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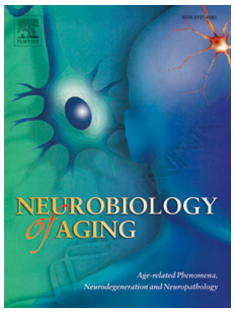

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PII: S0197-4580(16)30304-9

DOI: 10.1016/j.neurobiolaging.2016.11.017

Reference: NBA 9783

To appear in: *Neurobiology of Aging*

Received Date: 5 August 2016

Revised Date: 7 November 2016

Accepted Date: 25 November 2016

Please cite this article as: Janelidze, S., Hertze, J., Nägga, K., Nilsson, K., Nilsson, C., the Swedish BioFINDER study group, Wennström, M., van Westen, D., Blennow, K., Zetterberg, H., Hansson, O., Increased blood-brain barrier permeability is associated with dementia and diabetes, but not amyloid pathology or *APOE* genotype, *Neurobiology of Aging* (2017), doi: 10.1016/j.neurobiolaging.2016.11.017.

Increased blood-brain barrier permeability is associated with dementia and diabetes, but not amyloid pathology or *APOE* genotype

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ABSTRACT

Blood-brain barrier (BBB) dysfunction might be an important component of many neurodegenerative disorders. In this study, we investigated its role in dementia using large clinical cohorts. The cerebrospinal fluid (CSF)/plasma albumin ratio (Qalb), an indicator of BBB (and blood-CSF barrier) permeability, was measured in a total of 1015 individuals. The ratio was increased in patients with Alzheimer's disease (AD), dementia with Lewy bodies (DLB) or Parkinson's disease dementia (PDD), subcortical vascular dementia (VaD) and frontotemporal dementia (FTD) compared with controls. However, this measure was not changed during preclinical or prodromal AD and was not associated with amyloid PET or *APOE* genotype. The Qalb was increased in diabetes mellitus and correlated positively with CSF biomarkers of angiogenesis and endothelial dysfunction (VEGF, ICAM-1 and VCAM-1). In healthy elderly, high body mass index and waist-hip ratio predicted increased Qalb 20 years later. In summary, BBB permeability is increased in major dementia disorders, but does not relate to amyloid pathology or *APOE* genotype. Instead, BBB impairment may be associated with diabetes and brain microvascular damage.

Keywords: Blood-brain barrier, dementia, amyloid, *APOE ε4*, diabetes, vascular pathology.

1. INTRODUCTION

Cerebrovascular pathology is common in the spectrum of dementia disorders (Snyder et al., 2015). One of the manifestations of vascular disease in the brain is blood-brain barrier (BBB) dysfunction (Zlokovic, 2008). The BBB is a selective diffusion barrier at the level of the cerebral microvascular endothelium, that maintains homeostasis in the central nervous system (CNS) by regulating ion balance, facilitating nutritional transport and preventing influx of potentially neurotoxic molecules from the circulation (Chow and Gu, 2015). Thus, BBB failure may have detrimental effects on CNS function and animal studies have indicated that BBB breakdown could lead to secondary neuronal injury and neurodegeneration (Bell et al., 2010; Winkler et al., 2014).

Accumulating evidence suggests that BBB function is compromised in neurodegenerative disorders. Studies investigating BBB permeability in clinical cohorts of patients mainly utilize three experimental approaches: measurement of the cerebrospinal fluid (CSF)/blood albumin ratio, histologic assessment of the blood-derived proteins in the brain tissue and brain imaging, *e.g.*, magnetic resonance imaging (MRI) or positron emission tomography (PET). Such investigations have convincingly shown BBB dysfunction in vascular dementia (VaD) (Skoog et al., 1998; Taheri et al., 2011; Wada, 1998; Wallin et al., 1990). A recent meta-analysis reported elevated CSF/serum albumin ratio is in Alzheimer's disease (AD), however the effect size was small (Olsson et al., 2016). Studies subgrouping AD patients have found that AD cases with evidence of concomitant cerebrovascular pathology have high CSF/serum albumin ratio as a sign of impaired BBB function (Blennow et al., 1990; Blennow et al., 1991). In agreement, imaging techniques have mostly detected slight BBB impairments in AD patients and only in conjunction with vascular pathology (van de Haar et al., 2015). Although evidence of microvascular lesions has been reported in other dementias, *e.g.*, dementia with Lewy bodies (DLB), Parkinson's disease with dementia (PDD), and frontotemporal dementia (FTD) (De Reuck et al., 2012; De Reuck et al., 2013), little data is available with respect to BBB function in these conditions (Janelidze et al.,

2015; Llorens et al., 2015; Sjogren et al., 2004).

Multiple mechanisms have been suggested to underlie BBB dysfunction in dementia. Accumulation of β-amyloid in vascular wall may lead to endothelial cell damage and disrupt the BBB in AD (Burgmans et al., 2013; Erickson and Banks, 2013). Some studies have indicated that BBB breakdown may be linked to *APOE ε4*, a major genetic risk factor for non-familial AD (Bell et al., 2012; Halliday et al., 2013; Nishitsuji et al., 2011). We recently demonstrated that increased BBB permeability in PD and PDD is related to an abnormal angiogenic CSF profile (Janelidze et al., 2015). In dementia and other disorders linked to high risk of dementia (*e.g.*, diabetes, cardiovascular disease), BBB impairment has also been attributed to adverse effects of oxidative stress and chronic inflammation on the endothelial cell function (Di Marco et al., 2015; Raz et al., 2015). However, BBB dysfunction has so far been investigated in experimental models or in small patient cohorts and needs to be validated in larger clinical material.

In this study, we measured BBB permeability using the CSF/plasma albumin ratio (Qalb) in two different cohorts of in total 1015 individuals including cognitively healthy controls and patients with subjective cognitive decline (SCD), mild cognitive impairment (MCI) as well as with five major dementias types, AD, PDD, DLB, VaD and FTD. We assessed if the disruption of BBB was associated with amyloid pathology and the *APOE* genotype. We also investigated possible risk factors for BBB dysfunction and analyzed CSF biomarkers of angiogenesis, endothelial damage and neuroinflammation in order to determine if these factors are related to BBB breakdown in different dementias.

2. MATERIAL AND METHODS

The study was approved by the Regional Ethics Committee in Lund, Sweden, and the patients and controls gave their informed consent for research.

2.1 Study participants

Cohort 1: Seventy-five patients with AD, 34 patients with DLB/PDD, 28 patients with VaD, 41 patients with FTD and 65 healthy controls were recruited at the Memory Clinic of Skåne University Hospital in Malmö, Sweden. We also included 96 individuals with a baseline diagnosis of MCI. After an average clinical follow-up period of 5.7 years (3.0-9.6) 35 of these patients had converted to AD (MCI-AD), while 61 of them remained cognitively stable (sMCI).

All patients with a clinical syndrome of dementia met the DSM-IIIR criteria for dementia (American Psychiatric Association. Work Group to Revise DSM-III, 1987) combined with the NINCDS-ADRDA criteria for AD (McKhann et al., 1984), the NINDS-AIREN criteria for VaD (Roman et al., 1993), criteria of probable DLB according to the 2005 consensus criteria (Geser et al., 2005) or the 1998 consensus criteria for FTD (Neary et al., 1998). Patients with MCI at baseline had to fulfill the criteria advocated by Petersen (Petersen 2004), including: (1) memory complaint, preferably corroborated by an informant; (2) objective memory impairment adjusted for age and education, as judged by the physician; (3) preservation of general cognitive functioning, as determined by the clinician's judgment based on a structured interview with the patient and a Mini-Mental Status Examination (MMSE) score greater than or equal to 24; (4) zero or minimal impairment of daily life activities, and (5) not fulfilling the DSM-III-R criteria of dementia (American Psychiatric Association. Work Group to Revise DSM-III, 1987). The healthy participants were not allowed to have any cognitive complaints or any significant neurological or psychiatric illness and they needed to have a well-preserved general cognitive functioning. A careful clinical interview, together with an assessment of global function (Mini-Mental State Examination, MMSE), delayed recall (Alzheimer's Disease Assessment Scale Cognitive Subscale, ADAS Cog, item 3), attention (a quick test of cognitive speed, AQT) and visuospatial and executive function (cube-drawing test and clock test), was done to rule out mild cognitive impairment. All subjects were assessed by medical doctors with extensive experience in cognitive disorders. The characteristics of cohort 1 are given in Table 1.

Cohort 2: The study population stemmed from the prospective and longitudinal Swedish BioFINDER study (further information available at: www.biofinder.se). Cohort 2 included 292 cognitively normal elderly participants recruited from the population-based Malmö Diet Cancer Study (MDCS) (Berglund et al., 1993) and 384 patients with mild cognitive complaints enrolled consecutively at three memory outpatient clinics in Sweden. Cognitively normal elderly were eligible for inclusion if they 1) were aged ≥60 years old, 2) scored 28–30 points on the Mini-Mental State Examination (MMSE) (Folstein et al., 1975) at the screening visit, 3) absence of cognitive symptoms as evaluated by a physician, 4) were fluent in Swedish, 5) did not fulfill the criteria of MCI or any dementia. The exclusion criteria were 1) presence of significant neurologic or psychiatric disease (*e.g.*, stroke, Parkinson's disease, multiple sclerosis, major depression), 2) significant systemic illness making it difficult to participate, 3) refusing lumbar puncture (LP) 4) significant alcohol abuse. Data was collected between 2009 and 2014 in accordance with a standardised protocol. The patients with mild cognitive complaints were referred for assessment of their cognitive complaints and were included between 2010 and 2014. They were thoroughly assessed by physicians with special interest in dementia disorders. The inclusion criteria were: 1) cognitive symptoms; 2) not fulfilling the criteria for dementia; 3) MMSE score of 24–30 points 4) age 60–80 years; and 5) fluent in Swedish. The exclusion criteria were: 1) cognitive impairment that without doubt could be explained by another condition (other than prodromal dementia); 2) severe somatic disease; and 3) refusing lumbar puncture or neuropsychological investigation. These criteria resulted in a clinically relevant population where 45% were classified as SCD, 42% as amnestic MCI and 13% as non-amnestic MCI. The classification was based on a neuropsychological battery assessing the cognitive domains of verbal ability, visuospatial construction, episodic memory, and executive functions and the clinical assessment of a senior neuropsychologist. The characteristics of cohort 2 are given in Table 2.

In both cohorts, the diagnosis of hypertension, diabetes, hyperlipidemia and ischemic heart disease made by a medical doctor was available from the medical records.

2.2 CSF sampling and biological assays

For all patients and controls, blood plasma and CSF samples were drawn at some point between 8 a.m. and 12 a.m with the patients non-fasting. CSF was collected in polypropylene tubes and mixed gently to avoid gradient effects. All samples were centrifuged within 30 min at +4 °C at 2000 g for ten minutes to remove cells and debris. Samples were stored in aliquots at -80 °C pending biochemical analysis. The procedure followed The Alzheimer's Association Flow Chart for LP and CSF sample processing (Blennow et al. 2010). CSF Aβ42 and Aβ40 were analyzed by Euroimmun immunoassay (EUROIMMUN AG, Lübeck, Germany).

CSF levels of vascular endothelial growth factor (VEGF), soluble VEGF receptor 1 (sVEGFR-1), intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) were measured using Growth Factor I, and Vascular Injury II kits according to the manufacturer's protocol with some modifications (Meso Scale Discovery, Gaithersburg, Maryland, USA). Briefly, 10% bovine serum albumin was added to the blocking buffer for all the assays and in Growth Factor I assays, samples were incubated overnight at +4°C. Data was collected and analyzed using SECTOR Imager 6000 reader and Discovery Workbench® Software (www.mesoscale.com). All samples were measured in duplicates and the mean of the duplicated was used in the statistical analysis. Detection limits were: 8.1 pg/ml for VEGF, 213.6 pg/ml for sVEGFR-1, 5.6 pg/ml for ICAM-1 and 257.5 pg/ml for VCAM-1. The coefficient of variation (CV) was below 20% for all assays. The very few samples with CV >20% did not affect the results and were therefore included in the statistical analysis.

Albumin levels in plasma and CSF were measured by by immunoturbidimetry on a Roche Cobas Analyzer (Roche Diagnostics, Bromma, Sweden). The albumin ratio was calculated as CSF albumin (mg/L)/plasma albumin (g/L) and was used as a measure of BBB function.

2.3 [^{18}F]flutemetamol PET in cohort 2

Cerebral Aβ deposition was visualized with the PET tracer [^{18}F]flutemetamol (approved by the Food and Drug Administration, and the European Medical Agency). PET/CT scanning of the brain was conducted at two sites using the same type of scanner (Gemini, Philips Healthcare, Best, the Netherlands). Baseline sum images from 90-110 minutes post injection were analyzed using the software NeuroMarQ (provided by GE Healthcare, Cleveland, OH). A volume of interest (VOI) template was applied for the following 9 bilateral regions: prefrontal, parietal, lateral temporal, medial temporal, sensorimotor, occipital, anterior cingulate, posterior cingulate/precuneus and a global neocortical composite region (Lundqvist et al., 2013). The standardized uptake value ratio (SUVR) was defined as the uptake in a VOI normalized for either the cerebellar cortex or pons uptake. Amyloid PET data was available from 342 subjects (129 cognitively normal elderly, 102 SCD patients and 111 MCI patients).

2.4 Computed tomography and magnetic resonance imaging

In cohort 1, three hundred and twelve cases underwent computed tomography (CT, n=266) and magnetic resonance imaging (MRI, n=46).for assessment of white matter changes (WMC). Imaging was performed according to clinical protocols including acquisition of FLAIR images on a 1.5 Tesla scanner (Siemens) for MRI or of non contrast CT images using multiple CT scanners. Presence of WMC was visually assessed according to the Age Related WMC (ARWMC) rating scale developed by Wahlund et al. (Wahlund et al., 2001), that was developed for rating of both MRI and CT with high agreement between modalities. On CT images, WMC were rated in the left and right frontal and occipital-parietal lobes; temporal lesions were not included since this location is very rare for WMLs (Bronge, 2002). WMC were graded from 0 to 3 points: 0 = no lesions or lesions <5 mm, 1 = presence of lesion ≥5 mm, 2 = lesions beginning to aggregate, and 3 = confluent lesions involving almost the entire region.

In cohort 2, 618 study participants (269 controls, 159 SCD and 190 MCI) were examined using a single 3T MR scanner (Trio, Siemens). Automated segmentation of white matter lesions was performed using the Lesion Segmentation Tool (LST) implemented in SPM8 (http://www.applied-statistics.de/lst.html); this generated a total white matter lesion volume [mL] for each individual.

2.5 Statistical analyses

SPSS (IBM, Armonk, NY, US) was used for statistical analysis. Data for VEGF, sVEGFR-1, ICAM-1, VCAM-1 and albumin were skewed, therefore all variables were ln-transformed before analyses. In addition to CSF concentrations of angiogenesis biomarkers, we also used the VEFG/ sVEGFR-1 ratio when investigating associations with clinical data. The rationale for this is that sVEGFR-1 has direct antagonistic effect on VEGF by sequestering the ligands from the membrane receptors (Ambati et al., 2006; Kendall and Thomas, 1993; Qi et al., 2013). Consequently high VEGF/ sVEGFR-1 ratios provide an index of the bioactive levels of VEGF.

In cohort 1, the confounding effects of age, gender and body mass index (BMI) were tested with Pearson's correlation analysis and Student's T-tests. None of the measured analytes were associated with BMI. However, for most analytes we found correlations with age as well as gender differences. Therefore, all subsequent statistical analyses were controlled for age and gender.
For group-wise comparisons, we used univariate general linear models. Linear regressions were used to investigate associations between two continuous variables. The study participants were categorized into groups with normal and pathological PET status using the SUVR cutoff > 1.42 when normalized for the cerebellar cortex uptake (Palmqvist et al., 2014). The SUVR cutoff > 0.51 with the pons as a reference region was derived using mixture modeling.(Benaglia et al., 2009) We also categorized the study participants into groups with normal and pathological CSF signature using the CSF Aβ42/Aβ40 ratio cutoff≥0.1 (Janelidze et al., 2016). Associations between the Qalb

and [^{18}F]flutemetamol SUVR as well as vascular risk factors were tested in the total sample with univariate and linear regression models controlling for age, gender and diagnosis. Alpha-level of significance was set at $p<0.05$.

3. RESULTS

Demographic and clinical characteristics of cohorts 1 and 2 as well as raw untransformed concentrations of CSF analytes are shown in Table 1 and 2.

3.1 The Qalb in dementias

Cohort 1 (dementia)

We found that the Qalb differed significantly between the diagnostic groups. Specifically, the ratio was higher in AD (p=0.007), DLB/PDD (p=0.037), VaD (p=0.004) and FTD (p=0.004) patients compared with controls (Fig. 1A and Table 1). However, there were no differences between patients with stable MCI (p=0.690) or in patients with MCI who subsequently progressed to AD (p=0.186) when compared to controls. These results were similar in regression models additionally adjusting for the confounding effects of WML.

3.2 The Qalb and β-amyloid pathology

Cohort 2 (Biofinder)

Next we sought to determine if the Qalb was altered in prodromal and preclinical stages of AD in a large cohort of cognitively healthy individuals and patients with SCD or MCI (cohort 2). To this end, we compared diagnostic subgroups with pathological CSF signature (control-P, SCD-P and MCI-P; CSF Aβ42/Aβ40 ratio<0.1) with control subjects showing normal CSF status (control-N; CSF Aβ42/Aβ40 ratio≥0.1). In addition, we investigated if changes in the Qalb were associated with cortical amyloid deposition measured using [^{18}F]flutemetamol PET. There were no differences in the Qalb between any of the diagnostic subgroups (control-N, control-P, SCD-P and MCI-P; all

p>0.172, Table 3) and no significant correlations between the Qalb and composite [^{18}F]flutemetamol SUVR (β=-0.111, p=0.052 and β=-0.097, p=0.081 with the cerebellar cortex or pons as reference regions, respectively). Moreover, there were no differences in the ratio between study subjects with normal vs. pathological amyloid PET (p=0.315 and p=0.385 with the cerebellar cortex or pons as reference regions, respectively).

3.3 The Qalb and *APOE* genotype

Previous studies have indicated that *APOE* might play a role in maintaining the integrity of the BBB (Zhao et al., 2015). To explore that association, we compared the Qalb between different *APOE* genotypes in cohorts 1 and 2.

Cohort 1 (dementia)

The Qalb did not differ between the *APOE ε4* carriers and non-carriers in any of the diagnostic groups in cohort 1 (all p>0.081, Fig. 1B). We did not find significant differences in the Qalb between *APOE ε4* carriers and non-carriers (p=0.062) or between carriers of one *ε4* allele, two *ε4* alleles and non-carriers (all p>0.087) in the total sample using univariate regression model adjusting for age, gender and diagnosis.

Cohort 2 (Biofinder)

Confirming our findings in cohort 1, there were no differences in the Qalb between *APOE ε4* carrier and non-carriers in control (p=0.720), SCD (p=0.634) or MCI (p=0.874) groups in cohort 2. Furthermore, there were no differences when comparing carriers of one *ε4* allele, two *ε4* alleles and non-carriers (all p>0.228). The Qalb did not differ when comparing younger (≤ 65 years) and older (≥ 66 years) *APOE ε4* carrier and non-carriers in SCD and MCI groups (all p>0.380).

3.4 The Qalb and CSF biomarkers of angiogenesis or endothelial damage

Altered Qalb may be related to abnormal angiogenesis and endothelial cell function (Janelidze et al., 2015; Zlokovic, 2008). Therefore, we investigated associations between the ratio and CSF biomarkers of angiogenesis and endothelial dysfunction.

Cohort 1 (dementia)

High Qalb was associated with increased CSF levels of ICAM-1 and VCAM-1 (markers of endothelial dysfunction) in all diagnostic groups (Table 4). Moreover, the Qalb positively correlated with CSF levels of VEGF in control, sMCI, MCI-AD, AD, DLB/PDD and FTD groups and with the CSF VEGF/VEFGR1 ratio in sMCI, AD, DLB/PDD and FTD groups. The associations remained significant after additionally adjusting for WMLs.

When comparing with control subjects, we found that VEGF levels were increased in sMCI ($p=0.021$), MCI-AD ($p=0.008$), AD ($p=0.002$), VaD ($p=0.001$) and FTD ($p=0.001$) patients, whereas the VEGF/sVEGFR-1 ratio was increased across all the groups (all $p<0.001$, Fig. 2A, B and Table 1). There were no changes in CSF levels of ICAM-1 or VCAM-1 (Fig. 2C, D and Table 1).

3.5 Risk factors for abnormal Qalb

Finally, we examined associations between the Qalb and potential vascular risk factors in dementia cohorts 1 and 2.

Cohort 1 (dementia)

In cohort 1, the Qalb was increased in individuals with diabetes (diagnosed with diabetes or taking anti-diabetic medications) compared to those without diabetes ($p=0.015$) (Fig. 3A). Furthermore, diabetes was associated with high CSF levels of ICAM-1 ($p<0.001$), VCAM-1 ($p=0.007$) and

VEGF (p=0.024) (Fig. 3C-E). These results remained significant after additionally adjusting for WMLs. We did not find any associations with hypertension (p=0.633) or ischemic heart disease (p=0.801).

Cohort 2 (Biofinder)

Similar to findings in cohort 1, the Qalb was increased in individuals with diabetes in cohort 2 (p=0.041) (Fig. 3B). Whereas there was no association with ischemic heart disease (p=0.281), plasma homocysteine (p=0.608) and hyperlipidemia (p=0.414), the ratio was higher in patients with hypertension (p=0.012). In this cohort, linear regression models revealed no effects of WMLs on the Qalb (β=0.018, p=0.692), therefore we did not include WML variable in the statistical analysis. The cognitively healthy elderly in cohort 2 were recruited from the MDCS where the first assessments of the study participants were conducted 20.1±1.5 (mean±SD) years previous the present study. In this group, linear regression analysis revealed that high BMI (24.8±3.5, mean±SD) and the waist-hip ratio (0.8±0.09, mean±SD) in the middle age (53.9±4.7, mean±SD) predicted increase in the Qalb 20 years later (BMI, β=0.144, p=0.013; waist-hip ratio, β=0.304, p=0.003).

4. DISCUSSION

In the present study, we demonstrate that the Qalb is increased in patients with AD, DLB/PDD VaD and FTD but not during preclinical or prodromal AD stages. In two cohorts comprising a total of 1015 individuals we did not find any associations between the Qalb and *APOE* genotype. However, in both cohorts, the ratio was associated with coexisting diabetes mellitus. Further, the ratio positively correlated with CSF biomarkers of angiogenesis and endothelial dysfunction, including VEGF, the VEGF/VEFGR1 ratio, ICAM-1 and VCAM-1. VEGF or the VEGF/VEFGR1 ratio was increased in all dementias, as well as in MCI, whereas patients with diabetes showed increased CSF levels of VEGF, ICAM-1 and VCAM-1. Lastly, in the longitudinal cohort, a high Qalb was related to increased BMI and a higher waist-hip ratio at the middle age.

While an elevated Qalb is a consistent finding in vascular dementia (Skoog et al., 1998; Taheri et al., 2011; Wada, 1998; Wallin et al., 1990), the evidence is not clear-cut when it comes to AD (Erickson and Banks, 2013). Non-stringent clinical categorization of AD and VaD (especially in earlier investigations) bias related to sample size and effects of age on the ratio have been suggested to account for the conflicting results (Blennow et al., 1990; Blennow et al., 1991; Erickson and Banks, 2013). Nevertheless, a recent meta-analysis came out positive with a small but statistically significant 1.1-fold (95% CI 1.01-1.20) increase of Qalb in AD patients compared with control individuals (Olsson et al., 2016). In the present study, we compared the Qalb in a relatively large and well-characterized cohort of cognitively healthy controls and patients with major dementia disorders using statistical methods adjusting for confounding effect of age and gender. We found increased Qalb in VaD, but also in AD, DLB/PDD and FTD suggesting that dysfunction of the BBB is common across different dementia disorders (Janelidze et al., 2015; Sjogren et al., 2004). Compromised BBB has recently been demonstrated in the hippocampus of patients with MCI (Montagne et al., 2015). In contrast, we did not detect any differences in the Qalb between control and MCI groups. Furthermore, the ratio was not altered in cognitively healthy individuals who showed a pathological CSF AD biomarker profile and did not correlate with cortical amyloid deposition. Altogether these results speak against a significant causative link between amyloid pathology and BBB dysfunction at least during the early disease stages. In fact, the BBB appears intact in the murine models of AD displaying significant amyloid pathology (Bien-Ly et al., 2015).

Some evidence from preclinical models and from human disease implicated ApoE4 in BBB dysfunction in AD. In transgenic mice, production of human ApoE4 by astrocytes has been shown to induce BBB leakage and neurodegeneration (Bell et al., 2012). The Qalb was found to be higher in cognitively healthy individuals carrying *APOE ε4* than in non-carriers and to increase with age in *APOE ε4* carriers only (Halliday et al., 2013). However, analysis of the Qalb in the considerably

larger cohort in the present study, showed no differences between *APOE ε4* carriers and non-carriers or between younger and older *APOE ε4* carriers. In agreement with our findings, several studies reported no associations between *APOE ε4* and BBB breakdown (Bien-Ly et al., 2015; Bowman et al., 2007; Karch et al., 2013) thus suggesting that *APOE* genotype is unlikely to play a role in BBB dysfunction.

Our previous work indicated that in PD high Qalb was associated with increased CSF levels of angiogenic factors including VEGF (Janelidze et al., 2015). In addition to its central role in physiological and pathological angiogenesis, VEGF is known to regulate vascular permeability. In rats, administration of VEGF induces leakage of the BBB (Zhang et al., 2000). Increased production of VEGF has also been shown to cause BBB breakdown in experimental models of MS and cerebral ischemia (Argaw et al., 2012; Suzuki et al., 2015). In the present study, we found elevated VEGF or the VEGF/sVEFGR1 ratio (an index of the bioactive levels of VEGF) in the CSF of patients with different forms of dementia and positive correlation of between VEGF and the Qalb. Interestingly, CSF levels of VEGF were increased in MCI and MCI-AD patients who showed no difference in the Qalb compared with control individuals. These findings suggest that abnormal VEGF production may precede BBB breakdown in dementia and provide further support for the link between aberrant VEGF signaling and BBB dysfunction. We also found an association between elevated CSF levels of VEGF and coexisting diabetes. Notably, VEGF pathways have been recently shown to contribute to impaired BBB and functional recovery in experimental model of comorbid diabetes and stroke (Reeson et al., 2015).

In accordance with earlier studies (Hawkins et al., 2007; Starr et al., 2003), we observed associations between disrupted BBB and diabetes in two different cohorts. Obesity is a risk factor for type-2 diabetes and constitutes an important component of the metabolic syndrome and we also show that increased markers for obesity (BMI and the waist-hip ratio) at the middle age predicts

high Qalb 20 years later. Endothelial and vascular pathology induced by chronic inflammation and oxidative stress are major complications of diabetes (Tousoulis et al., 2013). Changes in endothelial cells include upregulation of adhesion molecules ICAM-1 and VCAM-1, which are considered biomarkers of peripheral vascular dysfunction in diabetes (Meigs et al., 2004; Tousoulis et al., 2013). Here, we demonstrate for the first time increased CSF levels of ICAM-1 and VCAM-1 as well as positive correlations between the Qalb and the CSF levels of adhesion molecules in diabetic patients indicating that diabetes may lead to endothelial damage in the cerebral vasculature. However, the number of individuals with diabetes was small in the present study and thus the results require further validation in larger population.

Another limitation of the present study is that we used Qalb as a measure for the permeability of the BBB, which is common practice (Nagga et al., 2014; Reiber, 1994; Tibbling et al., 1977). However, factors other than disruption of the barrier might affect levels of albumin in the CSF. In particular, the turnover rate of CSF is slowed with advancing age and in patients with AD that has been hypothesized to influence the CSF/serum albumin ratio, with resulting higher CSF levels of albumin (Erickson and Banks, 2013; Reiber and Peter, 2001). While we did adjust for the potentially confounding effects of age when analyzing our material, we cannot entirely rule out that changes in CSF turnover have contributed to the observed high Qalb. Some researchers also caution against describing Qalb as a BBB marker and state that it actually reflects the blood-CSF barrier at the choroid plexus (Reiber and Peter, 2001). We cannot rule out that for example vascular changes in the choroid plexus microvessels may impair function and thereby lower CSF production rate with a reduced CSF flow rate that would affect the CSF/serum albumin ratio. On the other hand, in for example stroke, leaving the choroid plexus intact but injuring cerebrovascular endothelial cells, the CSF/serum albumin ratio is increased (Brouns et al., 2011), suggesting that CSF/serum albumin ratio probably is a marker of both barriers. Altogether, direct assessments of BBB function, such as

using dynamic contrast enhanced MRI and labeled tracers, are warranted in order to confirm the results of the present study.

In conclusion, we show that a compromised BBB appears to be a feature of several different dementias, but is not directly associated with Aβ pathology or the *APOE ε4* genotype. Our data link BBB dysfunction with abnormal angiogenic pathways, endothelial damage and possibly with diabetes mellitus. These findings prompt for future studies investigating molecular mechanisms of BBB breakdown in dementia disorders and impact of therapeutic interventions that target these mechanisms.

5. ACKNOWLEDGEMENTS

The authors would like to thank the collaborators of this study and the entire BioFINDER Study group (www.biofinder.se), including Susanna Vestberg for classifying the MCI-AD patients into MCI subgroups, Per Wollmer and Douglas Hägerström for help with [^{18}F]flutemetamol PET imaging. Work in the authors' laboratory was supported by the European Research Council, the Swedish Research Council, the Strategic Research Area MultiPark (Multidisciplinary Research in Parkinson's disease) at Lund University, the Crafoord Foundation, the Swedish Brain Foundation, the Swedish Alzheimer Foundation, the Torsten Söderberg Foundation, Skåne Research Hospital research funds, Greta and Johan Kock Foundation, Koch's Foundation and the Swedish federal government under the ALF agreement.

6. AUTHOR DISCLOSURES

Drs Janelidze, Hertze, Nägga, Nilsson K., Nilsson C., Wennström, Van Westen and Hansson report no disclosures. Drs. Blennow and Zetterberg are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg. Dr.

Blennow has served at advisory boards or as a consultant for Eli-Lilly, Fujirebio Europe, Novartis, and Roche Diagnostics.

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8. FIGURE LEGENDS

Figure 1. The Qalb in different diagnostic groups and *APOE* genotypes in cohort 1.

(A) The Qalb in cognitively healthy controls and patients with stable mild cognitive impairment (sMCI), MCI that progressed to AD (MCI-AD), Alzheimer's disease (AD), dementia with Lewy bodies or Parkinson's disease with dementia (DLB/PDD), vascular dementia (VaD) and frontotemporal dementia (FTD). **(B**) The Qalb in different diagnostic groups stratified according to *APOE* genotype (*APOE ε4* carriers vs. non-carriers). Data are presented as mean±95% confidence interval; p values are from univariate general linear models controlling for age and gender; *p < 0.05; **p < 0.01; compared with controls. Abbreviations: Qalb, CSF/plasma albumin ratio.

Figure 2. CSF biomarkers of angiogenesis or endothelial damage in cohort 1.

CSF VEGF **(A)**, the VEGF/VEGF-R1 ratio **(B)**, ICAM-1 **(C)** and VCAM-1 **(D)** were measured in cognitively healthy controls and patients with stable mild cognitive impairment (sMCI), MCI that progressed to AD (MCI-AD), Alzheimer's disease (AD), dementia with Lewy bodies or Parkinson's disease with dementia (DLB/PDD), vascular dementia (VaD) and frontotemporal dementia (FTD). Data are presented as mean±95% confidence interval; p values are from univariate general linear models controlling for age and gender; *p < 0.05; **p < 0.01; ***p < 0.001 compared with controls. Abbreviations: ICAM-1, soluble Intercellular Adhesion Molecule 1; VEGF, Vascular Endothelial Growth Factor; VEGF-R1, Vascular Endothelial Growth Factor Receptor 1; VCAM-1, soluble Vascular Cell Adhesion Molecule 1.

Figure 3. The Qalb and CSF biomarkers of angiogenesis or endothelial damage in diabetes.

The Qalb in patients with and without diabetes in cohort 1 **(A)** and cohort 2 **(B**). CSF levels of ICAM-1 **(C)**, VCAM-1 **(D)** and VEGF **(E)** in patients with and without diabetes in cohort 1. Data are presented as mean±95% confidence interval; p values are from univariate general linear models controlling for age, gender and diagnosis; *p < 0.05; **p < 0.01; ***p < 0.001. Abbreviations: ICAM-1, soluble Intercellular Adhesion Molecule 1; Qalb, CSF/plasma albumin ratio; VEGF, Vascular Endothelial Growth Factor; VCAM-1, soluble Vascular Cell Adhesion Molecule 1.

9. TABLES

Table 1. Cohort 1, demographic data, clinical characteristics and CSF levels of biomarkers.

	Control **n=65**	**sMCI** **n=61**	**MCI-AD** **n=35**	**AD** **n=75**	**DLB/PDD** **n=34**	**VaD** **n=28**	**FTD** **n=41**
Sex F/M	42 / 23	34 / 27	23 / 12	51 / 24	13 / 21[a]	13 / 15	21 / 20
Age	75 (6)	69 (7)[d]	75 (8)	76 (7)	72 (6)	75 (8)	72 (6)[a]
MMSE	28.7 (1.7)	28.2 (1.2)	26.4 (1.7)[c]	19.5 (3.3)[c]	21.4 (5.1)[c]	21.5 (4.5)[c]	22.8 (6.1)[c]
APOE *1or 2 ε4 alleles*	34%	48%	80%[c]	65%[c]	55%[a]	21%	28%*
BMI	25.9 (3.7)	25.1 (3.2)	24.3 (4.2)	23.7 (3.6)	25.6 (3.4)	26.5 (5.8)	25.4 (4.1)
Qalb	6.3 (2.9)	6.3 (2.5)	7.1 (3.3)	7.6 (3.4)[b]	8.4 (4.6)[a]	8.7 (3.7)[b]	8.6 (5.5)[b]
Diabetes, yes/no	4 / 61	3 / 58	1 / 34	9 / 66	2 / 32	7 / 21	0 / 32

ICAM-1, *ng/ml*	2.0 (0.7)	1.9 (0.7)	2.1 (0.7)	2.2 (0.7)	2.0 (0.8)	2.1 (0.7)	2.0 (1.0)
VCAM-1, *ng/ml*	5.4 (1.4)	4.9 (1.5)	5.4 (1.6)	5.7 (1.6)	5.3 (1.4)	5.5 (1.5)	4.9 (1.6)
VEGF, *pg/ml*	58.0 (19.1)	65.0 (32.0)[a]	70.9 (21.8)[b]	73.2 (27.7)[b]	67.0 (29.0)	80.8 (33.5)[b]	74.6 (31.6)[b]
sVEGFR-1, *pg/ml*	149.2 (55.0)	111.4 (39.7)[c]	128.9 (44.7)[a]	130.3 (41.1)[b]	103.3 (37.4)[c]	100.3 (30.3)[c]	118.0 (38.7)[b]
VEGF/sVEFGR-1	0.4 (0.2)	0.6 (0.3)	0.6 (0.2)[c]	0.6 (0.2)[c]	0.7 (0.4)[c]	0.9 (0.4)[c]	0.7 (0.3)[c]
Aβ42, pg/ml	675.2 (289.8)	478.9 (195.0)	314.5 (78.9)	259.7 (105.0)	349.6 (172.7)	407.4 (187.0)	682.9 (290.5)
Aβ40, pg/ml	5241 (1487)	3786 (1360)	4219 (1327)	3899 (1376)	3240 (1200)	3218 (1345)	4530 (1536)

AD, Alzheimer's disease; Alb, albumin; BMI, body mass index; DLB/PDD, dementia with Lewy bodies or Parkinson's disease with dementia; F, female; FTD, frontotemporal dementia; ICAM-1, soluble Intercellular Adhesion Molecule 1; M, male; sMCI, mild cognitive impairment; MCI-AD,

MCI that progressed to AD; MMSE, Mini Mental State Examinations; Qalb, CSF/plasma albumin ratio; VEGF, Vascular Endothelial Growth Factor; VEGF-R1, Vascular Endothelial Growth Factor Receptor 1; VCAM-1, soluble Vascular Cell Adhesion Molecule 1; VaD, vascular dementia.

* *APOE* data was only available from 18 FTD patients.

Data are shown as mean (SD) unless otherwise specified. Demographic factors and clinical characteristics were compared using one-way ANOVA and chi-square tests. CSF biomarkers and the Qalb were analyzed with univariate general linear models controlling for age and gender; compared with controls [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$.

Table 2. Cohort 2, demographic and clinical data.

	Controls n=292	SCD n=171	MCI n=213
Gender F/M	176 / 116	94 / 77	94 / 119 [c]
Age, *years*	73 (5)	70 (6) [c]	71 (6) [c]
MMSE	29.1 (0.9)	28.4 (1.4) [c]	27.0 (1.9) [c]
APOE *1or 2 ε4 alleles*	29%	38% [a]	49% [c]
Qalb	5.9 (2.2)	6.0 (2.4)	6.5 (4.0)
Composite SUVR*	1.3 (0.3)	1.4 (0.4)	1.7 (0.5)
BMI	26.5 (4.3)	25.1 (3.6)	25.4 (4.2)
Homocysteine, µmol/L	13.5±4.1	12.6±3.9	13.5±4.1
Diabetes, yes / no	26 / 266	17 / 152	20 / 187
Hyperlipidemia, yes / no	124 / 168	22 / 147	19 / 188
Aβ42, pg/ml	561.9 (199.3)	589.6 (254.6)	470.5 (233.0)

Aβ40, pg/ml	4766 (1753)	4925 (1751)	4760 (1873)

* Normalized for the cerebellar cortex; amyloid PET data was available from 342 subjects (129 cognitively normal elderly, 102 SCD patients and 111 MCI patients).

BMI, body mass index; F, female; M, male; MCI, mild cognitive impairment; MMSE, Mini Mental State Examinations; N/A, not available; Qalb, CSF/plasma albumin ratio; SCD, subjective cognitive decline; SUVR, standardized uptake value ratio.

Data are shown as mean (SD) unless otherwise specified. Demographic factors and clinical characteristics were compared one-way ANOVA and chi-square tests. Qalb was analyzed with univariate general linear models controlling for age and gender; compared with controls [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$.

Table 3. The Qalb in individuals with normal (CSF Aβ42/Aβ40 ratio≥0.1) and pathological (CSF Aβ42/Aβ40 ratio<0.1) CSF signature.

	Controls-N n=214	Control-P n=77	SCD-P n=57	MCI-P n=121
Gender F/M	124 / 90	52 / 25	26 / 31	60 / 61
Age, *years*	72 (5)	74 (5) [b]	71 (5)	72 (5)
MMSE	29.2 (0.9)	29.0 (0.9)	28.1 (1.5) [c]	26.8 (1.8) [c]
APOE *1or 2 ε4 alleles*	20%	53% [c]	67% [c]	68% [c]
Qalb	6.0 (2.1)	5.8 (2.4)	5.7 (2.2)	6.6 (4.8)

Alb, albumin; F, female; M, male; MCI, mild cognitive impairment; MMSE, Mini Mental State Examinations; N, normal CSF signature; P, pathological CSF signature; SCD, subjective cognitive decline.

Data are shown as mean (SD) unless otherwise specified. Demographic factors and clinical characteristics were compared using one-way ANOVA and chi-square tests. Qalb was analyzed with univariate general linear models controlling for age and gender; compared with controls [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$.

Table 4. Cohort 1, associations between the Qalb and CSF biomarkers of angiogenesis or endothelial damage.

	Control	sMCI	MCI-AD	AD	DLB/PDD	VaD	FTD
ICAM-1	**β=0.637*** **	**β=0.733*** **	**β=0.776*** **	**β=0.650*** **	**β=0.756*** **	**β=0.729*** **	**β=0.833*** **
VCAM-1	**β=0.411** **	**β=0.470*** **	**β=0.620*** **	**β=0.512*** **	**β=0.431***	**β=0.627** **	**β=0.679*** **
VEGF	**β=0.402** **	**β=0.482*** **	**β=0.685*** **	**β=0.458*** **	**β=0.719*** **	β=0.240	**β=0.346***
sVEGFR-1	**β=0.240***	β=-0.021	**β=0.365***	β=0.092	β=-0.054	β=0.220	β=-0.075
VEGF/sVEFGR-1	β=0.060	**β=0.358** **	β=0.235	**β=0.370** **	**β=0.518** **	β=0.013	**β=0.305***

ICAM-1, soluble Intercellular Adhesion Molecule 1; Qalb, CSF/plasma albumin ratio; VEGF, Vascular Endothelial Growth Factor; VCAM-1, soluble Vascular Cell Adhesion Molecule 1.

β, standardized coefficient; p-values are derived from linear regression controlling for age and gender; significant results are shown in bold; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure 1

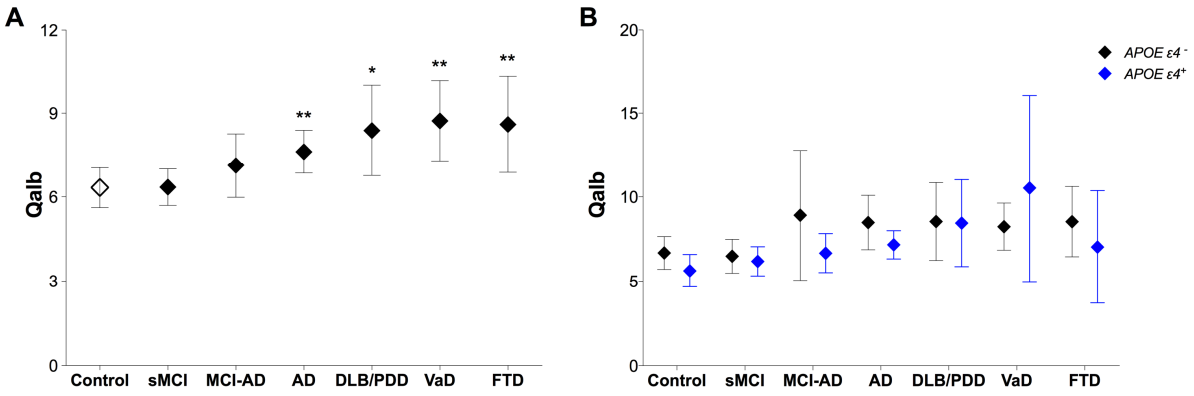


Figure 2

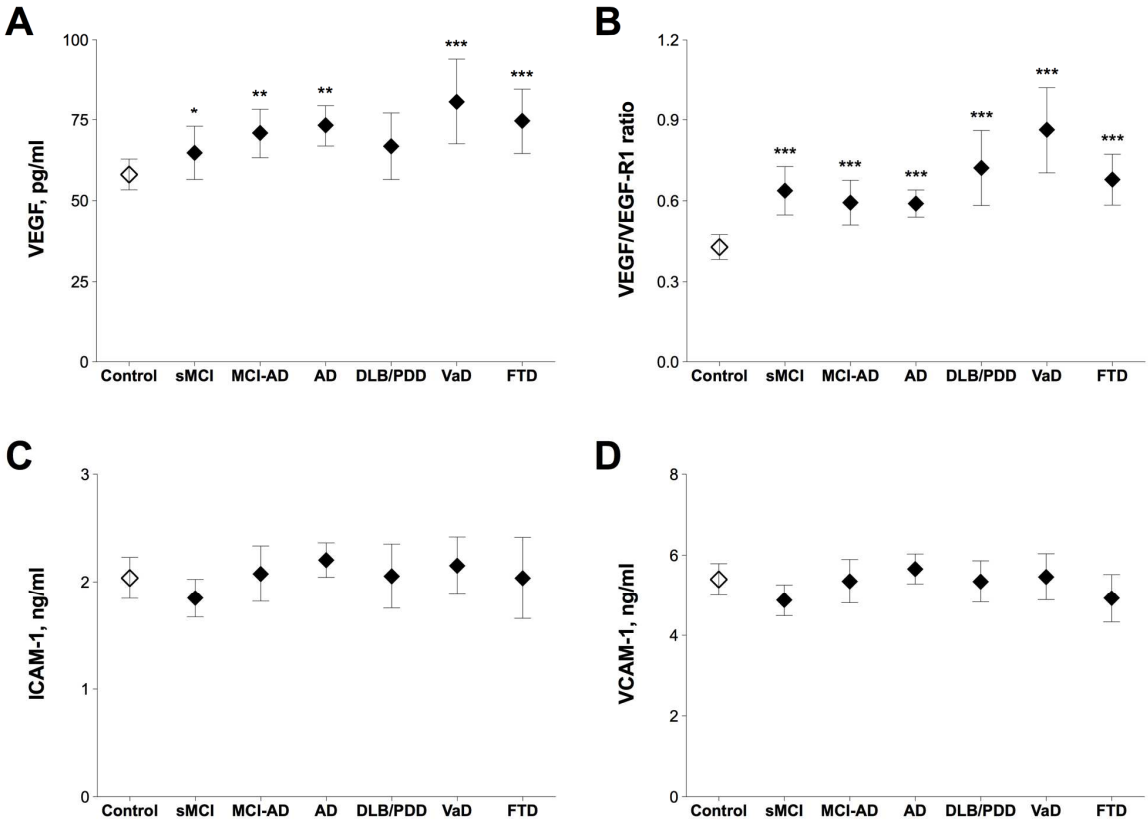
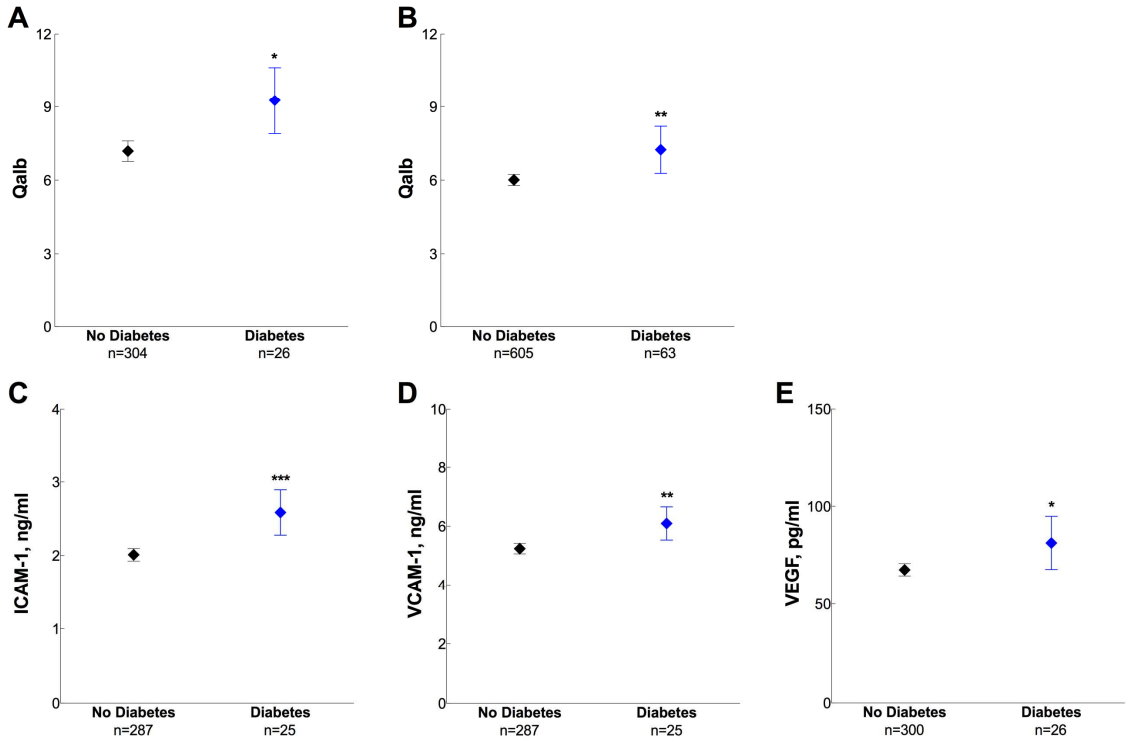


Figure 3



Increased blood-brain barrier permeability is associated with dementia and diabetes, but not amyloid pathology or *APOE* genotype

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HIGHLIGHTS

- BBB permeability is increased in major dementia disorders.
- BBB dysfunction is not directly related to amyloid pathology or *APOE* genotype.
- BBB dysfunction is associated with diabetes mellitus and brain microvascular damage.

VERIFICATION

1. Author disclosures

Drs Janelidze, Hertze, Nägga, Nilsson K., Nilsson C., Wennström, van Westen and Hansson report no disclosures. Drs. Blennow and Zetterberg are co-founders of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg. Dr. Blennow has served at advisory boards or as a consultant for Eli-Lilly, Fujirebio Europe, Novartis, and Roche Diagnostics.

2. Financial support

Work in the authors' laboratory was supported by the European Research Council, the Swedish Research Council, the Strategic Research Area MultiPark (Multidisciplinary Research in Parkinson's disease) at Lund University, the Crafoord Foundation, the Swedish Brain Foundation, the Swedish Alzheimer Foundation, the Torsten Söderberg Foundation, Skåne Research Hospital research funds, Greta and Johan Kock Foundation, Koch's Foundation and the Swedish federal government under the ALF agreement.

3. Manuscript submission

The data contained in the manuscript being submitted have not been previously published, have not been submitted elsewhere and will not be submitted elsewhere while under consideration at Neurobiology of Aging.

4. Ethical approval

The study was approved by the Regional Ethics Committee in Lund, Sweden, and the patients and controls gave their informed consent for research.