Research Article

Title:

Soluble TAM receptors sAXL and sTyro3 predict structural and functional protection in Alzheimer´s disease

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SUMMARY

There is an urgent need to improve the understanding of neuroinflammation in Alzheimer´s disease (AD). We analyzed cerebrospinal fluid inflammatory biomarker correlations to brain structural volume and longitudinal cognitive outcomes in the DELCODE study and a validation cohort of the ACE Alzheimer Center Barcelona. We investigated whether respective biomarker changes are evident before onset of cognitive impairment. YKL-40, sTREM2, sAXL, sTyro3, MIF, complement factors C1q, C4 and H, ferritin and ApoE protein were elevated in pre-dementia subjects with pathological levels of tau or other neurodegeneration markers, demonstrating tight interactions between inflammation and accumulating neurodegeneration even before onset of symptoms. Intriguingly, higher levels of ApoE and soluble TAM receptors sAXL and sTyro3 were related to larger brain structure and stable cognitive outcome at follow up. Our findings indicate a protective mechanism relevant for intervention strategies aiming to regulate neuroinflammation in subjects with no or subjective symptoms but underlying AD pathology profile.

INTRODUCTION

Neuroinflammation represents a pathological hallmark of Alzheimer´s disease (AD) and other neurodegenerative disorders, though the involved mechanisms are still being investigated (Heneka et al., 2015). No biomarkers of neuroinflammation are sufficiently established for use in clinical practice or studies. Though several promising candidate markers have emerged from research, there is still a lack of characterization regarding the exact time course of changes, interaction with other pathological features and prognostic potential. Only a few studies have investigated the relationship between cerebrospinal fluid (CSF) inflammatory markers and either gray matter integrity or cognitive decline in AD. Higher levels of CSF sTREM2 have been related to larger gray matter volume in temporal and parietal regions in patients with mild cognitive impairment (MCI) and to attenuated hippocampal structure and cognitive decline in AD patients when adjusting for markers of AD pathology (Ewers et al., 2019; Gispert et al., 2016a). These findings have been interpreted as a protective effect of inflammation or brain swelling at certain disease stages, which might be in line with potential bimodal trajectories of sTREM2 levels across disease stages (Suárez-Calvet et al., 2018). Similar results have been described for CSF YKL-40, whereas another study reported negative correlations between YKL-40 and cortical thickness of AD signature regions in amyloid positive (A+) but not amyloid negative (A-) controls or MCI subjects (Alcolea et al., 2015; Gispert et al., 2016b). These findings suggest that biomarkers represent different inflammatory mechanisms and relate to

increased or decreased brain structure depending on the clinical-pathological stage of disease, indicating the need for further characterizations of these interactions.

Here, we investigated experimental CSF biomarkers previously described by us and others to be linked to AD pathology: Inflammation & immune markers sTREM2, sAXL, sTyro3, YKL-40, MCP-1, IP-10, MIF, IL-6, IL-18, CRP, complement factors C1q, C3, C3b, C4, B, H; non-tau neurodegeneration markers neurogranin and FABP-3; and multifactorial markers ferritin and ApoE (Brosseron et al., 2019, 2020). The panel was determined in CSF samples from the DELCODE cohort of the German Center for Neurodegenerative Diseases (DZNE), a longitudinal observational study with focus on subjective cognitive decline (SCD) subjects, likely to represent patients prior to development of MCI (Jessen et al., 2018, 2020). CSF biomarker data were analyzed in relation to AD biomarkers beta-amyloid (Aβ) and tau isoforms, to brain structure (composite scores derived from Braak regions of interest, ROI) and longitudinal cognitive changes over up to 5 years (see online methods). To improve the current understanding on changes of inflammatory markers during early disease stages and identify those useful for future clinical trials, we first addressed the following questions in the DELCODE cohort: a) Which inflammatory markers are related to AD pathology and are these relationships detectable even before the onset of cognitive impairment; b) How do inflammatory markers relate to brain structure in the context of AD; c) Do these inflammatory markers predict longitudinal cognitive changes? Ultimately, analyses for the markers with the strongest relations to AD features were replicated within the framework of the EU-JPND funded PREADAPT project in a cohort of SCD and MCI subjects of the ACE Alzheimer Center Barcelona (ACE) Alzheimer Center, Barcelona (Boada et al., 2014).

RESULTS

DELCODE demographics

Descriptive statistics of the DELCODE cohort are provided in Data S1 AT1. We included a total of 309 subjects and baseline CSF samples from the DZNE DELCODE study (Jessen et al., 2018) consisting of healthy controls (HC), SCD and MCI subjects, supplemented by patients with dementia of the Alzheimer´s type (DAT) and cognitively normal first-degree relatives of DAT patients. Demographically, relatives were youngest and MCI and DAT subjects oldest. The *APOE* ε4 allele was enriched in DAT subjects. Sex was unequally distributed between screening diagnosis groups, and there was a trend towards differences in body mass index (BMI). All 4 variables (age, sex, *APOE* status, BMI) related to CSF panel biomarker levels in marker-specific manner (Data S1 AT2) and were consequently tested as potential covariates in following analyses. The routine AD biomarkers and their ratios, preclinical Alzheimer´s cognitive composite (PACC5)

score and Braak ROI structural imaging scores differed strongly between the screening diagnosis groups, always separating MCI and DAT subjects from the HC, SCD and relatives' groups (Data S1 AT1). Within the experimental biomarker panel, only FABP-3 showed elevation in DAT subjects compared to all other groups after correction for multiple testing (details provided in Data S1 AT3 and AF1).

Elevated inflammatory markers in pre-dementia tau pathology and neurodegeneration

To determine the inflammatory CSF markers most related to brain structure and cognition, we first examined their relationship with routine AD markers in DELCODE. We applied binary schematics approximating the amyloid/tau/neurodegeneration (A/T/N) classification by using DELCODE-specific cut-off values for pathological levels of Aβ and tau isoforms (see online methods): A/T (using CSF Aβ42/40 ratio and p-tau-181), as well as A/N (using CSF Aβ42/40 ratio and t-tau as neurodegeneration marker) (Jack et al., 2016). We did not apply the full A/T/N scheme as p-tau-181 and t-tau were highly correlated in the AD-focused DELCODE cohort. Instead, we compared results of A/T to A/N as complementary confirmative approaches.

Using the A/T scheme and adjusting for multiple testing and the biomarker-specific covariates, both neurodegeneration markers (FABP-3 and neurogranin), both multifactorial markers (ferritin and ApoE) as well as the inflammation markers sTREM2, sAXL, sTyro3, YKL-40, MIF and complement C1q were significantly elevated in A-T+ and A+T+, but not A+T- groups relative to A-T- (Figure 1, further results and details in supplement table S1 and figure S1). Application of A/T resulted in limited sample size of the A-T+ group (N = 7) which nonetheless showed significant differences compared to the other groups (N = 63 – 167). When using A/N, the smallest group was A-N+ (N = 20) compared to 51 to 154 subjects in other groups. All markers that had significant changes in A/T after multiple testing correction showed the same effect in A/N (Data S1 AT4 and AF2).

As these changes in CSF inflammatory markers were driven by subjects with elevated CSF tau isoform levels within the whole DELCODE cohort, we next tested whether this persisted in objectively cognitive normal (CN) individuals only (HC, SCD and DAT relatives). Furthermore, we compared the strength of biomarker level alterations in CN subjects to those with objective cognitive impairment (MCI or DAT). This resulted in a cognitive staging of CN, MCI and DAT, each either T+/T- or N+/N-. In this analysis, YKL-40, sTREM2, sAXL, sTyro3, MIF and C1q were again significantly elevated n all T+ groups (CN T+, MCI T+, DAT T+) against T- (CN T-, MCI T-, DAT T-) groups after correction for multiple testing (figure 1, supplement table S2 and figure S2). Of the multifactorial markers, ferritin showed the same pattern as

inflammation and neurodegeneration markers, while ApoE was elevated primarily in the CN T+ subjects against all other groups except MCI T+. These findings were mirrored when using the cognitive staging plus N schematic, with elevation of markers in any N+ group against the N- groups (Data S1 AT5 and AF3). The non-tau neurodegeneration markers neurogranin and FABP-3 were similarly elevated in all T+ and N+ groups, confirming that these groups indeed represent neurodegenerative phenotypes and are not just subjects with high levels of tau isoforms. In summary, we observed a robust elevation of specific inflammatory markers (YKL-40, sTREM2, sAXL, sTyro3, MIF and C1q) as well as non-tau neurodegeneration (neurogranin, FABP-3) and multifactorial markers (ferritin and ApoE) in subjects with pathological CSF tau isoform levels, even if these subjects by objective criteria were still classified as cognitively normal.

Continuous relations of inflammatory to sub-threshold AD pathology markers

We next tested interrelations between the different CSF biomarkers in DELCODE. Bivariate Spearman correlation matrices revealed a high degree of correlation between the experimental panel markers to each other and to the CSF routine AD markers (Data S1 AF4). We also tested correlations with neurofilament light chain (Nf-L) as an established neurodegeneration biomarker, with the limitation that Nf-L was determined in plasma, not CSF. Plasma Nf-L levels showed a weak, positive correlation to various experimental CSF markers. Overall, these correlations were robust against adjustment for age, sex, *APOE* status and BMI (Data S1 AF5) and were highly comparable to other cohorts in which we analyzed interrelations of similar marker panels (Brosseron et al., 2018, 2019).

To further characterize these relations between routine CSF AD biomarkers and the experimental markers, we used generalized additive models (GAM, figure 2, supplement table S3 and figure S3). GAM hold the advantage of a flexible modeling of non-linear relationships between variables (see online methods). These analyses revealed a significant relation of the Aβ42/40 ratio with ApoE, sAXl, sTyro3, YKL-40 and MIF after correction for multiple testing (supplement table S3). Interestingly, decreased CSF Aβ42/40 ratio was related to slightly lower marker levels when adjusting the model for levels of p-tau-181. Importantly, the same markers were positively related to p-tau-181. Herein, correlations of ApoE, sAXl, sTyro3, YKL-40 and MIF as well as ferritin, sTREM2, complement C1q, C4 and factor H withstood correction for multiple testing. When limiting the sample to cognitively unimpaired individuals, these findings were largely confirmed (Data S1 AT6). Notably, the relation between inflammatory markers and tau pathology was steepest at p-tau-181 levels below the pathological cut-off and leveled off after pathological p-tau-181 levels were reached (figure 2, supplement figure S3). In line with previous findings, the GAM models indicated that changes of inflammatory markers are primarily

accompanied by tau pathology. As illustrated by contour plots combining the relations with Aβ42/40 ratio and p-tau-181 (figure 2, supplement figure S3), inflammatory marker levels were highest in subjects with Aβ42/40 ratios close to or above the threshold for pathological levels. However, this group consisted only of a small number of subjects in DELCODE. In subjects with pathological Aβ42/40 ratio, inflammatory markers strongly increased with p-tau-181 levels, too.

The list of AD-related candidate markers identified by GAM included all markers that were found significant in the group-wise analyses of DELCODE described above. Since small group size in the group-wise analyses might have limited our power, we considered all markers as related to AD pathology that were identified by GAM analyses for further exploration of correlations to Braak ROI structural imaging scores and cognition. This selection thereby included 10 markers: YKL-40, sTREM2, sAXL, sTyro3, MIF, complement C1q, C4, factor H, ferritin, ApoE.

Braak ROI structure is positively related to sAXL, sTyro3, ApoE

Next, we assessed whether these AD-related CSF inflammatory markers are associated with brain structure. We utilized composite scores of six *a priori* structural ROIs that mirror Braak stages (figure 3A) (Baker et al., 2017; Schöll et al., 2016). In a first step, we performed a multivariate regression in DELCODE. Herein, mean structure (measures of volume for subcortical and of thickness for cortical ROI) in each Braak ROI was jointly predicted by all the 16 immune / inflammatory markers, adjusting for age and sex (figure 3B). Multivariate regression models were significant for all Braak ROI except of Braak VI (Braak I: $R^2 = 0.12$, $p < 0.01$; Braak II: $R^2 = 0.09$, $p = 0.01$, Braak III: $R^2 = 0.15$, $p < 0.001$, Braak IV: $R^2 = 0.11$, $p = 0.017$, Braak V: $R^2 = 0.13$, $p < 0.001$). As shown in figure 3B, the variance in structure (R^2) explained by all neuroinflammation markers was highest in the limbic Braak III region with 15%, followed by Braak V (covering frontal and parietal regions) with 13% of variance. Notably, when additionally adjusting for AD routine CSF markers Aβ42/40 ratio and p-tau-181, the results were similar with inflammatory markers explaining up to 13% additional variance in Braak ROI II – V (figure 3C, supplement table S4).

We further analyzed the individual AD-related experimental biomarker´s (ferritin, ApoE, sAXL, sTyro3, YKL-40, sTREM2, MIF, C1q, C4, factor H) correlations to Braak ROI I to V. To this end, we assessed bivariate, marker-specific relationships with structural Braak ROI measures in DELCODE by means of partial Spearman rank correlations using different models (figure 4). Experimental markers without significant correlation to AD CSF routine measures are depicted for comparison purposes. Model I used all available DELCODE data, adjusted for age and sex (Data S1 AT7). Model II

additionally adjusted for CSF AD pathology measures Aβ42/40 ratio and p-tau-181 (Figure 4A, supplement table S5). Lastly, Model II was run again excluding the MCI and DAT subjects from DELCODE (Data S1 AT8). Figure 4A shows the correlation strength (Spearman rho) for all correlations that were significant at $p < 0.05$ (uncorrected) in model II. As expected, more pathological CSF measures of AD pathology (Aβ42/40, p-tau-181, t-tau) and non-tau neurodegeneration markers (FABP-3, neurogranin) were correlated to lower structural measures, especially in earlier Braak ROIs I to III. In strong contrast, for CSF sTyro3, sAXL and ApoE positive relations with structure were found consistently throughout all Braak ROIs. Other results for both AD-marker related or non-related experimental markers were inconsistent regarding individual Braak ROIs (figure 4).

In line with this, GAM analyses of AD-related CSF markers confirmed significant positive correlations of ApoE, sTyro3 and sAXL with Braak ROIs that withstood correction for multiple testing (supplement table S6). When excluding MCI and DAT subjects, sTyro3 ApoE and sAXL were still significantly related to brain structure. The association of sTyro3 to Braak V structure remained significant after correction for multiple testing (Data S1 AT9). We also tested exclusion of subjects with suspected non-AD pathophysiology (SNAP), by excluding all A-T+ and A-N+ subjects from the whole cohort. Again, sTyro3 ApoE and sAXL were significantly related to brain structure. The association of sTyro3 to Braak ROI II – V, of sAXL to Braak ROI II, IV, V, and of ApoE to Braak ROI IV, V remained significant after correction for multiple testing (Data S1 AT10).

The regional pattern of brain structure relations to sAXL, sTyro3 and ApoE is visualized in Figure 4B in comparison to correlations with AD pathology and PACC5 score. Here, the correlations with individual brain regions within the Braak ROIs are displayed, showing that higher CSF sTyro3 is related to higher structural integrity in temporal, frontal and parietal regions. A similar pattern of regional relations was seen for Aβ and memory, with lower structural integrity being related to more advanced Aβ pathology and lower memory performance.

Elevated sAXL, sTyro3 relate to preserved cognitive performance

To test the correlation of the AD-related markers to cognitive performance, we used linear mixed models with a latent process in DELCODE to model both baseline differences and 5-year longitudinal changes. The PACC5 score was used as the outcome in the analyses. Results for the whole DELCODE cohort are depicted in Figure 5, detailed statistics provided in supplement table S7. We found a significant positive relation of CSF ApoE, sAXL and sTyro3 with cognitive performance at baseline, but the relation of ApoE did not pass correction for multiple testing. ApoE, sAXL, sTyro3 and

YKL-40 were associated with longitudinal change of cognition over time, though only the relation of sAXL passed correction for multiple testing.

Repeating the analysis in CN individuals (excluding MCI and DAT subjects; Data S1 AT11) did not result in significant associations. In contrast, exclusion of SNAP subjects (any A-T+ or A-N+ subjects) from the whole cohort lead to significant results for ApoE at follow up, for sTyro3 at baseline, and for sAXL at both baseline and follow up after correction for multiple testing, with trend level findings for all the three markers at both baseline and follow-up (Data S1 AT12).

Effect replication in the ACE cohort

To test if the findings made in DELCODE could be replicated in an independent cohort, we utilized samples and data of the ACE cohort including 59 subjects diagnosed with SCD and 723 MCI subjects. These groups did not differ by distribution of sex or BMI, but MCI subjects had higher median age and a larger proportion of *APOE* ε4 carriers (Data S1 AT13). Therefore, the distribution of demographic features in ACE was similar to DELCODE in terms of age and *APOE* status, but not for sex or BMI of subjects.

To examine replication of effects in the ACE cohort, we focused on those markers classified as immune- / inflammation related in the original panel that had a strong interrelation to AD CSF amyloid and tau markers in DELCODE (YKL-40, sTREM2, sAXL, sTyro3, MIF, complement factors C1q, C4, H) as well as ApoE - which we originally classified as "multifactorial" marker - due to its multiple correlations to AD routine CSF markers, structural imaging and cognition data in DELCODE. Of these 9 markers, between the MCI and SCD groups of ACE, only sTyro3 showed a tendency towards elevation in MCI ($p = 0.044$ (not adjusted for multiple testing), Data S1 AT13). This trend was not observed in DELCODE screening diagnosis group comparisons.

In contrast, application of the A/T scheme to the ACE data was consistent with the results from DELCODE, where all of the nine included markers showed highly significant elevation in T+ subjects (Data S1 AT14 and AF6). In part, there was a trend towards lower levels in A+ subjects (A+ T- slightly lower than A-T-, and A+T+ lower than A-T+), which is again in line with DELCODE results. Combination of diagnosis and T+ schematic in ACE (SCD T-, SCD T+. MCI T-, MCI T+) resulted in limitations of sample size for SCD T+ ($N = 7$) compared to SCD T- ($N = 52$) but yielded sufficient sample size for comparisons in both MCI groups ($N > 300$ each). Elevated levels were observed in MCI T+ against both T- groups,

and for sAXL and MIF also for SCD T+ against MCI T- or SCD T-, despite the limited sample size (Data S1 AT15 and AF7). These findings again were consistent with the results from DELCODE.

Furthermore, we tested GAM models of the correlation between the nine markers and amyloid (measured by CSF Aβ42 in ACE) as well as p-tau-181. In line with group-wise comparisons, GAM models showed lower inflammatory marker levels with lower Aβ42, but also a strong positive correlation to p-tau-181 levels. These results were highly significant for all nine markers, replicating DELCODE results (Data S1 AT16, AT17, AF8 and AF9).

We next analyzed the associations between our nine markers of interest with Braak ROI structural measures in the ACE data. Bivariate Spearman correlations showed again positive correlations of sAXL and sTyro3 throughout Braak ROI I-V, and for ApoE and C1q with Braak ROIs III – V (Data S1 AT18). With exception of C1q, these were the same associations as found in DELCODE. Most of these correlations persisted when excluding all SNAP (A-T+ or A-N+) subjects from the ACE data (Data S1 AT19). GAM models showed similar results for most Braak ROIs for sAXL and sTyro3 at trend level, while the results for sTyro3 passed multiple testing correction for Braak III and V in both models (Data S1 AT20 and AT21). Therefore, the DELCODE results on sAXLa nd sTyro3 could be replicated in ACE, while results on ApoE could not be confirmed in all models. A summary of the Braak ROI analysis in ACE is visualized in Data S1 AF10.

Finally, we calculated a cognitive composite score assessing memory and executive function in ACE. Due to differences in study design, this score used different items compared to the DELCODE PACC5 but reflected the same cognitive domains. In contrast to other analyses, we could not confirm cognition relations of sTyro3 and ApoE observed in DELCODE in ACE. Higher sAXL levels were related to better cognition at baseline when modelling the whole ACE cohort, though this effect did not withstand correction for multiple testing (unadjusted $p = 0.014$). However, when excluding SNAP subjects from the ACE data, this association of sAXL with higher baseline cognitive performance withstood correction for multiple testing (Data S1 AT22 and AT23, respectively).

In conclusion, results of striking inflammatory markers could be replicated in ACE for relations to routine Aβ and tau markers and structural imaging features, but only in part for cognitive decline.

DISCUSSION

Increasing attention is placed on neuroinflammation as an important pathomechanism of AD and thus target of pharmacological intervention (Heneka et al., 2015). However, there remains a need for readout biomarkers of the specific inflammatory processes that might be relevant for such interventions, and to understand their interactions with other disease

features. We investigated a panel of CSF biomarkers with a focus on different mechanisms of inflammation, immune regulation and signaling, which we established in previous studies (Brosseron et al., 2018, 2019, 2020). We found specific biomarkers (YKL-40, sTREM2, sAXL, sTyro3, MIF, C1q, C4, factor H, ferritin and ApoE) to be elevated in subjects with pathological p-tau and t-tau levels, even in the pre-dementia stages of apparently healthy controls or SCD subjects. The same subgroup also had higher levels of non-tau neurodegeneration markers FABP-3 and neurogranin, confirming that these subjects are likely to suffer neuronal damage already. Furthermore, the inflammatory markers positively correlated to CSF levels of tau isoforms, FABP-3 and neurogranin. This correlation was steepest for tau levels below the pathological cut-off and became less steep in subjects with pathological tau-profile. At the same time, the most significant markers were correlated to each other and to levels of Nf-L, which were available in DELCODE as plasma-based data. The relations between plasma Nf-L and CSF inflammatory markers were slightly weaker in effect and less robust against adjustment for covariates. Some previous studies found that trajectories of Nf-L throughout the course of disease differ between CSF and blood, depending on the disorder or subject group investigated (Alagaratnam et al., 2021; Andersson et al., 2020; Fortea et al., 2018; Meeter et al., 2016; Palmqvist et al., 2019; Pereira et al., 2017). Within these studies, the SIMOA® method used for our study achieved high plasma to CSF correlations (average r = 0.7), providing a close proxy of CSF Nf-L concentrations. Therefore, the observed differences between correlations to plasma Nf-L in comparison to CSF neurodegeneration markers might be due to other reasons, such as partly different cellular events, disease dynamics and trajectories.

In contrast, the inflammatory profile was less strongly correlated to the Aβ42/40 ratio. Here, higher marker levels were related to less pathological ratios of Aβ42/40 when adjusting for p-tau-181 levels. Although this seems counter-intuitive given the strong relations of the inflammatory markers to tau and neurodegeneration markers, the effect was observed in both cohorts and might be in line with previous findings. Studies using ADNI data found that higher CSF sAXL or sTREM2 were related to longitudinal reduction or reduced accumulation of Aβ42 (Ewers et al., 2020; Mattsson et al., 2013). Reduction of Aβ pathology has also been observed in combined experimental mouse models of AD and infection, where invading macrophages – themselves expressing relevant markers such as TREM2 – effectively degrade Aβ peptides (Möhle et al., 2016). Yet, it is well described that microglial phagocytosis of Aβ is reduced under conditions of neuroinflammation (Heneka et al., 2015). Hypothetically, at specific disease stages, reduction of phagocytic activity might coincide with transiently reduced expression of proteins like AXL or TREM2 and increased accumulation of Aβ pathology, reflected by the observed reduction of these markers CSF levels in A+ subjects. However, this concept is speculative, and

the respective changes in CSF biomarker levels might only be detectable in large cohorts including several hundred subjects, which have sufficient power to detect even smallest effects.

Overall, it appears reasonable to assume a pattern of inflammatory response to tau exists in AD and other neurodegenerative disorders, potentially with modulation related to Aβ accumulation. Tau isoforms have recently been demonstrated to induce key inflammatory signaling pathways in the context of AD (Ising et al., 2019). While our findings indicate that this reaction might not be specific to AD – due to the considerable elevation of inflammatory marker levels in SNAP subjects – we could show that the results were robust against exclusion of such SNAP subjects from the analyses. Therefore, this inflammatory profile likely represents a stage of neuroinflammation that parallels p-tau accumulation and onset of neurodegeneration even in asymptomatic or pre-dementia subjects, and continues to be present in later, symptomatic stages of disease.

When analyzing bivariate correlations between the AD biomarker-related candidate proteins and Braak ROI scores in DELCODE, levels of the soluble receptors sAXL and sTyro3 – as well as of ApoE protein– showed positive relations with structural integrity in Braak ROIs covering temporal, limbic and parietal regions, in particular if the model was adjusted for a given level of AD biomarker pathology. These findings were replicated in the ACE cohort, most of all for sAXL and sTyro3, and were also robust against exclusion of SNAP subjects in both cohorts. Furthermore, sAXL, sTyro3 and ApoE were positively related to longitudinal cognitive performance in DELCODE, though this could only be partly replicated for sAXL in ACE with regard to an effect on baseline cognitive performance. Taken together, this may indicate that sAXL, sTyro3 and ApoE, even though their CSF levels were elevated in relation to increased tau levels and neurodegeneration, represent mechanisms that arise in association with brain protection. It is also possible that these three markers relate to brain reserve (i.e. subjects with higher brain volume bear higher levels these markers while remaining cognitively normal). How exactly any of the involved pathways is mechanistically involved in neuroprotective process, must be elucidated in future studies.

The current understanding of Tyro3, AXL and Mertk (TAM) receptor signaling in neurodegeneration points towards a protective function, regulating inflammation and promoting phagocytosis or other beneficial effects (Tondo et al., 2019). AXL and Tyro3 have distinct cellular expression profiles and functions in the CNS, with both proteins found in astrocytes and oligodendrocytes, Tyro3 in neurons and radial glia, and AXL in microglia and Schwann cells (Tondo et al., 2019). Tyro3 has not been described in microglia, although it is expressed in human monocytes and macrophages and found on transcript and protein levels in microglia from a murine AD model (Rangaraju et al., 2018; Tondo et al., 2019). Microglial

toll-like receptor (TLR) signaling is regulated by AXL and its ligand Gas6 (Gilchrist et al., 2020). Recently, it was demonstrated that AXL and Mertk are essential for phagocytosis of Aβ plaques in murine AD models, resulting in an increase of dense-core plaques (Huang et al., 2021). The authors suggested that phagocytosed Aβ, after fibrillization in microglial lysosomes, is released again by microglial cell death or exocytosis, together resulting in densely packed but less propagative plaques.

At first glance, findings of our study seem to contradict this understanding of TAM biology. Soluble TAM receptors are usually considered to result from proteolytic cleavage (shedding) of the ectodomains from the membrane-anchored protein by alpha-secretases, which renders the receptor inactive. Furthermore, the soluble receptors capture free ligands such as Gas6 or Protein S. This two-sided regulatory mechanism can interfere with the protective functions of TAM receptors and aggravate inflammatory disorders if dysbalanced towards excessive shedding (Cohen and Shao, 2019; Falcone et al., 2020; Holstein et al., 2018; Miller et al., 2017). In this case, one would expect that soluble TAM levels correlate to neurodegeneration markers because excessive shedding renders the TAM signal pathway inactive, resulting in reduced phagocytosis and cell survival, which does not bode well with the protective relations of higher soluble TAM levels observed in our study. However, several receptor tyrosine kinases can also undergo regulated intramembrane proteolysis (RIP) mediated by ADAM10 and gamma-secretase, resulting in shedded soluble receptor ectodomains but also a functional intracellular domain that promotes cell growth. This mechanism has been demonstrated for AXL, whereas contradictory observations were made for Tyro3 (Lu et al., 2017; Merilahti et al., 2017). Aside of this, in cancer, cellular over-expression of TAM receptors results in excessive cell proliferation and contributes to cancerous cells evading clearance by the immune system (Falcone et al., 2020; Holstein et al., 2018). Importantly, serum sAXL levels reflect the increased cellular AXL levels found in melanoma and hepatocellular carcinoma, and higher sAXL relates positively to melanoma disease severity and negatively to treatment outcome (Falcone et al., 2020; Flem-Karlsen et al., 2020; Holstein et al., 2018; Reichl and Mikulits, 2016). These observations indicate that soluble TAM levels can be proxies of TAM cellular expression and activity. In this scenario, soluble TAM receptors are derived from shedding by regular receptor turnover, but not from signal pathway inactivation. In conclusion, we could hypothesize that cellular TAM expression levels are increased as part of a damage response reaction in neurodegenerative diseases and brain injury. TAM receptor turnover then results in soluble TAM ectodomains, which can be detected as biomarkers in CSF. The increase of sTAM levels is a proxy of the cellular danger response to tau pathology and neurodegeneration, and therefore sTAM levels correlate positively to neuronal damage markers. However, as the soluble TAM levels reflect increased expression of the cellular TAM, those

subjects with higher levels will be those in which the TAM system exerts stronger immune regulation, promotion of phagocytosis and cell survival, resulting in preserved brain structure and delayed cognitive decline. While this mechanism would be in line with both current knowledge on TAM biology and our findings in this study, verification of this hypothesis will require further investigation in neurodegeneration models.

In DELCODE, the third biomarker associated to protection was ApoE, though without replication in ACE. Previous studies also found contradictory results for ApoE protein levels in relation to cognitive outcome (van Harten et al., 2017; Toledo et al., 2014). Similar to our approach, Toledo et al. adjusted their analyses for age, sex, *APOE* status and AD pathology, whereas van Harten et al. did not adjust their model in this way. This type of adjustment might be critical to recognize protective effects at a given stage of AD pathology. However, we utilized the same statistical model on both DELCODE and ACE data. As both cohorts used in this study contain data of several hundred subjects, it seems unlikely that discrepancies arise from overfitting or over-estimation of small effects. Speculatively, other factors, such as cohort composition or stage-dependent effects, could be the reasons behind differences in findings. There is a potential difference on the effect of *APOE* as a genetic risk factor in different ethnic backgrounds or ancestries present in the Iberian peninsula region due to historic population movements, and smaller effects of *APOE* genotypes have been observed in previous data of ACE (Beydoun et al., 2021; Bycroft et al., 2019; Ramirez-Lorca et al., 2009; de Rojas et al., 2021). Such differences might create discordances among DELCODE and ACE results. ApoE has gained increasing attention for its role in neuroinflammation within the last years, as reviewed in detail by others, and is involved in regulation of microglia-mediated neuroinflammation as well as phagocytosis of toxic protein aggregates (Kloske and Wilcock, 2020; Loving and Bruce, 2020; Perea et al., 2020; Schwabe et al., 2020). ApoE and AXL belong to a cluster of genes co-regulated in AD disease-associated microglia (Gao et al., 2019; Keren-Shaul et al., 2017; Yin et al., 2017). Recently, it has been shown that the AXL kinase inhibitor AZ7235 specifically enhances ApoE secretion in microglia, astrocytes and pericytes (Zhao et al., 2020). The same study also found that AXL elevates ApoE expression in astrocytes independent of its kinase domain and non-responsive to Gas6 stimulation, suggesting a non-canonical mechanism of AXL-dependent ApoE homeostasis. Therefore, similar (protective) effects observed for AXL and ApoE CSF levels would be in line with current knowledge on their mechanistic interactions. Yet, if this holds true and if the reasons behind discrepancies between cohorts can be explained, remains to be resolved.

In contrast to some previous studies, we did not observe significant relations between two of the most frequently investigated markers, YKL-40 and sTREM2, with structural features or cognition scores in most of the models. These

discrepancies could be caused by differences in study cohorts, stage of disease and methodology and warrant further observations, as studies on such interactions are still limited in number (Alcolea et al., 2015; Ewers et al., 2019; Gispert et al., 2016a, 2016b).

This study is not without limitations. First, our results show that subjects with A+T+ AD biomarker profile, but symptomatically still within the SCD or even control range, might be the most interesting group to study for inflammatory marker profiles. The subgroup of subjects in predementia stage that had a positive AD biomarker profile and also showed elevation of inflammatory markers was still limited in size, including around 10-20% of subjects depending on the cohort, biomarker and cut-off used. This spectrum of subjects could be investigated in more detail with specific screening and enrichment strategies to achieve significantly larger cohorts with this type of biomarker-positive, symptomatic pre-dementia profile. Second, it is likely that the same reaction can be observed in the SNAP spectrum of neurodegenerative disorders. In particular, cohorts with primary tauopathy subjects might be of interest for this question, although these were not in the focus of this study. Third, DELCODE and ACE cohorts are ongoing and both will be collecting and releasing biomaterial and follow-up data throughout the next years. Therefore, longitudinal data on cognitive performance from both cohorts used in this study is not yet complete for follow-up times. This adds uncertainty to the modelling and might limit the power to detect associations with cognitive change. Likewise, neuroimaging data was not yet available for the follow-up visits of DELCODE and although findings were quite reproducible in ACE, higher values in thickness or volume can resemble attenuated structure, brain swelling or simply individual differences in brain structure. Within the next years, longitudinal samples and data will be more complete for the DELCODE cohort and enable improved longitudinal outcome modelling with the baseline inflammatory marker data described here.

In summary, our study provides evidence that the increase of specific immune biomarkers in the CSF is tightly related to tau isoform levels and neurodegeneration, that this elevation is steepest before pathology markers reach cut-off value range and that subjects could reach elevated inflammation levels in biomarker-positive, but still pre-dementia stages of disease. It is likely that future studies can be enriched for such subjects, e.g. by pre-screening using advanced technologies for blood-based neurodegeneration marker detection (Alawode et al., 2021). Based on our results, we expect a tight link to neuroinflammation in these stages of disease, representing a potential intervention target aside of the classical AD hallmarks amyloid and tau. The biomarkers described in this study – most of all sAXL and sTyro3 - might serve as established readouts in such trials. Despite this potential, the exact mechanisms behind the inflammatory biomarker regulation as well as development and approval of effective drugs will require further investigation.

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AUTHOR CONTRIBUTIONS

Conceptualization, generation, analysis and interpretation of experiments and data for this manuscript were done by FB, AM, LK, KAR, CCK, RM, CI, LMH, FS, EL, MJ, CET, NLM, AR, ARL and MTH. Braak ROI scores were generated from Freesurfer scores and analyzed by AM. Data from neuropsychologic / neurocognitive assessments for PACC5 score were generated and analyzed by LK. Overall design, implementation, and collection of data for the DELCODE study at the different study sites was provided by FJ, AR, ASp, KB, DJ, MD, MTH, CL, MHM, OP, NC, AC, XW, JP, EJS, SA,

ASc, KF, RP, BSR, ST, IK, DG, JW, BHS, MW, JDH, PD, ME, KS, ED, SR, LD, RY, CDM and WG. Plasma Nf-L data for DELCODE were provided by DM and MS. Furthermore, FJ, ASp, NR, FB, MTH, OP, AR, SW, MW, and ED were responsible for DELCODE methodological core central data management and data analyses. Data of ACE were provided by PGG, MM, MB, AO, IdR and ARL, within the framework of the EU-JPND project PREADAPT coordinated by AR. FB, AM and LK contributed equally to data analysis strategy, conduction of statistics and drafting of the manuscript. All authors read and approved the final version of this manuscript.

DECLARATION OF INTERESTS

The authors disclose any financial or other interests related to the submitted work.

MAIN FIGURE TITLES AND LEGENDS

Figure 1. Inflammatory biomarkers are elevated in cognitively normal individuals with tau pathology

Elevation of CSF inflammatory biomarkers in T+ groups when using the A/T scheme on the whole cohort (left column) and when combining a cognitive staging with p-tau-181 positivity (right column). Violin plots with median and interquartile range. Groups colored red were elevated against at least one group colored green, as indicated by capped lines. Groups colored yellow were elevated against at least one group colored green, but still lower than at least on group in red. Groups colored grey were not significantly different. Significant effects after multiple testing correction are displayed for sAXL (**A, B**), sTyro3 (**C, D**) and ApoE (**K, L**) as these markers were most relevant in later analyses. Results for further significant markers and details on statistics are displayed in supplement tables and figures S1 and S2, respectively.

Figure 2. Nonlinear interrelation analysis between inflammatory and AD hallmark markers

Nonlinear correlation analysis between key CSF inflammatory markers and AD pathological hallmark markers p-tau-181 and Aβ42/40 ratio. Results are displayed top to bottom for sAXL (**A**), sTyro3 (**B**) and ApoE (**C**), as these markers were most relevant in later analyses. Further results for other significantly altered markers are displayed in supplement table and figure S3. Left panel: Heat map displaying inflammatory marker levels by color code (green, lower levels; red, higher levels; grey, no data for these coordinates) over the spectrum of p-tau-181 levels (y axis) and Aβ 42/40 ratio (x axis). Black lines indicate cut-offs for Aβ42/40 ratio and p-tau-181 levels. Inflammatory marker levels increased most of all depending on p-tau-181 levels, but were also influenced to lesser extend by Aβ42/40 ratio.

Middle and right panel: Bivariate nonlinear correlation modelling between inflammatory markers and either Aβ42/40 ratio or p-tau-181. The GAM models showed nonlinear relations, were most pronounced for p-tau-181 and steeper at sub-threshold levels, before p-tau-181 reached pathological levels.

Figure 3. Braak region structure predicted by inflammation markers

A) Structural measures were derived from composite regions that follow Braak stages of neurofibrillary tangle pathology. The six Braak composite regions are color-coded with warmer colour denoting regions earlier affected by AD pathology. For each Braak composite region, thickness/volume measures were adjusted for head size and averaged across individual

cortical/subcortical FreeSurfer regions after segmentation of the T1 MR images. **B)** Relations between CSF inflammatory markers and brain structure across Braak composite regions in all samples with available MRI data (N=266) of cognitively normal older adults and impaired subjects. Multivariate Regression models were run, in which mean structure in each Braak ROI was predicted by the 16 neuroinflammation markers, after adjusting for age and sex. Models were significant for all Braak ROI except Braak VI (see Results section). The percental amount of variance in structure (R^2) predicted by inflammatory markers is color coded, with strongest associations seen for the limbic Braak III ROI with 15% (red colors). **C)** Relations of inflammatory markers to Braak ROI after additional adjustment for AD pathology markers Aβ42/40 and p-tau-181. Statistics for this model are provided in supplement table S4. In this model, results were significant for Braak ROI II – V, with Braak ROI I marginally missing significance threshold (p = 0.058). The percental amount of variance in structure predicted was slightly lower compared to the model without adjustment for AD pathology markers, with the strongest association in Braak ROI III reaching approx. 13%.

Figure 4. Relationships between neuroinflammation markers and regional brain structure.

A) Bivariate Spearman rank correlations were run between each CSF marker and Braak structural measures. The correlation matrix shows the strength (rho) of all correlations that were significant at uncorrected p-value < 0.05. Detailed statistics for Braak ROI bivariate correlation models are provided in supplement table S5.

1) Reference panel including routine AD and neurodegeneration markers, adjusted for age and sex. Aβ42/40 ratio was inverted, displaying increasing pathology negatively related to structure. 2) Panel of inflammatory markers with strong relation to tau isoform levels, adjusted for age, sex, p-tau-181 and Aβ42/40 ratio. Red boxes indicate results confirmed by GAM modelling, adjusted for multiple testing for the markers within the respective panel. 3) Panel of inflammatory markers with no or weak correlation to tau isoform levels, displayed for comparison purposes, adjusted for age, sex, p-tau-181 and Aβ42/40 ratio. Positive relations were found for ApoE, sTyro3 and sAXL with volumetric measures of several Braak ROIs.

B) Region-specific relations of PACC score, CSF routine AD markers and specific neuroinflammation markers with structure. Correlation strength (rho) is plotted for each individual Freesurfer ROI (rendered on the brain surface) for significant correlations at an uncorrected p-value < 0.05. All correlations were adjusted for age and sex. Correlations with sTyro3, sAXL and ApoE were also adjusted for AD markers and p-tau and Aβ 42/40. Noteworthy, similar brain regions

showed lower volume/thickness with more AD pathology (temporal, frontal and parietal regions) but higher volume/thickness with higher levels of sAXL, sTyro3 and ApoE.

Figure 5. Longitudinal outcome depending on baseline biomarker levels

Available data from baseline and follow-up years 1 to 5 of the DELCODE cohort was used to calculate a Preclinical Alzheimer disease Cognitive Composite (PACC) score, based on items like FCSRT, MMSE and other neuropsychological testing features. Baseline CSF biomarker levels were correlated against longitudinal PACC score values and compared between levels one standard deviation (SD) higher (orange) and lower (green) than the mean standardized biomarker level. Results are adjusted for age, sex and also AD hallmark markers Aβ42/40 ratio and tau. In this analysis, sAXL (**A**) and sTyro3 (**B**) were most robust in significance and higher baseline levels were related to more stable cognitive performance in follow up; whereas lower baseline levels related to decrease of cognitive performance over years. A similar effect was observed for ApoE levels, though this was not robust against adjustment for multiple testing (**C**). Available subject number throughout follow-ups were as follows: Baseline, N = 288; Y1, 245; Y2, 213; Y3, 164; Y4, 89; Y5, 20. Statistical details are provided in supplement table S7. Correlations are displayed with confidence interval.

STAR METHODS

Resource availability

Lead contact:

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Dr. Michael T. Heneka (michael.heneka@ukbonn.de).

Materials availability

This study did not generate plasmids, mouse lines or unique reagents. There are restrictions to the availability of biomaterial from the DELCODE study as well as from ACE due to study regulations. For details and contact, please see https://www.dzne.de/en/research/studies/clinical-studies/delcode/ or contact ACE (https://www.fundacioace.com/en/contact-us.html), respectively.

Data and code availability

No code was generated during this study. There are restrictions to the availability of data from the DELCODE study as well as from ACE due to study regulations. For details and contact, please see https://www.dzne.de/en/research/studies/clinical-studies/delcode/ or contact ACE (https://www.fundacioace.com/en/contact-us.html), respectively.

Experimental model and subject details

Ethics approval and consent to participate

The general study protocol for DELCODE was approved by the ethical committees of the medical faculties of all participating sites: the ethical committees of Berlin (Charité, University Medicine), Bonn, Cologne, Göttingen, Magdeburg, Munich (Ludwig-Maximilians-University), Rostock, and Tübingen. The process was led and coordinated by the ethical committee of the medical faculty of the University of Bonn. The registration number of the trial at the ethical committee in Bonn is 117/13. Use of data and biomaterial for the specific work described in this manuscript was furthermore approved by the ethical committee of the medical faculty of the University of Bonn, reference No. 122/18. Use of data and biomaterial of the Fundacío ACE (ACE) cohort for the work described in this manuscript was approved by the Ethical Committee of the Hospital Clínic I Provincial de Barcelona (HCB/2014/0494, HCB/2016/0571,

HCB/2016/0835, HCB/2017/0125 and HCB/2018/0333). Protocols of ACE had been designed in agreement with the indications of the Sociedad Española de Neurología (www.sen.es), according to the current regulations for the use of clinical data and biological material and surplus of the assisted process for the biomedical research of neurodegenerative diseases.

Study design

This study included a total of 309 CSF samples from the baseline subject group of the DZNE DELCODE study, for which general study design and other baseline data have been described elsewhere (Jessen et al., 2018). DELCODE includes subjects at a minimum age of 60 years, recruited from German residents. The main subject groups by screening diagnosis were healthy controls (N = 74), subjective cognitive decline (N = 99) and amnestic mild cognitive impairment (MCI) subjects (N = 75), supplemented by smaller groups of DAT subjects (N = 38) and cognitively normal first-degree relatives of DAT patients (N = 23). Descriptive statistics on the cohort are provided in Data S1 AT1. CSF samples underwent biomarker measurement for determination of the inflammatory marker panel. Additional data retrieved from the DELCODE study included demographic data (age, sex, *APOE* genotype and body mass index (BMI), previously determined routine AD biomarker levels (Aβ40, Aβ42 and ratio Aβ42/40, phospho(p)-tau-181, total(t)-tau and the ratio Aβ42/p-tau-181), neuropsychological test results and structural T1 MRI data. Data analysis was focused on the relation of inflammatory markers towards structural features as well as cognitive decline, emphasizing those markers that were at the same time linked to routine AD pathology biomarkers.

For validation of effects observed in DELCODE, we included samples and data of 59 SCD and 723 amnestic and non-amnestic MCI subjects provided by Fundacío ACE (ACE) within the framework of the JPND-funded project PREADAPT and replicated biomarker measures as well as statistical analyses for those proteins with strongest effects in the DELCODE dataset. Data obtained from ACE included age, sex, APOE genotype, BMI, CSF Aβ42, p-tau and t-tau, neuropsychological test results and segmented T1-weighted MR images data (see "Structural measures" section below). General information on the ACE cohort criteria and procedures has been described elsewhere (Boada et al., 2014; Espinosa et al., 2013; Rodriguez-Gomez et al., 2017).

Method details

Biomarker measurements

609 The panel of candidate CSF biomarkers consisted of immune- / inflammatory markers (sTREM2, sAXL, sTyro3, YKL-
610 40, MCP-1, IP-10, MIF, IL-6, IL-18, CRP, complement factors C1q, C3, C3b, C4, B, H), non-tau neurodegeneration
611 markers (neurogranin, FABP-3) and multifactorial markers related to inflammation plus other pathomechanisms (ferritin,
612 ApoE). The panel was determined by enzyme-linked immunosorbent assays (ELISA) as described previously (Brosseron
613 et al., 2019, 2020). Details on assay specifications are provided in Data S1 AT24 as well as in the key resources table. In
614 brief, samples were retrieved from the biorepository of the DELCODE study and initially underwent one additional freeze-
615 thaw-cycle on ice to split samples into smaller aliquots of 10 to 60 µl, depending on the requirements of the respective
616 immunoassays. For this purpose, samples were processed by a robot, pipetted into 96 well V-bottom storage plates (Greiner
617 Bio-One, ref. 651101), sealed using a freezing-resistant aluminum foil (Greiner Bio-One, ref. 676090), placed on dry ice
618 for fast re-freezing and finally stored at -80 °C until analysis. This way, from lumbar puncture to immunoassay
619 measurement, the samples underwent a total of 2 freeze-thaw-cycles. Samples were processed in arbitrary order using
620 pseudonymized identification numbers and laboratory personal was blinded to subject groups or any other data relevant
621 for statistical analysis. All samples were measured in duplicates with a maximum coefficient of variance (CV) of 20%.
622 Samples with higher CV underwent repeated measurement. An internal, aliquoted reference CSF sample was included in
623 each immunoassay run to control for inter-run variances.
624
625 *Structural measures*
626 Thickness and volume measurements were obtained by segmentation of the T1-weighted MR images with FreeSurfer 6.0
627 ((Fischl, 2012), http://surfer.nmr.mgh.harvard.edu/) using the Desikan–Killiany atlas (Fischl et al., 2002, 2004). MRI data
628 with FreeSurfer segmentations were available for 266 subjects (87%). Six measures of "structural integrity" were derived
629 by combining individual regions into a priori composite regions of interest (ROI) (Baker et al., 2017; Schöll et al., 2016)
630 that follow Braak stages of neurofibrillary tangle pathology (Braak and Braak, 1991). Earlier Braak regions are expected
631 to show earlier atrophy in the course of AD and thus should most strongly correlate with AD biomarkers. The six Braak
632 ROIs covered the following regions: I: Entorhinal Cortex; II: Hippocampus; III: Amygdala, Fusiform, Parahippocampal
633 Cortex, lingual gyrus: temporal regions, cingulate, retrosplenial cortex, insula; V: frontal and parietal regions; VI: primary
634 visual, motor and sensory areas; exact list of individual regions described by Baker et al. (Baker et al., 2017). We used
635 volume measures for subcortical ROIs (Hippocampus, Amygdala), which were adjusted for total intracranial volume and
636 thickness measures for all cortical regions. For each Braak composite region, individual thickness/volume measures were

Z-scored across the whole sample and then averaged. Bilateral means were calculated as we had no hemisphere-specific hypotheses.

Cognition score calculation

For DELCODE, we used a preclinical Alzheimer´s cognitive composite (PACC5) to model cognitive performance (Papp et al., 2017). The PACC5 is a neuropsychological composite measure that was designed to index cognitive changes in the early phase of AD. To construct the PACC, we z-standardized and averaged the following tests: Free cued and selective reminding test (FCSRT) total and free recall, symbol digit modalities test (SDMT), logical memory delayed recall, semantic fluency (sum of animals named in one minute and grocery named in one minute) and the mini mental state examination (MMSE).

For ACE, not all items required for PACC5 score calculation were available. We therefore constructed a similar composite using a z-composite score of Wechsler memory scale III immediate and delayed word list recall, semantic fluency (animals named in one minute) and the automatic inhibition subtest from Syndrom-Kurz-Test (time to complete) (Alegret et al., 2012, 2013).

Statistical analysis

To address the questions outlined in the study design, we started by screening the CSF inflammatory and multifactorial markers in DELCODE for those with most significant relations with AD features, with the non-tau neurodegeneration markers as comparators. Here, we began with group-wise comparisons based on clinical staging, A/T/N schematic and combinations of both. Cut-off values for A/T/N biomarkers were based on Gaussian mixture modeling of the DELCODE data independent of any group assignments using the R package flexmix (version 2.3-15) (Bertens et al., 2017): Amyloid ratio (A) Aβ42/Aβ40 0.08; tau pathology (T) by p-tau-181 73.65 pg/ml; neurodegeneration (N) by t-tau 510.9 pg/ml. As T+ and N+ subjects within this AD-focused cohort were largely redundant (96% of T+ also N+; 66% of N+ also T+), we reduced the schematic to A/T and used A/N as comparator for statistical analysis. For ACE, we replicated this analysis using an A/T scheme based on available Aβ42 and p-tau-181 CSF level data. ACE contained data obtained by use of different immunoassays for routine AD CSF markers: Assay 1, manual ELISA (Innotest®); Assay 2, automated CLEIA assay (LUMIPULSE®). The following study-specific cut-off values were used for ACE data: Assay 1, Aβ42: 676 pg/ml, t-tau: 367 pg/ml, p-tau-181: 58 pg/ml; Assay 2, Aβ42: 796 pg/ml, t-tau: 412 pg/ml, p-tau-181: 54 pg/ml. In the A/T scheme,

we did not differentiate between assays, but categorized data according to the assay-specific cut-offs. Prism 8 (GraphPad Software Inc., La Jolla, USA) and IBM SPSS Statistics 21 (IBM Corporation, Armonk, USA) were used to visualize data and calculate non-parametric Kruskal-Wallis tests without exclusion of outliers. To control for multiple testing, Bonferroni-correction was performed. To perform a sensitivity assessment for influence of potential covariates on these comparisons, biomarker data were log-transformed to approximate normal distribution for use in ANCOVA as described previously (Brosseron et al., 2018). Associations of marker levels were tested for age, sex, *APOE* ε4 positivity (carriers of at least one ε4 allele Vs. non-carriers) and BMI, as these features differed between the subject groups and correlated to biomarker levels (Data S1 AT1 and AT2). For final ANCOVA models, only those covariates with significant influence on marker levels were included for each individual biomarker.

As these group-wise comparisons showed that CSF inflammatory markers were primarily changing depending on tau pathology or neurodegeneration, rather than from Aβ42/40 ratio or clinical staging, we aimed to test if this effect was driven by late-stage, cognitively impaired subjects or also present in pre-dementia subjects. As T+ or N+ subjects were less frequent in the pre-dementia groups, we combined all HC, SCD and relatives as "cognitive normal by objective criteria" (CN) and assessed the difference between T+/-, resulting in a comparison of CN with MCI and DAT subjects, each either T+ or T-. This approach was repeated using of N+/-, and using the non-tau neurodegeneration markers as positive controls to ensure that findings for smaller subgroups were representative and not driven by potential artefacts of tau levels. This approach was reflected in the ACE data by using SCD T+/T- and MCI T+/T-.

We also analyzed the effect of continuous changes in Aβ42/40 ratio and p-tau-181 on inflammatory markers using generalized additive models (GAM) (Wood, 2011). GAM allows assessing the relationship between two variables without assuming a specific function form of the relationship, such as linear functions. To estimate the non-linear association, we used thin plate regression splines and maximum likelihood estimation adjusted for age and sex as implemented in the mgcv package in R (Wood, 2003, 2011). Analyses were corrected for multiple comparisons using Bonferroni correction. In ACE, we replicated this analysis considering the two distinct immunoassays used for routine AD CSF markers and their potentially different dynamics, thereby splitting results for assay 1 and 2, respectively.

As we were interested in the role of inflammatory markers in the context of AD, only those markers showing an association with either Aβ42/40 ratio or p-tau-181 were further tested for association with a) Braak stages I-V and b) cognitive function at baseline and cognitive decline over time. GAM modelling, in line with group-wise comparison results, identified a total of 10 immune / inflammatory and multifactorial markers to be strongly related to tau pathology and neurodegeneration,

and in part and to lesser extend to Aβ42/40 ratio. These 10 markers (ferritin, ApoE, sAXL, sTyro3, YKL-40, sTREM2, MIF, complement C1q, C4, factor H) were included for further analysis, whereas others were excluded. Again, Bonferroni correction for multiple testing was applied.

Next, we screened all inflammatory biomarkers for relationships with structural measures to define which of the six Braak ROI were most relevant for further analysis. Following this outline, we first tested whether variance in structure in any of the six Braak ROIs was explained by CSF neuroinflammation markers by calculating a multivariate regression using the 16 immune / inflammatory markers (excluding the non-tau neurodegeneration and multifactorial markers from the panel), adjusted for age and sex as critical covariates for structure (figure 3). Only those Braak ROI with significant relations to inflammatory markers were considered for further analysis.

To assess the relations of the AD-related inflammatory markers to Braak ROI structural measures at a given level of AD pathology, bivariate Spearman rank correlations were run between each of the markers and Braak structural measures, adjusted for age, sex, Aβ42/40 ratio and tau pathology. The 95% confidence intervals of the correlations were calculated based on Fisher's z-transformation using VassarStats (http://vassarstats.net/rho.html). For comparison of effect strength, this analysis was also performed for the established Aβ42/40, tau and non-tau neurodegeneration biomarkers. This way, we could also analyze effect of AD pathology adjustment on the modelling (figure 4A). In addition, we estimated the effects of the inflammatory markers on Braak regions I – V using GAM adjusting for age, sex and Aβ42/40 ratio and p-tau-181. This analysis was repeated using data available for ACE, including ApoE, sAXL, sTyro3, YKL-40, sTREM2, MIF, complement C1q, C4, factor H. For ACE, we considered the distinct immunoassays used for routine AD biomarkers by adjusting for the assay in the model. Furthermore, we fitted different smooth functions for Aβ42 and p-tau-181 for each array, thereby allowing for a differential association of DA biomarkers depending on utilized assay.

Finally, the association of the cognitive function was assessed using linear mixed models with a latent process as implemented in the R package lcmm (Proust-Lima et al., 2011, 2017). In contrast to standard linear mixed models, linear mixed models with a latent process allow adjusting for the frequently observed unequal interval scaling cognitive tests by modeling non-linear link functions. Herein, a beta cumulative distribution function was used as previously recommended (Proust-Lima et al., 2011). A random intercept and random slope of time from baseline was modeled. We examined the effect of markers on the PACC5 at baseline (main effect), the interaction of time from baseline and marker controlling for age, sex and Aβ42/40 ratio and p-tau-181 and their interaction with time. Again, we repeated this analysis with the ACE

data with additional adjustment for the assay type (assay indicator and interaction of AD biomarker (Aβ42 and p-tau-181) with assay indicator as well as their interactions with time), and using the cognition score generated for ACE.

Imaging and cognition analyses were repeated for DELCODE excluding patients with MCI or DAT to test if observations were mainly driven by patients in later stages of AD. Furthermore, cognition and imaging analyses for DELCODE and ACE were repeated excluding suspected non-AD pathology (SNAP, all A-T+ and A-N+ subjects), to test if effects were driven by SNAP subjects in the cohorts.

SUPPLEMENTAL ITEM TITLES

Data and analyses related to the figures in this article are provided as supplement tables & figures:

Additional tables and figures are provided in Data S1:

Additional Data – ACE validation cohort

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