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ORIGINAL PAPER

Heart-type fatty acid binding protein and vascular endothelial growth factor: cerebrospinal fluid biomarker candidates for Alzheimer's disease

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Abstract The main objective of the study was to validate the findings of previous cerebrospinal fluid (CSF) proteomic studies for the differentiation between Alzheimer's disease (AD) dementia and physiological ageing. The most consistently significant proteins in the separation between AD dementia versus normal controls using CSF proteomics were identified in the literature. The classification performance of the four pre-selected proteins was explored in 92 controls, 149 patients with mild cognitive impairment (MCI), and 69 patients with AD dementia. Heart-type fatty acid binding protein (hFABP) and vascular endothelial growth factor (VEGF) CSF concentrations distinguished between healthy controls and patients with AD dementia with a sensitivity and specificity of 57 and 35 %, and 76 and 84 %, respectively. The optimal classification was achieved by a combination of the two additional CSF biomarker candidates in conjunction with the three established markers Amyloid-β $(Aβ)_{1–42}$, total-Tau (tTau), and phosphorylated-Tau $(pTau)_{181}$, which resulted in a sensitivity of 83 % and a specificity of 86 %. hFABP also predicted the progression from MCI to AD dementia. The present study provides evidence in support of hFABP and VEGF in CSF as AD biomarker candidates to be used in combination with the established markers $Aβ_{1–42}$, tTau, and $pTau_{181}$.

Keywords Alzheimer's disease · Biomarker · Cerebrospinal fluid · Diagnosis · Proteomics

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Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.ucla.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at: http://adni.loni.ucla.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf.

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Introduction

The accurate and reliable diagnosis of Alzheimer's disease (AD) at an early clinical stage is of high public interest and a prerequisite for the selection of appropriate candidates for clinical treatment trials. Neuropsychological tests are able to predict in vivo AD pathology to a certain degree [43], and great hopes are being placed on biomarkers such as cerebrospinal fluid (CSF) proteins and neuroimaging studies. The CSF reflects biochemical processes in the brain, and concentrations of total-Tau (tTau), phosphorylated-Tau $(pTau)_{181}$ and Amyloid-β $(Aβ)_{1–42}$ in CSF are routinely assessed as part of the dementia diagnostics process. Decreased CSF levels of $Aβ_{1–42}$, due to its cerebral deposition into Aβ plaques, and increased levels of tTau/

$pTau_{181}$, related to axonal damage, discriminate with reasonable accuracy between AD dementia and physiological ageing [4, 34]. However, a substantial overlap between control and AD groups has been repeatedly reported [17, 42], and altered concentrations of these biomarkers in CSF have also been observed in other neurodegenerative disorders such as frontotemporal lobar degeneration [4, 5, 26]. Furthermore, markers of Aβ pathology and axonal injury only provide information on two selected, albeit central, aspects of a multi-factorial pathological process; other important factors are being disregarded. Consequently, an urgent need remains for a more comprehensive set of markers covering different aspects of AD.

In theory, a hypothesis-driven knowledge-based approach to biomarker development can be distinguished from a hypothesis-generating unbiased approach. While the knowledge-based approach relies on the understanding of the central pathomechanisms of AD, an unbiased procedure such as proteomics potentially increases the predictive power, relating to multiple interacting biological processes, which has led to the identification of a number of novel candidate AD biomarkers [21] using various technologies [5]. In the past, small sample sizes and technological shortcomings made it difficult to produce reliable results. A recently developed bead-based Multi-Analyte Profiling (MAP) panel (Human Discovery MAP, Myriad RBM Inc., Austin, TX, USA) allows for the simultaneous measurement of multiple analytes. This Luminex multiplex platform has been associated with less intra- and inter-assay variability [14] and appears to present certain advantages over traditional enzyme-linked immunosorbent assays (ELISA) with respect to analytical precision [33, 40].

Despite the clear advantages of multiplex approaches to candidate biomarker discovery, problems with the replication of intriguing results in independent samples may arise due to between-cohort heterogeneity and different technologies used. Without replication, however, the clinical relevance of such findings is in question. Therefore, the main aim of the present study was to apply rigorous selection criteria to published CSF proteomic studies differentiating AD dementia from healthy controls in order to identify the most consistent findings to be replicated in a large independent cohort from the Alzheimer's disease neuroimaging (ADNI) study.

Methods

Study design and sample

All studies in PubMed/MEDLINE (http://www.ncbi.nlm.nih.gov/pubmed/) that provided information on CSF protein concentrations in AD using the Human Discovery MAP Myriad RBM multiplex assay were identified. CSF proteins were selected for replication in the present study if they were significant in the differentiation between AD dementia and healthy controls in at least two previous reports. Applying this selection procedure, four studies involving five independent cohorts were found [6, 12, 15, 25], resulting in a total of 41 significant proteins, eight of which were mentioned in at least two reports. Three of these eight proteins, heart-type fatty acid binding protein (hFABP), interleukin-7 (IL7), and vascular endothelial growth factor (VEGF), were reported in three studies confirmed by at least two different statistical procedures; the other five proteins, alpha-fetoprotein (AFP), Eotaxin/CCL11, C-reactive protein (CPR), interleukin-17 (IL17), and TNF-related apoptosis-inducing ligand receptor 3 (TRAIL-R3), were reported in two studies using a single statistical method (see Supplemental Table 1 for further characteristics of the study samples and the PubMed/MEDLINE search strategy).

The data used in this study were obtained from the ADNI database at www.loni.ucla.edu/ADNI on 17 January 2012. Information from 311 subjects was available, including 92 healthy elderly control subjects (CON), 149 patients with mild cognitive impairment (MCI), and 69 patients with AD dementia. In the MCI group, clinical data were available from follow-up visits conducted up to 60 months after the baseline assessment. The study was approved by the institutional review boards of all participating centres, and written informed consent was obtained from all participants or authorised representatives after extensive description of the ADNI according to the 1975 Declaration of Helsinki. The study is registered at ClinicalTrials.gov (registration number NCT00106899, http://clinicaltrials.gov).

The ADNI recruitment and inclusion procedures are described in detail at www.adni-info.org. Briefly, at baseline, subjects in ADNI were between 55 and 90 years of age, had a modified Hachinski score $\leq$4, and had at least 6 years of education. Patients with AD met the National Institute of Neurological and Communicative Disorders and Stroke-AD and Related Disorders Association (NINCDS-ADRDA) criteria and had a Mini-Mental-State Examination (MMSE) score between 20 and 26 (inclusive) and a Clinical Dementia Rating (CDR) score of 0.5 or 1. Patients with amnestic MCI had MMSE scores between 24 and 30, a CDR score of 0.5, memory complaints but no significant functional impairment, and objective memory deficits on the Wechsler Memory Scale Logical Memory II test. Cognitively normal subjects had MMSE scores between 24 and 30, a CDR score of 0, no evidence of depression, and no memory complaints. After the baseline visit, follow-up visits were conducted at six- or 12-month intervals up to a maximum of 6 years (see Supplemental

Table 2 for a listing of individual follow-up times in the MCI group). The full list of inclusion/exclusion criteria can be accessed at http://www.adni-info.org.

Multiplex protein assays

Baseline CSF samples were obtained from the study participants and analysed at the ADNI biomarker core laboratory at University of Pennsylvania according to published methods [19, 32]. Briefly, CSF samples were obtained from the participants in the morning and put into the freezer at −80 °C; aliquoting and processing were conducted according to ADNI standardised operating procedures. The CSF concentrations of $A\beta_{1–42}$, tTau, and $pTau_{181}$ were measured using the multiplex xMAP Luminex platform with Innogenetics immunoassay kit-based reagents (INNOBIA AlzBio 3; Ghent, Belgium; for research use–only reagents). In addition, a 159 analytes panel was developed for the Luminex xMAP platform (Luminex Corp., Austin, TX, USA) by Myriad RBM at the service providers' facilities. The detailed quality control procedures and results are available from the ADNI website (http://adni.loni.ucla.edu/2012/01/biomarkers-consortium-adni-csf-multiplex-immunoassay-proteomics-data-available-Tuesday-January-3rd-2012-2/). Data of 83 analytes, which had passed the strict ADNI quality control procedures, were considered for the present study (see Supplemental Table 3 for a complete listing). CSF concentrations of AFP, Eotaxin/CCL11, IL7, and IL17 were not available from ADNI; the final dataset for the present analysis therefore included hFABP, VEGF, CRP, and TRAIL-R3.

Statistical analysis

All statistical analyses were performed using SPSS, v19.0 (IBM corp., Somers, NY, USA) and R-Software, v2.13.0 with the *Q* value package (http://genomics.princeton.edu/storeylab/qvalue/) [37]. Data are expressed as means and standard deviation (SD). All tests were two-sided, and a p value less than 0.05 was considered significant. The false discovery rate (FDR) [1], which controls the expected proportion of incorrectly rejected null hypotheses (type-I errors), was applied when appropriate to adjust for multiple comparisons, that is, results at $q < 0.05$ were regarded significant. All protein concentrations were normally distributed.

Univariate analysis of covariance (ANCOVA) was used to determine the CSF analytes that differed between the CON and AD dementia groups; the models were adjusted for the following variables: age, gender, education, Apolipoprotein E (*ApoE*) *ε4* carrier status, presence of cardiovascular disease or diabetes mellitus, and history of stroke or malignancies. A binary, stepwise forward logistic regression (LR) assessed the ability of the pre-specified biomarker models to differentiate between AD dementia and CON, using the significant proteins from the ANCOVA as predictors. A receiver operating characteristic curve (ROC) analysis was applied to determine the sensitivity, specificity, positive and negative predictive values (PPV, NPV, respectively), and accuracy of the best model. In order to compare the accuracy of the additional CSF biomarker candidates with the traditional AD markers, concentrations of $A\beta_{1–42}$, tTau, and $pTau_{181}$ were tested in additional LR models.

In addition, a Cox proportional hazard model, with covariates as specified for the ACONVA, was applied to assess the ability of baseline biomarkers to predict the progression from MCI to AD dementia. Data from patients who did not convert during the follow-up period were statistically censored at the date of the last assessment.

Results

The characteristics of the study sample are presented in Table 1. Compared with the controls, patients with AD dementia exhibited an AD-typical profile characterised by a higher proportion of *ApoE ε4* carriers, lower Mini-Mental-State Examination (MMSE), and higher AD Assessment Scale-cognitive subscale (ADAS-cog) scores, as well as lower $A\beta_{1–42}$, higher tTau, and higher $pTau_{181}$ concentrations in CSF. After adjustment for the covariates specified above, the ANCOVA analysis showed significant differences between the AD dementia and CON groups for hFABP ($p < 0.001$) and VEGF ($p = 0.03$) but not for CRP and TRAILR3 levels in CSF (Table 2 and Fig. 1). Age showed significant effects in these ANCOVA models (for hFABP: $p = 0.03$, for VEGF: $p < 0.001$). The other covariates were not significant.

According to the results of the ANCOVA, hFABP and VEGF were used as predictors in the subsequent binary LR models for the differentiation between CON and AD dementia. The single protein LR models showed a sensitivity of 56.52 % and a specificity of 76.09 % for hFABP, and a sensitivity of 34.78 % and a specificity of 83.70 % for VEGF. The LR model including both Myriad RBM analytes ($LR_{CSF\text{-}new}$) showed comparable results with a sensitivity of 71.01 % and a specificity of 80.43 %. The overall classification performance for the diagnosis of AD dementia was improved compared to the single marker models (area under the curve (AUC): hFABP 0.71, VEGF 0.63, $LR_{CSF\text{-}new}$ 0.84). In comparison, the regression model restricted to the three traditional CSF biomarkers ($LR_{CSF\text{-}trad}$) resulted in a sensitivity of 78.26 % and a specificity of 79.12 % for distinguishing between AD dementia and CON. A final LR model ($LR_{CSF\text{-}comb}$) including both Myriad RBM analytes

Table 1 Characteristics of the study sample

	CON	MCI-stable	MCI-progressive	AD dementia
N[a]	92	76	73	69
Age (years)	75.75 (5.45)	74.78 (7.42)	75.01 (7.17)	75.01 (7.62)
MMSE (points)	29.09 (1.01)	27.26 (1.67)	26.51 (1.81)	23.53 (1.91)*
ADAS-cog (points)	9.38 (4.22)	17.06 (6.77)	20.85 (5.87)	28.60 (9.11)*
Education (years)	15.58 (2.93)	16.42 (2.72)	15.56 (3.11)	15.16 (2.98)
Sex (male/female)	46/46	55/21*	47/26*	39/30
ApoE ε4 (% carrier)	23.91	42.11*	65.75*	71.01*
$A\beta_{1–42}$ (pg/mL)	207.74 (53.51)	172.20 (54.71)*	146.73 (39.29)*	141.91 (34.87)*
tTAU (pg/mL)	68.96 (26.55)	94.09 (49.85)*	116.71 (55.60)*	121.63 (60.30)*
$pTAU_{181}$ (pg/mL)	24.24 (12.70)	32.26 (14.82)*	40.22 (15.66)*	41.12 (20.13)*

Data presented as mean (SD) where appropriate

CON cognitively normal controls, *MCI* mild cognitive impairment, *MCI-progressive* patients with MCI who progressed to AD dementia, *MCI-stable* patients with MCI who remained cognitively stable, *AD* Alzheimer's disease, *MMSE* mini-mental-state examination, *ADAS-cog* Alzheimer's Disease Assessment Scale—cognitive subscale; *ApoE* apolipoprotein E, $A\beta_{1–42}$ Amyloid-$\beta_{1–42}$, *tTau* total-Tau, $pTau_{181}$ phosphorylated-Tau_{181}

* Significant differences compared with the CON group at $q < 0.05$

[a] N for the sample with CSF results

Table 2 Cerebrospinal fluid protein concentrations in Alzheimer's disease and healthy controls

	Group (*N*)	Concentration	*p* value*
hFABP (pg/mL)	CON ($N = 92$)	1503.78 ± 427.64	<0.001
	AD ($N = 69$)	1837.68 ± 474.19	
VEGF (pg/mL)	CON ($N = 92$)	15.30 ± 1.87	0.03
	AD ($N = 69$)	14.49 ± 1.90	
CRP (ug/mL)	CON ($N = 92$)	0.07 ± 0.04	0.89
	AD ($N = 69$)	0.08 ± 0.08	
TRAILR3 (ng/mL)	CON ($N = 92$)	0.81 ± 0.12	0.95
	AD ($N = 69$)	0.81 ± 0.13	

Data presented as mean ± SD

CON cognitively normal controls, *AD* Alzheimer's disease dementia, *CRP* C-reactive protein, *hFABP* heart-type fatty acid binding protein, *TRAILR3* TNF-related apoptosis-inducing ligand receptor 3, *VEGF* vascular endothelial growth factor

* *p* values were obtained by exploring log-transformed biomarker concentrations by analysis of covariance (ANCOVA), adjusting for age, gender, education, *ApoE ε4*, cardiovascular disease, diabetes mellitus, stroke, and malignancies

in addition to the three traditional markers resulted in the best classification with a sensitivity of 82.61 %, a specificity of 85.71 %, and an AUC of 0.91 (Table 3). A comparison of the AUCs of the models $LR_{CSF\text{-}comb}$ and $LR_{CSF\text{-}trad}$ using the tool StAR (http://protein.bio.puc.cl/cardex/servers/roc/roc_analysis.php) showed a trend towards a statistically significant difference ($p = 0.06$).

Within the follow-up period (mean 2.81, SD 0.97 years), 73 patients with MCI progressed to AD dementia, whereas 76 remained in the MCI stage; the length of the follow-up time did not significantly differ between the two MCI sub-groups (Table 1). CSF hFABP, but not VEGF, concentrations differed between the two MCI sub-groups (Fig. 1); therefore, only hFABP was included in the univariate Cox proportional hazards model, which indicated that the progressive MCI sub-group had significantly higher baseline CSF hFABP concentrations than the stable MCI sub-group (hazard ratio [HR] 1.001, $p = 0.04$) (Table 4). However, this result was not significant anymore (HR = 1.001; $p = 0.13$) when additional covariates as specified above were entered into the model.

Discussion

We aimed to provide a robust replication of previous CSF proteomic studies in AD presenting the first report on data from the ADNI. With a strong a priori hypothesis derived from published studies, we corroborate the significant concentration differences between AD dementia and physiological ageing for hFABP and VEGF in CSF. Our results confirm the good diagnostic accuracy of the combined use of the three established CSF biomarkers $A\beta_{1–42}$, tTAU, and $pTau_{181}$ ($LR_{CSF\text{-}trad}$ model), which was improved by adding the two analytes hFABP and VEGF ($LR_{CSF\text{-}comb}$). In addition, hFABP was also useful in predicting clinical progression in MCI.

Sporadic AD is a biologically complex neurodegenerative disease that is unlikely to be caused by any single pathogenic event (or cascade of events). Therefore, the traditional biomarkers $A\beta_{1–42}$ and $tTau/pTau_{181}$ only represent two selected aspects of the multi-factorial nature of AD, which restricts their diagnostic utility [29, 32, 39].

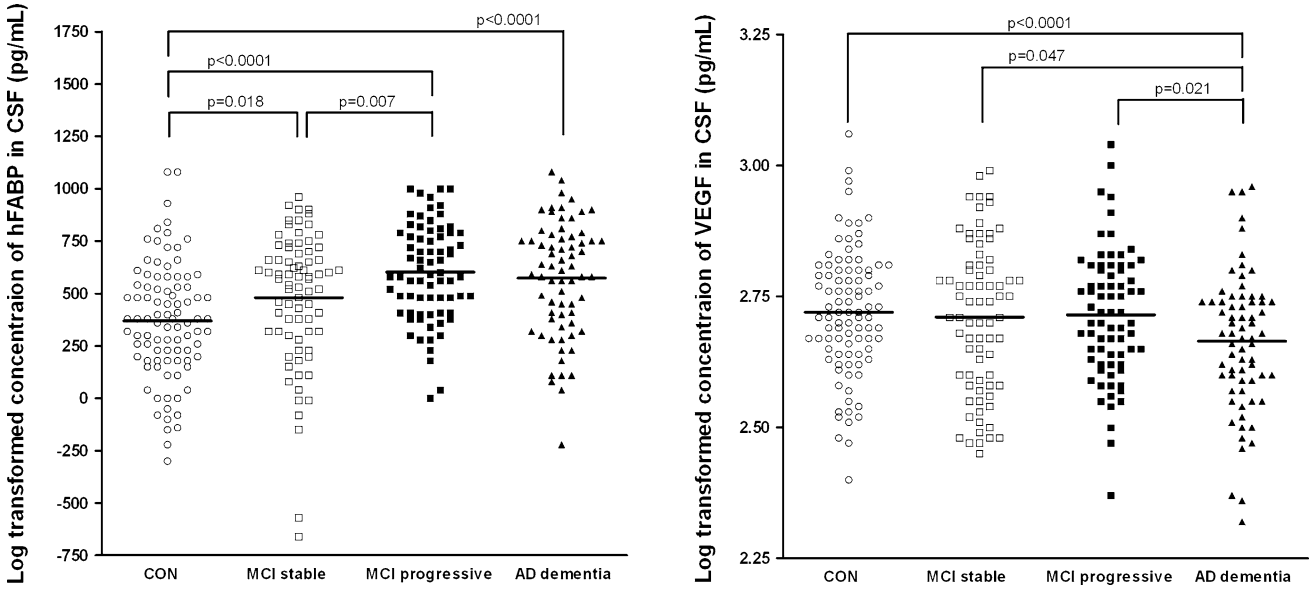

Fig. 1 Cerebrospinal fluid concentrations of the two new biomarkers in the study groups *CSF* cerebrospinal fluid, *CON* cognitively normal controls, *MCI* mild cognitive impairment, *MCI-progressive* patients with MCI who progressed to AD dementia, *MCI-stable* patients with MCI who remained cognitively stable, *AD* Alzheimer's disease, *hFABP* heart-type fatty acid binding protein, *VEGF* vascular endothelial growth factor

Table 3 Performance of the biomarker sets in the differentiation between healthy controls and the Alzheimer's disease dementia group

	$A\beta_{1–42}$	tTAU	$pTAU_{181}$	$LR_{CSF\text{-}trad}$	hFBAP	VEGF	$LR_{CSF\text{-}new}$	$LR_{CSF\text{-}comb}$
ROC AUC	0.83	0.80	0.80	0.88	0.71	0.63	0.84	0.91
Sensitivity (%)	79.71	60.87	60.87	78.26	56.52	34.78	71.01	82.61
Specificity (%)	73.91	84.78	85.71	79.12	76.09	83.70	80.43	85.71
Cut-off (pg/mL)	160.96	97.60	34.25	NA	1768.36	13.67	NA	NA
ACC (%)	76.40	74.53	75.00	78.75	67.70	62.73	76.40	84.38
PPV (%)	69.62	75.00	76.36	73.97	63.93	61.54	73.13	81.43
NPV (%)	82.93	74.29	74.29	82.76	70.00	63.11	78.72	86.67

ROC receiver operating characteristic, *AUC* area under the curve, *ACC* classification accuracy, *PPV* positive predictive value, *NPV* negative predictive value, $A\beta_{1–42}$ amyloid-$\beta_{1–42}$, *tTau* total-Tau, $pTau_{181}$ phosphorylated-Tau_{181}, *hFABP* heart-type fatty acid binding protein, *VEGF* vascular endothelial growth factor, $LR_{CSF\text{-}new}$ logistic regression model with the 2 novel CSF markers hFABP and VEGF as the independent variables, $LR_{CSF\text{-}trad}$ logistic regression model with traditional CSF biomarkers $A\beta_{1–42}$, tTau, and $pTau_{181}$ as the independent variables, $LR_{CSF\text{-}comb}$ logistic regression model with the combination of 5 CSF proteins as the independent variables, *NA* not applicable

Proteomics in AD allows a large number of proteins to be studied simultaneously in order to obtain accurate and comprehensive data about their structure, functional characterisation, and quantification [47]. One major obstacle of applying a proteomic approach to disease classification is the poor generalizability of the results across various datasets; often only a minimal overlap between studies is obtained, probably due to different experimental designs and diverse analytical methods as well as heterogeneous cohorts [49]. The validation phase of proteomic studies seems to be more challenging but also more valuable than the discovery phase since there are a limited number of replication studies compared with the large number of profiling works focused on discovery [47, 48]. In three previous independent proteomic studies, CSF hFABP is suggested as a potential biomarker for AD using the same multi-analytes platform that has also been applied in ADNI [6, 15, 25]. A fourth study also found a statistical trend for concentration differences of hFABP in CSF between AD dementia and physiological ageing [12]. Since the Bonferroni correction procedure applied in the latter study may lead to loss of statistical power [8], we recalculated the published *p* value using an FDR correction, which resulted in a significant finding at $q = 0.04$. After this recalculation, the present study is the fifth report, or sixth independent cohort, in which hFABP has been successfully used to differentiate between AD dementia and normal ageing. Similarly to hFABP, VEGF has been suggested as a

Table 4 Univariate Cox regression model of cerebrospinal fluid biomarkers in the differentiation between the progressive and stable mild cognitive impairment sub-groups

	Regression coefficient [B]	*p* value	Estimated hazard [Exp (B)]	95.0 % confidence interval for hazard ratio [Exp (B)]	
				Lower	Upper
hFABP	0.001	0.04*	1.001[a]	1.000	1.002
$A\beta_{1–42}$	−0.008	<0.01*	0.992[a]	0.987	0.998
tTAU	0.004	0.04*	1.004[a]	1.000	1.008
$pTAU_{181}$	0.019	<0.01*	1.019[a]	1.005	1.033

hFABP heart-type fatty acid binding protein, $A\beta_{1–42}$ amyloid-β1–42, *tTau* total-Tau, $pTau_{181}$ phosphorylated-Tau_{181}

* Significant at $p < 0.05$

[a] The estimated hazard refers to the risk increase associated with a 1 unit increase in the value of the covariate, that is, a 1 pg/mL concentration increase for the tested proteins

potential biomarker of AD in a total of four studies with five independent cohorts. Previous findings in relation to the protein CRP and TRAIL-R3 could not be replicated in our study.

Heart-type fatty acid binding protein (hFABP) (also known as FABP3) is a low molecular mass (15 kDa) lipid-binding protein highly expressed in the adult human brain, particularly in pons and frontal lobe, which participates in neurite formation and synapse maturation [27, 41, 42]. Considering its ability to change the lipid composition and fluidity of the cell membrane through the regulation of long-chain fatty acids [42], hFABP may facilitate signal transduction, membrane functionality, and maintaining the balance between phospholipid and arachidonic acid in the adult brain; consequently, the involvement of hFABP in cellular dysfunction related to neurodegenerative disorders such as AD has been suggested [36]. Central and peripheral hFABP has previously been proposed as an effective marker for mild traumatic brain injury and stroke [20, 44, 46]. Several recent works using conventional ELISA methods have demonstrated increased hFABP CSF concentrations in patients with AD dementia [2, 10, 46]. However, decreased hFABP has also been reported in AD brain samples in a single study [9]. Heretofore, the cerebral physiological function of hFABP remains elusive. In the present study, both patients with AD dementia and progressive MCI had higher hFABP levels in CSF when compared to cognitively healthy controls, but no difference was seen between the AD dementia group and the progressive MCI sub-group. This finding may suggest that hFABP CSF concentrations are already increased in early clinical stages of AD and that they remain unchanged thereafter; this finding is also supported by the contribution of hFABP to the prediction of clinical progression in MCI. Since no association between hFABP and $A\beta_{1\text{-}42}$ was noted in our study, hFABP may be an indicator of a different pathophysiological aspect related to the clinical signs of sporadic AD such as concomitant cerebrovascular disease [2, 10]. In this scenario, disrupted cellular metabolism due to elevated hFABP levels may have detrimental effects in individuals with altered Aβ metabolism. Recent evidence also indicates that increased hFABP in CSF is also found in Creutzfeldt–Jakob disease, Parkinson disease dementia, and dementia with Lewy bodies [10, 22, 23, 35], suggesting that hFABP is an unspecific marker of brain damage.

Vascular endothelial growth factor (VEGF) is a hypoxia-induced signalling protein involved in vasculogenesis and angiogenesis that is closely related to central and peripheral inflammation, injury, diabetes, malignancy, and cardiovascular disorders [31]. Recent reports suggest that VEGF might also be relevant to AD [28]. In the brain of patients with AD, increased expression of VEGF was detected not only in clusters of reactive astrocytes but also co-localised with senile plaques; in vitro, VEGF binds with high affinity to pre- and co-aggregated Aβ and is released slowly from co-aggregated complexes, suggesting a role of VEGF in the AD pathophysiological process [7, 18, 45]. In CSF studies on patients with AD dementia, both increased [30, 38] and unaltered concentrations of VEGF have been reported [3]. In contrast to these observations, we report decreased CSF concentrations of VEGF in AD dementia compared with healthy controls and patients with MCI, which may be explained by the trapping of VEGF in cerebral Aβ plaques.

Limitations of our study include the lack of histopathological confirmation of AD diagnoses; however, the validity of the clinical diagnoses at specialised centres has repeatedly been confirmed by autopsy series [24]. The patients in ADNI were recruited from specialised university centres and may therefore not truly represent the whole population with AD. hFABP and VEGF are also vascular risk factors related to cardiovascular disorders. We did not detect associations of these two factors with cardiovascular risk in the current cohort, which may be attributed to the exclusion of vascular dementia in ADNI or to the confounding effects of the presence of AD pathology; therefore, the effects of vascular changes should be studied in more appropriate cohorts. Moreover, proteins from previous studies were selected according to pre-specified criteria but irrespective of other sample characteristics such as ethnicity. Some of these between-group differences may have affected the biomarker candidate findings [11]. Finally, in our analyses, age was a significant factor, which will therefore also have to be accounted for when using hFABP and VEGF in future diagnostic settings.

To sum up, our study supports hFABP and VEGF in CSF as AD biomarker candidates to be used in conjunction

with the more established markers $A\beta_{1-42}$, tTau, and $pTau_{181}$. Including the two Myriad RBM analytes in the diagnostic algorithm seemingly provides an added value over the traditional CSF markers that may be of clinical relevance. Our findings also stress the notion that treating AD as a complex disorder not exclusively related to Aβ pathology may have important diagnostic, and probably also therapeutic, implications. In line with the studies conducted to establish $A\beta_{1-42}$ and tau as clinical biomarkers for AD [13, 16], we propose a multi-centre study including patients with different neurodegenerative disorders and healthy controls in order to define cut-off values for hFABP and VEGF to be applied within the framework of an optimised diagnostic algorithm.

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Conflict of interest The authors declare that they have no conflict of interest.

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