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Cerebrospinal fluid biomarkers for Alzheimer disease and subcortical axonal damage in 5,542 clinical samples

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Abstract

Introduction: The neuronal loss in Alzheimer disease (AD) has been described to affect grey matter in the cerebral cortex. However, in the elderly, AD pathology is likely to occur together with subcortical axonal degeneration on the basis of cerebrovascular disease. Therefore, we hypothesized that biomarkers for AD and subcortical axonal degeneration would correlate in patients undergoing testing for dementia biomarkers, particularly in older age groups.

Methods: We performed correlation and cluster analyses of cerebrospinal fluid (CSF) biomarker data from 5,542 CSF samples analyzed in our routine clinical neurochemistry laboratory in 2010 through 2012 for the established CSF AD biomarkers total tau (T-tau), phosphorylated-tau (P-tau), amyloid β 1-42 (A β 42), and for neurofilament light (NFL), which is a protein expressed in large-caliber myelinated axons, the CSF levels of which correlate with subcortical axonal injury.

Results: A β 42, T-tau, and P-tau correlated with NFL. By cluster analysis, we found a bimodal data distribution in which a group with a low A β 42/P-tau ratio (suggesting AD pathology) had high levels of NFL. High levels of NFL also correlated with the presence of an AD biomarker pattern defined by A β 42/P-tau and T-tau. Only 29% of those with an AD biomarker signature had normal NFL levels. Age was a possible confounding factor for the associations between NFL and established AD biomarkers, but in a logistic regression analysis, both age and NFL independently predicted the AD biomarker pattern.

Conclusions: The association between an AD-like signature using the established biomarkers A β 42, T-tau, and P-tau with increased levels of NFL provides *in vivo* evidence of an association between AD and subcortical axonal degeneration in this uniquely large dataset of CSF samples tested for dementia biomarkers.

Introduction

A growing interest exists in the role of mixed pathologies for development of symptomatic Alzheimer disease (AD). The canonic brain hallmarks of AD are extracellular β -amyloid (A β) deposits and intracellular tangles composed of tau proteins, but the correlations between these findings and dementia symptoms decline with increasing age [1]. Although pathologic A β metabolism is clearly the initiating event in autosomal dominant AD, sporadic AD may be a more heterogeneous etiology, with, for example,

cerebrovascular pathology contributing to symptoms in some patients [2]. A large overlap in pathology is noted between elderly AD patients and individuals dying of other causes [3-5], which might be explained by combined effects of Alzheimer-type and cerebrovascular pathology in the form of white-matter lesions actually leading to dementia in elderly AD patients [6]. This is supported by neuropathologic studies showing increased frequency of mixed pathologies rather than pure AD pathology in dementia patients in older age groups [7].

Cerebrospinal fluid (CSF) biomarkers have become increasingly important in dementia research and have been included in research diagnostic criteria for AD [8-10]. The most well-established CSF AD biomarkers are the 42-amino-acid isoform of A β (A β 42), total-tau (T-tau),

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and phosphorylated tau (P-tau), which are believed to reflect the presence of amyloid plaque deposition, axonal degeneration, and neurofibrillary tangles, respectively [11]. Another CSF biomarker for neuroaxonal injury is neurofilament light (NFL) protein. NFL is expressed predominantly in large-caliber myelinated axons [12], and its CSF levels correlate with white-matter lesions and other injuries to subcortical brain regions [13-18]. NFL is often normal in clinically pure AD [19] but abnormal in the presence of vascular pathology (for example, small-vessel disease [20], which mainly affects subcortical brain regions). NFL is also elevated in several other pathologic conditions, such as frontotemporal dementia (FTD) [21,22], MS [23], idiopathic normal-pressure hydrocephalus [24], ALS [25], and various CNS infections [26-28]. Mutations in the NFL gene cause Charcot-Marie-Tooth disease [29].

Here, our main goal was to explore *in vivo* relations between pathologic hallmarks of AD and subcortical axonal damage, by using CSF biomarkers. Because the influence of vascular pathology and other causes of injury to subcortical brain regions in the general AD population may be underestimated in small cohorts with selected AD patients, we performed a study of CSF biomarkers in a uniquely large sample set from our clinical routine practice. This included mainly samples from memory clinics, geriatric clinics, and neurology clinics. In this unselected material, we hypothesized that a positive CSF AD biomarker signature, with reduced A β 42 and elevated T-tau and P-tau, would often be seen in combination with elevated NFL, because of the presence of mixed pathology, particularly in the older age groups. We assumed that the main cause of elevated NFL was subcortical vascular disease, but it is possible that NFL may also be elevated because of increased axonal loss in general, or secondary to amyloid angiopathy, which may accompany and correlate with AD pathology, and lead to cerebrovascular pathology.

Methods

This was a cross-sectional study, using archived data on CSF A β 42, T-tau, P-tau, and NFL measurements from January 1, 2010, to June 1, 2012, extracted from the database of clinical routine test results at the Mölndal site of the Sahlgrenska University Hospital, Sweden. All test results were obtained for diagnostic purposes. The inclusion criteria were that patients were older than 30 years at sampling, and that all four CSF analyses had been requested by the clinician. The majority of the samples were ordered from geriatric clinics, neurology clinics, and memory clinics. For patients tested more than once during the study period ($N = 126$), only the first sample was used. In all, the study included 5,442 samples. Because this was a registry study, the concept of informed consent was not applicable. The laboratory receives samples from many different clinical sites in Sweden, so we did not

have access to medical records, CSF cell counts, or total protein levels. This makes us unable to rule out cases of infectious, inflammatory, or acute cerebrovascular disease in this cohort.

Biochemical measurements

CSF A β 42, T-tau, and P-tau were measured by using enzyme-linked immunosorbent assay (ELISA) assays (INNOTEST β -amyloid [1-42], hTau Ag, and Phospho-tau [181P]; Innogenetics) as previously described [30,31]. The between-assay coefficients of variation (CV) for the A β 42, T-tau, and P-tau tests were 13.4%, 11.3%, and 9.70%, respectively (as determined by internal control samples during the entire study period). CSF NFL was measured with a novel, sensitive sandwich ELISA method (NF-light ELISA kit; UmanDiagnostics AB, Umeå, Sweden), as described by the manufacturer. The lower limit of quantification for this assay was 50 ng/L. The between-assay CV for the NFL assays was 14.0%. For further details on these measurements, see Appendix A.

For the AD biomarkers, we used cut-offs previously generated at our laboratory, in a study with long follow-up time of early-stage AD patients, in which the combination of T-tau and A β 42/P-tau ratio had 95% sensitivity and 87% specificity [32]. With these cut-offs (T-tau > 350 ng/L; A β 42/P-tau < 6.5), our dataset was divided into groups of sample profiles matching the AD biochemical profile (26.9%) or not (73.1%).

For NFL, we defined a threshold value for subcortical axonal degeneration by using an independent dataset on 108 CSF samples from clinically examined neurologically healthy volunteers (age median, 38 years; range, 18 to 76 years). The 95th percentile for CSF NFL in this group gave us a threshold value of 1,400 ng/L. This dataset forms the basis for the clinical normal reference limits for the UmanDiagnostics NFL assay at the Sahlgrenska Mölndal laboratory.

All analyses were performed in clinical routine by board-certified laboratory technicians with procedures accredited by the Swedish Board for Accreditation and Conformity Assessment (SWEDAC). Longitudinal stability in the measurements over years was ascertained by using an elaborate system of internal quality control samples and testing of incoming reagents (Appendix A).

Variables

The outcomes were relations between NFL levels and the AD biomarkers. In particular, we were interested in the frequency of patients with increased NFL levels among patients with a positive AD biomarker pattern. Age and sex were potential confounders.

Statistics

Correlations were calculated by using the Spearman rank correlation coefficient, and statistical hypothesis

testing was performed by using Pearson χ^2 test, logistic regression, ANOVA, multiple regression, or Mann–Whitney *U* tests. Outliers in data were identified by the Grubbs test for outliers and excluded from the dataset before analysis. These general statistics and all charts and tables were produced in SPSS version 20 (IBM, New York, NY, USA). Logarithmic transformations were used to fit the significantly skewed NFL data for logistic regression analysis and multiple regression. Figure axes are presented with logarithmic axes where specified in axis title.

Clustering was performed in JMP version 10.0.0 (SAS Institute Inc., Cary, NC, USA) by using the K-means clustering algorithm. K-means clustering is a special case of a general approach called the EM algorithm, where E stands for Expectation (the cluster means in this case), and M stands for Maximization, which in this case means assigning points to the closest clusters. Distance is measured by the squared euclidian distance of each point, assigning each data point to the cluster that yields the least within-cluster sum of squares. To choose the optimal number of clusters, we used the cubic clustering criterion

(CCC), in which the possible numbers of clusters are compared with each other in terms of their within-cluster sum of squares [33].

Ethics

The study was approved by the regional ethics committee at the University of Gothenburg.

Results

Dataset description

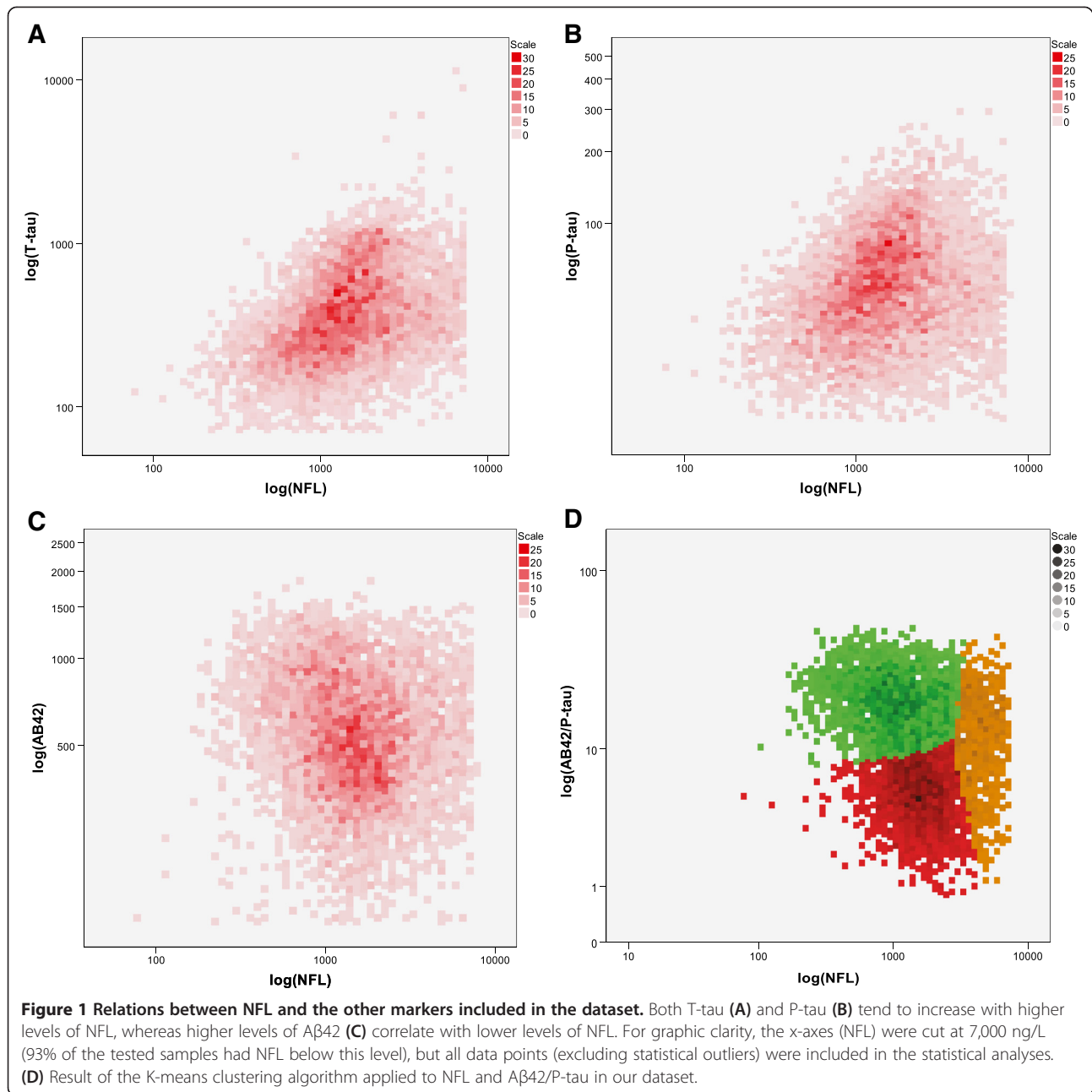
Demographics are summarized in Table 1. The majority of patients were from 60 to 90 years old (M, 70.1; SD, 11.0), and the sex distribution was even.

CSF AD biomarkers in relation to NFL levels

We first analyzed the relation between NFL and the other biomarkers in the overall study population (Figure 1). Both T-tau and P-tau had positive correlations with NFL (Figure 1A,B; $R = 0.416$; $P \leq 0.001$ for T-tau and $r = 0.231$, $P \leq 0.001$ for P-tau), whereas A β 42 had an inverse correlation with NFL (Figure 1C; $r = -0.130$; $P \leq 0.001$).

Table 1 Demographics

Age group		Sex		A β 42	T-tau	P-tau	NFL
		F	M				
All	Valid N	50.8%	49.2%	5,542	5,542	5,542	5,542
	Mean (SD)			602 (286)	504 (953)	60 (33)	3,007 (6,935)
	Median (range)			544 (1,725)	360 (32,125)	52 (283)	1,530 (226,920)
31-40	Valid N	51.3%	48.7%	78	78	78	78
	Mean (SD)			733 (309)	262 (229)	38 (20)	1,974 (6,056)
	Median (range)			704 (1,420)	194 (1,325)	35 (122)	460 (45,790)
41-50	Valid N	52.0%	47.7%	237	237	237	237
	Mean (SD)			739 (304)	302 (494)	39 (18)	1,856 (5,790)
	Median (range)			707 (1,725)	210 (6,025)	35 (122)	520 (66,220)
51-60	Valid n	52.2%	47.8%	688	688	688	688
	Mean (SD)			723 (297)	458 (1,354)	49 (30)	2,788 (8,719)
	Median (range)			700 (1,612)	258 (27,525)	42 (206)	870 (100,810)
61-70	Valid n	48.9%	51.1%	1,539	1,539	1,539	1,539
	Mean (SD)			626 (289)	464 (947)	55 (30)	2,931 (6,609)
	Median (range)			580 (1,455)	317 (30,425)	48 (283)	1,280 (89,820)
71-80	Valid n	49.6%	50.4%	2,069	2,069	2,069	2,069
	Mean (SD)			559 (271)	546 (1,027)	64 (32)	3,026 (7,203)
	Median (range)			500 (1,715)	409 (32,125)	57 (240)	1,710 (226,770)
81-90	Valid n	55.1%	44.9%	905	905	905	905
	Mean (SD)			523 (247)	581 (349)	72 (34)	3,573 (5,422)
	Median (range)			465 (1,535)	479 (3,493)	65 (235)	2,200 (93,130)
91+	Valid n	53.8%	46.2%	26	26	26	26
	Mean (SD)			487 (161)	778 (549)	90 (53)	5,605 (8,049)
	Median (range)			496 (785)	590 (2,413)	76 (250)	2,845 (42,000)



The combination of the markers A β 42, P-tau, and NFL had interesting features (Figure 1D). The data distribution contained three separate clusters, with one dense group consisting of samples with a high A β 42/P-tau ratio in combination with low levels of NFL (green cluster), another group with lower A β 42/P-tau ratio and higher levels of NFL (red cluster), and a third cluster of outliers with substantially higher levels of NFL (brown cluster). By k-means cluster analysis, we could successfully identify these three groups. By applying CCC, we could confirm that the division into three clusters was the most efficient

way to model these data (CCC = 0.62, which was the largest CCC (optimal) in the range of one to 20 clusters [two-cluster CCC = -13.98; four-cluster CCC = -15.23]). The identified clusters showed the following properties: (I, green cluster) high A β 42/P-tau (median = 18.9; mean = 20.0) and low NFL (median = 1,010; mean = 1,158); (II, red cluster) low A β 42/P-tau (median = 5.4; mean = 5.5) and high NFL (median = 1,600; mean = 1,697); (III, brown cluster) outliers with very high NFL values (median = 4,440; mean = 4,609) and intermediate A β 42/P-tau (median = 13.4; mean = 12.1). The age distribution varied across the clusters

(M, SD, I: 65.7, 11.5; II: 74.0, 8.6; III: 73.2, 10.0), with significant differences when tested with one-way ANOVA ($F(2, 5,154) = 413, 193; P < 0.001$).

Biochemical AD criteria versus NFL levels

To search further for associations between AD and subcortical pathology, we classified the study subjects by using NFL as a marker of subcortical axonal degeneration, and A β and tau markers for AD, by cut-offs, as explained earlier (Figure 2). The relative number of NFL-positive patients was significantly higher in the group of AD biomarker-positive patients than in the AD biomarker-negative group ($\chi^2 = 197.0; P \leq 0.001$).

CSF biomarker patterns in different ages

Age is a strong risk factor for both AD and cerebrovascular pathology, which is a main cause of subcortical axonal degeneration, and a possible confounder for the relation between the AD biochemical profile and elevated NFL. We therefore explored the relation between age and biomarkers in detail.

First, pathologic levels of all the biomarkers were explored in the different age groups (Figure 3). The frequency of pathologic levels of A β 42 was about 30% in the youngest cohort, started to increase in subjects from about 60 years of age, and plateaued at about 60% in patients older than 80 years (Figure 3A). In contrast, the frequency of pathologic levels of T-tau was only about 15% in the youngest cohort, and increased linearly in subjects from about age 40 years, reaching 90% in the oldest cohort (Figure 3B). P-tau had a similar pattern, but with an even more dramatic increase in the oldest

old (Figure 3C). Pathologic levels of the A β 42/P-tau ratio were seen in only about 5% of subjects in the youngest cohort and increased linearly in subjects from about 40 years of age, reaching 55% in the oldest cohort (Figure 3D). The pathologic combination of all three AD biomarkers (AD biomarker profile) had a similar pattern (Figure 3E). Pathologic levels of NFL were seen in about 20% in the youngest cohort and showed a sigmoidal increase, reaching about 90% in the oldest cohort (Figure 3F).

Figure 4 displays the groups of AD biomarker-positive and -negative patients, and the prevalence of increased NFL levels in different age groups. It was more common among AD biomarker-positive patients to have low NFL levels in younger than in older patients (although the number of young patients with the AD biochemical profile was small), but the fraction of NFL-positive subjects increased with age, in both the AD-positive and AD-negative groups. To adjust for the effect of age, we did a logistic regression analysis with age and NFL as predictors of the AD biomarker profile (dichotomized as positive or negative). Both age ($B = -0.063; P \leq 0.001; \exp(B) = 0.939$) and $\log(\text{NFL})$ ($B = -0.685; P \leq 0.001, \exp(B) = 0.504$) were significant predictors when used as single predictors, and when used together in the model (Age, $B = -0.060, P < 0.001, \exp(B) = 0.941, \log(\text{NFL}): B = -0.246, P = 0.005, \text{Exp}(B) = 0.782$).

Figure 5 shows NFL values versus age, and LOESS fit lines for the AD biomarker profile- positive and -negative groups. In younger ages, NFL levels were higher in the biomarker-positive group (although the few available AD biomarker-positive young subjects makes these estimates uncertain), but the fitted lines for the positive and negative groups converged around age 65 years.

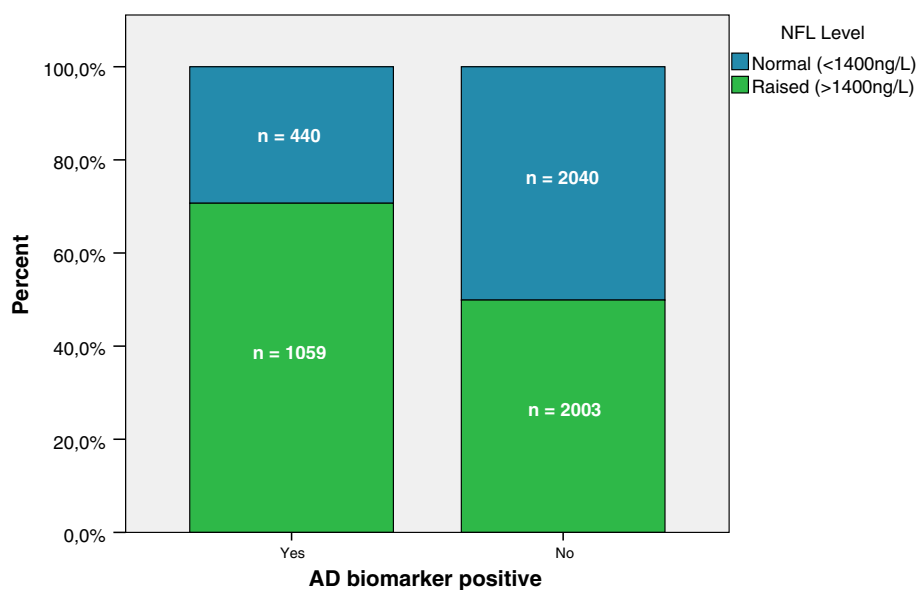
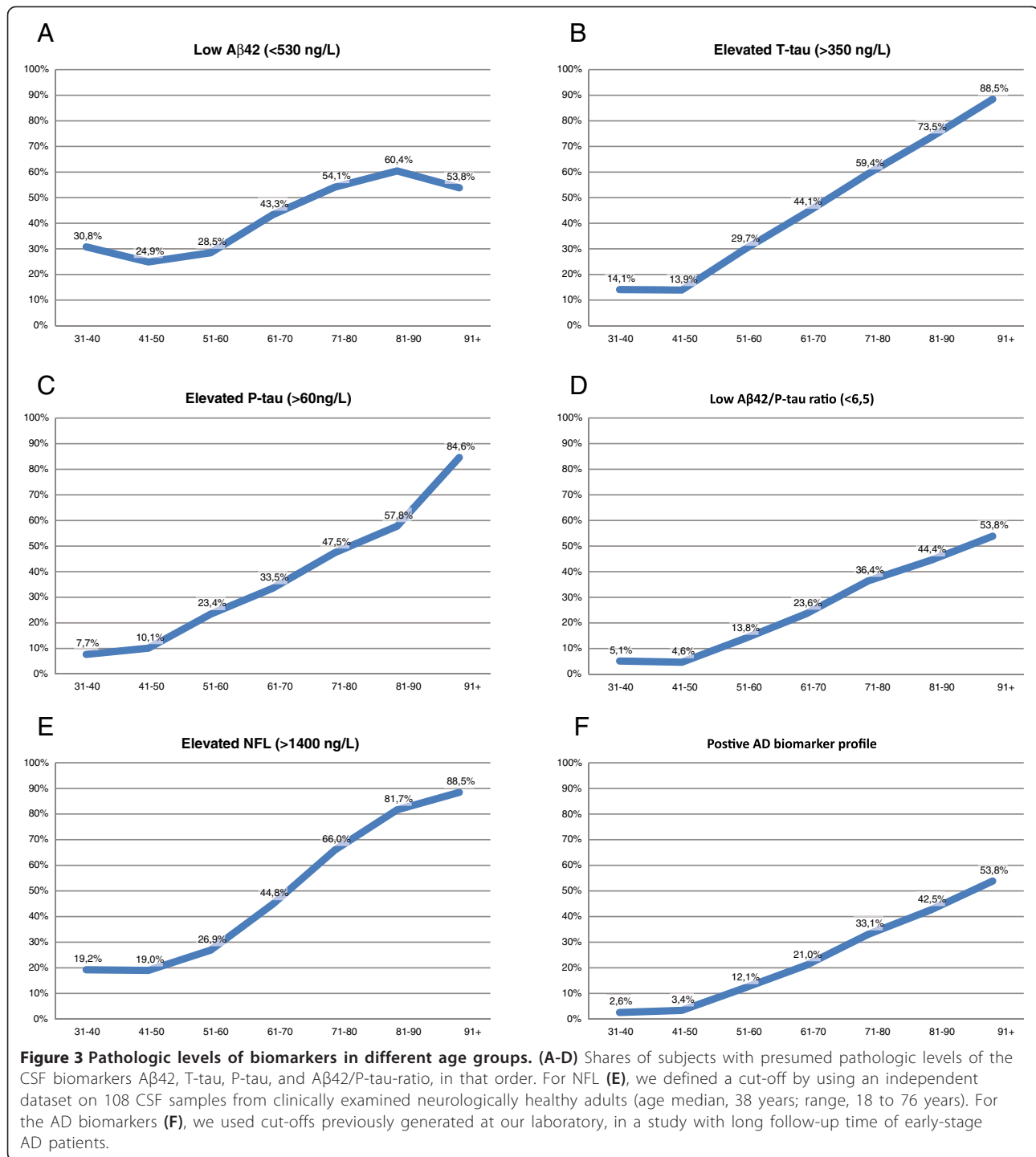


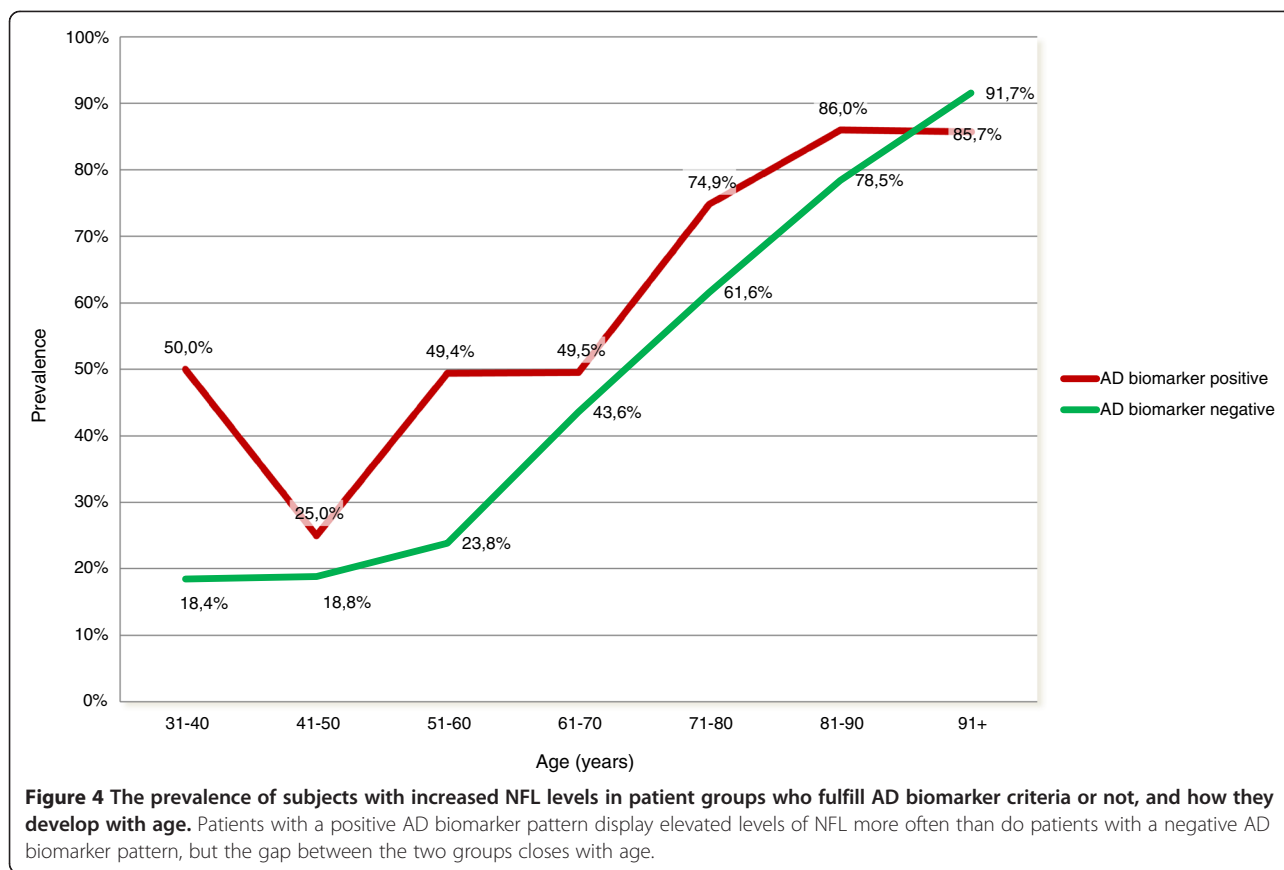
Figure 2 More patients in the group with positive AD biomarker pattern also display increased NFL levels. The chart shows the percentage relations between the prevalence of normal or increased NFL levels in those who fulfill AD biomarker criteria or not.



To verify that the differences in NFL values remained and did not solely rely on the relatively high levels of NFL among younger subjects (as shown in Figures 4 and 5), the data were split into two age categories, with 65 years as break point. A Mann–Whitney *U* test confirmed that the NFL levels in the AD biomarker-positive groups was significantly higher for both age groups ($H(1) = 68.6$,

$P < 0.001$ for ages younger than 65), and ($H(1) = 41.53$; $P < 0.001$ for ages older than 65).

To assess the importance of different factors in an adjusted model, a multiple linear regression was performed to predict NFL from sex, age, and AD biomarker profile (dichotomized as positive or negative). These variables statistically significantly predicted NFL ($F(3, 5,538) = 320.31$;



$P < 0.001$, $R_2 = 0.148$). All three variables were independently statistically significant (age (years): $\beta = 0.352$, $P < 0.001$; sex (female): $\beta = 0.128$; $P < 0.001$; AD-biomarker profile (positive): $\beta = 0.043$; $P = 0.001$).

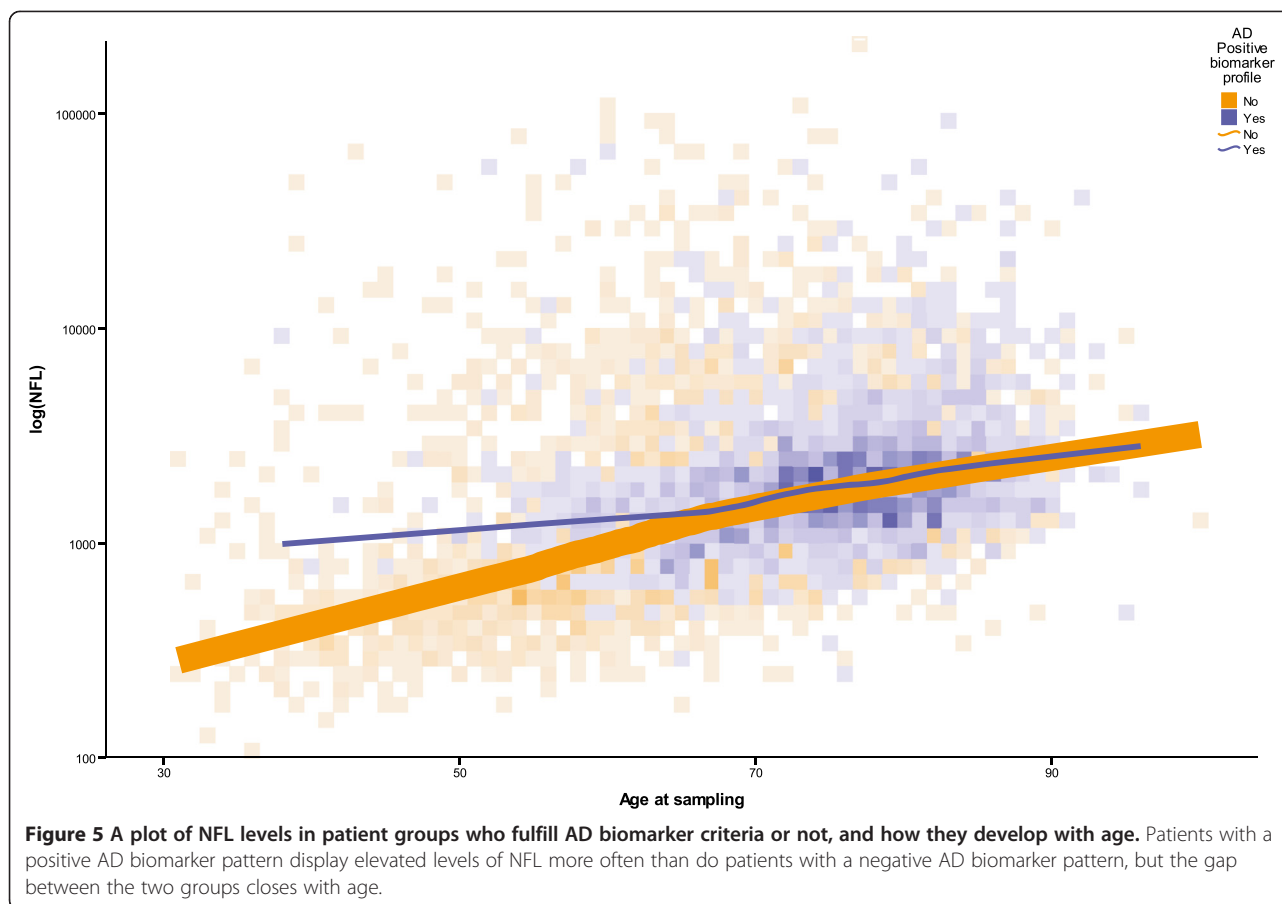
Discussion

In a uniquely large dataset of clinical routine samples, we tested associations between established CSF biomarkers for AD pathology ($A\beta_{42}$, T-tau, and P-tau) and a CSF biomarker for subcortical axonal degeneration (NFL). In this unselected population of patients from memory clinics, geriatric clinics, and neurology clinics, we expected to find a large proportion of subjects with positive AD biochemical profile, suggesting the presence of AD, and a large proportion with elevated NFL levels, suggesting the presence of cerebrovascular or other white-matter disease. This was confirmed, but we also found an association between white-matter disease, as reflected by NFL levels and AD, as reflected by $A\beta_{42}$, T-tau, and P-tau levels. Subjects with a positive AD biomarker profile often also had high NFL levels. Only 29% of the subjects with positive AD biomarker profile had normal NFL levels, suggesting that a pure AD biochemical profile without signs of NFL leakage is unusual in subjects undergoing testing for dementia biomarkers. In contrast, elevated NFL was rare in the AD biomarker-negative group. It should be

noted that NFL is believed to be a marker of white-matter disease because it is predominantly expressed in myelinated large-caliber axons [12]. However, other types of injury to such neurons (not only white matter lesions) could also lead to higher NFL levels in CSF.

Cluster analysis showed three clusters in our data; the first large group had high $A\beta_{42}$ /P-tau quota and low NFL (that is, weak biochemical support for either AD or subcortical pathology). The second equally large cluster had low $A\beta_{42}$ /P-tau quota and high NFL (that is, for concomitant AD and subcortical pathology), whereas the third, considerably smaller cluster had the highest levels of NFL and intermediate $A\beta_{42}$ /P-tau quota, suggesting predominant subcortical pathology or other reasons for NFL leakage. This was a cross-sectional study, so we cannot make conclusions on the order of pathologic events underlying these biomarkers, but a possible explanation for the identified biomarker distributions is that AD pathology is strongly associated with vascular pathology, because NFL levels have previously been found to correlate with vascular pathology [15,19,34]. Alternative explanations to our findings may be that the increased NFL levels are caused by high cell loss or increased amyloid angiopathy in AD, or a combination of all these factors.

The cluster with the most normal pattern of $A\beta_{42}$, P-tau, and NFL (indicating least brain engagement) had the



youngest mean age, which may be expected in an unselected group of patients. In a detailed analysis of the relation between biomarkers and age, we observed that the prevalence of high NFL levels increased with age, and we noted that this was seen in patients both with and without the AD biomarker profile. The group with a positive AD biomarker profile had the highest levels of NFL. This difference was greater among the younger patients, and the gap between the groups closed with increasing age, but age and NFL were still independent predictors of the AD biomarker profile in a logistic regression analysis. When dichotomized into subjects younger or older than 65 years, AD biomarker-positive subjects had significantly higher NFL levels in both age groups, tested by using the Mann–Whitney *U* test.

The main strength of this study is its unique size; to our knowledge, it is the largest study ever published on CSF biomarkers. The main study limitation is the limited access to clinical data. The absolute majority of analyses were requested in investigations of cognitive decline. The study population may therefore be considered to represent a group of patients with different degrees of cognitive dysfunction. However, at some centers lumbar puncture and NFL analysis may preferentially be performed in clinically

complicated or ambiguous cases, which could bias the population toward rare or mixed brain pathologies, and increase the likelihood of finding atypical biomarker patterns. Because of lack of CSF cell count and total protein data, we cannot exclude that a few patients with inflammatory or infectious conditions were included. However, this limitation is unlikely to have influenced the positive association between high NFL levels and a positive AD biomarker profile, as neuroinflammation might increase NFL levels but not the prevalence of a positive AD biomarker status [35,36]. The lack of clinical data may partly be compensated by the known strong association of positive CSF AD biomarkers with AD pathology [37] and the fact that CSF AD biomarkers are strong predictors of clinical AD in patients undergoing cognitive evaluation [11]. The lack of clinical or imaging data on cerebrovascular disease prevents firm conclusions regarding the cause of NFL elevations. We based the association of elevated CSF NFL with cerebrovascular disease on previous studies [15,19,34].

Conclusions

Biochemical evidence of AD was most often seen together with elevated NFL levels, which is a biomarker sign compatible with subcortical vascular pathology. However, we

acknowledge that several factors may have contributed to NFL elevations in this patient group, including general neuronal death or amyloid angiopathy, which may accompany AD pathology and lead to cerebrovascular pathology. The *in vivo* relation between AD and subcortical axonal degeneration must be further explored in other cohorts with access to clinical, neuroimaging, and fluid biomarker data. The results of this study, if replicated, may be relevant to clinical trials in which drugs developed against AD pathology are evaluated, because we show that patients with an AD biochemical profile are likely to have subcortical axonal degeneration, which may contribute to the symptoms and explain difficulties in obtaining desired drug effects.

Appendix A

The coefficients of variation (CVs) of the assays for NFL, A β 42, T-tau, and P-tau were determined by analyzing records of measurements of internal control samples that are routinely carried out at the laboratory at Mölndal at least twice a week. Control samples are kept in frozen aliquots and reused until depleted and then exchanged. Standard deviations of the averages of all measurements from each control sample were calculated, and an average of these standard deviations was used to represent a CV for every analysis.

For NFL, one low and one high control sample was used. The first low control was used from January 2010 until May 2010, whereas the first replaced control was used until January 2011, and the last replaced control was used until June 2012. The three low controls showed standard deviations of 15.15%, 15.24%, and 13.24%, respectively. The first two high controls were used during the same periods as the first two low controls and measured deviations of 14.41% and 16.60%, respectively. The third control was used until May 2012 and had a deviation of 15.32%. The last control was used between May and June 2012 and had a deviation of 7.84%. The average of all these deviations was calculated and resulted in a CV of 14.04%.

For A β 42, three different low and three different high controls were used during the study. The first low control was used from January 2010 until January 2011; the second one was replaced in May 2012, and the third control was used the remaining time of the test period. The three low controls measured standard deviations of 19.42%, 12.76%, and 12.46%, respectively. The first high control was used from January 2010 until August 2010; the second one, until November 2010; the third, until June 2011; and the last control was used for the remainder of the test period. The high controls measured standard deviations of 10.45%, 13.14%, 12.62%, and 11.54%. The total average of the A β 42 measurements was calculated as 13.41%.

For P-tau, three different low, and three different high controls were used. The first low control was used from

January 2010 until January 2011; the second one was replaced in May 2012; and the third control was used the remaining time of the test period. The three low controls measured standard deviations of 8.60%, 11.68%, and 11.04%, respectively. The first high control was used from January 2010 until November 2010; the second one, until June 2011; and the last control were used for the remainder of the test period. The high controls measured standard deviations of 8.62%, 8.85%, and 9.38%. The total average of the P-tau measurements was calculated to be 9.70%.

For T-tau, three different low, and three different high controls were used. Both the high and low controls were replaced at the same time as the ones for P-tau. The three low controls measured standard deviations of 9.35%, 9.88%, and 9.05%, respectively. The high controls measured standard deviations of 19.15%, 10.73%, and 9.63%. The total average of the T-tau measurements was calculated to be 11.30%.

No measureable longitudinal drift was registered for any of the analyses.

Abbreviations

AD: Alzheimer disease; A β : β -amyloid; A β 42: Amyloid β 1-42; CCC: Cubic clustering criterion; CSF: Cerebrospinal fluid; CV: Coefficient of variation; NFL: Neurofilament light; P-tau: Phosphorylated tau; T-tau: Total tau.

Competing interests

None of the authors of this article report any disclosures.

Authors' contributions

TS performed study design, data analysis, interpretation, and writing of the manuscript draft. HZ contributed to the study concept and design, and to critical revision of the manuscript for important intellectual content. KB added critical revision of the manuscript for important intellectual content. NM performed study supervision, concept, design, interpretation, and critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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