

Promising Diagnostic Accuracy of Plasma GFAP and NfL Within The AD Continuum

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Research

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Abstract

Background: Blood-based biomarkers may add a great benefit in detecting the earliest neuropathological changes in patients with Alzheimer's disease (AD). We examined the utility of neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) in plasma and cerebrospinal fluid (CSF) regarding clinical diagnosis and amyloid positivity in an outpatient memory clinic - based cohort.

Methods: In this retrospective analysis, we included a total of 185 patients, 141 patients along clinical the AD continuum, i.e. subjective cognitive decline (SCD, n=18), mild cognitive impairment (MCI, n=63), AD (n=60) and 44 age-matched healthy controls (HC). CSF and plasma concentrations of NfL and GFAP were measured with single molecule array (SIMOA) technology using the Neurology 2-Plex B kit from Quanterix. Amyloid-PET was performed in 75 patients and graded as amyloid positive and negative by visual rating. To assess the discriminatory potential of different biomarkers, age- and sex-adjusted receiver operating characteristic (ROC) curves were calculated and the area under the curve (AUC) of each model was compared using DeLong's test for correlated AUC curves.

Results: We constructed a panel combining plasma NfL and GFAP with known AD risk factors (age+sex+APOE4+GFAP+NfL panel). Using this panel, AUC was 91.6% for HC vs. AD, 81.7% for HC vs. MCI, 85% for SCD vs. AD, 81.3% for SCD vs. MCI, 77.7% for HC vs. SCD and 72.3% for MCI vs. AD. In terms of predicting amyloid PET status, we computed an AUC of 88.4%.

Conclusion: The combination of plasma GFAP and NfL with well-established risk factors could contribute crucially to the identification of patients at risk, and thereby facilitate inclusion of patients in clinical trials for disease modifying therapies.

Background

Alzheimer's disease (AD) represents a frequent neurodegenerative disorder, which leads to a progressive decline in cognitive functions^{1,2}. In the last decades, emerging evidence indicates the beginning of neuropathological changes with cerebral accumulation of amyloid-beta (Ab) and neurofibrillary tangles (NFT) 10 to 20 years before manifestation of obvious clinical signs³⁻⁵. Based on this knowledge, the definition of AD shifted towards a rather biological construct with a better understanding of AD as a disease continuum^{4,5}. The preclinical phase of AD defines a stage where early neuropathological changes are present but cognitive deficits are not yet detectable. Regarding the inclusion in clinical trials and the development of disease modifying therapies, it is crucial to identify patients at-risk as early as possible in the evolution of the neuropathological cascade. Therefore, the diagnosis of early phases of AD, such as mild cognitive impairment (MCI) due to AD and even stages of subjective cognitive decline (SCD) as a possible late stage of preclinical AD, is of particular interest.

Neurodegeneration with early synaptic and consecutive neuronal loss represents an important neuropathological component in the pathophysiology of AD and thus correlates even better with the main symptoms of cognitive decline than do amyloid and tau pathology, although both proteins contribute to

the neurodegenerative process^{6,7}. Considering that positron emission tomography (PET) imaging is a cost-intensive diagnostic biomarker and lumbar puncture is an invasive examination, recent studies have been looking for the possibility to identify reliable fluid biomarkers by conventional blood analysis. The establishment of new and sensitive analytical methods may facilitate this approach. In comparison to already established enzyme-linked immunosorbent assay (ELISA), the usage of ultrasensitive single molecule array (SIMOA→) has proven a superior value for several molecules⁸.

Neurofilament light chain (NfL), a subunit of specific cytoskeletal proteins of neurons, represents a highly proposed biomarker regarding detection of neuronal loss. NfL is released through axonal damage into the cerebrospinal fluid (CSF) and eventually into the blood⁹. Accumulating data have shown that plasma NfL could be used as a non-invasive biomarker for neurodegeneration, which correlates well with cognitive decline and brain atrophy^{10–12}. Studies have found higher plasma NfL levels in patients with MCI and AD in comparison to cognitively unimpaired controls¹³, and estimated a beginning increase in plasma NfL levels about 10 years before AD diagnosis compared to those who remain dementia-free¹⁴. Another promising biomarker for tracking neurodegenerative changes could be glial fibrillary acidic protein (GFAP), an intermediate filament protein of astrocytes. Neuropathological data have shown a close spatial relationship between reactive astrocytes and amyloid plaques in brain tissue of patients with AD^{15,16}. GFAP is known to be involved in the neuroinflammatory cascade of AD pathophysiology. High GFAP concentrations have been detected in the CSF of patients with dementia of various aetiologies^{17–19}, and increased blood GFAP levels were also found in patients with AD in comparison to healthy controls^{20,21}. Furthermore, an association between plasma GFAP levels and amyloid load in AD patients has been recently observed^{20–23}, corroborating neuropathological findings.

The aim of this study was to examine GFAP and NfL levels in CSF and plasma in various stages of the clinical AD continuum compared to healthy controls. Furthermore, we investigated the predictive value of these blood biomarkers in combination with well-established risk factors in relation to clinical diagnosis and amyloid positivity in an outpatient memory clinic setting,

Methods

Study population

185 patients were enrolled in this retrospective study at the Memory Clinic of the Department of Neurology, Medical University of Vienna. Using two existing registries, i.e. the Dementia Registry RDA MUV (EK 1323/2018) and the BIOBANK MUV (EK 2195/2016), we identified 141 patients with a diagnosis along the clinical spectrum of cognitive decline, i.e. SCD (n = 18), MCI (n = 63) and AD (n = 60). Additionally, 44 age-matched healthy controls (HC) without signs of neurodegenerative disorder or cognitive decline were included.

All 141 patients with cognitive complaints (SCD, MCI, AD) underwent a thorough standardized diagnostic examination including physical and neurological evaluation, neuropsychological testing, magnetic resonance imaging (MRI) of the brain and basic laboratory testing. For a subset of patients, we extended our diagnosis with a biomarker-based approach. CSF analysis of established AD biomarkers were available (e.g. Ab42, tTau, pTau) in 75 patients, amyloid-PET imaging was performed in 75 patients, and 54 patients underwent both diagnostic methods.

Diagnosis of AD and MCI were based on the recommendation of the National Institute of Ageing and Alzheimer's Association (NIA-AA)^{4,24}. The diagnosis of SCD was applied when no abnormalities on cognitive tests were observed and diagnosis criteria for MCI, AD or other major neurological or psychiatric disorders were not met^{25,26}.

All 185 study participants were required to have a plasma EDTA sample stored in the Biobank MUV, for 103 study participants CSF samples were available as well.

The project was approved by the Ethics Committee of the Medical University of Vienna (EK 1965/2019) on November 28th, 2019.

Neuropsychological assessment

The Neuropsychological Test Battery Vienna (NTBV) was administered to assess cognitive function, including domains of attention, language, executive functioning and episodic memory and is described elsewhere^{27,28}. Adequate normative data from cognitively unimpaired individuals were available and z-scores for each variable were calculated, corrected for age, education and sex. Global cognition was computed by Mini Mental State Examination (MMSE) and Wortschatztest (WST), a standardized vocabulary test providing an estimate of premorbid IQ. Depressive symptoms were measured via Beck Depression Inventory (BDI-II)²⁹.

MR Imaging

All patients underwent at least a T1-weighted MR sequence, a T2-weighted or a Fluid-attenuated inversion recovery (FLAIR) MR sequence and a diffusion-weighted MR sequence within the routine diagnostic setting for the evaluation of the extent and pattern of atrophy, the presence and degree of vascular lesions and to exclude other underlying pathologies causing cognitive decline (e.g. normal pressure hydrocephalus, subdural hematomas or brain tumors) and diffusion restricted areas (representing acute ischemia, inflammation or signal changes indicating Creutzfeldt-Jakob disease).

Amyloid-PET Imaging

75 patients underwent an Amyloid-PET scan with [¹⁸F]flutemetamol (n = 25) or [¹¹C]Pittsburgh compound-B (PiB, n = 50). Amyloid PET imaging was performed on one of two possible PET scanner systems (Siemens Biograph 64 True Point, Erlangen, Germany or GE Advances PET, GE Healthcare Institute, Waukesha, Wisconsin, USA) belonging to the Division of Nuclear Medicine of the Medical University of Vienna. All studies were performed under strictly controlled conditions. Either ~ 400 MBq of

[11C]PiB (in house production according to previously published recommendations³⁰) or 185MBq of [18F]flutemetamol (Vizamyl®, GE Healthcare) were injected intravenously into a peripheral vein with starting image acquisition 40 minutes p.i. for [11C]PiB and 90 minutes p.i. for VizamyI® for 20 minutes, where the tracer accumulation in the brain is reaching the maximum. Using the GE Advances PET scanner, a 3D ordered subset expectation maximization (OSEM) filtered backprojection (FBP) reconstruction was done (128x128 matrix, Hanning filter, cut-off 6.2mm). For [18F]flutemetamol, using the Siemens Biograph 64 True Point the reconstruction was performed using a 3D OSEM with 4 iterations and 21 subsets into a 168x168 matrix with a Zoom of 2 (2 x 2 mm pixel size), and with a 5 mm full width at half-maximum (FWHM) Hann post reconstruction filter applied to the final images. Subsequently, the image acquisition was performed for about 20 min following a computed tomography (CT) acquisition for attenuation correction using Siemens Biograph 64 True Point.

APOE genotyping

Genomic DNA was extracted from peripheral blood leucocytes of individuals with available whole blood samples (n = 159) using standard DNA isolation methods. Apolipoprotein E (*APOE*) genotyping was performed using quantitative polymerase chain reaction (qPCR) with TaqMan probes (Thermofisher) evaluating two single nucleotide polymorphisms (SNPs) in the *APOE* gene (rs429358 and rs7412). Each sample was tested for both SNPs in triplicates using 20 ng DNA. Allelic discrimination analysis was used to determine the *APOE* genotype of the study participants.

CSF biomarkers

CSF was obtained by lumbar puncture between the L3/L4, L4/5 or L5/S1 intervertebral space, collected in polypropylene tubes and further stored at -20°C until biomarker analysis (as for Ab42, pTau 181 and tTau), or immediately at -80°C for future research purposes^{31,32}. Levels of Ab42, pTau 181 and tTau were measured with commercially available enzyme-linked immunosorbent assays (ELISA) (Innotest hTAU-Ag, Innotest phosphoTAU 181p, Innotest beta-amyloid 1-42)^{33,34}.

Plasma biomarkers

EDTA plasma was collected through venepuncture and stored at -80°C in our local biobank. Concentrations of NfL and GFAP were quantified with ultrasensitive single molecule array (SIMOA) using the Neurology 2-Plex B kit from Quanterix in CSF and plasma. Detailed analyses are described elsewhere³⁵. In short, equilibrated calibrators, samples and controls were diluted (1:4 for plasma and 1:100 for CSF) and incubated with detector and paramagnetic reagents provided by the manufacturer. Streptavidin β-galactosidase was added to each well before samples were transferred to the Quanterix SR-X analyser for measurement of protein levels. All samples were analysed as duplicates and all assay materials were obtained from the same kit lot. Intra-assay CV was < 12% for GFAP and < 8% for NfL. Inter-assay CV for two samples measured repeatedly on 10 plates was well acceptable (< 13% for GFAP and 9% for NfL).

Statistical Analysis

Data is presented as n (percent) or median (interquartile range) as appropriate. Testing for differences between groups was performed using chi-square test, the Mann-Whitney-U-test or the Kruskal-Wallis test. Correlation was assessed using Spearman's rank correlation coefficient. To evaluate the discriminatory performance of the biomarkers assessed herein, the cohort was split into pairs of two diagnosis (e.g. AD and HC) and the response variable was coded as existing for the more severe diagnosis (i.e. SCD when assessing SCD vs HC). Next, a baseline model consisting of sex, age and *APOE4* status (increased risk for carriers of the *APOE4* allele) was constructed using logistic regression. A receiver operating characteristic (ROC) curve was plotted and the area under the curve (AUC) was measured. Optimal cutoffs were calculated using Youden's J-Statistic³⁶ and sensitivity and specificity are reported as percentage. The baseline model was then supplemented by either levels of plasma GFAP, plasma NfL, or both and the AUC of each model was compared using DeLong's test for correlated AUC curves³⁷. A p value of < 0.05 was interpreted as statistically significant. All calculations were performed in R (Version 4.0.4) and the pROC package was used for ROC calculations³⁸

Results

Table 1
Demographics and clinical characteristics

	HC	SCD	MCI	AD	<i>p</i> value
N	44	18	63	60	
Sex (f)	24 (54.5%)	8 (44.4%)	29 (46.0%)	36 (60.0%)	<i>p</i> = 0.399 over all groups
Age	61.2 (55.8,69.5)	68.3 (60.1, 77.9)	69.9 (59.3, 77.8)	69.0 (61.4, 75.1)	<i>p</i> < 0.05 for HC vs. SCD, <i>p</i> < 0.01 for HC vs. MCI and AD
MMSE	n.a.	29 (28, 30)	27 (25, 28)	20 (14, 23)	<i>p</i> < 0.001 for AD vs. SCD and MCI, <i>p</i> < 0.01 for SCD vs. MCI
APOE4 carrier	12 (33.3%)	3 (18.8%)	22 (40.7%)	33 (62.3%)	<i>p</i> < 0.01 over all groups
CSF Ab42 (pg/ml)	n.a.	n.a.	354 (248, 479.5)	332.5 (231.8, 454.8)	<i>p</i> = 0.370 for MCI vs. AD
CSF tTau (pg/ml)	n.a.	n.a.	310 (188, 504.5)	600.5 (404.3, 1106.8)	<i>p</i> < 0.001 for MCI vs. AD
CSF pTau (pg/ml)	n.a.	n.a.	53 (33.5, 79.5)	77.5 (51.3, 96.3)	<i>p</i> < 0.05 for MCI vs AD
Positive amyloid PET	n.a.	n.a.	20 (55.6%)	37 (94.9%)	<i>p</i> < 0.001 for MCI vs. AD
Data is presented as median and interquartile range (IQR, 25th – 75th percentile) or n (%). Demographic and clinical differences were measured using Kruskal-Wallis test or chi-square tests as appropriate. <i>APOE</i> status was available for 159 patients (36 HC, 16 SCD, 54 MCI, 53 AD), CSF AD biomarkers (Ab42, tTau, pTau) for 75 patients (37 MCI, 38 AD) and amyloid-PET for 75 patients (36 MCI, 39 AD).					
<i>HC</i> healthy control, <i>SCD</i> subjective cognitive decline, <i>MCI</i> mild cognitive impairment, <i>AD</i> Alzheimer's disease, <i>f</i> female, <i>MMSE</i> mini mental state examination, <i>CSF</i> cerebrospinal fluid, <i>Ab42</i> amyloid-beta 42, <i>tTau</i> total tau, <i>pTau</i> phosphorylated tau, <i>n.a.</i> not available					

Demographic and clinical characteristics are listed in Table 1.

We observed no significant difference in sex distribution between the groups (*p* = 0.399).

HC were significantly younger than the patient groups (*p* < 0.05 for HC vs. SCD, *p* < 0.01 for HC vs. MCI and AD), but there was no difference in age between the three different patient cohorts. MMSE decreased significantly with progressing disease with the lowest score in the AD group (*p* < 0.001 for AD vs. SCD and MCI, *p* < 0.01 for SCD vs. MCI).

Data of *APOE4* carriership (carriers of at least one *APOE4* allele) was available for 159 patients, i.e. in 36 controls, 16 patients with SCD, 54 with MCI and 53 with AD, with the highest occurrence of *APOE4* alleles in AD patients (33 of 53 patients, 62.3%). A chi-square test of independence was performed to examine the relation between the *APOE4* status and the diagnosis. As can be seen by the frequencies cross tabulated in Table 1, there was a significant relationship between *APOE4* status and diagnosis (X^2 (3, N = 185) = 13.21, $p < 0.01$).

For a subset of patients ($n = 75$) CSF analysis of established AD biomarkers were available (e.g. Ab42, tTau, pTau). Regarding the concentration of the AD biomarkers, median levels of tTau in MCI and AD were 310 pg/ml (IQR 188, 504.5) and 600.5pg/ml (IQR 404.3, 1106.8), respectively, pTau 53 pg/ml (IQR 33.5, 79.5) and 77.5 pg/ml (IQR 51.3, 96.3), respectively and amyloid-beta 354pg/ml (IQR 248, 479.5) and 332.5pg/ml (231.8, 454.8), respectively. While CSF tTau and pTau levels increased significantly with progression from MCI to AD ($p < 0.001$ for MCI vs. AD for tTau and $p < 0.05$ for MCI vs. AD for pTau), the difference of Ab42 concentration between MCI and AD reached no statistical significance ($p = 0.370$ for MCI vs. AD).

Amyloid-PET imaging was performed in 36 patients with MCI and 39 patients with AD. Amyloid-PET detected the cerebral cortical accumulation of amyloid-beta in 20 MCI patients (55.6%). Positive amyloid - PET imaging was significantly higher in AD patients with a total of 37 (94.9%) positive subjects ($p < 0.001$ for MCI vs. AD).

Table 2
Plasma and CSF Results for NfL and GFAP

	HC	SCD	MCI	AD	<i>p</i> value
N	44	18	63	60	
Plasma NFL (pg/ml)	8.1 (5.9, 12.2)	15.8 (8.2, 18.6)	12.9 (8.5, 20.4)	15.5 (11.8, 23.2)	<i>p</i> < 0.001 for HC vs. MCI and AD, <i>p</i> < 0.01 for HC vs. SCD, <i>p</i> < 0.05 for MCI vs AD
Plasma GFAP (pg/ml)	79.0 (53.7, 120.6)	111.0 (71.0, 154.0)	167.5 (93.8, 256.3)	181.9 (129.6, 269.6)	<i>p</i> < 0.001 for HC vs. MCI and AD, <i>p</i> < 0.01 for SCD vs. AD, <i>p</i> < 0.05 for SCD vs. MCI
N	36	0	30	37	
CSF NFL (pg/ml)	584.1 (449.6, 832.8)	n.a.	807.7 (507.6, 1103.2)	1559.1 (1026.6, 2513.9)	<i>p</i> < 0.001 for HC vs. AD and MCI vs. AD
CSF GFAP (pg/ml)	11145.3 (6980.5, 14373.8)	n.a.	8946.2 (7028.8, 13842.7)	13663.5 (9945.4, 21059.1)	<i>p</i> < 0.01 for MCI vs. AD, <i>p</i> < 0.05 for HC vs. AD

Data is presented as median and interquartile range (IQR, 25th – 75th percentile) or n. Differences of biomarker concentration were calculated using Kruskal-Wallis test. CSF data for GFAP and NFL were available for 103 patients.

HC healthy control, *SCD* subjective cognitive decline, *MCI* mild cognitive impairment, *AD* Alzheimer's disease, *CSF* cerebrospinal fluid, *NfL* neurofilament light chain, *GFAP* glial fibrillary acidic protein, *n.a.* not available

Plasma GFAP showed a gradual increase along the four cohorts, with the lowest concentration in HC (median 79pg/ml, IQR 53.7, 120.6) and the highest in patients with AD (median 181.9pg/ml, IQR 129.6, 269.6) (Table 2). Besides significantly higher levels of plasma GFAP in patients with MCI and AD compared to healthy controls (Fig. 1, *p* < 0.001), we observed a significant difference between patients with SCD and AD (*p* < 0.01) and patients with SCD and MCI (*p* < 0.05). The difference between HC and SCD, as well as between MCI and AD missed statistical significance (*p* = 0.092 and *p* = 0.098, respectively).

Plasma NfL performed similar to GFAP regarding the difference in concentrations between HC and MCI/AD (*p* < 0.001, Table 2 and Fig. 1). However, NfL levels showed a significant difference between HC and SCD (*p* < 0.01) as well as between MCI and AD (*p* < 0.05), but not between SCD and MCI/AD. Interestingly, we found the highest concentration of NfL in patients with SCD (median 15.8 pg/ml, IQR 8.2, 18.6).

For 103 patients, CSF samples in our local biobank were available. While levels of CSF NfL increased gradually (HC 584.1 pg/ml IQR 449.6, 832.8; MCI 807.7pg/ml IQR 507.6, 1103.2; AD 1559.1 pg/ml IQR 1026.6, 2513.9), CSF GFAP presented the lowest concentration in the MCI group (8.946.2pg/ml IQR 7028.8, 13842.7), followed by HC (11.145.3pg/ml IQR 6980.5, 14373.8) and AD (13.663.5pg/ml IQR 9945.4, 21059.1). Concerning the performance of these two CSF biomarkers in distinguishing between HC and AD or MCI and AD, CSF NfL showed slightly better results (*p* < 0.001) in comparison to CSF GFAP (*p* < 0.05 and *p* < 0.01, respectively).

Using Spearman correlation coefficient, the correlation of NfL and GFAP in CSF and plasma were analysed (Fig. 2a and 2b). Correlation between NfL in CSF and plasma was calculated with *R* = 0.64 (*p* <

0.001, Fig. 2a) and GFAP in CSF and plasma with $R = 0.4$ ($p < 0.001$, Fig. 2b).

To assess the clinical utility of GFAP and NfL in plasma, particularly in distinguishing healthy controls from patients with cognitive complaints (e.g. SCD, MCI and AD) and potentially predicting cerebral amyloid status as measured by amyloid-PET imaging, ROC analyses were performed and adjusted for sex and age. We constructed a diagnostic panel, consisting of well – established risk factors such as age, sex (defined as female > male), and *APOE4* carriership (defined as carrying at least one copy of the *APOE4* allele) (i.e. age + sex + *APOE4* panel) and compared it with a panel of age, sex, *APOE4* carriership added by plasma NfL and plasma GFAP, called age + sex + *APOE4* + GFAP + NfL panel (Fig. 3a-g). Additionally, we analysed in this model each biomarker separately to assess the potential benefit of GFAP or NfL alone (i.e. age + sex + *APOE4* + GFAP panel and age + sex + *APOE4* + NfL panel).

The age + sex + *APOE4* + GFAP + NfL panel performed better in discriminating HC from AD (AUC 91.6%) than HC from MCI (AUC 81.7%) and outperformed the age + sex + *APOE4* panel alone ($p < 0.001$ and $p = 0.004$, respectively) (Fig. 3a and 3b). When assessing each biomarker separately, the age + sex + *APOE4* + GFAP panel outperformed the age + sex + *APOE4* + NfL panel in distinguishing HC from MCI and AD. The age + sex + *APOE4* + GFAP panel distinguished HC from MCI and AD with an AUC of 81.3%, $p = 0.005$ and an AUC of 91.3%, $p < 0.001$, respectively, compared to the age + sex + *APOE4* panel, whereas the age + sex + *APOE4* + NfL panel showed no additional benefit in differentiating HC from MCI (AUC 68.8%, $p = 0.2936$), and differentiating HC vs. AD was inferior to the age + sex + *APOE4* + GFAP panel and the age + sex + *APOE4* + GFAP + NfL panel (AUC 84.5%, $p = 0.003$).

When comparing patients with MCI and AD, the age + sex + *APOE4* + GFAP + NfL panel (AUC 72.3%) and the age + sex + *APOE4* + NfL panel (AUC 72%) performed slightly better than the age + sex + *APOE4* panel (AUC 66.4%) and the age + sex + *APOE4* + GFAP panel (AUC 66.7%), but no significant difference was observed (Fig. 3c).

In terms of distinguishing SCD from the other cohorts, the age + sex + *APOE4* + GFAP + NfL panel outperformed the age + sex + *APOE4* panel significantly ($p < 0.05$). The best results were obtained for SCD vs. AD (AUC 85%, Fig. 3d), followed by SCD vs. MCI (AUC 81.3%, Fig. 3e) and SCD vs. HC (77.7%, Fig. 3f). Again, adding GFAP alone outperformed adding NfL alone. The age + sex + *APOE4* + NfL panel showed no significant improvement compared to the age + sex + *APOE4* panel (SCD vs. AD AUC 78.8%, SCD vs. MCI AUC 64.7%, SCD vs. HC AUC 73.8%), while the age + sex + *APOE4* + GFAP panel demonstrated a significantly higher AUC compared to the age + sex + *APOE4* panel for SCD vs. AD (84.7%, $p < 0.05$), whereas the distinction between SCD vs. MCI (AUC 74.4%) and SCD vs. HC (AUC 76.4%) missed statistical significance.

Regarding the diagnostic accuracy in predicting amyloid-PET status and the distinction of amyloid-negative (Ab-) from amyloid-positive (Ab+) individuals, AUC for the age + sex + *APOE4* + GFAP + NfL panel was calculated with 88.4% and was therefore superior than the age + sex + *APOE4* panel alone (AUC 75%, $p < 0.05$, Fig. 3g). The age + sex + *APOE4* + GFAP panel obtained an AUC of 86.9% but missed statistical

significance compared to the age + sex + *APOE4* panel alone ($p = 0.07$). Results for age + sex + *APOE4* + NfL were similar to the age + sex + *APOE4* panel (AUC 77.4%).

Discussion

In this outpatient memory clinic-based study we examined the performance of two promising biomarkers of neurodegeneration and neuroinflammation, e.g. NfL and GFAP, for the diagnostic work-up of patients along the continuum of AD-related cognitive decline. We aimed to develop a practical and reproducible model for a quick and accurate patient diagnosis in an easy-to-handle way for an outpatient memory clinic setting. As age, sex and *APOE4* status are the most well-known risk factors for AD^{39,40} and are already part of an established diagnostic work – up in many memory clinic settings, we contemplated an extended risk model that included these risk factors in addition to plasma GFAP and NfL levels (age + sex + *APOE4* + GFAP + NfL panel). To assess the best performance of each single biomarker, we furthermore analysed the impact of adding GFAP (age + sex + *APOE4* + GFAP panel) or NfL alone (age + sex + *APOE4* + NfL panel).

The combined age + sex + *APOE4* + GFAP + NfL panel could differentiate healthy controls from patients with MCI or AD and significantly outperformed the age + sex + *APOE4* panel alone. For discriminating the disease states of MCI and AD, the age + sex + *APOE4* + GFAP + NfL panel could not add a significant benefit to the age + sex + *APOE4* panel.

We further investigated the potential of the age + sex + *APOE4* + GFAP + NfL panel in regard of discriminating patients with SCD from the other cohorts and found promising results, especially in differentiating SCD from patients with an objective cognitive decline (MCI and AD), representing therefore a potential useful complementary diagnostic tool in an outpatient setting. In terms of predicting amyloid positivity on PET-imaging the age + sex + *APOE4* + GFAP + NfL panel performed significantly better than the age + sex + *APOE4* panel alone and showed a good distinction between Ab + and Ab- patients. When analysing the performance of a combination of each biomarker alone, the age + sex + *APOE4* + GFAP panel outperformed the age + sex + *APOE4* + NfL panel in all examined patient cohorts, except for MCI vs. AD.

Focusing on plasma GFAP alone, its levels showed a gradual increase along the four cohorts, with the lowest concentration in HC and the highest in patients with AD, thereby allowing a good biological interpretation of a gradual rise of this biomarker along the progressing neuropathological process. Plasma GFAP alone achieved the most prominent discrimination between HC and patients with an objective cognitive decline (MCI and AD). Moreover, the SCD group could be discriminated significantly from MCI and AD, however, MCI and AD as well as HC and SCD could not be differentiated by the use of plasma GFAP alone.

Plasma NfL alone could best discriminate between HC and MCI/AD patients and showed also promising demarcation between the HC and SCD group and even more interesting between the MCI and AD group.

Intriguingly, we found the highest concentration of plasma NfL in patients with SCD, which might not be solely explained by the small sample size. Therefore, these results must be interpreted cautiously.

Diagnosis of early phases of AD is crucial in regard of detecting patients at-risk as early as possible in the development of the neuropathological cascade. Besides the abnormal aggregation of Ab peptide and tau protein, neuroinflammation and neurodegeneration represent major components in the pathophysiology of AD⁴. Due to the recent evolvement of more sensitive analytical methods, non-invasive blood-based biomarkers could serve as a reliable approximation to this early pathogenic process. In recent years, the role of neuroinflammation in the pathogenesis of AD has been increasingly focused on in the literature. Neuropathological data have shown a close spatial relationship between Ab plaques and reactive astrocytes, which along with microglia, may trigger a pro-inflammatory cascade and eventually lead to neurodegeneration, which in turn activates astrocytes and microglia^{41,42}. As a cytoskeletal component of astrocytes, GFAP could serve as a promising biomarker reflecting astrocytic activation and proliferation during the neurodegenerative processes, including AD, particularly in its earliest stages^{43,44}. On the other hand, NfL represents a rather unspecific biomarker for neurodegeneration, as it is released by axonal damage in multiple neurological disorders^{45,46}. While the importance of NfL as a blood-based biomarker has been already reported in several studies^{12,13,47,48}, the significance of GFAP is currently still evolving. To our knowledge, only few studies have evaluated the combination of GFAP with other biomarkers so far, and presented the utility of plasma GFAP not just in discriminating healthy controls from patients with AD but also in distinguishing Ab + from Ab- individuals²¹⁻²³. Furthermore, higher GFAP levels have been associated with an increased risk for future progression to dementia and a steeper cognitive decline^{43,49}. Our data encourage the application of a blood-based biomarker model for an outpatient memory clinic setting, with even more promising results for GFAP than NfL for the discrimination of early stages along the AD continuum, whereas NfL might be more helpful in discriminating later stages. This could be in line with data, in which NfL has been reported to be rather a marker for progression of disease, whereas GFAP could be superior in identifying patients at-risk for developing dementia⁴³. Our data may support this hypothesis. Regarding the heterogeneity of AD pathology, a panel of well-combined blood-based biomarkers could aid in the early detection as well as disease monitoring in the future. The failure to show a discriminatory superiority of the age + sex + *APOE4* + GFAP + NfL panel in regard of differentiation of MCI and AD, while showing a slightly better performance of plasma NfL in this matter, might further underline this concept.

While recent studies increasingly focus on the evaluation of biomarkers in blood, especially in terms of feasibility, CSF biomarkers in AD have partially faded from the spotlight. In our cohort, we found the highest concentration of CSF GFAP in patients with AD followed by the HC group and the lowest concentration in MCI.

CSF NfL demonstrated a gradual increase over the three cohorts with the lowest levels in HC and the highest in AD and concentration of NfL in CSF and plasma correlated well with each other, which is in line

with already published data^{9,12,50-52}, suggesting that plasma levels might be considered as an acceptable proxy of CSF levels.

We found the lowest concentration of CSF GFAP in patients with MCI, followed by the HC and AD group, therefore results must be interpreted cautiously. In contrast to NfL, levels of GFAP in CSF and plasma showed a lower correlation, which was already described by another study²¹. While other studies analysed levels of GFAP with ELISA^{18,19,21,53}, we found no comparable results for measurement of CSF GFAP with SIMOA→ technology in patients along the AD continuum. Thus, further investigations are needed to better determine the role of CSF GFAP and its correlation with GFAP levels in blood in these patient cohorts.

Nevertheless, both measurements- CSF GFAP and CSF NfL- allowed a good discrimination between HC and AD as well as MCI and AD, with better results for CSF NfL.

Limitations

A limitation of our study is its retrospective and cross-sectional nature. Therefore, we lack longitudinal follow-up results and miss data in some of the patients. Positive amyloid-PET and *APOE4* carriership were significantly less common in the MCI group, which might lead to the notion, that at least some of the MCI patients are not along the AD continuum. Neuropathological data could not be attained in our in vivo patient cohort. The fact that the highest concentration of plasma NfL was found in patients with SCD could be interpreted as a sign of inclusion of a too diverse population, caused by the known heterogeneity of this diagnostic entity. Still, since we focused on patients with putative AD pathology, we excluded patients with other causes of dementia or high vascular burden, which led to a smaller sample size. Moreover, healthy controls were significantly younger than the patient groups, which might influence results of these biomarkers and corresponding analysis. To counteract this potential bias in our data, ROC analyses were adjusted for sex and age.

Conclusion

Blood-based biomarkers for AD may represent a valuable complementary tool for the clinical diagnosis and patient management in the near future. As to the increasing incidence of patients suffering from neurodegenerative disorders, developing minimally invasive and affordable biomarkers might confer great benefit in a quick and accurate diagnostic work -up. The outstanding benefits of blood-based biomarkers are the good availability and the potential of repetitive analysis in an individual patient without major efforts. In a time, where clinical trials are increasingly focusing on early stages of AD, it is crucial to develop a reliable diagnostic method that can be used as an easy-to-apply screening tool. We suggest that plasma GFAP could aid in a better distinction of patients along different predementia stages and that the combination of GFAP and NfL plasma levels with conventional risk factors could serve as a good “at-risk” model for early patient selection and assessment of progression to AD. Regarding the

heterogeneity of AD pathology, the implementation of a panel of well combined blood-based biomarkers could serve as a valuable adjunct during the diagnostic process in an outpatient memory clinic setting.

Abbreviations

Ab	Amyloid-Beta
AD	Alzheimer's disease
APOE	Apolipoprotein E
AUC	Area under the curve
BDI-II	Beck Depression Inventory
CSF	Cerebrospinal fluid
CT	Computed tomography
e.g.	Exempli gratia
ELISA	Enzyme-linked immunosorbent
f	Female
FBP	Filtered backprojection
FLAIR	Fluid-attenuated inversion recovery
FWHM	Full width at half-maximum
GFAP	Glial Fibrillary Acidic Protein
HC	Healthy control
i.e.	id est
IQR	Interquartile range
MCI	Mild cognitive impairment
MMSE	Mini-Mental State Examination
MRI	Magnetic resonance imaging
MUV	Medical University of Vienna

n.a.	not available
NfL	Neurofilament light chain
NTBV	Neuropsychological Test Battery Vienna
NFT	Neurofibrillary tangles
NIA-AA	National Institute on Aging and Alzheimer's Association
ns	Not significant
OSEM	Ordered subset expectation maximization
PET	Positron emissions tomography
PiB	Pittsburgh Compound B
pTau	Phosphorylated Tau
qPCR	Quantitative polymerase chain reaction
RDA	Research Documentation and Analysis
ROC	Receiver operating characteristic
SCD	Subjective cognitive decline
SIMOA	Ultrasensitive single-molecule array
SNP	Single nucleotide polymorphisms
tTau	Total Tau
WST	Wortschatztest

Declarations

Ethics approval and consent to participate

The project was approved by the Ethics Committee of the Medical University of Vienna (EK 1965/2019) on November 28th, 2019 and renewed on November 27th, 2020. The study was conducted in accordance with the Helsinki Declaration.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Not applicable.

Author's contributions

TP, RW and ES devised the protocol. TP collected and managed the data with contribution of RW, SS and ES. TP and TK performed SIMOA analyses in CSF and plasma. TP and RW performed the statistical analysis. TP and ES interpreted the data and prepared the manuscript. RW, TK, PR, SK, EG, PA, TTW and JF provided feedback and major contribution to the manuscript. All authors read and approved the final manuscript.

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Figures

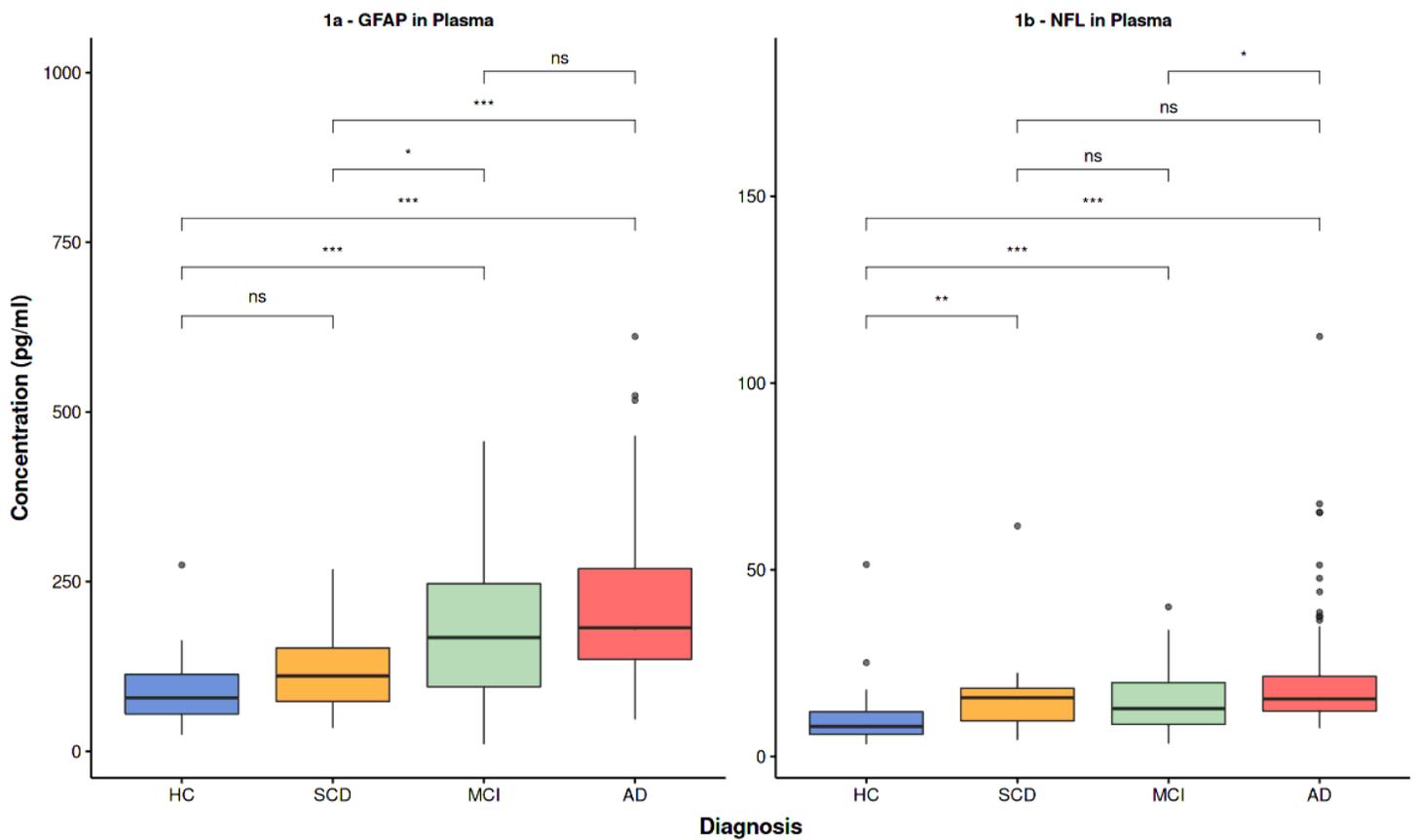


Figure 1

Concentration of GFAP (a) and NfL (b) in plasma among the four cohorts (HC, SCD, MCI, AD) Differences of biomarker concentration were calculated using Kruskal-Wallis Test, p value is displayed as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns not significant HC healthy control, SCD subjective cognitive decline, MCI mild cognitive impairment, AD Alzheimer’s disease, CSF cerebrospinal fluid, NfL neurofilament light chain, GFAP glial fibrillary acidic protein

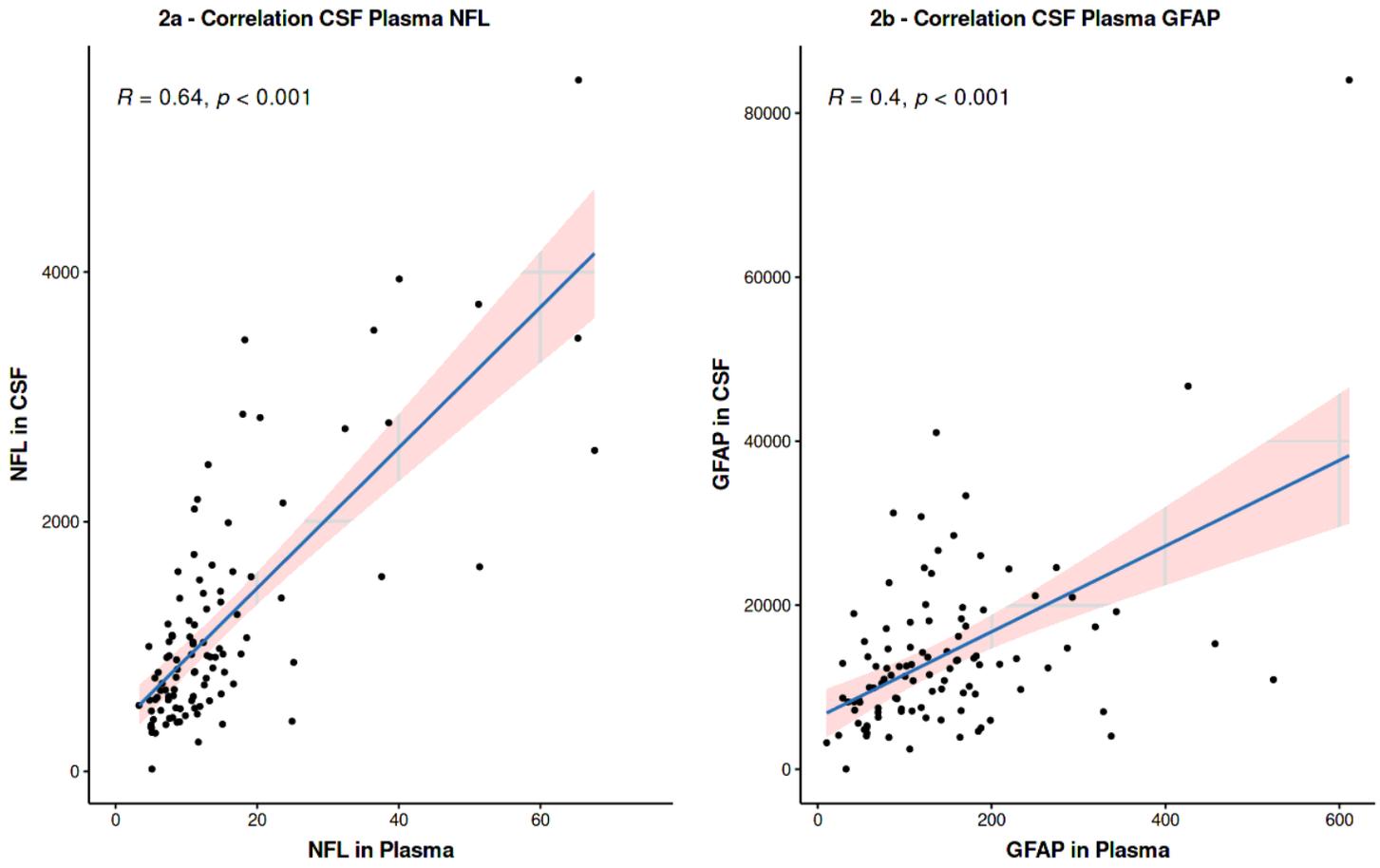


Figure 2

Correlation of NfL in CSF and plasma (a) and GFAP in CSF and plasma (b). Correlation was assessed using Spearman's rank correlation coefficient. CSF cerebrospinal fluid, NfL neurofilament light chain, GFAP glial fibrillary acidic protein

Figure 3

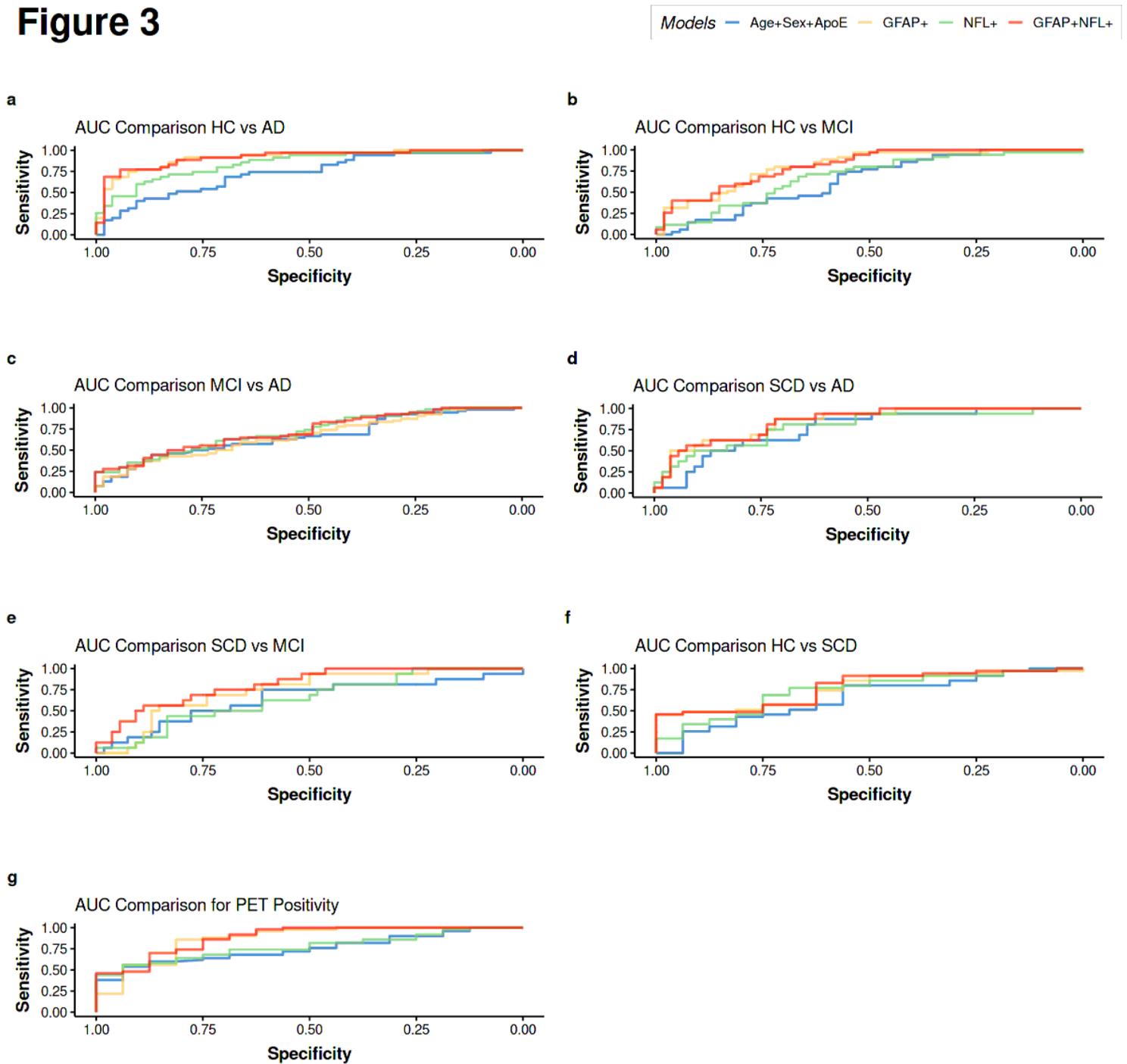


Figure 3

Receiver operating characteristic (ROC) curves for the diagnostic performance in distinguishing HC from AD (a), HC from MCI (b), MCI from AD(c), SCD from AD (d), SCD from MCI (e), SCD from HC (f), the prediction of amyloid positivity in our cohort (g). The area under the curve (AUC) of each model was compared using DeLong’s test for correlated AUC curves. The four panels analysed are called age+sex+APOE4 (blue), GFAP+ (i.e. age+sex+APOE4+GFAP, orange), NfL+ (i.e. age+sex+APOE4+NfL, green) and GFAP+NFL+ (age+sex+APOE4+GFAP+NfL, red) in this figure. HC healthy control, SCD

subjective cognitive decline, MCI mild cognitive impairment, AD Alzheimer's disease, NfL neurofilament light chain, GFAP glial fibrillary acidic protein