

CSF neurofilament light levels predict hippocampal atrophy in cognitively healthy older adults

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Abbreviations: A β 42 – β -amyloid 1-42, AD – Alzheimer’s Disease, AIC – Akaike Information Criterion, APOE – Apolipoprotein E, GAMM – Generalized Additive Mixed Models, MCI – Mild Cognitive Impairment, MMSE – the Mini Mental Status Examination, MS – Multiple

Sclerosis, NFL – Neurofilament Light, P-tau – Phosphorylated Tau, SNAP – Suspected Non-Alzheimer Pathology, T-tau – Total Tau, WM-hypointensities – White Matter Hypointensities.

Keywords: cerebrospinal fluid; magnetic resonance imaging; normal aging; neurofilament light; hippocampal atrophy rate

ABSTRACT

Cerebrospinal fluid (CSF) neurofilament light (NFL) is a marker of axonal degeneration. We tested whether CSF NFL levels predict hippocampal atrophy rate in cognitively healthy older adults independently of the established CSF Alzheimer's disease (AD) biomarkers, β -amyloid 1-42 (A β 42) and phosphorylated tau (P-tau). We included 144 participants in a 2-year longitudinal study with baseline CSF measures and two magnetic resonance images. 88 participants had full data available. A subgroup of 36 participants with very low AD risk was also studied. NFL predicted hippocampal atrophy rate independently of age, A β 42 and P-tau. Including NFL, P-tau and age in the same model, higher NFL and lower P-tau predicted higher hippocampal atrophy ($R^2=.20$, NFL: $\beta=-.34$; $p=.003$, P-tau: $\beta=.27$; $p=.009$). The results were upheld in the participants with very low AD risk. NFL predicted neurodegeneration in older adults with very low AD probability. We suggest that factors previously shown to be important for brain degeneration in mild cognitive impairment may also impact changes in normal aging, demonstrating that NFL is likely to indicate AD-independent, age-expected neurodegeneration.

1. Introduction

Hippocampal atrophy rates are higher in patients with Alzheimer's disease (AD) than in cognitively healthy older adults (Barnes, et al., 2009). However, hippocampal atrophy is known to accelerate from midlife onwards also in persons with low AD-risk (Fjell, et al., 2013), and hippocampus is one of the brain areas with highest atrophy rate in aging (Fjell, et al., 2013), reported to be around 1% annually (Fjell, et al., 2013, Fraser, et al., 2015). Thus, identification of biomarkers predicting hippocampal atrophy is critical for understanding brain changes both in normal aging and early AD. Interestingly, a recent study showed that cerebrospinal fluid (CSF) neurofilament light subunit (NFL) levels predicted hippocampal atrophy in mild cognitive impairment (MCI) patients (Zetterberg, et al., 2016), indicating that CSF NFL could be a progression marker in AD.

Neurofilaments are important cytoskeletal components of neuronal axons, and CSF NFL levels are believed to reflect axonal degeneration (Petzold, 2005, Zetterberg, et al., 2006). CSF NFL levels are associated with age (Rosengren, et al., 1996, Skillback, et al., 2014, Vagberg, et al., 2015), white matter lesions (Sjogren, et al., 2001), AD (Olsson, et al., 2016, Petzold, et al., 2007, Skillback, et al., 2014, Zetterberg, et al., 2016), and other neurodegenerative diseases (Backstrom, et al., 2015, Petzold, et al., 2007, Skillback, et al., 2014, Steinacker, et al., 2016, Teunissen, et al., 2005). Previous studies have indicated a relationship between high CSF NFL and lower brain volume in frontotemporal lobe dementia (Scherling, et al., 2014), and nondemented older adults (Bjerke, et al., 2014, Vagberg, et al., 2015), but studies are mostly cross-sectional and results have not been consistent (Bendlin, et al., 2012, Khalil, et al., 2013). The

relationship between CSF NFL levels and hippocampal atrophy in cognitively healthy older adults has never been tested, but is critical for understanding whether NFL is a general or disease-specific atrophy marker. Thus, the objective of this study was to test whether CSF NFL levels predict hippocampal atrophy rate in cognitively healthy older adults independently of the established CSF AD biomarkers β -amyloid 1-42 (A β 42) and phosphorylated tau (P-tau) (Blennow, et al., 2010).

2. Methods

2.1 Participants

We recruited patients scheduled for elective gynecological (genital prolapse), urological (benign prostate hyperplasia, prostate cancer or bladder tumor/cancer) or orthopedic (knee or hip replacement) surgery in spinal anesthesia turning 65 years or older the year of inclusion. Dementia, previous stroke with sequela, Parkinson's disease and other neurodegenerative diseases likely to affect cognition were exclusion criteria. Participants were assessed with a multi-domain battery of cognitive tests before surgery, comprising the Mini Mental Status Examination (MMSE) (Folstein, et al., 1975), Clock Drawing Test (Shulman, 2000), Word List Memory Task (Morris, et al., 1989), Trail Making Test A and B (Reitan, 1955), Kendrick Object Learning Test (Kendrick, et al., 1979), and verbal fluency (FAS test and Animal Naming) (Spreen and Strauss, 1991), giving 11 test scores. Blood and CSF samples were collected by the anesthesiologist in conjunction with spinal anesthesia, and participants underwent magnetic resonance imaging (MRI) after surgery. The mean time between CSF sampling and MRI at baseline was 8 weeks (standard deviation [SD] [range]: 6 [-20 to 24]). Participants underwent a

second MRI and were tested with the same battery of cognitive tests at two-year follow-up (mean time between MRIs 2.2 years, SD [range]: 0.3, [1.6 to 2.9]) (see Table 1).

Table 1. Demographics, CSF biomarkers and hippocampal measures.

| | All participants (Sample A, n=144) | Participants with MRI at both time points and CSF NFL analyses (Sample B, n=88) |
|---|---------------------------------------|---|
| Age at baseline, years | 73 (6), 64 to 91 | 73 (6), 64 to 89 |
| Sex, male | 68 (47) | 43 (49) |
| Education, years | 14 (4), 7 to 23 | 15 (3), 8 to 23 |
| Hypertension ^a | 61 (42) | 29 (33) |
| MMSE score, baseline | 29 (1.2), 25 to 30 | 29 (1.3), 25 to 30 |
| MMSE score, 2-year follow-up | 29 (1.4), 21 to 30 ^b | 29 (1.2), 24 to 30 |
| APOE genotype ^c | | |
| E3/E2 | 12 (9) | 4 (5) |
| E3/E3 | 68 (53) | 44 (53) |
| E4/E2 | 1 (1) | 1 (1) |
| E4/E3 | 44 (34) | 31 (37) |
| E4/E4 | 4 (3) | 3 (4) |
| CSF A β 42, pg/mL | 718 (208), 275 to 1179 ^d | 724 (203), 275 to 1175 |
| CSF P-tau, pg/mL | 60 (20), 25 to 115 ^d | 61 (19), 26 to 110 |
| CSF NFL, pg/mL | 1163 (507), 487 to 3123 ^e | 1141 (558), 510 to 3123 |
| A β 42+ (< 550 pg/mL) | 34 (26) ^c | 24 (27) |
| Months between MRIs | - | 26 (3), 19 to 35 |
| Hippocampal volume, baseline, mm ³ | - | 3505 (396), 2337 to 4544 |
| Hippocampal volume, 2-year follow up, mm ³ | - | 3464 (407), 2425 to 4514 |
| Hippocampal volume, % annual change | - | -.55 (1.08), -4.24 to 2.14 |

Legend: Values are n (%) and mean (SD), range. ^aBased on information from the participant and patient records, ^bn=115, ^cn=129 and n=83, respectively, ^dn=130, ^en=128. MMSE = Mini Mental Status Examination. APOE = Apolipoprotein E. CSF = cerebrospinal fluid. A β 42= β -amyloid 1-42. P-tau = phosphorylated tau. NFL = neurofilament light. MRI = magnetic resonance imaging.

We selected participants as shown in Figure e-1. CSF was available from baseline only, while the majority of the participants had two MRI scans. Only participants with CSF data and/or brain MRI(s) were included. We selected only cognitively healthy participants based on the following procedure: First, participants offered referral to cognitive assessment were excluded. Next, we included all participants with MMSE score ≥ 27 . Last, for participants with MMSE score < 27 , only those with none or one other abnormal test score(s) when last tested were included.

Abnormal score was defined as more than 1.5 SD below the mean normal value for age, sex, and educational level. 4 participants with CSF NFL levels > 4000 pg/mL (i.e. more than ± 3 SD from the mean value) were excluded. This resulted in 144 participants with CSF analyses or MRI at baseline (sample A) and 88 participants with CSF NFL analyses and MRI at both time points (sample B). After further screening of sample B, some participants with additional conditions (details Table e-1) were excluded, resulting in sample C. From sample B and C, we created subgroups with very low risk of AD by excluding participants in a hierarchical manner: 1) no apolipoprotein E (APOE) 4 alleles (sample D and H), 2) also A β 42 > 550 pg/mL (Mulder, et al., 2010) and stable or improved delayed recall score on Word List Memory Task at two-year follow-up compared to baseline (sample E and I), 3-1) also P-tau < 60 pg/mL (sample F and J), and 3-2) also A β 42 > 650 pg/mL (sample G and K). It is possible to effectively define low risk group based on APOE status only, but we created additional low risk groups to further reduce AD risk by including A β 42 and memory function as further criteria. Several cut-off values for CSF A β 42 levels are described in the literature, ranging from 500 to 650 pg/mL (Fagan, et al., 2009, Mulder, et al., 2010, Niemantsverdriet, et al., 2016, Zwan, et al., 2016). We used 550 pg/mL

as our cut-off, and in addition we increased the cut-off to 650 pg/mL for one subgroup to be more conservative.

2.2 Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki and approved by the Regional Committee for Ethics in Medical Research in Norway (REK 2011/2052). All participants provided written informed consent.

2.3 Magnetic resonance imaging acquisition and processing

T1-weighted MPRAGE 3D images were acquired with a 1.5 T Siemens Avanto scanner using a 12-channel head coil (TR=2400 ms, TE=3.79ms, Field of View=240mm, slice thickness=1.20mm, pixel size=1.25x1.25mm).

Images were processed with FreeSurfer (version 5.3) and its specific longitudinal stream (<https://surfer.nmr.mgh.harvard.edu>). For each MRI, the FreeSurfer pipeline performs a set of automated procedures for the cortical reconstruction and volumetric segmentation, documented elsewhere (Dale, et al., 1999, Fischl, et al., 2002). We used hippocampi volume measures and white matter hypointensities (WM-hypointensities) estimations obtained from the automated segmentation. More specifically, the FS-segmentation algorithm assigns labels to all the brain regions of each individual scan, based on an available probabilistic atlas obtained from a training set of subjects which has been accurately manually labeled (Fischl, et al., 2002). Both the

hippocampal volume and the WM-hypointensities are defined from this available atlas.

Hippocampal volume was not normalized by estimated intracranial volume, since the main analyses were done on rate of atrophy, where normalizations are not recommended. WM-hypointensities appear as dark white matter on the T1-weighted image, and are obtained from the overall sum of regions within the white matter with T1-intensity values within a certain range defined from the probabilistic atlas. This measure is related to WM lesions, but is considered less sensitive than WM hyperintensities based on T2 or FLAIR images. The Freesurfer longitudinal stream includes methods designed to minimize the bias to any time point in a participant and which lead to increased statistical power, better separation of groups based on atrophy, and higher reproducibility. These include the generation of a subject-specific intermediate template followed by a projection of each time point to this template (Jovicich, et al., 2013, Reuter, et al., 2012). For both the individual and longitudinal processing steps, reconstructed surfaces and volumes were visually inspected and manually corrected when necessary.

2.4 APOE genotyping

Blood samples were genotyped for *APOE* (gene map locus 19q13.2) using TaqMan Allelic Discrimination technology (Applied Biosystems, Carlsbad, CA, USA). Genotypes were obtained for the two SNPs that are used to unambiguously define the $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ alleles (rs7412 and rs429358).

2.5 CSF collection and analyses

CSF was collected in polypropylene tubes, centrifuged at room temperature for 10 minutes, the supernatant aliquoted into polypropylene tubes, and frozen at -80 °C pending analyses. Mean time from CSF sampling to freezing was 83 minutes (SD, [range]: 21, [30 to 127]). Samples were sent on dry ice to the Clinical Neurochemistry Laboratory at Sahlgrenska University Hospital, Mölndal, Sweden, for analyses. CSF A β 42, total tau (T-tau) and P-tau concentrations were determined using INNOTEST enzyme-linked immunosorbent assays (Fujirebio, Ghent, Belgium) and CSF NFL concentrations using a commercial ELISA (UmanDiagnostics, Umeå, Sweden). Analyses were performed by board-certified laboratory technicians masked to clinical data. Intra-assay coefficients of variation were 9-13% and the lower limit of detection for NFL was 50 pg/mL. The ELISA method for CSF A β 42 has been fully validated analytically (Vanderstichele, et al., 2000), and also validated against the Joint Committee for Traceability in Laboratory Medicine (JCTLM) approved mass spectrometry Reference Measurement Procedure (RMP) for CSF A β 42 (Leinenbach, et al., 2014), and show high consistency in results over time and between batches when adhering to strict laboratory analytical procedures (Palmqvist, et al., 2014). CSF T-tau and P-tau levels were strongly correlated ($r=.96$, $p<.001$), thus we only used CSF P-tau in the main statistical analyses.

2.6 Statistical analysis

We calculated hippocampal atrophy rate as the annual percent change in hippocampal volume (average of both hemispheres), normalized by the average volume across time points and divided

by years between scans. We also calculated the average WM-hypointensities volume across time points for use as a control variable.

We tested associations between CSF biomarkers, age and hippocampal atrophy rate using SPSS (version 22). Generalized Additive Mixed Models (GAMM) implemented in R (www.r-project.org) using the package “mgcv”(Wood, 2006) was used to derive the age-function for hippocampal atrophy and for the relationship between hippocampal change and CSF NFL levels, taking advantage of all longitudinal and cross-sectional observations, run through the PING data portal (<http://pingstudy.ucsd.edu/welcome.html>) (Bartsch, et al., 2014). Akaike Information Criterion (AIC) (Akaike, 1974) was used to guide model selection and help guard against over-fitting. For analyses including CSF biomarkers and not MRI measures, we used age at the day of CSF sampling. For analyses including MRI measures, we used the age at the day of baseline MRI. Significance was set at $P < .05$.

We tested correlations between CSF biomarkers, age and hippocampal atrophy rate using Pearson correlations, and hippocampal volume change using paired samples T-test. We performed multiple linear regression analyses to test associations between age, CSF biomarkers and hippocampal atrophy rate. Regressions were performed in several steps. The first model included NFL and age as predictors of hippocampal atrophy rate. Next, we tested the predictive power of A β 42 and P-tau levels separately in conjunction with NFL in the model. The resulting regression model was tested for stability by including sex and WM-hypointensities separately. All analyses were done in the main sample (sample B). The most important analyses were also

repeated in sample C (details Table e-2). We also tested the final regression model within the very low AD risk subgroups (samples D-K). Sensitivity analyses were performed with and without outliers (defined as studentized deleted residuals $> \pm 2$) for all regression models. Although we expected correlations between the explanatory variables, we chose not to use data reduction methods, such as principal component or cluster analysis, to be able to evaluate the contributions from each biomarker separately. Finally, we ran mediation analyses in sample B using the SPSS macro INDIRECT (Preacher and Hayes, 2008). Mediation is present if the relationship between the predictor variable and the dependent variable (c) attenuates when accounting for a third variable (the mediator) (c'). The % reduction was calculated as $(c-c')/c$. The significance of the indirect effect ($a*b$) was tested using bootstrapped confidence intervals (CI). Standardized coefficients were obtained using z-scores.

3. Results

3.1 CSF biomarkers, hippocampal volume and demographic factors

Demographics, and CSF biomarker and MRI characteristics are shown in Table 1 and Table e-3. NFL levels correlated positively with age ($r=.45$, $p<.001$), while P-tau ($r=.09$ $p=.41$) and A β 42 levels ($r=.05$, $p=.67$) did not correlate with age. NFL levels correlated positively with P-tau levels ($r=.23$, $p=.03$), but not with A β 42 levels ($r=.07$, $p=.55$).

3.2 CSF NFL levels and hippocampal atrophy rate

CSF NFL levels were negatively correlated with baseline hippocampal volume, averaged bilaterally, ($r=-.25$, $p=.02$). Hippocampal atrophy, measured as the difference in hippocampal volumes between baseline and follow-up was significantly different from zero (mean [SD], range: -40.49 mm^3 [74.71], -24.66 to -56.32 , $t=5.09$, $p<.001$), and the mean annual atrophy rate was $-.55\%$. Age correlated with higher atrophy rate ($r=-.26$, $p=.01$), indicating accelerated atrophy with increasing age. This relationship was confirmed with GAMM for the full sample, as illustrated in Figure 1. We ran a multiple regression analysis using NFL and age as predictors of hippocampal atrophy rate. Higher NFL levels predicted higher hippocampal atrophy rate ($p=.02$) (see Table 2). Age was not a significant predictor in this model. Next, $A\beta_{42}$ level was also introduced as a possible predictor, and did not predict hippocampal atrophy rate independently of NFL, while NFL was still significant (Table 2). The last step included P-tau as a predictor together with age and NFL, and we obtained a model with higher NFL levels and lower P-tau levels predicting higher hippocampal atrophy rate (Table 2) independently of age. Regression analyses results were unchanged when excluding 5-6 outliers per analysis. Substitution of P-tau with T-tau in this last step gave the same results (Table e-4), while only NFL was a significant predictor when P-tau was substituted with $A\beta_{42}/P\text{-tau}$ ratio (data not shown).

The relationship between NFL and hippocampal volume was also tested with GAMM to take advantage of all data points, obtaining an optimal fit based on both cross-sectional and longitudinal information. The sample was divided into NFL+ and NFL- by a median split, and the relationship between hippocampal volume and age was plotted in each group, with sex as a

covariate. AIC for the model was 2587 and NFL status yielded a highly significant contribution ($t = -2.96$, $p < .005$). Removing NFL increased AIC to 2595, indicating a worse fit. Adding P-tau as a covariate did not improve the model fit (AIC = 2588), and P-tau did not contribute significantly ($t = -0.18$, $p = 0.85$) while NFL still did ($t = -2.66$, $p < .01$). Thus, the initial model was preferred and plotted in Figure 2.

Table 2. Multiple linear regression with hippocampal atrophy rate as dependent variable (full sample).

| Independent variables | R ² | B | 95 % CI | β | P value |
|-----------------------|----------------|-------|------------------|---------|---------|
| Age | .13 | -.024 | -.064 to .016 | -.14 | .23 |
| NFL | | -.001 | -.001 to -.00001 | -.28 | .02 |
| Age | .16 | -.025 | -.064 to .015 | -.14 | .22 |
| NFL | | -.001 | -.001 to -.0001 | -.29 | .01 |
| A β 42 | | .001 | -.0002 to .002 | .16 | .11 |
| Age | .20 | -.023 | -.062 to .015 | -.13 | .23 |
| NFL | | -.001 | -.001 to -.0002 | -.34 | .003 |
| P-tau | | .016 | .004 to .027 | .27 | .009 |

Legend: Sample B (n=88). CI= confidence interval. A β 42= cerebrospinal fluid β -amyloid 1-42.

P-tau = cerebrospinal fluid phosphorylated tau. NFL = cerebrospinal fluid neurofilament light.

3.3 Adjusting for effect of white matter hypointensities and sex on hippocampal atrophy rate

Since vascular brain pathology may affect the relationship between NFL and hippocampal atrophy (Sjogren, et al., 2001), we entered WM-hypointensities into the regression model including age, NFL and P-tau levels as predictors of hippocampal atrophy rate. NFL and P-tau levels were still significant predictors of hippocampal atrophy rate, whereas WM-hypointensities were not predictive (data not shown). Results were unchanged after exclusion of 6 outliers. We

adjusted for sex in the same way as for WM-hypointensities, and NFL and P-tau levels were the only significant predictors of hippocampal atrophy rate (data not shown). Sex was not a significant predictor, however after exclusion of 6 outliers, sex was also a significant predictor (higher atrophy rates in males).

3.4 CSF NFL levels and hippocampal atrophy in low risk subgroups

The most important analyses were repeated in sample C (exclusions after further screening of sample B). NFL did not correlate with age in this sample ($r=-.18$, $p=.13$), however, when excluding 4 statistical outliers (studentized deleted residuals $> \pm 2$) the correlation was significant ($r=-.36$, $p=.002$). All other correlation results were unchanged from sample B, and hippocampal volume change was significantly different from zero. In linear regression analyses, NFL was the only significant predictor (borderline significant in analyses adjusted for sex) (Table e-5), however, after excluding 4-5 outliers per analysis, all results were unchanged from sample B, except that A β 42 was also a significant predictor of hippocampal atrophy rate (Table e-6). We further applied our final regression model including age, NFL, and P-tau levels as predictors of hippocampal atrophy rate in the very low AD risk subgroups from sample B (samples D-G). In the first subgroup, participants without APOE4 alleles, higher NFL levels and lower P-tau levels predicted higher hippocampal atrophy rate independent of age as in the full sample (Table e-7). The results were unchanged when also excluding A β 42 positive participants and those with declining memory function (Table e-7). Further, exclusion of participants with P-tau levels ≥ 60 pg/mL, increase of the A β 42 cutoff from 550 to 650 pg/mL, and also exclusion of

2-3 outliers per analysis did not alter the results (data not shown). Results were the same in the very low AD risk subgroups from sample C (samples H-K).

3.5 Mediation analyses

We tested the mediating (indirect) effect of NFL on the relationship between age and hippocampal atrophy rate (Fig. 3). NFL was a significant mediator, with confidence interval of -.24 to -.01, and accounted for 36 % of the age effect on hippocampal atrophy rate. In our model, the total effect of age on hippocampal atrophy was $\beta = -.23$ equal to the sum of the direct effect of age ($\beta = -.15$) and the indirect effect through the relationship with NFL ($\beta = -.08$).

[Insert Figure 3 around here]

4. Discussion

High CSF NFL levels predicted higher hippocampal atrophy rate in cognitively healthy older adults. While previous studies have demonstrated this in samples of high-risk participants, i.e. MCI patients (Zetterberg, et al., 2016), here we show that the relationship was replicated in a sample with very low AD-risk, and that NFL predicted hippocampal atrophy independently of the established AD CSF biomarkers A β 42 and P-tau. This suggests that CSF NFL may be an important marker of neurodegeneration both in normal aging and in age-related neurodegenerative diseases.

The only previous study assessing CSF NFL in relation to longitudinal volume change in older adults found that higher NFL levels were associated with deterioration in whole-brain, ventricular and hippocampal volume in MCI patients (Zetterberg, et al., 2016). However, cross-sectional studies in non-demented adults have been more inconsistent. One study found that CSF NFL correlated with ventricular size, but not with sulcal atrophy (Bjerke, et al., 2014), a second study found a correlation between brain parenchymal fraction and CSF NFL that did not survive adjustment for age (Vagberg, et al., 2015), while a third study found no relationship between baseline CSF NFL levels and gray matter volumes 3.5 years later (Bendlin, et al., 2012). In frontotemporal dementia, higher CSF NFL is associated with lower gray and white matter volumes, including in the temporal lobe (Scherling, et al., 2014), while findings in multiple sclerosis (MS) and related disorders are less straightforward (Eikelenboom, et al., 2003, Khalil, et al., 2013). Thus, previous literature on the association between CSF NFL and brain volumes is scarce and inconsistent, but the only longitudinal study in older adults is in line with our findings (Zetterberg, et al., 2016), showing that high CSF NFL predicts more hippocampal atrophy in MCI patients. The present study takes these results further by showing that the NFL-atrophy association is likely not caused by AD-specific mechanisms, but is important also in AD-independent, age-expected hippocampal decline.

Neurofilaments are abundant in neuronal axons where they are essential for axon radial growth (Petzold, 2005), but are also found in soma and dendrites of neurons (Trojanowski, et al., 1986). NFL is expressed in neurons in both the central and peripheral nervous system (Trojanowski, et

al., 1986), including hippocampus.(Vickers, et al., 1994) Thus, following neuronal damage, NFL is believed be released into the extracellular compartment resulting in increased CSF NFL levels (Petzold, 2005). Age is associated with increasing CSF NFL levels in several studies (Khalil, et al., 2013,Rosengren, et al., 1996,Skillback, et al., 2014,Steinacker, et al., 2016,Vagberg, et al., 2015), suggesting that CSF NFL levels increase with normal aging. Interestingly, we found that NFL levels could explain more than one third of the age-related increase in hippocampal atrophy rates. As accelerated decline of the hippocampus also in normal aging is observed independently of AD-related pathology (Fjell, et al., 2013), this is an important finding. Thus, our results indicate that CSF NFL levels reflect processes characterizing normal aging.

There has recently been increasing focus on amyloid-independent neurodegeneration in aging, often referred to as suspected non-Alzheimer pathology (SNAP), making it important to map out correlates of atrophy also in AD-typical areas in A β 42 negative older adults (Jack, et al., 2016). Thus, we created a subgroup with very low AD risk (only A β 42 negative participants), in which our finding was upheld. This bolsters that AD brain pathology is not a confounder of the relationship between CSF NFL and hippocampal atrophy rate, and suggests that CSF NFL most likely reflects neurodegeneration processes in normal aging. Further, in another subgroup analysis, CSF NFL predicted hippocampal atrophy rate after exclusion of participants with additional risk conditions, supporting that CSF NFL likely reflects normal aging processes. Previous studies suggest that CSF NFL may reflect the rate of ongoing neurodegeneration. High CSF NFL levels are seen days after a bout in amateur boxing (Zetterberg, et al., 2006), with subsequent decrease during the next months, CSF NFL levels are highest in MS patients with an ongoing relapse (Malmstrom, et al., 2003), and high CSF NFL levels are associated with

progression of neurodegenerative diseases (Backstrom, et al., 2015, Skillback, et al., 2014, Steinacker, et al., 2016, Zetterberg, et al., 2016). Accordingly, CSF NFL levels are higher in the rapidly progressing neurodegenerative disease amyotrophic lateral sclerosis than in AD which progresses more slowly (Steinacker, et al., 2016), and MCI patients have CSF NFL levels intermediate between those of AD patients and controls (Zetterberg, et al., 2016). Thus, CSF NFL may reflect that similar neurodegenerative processes are ongoing in both normal aging and diseases, and the CSF NFL levels may reflect the progression rate of the processes.

The etiologies of neuronal damage and neurodegeneration, and thus high NFL levels, can be manifold. Cerebrovascular pathology, including stroke (Norgren, et al., 2003) and white matter lesions (Sjogren, et al., 2001), has been associated with elevated CSF NFL levels. Since clinically silent cerebrovascular pathology is prevalent in older adults without dementia (Ikram, et al., 2008, Vermeer, et al., 2002), cerebrovascular pathology may be one cause of elevated NFL levels in our study. Associations between white matter lesions and hippocampal atrophy have been shown previously, although not consistently (Appelman, et al., 2009). It is still unknown whether this represents a causal link or is due to shared risk factors (Appelman, et al., 2009). Because vascular brain pathology may affect the relationship between NFL and hippocampal atrophy, we adjusted our final regression model for WM-hypointensities. NFL was still a significant predictor of hippocampal atrophy rate, indicating that NFL predicts hippocampal atrophy rate independently of cerebrovascular pathology, although it cannot be ruled out that more sensitive measures of WM lesions could yield other results.

Unexpectedly (Tosun, et al., 2010), in the final model, higher P-tau levels predicted lower hippocampal atrophy rates. However, P-tau was not significantly related to atrophy in GAMM, neither when no covariates but P-tau were included in the regression model. We can thus not exclude the possibility that the unexpected relationship with hippocampal atrophy is due to shared variance with the other covariates NFL and age. One explanation for the finding may be that our study could have excluded individuals with high CSF P-tau levels and high hippocampal atrophy rates, as they are more likely to have dementia or cognitive impairment. Thus, this result should be interpreted with caution. Since there were correlations among the biomarkers, data reduction methods such as principal component or cluster analysis could have been used to optimize classification accuracy. In this study, this was not done because we aimed to evaluate the contributions from the different biomarkers separately. However, this would be an important step for future studies to develop optimal combinations of variables in terms of classification accuracy.

Several limitations should be addressed. The main limitation is that although the likelihood of confounding by presymptomatic AD is low, we cannot rule out the possibility that presymptomatic neurodegenerative pathology of other etiologies may in part account for some of the relationship between CSF NFL and hippocampal atrophy. Further, although participants were followed for two years, we cannot be sure that they do not develop neurodegenerative diseases later. A second limitation is the method used to measure white matter lesion load. We obtained a measure based on the automated labeling of the T1 signal, but we believe that this measure could be more accurate if other modalities, designed specifically to evaluate white matter, such as T2 or FLAIR were available. Finally, a third limitation is the fact that our study had a mainly

exploratory aim, in which it was difficult to address the issue of multiple comparisons. However, we believe that our results were consistently supported by the different analyses undertaken.

6. Conclusion

CSF NFL predicted neurodegeneration in older adults with very low probability of AD. The present results suggest that factors previously shown to be important for brain degeneration in MCI may also impact brain changes in normal aging, demonstrating that NFL is likely to be a marker of AD-independent, age-expected neurodegeneration. Future research needs to explore the predictive value of this biomarker with regard to onset and progression of prodromal AD. For this purpose, even longer follow ups than the current two-year interval are necessary.

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Disclosure statement

Dr. Watne has given a lecture on delirium for Lilly. Dr. Bruun Wyller has given lectures on delirium for Pfizer, Roche, AstraZeneca and Nycomed. Dr. Blennow has served on Advisory Boards for IBL International and Roche Diagnostics. Dr. Walhovd has given a lecture on lifespan changes in brain and cognition for Shire International GmbH (2015) and has served in an expert group for ILSI Europe, for both of which honoraria were paid. The other authors report no conflicts of interest.

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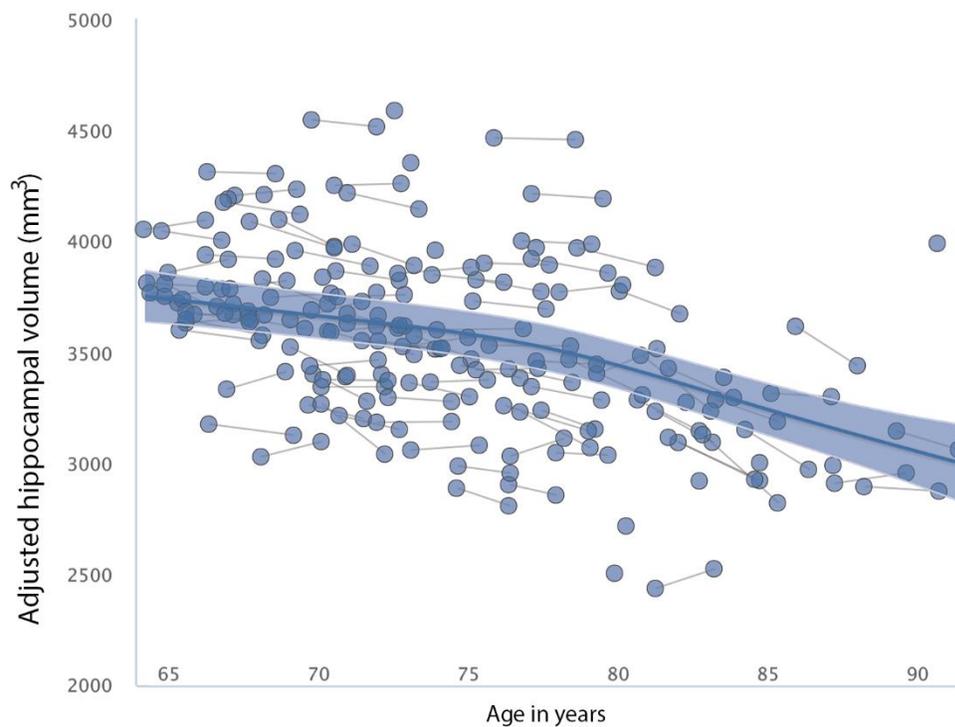
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Figure legends

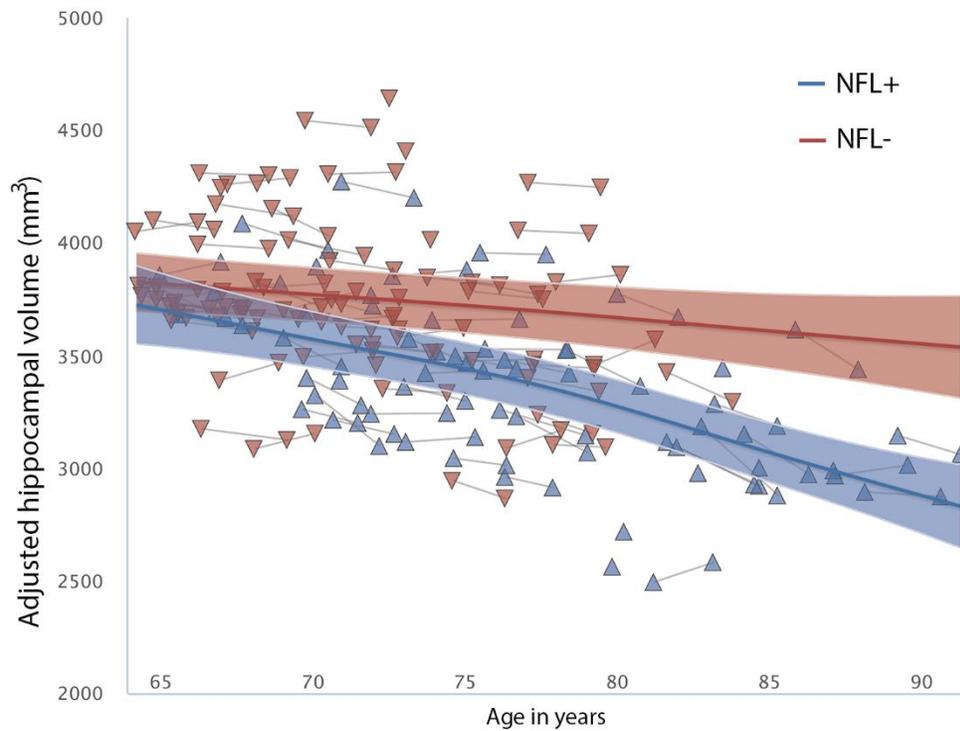
Figure 1. Relationship between age and hippocampal volume.



Detailed legend: Adjusted for sex. The graph shows mean slope with 95 % confidence interval.

Data points from participants with MRI are displayed, including within-person changes for those with MRI at both time points. MRI = magnetic resonance imaging.

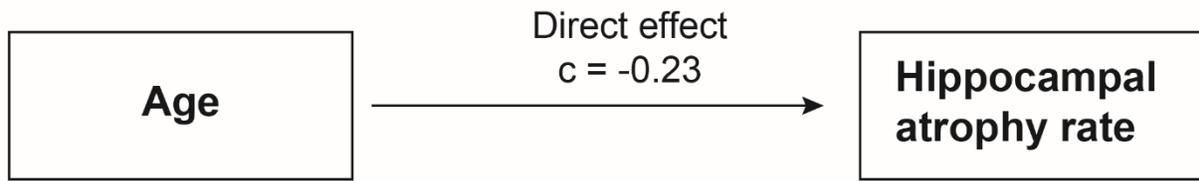
Figure 2. Relationship between age and hippocampal volume in NFL+ and NFL- participants.



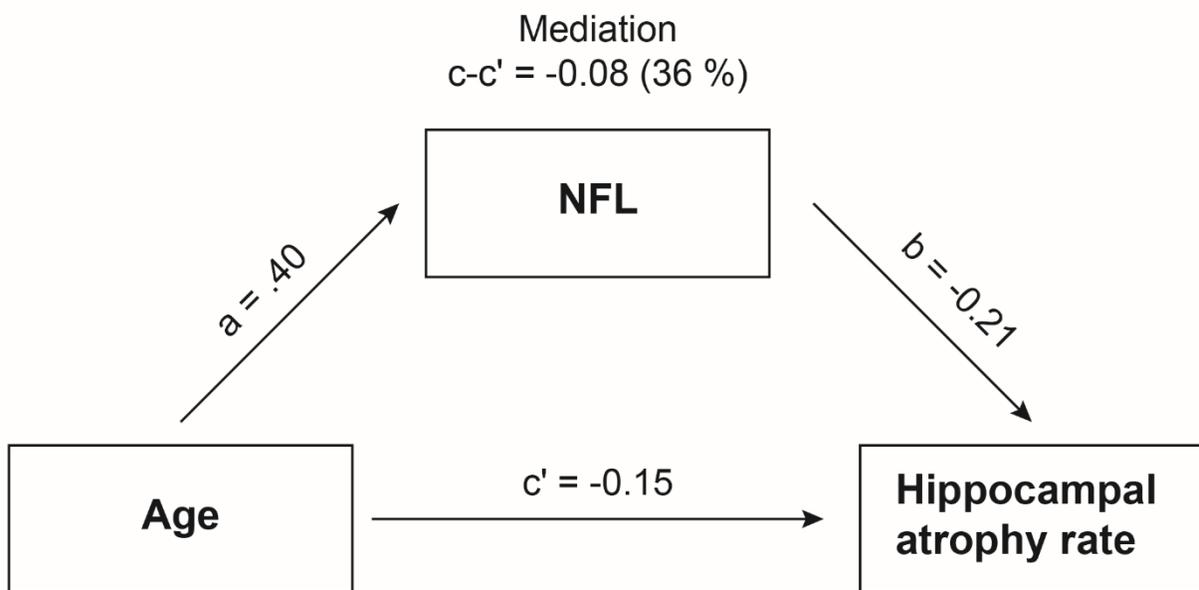
Detailed legend: Adjusted for sex. NFL+ (> 902 pg/mL) and NFL- (≤ 902 pg/mL) participants are defined by a median split. Estimated group slopes with 95 % confidence intervals are displayed. Data points from participants with MRI are displayed, including within-person changes for those with MRI at both time points. NFL = cerebrospinal fluid neurofilament light. MRI = magnetic resonance imaging.

Figure 3. NFL mediates the effect of age on hippocampal atrophy rate.

A



B



Detailed legend: Path analyses showing that NFL mediates the effect of age on hippocampal atrophy rate. Standardized regression coefficients for the paths are presented; A) c = the direct association between age and hippocampal atrophy rate, B) a = the association between age and NFL, b = the association between NFL and hippocampal atrophy rate adjusted for age, and c' the association between age and hippocampal atrophy rate adjusting for NFL. The regression coefficient for the mediation effect ($c - c' = a * b$) and the % reduction of the effect of age on hippocampal atrophy rate are also presented. The bootstrapped 95 % confidence interval for the

mediation effect was $-.24$ to $-.01$, showing that the mediation effect is significant. NFL = cerebrospinal fluid neurofilament light level.