Smoker incl. occasional	14 (13)	3 (30)	6 (12)	$p = 0.3$[h]
Nonsmoker	95 (86)	7 (70)	41 (84)	
No. of years of smoking (median [IQR])[e]	10.0 (6.0–19.0)	10.0 (4.8–18.3)	12.0 (9.0–20.0)	$p = 0.8$[g]
No. of alcohol units per wk[f] **(median [IQR])**	7.0 (3.0–12.0)	6.5 (4.5–8.0)	4.0 (2.0–7.0)	$p = 0.09$[i]

Abbreviations: BMI = body mass index; IQR = interquartile range; NfL = neurofilament light chain.
[a] BMI data were missing for 1 multiple sclerosis donor without observed increased NfL levels.
[b] Physical component summary data were missing for 4 healthy donors and 4 multiple sclerosis donors without observed increased NfL levels.
[c] Mental component summary data were missing for 2 healthy donors and 4 multiple sclerosis donors without observed increased NfL levels.
[d] Current smoking status data were missing for 1 healthy donor and 2 multiple sclerosis donors without observed increased NfL levels.
[e] Smokers only.
[f] No. of alcohol units/week data were missing for 44 healthy donors, 20 multiple sclerosis donors without observed increased NfL levels and 6 multiple sclerosis donors with increased NfL levels.
[g] Kruskal-Wallis test.
[h] Pearson χ^2 test.
[i] Welch ANOVA.

In our study, we observed that 17% of presymptomatic MS donors had peaks. However, this may be explained by the variation in the number of samples we had access to for each presymptomatic MS donor.

We therefore applied a Bayesian approach, accounting for the variation in sampling frequency and for the hierarchical nature of the data, which allowed us to estimate that the most probable frequency of presymptomatic MS donors with peaks during the observation period was 55% (with a minimum of 10%); however, a model which assumed that all donors had peaks during the observation period also fitted well. Thus, although our data provide very strong evidence that at least some patients with MS show intermittent, preclinical disease activity with axonal damage, we cannot determine whether this pattern applies to all patients with MS or if it reflects only a subgroup. Further studies answering this question could contribute to the discussion on whether different mechanisms of disease onset exist and influence the course of disease.

We did not detect any statistically significant difference in the postonset clinical course between the group with and without peaks. However, we had a limited number of observations and were therefore likely underpowered to show any such differences between the groups. Furthermore, as the Bayesian analyses illustrated, it is a possibility that all patients with MS experience presymptomatic NfL peaks and that the observed variation in detected peaks among the presymptomatic MS donors accordingly reflects a continuum of degrees of axonal injury, rather than 2 distinct subgroups. This may hamper group comparisons.

Our study has a unique basis for reliably assessing presymptomatic NfL dynamics. This includes access to many consecutive, preclinical samples in healthy individuals, which enabled us to show intraindividual fluctuations in presymptomatic MS donors and build on the current knowledge about the presymptomatic phase of MS. The male-to-female ratio in our study population was representative of the real life clinical MS population. Furthermore, we were able to select our cases based on the DMSR, which encompasses data on all patients with MS in Denmark. As such, we had detailed, high-quality follow-up data of our MS population for on average 5.9 of years. Finally, we had unique questionnaire data on self-reported wellbeing, providing a rare opportunity to validate the clinically silent nature of the NfL elevations. The DBDS questionnaire data were collected at the same time for cases and controls, all before MS onset and often many years before the first symptom. As such, the data were less likely to be

biased by intermediary effects. We believe that our cases and noncases were comparable because they exhibited very similar lifestyles.

Our study was limited by the fact that the samples were gathered at random times, with great interindividual variation in frequency and timing, which made it more difficult to compare samples. Although this was accounted for in the Bayesian approach, we suspect that these factors may have contributed to underestimation of the frequency and magnitude of the NfL elevations.

To conclude, our study shows that some patients with MS have intermittent and asymptomatic axonal injury up to 9 years before the first demyelinating symptom. This included 2 donors with later primary progressive MS, suggesting that some patients with primary progressive MS may experience clinically silent, episodic disease activity with axonal injury before onset. Collectively, our results highlight the need for earlier diagnosis and therapeutic intervention.

Acknowledgment

The authors thank Ulla Abildtrup and Linda W. Christensen for excellent laboratory assistance.

Study Funding

This study has received funding from The Multiple Sclerosis Foundation, The Jascha Foundation, The Dagmar-Marshall Foundation, The Direktør Emil C. Hertz og Hustru Inger Hertz Foundation, The Torben og Alice Frimodts Foundation, and the Axel Muusfeldts Foundation.

Disclosure

The authors report no relevant disclosures. Go to Neurology.org/NN for full disclosures.

Publication History

Received by *Neurology: Neuroimmunology & Neuroinflammation* June 12, 2024. Accepted in final form September 20, 2024. Submitted and externally peer reviewed. The handling editor was Deputy Editor Scott S. Zamvil, MD, PhD, FAAN.

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Appendix *(continued)*

Name	Location	Contribution
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