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FEATURED ARTICLE

Amyloid beta, tau, synaptic, neurodegeneration, and glial biomarkers in the preclinical stage of the Alzheimer's *continuum*

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Abstract

Introduction: The biological pathways involved in the preclinical stage of the Alzheimer's *continuum* are not well understood.

Methods: We used NeuroToolKit and Elecsys® immunoassays to measure cerebrospinal fluid (CSF) amyloid-β (Aβ)42, Aβ40, phosphorylated tau (p-tau), total tau (t-tau), neurofilament light (NfL), neurogranin, sTREM2, YKL40, GFAP, IL6, S100, and α-synuclein in cognitively unimpaired participants of the ALFA+ study, many within the Alzheimer's *continuum*.

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Results: CSF t-tau, p-tau, and neurogranin increase throughout aging only in Aβ-positive individuals, whereas NfL and glial biomarkers increase with aging regardless of Aβ status. We modelled biomarker changes as a function of CSF Aβ42/40, p-tau and p-tau/Aβ42 as proxies of disease progression. The first change observed in the Alzheimer's *continuum* was a decrease in the CSF Aβ42/40 ratio. This is followed by a steep increase in CSF p-tau; t-tau; neurogranin; and, to a lesser extent, in NfL and glial biomarkers.

Discussion: Multiple biological pathways are altered and could be targeted very early in the Alzheimer's *continuum*.

KEYWORDS
Alzheimer's disease, biomarker, neurodegeneration, neuroinflammation, preclinical

1 | BACKGROUND

The natural history of Alzheimer's disease (AD) comprises a long asymptomatic or preclinical stage characterized by pathophysiological changes that start decades before symptoms arise.[1-3] In the new 2018 research framework, AD is defined based on biomarker evidence of amyloid-β (Aβ) and tau pathology, while clinical manifestations are used for grading severity.[4] According to this framework, the term "Alzheimer's disease" is applied whenever there is evidence of Aβ and tau pathology, regardless of the clinical manifestations. When there is evidence of Aβ pathology but not tau, the term "Alzheimer's pathologic change" is used. Together, individuals with either "Alzheimer's pathologic change" or "Alzheimer's disease" belong to the so-called "Alzheimer's *continuum*.".

AD cerebrospinal fluid (CSF) core biomarkers allow an accurate diagnostic and early identification of AD pathology.[5] AD CSF core biomarkers comprise Aβ42 and the Aβ42/40 ratio, phosphorylated tau (p-tau), and total tau (t-tau), which reflect Aβ pathology, tau pathology, and neurodegeneration, respectively. However, multiple additional pathophysiological processes occur in these early stages of the Alzheimer's *continuum* such as neuronal and axonal damage,[6-9] synaptic dysfunction,[10-12] neuroinflammation and glial response,[13-15] and α-synuclein or TDP-43 co-pathology.[16-18] In this context, the development of drugs targeting these processes may potentially modify the evolution of the disease.

The pathophysiological events that occur in this early stage remain to be fully elucidated. This can be explained by several reasons. (1) It is particularly challenging to recruit individuals in the earliest extreme of the Alzheimer's *continuum* (Aβ-positive but still tau-negative, ie, preclinical Alzheimer's pathologic change). (2) Most cohorts include elders but not middle-age adults, when AD pathology most likely starts. (3) Most studies include a single or very low number of biomarkers and therefore it is difficult to assess the relationships between them. Recently, a very interesting study in the BioFINDER cohort modelled the changes in CSF and plasma biomarkers in cognitively unimpaired, subjective cognitive decline, and mild cognitively impaired individuals.[19] They proposed a model with a sequence of events starting with changes in Aβ; followed by tau biomarkers; and, only after Aβ positron emission tomography (PET) became abnormal, changes in neuronal injury, and synaptic and glial biomarkers.

Herein, we focused in cognitively unimpaired individuals and, very particularly, at the earliest stage of the Alzheimer's *continuum*. The main aim of our study is to define the pathophysiological events that occur in the preclinical stage of the *continuum*. We addressed the challenges described above by studying the well-characterized ALFA+ cohort, which includes cognitively unimpaired individuals, mainly in their middle age, and with a high prevalence of individuals that are Aβ positive but still tau negative.[20] Moreover, we measured several CSF biomarkers that mark the main pathogenic events described in AD: Aβ pathology (Aβ42, Aβ42/40 ratio), tau pathophysiology (p-tau), neurodegeneration (t-tau), axonal damage (neurofilament light [NfL]), synaptic dysfunction (neurogranin), microglial (sTREM2) and astroglial-related response (GFAP, YKL40, S100), other neuroinflammatory biomarkers (interleukin 6 [IL6]), and α-synuclein. We investigated how these CSF biomarkers change with age, sex, and Aβ pathology. Importantly, we modelled the sequence of biomarker changes during the preclinical stage of the Alzheimer's *continuum* to provide a model of the main pathophysiological changes in the earliest stage of the disease *continuum*.

2 | METHODS

2.1 | ALFA participants and study design

The ALFA+ cohort is a nested longitudinal study of the ALFA (for ALzheimer's and FAmilies) study.[20] The ALFA cohort was established as a research platform to characterize preclinical AD in 2743 cognitively unimpaired individuals, aged between 45 and 75 years old, and enriched for family history of AD (excluding autosomal-dominant AD). In the nested ALFA+ study, participants are longitudinally followed up and undergo a more comprehensive evaluation. CSF samples in

these participants were obtained by lumbar puncture following standard procedures (see supporting information). Herein, we included the first consecutive 381 participants of ALFA+.

2.2 | CSF biomarker measurements

CSF t-tau and p-tau were measured using the electrochemiluminescence immunoassays Elecsys® Total-tau CSF and phosphor-tau(181P) CSF on a fully automated cobas e601 instrument (Roche Diagnostics International Ltd.). The rest of the biomarkers were measured with the prototype NeuroToolKit (Roche Diagnostics International Ltd.) on a cobas e411 or e601 instrument (supporting information). All measurements were performed at the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden.

2.3 | CSF amyloid-β and p-tau cutoffs derivation

Aβ pathology positivity (A+) and tau pathology positivity (T+) were defined by CSF Aβ42/40 ratio and CSF p-tau, respectively.[21] We derived the cutoffs for each of these biomarkers using a two-Gaussian mixture modelling (GMM; supporting information). The cutoff was defined as the mean plus two standard deviations of the non-pathologic Gaussian distribution (ie, the Gaussian with the higher mean for CSF Aβ42/40 ratio and the Gaussian with the lower mean for p-tau and p-tau/Aβ42 ratio). The resulting cutoffs were 0.071 for the Aβ42/40 ratio, 24 pg/mL for p-tau, and 0.013 for the p-tau/Aβ42 (Figure S1 in supporting information).

2.4 | Statistical analyses

For each of the CSF biomarkers, we excluded the extreme values defined as either those that fell outside of three times the interquartile range below the first quartile (Q1) or those above the third quartile (Q3). In the main text, all the analyses were performed excluding extreme values, but including them rendered similar results (see Tables S2–S4 in supporting information). We tested for normality of the distribution for each biomarker using the Kolmogorov-Smirnov test and visual inspection of histograms. CSF Aβ42, p-tau, t-tau, NfL, neurogranin, YKL-40, GFAP, IL6, S100, α-synuclein, and the p-tau/Aβ42 ratio did not follow a normal distribution and were thus $\log_{10}$-transformed. CSF Aβ40, Aβ42/40 ratio, and sTREM2 followed a normal distribution and were not transformed.

We conducted a one-way analysis of variance (ANOVA) to test statistically significant differences on age and education among AT groups. The mean levels of CSF biomarkers among AT (Aβ/tau pathology) groups were assessed by a one-way analysis of covariance (ANCOVA) adjusting for age and sex. Cognitive performance (Mini-Mental State Examination [MMSE] and Free and Cued Selective Reminding Test [FCSRT]) was assessed by an ANCOVA adjusting for age, sex, and education. These comparisons were followed by Tukey corrected *post hoc* pairwise comparisons. Differences in the frequencies of sex and apolipoprotein E (*APOE*)-*ε4* categories were assessed by the Pearson's χ^2 test. Correlations between CSF biomarkers were tested by partial correlations adjusted by age.

To study the association of each CSF biomarker with demographic characteristics and *APOE-ε4* status, we computed a linear regression model with age, sex, and *APOE-ε4* status as predictor variables. We conducted these analyses stratifying by Aβ status and, additionally, including in the linear regression an "Aβ42/40 ratio x age" interaction term.

We plotted CSF biomarkers levels as a function of CSF Aβ42/40, p-tau, and p-tau/Aβ42. We corrected each of the CSF biomarker values by age and sex and computed the mean and standard deviation of each biomarker in the A–T– group (reference group). We next

HIGHLIGHTS

- Multiple cerebrospinal fluid (CSF) biomarkers are altered very early in the Alzheimer's *continuum*.
- CSF Aβ42/40 ratio changes first in the preclinical stage of the Alzheimer's *continuum*.
- CSF p-tau, t-tau, and neurogranin increase after the decrease in CSF Aβ42/40 ratio.
- CSF p-tau, t-tau, and neurogranin change specifically in Aβ-positive individuals.
- CSF neurofilament light (NfL) and glial biomarkers increase with age regardless of Aβ status.

RESEARCH IN CONTEXT

1. Systematic review: The authors reviewed the literature using traditional sources. Publications describing the natural history of Alzheimer's disease (AD) and its associated cerebrospinal fluid amyloid-β, tau, synaptic, neurodegeneration, and glial biomarkers are cited throughout the manuscript.
2. Interpretation: Our findings show that multiple biological pathways are altered in the earliest stages of the preclinical Alzheimer's *continuum* and thus could be targeted in asymptomatic individuals.
3. Future directions: This article proposes a framework for the generation of new hypotheses and the conduct of further studies. Some of these include, for example, the gathering of longitudinal data to assess biomarker profile progression, the addition of biomarkers related to other pathophysiological processes as well as neuroimaging biomarkers.

converted CSF biomarker values to z-scores by subtracting the mean and dividing by the standard deviation of the reference group. The relationship between each CSF biomarker and the proxies of disease progression (ie, CSF Aβ42/40, p-tau, and p-tau/Aβ42) were modelled using a robust local weighted regression method (rlowess; "smooth" function in Matlab and a span of 300) and we plotted the resulting model.[22,23] Moreover, we calculated the linear regression slopes for each of the biomarkers in each of the negative or positive groups, using the previously described cutoffs. We computed the *P*-values testing the null hypothesis of whether the regression slope being equal to zero. In addition, the probability of the two regression slopes being equal was also calculated.

For all the analyses, we applied a false discovery rate (FDR) multiple comparison correction following the Benjamini-Hochberg procedure.[24] All tests were two-tailed, with a significance level of $\alpha = 0.05$. Statistical analyses were performed in SPSS IBM 20.0 and R software (http://www.r-project.org/). Figures were built using R and Matlab (v2018b).

3 | RESULTS

3.1 | Participants' characteristics

Table 1 summarizes the demographic characteristics and CSF biomarkers measurements of ALFA+ study participants. Of note, 13 (3.4%) participants fell into the A−T+ group (non-AD pathologic change). Because our aim was to study the Alzheimer's *continuum*, we excluded these 13 participants from all analyses, and they are only depicted in Table 1 and Figure 1 for descriptive purposes. AT groups differed in years of age and education and prevalence of *APOE-ε4* status, but not in sex distribution or MMSE and FCSRT cognitive scores (Table 1).

3.2 | CSF biomarkers and AT groups. Correlations between CSF biomarkers

We observed significant differences in the mean values of CSF NfL, neurogranin, sTREM2, YKL40, GFAP, S100, and α-synuclein between AT groups (Figure 1). Specifically, CSF NfL, neurogranin, sTREM2, YKL-40, GFAP, and α-synuclein were significantly higher in the A+T+ group compared to the A−T− and the A+T− groups (Table 1 and Figure 1). Adding years of education in the analyses as a covariate did not modify the results. Of note, none of the studied CSF biomarkers was increased in the A+T− group compared to the A−T− group.

We also tested the correlations between the CSF biomarkers. In a partial correlation adjusting by age, all CSF tau-related, synaptic dysfunction, neuronal injury, and glial markers significantly and positively correlate with each other. There was a negative correlation between CSF Aβ42/40 and CSF p-tau, t-tau, NfL, neurogranin, GFAP, and S100, but not with the rest of the biomarkers (Figure S2 in supporting information).

3.3 | Associations of CSF biomarkers with age, sex, and *APOE-ε4* status

In the whole sample, CSF Aβ42, Aβ42/40, p-tau, t-tau, NfL, neurogranin, sTREM2, YKL-40, GFAP, and S100 were significantly associated with age (Table 2). We stratified the sample into those participants with normal AD biomarkers (A−T−; n = 237) and those within the Alzheimer's *continuum* biomarkers group (A+T*; n = 131). In the A−T− group, there was a significant association with a positive direction between age and CSF NfL, YKL-40, and GFAP (Figure 2). In contrast, in the A+T* group, there was a significant association with a positive direction between age and CSF p-tau, t-tau, NfL, neurogranin, sTREM2, YKL-40, GFAP, and α-synuclein (Figure 2). Importantly, we found a significant interaction between age and CSF Aβ42/40 only in the models with CSF p-tau, t-tau, and neurogranin as outcomes (Figure 2).

Interestingly, there were sex differences in several CSF biomarkers, adjusting by the effect of age and *APOE-ε4* status (Table 2). CSF NfL was higher in men, while CSF neurogranin was higher in women (Table 2). Minor (< 10%) but significant differences were observed in CSF GFAP and IL6 (higher in men) and CSF Aβ40 and YKL-40 (higher in women). Including the CSF Aβ42/40 ratio as a covariable did not change the results, indicating that the observed differences in CSF biomarkers between sexes are not driven by Aβ pathology. In the studied sample, men had a higher education compared to women (Table S1 in supporting information). Adding education as a covariable in the aforementioned analyses only changed the observed sex differences to non-significant for Aβ40 ($P = .075$). Unlike age and sex, *APOE-ε4* status was only significantly associated with CSF Aβ42 and Aβ42/40 ratio (Table 2).

3.4 | Pathophysiological model of changes in the preclinical Alzheimer's *continuum*

Finally, we modelled the trajectories of the standardized (z-scores) CSF biomarkers in the preclinical Alzheimer's *continuum* applying a robust local weighted regression method. Cognizant that this is a cross-sectional analysis and to understand the changes against Aβ and tau, we anchored the model to: (1) Aβ42/40 ratio, (2) p-tau, and (3) p-tau/Aβ42 ratio, as proxies of disease progression. Figure 3 shows the resulting plots. Moreover, for each CSF biomarker and each model, we computed the slopes of a given CSF biomarker before and after the cutoff for the CSF Aβ42/40 ratio, p-tau, or p-tau/Aβ42 ratio, respectively, and tested whether these slopes were statistically significantly different.

Anchoring to CSF Aβ42/40 as a proxy of disease progression, we observed that CSF p-tau, t-tau, and neurogranin start to significantly increase as soon as the CSF Aβ42/40 ratio becomes positive and continue to increase across the preclinical Alzheimer's *continuum*, eventually reaching the highest z-scores of all CSF biomarkers (3 z-scores for CSF p-tau and t-tau and 2.5 z-scores for CSF neurogranin, compared to their basal levels, Figure 3A). In the CSF

TABLE 1 Participants' characteristics and CSF biomarkers by AT group

	Total (n = 381)	A−T− (n = 237, 62.2%)	A+T− (n = 100, 26.2%)	A+T+ (n = 31, 8.1%)	A−T+ (n = 13, 3.4%)	*P*-Value
Age, years	61.2 (4.68)	60.6 (4.44)	61.9 (5.01)	63.8 (4.41)*	61.2 (4.51)	.0004[a]
Female, n (%)	232 (60.9)	146 (61.6)	56 (56.0)	21 (67.7)	9 (69.2)	.56
Education, years	13.4 (3.51)	13.5 (3.46)	13.7 (3.50)	11.9 (3.52)†	14.8 (3.59)	.044[a]
APOE-ε4 carriers, n (%)	201 (52.8)	97 (40.9)	81 (81.0)‡	18 (58.1)	5 (38.5)	<.0001[a]
MMSE	29.1 (0.95)	29.1 (0.92)	29.2 (0.93)	28.8 (1.13)	29.2 (1.17)	.32
FCSRT	15.2 (1.15)	15.2 (1.16)	15.3 (1.11)	15.0 (1.37)	15.7 (0.63)	.45
CSF biomarkers						
Aβ42 (pg/mL)	1302 (564)	1469 (514)	858 (277)‡	1016 (370)‡	2454 (383)	<.0001[a]
Aβ42/40	0.075 (0.020)	0.086 (0.009)	0.054 (0.010)‡	0.044 (0.012)‡,§	0.097 (0.019)	<.0001[a]
Aβ40 (ng/mL)	17.4 (5.03)	16.8 (4.76)	15.9 (3.63)	22.9 (3.59)‡,§	27.0 (3.23)	<.0001[a]
p-tau (pg/mL)	15.9 (6.28)	13.8 (4.23)	15.6 (4.18)*	29.8 (4.86)‡,§	27.6 (4.58)	<.0001[a]
t-tau (pg/mL)	196 (68.1)	175 (48.6)	191 (44.9)‖	338 (52.8)‡,§	317 (51.6)	<.0001[a]
p-tau/Aβ42	0.014 (0.008)	0.010 (0.002)	0.020 (0.007)‡	0.032 (0.011)‡,§	0.011 (0.002)	<.0001[a]
NfL (pg/mL)	81.5 (26.3)	75.7 (23.4)	82.1 (22.6)	115 (33.8)‡,§	103 (28.1)	<.0001[a]
Neurogranin (pg/mL)	796 (326)	712 (250)	743 (224)	1366 (293)‡,§	1423 (328)	<.0001[a]
sTREM2 (ng/mL)	7.91 (7.57)	7.65 (1.95)	7.50 (2.06)	9.85 (2.69)‡,§	11.24 (2.84)	<.0001[a]
YKL-40 (ng/mL)	147 (52.9)	138 (44.0)	142 (46.4)	212 (64.9)‡,§	206 (74.3)	<.0001[a]
GFAP (ng/mL)	7.54 (2.29)	7.20 (2.10)	7.44 (2.25)	10.3 (2.17)‡,§	7.93 (2.01)	<.0001[a]
IL6 (pg/mL)	3.89 (1.46)	3.84 (1.34)	3.94 (1.65)	4.17 (1.76)	3.62 (1.31)	.54
S100 (ng/mL)	1.02 (0.23)	0.99 (0.20)	1.03 (0.24)	1.14 (0.27)¶	1.13 (0.33)	.010[a]
α-synuclein (pg/mL)	198 (80.8)	187 (79.2)	181 (53.9)	298 (66.9)‡,§	322 (57.9)	<.0001[a]

Notes: Data are expressed as mean (M) and standard deviation (SD) or percentage (%), as appropriate. One-way ANOVA followed by Tukey corrected *post hoc* comparisons was used to compare age and education and Pearson's χ^2 test to compare sex and *APOE-ε4* status between AT groups. MMSE and FCSRT scores were compared with an ANCOVA adjusted by age, sex, and education. CSF biomarkers were compared with an ANCOVA adjusted by age and sex followed by Tukey corrected *post hoc* comparisons. The *P*-values indicated in the last column refer to the AT group effect. We did not include the A-T+ group in the analyses, but this group is included in the table for the sake of completeness. *P*-values are corrected for multiple comparisons using FDR approach.

Abbreviations: Aβ40, amyloid-β 40; Aβ42, amyloid-β 42; ANCOVA, analysis of covariance; ANOVA, analysis of variance; APOE, apolipoprotein E; CSF, cerebrospinal fluid; FCSRT, Free and Cued Selective Reminding Test (total recall); GFAP, glial fibrillary acidic protein; IL6, interleukin 6; MMSE, Mini-Mental State Examination; NfL, neurofilament light; p-tau, phosphorylated tau; sTREM2, soluble triggering receptor expressed on myeloid cells 2 (TREM2); t-tau, total tau.

[a]Significant values.

Pairwise *post hoc* comparisons:

*$P < .001$ versus A-T-.

†$P < .05$ versus A+T-.

‡$P < .0001$ versus A-T-.

§$P < .0001$ versus A+T-.

‖$P < .05$ versus A-T-.

¶$P < .01$ versus A-T-.

Aβ42/40-positive participants, the higher absolute slopes are those of CSF p-tau and t-tau, followed by CSF neurogranin (Table 3). These slopes in the CSF Aβ42/40-positive participants were significantly different from the slopes of the CSF Aβ42/40-negative participants (Figure 3A; Table 3). The rest of the CSF biomarkers, except for CSF IL6 and S100, also increased in the CSF Aβ42/40-positive participants, but that increase was considerably less pronounced (as shown by lower absolute slopes). The slopes of CSF sTREM2 and YKL40 in Aβ-positive participants were also significantly different from those that were Aβ-negative. Remarkably, CSF NfL significantly increased as a function of CSF Aβ42/40 in Aβ-positive individuals, although the slope was not significantly different than that of Aβ-negative individuals (Table 3).

When we used CSF p-tau as a proxy of disease progression, we observed that CSF Aβ42/40 dramatically decreases before CSF p-tau becomes positive. Importantly, CSF Aβ42/40 values already reach a decrease of four z-scores from the basal levels at the point when CSF p-tau becomes positive, and they plateau after (Figure 3B, Table 3). This result strongly suggests that changes in soluble Aβ precede those of tau pathophysiology. Remarkably, CSF neurogranin also started to increase before the CSF p-tau cutoff, reaching almost two z-scores from the basal levels at the point when CSF p-tau becomes positive,

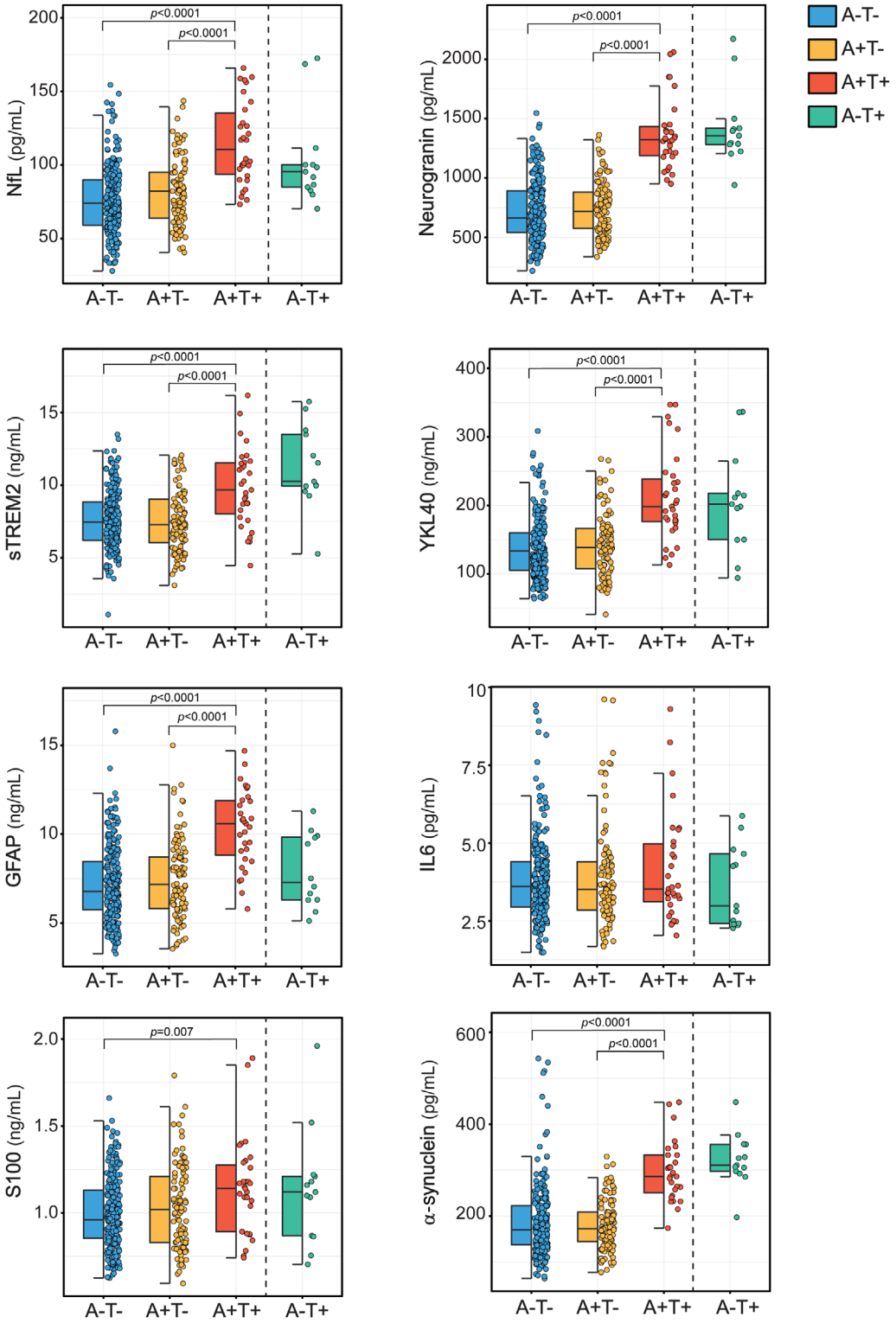

FIGURE 1 Comparison of cerebrospinal fluid (CSF) biomarkers between AT groups. Dot and box plots depicting the levels of each CSF biomarker in each of the AT groups. The box plots depict the median (horizontal bar), interquartile range (IQR, hinges), and 1.5 × IQR (whiskers). Because our goal is to assess CSF biomarkers in the Alzheimer's *continuum*, we did not include the A−T+ group (ie, non-AD pathologic change) in the analyses, but this group is included in the figure for the sake of completeness. *P*-values were assessed by a one-way analysis of covariance adjusted by age and sex, followed by Tukey corrected pair-wise *post hoc* comparisons

TABLE 2 Effect of age, sex, and *APOE-ε4* status on CSF biomarkers

	Age		Sex			*APOE-ε4*		
Biomarker	*β* (SE)	*P*-value	Male, M (SD)	Female, M (SD)	*P*-value	Non-carriers, M (SD)	Carriers, M (SD)	*P*-value
Aβ42	−0.12 (0.050)	.023[a]	1203 (529)	1306 (526)	.26	1437 (563)	1114 (446)	<.0001[a]
Aβ42/40	−0.24 (0.047)	<.0001[a]	0.073 (0.019)	0.074 (0.019)	.47	0.081 (0.018)	0.067 (0.019)	<.0001[a]
Aβ40	0.090 (0.052)	.092	16.3 (4.82)	17.6 (4.63)	.043[a]	17.6 (5.03)	16.6 (4.42)	.33
p-tau	0.24 (0.051)	<.0001[a]	15.1 (5.79)	15.7 (6.02)	.20	15.4 (6.26)	15.5 (5.64)	.81
t-tau	0.23 (0.052)	<.0001[a]	188 (65.1)	195 (64.4)	.20	193 (69.5)	191 (60.2)	.93
NfL	0.41 (0.046)	<.0001[a]	88.7 (24.5)	75.5 (25.4)	<.0001[a]	79.5 (25.8)	81.7 (25.9)	.81
Neurogranin	0.15 (0.052)	.008[a]	723 (287)	808 (310)	.013[a]	782 (314)	767 (294)	.94
sTREM2	0.21 (0.051)	<.0001[a]	7.81 (2.22)	7.78 (2.09)	.94	8.03 (2.17)	7.59 (2.09)	.27
YKL-40	0.41 (0.048)	<.0001[a]	140 (50.3)	148 (51.1)	.043[a]	147 (50.9)	144 (51.0)	.94
GFAP	0.33 (0.049)	<.0001[a]	7.93 (2.29)	7.27 (2.28)	.043[a]	7.50 (2.43)	7.55 (2.19)	.91
IL6	−0.068 (0.053)	.20	4.09 (1.51)	3.77 (1.42)	.043[a]	3.94 (1.52)	3.86 (1.42)	.76
S100	0.12 (0.052)	.029[a]	1.04 (0.24)	1.00 (0.21)	.20	1.00 (0.22)	1.03 (0.23)	.76
α-synuclein	0.092 (0.053)	.092	194 (83.3)	193 (74.2)	.86	196 (81.4)	192 (60.2)	.91

Notes: Each CSF biomarker was assessed by a linear model with age, sex, and *APOE-ε4* status as main effects. The standardized regression coefficients (*β*) and standard errors (SE) are depicted for age and mean (M) and standard deviation (SD) for sex and *APOE-ε4*. *P*-values are corrected for multiple comparisons using FDR approach.
Abbreviations: Aβ40, amyloid-*β* 40; Aβ42, amyloid-*β* 42; APOE, apolipoprotein E; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; IL6, interleukin 6; NfL, neurofilament light; p-tau, phosphorylated tau; sTREM2, soluble TREM2; t-tau, total tau.
[a]Significant values.

and plateaus in individuals that are already tau positive (Figure 3B, Table 3). The rest of CSF biomarkers (except CSF IL6) also increase as a function of CSF p-tau but their increase is less pronounced (< 0.5 z-scores). As expected, CSF t-tau levels parallel those of CSF p-tau. When we used CSF p-tau/Aβ42 as a proxy of disease progression, the results were very similar to those of CSF p-tau. Before CSF p-tau/Aβ42 becomes positive (Figure 3C), CSF Aβ42/40 significantly decreases to later plateau, and eventually reach a decrease of six z-scores. In contrast, the rest of CSF biomarkers do not significantly change before the CSF p-tau/Aβ42 cutoff, but they significantly increase after surpassing this cutoff, except for CSF IL6 and S100 (Table 3). CSF p-tau and t-tau have the steeper slopes and reach >4 z-scores of its basal levels. CSF neurogranin reaches two z-scores from its basal levels and the rest of CSF biomarkers showed a more moderate increase (< 2 z-scores).

4 | DISCUSSION

In this cross-sectional study we aimed at determining CSF biomarker changes in the preclinical Alzheimer's *continuum* in a well-characterized cohort of cognitively unimpaired individuals. Our main results are the following. (1) Changes in soluble Aβ occur earlier than in any other biomarker studied. (2) After soluble Aβ biomarkers become positive, there is a steep increase in tau-related (p-tau and t-tau) and synaptic dysfunction (neurogranin) CSF biomarkers and, to a lesser extent, in axonal injury (NfL) and glial (sTREM2, YKL40, GFAP) biomarkers. (3) Tau-related and synaptic dysfunction biomarkers increase across age only in Aβ-positive individuals, whereas axonal damage and glial biomarkers increase during aging in both Aβ-positive and -negative individuals. Altogether, our results show that the first observable changes in the Alzheimer's *continuum* are those in soluble Aβ, and as soon as individuals become Aβ positive, changes in tau pathology and synaptic dysfunction occur. Changes in CSF NfL and glial biomarkers also occur during disease progression, but these are less pronounced.

We initially observed that all CSF biomarkers (except IL6) increased in the A+T+ group, but not in the A+T− group. However, this approach has the limitation that it simplifies the preclinical stage of the Alzheimer's *continuum* in only two stages, that is A+T− and A+T+. Therefore, we applied two additional approaches in order to define more precisely the CSF biomarker changes in the Alzheimer's *continuum*. We first assessed the changes of CSF biomarkers as a function of age and, second, as a function of Aβ (as defined by the CSF Aβ42/40 ratio) and tau (as defined by CSF p-tau) pathophysiology.

We observed that all biomarkers (except CSF Aβ40, IL6, and α-synuclein) increase with age. Interestingly, for CSF p-tau, t-tau, and neurogranin, this increase is specifically linked to Aβ pathology. When we used the CSF Aβ42/40 ratio as a proxy of disease progression, CSF p-tau, t-tau, and neurogranin are the biomarkers that change more markedly, and before the Aβ-positivity cutoff. This finding is consistent with the notion that CSF p-tau and neurogranin are biomarkers mainly related to AD, and less linked to other forms of neurodegeneration.[6,25,26] In contrast, CSF NfL and glial biomarkers CSF YKL40, GFAP, and sTREM2 (the latter approaching significance) change through aging in both Aβ-positive and -negative individuals. Although this increase is more pronounced in Aβ-positive than in negative individuals (as shown by the *β* slopes), the fact that there is no interaction between Aβ and age indicates that Aβ does not

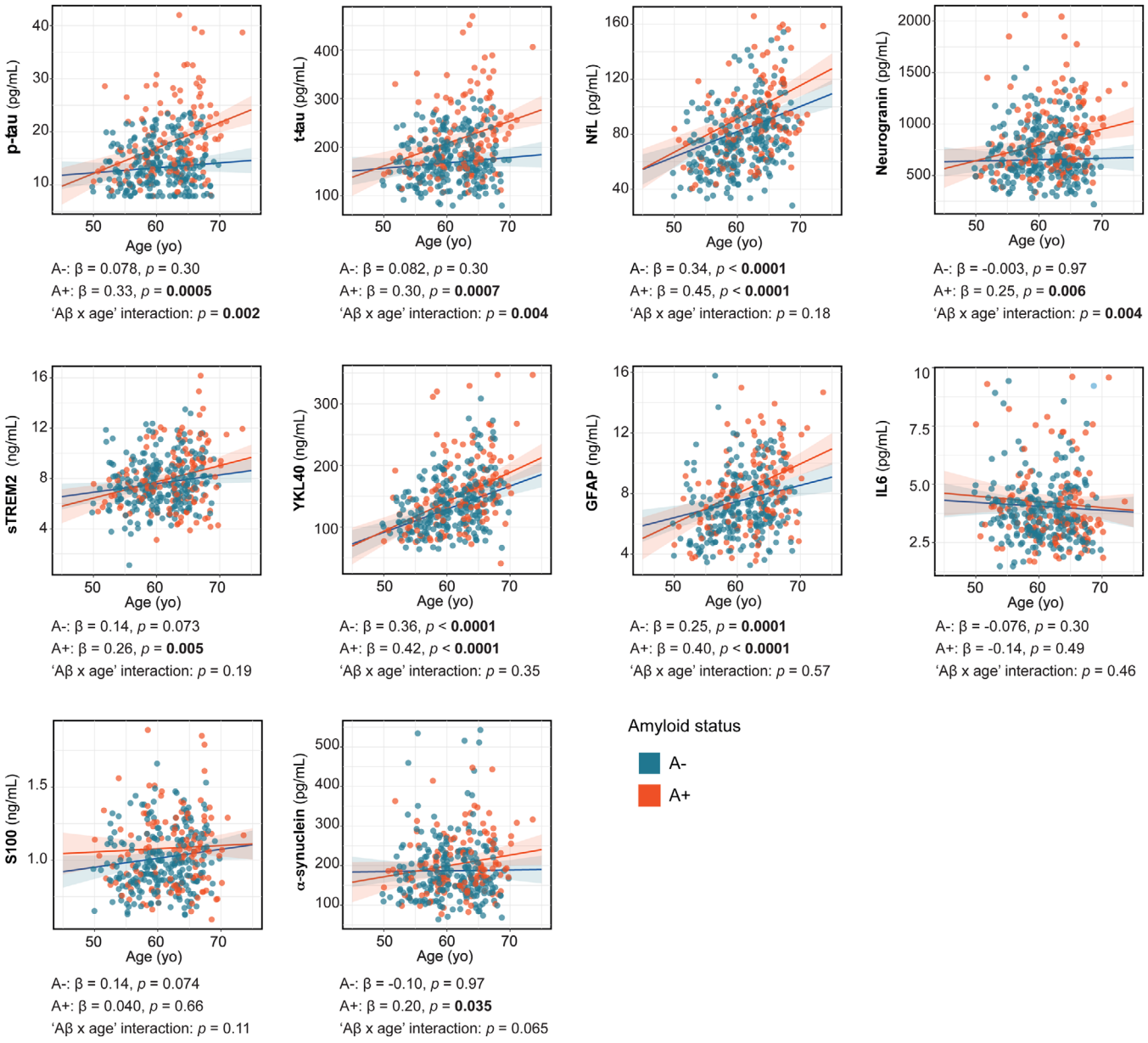

FIGURE 2 Association of cerebrospinal fluid (CSF) biomarkers with age. Scatter plots representing the associations of each of the CSF biomarkers with age in the A–T– (ie, normal AD biomarkers) and the A+T* (ie, Alzheimer's *continuum*) groups. Each point depicts the value of the CSF biomarker of an individual and the solid lines indicate the regression line for each of the groups. The standardized regression coefficients (*β*) and the *P*-values are shown and were computed using a linear model adjusting for age, sex, and apolipoprotein E (*APOE*)-*ε4*. Additionally, we also computed the "Aβ42/40 x age" interaction term. All *P*-values are corrected for multiple comparisons using the FDR approach

significantly modify the association between age and the specific CSF biomarker. These results are in line with previous studies that show that glial biomarkers increase with aging and also in other neurological diseases besides AD.[27-36] Of note, our sample comprises a higher prevalence of A+T– than A+T+ individuals. We speculate that the increase of CSF NfL and glial markers throughout age would be more pronounced in the latter stages of the preclinical Alzheimer's *continuum* (ie, A+T+) and in early symptomatic stages. When we modeled the changes of CSF biomarkers as a function of the CSF Aβ42/40 ratio, we observe similar results, that is CSF NfL and glial biomarker changes are less pronounced than those of tau-related and synaptic biomarkers. It is remarkable that CSF NfL behaves differently from CSF t-tau, despite the fact that both are biomarkers of neurodegeneration. Unlike CSF t-tau, CSF NfL may reflect a degree of age-related neuronal and axonal injury independent from A*β* pathology. Moreover, CSF NfL less marked increase as a function of CSF Aβ42/40 may reflect that neuronal and axonal injury may be more prominent in later stages of preclinical AD, where A*β* and tau pathology are already present. These findings are also consistent with previous studies showing that CSF t-tau and NfL might provide different information regarding neurodegeneration. While CSF t-tau mainly reflects an A*β*-related change in tau metabolism and secretion that eventually

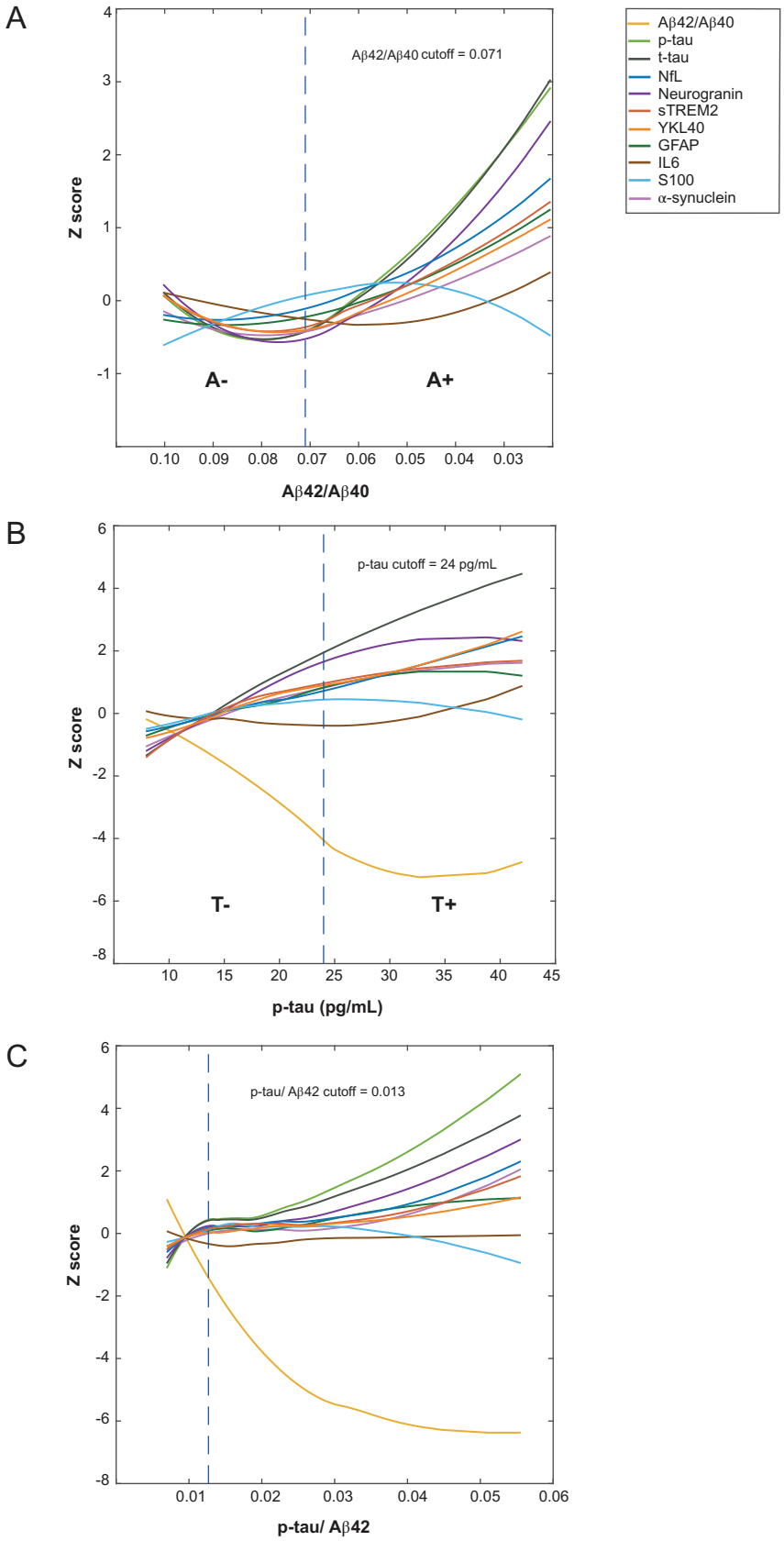

FIGURE 3 Cerebrospinal fluid (CSF) biomarker trajectories. The graphs represent the z-scores changes of each CSF biomarker using the mean and the standard deviation of that CSF biomarker in the A−T− group as a reference. The resulting z-scores are shown as a function of CSF Aβ42/40 (A) p-tau (B) or p-tau/Aβ42 (C) using a robust local weighted regression method. The solid lines depict the trajectory of each CSF biomarker. The dashed lines depict the cutoff for CSF Aβ42/40, p-tau, and p-tau/Aβ42, respectively. The horizontal axis direction of CSF Aβ42/40 (A) was inverted.

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TABLE 3 Association between each CSF biomarker and Aβ42/40, p-tau, and p-tau/Aβ42

Model 1: CSF Aβ42/40 as proxy of disease progression					
	Aβ42/40 negative		Aβ42/40 positive		Slopes difference
	B (SE)	*P*-value	B (SE)	*P*-value	*P*-value*
p-tau	13.8 (8.30)	.46	−66.2 (11.2)	.002[a]	<.0001[a]
t-tau	15.0 (8.24)	.24	−52.3 (9.94)	<.0001[a]	<.0001[a]
NfL	−0.82 (11.8)	.9	−29.1 (9.24)	.004[a]	.085
Neurogranin	21.9 (7.91)	.064	−39.2 (8.94)	.0001[a]	<.0001[a]
sTREM2	14.5 (11.2)	.46	−20.9 (7.03)	.005[a]	.015[a]
YKL-40	9.83 (9.80)	.46	−31.2 (9.09)	.002[a]	.005[a]
GFAP	−6.22 (11.7)	.66	−24.7 (8.74)	.007[a]	.23
IL6	8.95 (11.2)	.53	0.064 (10.7)	.10	.57
S100	−23.0 (11.2)	.21	−2.75 (10.1)	.87	.22
α-synuclein	11.0 (11.1)	.46	−21.7 (6.56)	.003[a]	.019[a]
Model 2: CSF p-tau as proxy of disease progression					
	p-tau negative		p-tau positive		Slopes difference
	B (SE)	*P*-value	B (SE)	*P*-value	*P*-value*
Aβ42	0.12 (0.013)	<.0001[a]	0.023 (0.041)	.71	.066
Aβ42/40	−0.24 (0.032)	<.0001[a]	−0.041 (0.056)	.63	.028[a]
Aβ40	0.13 (0.008)	<.0001[a]	0.076 (0.024)	.022[a]	.066
t-tau	0.19 (0.006)	<.0001[a]	0.14 (0.022)	<.0001[a]	.070
NfL	0.076 (0.016)	<.0001[a]	0.12 (0.053)	.083	.58
Neurogranin	0.17 (0.007)	<.0001[a]	0.057 (0.041)	.31	.055
sTREM2	0.12 (0.013)	<.0001[a]	0.061 (0.022)	.029[a]	.066
YKL-40	0.11 (0.014)	<.0001[a]	0.12 (0.061)	.13	.96
GFAP	0.074 (0.015)	<.0001[a]	−0.012 (0.055)	.90	.19
IL6	−0.014 (0.018)	.43	0.006 (0.071)	.93	.94
S100	0.073 (0.018)	<.0001[a]	−0.064 (0.061)	.47	.066
α-synuclein	0.085 (0.013)	<.0001[a]	0.089 (0.029)	.022[a]	.96
Model 3: CSF p-tau/Aβ42 as proxy of disease progression					
	p-tau/Aβ42 negative		p-tau/Aβ42 positive		Slopes difference
	B (SE)	*P*-value	B (SE)	*P*-value	*P*-value*
Aβ42	−48.2 (44.1)	.51	−55.7 (9.21)	<.0001[a]	.87
Aβ42/40	−387 (59.3)	<.0001[a]	−152 (13.5)	<.0001[a]	.001[a]
Aβ40	−71.6 (31.0)	.22	32.0 (9.21)	.001[a]	.033[a]
p-tau	84.9 (36.2)	.13	109 (11.2)	<.0001[a]	.75
t-tau	54.4 (35.1)	.40	86.2 (10.8)	<.0001[a]	.65
NfL	63.0 (54.5)	.51	46.3 (10.9)	.0001[a]	.83
Neurogranin	12.3 (35.2)	.86	59.8 (10.2)	<.0001[a]	.58
sTREM2	−31.9 (51.9)	.86	30.9 (8.7)	.0008[a]	.58
YKL-40	−12.6 (44.9)	.86	43.1 (10.8)	.0002[a]	.58
GFAP	6.12 (53.6)	.91	36.8 (10.5)	.0008[a]	.75
IL6	68.6 (51.0)	.47	10.6 (12.7)	.44	.58
S100	24.3 (56.0)	.86	3.28 (11.6)	.78	.83
α-synuclein	−13.6 (52.6)	.86	31.2 (7.83)	.0002[a]	.65

Notes: For each CSF biomarker we computed the linear regression unstandardized coefficients (B) of the z-scores and standard errors (SE) in each of the negative or positive groups. *P*-values are corrected for multiple comparisons using FDR approach.

Abbreviations: Aβ40, amyloid-β 40; Aβ42, amyloid-β 42; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; IL6, interleukin 6; NfL, neurofilament light; p-tau, phosphorylated tau; sTREM2, soluble TREM2; t-tau, total tau.

[a]Significant values.

*P- value tests whether the regression slopes of the two groups are equal.

may translate into AD-type neurodegeneration, CSF NfL also reflects neurodegeneration not due to Aβ pathology.[37] The underlying cause of that increase on CSF NfL in Aβ-negative cognitively unimpaired individuals still needs to be clarified, but we speculate that age-related factors such as vascular and/or other neurodegeneration-related factors (eg, co-pathology with Lewy body disease, TDP-43, or hippocampal sclerosis) may at least partially explain those differences. Together, these findings also favor the use of CSF NfL as marker of neurodegeneration ("N") in the amyloid/tau/neurodegeneration (ATN) framework, instead of CSF t-tau. The CSF glial markers parallel the increase of CSF NfL in the model using CSF Aβ42/40 as a proxy of disease progression. Both CSF NfL and CSF glial biomarkers increase as a function of Aβ pathology (as shown as decreased CSF Aβ42/40 ratio) in the Aβ-positive but not the Aβ-negative group. This may indicate that Aβ pathology underlies the neuronal injury and glial response in these individuals. Yet, this idea does not exclude that, in other individuals, other mechanisms different from Aβ pathology may also trigger neuronal injury and glial response.

Interestingly, we found sex differences in some of the studied CSF biomarkers. Men showed higher levels of NfL in CSF, which is consistent with previous studies,[38,39] and with the fact that the prevalence of A−T−N+ individuals (ie, neurodegeneration positive, as measured by hippocampal volume or cortical thickness, but Aβ- and tau-negative) is higher in men than in women.[40] An unexpected finding was that CSF neurogranin is overall higher in women than in men. Whether this indicates that women may have a greater susceptibility to synaptic dysfunction remains to be clarified in further studies. The effect of sex, often overlooked, should be further studied to better understand AD pathogenesis and design preventive strategies.

The main limitation of cross-sectional biomarker studies in the sporadic preclinical Alzheimer's *continuum* is the lack of a proxy for the temporal evolution of the disease, such as the concept of estimated years from symptom onset in autosomal-dominant AD. We chose the CSF Aβ42/40 ratio to understand how biomarkers evolve during the *continuum* because changes in soluble Aβ are those that occur earlier in the Alzheimer's *continuum*, both in autosomal-dominant and sporadic AD, and occur before than Aβ PET becomes positive.[41,42] Moreover, CSF Aβ42/40 ratio is less affected by pre-analytical factors and inter-individual differences than CSF Aβ42. Remarkably, the CSF Aβ42/40 ratio cutoff used here is higher (and thus expected to be more sensitive) than the one commonly used for diagnostic purposes, given that our goal is to sensitively detect very early changes in Aβ pathology. This is the reason why we used a two-Gaussian mixture modelling to determine the cutoff instead of conducting a comparison with a gold-standard (such as Aβ PET), which would have rendered a more specific cutoff for deposited Aβ but less sensitive for early soluble Aβ-related pathophysiological changes. Being aware that using the CSF Aβ42/40 ratio as a proxy of disease progression may seem that we are assuming a sequence of events in which soluble Aβ comes first, we also explored CSF biomarker changes using CSF p-tau and the p-tau/Aβ42 ratio as proxies, the latter being highly correlated with Aβ PET load.[43] We similarly computed the CSF p-tau and p-tau/Aβ42 cutoffs using a two-Gaussian mixture modelling and these resulted lower (thus expected to be more sensitive) than those usually used in the clinical setting. Even with such sensitive cutoffs, changes in CSF Aβ42/40 always come first, before CSF p-tau or the p-tau/Aβ42 become positive or before any other biomarker changes. Using these models, we also observed the initial increase in CSF neurogranin, earlier than changes in neuronal and axonal injury and glial-related biomarkers. Overall, these results are similar to those that have been reported in autosomal-dominant AD in both cross-sectional studies and longitudinal studies, in which initial changes in Aβ are followed by tau-related biomarkers and, afterward, neurodegeneration and glial markers.[1,2,15,44-47] Recently, a very interesting study in the BIOFINDER cohort modelled the changes in several CSF and plasma biomarkers in predementia individuals.[19] The authors used Aβ PET as a proxy of the disease progression, instead of CSF Aβ42/40, but their results were similar. The main difference with our study is that we observe earlier changes in CSF neurogranin, while they observed an early inflection of CSF NfL. We may argue that changes in soluble Aβ are more linked to early synaptic dysfunction, while Aβ deposition (as measured by Aβ PET) captures a slightly later event that may be more associated to neuronal injury. Moreover, our study includes a high number of cognitively unimpaired individuals that are Aβ positive but still tau negative, which may have allowed us to observe these early changes in CSF neurogranin.

It is worth noting the local regression methods we applied to model the association between CSF biomarker changes as a function of CSF Aβ42/40, p-tau, or p-tau/Aβ42. These methods are particularly suited to model non-linear associations without the need to specify any function or fit a model a priori. Still, the smoothing parameter ("span") needs to be specified. To this end, we chose a smoothing parameter that produced curves with a maximum of two inflection (change) points to avoid overfitting. Similar methods have been applied in previous reports displaying the cross-sectional variation of CSF and other biomarkers against proxies of AD progression.[2,41,45]

The main limitations of our study are the following. (1) It is a cross-sectional analysis and longitudinal studies are needed to confirm the results. (2) Aβ- and tau-pathology cutoffs derived herein might not be applied to clinical cohorts because ALFA+ is a very specific cohort aimed at studying preclinical AD and with a high percentage of *APOE-ε4* carriers and Aβ-positive individuals. Moreover, the diagnostic and prognostic value of these cutoffs in clinical population has not been assessed. (3) Although it includes CSF biomarkers related to different pathophysiological processes, we do not include biomarkers related to vascular function or TDP-43 pathology. (4) We did not include neuroimaging biomarkers such as structural magnetic resonance imaging or Aβ PET. (5) We measured total levels of α-synuclein, which probably does not reflect Lewy body disease or α-synuclein deposition as phosphorylated or oligomeric forms do,[48-51] but most likely reflects neuronal injury.

In conclusion, our study shows that biomarkers reflecting multiple pathophysiological pathways change very early in the Alzheimer's *continuum*. tau-related and synaptic biomarkers are those that change earlier and more markedly, as soon as there is evidence of incipient Aβ pathology. In order to develop therapeutic strategies targeting this early stage, it is fundamental to understand "which" are the

biological pathways involved and "when" in the long preclinical Alzheimer's *continuum* they are involved. Our results favor the idea of targeting tau and synaptic dysfunction in the earliest stages of the preclinical Alzheimer's *continuum*, as soon as alterations in Aβ occur.

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CONFLICTS OF INTEREST

JLM has served/serves as a consultant or at advisory boards for the following for-profit companies, or has given lectures in symposia sponsored by the following for-profit companies: Roche Diagnostics, Genentech, Novartis, Lundbeck, Oryzon, Biogen, Lilly, Janssen, Green Valley, MSD, Eisai, Alector, BioCross, GE Healthcare, ProMIS Neurosciences. KB has served as a consultant or at advisory boards for Abcam, Axon, Biogen, Lilly, MagQu, Novartis, and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. HZ has served at scientific advisory boards for Roche Diagnostics, CogRx, Samumed, and Wave, and has given lectures in symposia sponsored by Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. GK is a full-time employee of Roche Diagnostics GmbH. MS is a full-time employee of Roche Diagnostics International Ltd. The remaining authors declare that they have no conflicts of interest.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The ALFA+ study (ALFA-FPM-0311) was approved by the Independent Ethics Committee "Parc de Salut Mar," Barcelona, and registered at Clinicaltrials.gov (Identifier: NCT02485730). All participating subjects and signed the study's informed consent form that had also been approved by the Independent Ethics Committee "Parc de Salut Mar," Barcelona.

AUTHOR CONTRIBUTIONS

Marta Milà-Alomà, Gemma Salvadó, Juan Domingo Gispert, Natalia Vilor-Tejedor, José Maria González-de-Echávarri, Marc Suárez-Calvet, and José Luis Molinuevo analyzed and interpreted the data. Kaj Blennow, Henrik Zetterberg, and Marc Suárez-Calvet analyzed the CSF samples. Maryline Simon and Gwendlyn Kollmorgen developed and provided the NeuroToolKit (Roche). Oriol Grau-Rivera, Aleix Sala-Vila, Gonzalo Sánchez-Benavides, Eider M. Arenaza-Urquijo, Marta Crous-Bou, José Maria González-de-Echávarri, Carolina Minguillon, Karine Fauria, Marc Suárez-Calvet, and José Luis Molinuevo contributed with ALFA+ participants' data. Marta Milà-Alomà, Juan Domingo Gispert, Marc Suárez-Calvet, and José Luis Molinuevo designed the study and wrote the manuscript. All authors critically reviewed and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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