Technical and clinical validation of glial reactivity biomarkers sTREM2 and YKL-40 in Alzheimer's disease

Human Biology Final Degree Project 2024

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SUMMARY

Glial reactivity plays an important role in the pathogenesis of Alzheimer's disease (AD). Biomarkers associated with this process have the potential for understanding AD pathogenesis and facilitating the development of novel therapeutic strategies. Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) and YKL-40, also known as chitinase 3-like protein 1 (CHI3L1), are involved in the modulation of these glial responses. Our study aimed to conduct both technical and clinical validation of an in-house Meso Scale Discovey (MSD) assay and a commercial enzyme-linked immunosorbent assay (ELISA) for quantifying these biomarkers in cerebrospinal fluid (CSF) of AD patients. Technical validation included spike recovery, parallelism and precision tests. Clinical validation involved investigating the biomarker correlations with demographic factors within the cohort and the differences between biologically diagnosed AD and non-AD patients. Both assays showed high accuracy, robustness, and consistency in measuring CSF sTREM2 and YKL-40. Both biomarkers increased with age and CSF YKL40 was higher in patients with biological defined AD. Additionally, significant correlations were observed between CSF sTREM2, YKL-40, and the core CSF biomarkers Aβ40, Aβ42, and pTau181. No significant associations were found between CSF sTREM2, YKL-40, sex, *APOE ε4* profile and MMSE scores.

INTRODUCTION

Alzheimer's disease (AD) is a chronic neurodegenerative disorder and the leading world cause of dementia. Patients experience gradual memory decline, impaired executive function and inability to complete daily life activities (1). AD is histologically characterized by intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein and extracellular plaques of amyloid-β peptide (Aβ) in the brain (2). These pathological hallmarks develop decades before the onset of clinical symptoms, causing synapse and neuron loss (3).

Neuronal death and dysfunction, alongside insoluble Aβ deposits and NFTs, trigger glial reactivity in AD brains (4). Genome-wide association studies highlight the key role of microglia in AD pathogenesis (5). The activation of astrocytes and microglia in the central nervous system (CNS) can contribute to Aβ clearance in early neurodegeneration, but this ability diminishes with age (6). In late-stage AD, microglia may worsen the disease by releasing pro-inflammatory molecules, including cytokines, chemokines, complement proteins, growth factors, and cell adhesion molecules, in response to Aβ (7,8).

Biomarkers reflecting glial reactivity could offer new ways to evaluate AD patients. This study investigates the association between AD and two candidate glial CSF biomarkers: sTREM2 and YKL-40.

Triggering receptor expressed on myeloid cells 2 (TREM2) is an innate immune receptor on myeloid lineage cells, including dendritic cells, granulocytes, monocytes, and tissue-specific macrophages. In the CNS, TREM2 is expressed exclusively by microglia and regulates phagocytosis, apoptotic neuron removal, and inhibition of microglial pro-inflammatory responses (9,10). Loss-of-function mutations of *TREM2* increase susceptibility to late-onset AD by impairing microglial phagocytosis and reducing Aβ clearance. This is similar to the risk conferred by the ε4 allele of the apolipoprotein E (*APOE*) gene, the strongest common genetic risk factor for late-onset AD (11). The importance of TREM2 in brain function is also highlighted by the fact that rare biallelic mutations in the gene cause Nasu-Hakola disease and, in some cases, a frontotemporal dementia-like syndrome (12).

TREM2 ligands include lipopolysaccharide, phospholipids, and lipoproteins (13). TREM2 signaling involves the DAP12 adaptor protein and the tyrosine-protein kinase SYK, which lead to protein phosphorylation, Ca2+ mobilization, and activation of mitogen-activated protein kinase (MAPK) cascades (14,15). Additionally, TREM2 undergoes ectodomain shedding by ADAM10/17, resulting in the release of soluble TREM2 (sTREM2) (16). The precise role of sTREM2 in AD is not fully understood; however, some studies indicate that upon its release, sTREM2 preferentially binds to Aβ oligomers, influencing their aggregation state. This interaction refolds the aggregates into soluble forms, thereby mitigating the harmful effects of Aβ on the CNS and conducting a potential neuroprotective effect in AD (17–19). Most studies show that CSF sTREM2 levels rise along the AD continuum; however, findings vary based on the stage of the disease (20). Some studies indicate an increase of sTREM2 during the preclinical phase, while others observe the highest levels in the mild cognitive impairment (MCI) stage, suggesting TREM2-related microglial activation early in the disease (10,16,21,22).

YKL-40, also known as chitinase 3-like protein 1 (CHI3L1), is a chitin-binding lectin of the glycosyl hydrolase family 18 (23). It is involved in connective tissue cell growth, endothelial cell migration, inhibition of mammary epithelial cell differentiation, and promotion of tumor angiogenesis (24). YKL-40 interacts with cell membrane receptors, particularly interleukin-13 receptor subunit alpha-2 (IL-13Rα2), triggering intracellular signaling pathways that affect cellular responses (25,26).

Although the exact role of YKL-40 in the CNS is not fully understood, it plays a significant role in astrocytic reactivity. It is expressed in various tissues, including microglia, perivascular macrophages, and some white matter neurons (27). In AD, YKL-40 is found in astrocytes near Aβ plaques (28) and its CSF levels correlates with tau pathology and neuroimaging variables, such as cortical thickness in Aβ-positive patients and gray matter volume in *APOE* ε4 carriers (29). This suggests that YKL-40 is involved in the inflammatory response in AD and other tauopathies (28).

Previous studies have found that YKL-40 levels are elevated in CSF of AD patients (30,31). Interestingly, elevated CSF YKL-40 have also been detected in the preclinical stages of AD, suggesting that immune system activation occurs early in the disease process (32). These studies have also shown that CSF YKL-40 and tau protein strongly correlate (24).

Considering the potential roles of CSF sTREM2 and YKL-40 as biomarkers in the AD continuum, we performed an analysis of these glial factors to study their specific function in the glial response of AD. Therefore, the overall aim of this Human Biology Final Degree Project is to technically and clinically validate both a Meso Scale Discovery assay (MSD) and an enzyme-linked immunosorbent assay (ELISA) to measure sTREM2 and YKL-40 levels, respectively, in human CSF of the BIODEGMAR cohort from the Hospital del Mar AD patients.

RESULTS

The aim of this study was to technically and clinically validate two different immunoassays for the accurate and precise measurement of sTREM2 and YKL-40 levels in cerebrospinal fluid (CSF) from patients with Alzheimer's disease, part of the BIODEGMAR clinical cohort from Hospital del Mar. For sTREM2, we applied an in-house developed and optimized immunoassay, using the Meso Scale Discovery (MSD) platform. This platform uses electrochemiluminescence (ECL) labels, referred to as SULFO-TAG, which are conjugated to detection antibodies, enabling ultra-sensitive detection. When electricity is applied to the plate electrodes, the SULFO-TAG labels emit light, and the light intensity is measured to quantify the analytes in the sample. For YKL-40, we employed a commercial enzyme-linked immunosorbent assay (ELISA). ELISA is based on the specific binding of an antibody to the target protein, capturing the protein from the sample, and detecting it with a secondary antibody conjugated to an enzyme that produces a measurable signal.

1. Technical validation

We performed a partial technical validation for both the in-house MSD for sTREM2 and the commercial ELISA for YKL-40, to assess the accuracy and consistency of the assays and whether they were suitable for the measurement of the clinical samples. Our validation focused on key criteria: parallelism, recovery, lower limit of detection (LLOD), lower limit of quantification (LLOQ) and precision (validation parameters are explained in the methods section). Additionally, we conducted a pilot assay using spare clinical samples and quality controls (QCs) to evaluate the feasibility, reliability and reproducibility of the assays for clinical measurements. These validation studies were carried out across different biological matrices, including CSF, plasma and serum, ensuring the robustness of the assays in diverse sample types.

a. sTREM2

First, we undertook an initial validation of the sTREM2 ELISA immunoassay. The detailed data obtained from this validation process is presented in **table 1 and 2** for comprehensive analysis.

To assess the assay's accuracy and consistency, as well as to determine the minimal required dilution (MRD) for clinical validation of the samples, we conducted recovery and parallelism tests across various sample matrices, using known concentrations of recombinant human protein. Specifically, two CSF samples, two plasma samples, and one serum sample were tested in duplicates. Additionally, four different dilutions (1:2, 1:4, 1:8, and 1:16) were analyzed. The percentage of recovery and linearity was calculated accordingly to the methods section.

In the case of CSF, the MRD was delineated as the 1:4 dilution, as it exhibited optimal recovery (102.48%) and parallelism (83% linearity). Differently, for plasma and serum, the highest dilution (1:16) demonstrated the best performance (**Table 1**). These findings suggest that the immunochemical properties of the analyte across all matrices remained consistent throughout the tested dilution ranges

An advanced spike recovery test was conducted to assess the accuracy of the assay across an extended dynamic range, mimicking the variability of clinical samples. Specifically, three CSF samples were analyzed, each spiked with varying concentrations: high (1292 pg/mL), medium (310 pg/mL), and low (61 pg/mL). The MRD previously established for the CSF matrix was employed for these evaluations.

The data obtained showed some inconsistencies. While the mean recovery rates ranged from 80% to 96% for the three spike dilutions, the coefficient of variation (CV%) for the low and medium spike dilutions exceeded the acceptable threshold of 20%, with values of 205.3% and 38.15%, respectively. In particular, the low spike dilution demonstrated significantly poor performance (**Table 1**). Ideally, the assay should be repeated to address these inconsistencies; however, due to limited sample availability, these were the only determinations that could be made.

In addition to the experimental findings, theoretical estimates of the LLOD and the LLOQ were determined to provide further insight into the sensitivity and detection limits of the sTREM2 assay. The LLOD represents the lowest concentration of sTREM2 that can be reliably detected above background noise, while the LLOQ signifies the lowest concentration that can be accurately quantified with acceptable precision and accuracy. Both were calculated as explained in the methods section. The overall LLOD of the assay was 25.93 pg/mL; whereas the LLOQ had a value of 50.52 pg/mL (**Table 1**).

The sTREM2 pilot assay was conducted to further evaluate the performance and reliability of the assay. Specifically, we tested four QCs for CSF and plasma, as well as eight distinct CSF samples, all in triplicates. We employed the previously determined MRD for the CSF matrix and a 1:10 dilution for plasma samples. To assess the data obtained from the pilot measurements, the coefficients of variation (CV%) were calculated for each of the samples and QCs. All calculated CV% values were within the acceptable range (below 20%): the CSF samples exhibited a mean CV% of 1.32, CSF QCs had a mean CV% of 2.89, and plasma QCs demonstrated a mean CV% of 2.57 (**Table 2**).

The technical validation data and the low CV% values across all tested samples and QCs suggest excellent precision and suitability of the sTREM2 MSD immunoassay for clinical validation, aiming to assess sTREM2 levels in AD patients within the clinical cohort.

b. YKL-40

Subsequently, a partial validation of the MicroVue YKL-40 ELISA was conducted to verify the reliability of the analysis. In contrast to the sTREM2 assay, the YKL-40 assay has already been validated by the manufacturer. However, all commercial ELISA assays also need to be validated, at least partially, in each Lab. For this biomarker, we tested three CSF samples and two plasma samples in duplicates and three different dilutions (neat, 1:2 and 1:4). Standard curves were generated using the provided kit standards. The data obtained are presented in **tables 2 and 3**.

To determine the consistency and efficiency of the assay, a recovery and parallelism test was conducted. Samples were spiked with a known amount of MSD recombinant proteins, but due to the availability of reagents we only tested the medium spike concentration. Linearity was assessed by diluting the samples in the buffer provided by the kit and then comparing the observed values with the expected ones. Both matrices showed great recovery performance and excellent % of linearity aligning with the expected values. Recovery rates for CSF samples ranged between 85% and 115%; and, plasma samples showed slightly more variability, with recovery rates ranging from 80% to 110% (**Table 3**). Overall, the recovery rates and parallelism across different samples and dilutions were within acceptable ranges, confirming the assay's accuracy and robustness for the measurement of YKL-40 in both CSF and plasma matrices.

Differing from the sTREM2 validation, the determination of LLOD and the LLOQ values for the YKL-40 assay were extracted from the manufacturer's technical specifications. The LLOD was recorded at 5.4 ng/mL, while the LLOQ was established at 15.6 ng/mL (**Table 3**). Our findings corroborate the manufacturer's recommendation that the optimal working dilution for the samples is neat.

Although this assay has already been validated by the manufacturer, we conducted a YKL-40 pilot study to further verify its performance. This study included the analysis of two CSF QCs, four plasma QCs, two serum QCs, and eight clinical samples, each analyzed in triplicates. The assay's variability was assessed using the coefficient of variation (CV%). All CV% values were within the acceptable range, indicating consistent performance. Specifically, the mean CV% for CSF was 4.94, for plasma 4.86, and for serum 4.59. The clinical samples demonstrated a mean CV% of 4.83, further confirming the assay's reliability and precision **(Table 2)**.

The YKL-40 ELISA demonstrated robust performance across various matrices and dilutions. The validation process confirmed the assay's accuracy, precision, and consistency, matching the manufacturer's specifications and supporting its suitability for clinical sample validation. The pilot study further reinforced these findings, ensuring the reliability of the assay.

2. Clinical validation

Following the technical validation of the immunoassays, a clinical validation using CSF samples from patients in the BIODEGMAR cohort at Hospital del Mar was performed. This validation involved a comprehensive analysis of 294 participants, with their demographic characteristics detailed in **Table 4**. The primary objective of this clinical validation was to assess whether CSF sTREM2 and YKL-40 differed between biologically-defined AD versus non-AD. Biological definition of AD relies on the core AD CSF biomarkers (CSF Aβ42/pTau181 < 10.25) (33). Measurements of sTREM2 and YKL-40 levels were performed in CSF samples using the previously validated MSD and ELISA protocols. All samples, except for two (one for each biomarker), met the coefficient of variation acceptance criteria (CV% < 20). Therefore, these two samples were excluded from the analysis. Notably, all other samples had a CV% below 10%, indicating a high precision of the assays.

We also evaluated the impact of various demographic and clinical factors on the concentrations of sTREM2 and YKL-40 in CSF. Factors included age, sex, *APOE* ε4 status, Mini-Mental State Examination (MMSE) scores, and other AD-related biomarkers, such as CSF Aβ40, Aβ42, and pTau181. This analysis tried to identify potential correlations between these variables to understand their relationships and clinical significance in the context of AD.

To achieve this, we employed a series of statistical analyses, including linear regression models and correlation matrices. As previously mentioned, participants were stratified into two groups based on their Aβ status, determined by the Aβ42/pTau181 ratio. A ratio below 10.25 classified participants as Aβ positive (AD CSF profile), indicative of AD. Conversely, a ratio above this threshold classified participants as Aβ negative (non-AD CSF profile), suggesting the absence of AD from a biomarker perspective.

Among the 294 participants included in this analysis, the distribution was as follows: 186 (63.27%) exhibited an AD CSF profile, while 108 (36.73%) displayed a non-AD CSF profile. Age was similar across groups, with an overall mean of 72.73 ± 5.91. Female participants were predominant, especially in the AD CSF profile group (61.8% women), whereas in the non-AD CSF profile group, the distribution of sexes was more balanced (52.8% women). Overall, 28.9% of participants were carriers of *APOE* ε4, with the lowest prevalence observed in the non-AD CSF profile group (13.9%). The mean score on the MMSE test, explained in the methods section, was 20.92 ± 5.39. Patients with an AD CSF profile exhibited lower MMSE scores (19.62 ± 5.29) compared to the non-AD CSF profile group (23.22 ± 4.76).

Given that the data was not normally distributed, we conducted non-parametric analyses (**Figure 1 and 2**). CSF YKL40 was significantly higher in AD CSF profile group compared to the non-AD CSF profile group (p-value = 0.0171), but no differences were found in CSF sTREM2 (**Fig.1c and 2c**). We also observed that age exhibited significant positive correlations with sTREM2 (p-value = 0.0037, Spearman's r = 0.1692). Therefore, we stratified by Aβ status, and saw a significant positive correlation between CSF sTREM2 and age in the CSF AD profile group (p-value = 0.012, Spearman's r = 0.184), but we did not observe a significant correlation with the CSF non-AD profile one (p-value = 0.199, Spearman's r = 0.125) (**Fig.1d**). YKL-40 also showed a significant association with age (p-value = 0.0002, Spearman's r = 0.2183), in both the AD CSF profile group (p-value = 0.012, Spearman's r = 0.187) and non-AD CSF group (p-value = 0.019, Spearman's r = 0.226) (**Fig.2d**). This indicates that as age increases, the levels of these biomarkers also increase. However, no statistically significant associations were observed between the concentrations of these biomarkers and sex, *APOE* ε4 status and MMSE scores, suggesting that these factors don't have a direct impact on sTREM2 and YKL-40 levels in this cohort.

After observing a significant association between YKL-40 levels and Aβ status, we examined whether this outcome was influenced by age, given the known association between CSF YKL-40 and age reported in the literature and in our sample. To investigate this, we employed an ANCOVA, adjusting for age as a covariate. The analysis revealed a trend of significance between YKL-40 and AD CSF profile (p-value = 0.0597) even after correcting for age, suggesting that the relationship between the biomarker and an AD CSF profile is partially influenced by the age difference between the two groups. We also assessed the normality of the residuals from the linear regression model using the Shapiro-Wilk test, as it is a parametric test. The non-significant results of this test (p-value = 0.1177) indicate that there is insufficient evidence to reject the null hypothesis of normality, suggesting that the residuals are likely normally distributed. This confirms that our linear regression analysis and its interpretation is reliable.

We conducted a statistical analysis to examine the associations between CSF sTREM2 and YKL-40 with the CSF Aβ42/Aβ40 ratio and pTau181, which are core CSF biomarkers previously measured in the cohort (**Figure 3**). The Aβ42/Aβ40 ratio was used instead of the individual biomarkers to account for Aβ42 levels with Aβ40, as the latter is considered a "housekeeping protein" that remains stable across AD.

CSF sTREM2 exhibited statistically significant correlations with both biomarkers. Specifically, it showed a negative correlation with CSF Aβ42/Aβ40 ratio (p-value = 0.016, Spearman's r = -0.139) (**Fig.3a**) and a positive correlation with CSF pTau181 levels (p-value < 0.0001, Spearman's r = 0.294) (**Fig.3c**). When stratifying the data by Aβ status, we observed that only the AD CSF profile group exhibited a significant correlation between CSF sTREM2 and the CSF Aβ42/Aβ40 ratio (p = 0.003, Spearman's r = -0.219), whereas the non-AD CSF profile group did not show a significant correlation (p = 0.549, Spearman's r = -0.059) (**Fig.3a**).

Similarly, CSF YKL-40 demonstrated significant correlations: a negative correlation with CSF Aβ42/Aβ40 ratio (p-value < 0.0001, Spearman's r = -0.2749) (**Fig.3b**) and a positive correlation with CSF pTau181 levels (p-value < 0.0001, Spearman's r = 0.4080) (**Fig.3d**). When stratified by Aβ status, we found that, similar to sTREM2, the correlation between CSF YKL-40 and the CSF Aβ42/Aβ40 ratio was significant only in the AD CSF profile group (p < 0.0001, Spearman's r = -0.403) and not in the non-AD CSF profile group (p = 0.740, Spearman's r = -0.032) (**Fig.3b**).

These findings indicate that higher burden of Aβ pathology (as indicated by lower CSF Aβ42/Aβ40 ratios) are associated with higher levels of both CSF sTREM2 and YKL-40, while higher Tau pathology (as indicated by CSF pTau181) are positively correlated with increased levels of CSF sTREM2 and YKL-40.

The findings of this clinical validation underscore the influence of age on biomarker levels and highlight a specific correlation between CSF YKL-40 and AD pathology, while other factors such as sex, genetic risk, and cognitive performance seem to be unrelated to the biomarker levels in this study. Additionally, the significant associations observed between CSF sTREM2, YKL-40, and the core CSF biomarkers Aβ42 and pTau181 suggest that these glial markers may serve as valuable tools in assessing and monitoring AD progression and pathology.

DISCUSSION

In this study, we conducted a comprehensive technical and clinical validation of two immunoassays to quantify sTREM2 and YKL-40 biomarkers in MSD and ELISA-based platforms, respectively, in CSF samples from patients with cognitive impairment at Hospital del Mar. The primary objective was to evaluate the reliability of these assays in measuring biomarker levels, and subsequently, to investigate the associations between these CSF biomarkers and presence of Aβ pathology and other clinical characteristics.

The technical validation demonstrated that both MSD and ELISA assays met the required performance criteria. Parameters such as spike recovery, parallelism (% of linearity), and precision (CV%) were assessed. The minimal required dilution (MRD) was determined to be 1:4 for MSD CSF sTREM2 and neat for ELISA CSF YKL-40. Additionally, lower limits of detection and quantification were established for each assay. Pilot assays using samples and quality controls exhibited CV% values below the acceptable threshold (<20%), indicating high assay precision.

For the clinical validation, CSF samples from 294 individuals in the BIODEGMAR cohort were analyzed. BIODEGMAR cohort comprises patients with cognitive and/or conductual symptoms seen in the Cognitive Disorders Unit at Hospital del Mar. Participants were categorized based on their CSF Aβ42/pTau181 ratio, which defines biologically the presence of AD. This is a key aspect of our study; many studies include patients with clinical diagnoses and not biological diagnoses. Statistical analyses examined associations between biomarker concentrations and demographic characteristics of the cohort.

CSF sTREM2 showed a significant correlation with age. This age-related increase in CSF sTREM2 levels is thought to result not only from increased TREM2 expression by microglia but also from other mechanisms, such as increased shedding of the full-length form of TREM2 or reduced clearance during aging (22). Additionally, we observed a positive correlation between CSF sTREM2 and CSF pTau181 levels, suggesting that enhanced microglial reactivity is associated with tau deposition and neurodegeneration (34). Contrary to some studies (16,34), CSF sTREM2 demonstrated a significant correlation with the CSF Aβ42/Aβ40 ratio in the AD CSF profile group. Related to this, it has been seen that in individuals with advanced Aβ accumulation, CSF sTREM2 was no longer associated with Aβ levels; instead, CSF pTau181 and CSF sTREM2 levels increased in a similar manner (35). Moreover, the role of sTREM2-mediated microglial response in AD remains unclear, with some evidence suggesting a protective effect (36) rather than a detrimental one (37), with the need of further investigation in future studies.

CSF YKL-40 also exhibited a significant association with age and was increased in the AD CSF profile group. A positive correlation between CSF YKL-40 and increasing age has been documented in previous studies (31,32,38), indicating that CSF YKL-40 levels may be sensitive to physiological aging processes. Additionally, elevated CSF YKL-40 levels have been detected in prodromal AD, suggesting that inflammatory processes associated with AD appear in the early clinical stages (32). This could be a focus for future analyses to further investigate the role of YKL-40 in the early diagnosis and progression of AD.

Moreover, a positive correlation between CSF YKL-40 and CSF pTau181 was identified, suggesting that astrocyte reactivity and tau-associated neurodegeneration are related pathophysiological processes, as reported in other studies (31,32). The significant association between CSF YKL-40 and the CSF Aβ42/Aβ40 ratio within the AD CSF profile group supports the findings of *Craig-Schapiro et al.*, which suggest that YKL-40, coupled with Aβ42, can predict cognitive decline with a predictive value comparable to the best current CSF biomarkers (CSF tau/Aβ42 and p-tau181/Aβ42) (31).

Overall, this study aimed to address significant controversies surrounding the roles of sTREM2 and YKL-40 in AD pathology. Our findings highlight the potential utility of both MSD and ELISA assays for quantifying CSF sTREM2 and YKL-40 levels, to keep enhancing our understanding of their specific roles in microglial and astrocyte reactivity. Although they may not be useful as a diagnostic biomarkers of AD, these biomarkers show promise in improving our understanding of disease progression, facilitating early diagnosis, and developing novel therapeutic strategies. Together with other AD-related biomarkers, they have the potential to enhance patient outcomes and their quality of life in the future. Further research is necessary to fully explore their clinical potential.

However, our study has some limitations, especially due to the small sample size. Additionally, our comparison between Aβ-positive and Aβ-negative groups is based on a cohort of cognitively impaired patients, lacking a control group of healthy individuals. In the future, these results should be validated in an independent cohort and include longitudinal data in order to assess whether levels of CSF sTREM2 and YKL40 modify the progression to dementia.

MATERIALS AND METHODS

1. Sample collection

CSF samples of this study were collected from the BIODEGMAR cohort, an observational longitudinal study that enrolls individuals with cognitive decline and/or neurodegenerative diseases visiting the Cognitive Decline and Movement Disorders Unit of Hospital del Mar (Barcelona, Spain). A lumbar puncture was performed in these individuals in the intervertebral space L3/L4, L4/L5 or L5/S1 between 8 and 11 am. Participants had fasted for at least 8h. CSF was collected into a polypropylene sterile tube. Following centrifugation at 2000g for 10 minutes at 4ºC, the resulting supernatant was carefully aliquoted into 1.8 ml volumes and promptly stored at −80°C, for future analyses.

The patients of the BIODEGMAR cohort underwent a detailed neuropsychological evaluation, magnetic resonance imaging (MRI) scans, apolipoprotein ε4 (*APOE)* genotyping, lumbar puncture for CSF collection and blood sampling, as a part of the study protocol. Clinical evaluation was conducted by a neurologist, including anamnesis, physical examination and clinical diagnosis. Neuropsychological evaluation was performed by a neuropsychologist and consisted of a series of standardized cognitive tests and functional scales. The BIODEGMAR cohort has been described in detail in a previous publication, which outlines the specific criteria for participant inclusion and exclusion (33,39).

2. Biomarker measurements

a. sTREM2 MSD

Kleinberger G, Suárez-Calvet M, Haass C *et al* (40) previously developed MSD assay was employed to quantify sTREM2 levels in human CSF. The protocol used in this study is as follows:

MSD GOLD Small Spot Streptavidin coated SECTOR 96-well plates were blocked overnight at 4°C in blocking buffer [3% bovine serum albumin (BSA) and 0.05% Tween 20 in PBS (pH 7.4)]. To detect human sTREM2, the plates underwent a 90-minute incubation at room temperature (RT) on a shaker (ca. 700 rpm) with biotinylated polyclonal goat antihuman TREM2 capture antibody (0.25 mg/ml) (R&D Systems), diluted in blocking buffer. Subsequently, 4 washing steps were performed on the plates with washing buffer (0.05% Tween 20 in PBS), followed by 2-hour incubation at RT on a shaker (ca. 700 rpm) with samples diluted 1:4 in sample dilution buffer [1% BSA and

0.05% Tween 20 in PBS (pH 7.4)] and supplemented with protease inhibitors (Roche). A recombinant human TREM2 protein (Abyntek) was also diluted in sample dilution buffer in a twofold serial dilution and used for the standard curve (concentration range, 8000 to 62.5 pg/ml). Plates underwent 2 washing steps, with 3 cycles each, before incubation with monoclonal mouse IgG anti-human TREM2 detection antibody (1 mg/ml) (Santa Cruz Biotechnology) diluted in sample dilution buffer, for 1 hour at RT on a shaker (ca. 700 rpm). Plates were washed twice, with 3 cycles each, and incubated with a SULFO-TAG-labeled amti-mouse secondary antibody (1:1000; Meso Scale Discovery) for 1 hour on a shaker (ca. 700 rpm). Lastly, plates were washed two times, with 3 cycles each, with wash buffer followed by two washing steps in PBS and developed by adding 1X Meso Scale Discovery Read buffer. Meso Scale Discovery SECTOR Imager 2400 reader was used to quantify light emission at 620 nm after electrochemical stimulation.

b. YKL-40 ELISA

YKL-40 concentration in CSF was measured by the MicroVue YKL-40 ELISA Kit from Quidel Corporation, following the manufacturer's instructions.

Prior to the assay, the coated strips were allowed to equilibrate to RT. Subsequently, the standards, controls and samples were pipetted into the designated assay wells within a 30-minute timeframe. Capture solution was added to each assay well, followed by a 60-minute incubation at RT. Following incubation, plates were washed four times with 1x wash buffer. Next, enzyme conjugate containing the anti-YKL-40 antibody was added. The plates were then incubated for an additional 60 minutes at RT. Afterward, the washing procedure was repeated four times as previously described. A chromogenic substrate solution was prepared by dissolving one substrate tablet per bottle of substrate buffer within one hour of use. After allowing sufficient time for dissolution and vigorous shaking, 100 µL of substrate solution was added to each well. The plate was then incubated for 60 minutes at room temperature, after which 100 µL of stop solution was pipetted into each well. The strips were read within 15 minutes of stop solution addition using a microplate reader at an optical density of 405 nm.

c. Recombinant protein preparation.

The recombinant human TREM2 protein was obtained in a lyophilized form at a concentration of 0.1 mg. The lyophilized material was reconstituted in 500 µL of sterile double-distilled water to achieve a pre-stock concentration of 0.2 mg/mL. The pre-stock solution was then diluted in two steps to achieve a working stock concentration of 200 ng/mL. 10 µL of the pre-stock solution was added to 990 µL of sample dilution buffer, and then 200 µL of the resulting solution was added to 1800 µL of sample dilution buffer.

3. **Assay validation**

a. **Spike and recovery**

To determine whether analyte detection was affected by a difference between the diluent used to prepare the standard curve and the sample matrix, a recovery spike assay was performed for both validations. The acceptance range for the recovery is between 80 and 120%, calculated using the following formula:

$$\%\,Recovery = \frac{Mesured\ concentration_{spiked\ sample} - Mesuered\ concentration_{neat\ sample}}{Theoretical\ concentration_{spiked}} \text{ x } 100$$

b. **Parallelism**

Parallelism was evaluated by assessing the dilutional linearity across the tested dilution ranges for each matrix. This ensures that the binding characteristics of the endogenous analyte in the sample are consistent with those of the standard. The percentage of linearity, which measures how well the assay results conform to a linear response across the dilutions was determined. The acceptance range for parallelism is between 80 and 120%. The percentage of linearity is calculated using the formula:

$$\%\,linearity = \frac{observed\ value}{expected\ value} \text{ x } 100$$

c. **Precision**

Precision was assessed by examining the closeness of agreement between independent test results under stipulated conditions. Three types of precision were evaluated: repeatability (r), intermediate precision (Rw), and reproducibility (R).

- Repeatability: measures variability when conditions such as laboratory, technician, days, instrument, and reagent lot are held constant.
- Intermediate precision: measures variability when all factors except laboratory are allowed to vary.
- Reproducibility: measures variability when all factors are varied over several days.

Precision is evaluated by calculating the coefficient of variation (CV%). The acceptance criteria for CV% should be below 20%. The CV% was calculated with the following formula:

$$\%CV = \frac{Standard\ Deviation\ (SD)}{Average\ Measured\ Signal} \text{ x } 100$$

d. Lower Limit of Detection (LLOD) and Quantification (LLOQ)

The LLOD represents the lowest concentration of analyte that can be reliably detected above background noise, while the LLOQ signifies the lowest concentration that can be accurately quantified with acceptable precision and accuracy. Both parameters were determined by analyzing the mean signal of 16 blank samples. Specifically, the mean ($\bar{x}$) and standard deviation (SD) of the signal from these blanks were used in the following formulas to compute the LLOD and LLOQ:

$$LLOD = \bar{x} + 3SD \qquad LLOQ = \bar{x} + 10SD$$

4. Statistical analysis

For these analyses, all participants from the BIODEGMAR cohort at Hospital del Mar with CSF results were included. Participants were stratified based on their Aβ status using the Aβ42/pTau181 ratio, with a threshold of 10.25. A ratio below the cutoff classified participants as Aβ positive, indicative of AD, while a ratio above this threshold classified them as Aβ negative (33). Demographic variables such as age, sex, *APOE* ε4 carrier status and MMSE were included in the analysis as well as three other AD related CSF core biomarkers (Aβ40, Aβ42 and pTau181).

We summarized participant characteristics across the groups using means and standard deviations for continuous variables and counts and percentages for categorical variables in **table 4**. Comparisons of biomarkers between categorical variables were performed with Mann U Whitney test and ANCOVA adjusted by age; Shapiro-Wilk test was used to test the assumption of normality. Correlations were tested with a Spearman's correlation coefficient.

a. Mini-Mental State Examination (MMSE)

The MMSE test was used to assess cognitive impairment in participants. It is a standardized tool that evaluates cognitive functions such as orientation, registration, attention, memory, language, and ability to follow simple commands. The MMSE was scored based on the number of correct responses, with higher scores indicating better cognitive function. The standard MMSE form includes the following cut-off levels to classify the severity of cognitive impairment: no cognitive impairment [30], questionable dementia [26-29], mild dementia [21-25], moderate dementia [11-20], and severe dementia [0-10] (41).

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FIGURES AND TABLES

Basic validation					
Matrix	**Dilution**	**Recovery (%)**	**Parallelism (% linearity)**	**LLOD (pg/mL)**	**LLOQ (pg/mL)**
CSF	1:2	78.76	-	25.93	50.52
	1:4	102.48	82		
	1:8	113.62	70		
	1:16	117.83	73		
Plasma	1:2	34.57	-		
	1:4	55.47	129		
	1:8	80.02	104		
	1:16	98.86	73		
Serum	1:2	19.52	-		
	1:4	45.59	185		
	1:8	70.14	120		
	1:16	96.21	88		

Advanced validation						
Matrix	**Dilution**	**Spike dilution**	**Spike (pg/mL)**	**Recovery (%)**	**SD**	**CV%**
CSF	1:4	Low	61	88,21	205,3	232,76
		Medium	310	95,70	38,15	39,86
		High	1292	80,65	6,93	8,59

Table 1. sTREM2 technical validation parameters. Three different matrices (CSF, plasma, and serum) were tested using four dilutions (1:2, 1:4, 1:8, 1:16), each analyzed in duplicates. The best-performing dilution for each matrix is highlighted in green. The table provides comprehensive information on spike recovery (%), parallelism (as % of linearity), Lower Limit of Detection (LLOD), and Lower Limit of Quantification (LLOQ). Data from the advanced recovery test are also included, reporting mean recovery spike concentrations, standard deviation (SD), and coefficient of variation (CV%) for three spike concentrations (low, medium, and high). These validation parameters were used to determine the optimal assay conditions for sTREM2 measurements.

	Matrix	Mean (pg/mL)	SD	CV%
sTREM2	Samples	3195,42	43,126	1,32
	CSF QCs	2684	77,209	2,89
	Plasma QCs	2519,63	60,873	2,57
YKL-40	Samples	257,00	11,33	4,83
	CSF QCs	250,82	12,17	4,94
	Plasma QCs	173,69	6,45	4,59
	Serum QCs	39,78	1,96	4,86

Table 2. sTREM2 and YKL-40 pilot test parameters. Two different QC matrices (CSF and plasma) were tested for sTREM2, and three matrices (CSF, plasma, and serum) were tested for YKL-40. Additionally, CSF samples were analyzed in both pilot assays. The table provides comprehensive information on mean concentrations (pg/mL), standard deviation (SD), and coefficient of variation (CV%). These parameters validate the accuracy of the assays in measuring both biomarkers.

Matrix	Dilution	Recovery (%)	Parallelism (% linearity)	LLOD (ng/mL)	LLOQ (ng/mL)
CSF	1:1	92.56	-	5.4	15.6
	1:2	88.26	101		
	1:4	102.00	105		
Plasma	1:1	90.01	-		
	1:2	99.04	98		
	1:4	85.72	104		

Table 3. YKL-40 technical validation parameters. Two different matrices (CSF and plasma) were tested using three dilutions (1:1, 1:2, 1:4) each analyzed in duplicates. The best-performing dilution for each matrix is highlighted in green. The table provides comprehensive information on spike recovery (%), parallelism (as % of linearity), Lower Limit of Detection (LLOD), and Lower Limit of Quantification (LLOQ). These validation parameters were used to determine the optimal assay conditions for YKL-40 measurements.

Characteristics	Total (n = 294)	AD CSF Profile (n = 186)	Non-AD CSF Profile (n = 108)
Age	72.73 ± 5.91	73.39 ± 5.63	71.59 ± 6.23
Sex			
Men	122 (41.5%)	71 (38.2%)	51 (47.2%)
Women	172 (58.5%)	115 (61.8%)	57 (52.8%)
Apoε 4			
Carrier	85 (28.9%)	70 (37.6%)	15 (13.9%)
Non-Carrier	124 (42.2%)	64 (34.4%)	60 (55.6%)
MMSE	20.92 ± 5.39	19.62 ± 5.29	23.22 ± 4.76
CSF Core Biomarkers			
CSF Aβ40	12213.37 ± 3918.63	12265.83 ± 3893.99	12123.03 ± 3977.31
CSF Aβ42	736.47 ± 384.03	546.33 ± 174.23	1063.92 ± 424.6
CSF pTau181	90.95 ± 57.04	116.82 ± 55.08	46.39 ± 22.3
CSF sTREM2	3762.58 ± 1015	3790.68 ± 933.14	3713.72 ± 1146.51
CSF YKL-40	295.88 ± 83.84	304.66 ± 82.36	280.62 ± 84.6

Table 4. Demographics of the cohort. Demographic and clinical characteristics of the 294 patients of the cohort, stratified by total participants, those with an AD CSF profile, and those with a non-AD CSF profile. Continuous variables, including age, MMSE scores and core CSF biomarkers (Aβ40, Aβ42, pTau181, sTREM2 and YKL-40), are reported as mean ± standard deviation (SD). Categorical variables, such as sex and *APOE* ε4 status, are presented as counts and percentages (n, %). For the *APOE* ε4 status variable, information was not available for all participants; *APOE* ε4 profile was described for 209 participants.

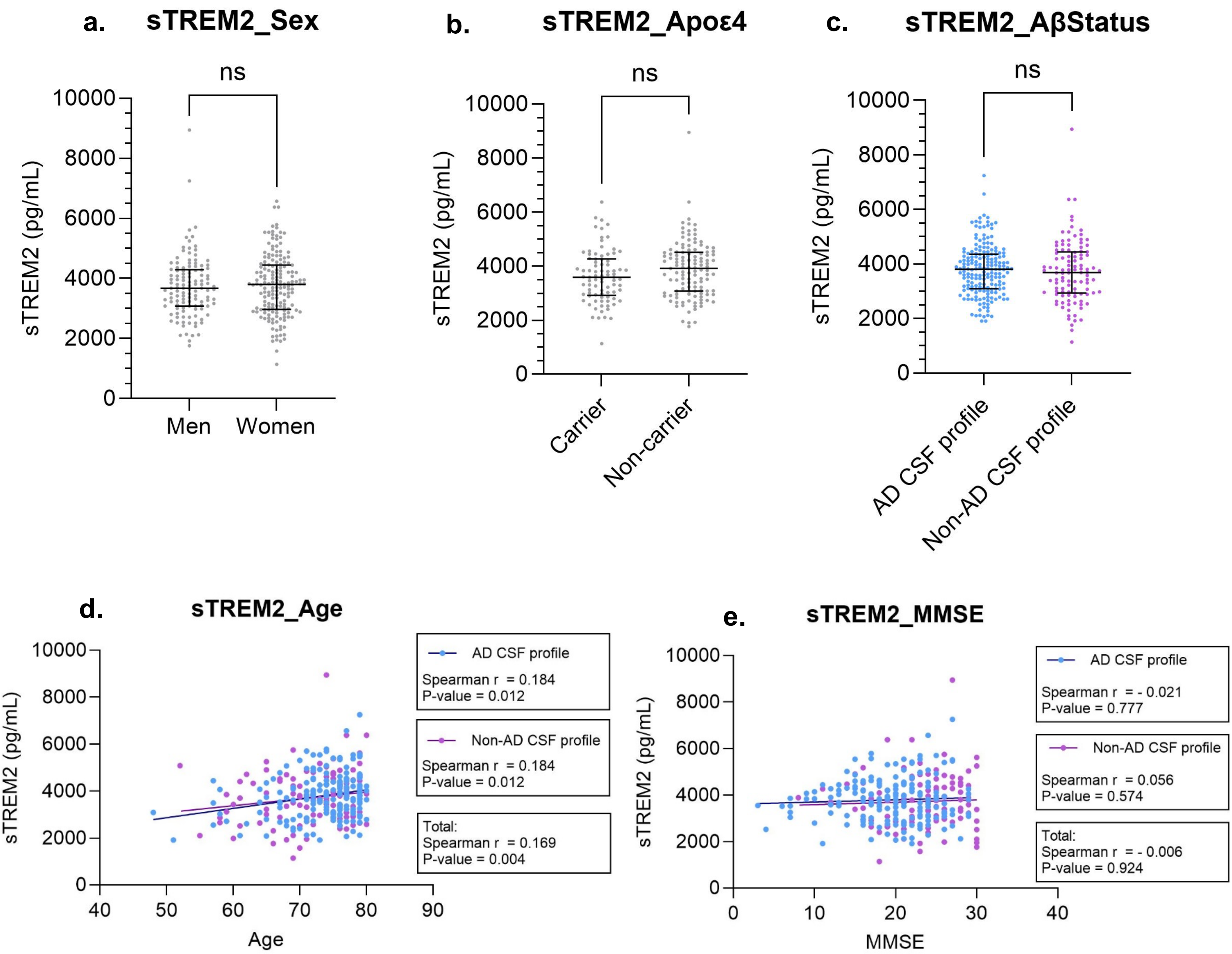

Figure 1. sTREM2 correlations. For categorical variables (Sex, *APOE* ε4 status, and Aβ profile), the Mann U Whitney test was performed. For continuous variables (Age and MMSE scores), Spearman correlation analysis was conducted. Significance was determined by a p-value < 0.005. Black lines represent the median with 95% confidence intervals (CI) and colored lines represent linear regression. P-value and Spearman's r value are shown for the AD CSF profile group, non-AD CSF profile group and total of participants.

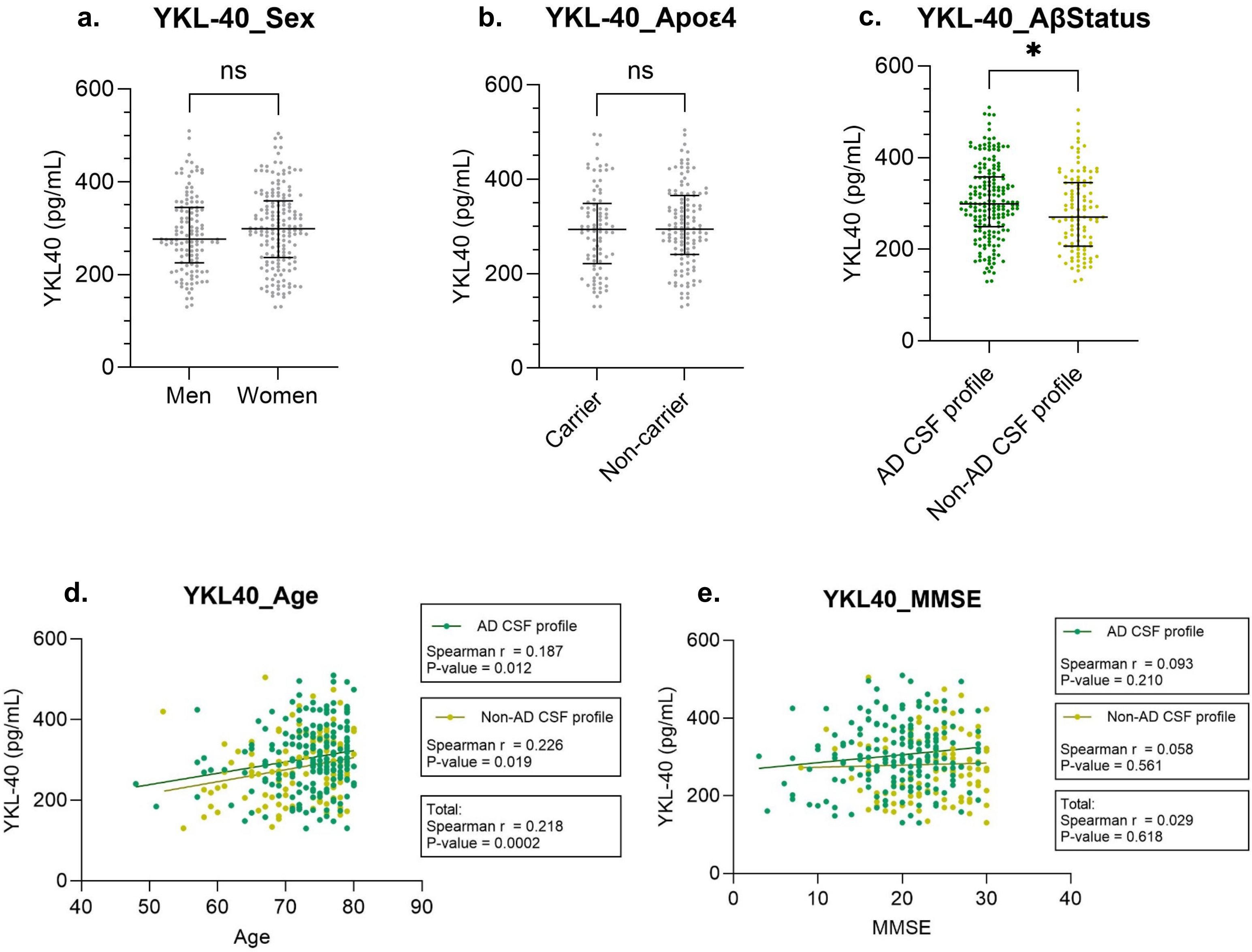

Figure 2. YKL-40 correlations. For categorical variables (Sex, *APOE* ε4 status, and Aβ profile), the Mann U Whitney test was performed. For continuous variables (Age and MMSE scores), Spearman correlation analysis was conducted. Significance was determined by a p-value < 0.005. Black lines represent the median with 95% confidence intervals (CI) and colored lines represent linear regression. P-value and Spearman's r value are shown for the AD CSF profile group, non-AD CSF profile group and total of participants.

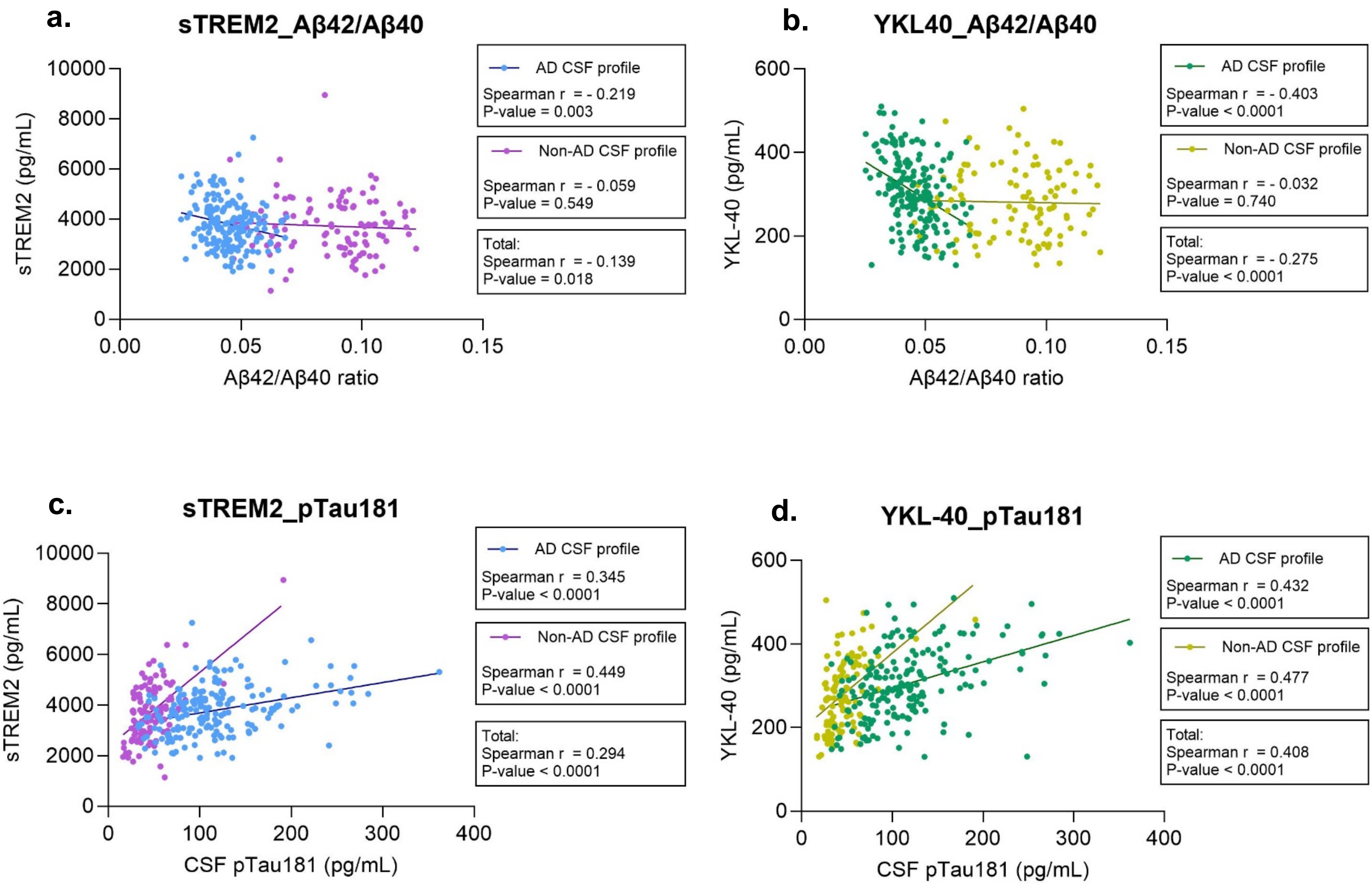

Figure 3. sTREM2 and YKL-40 correlations with CSF core biomarkers. Correlations with CSF core biomarkers Aβ40, Aβ42, and pTau181 were analyzed by Spearman correlation analysis and ANCOVA. Significance was determined by a p-value < 0.005. P-value and Spearman's r value are shown for the AD CSF profile group, non-AD CSF profile group and total of participants. Lines represent linear regression.