Chapter 8

BRI2 correlates with markers reflecting Alzheimer's disease pathology in human cerebrospinal fluid.

In preparation.

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Abstract

BRI2 can regulate the amyloidogenic processing of amyloid precursor protein (APP) as well as the oligomerization and clearance of amyloid β (Aβ), which are key processes in Alzheimer's disease (AD) pathogenesis. Deposits of BRI2 containing the BRICHOS domain were found in human AD hippocampus in early stages of the disease, which correlated with amyloid plaque and neurofibrillary tangle (NFT) formation. In this study, we aimed to unravel whether the BRI2 changes observed in AD in tissue were reflected in CSF. We have developed and validated an specific BRI2 ELISA detecting an epitope within the BRI2 BRICHOS domain. The levels of BRI2 CSF were determined in non-demented cases with subjective memory complaints (SMC; n = 50) and AD (n = 54). In addition, we performed a longitudinal study on the predictive value of BRI2 for conversion to AD, including patients with mild cognitive impairment (MCI) who either converted to AD (MCI-AD; n = 17) or remained stable (MCI-S; n= 15) after two-years of follow-up. We analyzed the relationship of BRI2 CSF with different CSF biomarkers reflecting AD pathology such as $A\beta_{40}$, $A\beta_{42}$, $A\beta_{42}$/$A\beta_{40}$ ratio, total tau (t-Tau), phosphorylated tau (p-Tau) and a panel of neuroinflammatory markers. No differences in the levels of BRI2 CSF were detected between the different clinical groups. Nevertheless, the levels of BRI2 CSF correlated positively with the CSF concentration of $A\beta_{40}$ ($r = 0.53$, $p < 0.0001$), $A\beta_{42}/A\beta_{40}$ ratio ($r = -0.305$, $p = 0.021$), t-Tau ($r = 0.37$, $p = 0.0001$) and p-Tau ($r = 0.31$, $p = 0.001$). In addition, BRI2 also correlated with ten different cytokines (r between 0.28 and 0.68). In conclusion, despite BRI2 cannot be used as a diagnostic AD biomarker using this specific assay, this study supports that BRI2 is related to several processes involved in neurodegeneration in AD.

Key words: Alzheimer's disease, BRI2, biomarker, CSF.

Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative dementia with unknown etiology[1]. Early diagnosis is of great importance since it opens opportunities to slow down or halt the disease before extensive neuronal damage has taken place[2] and thus, interest in biomarkers able to detect the earliest phase is high. Ideally, biomarkers should reflect the underlying molecular pathology related to the disease[3]. The pathophysiological changes leading to AD starts decades before clinical symptoms appear[4], which provides a good time-frame to detect biomarkers predicting the development of AD (preclinical AD). The current core CSF biomarkers for AD diagnosis represent the classical pathological AD hallmarks: a decrease in $A\beta_{42}$ levels reflects senile plaque pathology, and the increase in total tau (t-Tau) and phosphorylated tau (p-Tau) levels reflect axonal degeneration and NFT formation[5–8]. Their sensitivity and specificity for AD is high and they can also reasonably predict the transition of mild cognitive impairment (MCI) to AD[9–11]. Nevertheless, AD CSF biomarker patterns are also present in cognitive normal subjects and influenced by the age of the patient, resulting in loss of sensitivity at higher age; they are far from dynamic and do moderately correlate with disease progression, which are prerequisites for monitoring treatment effects[12,13]. Thus, the quest for the earliest and dynamic markers is ongoing.

We recently discovered the presence of BRI2 deposits associated with amyloid plaques in the hippocampus of AD patients[14]. Several studies have suggested that BRI2 may play a role in AD pathology[15] since it regulates the homeostasis of critical processes involved in AD pathogenesis such as APP processing[16–22], Aβ formation, fibrillation and degradation[23–26], or the expression of β-secretase[127]. Recently, we described that BRI2 deposition in post-mortem hippocampus started in early stages of AD and significantly correlated with the formation of both plaques and NFT[14]. Deposition of BRI2 may also participate in the neurodegenerative process in AD since BRI2 aggregates promoted apoptosis and the truncation of tau[28]. In addition, a pilot hypothesis-free proteomics study revealed that the levels of BRI2 in CSF increased along with clinical disease development (Supplementary material).

Given the early changes of BRI2 found in human AD tissue, its association with plaques and NFT development and the proteomics data, we hypothesized that BRI2 is a potential early biomarker reflecting subclinical AD pathology. Thus, here we aimed to analyze the potential of BRI2 in CSF as an early AD diagnostic marker, for which we have developed a specific ELISA against the BRI2-BRICHOS domain. We clinically validated the assay by the analysis of BRI2 levels in CSF of AD patients and controls (subjective memory complaints, SMC), and in subjects with mild cognitive impairment (MCI) who after two years follow-up

either converted to AD (MCI-AD) or remained stable (MCI-S). Moreover, we studied the association of BRI2 with other CSF biomarkers related to AD pathology such as $A\beta_{42}$, $A\beta_{40}$, t-Tau, p-Tau, and a panel of neuroinflammatory markers.

Materials and Methods

Human CSF samples

CSF material was obtained from Alzheimer Center Memory Cohort[29], NUBIN/ (NeuroUnit Biomarkers for Inflammation and Neurodegeneration) VUmc biobank (Amsterdam, The Netherlands). For the initial pilot proteomic study we selected AD patients (n =5; MMSE: 20.8 ± 5.6) and non-demented subjects with subjective memory complaints (SMC; n = 4; MMSE: 29.5 ± 1), patients with MCI who after two years follow-up converted to AD (MCI-AD; n = 5; MMSE: 25 ± 2.5) or remained stable (MCI-S; n = 4; MMSE: 28.4 ± 2). For the analysis of BRI2 in CSF using our specific immunoassay, two different studies were performed. For study I or AD-cohort, SMC subjects (n = 50; MMSE: 28.2 ± 1.65) and AD patients (n = 54; MMSE: 18.7± 4.3) were selected. In order to understand whether changes in BRI2 CSF occurred in early stages, we performed a second analysis (MCI-cohort) in which SMC cases (n = 13; MMSE: 27.9 ± 0.99), MCI-S (n = 15; MMSE: 26.9 ± 1.6), MCI-AD AD (n = 17; MMSE: 26.06 ± 2.63) and AD patients (n = 15; MMSE: 25.4± 3.18) were selected. Diagnoses were defined in a multidisciplinary meeting as previously described[29] and patients were matched for age and sex. Analysis of CSF biomarkers ($A\beta_{1-42}$, t-Tau and p-Tau) was done as previously described[11]. CSF samples were collected and stored in agreement with JPND-BIOMARKAPD guidelines[30]. The levels of $A\beta_{40}$ as well as a panel of neuroinflammatory markers were measured in a subset of the CSF samples from the AD-cohort (SMC = 20; AD = 37) using our in-house $A\beta_{40}$ ELISA (intra-assay CV = 0.7%)[31] and the Neuroinflammation panel 1 (human) kits (Mesoscale discovery, MSD Rockville, MD, USA; intra-assay CV = 9.8%)) respectively. Age, sex, biomarker levels as well as Mini Mental State Examination (MMSE) scores of all cases used are listed in Table 1. For assay validation and internal controls, pooled CSF samples from AD and SMC cases were prepared, aliquoted and stored at -80 ºC until further analysis. The ethical review board of the VUmc approved the study and all subjects gave written informed consent.

BRI2 immunoassay and CSF analyses

EIA/RIA Plates 96 well (Corning, Kentucky, USA) were coated with a specific antibody against BRI2 (1.5 µg/ml, Monoclonal Protein G-purified anti-$BRI2_{140-153}$, clone BG1[14]) overnight at 4ºC in coating buffer (0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH 9.6). Plates were next

Table 1. Demographic data of CSF samples for proteomics analysis.

	Proteomics analysis				Study I		Study II			
	SMC	MCI-S	MCI-AD	AD	SMC	AD	SMC	MCI-S	MCI-AD	AD
Age (mean ± SD)	60.3 ± 4.5	62.1 ± 3.2	66.2 ± 6.4	63.9 ± 6.6	59.7 ± 2.65	60.3 ± 2.94	61.56 ± 8.93	66.57±9.85	67.29±7.87	63.38 ± 8.82
No (M/F)	4(2/2)	4(1/3)	5(2/3)	5(2/3)	50(29/21)	54(28/26)	13(5/8)	15(10/5)	17(9/8)	15(11/4)
MMSE baseline (mean ± SD)	29.5 + 1.0.	27.4 ± 2.2	27.0 ± 1.4	21.4 ± 6.3	28.2 ± 1.65	18.7± 4.33[a]	27.85 ± 0.99	26.87 ± 1.6	26.06 ± 2.63	25.40 ± 3.18
MMSE follow-up (mean ± SD)	/	28.4 ± 2.0	25.0 ± 2.5	20.8 ± 5.6	n.a.	n.a.	28.46 ± 1.27	25.87 ± 2.36[a]	21.41 ± 4.47[a,b]	22.87 ± 3.87[a]
Aβ_{42} (pg/mL)	838 ± 133	875 ± 201	499 ± 78	384 ± 146 [a, b]	950 ± 263	458 ± 183[a]	957 ± 293	571 ± 485	476 ± 222[a]	454 ± 143[a]
t-tau (pg/mL)	200 ± 76	421 ± 347	1071 ± 248[a]	526 ± 120	228 ± 177	669 ± 579 [a]	247 ± 104	413 ± 255	891 ± 501[a,b]	664 ± 500[a]
p-tau (pg/mL)	47 ± 16	73 ± 48	138 ± 37 [a]	102 ± 40	45 ± 24	90 ± 24[a]	42 ± 20	73 ± 23[a]	121 ± 68[a,b]	87 ± 37[a]

Data are reported as medians and 25-75% percentiles unless indicated. SMC = subjective memory complaints, MCI-S = MCI with stable disease, MCI-AD = MCI converting to AD, AD = probable AD. a = at least p<0.05 from SMC, b = at least p<0.05 from MCI-S.

washed with phosphate saline buffer (PBS) and blocked with 2% of bovine serum albumin (BSA, Sigma Aldrich, St. Louise, MO, USA) in PBS during 1 hour at room temperature. Washing steps were performed 3 x with washing buffer (20mM Tris-HCl, 0.05 % Tween20, pH 7.5). The synthetic peptide corresponding to the human BRI2 amino acids 140–153 ($sBRI2_{140-153}$, BioGenes GmbH, Berlin, Germany) belonging to the BRICHOS domain[34] was used for the standard curve. A stock solution of $sBRI2_{140-153}$ peptide was prepared in assay buffer (4 µg/mL, 20mM Tris-HCl, 50 mM NaCl, 0.1% BSA, 0.1% Tween20, pH 7.5) and aliquoted and stored at -80ºC. Eight different standard dilutions were prepared in assay buffer to a final concentration of 16, 10, 6, 4, 2, 1 and 0.5 ng/mL. CSF samples were diluted 1:10 in assay buffer. For every analysis one control sample (pool CSF) were used in two different concentrations (1:4 and 1:10 in assay buffer). For the analysis, 100 µl of the standards, controls and CSF samples were loaded per well in duplicates and incubated for 1 hour at room temperature at 600 rpm on a plate mixer. Plates were washed and incubated for 45 minutes at room temperature in a plate mixer with the detection antibody produced against $BRI2_{111-153}$ (0.5 µg/mL, biotinylated monoclonal IgM-purified anti-$BRI2_{111-153}$, clone CK1[14]). Plates were washed with washing buffer (20 mM Tris-HCl, 0.05% Tween20, pH 7.5) and incubated with Streptavidine Poly- Horseradish Peroxidase (1:10,000 in assay buffer, Sanquin, Amsterdam, The Netherlands) containing normal mouse serum (1:500, Dako, Glostrup, Denmark) for 20 minutes at room temperature in the plate mixer. Plates were next washed 4 x with washing buffer and incubated with 100 µL of 3,3',5,5'-tetramethylbenzidine/Dimethylsulfoxide (TMB/DMSO, 10mg/mL) and 0.03% H_2O_2 in substrate buffer (0.1 M $C_6H_8O_7$, 0.1 M NaOAc, pH 4). After 10 minutes incubation at room temperature on a plate mixer, the color reaction was stopped with 100 µl 1M H_2SO_4. The absorbance (optical density, OD) of each well was read at 450 nm. Because the material used for standard may differ from the protein measured in CSF the results were expressed in units per millilitre (U/mL). One unit is defined as the amount of BRI2 protein that equals the immune reactive signal from 1 ng of $sBRI2_{140-153}$ peptide when diluted in assay buffer. Data were presented as medians and interquartile ranges (IQR).

BRI2 assay and protein characterization

For assay development and optimization different variables were analyzed as linearity, lower limit of detection (LLOD), sample stability according to time of processing and temperature and the effect of freeze/thaw cycles using both $sBRI_{140-153}$ and CSF samples. LLOD was calculated by the extrapolation of the mean blank values + 10 times the corresponding standard deviation (SD) of twenty different blank samples. The coefficient of variation (CV) was calculated for each duplicate sample as the standard-deviation divided by the mean, expressed as percentage. The mean CV of all the samples was

calculated to establish the intra-assay CV in the two different studies. Intra-assays CVs in the AD-cohort (n = 104) and the MCI-cohort (n = 60) were 1.8% and 6.2% respectively. Inter-assay CVs were calculated using two internal quality control of pooled CSF samples at high and low concentration of BRI2 in different plates. The mean inter-assay CVs were 3% for the AD cohort. The impact of repeated freeze/thaw cycles after processing was analyzed in 5 individual CSF samples or $sBRI2_{140-153}$ (4 mM) by freezing the samples directly after centrifugation and thawing the sample up to 5 additional times by keeping them for 2 hours at room temperature and freezing again at -80ºC for at least 24 hours. To analyze the stability of $sBRI2_{140-153}$ in PBS or assay buffer, stock solutions (4 mM) in the respective buffers were kept during 1, 2, 3 and 4 days at room temperature and stored at -80 ºC until further analysis. For analysis of the effect of different storage conditions on the stability of BRI2, 5 different CSF samples or stock solutions of $sBRI2_{140-153}$ (4 µM in assay buffer) were stored at –20 °C, 4 °C, room temperature and 37°C during 1, 2, 4, 24, 72, 120 and 168 hours. Next, samples were stored at -80ºC until final analysis. A sample immediately stored at -80 ºC served as reference samples.

Statistical analysis

Statistical analyses were performed on SPSS version 20 (Chicago, IL, USA). Comparisons between the different clinical groups were performed using nonparametric Mann-Whitney or Kruskal-Wallis test due to the skewed distribution of some biomarkers. Spearman's rank correlation coefficient test was used for assessment of correlations.

Results

Label-free GeLC-MS/MS-based proteomics analysis.

Demographic data for the 18 patients are presented in Table 1. The spectral count of BRI2 peptides in CSF increased along with disease progression. The levels of BRI2 CSF were significantly changed between SMC and MCI (p = 0.05), SMC and MCI-AD (p = 0.01) and between SMC and AD (p = 0.02). Significant differences were also found when comparing SMC, MCI and MCI-AD (p = 0.02) and when patients with SMC and MCI-S where compared to patients with MCI-AD and AD (p = 0.05) (Supplementary figure 1*A*). The peptides found belong to the extracellular domain of BRI2 and all are located between amino acids 110 and 221, which contains the BRICHOS domain[33]. These results supported the potential of BRI2 as an early AD biomarker.

BRI2 assay optimization

The standard curve using the sBRI2 peptide (140-153) for the final assay ranged from 0.5 to 16 ng/mL (Fig. 1A). The corresponding LLOD was 1 u/mL. A variability was found between the standard curves when the assays were performed on different days (here the AD cohort vs MCI cohort) but not within the same study. Dilution of CSF samples showed optimal linearity between dilutions 1:7 and 1:15 (Fig. 1B). The levels of BRI2 in CSF or $sBRI2_{140-153}$ were not significantly affected by freezing/thaw cycles (Fig. 1C, E). The concentration of BRI2 in five different CSF samples stored at -20ºC, 4ºC and room temperature during up to 7 days remained similar to the concentration of the reference sample, which was stored at -80ºC (time 0) (Fig. 1D). However, when samples were stored at 37ºC, the BRI2 signal in CSF was completely lost after 168 hours (Fig. 1D). Analysis of $sBRI2_{140-153}$ revealed that the recombinant peptide diluted in PBS was highly unstable since the remaining concentration was 63, 81, 44 and 0% when samples were stored at room temperature for 1, 2, 3 and 4 days respectively. When $sBRI2_{140-153}$ was diluted in assay buffer the remaining concentration was slightly higher but still remarkable decreased with percentages of 84, 73, 63 and 0% respectively after 168 hours (Fig. 1F). Nevertheless, no changes on $sBRI2_{140-153}$ signal were detected when it was diluted in assay buffer and kept at either -20ºC or 4ºC for up to 7 days (Figure 1G). In conclusion, BRI2 in CSF is stable for up to 7 days during normal laboratory conditions. In contrast, $sBRI2_{140-153}$ should always be kept at 4ºC or lower since it is highly unstable, especially when diluted in PBS. Storage at -80ºC and -20ºC is recommended for periods longer than one week.

Study I: BRI2 CSF in non-demented controls and AD patients (AD-cohort)

Demographic data for the 104 patients used in this case-control study are presented in Table 1. Groups significantly differed in the MMSE score as well as in the CSF concentrations of $A\beta_{42}$, t-Tau and p-Tau, but not in age or gender. No interaction of BRI2 CSF with either age or gender was detected. No significant differences in BRI2 CSF concentration were observed between SMC and AD patients (Fig. 2A). No significant correlation was observed between BRI2 CSF and either MMSE or the concentration of $A\beta_{42}$ in CSF (data not shown). However, a positive correlation was observed between the CSF concentration of BRI2 with t-Tau (r = 0.372, p = 0.0001, Fig. 3A) and p-Tau (r = 0.316, p = 0.001, Fig. 3B). $A\beta_{40}$ and a panel of pro-inflammatory markers were also measured in a subset of cases (n = 60). Interestingly, significant correlations were found between the CSF concentration of BRI2 with $A\beta_{40}$ (r = 0.528, $p < 0.0001$, Fig. 3C) with the ratio $A\beta_{42}/A\beta_{40}$ (r = -0.305, p = 0.021, Fig. 3D) and in 10 out of the 37 neuroinflammatory markers (r between 0.28 and 0.68; Table 2), which included vascular factors (i.e. VEGF), cell adhesion molecules (VCAM, ICAM) as

well as several pro-inflammatory molecules involved in the regulation of immune cells (IL-12p40, IL-15, IL-16, IL-8, CRP) or the secretion of immunoglobulin's (IL-5 and MIP1α).
We used the previously determined cut-off values for CSF biomarkers levels to divide our cohort between patients with an AD biomarker profile (n = 52, at least two biomarkers are abnormal: $A\beta_{42}$ (< 495 pg/mL), t-Tau (> 356 pg/mL) and p-Tau (> 54 pg/mL)) or a normal biomarker profile (n = 52, none or only 1 biomarker is abnormal)[34]. Six out of the 60 SMC cases (10%) were classified in the AD-profile group, while eight out of 54 AD patients (14.8%) were classified into the normal biomarker profile. Age but not sex was significantly different between the two new groups (*p* = 0.038) and thus analysis of BRI2 CSF in these cases were corrected for age. No difference in the levels of BRI2 CSF was observed between the cases with a normal- and AD-biomarker profile (Fig. 2B).

Study II: BRI2 CSF in non-demented controls, MCI and AD (MCI-cohort)

A second analysis of BRI2 CSF in a different cohort of samples (MCI-cohort) was performed in order to confirm the findings from the AD cohort and to include patients with MCI who either convert to AD (MCI-AD) or remained stable (MCI-S) within two years follow-up. Demographic data of these 64 patients are presented in Table 1. Similarly to the AD-cohort and as expected, groups significantly differed in the MMSE scores as well as in the CSF concentrations of $A\beta_{42}$, t-Tau and p-Tau, but not in age or gender. The results from the AD-cohort were replicated in the second study since we did not detect significant differences between the AD and SMC (Fig. 2C) and a positive correlation between the CSF concentration of BRI2 with t-Tau (r = 0.257, *p* = 0.05, Fig. 3E) and p-Tau (r = 0.37, *p* = 0.004, Fig. 3F) was again observed. However, we observed that the levels of BRI2 CSF tended to be increased in MCI-AD patients compared to SMC, MCI-S and AD (p = 0.14, 0.09 and 0.16 respectively).

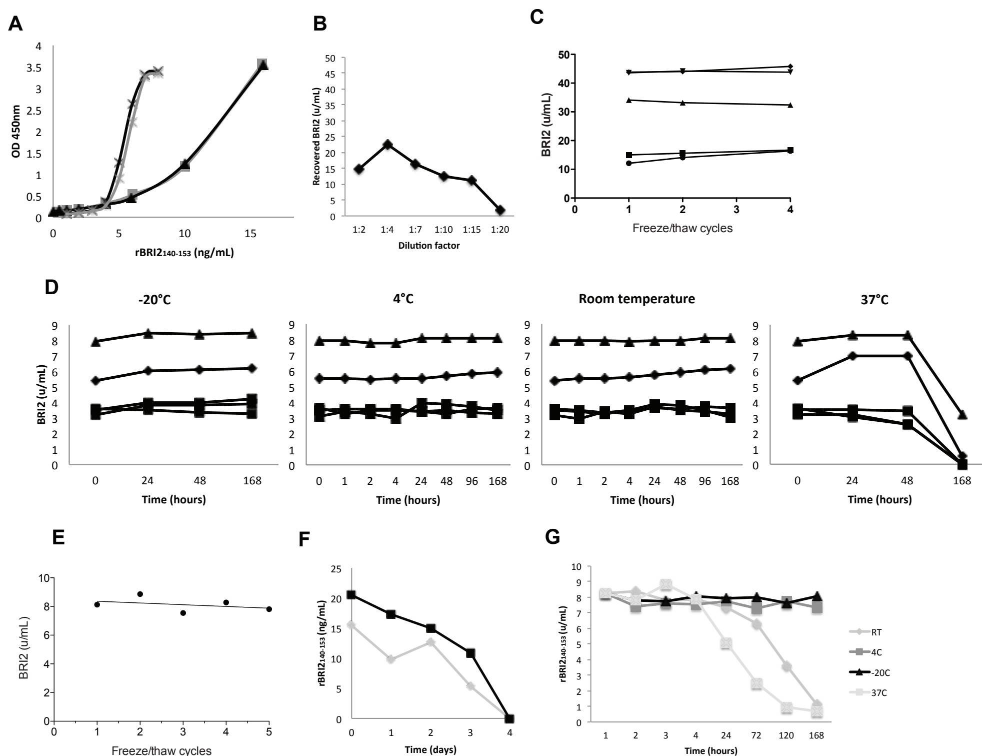

Figure 1. BRI2 assay optimization.

A, Standard curve of $sBRI2_{140\text{-}153}$ used in the BRI2 ELISA assays for the AD cohort (square, triangle and diamond) and MCI-cohort (crosses). The standard curve was obtained by using seven $sBRI2_{140\text{-}153}$ standards at the concentrations between 16 and 0.5 ng/mL diluted in assay buffer. ***B,*** To unravel the optimal dilution factor, 10 different dilutions of CSF samples in assay buffer were prepared ranging from 1:2 to 1:100. The concentration was calculated based on the dilution factor. A linear tendency in BRI2 units was observed between dilutions 1:7 and 1:15. ***C,*** Effect of freeze–thaw cycles on BRI2 in CSF. The effect of repeated freeze–thaw cycles was studied by freezing the samples directly after centrifugation and thawing the samples up to 4 times extra by keeping them for 2 h at RT and freezing them again at –80 °C for minimal 24 h. ***D,*** Effect of temperature on BRI2 stability in five different CSF samples. Aliquots were stored for up to 7 days at -20°C, 4°C, room temperature or 37°C***. E,*** Effect of repeated freeze–thaw cycles on $sBRI2_{140\text{-}153.}$ $sBRI2_{140\text{-}153}$ (4 mM) was repeatedly frozen and thawed up to 5 times. ***F,*** Stability of $sBRI2_{140\text{-}153}$ diluted in either assay buffer (black) or PBS (grey) and stored for up to 4 days at room temperature. ***G,*** Stability of $sBRI2_{140\text{-}153}$ diluted in assay buffer and stored for up to 168 hours at -20°C, 4°C, room temperature and 37°C**.**

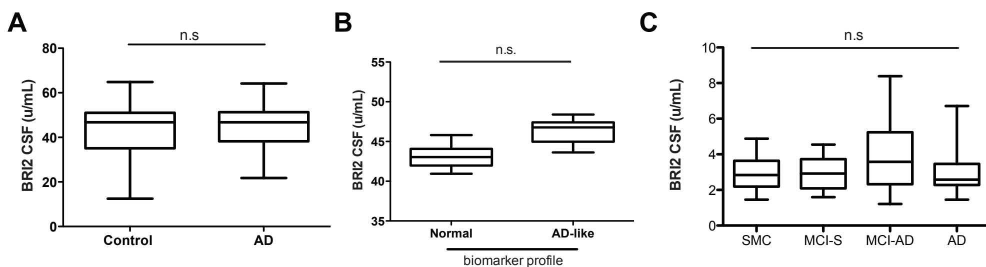

Figure 2. The levels of BRI2 in CSF are not modified in patients with MCI-S, MCI-AD or AD.
A, BRI2 levels in CSF from SMC (n= 50), AD (n = 54). No significant difference was observed between groups. ***B,*** BRI2 levels in CSF from cases with a normal (n = 52) or an AD CSF biomarker profile. No significant difference was observed between groups. ***C,*** BRI2 levels in CSF from SMC (n= 13), MCI-S (n =15), MCI-AD (n = 17) and AD (n = 15). No significant differences were observed between groups. Box plots show median values and interquartile ranges. n.s: non significant.

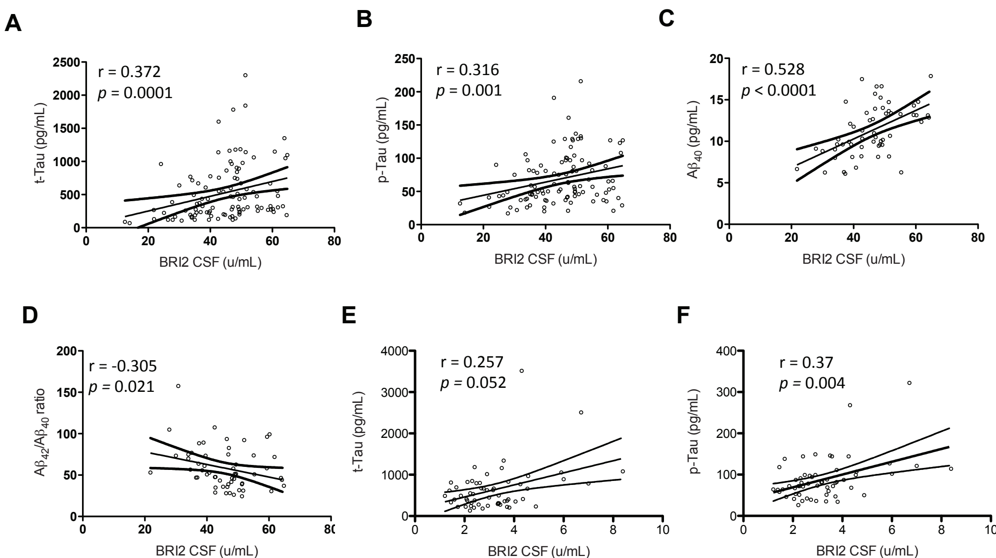

Figure 3. The levels of BRI2 in CSF correlate with t-Tau, p-Tau and Aβ_{40}
A-C, Correlation analysis between the levels of BRI2 CSF with the concentration of t-Tau (***A***), p-Tau (***B***),Aβ_{40} (***C)*** and Aβ_{42}/Aβ_{40} ratio (***D***) in the AD-cohort***. E-F,*** Correlation analysis between the levels of BRI2 CSF with the concentration of t-Tau (***E***), p-Tau (***F***) in the MCI-cohort. Correlation coefficients and *p* values are presented. Regression line is shown for the correlation found in all samples Dotted lines represent the 95% confident intervals.

Table 2. Inflammatory markers correlating with BRI2 levels in CSF

Inflammatory marker	Correlation coefficient	*p* value
Intercellular adhesion molecule 1 (ICAM-1)	0.657	<0.0001
Vascular cell adhesion molecule 1 (VCAM-1)	0.548	<0.0001
IL12p40	0.456	<0.0001
IL5	0.586	<0.0001
IL15	0.573	<0.0001
IL16	0.504	<0.0001
Vascular endothelial growth factor D (VEGFD)	0.590	<0.0001
C-reactive protein (CRP)	0.340	0.01
Macrophage inflammatory protein-1α (MIP1α)	0.316	0.017
Vascular endothelial growth factor (VEGF)	0.289	0.029
IL8	0.28	0.035

Data obtained using Spearman's correlation analysis.

Discussion

In this study we developed a specific immunoassay to investigate the potential of BRI2 as a CSF biomarker for early diagnosis of AD. Tandem mass spectrometry analysis had shown a significant increase of BRI2 peptides together with disease progression. However, no difference in BRI2 CSF concentration was found between controls, MCI-S, MCI-AD and AD and controls using the novel BRI2 immunoassay. Nevertheless, the levels of BRI2 in CSF positively correlated with the levels of t-Tau, p-Tau and $A\beta_{40,}$ but not with $A\beta_{42.}$ The data additional revealed a positive interaction between BRI2 CSF and ten different cytokines.

Our proteomic data are in agreement with another previous proteomic study which found an increase of BRI2 C-terminal domain in AD CSF patients compared to controls[35]. Our analysis additionally included cohorts that were not included in that study such as patients with MCI who either converted to AD within two years or remained stable and indicated that BRI2 is a potential early biomarker which may reflect the pathological process in AD similarly to other proteins such as Aβ or tau[36].

To further explore this hypothesis we have developed a specific BRI2 ELISA. Assay optimization has revealed that BRI2 CSF is very stable since its concentration was not changed after 4 freeze/thaw cycles or when CSF was kept up to 7 days at -20°C, 4°C or room temperature. However, the current pre-analytical guidelines for the analysis of the classical AD CSF biomarkers recommend that CSF samples should not undergo more than 2 freeze/thaw cycles and should not be stored more than 5 days at 4°C before storage[30]. Thus, no especial pre-analytical procedures are needed when analyzing BRI2 CSF. Noteworthy, it is important to use fresh aliquots of $sBRI2_{140-153}$ stored at -80°C or -20°C for

the standard curve since a considerable drop of signal was already observed after one day when sBRI2$_{140-153}$ was kept at room temperature. Aggregation of different recombinant BRI2 fragments has been previously reported[14,24]. Thus, aggregation of sBRI2$_{140-153}$ may account for the observed loss of signal. This is further supported by the lower signal detected when sBRI2$_{140-153}$ is diluted in PBS instead of assay buffer, which contains detergents that may prevent the aggregation of the peptide[37]. The comparison of the standard curves obtained in the analysis of the AD cohort to those of the MCI-cohort revealed a great variability in BRI2 signal when BRI2 analyses are performed on different days. Although samples can be compared within the individual studies (pre-analytical effects, AD or MCI-cohorts) due to the low intra- and inter- assay CVs, the large variability between different days reveal that the assay is not completely optimal and further experiments are needed to understand the causes of this inconstancy.

The present study revealed no differences in the concentration of BRI2 CSF between the SMC, MCI-S, MCI-AD and AD in the two cohorts analyzed, indicating that BRI2 can not be used as a diagnostic AD biomarker using this immunoassay. However, the slightly higher values of BRI2 CSF found in MCI-AD patients suggest that BRI2 changes take place in early stages, which agrees with the data obtained in human hippocampal tissue[14]. Similar pattern of transient higher protein levels in early stages of the disease have also been previously reported for CSF biomarkers of neuronal injury/death in patients with autosomal-dominant AD, highlighting the importance of longitudinal approaches in biomarkers studies[38]. However, one limitation of our MCI-cohort study is the small sample size used (n » 15/group). The discrepancy found between the ELISA results and the proteomics data might be explained by the small sample size in the latter (n=5/group). However, the case-control proteomics study of Jahn and colleagues, which also identified BRI2 as a potential AD biomarker, used two cohorts of patients and a similar sample size as the current study (n = 51). It could also be possible that only specific BRI2 fragments are changed in AD as we observed in human hippocampal tissue, where only specific larger BRI2 forms were increased in AD by western blot[14]. Thus, the BRI2 ELISA assay may lack the sensitivity or specificity needed to detect the specific fragments changed in AD. For instance, the full length recombinant BRI2 ectodomain (rBRI2$_{76-266}$) could not be detected with this ELISA indicating that only short BRI2 fragments containing the BRI2-BRICHOS domain are detected with this assay.

The levels of BRI2 CSF positively correlated with the levels of t-Tau and p-Tau in both studies, which suggests a relationship between BRI2 and tau dysfunction. It is well established that while the levels of t-Tau reflect axonal neurodegeneration, the levels of p-Tau likely mirrors NFT formation[5,6,8]. In line with this data, the increase in BRI2 deposition in human AD hippocampus strongly correlated with the formation of NFT, thus the levels of BRI2 in CSF may reflect tau pathology[14]. A relation of BRI2 with tau pathology was previously shown

by the finding that aggregates of BRI2 activated apoptotic pathways in neuronal cells and induced truncation of tau[28], indicating that BRI2 aggregation can induce neuronal death and promote NFT formation. Thus, the positive correlation observed in human CSF between BRI2, t-Tau and p-Tau further support an involvement of BRI2 in the underlying AD pathological pathways leading to NFT formation and neurodegeneration.

BRI2 concentration in CSF positively correlated with the levels of $A\beta_{40}$ but not with the levels of $A\beta_{42.}$ A similar positive associations with $A\beta_{40}$ but not $A\beta_{42}$ have been previously reported for other variables measured in CSF such as the activity of the beta-site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1)[34,39], an enzyme involved in the amyloidogenic processing of APP and thus, in Aβ production. Noteworthy, although $A\beta_{42}$ reflects plaque formation, it may not reflect Aβ production since it is more prone to aggregate and fibrillize than other Aβ species[40-41]. Thus, the reduced solubility of $A\beta_{42}$ compared to $A\beta_{40}$ in tissue may explain different associations observed with $A\beta_{42}$ and $A\beta_{40}$. Therefore, the positive correlation between BRI2 and $A\beta_{40,}$ the most abundant Aβ form, may reflect a relationship between BRI2 and Aβ production. This is in agreement with previous studies that revealed that BRI2 is able to bind APP downregulating the production of Aβ *in vitro*[16,17,19] and *in vivo*[21,23,26]. In addition, BRI2-APP complexes have been mainly detected in control human hippocampus but not in AD, suggesting that indeed Aβ production might be influenced by changes in BRI2[14]. Taking together, the current CSF data further support a role of BRI2 in the amyloidogenic processing of APP and Aβ production in humans.

Interestingly, we also found a positive correlation between BRI2 with a panel of inflammatory markers, indicating an association between BRI2 and inflammation, an early event in AD pathogenesis[42]. Although a role of BRI2 with inflammatory processes has not been reported yet, the C-terminal fragment of BRI2, $BRI2_{1-23}$, was significantly reduced in the CSF from patients with multiple sclerosis (MS), and BRI2 protein levels were also decreased in the cerebellum of MS, an inflammatory demyelinating disease[43]. However, the exact relationship between BRI2 and the autoimmune response as well as the potential of BRI2 as a biomarker for neuroinflammatory disorders such as MS remains to be investigated. Especially interesting is the correlation of BRI2 with the cytokine IL12p40, since besides the role of IL12p40 in adaptive immunity[44], this cytokine was able to regulate Aβ load and cognitive performance in transgenic models of AD or aging[45,46]. IL12 expression is triggered by the intracellular domain of TNFα (ICD-TNFα), which is released by the novel intramembrane protease SPPL2b[44,47], which also processes BRI2[48] and was found to be drastically increased in AD hippocampus[14]. Thus, the SPPL2b increase may lead to parallel modifications on BRI2 and IL12 (via the ICD-TNFα), which may explain the subsequent correlations seen in the CSF.

In summary, the lack of significant differences of BRI2 CSF between the different clinical groups indicate that BRI2 can not be used as an early AD CSF biomarker using the current BRI2 immunoassay. Other studies need to be performed in order to unravel the potential of BRI2 as a biomarker for neuroinflammatory disorders such as MS. Nevertheless, the positive correlation of BRI2 with $A\beta_{40}$ and tau markers in human CSF, further suggest that BRI2 is a contributing factor in critical aspects of AD pathogenesis such as Aβ production, NFT formation and neurodegeneration.

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Disclosure statement

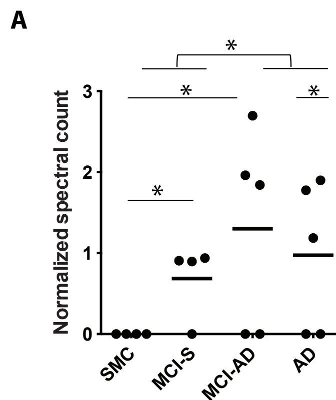
Dr. Teunissen serves on the advisory board of Fujirebio and Roche, performec studies for Probiodrug and received research consumables from Euroimmun, IBL, Fujirebio, Invitrogen and Mesoscale Discovery. Dr Scheltens serves/has served on the advisory boards of: Genentech, Novartis, Pfizer, Roche, Danone, Nutricia, Jansen AI, Baxter and Lundbeck. He has been a speaker at symposia organized by Lundbeck, Lilly, Merz, Pfizer, Jansen AI, Danone, Novartis, Roche and Genentech. He serves on the editorial board of Alzheimer's Research & Therapy and Alzheimer's Disease and Associated Disorders, is a member of the scientific advisory board of the EU Joint Programming Initiative and the French National Plan Alzheimer. The Alzheimer Center receives unrestricted funding from various sources through the VUmc Fonds. Dr Scheltens receives no personal compensation for the activities mentioned above.
None of the other authors has any competing interest.

Supplementary information

Methods

Mass spectrometry analysis of CSF

CSF samples were analyzed by label-free GeLC-MS/MS-based proteomics and normalized spectral counting as previously described[32]. The data obtained were processed and analyzed as described before[33]. The global protein profiling results of the CSF proteomics screen will be reported elsewhere (Chiasserini et al., manuscript submitted). BRI2 was one of the proteins identified.



Supplementary figure 1. The spectral counts of BRI2 peptides in CSF increased along with disease progression. Proteomic analysis of CSF samples from SMC (n = 4), MCI-S (n = 4), MCI-AD (n = 5) and AD (n =5) cases. *$p \leq 0.05$

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