

# Peripheral Markers of Neurovascular Unit Integrity and Amyloid- $\beta$ in the Brains of Menopausal Women

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## Abstract.

**Background:** The identification of blood-borne biomarkers for the diagnosis and prognosis of Alzheimer's disease and related dementias is more feasible at the population level than obtaining cerebrospinal fluid or neuroimaging markers.

**Objective:** This study determined the association of blood microvesicles, derived from cells of the neurovascular unit, with brain amyloid- $\beta$  deposition in menopausal women.

**Methods:** A subset of women from the Kronos Early Estrogen Prevention Study underwent brain amyloid- $\beta$  positron emission tomography three years following cessation of study treatment with placebo (PL,  $n = 29$ ), transdermal 17 $\beta$ -estradiol (tE2;  $n = 21$ ), or oral conjugated equine estrogen (oCEE;  $n = 17$ ). Isolated peripheral venous blood microvesicles were analyzed by digital flow cytometry using fluorophore conjugated antibodies directed toward total tau, amyloid- $\beta$  1–42 (A $\beta$ <sub>1–42</sub>), neuron specific class III  $\beta$ -tubulin (Tuj1), microglia ionized calcium-binding adaptor molecule 1 (Iba1), glial fibrillary acid protein (GFAP), and low density lipoprotein receptor-related protein 1 (LRP1). Principal components analysis reduced the dimensionality of these selected six markers to two principal components (PCs). Proportional odds ordinal logistic regression analysis was used with amyloid- $\beta$  deposition regressed on these PCs.

**Results:** Only the number of microvesicles positive for A $\beta$ <sub>1–42</sub> differed statistically among prior treatment groups (median [IQR]: 6.06 [2.11, 12.55] in PL; 2.49 [0.73, 3.59] in tE2; and 4.96 [0.83, 10.31] in oCEE;  $p = 0.032$ ). The joint association between the 2 PCs and brain amyloid- $\beta$  deposition was significant ( $p = 0.045$ ).

**Conclusion:** Six selected markers expressing peripheral blood microvesicles derived from cells of the neurovascular unit, when summarized into two principal components, were associated with brain amyloid- $\beta$  deposition.

Keywords: Alzheimer's disease, 17 $\beta$ -estradiol, conjugated equine estrogen, extracellular vesicles, KEEPS, PET imaging

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## INTRODUCTION

The development of diagnostic and prognostic blood-borne biomarkers for Alzheimer's disease

(AD) pathology and dementia would be more feasible and more cost-effective at the population level compared to cerebrospinal fluid or neuroimaging measures. Studies suggest that it may now be possible to measure the hallmark characteristics of AD, amyloid- $\beta$  and tau, in the blood [1–8]. However, previous studies have not considered that the ability to measure these proteins in blood not only reflects changes in the activation or function of neurons and glial cells, but also may reflect changes in cerebral microvasculature of the blood-brain barrier. The concept of the neurovascular unit composed of cerebrovascular endothelial cells, glia, and neurons conveys the dynamic interaction among these cellular elements in affecting brain function and repair in relationship to cerebral ischemia, brain injury due to trauma or irradiation, and neurological diseases such as multiple sclerosis and AD [9–15]. An integrative approach which includes the collective analysis of blood-borne markers of both AD pathology and the neurovascular unit may provide insight into early changes in neurovascular cellular activation and function that may precede development of cognitive impairment.

Injured and activated neurovascular cells shed two distinct major populations of biologically-active extracellular vesicles into the blood: 1) Exosomes (shed from endosomes) and 2) Microvesicles (MV; shed from cell membranes). Both are involved in cell-cell communication and the specific exchange of bioactive molecules in pathophysiological processes. Several studies highlighted the importance of these vesicles and their bioactive molecules to identify and understand the early pathogenesis of AD, and the potential for their use as a diagnostic or prognostic biomarker [16–20]. The concentration and content of bioactive molecules of MV in body fluids depend upon their cellular origin and the pathophysiological processes that trigger their production. Thus, the absolute and relative population of MV and their content of bioactive molecules may reflect early or late pathophysiological processes in the brain [16–21]. Therefore, this study was designed to assess the ability to measure MV derived from cells of the neurovascular unit in peripheral blood as potential markers of amyloid- $\beta$  deposition in the brain. This analysis was performed on blood from a cohort of middle-aged, post-menopausal women who participated in the ancillary study of Kronos Early Estrogen Prevention Study (KEEPS) for measures of brain amyloid- $\beta$  by positron emission tomography [22].

## METHODS

### *Study participants*

Women enrolled in the KEEPS (NCT000154180) ancillary neuroimaging study at Mayo Clinic were eligible for this study. In brief, women were between 42–58 years old and within 6 months to 3 years past their last menses at the time of enrollment for the initial trial. Women were excluded if they had a coronary artery calcium score of  $>50$  Agatston Units (AU), smoked over 10 cigarettes per day, body mass index (BMI)  $>35$  kg/m<sup>2</sup>, a history of cardiovascular disease, LDL cholesterol  $>190$  mg/dl, triglycerides  $>400$  mg/dl, diagnosis of diabetes, uncontrolled hypertension (systolic blood pressure  $>150$  mmHg and/or diastolic blood pressure  $>95$  mm Hg) and current or recent (6 months) use of cholesterol lowering medications (statins, fibrate, or  $>500$  mg/day niacin). Women were randomized for four years to: oral conjugated equine estrogens (oCEE; Premarin, 0.45 mg/day); transdermal 17 $\beta$ -estradiol (tE2, Climera, 50  $\mu$ g/day; or placebo (PL) pills and patch. Progesterone was given orally (Prometrium; micronized progesterone, 200 mg/day) for the first 12 days each month to both active treatment groups [23]. The study was approved by the Mayo Clinic Institutional Review Board and all women gave written informed consent.

### *Neuroimaging*

Neuroimaging for the current study was conducted from December 2012 through July 2014 and included the subset of women enrolled in KEEPS at the Mayo Clinic Rochester, MN who agreed to continue the study three years after the end of the trial. Exclusion criteria for the imaging studies were contraindications for safety and neurologic disorders such as brain tumors, multiple sclerosis, neurodevelopmental abnormalities, or treatments (e.g., systemic chemotherapy) that would affect the brain structure.

Positron emission tomography (PET) with Pittsburgh compound-B (PiB) was acquired using a PET/CT scanner (DRX; GE Healthcare) operating in 3D mode three years after the end of hormone therapies. An average of 596 MBq (range, 292–729 MBq) <sup>11</sup>C-PiB was used. Following the injection of <sup>11</sup>C-PiB, a 40 min <sup>11</sup>C-PiB uptake period, a 20 min PiB scan consisting of four 5 min dynamic frames was obtained. In addition, MRI examinations were performed at 3 Tesla (GE Healthcare). A 3-D high

resolution magnetization prepared rapid gradient echo was obtained for anatomical segmentation and labeling. Standardized uptake value ratios (SUVr) were calculated from the PiB PET images as previously described [24]. Briefly, using an automated image processing pipeline, PET image volumes were registered to each subject's own 3-dimensional T1-weighted MRI using SPM12. Regional cortical uptake of PiB was determined using the Mayo Clinic Adult Lifespan Template (MCALT\_AD1R122) [25]. The global cortical PiB retention standardized uptake value ratio (SUVr) was obtained from the bilateral parietal (including posterior cingulate and precuneus), temporal, prefrontal, orbitofrontal, and anterior cingulate regions referenced to the cerebellar crus region.

### *Cognitive function*

A battery of neuropsychological tests was administered within three weeks of the neuroimaging examinations in the Research Psychometrics Resource Laboratory within the Division of Neurocognitive Disorders in the Department of Psychiatry and Psychology at Mayo Clinic. Cognitive performance was investigated in four domains: 1) learning and memory (California Verbal Learning Test, New York University Paragraphs, and Benton Visual Retention Test); 2) auditory attention and working memory (Wechsler Memory Scale-III Letter-Number Sequencing and Digit Span subtests); 3) visual attention and perceptual speed (Trail Making Test part A, Color and Word trials of the Stroop test, and Wechsler Adult Intelligence Scale-III Digit Symbol Coding subtest); and 4) speeded language/fluency (phonemic [F, A, S] and category [animals, fruits, vegetables]) and flexibility (Trail Making Test part B, and Color-Word Interference Trial of the Stroop). The individual test scores were summarized within general domains representing global cognitive function as previously described [26].

### *Blood collection and analysis*

Women were asked to refrain from aspirin two weeks prior to blood collection. Fasting venous blood was collected in tubes containing a mixture of thrombin inhibitor (1  $\mu$ M hirudin) plus coagulation factor Xa inhibitor (10  $\mu$ M soybean trypsin inhibitor) within a week of the brain imaging as previously described [27, 28]. Total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein

Table 1

Fluorophore conjugated antibodies used in the analyses of microvesicles in plasma collected from postmenopausal women

Markers used for the cells of neurovascular unit

Alzheimer Disease:

Total tau

Amyloid- $\beta$  1–42 ( $A\beta_{1-42}$ )

Cerebrovascular endothelial cells: Low density lipoprotein receptor-related protein 1 (LRP1), Amyloid- $\beta$  clearance receptor

Astrocytes: Glial fibrillary acidic protein (GFAP)

Microglia: Ionized calcium-binding adaptor molecule 1 (Iba1)

Neurons: Neuron specific class III beta-tubulin (Tuj-1)

cholesterol (HDL-C), triglycerides, blood glucose and 17 $\beta$ -estradiol were measured by the Mayo Clinic Department of Laboratory Medicine and Pathology (Rochester, MN). Apolipoprotein E (*APOE*) genotyping was performed after randomization [26].

Blood-borne microvesicles were isolated from platelet-free plasma by high-speed centrifugation (20,000 g for 30 min) and characterized by digital flow cytometer using fluorophore conjugated antibodies directed toward cells of the neurovascular unit (Table 1): total tau, amyloid- $\beta$  1–42 ( $A\beta_{1-42}$ ), neuron specific class III  $\beta$ -tubulin (Tuj1), microglia ionized calcium-binding adaptor molecule-1 (Iab-1), glial fibrillary acid protein (GFAP), and low density lipoprotein receptor-related protein1 (LRP1) as described previously [28].

### *Statistical analysis*

Continuous variables were described with medians and interquartile ranges (IQR). Differences among groups defined by previous hormone treatment were assessed using the proportional odds (PO) ordinal logistic model applied to the ranked versions of the continuous variables. Its two degree-of-freedom likelihood ratio test for any group difference provides a generalization of the Kruskal-Wallis test but also provides a more consistent framework for pairwise treatment comparisons, as derived by Wald tests on the individual parameter estimates. To limit multiple comparisons, pairwise group contrasts were performed only when the overall test of association for treatment was significant. The marginal relationship of each individual blood-borne biomarker with the PET SUVr measure of amyloid- $\beta$  deposition was examined using Spearman's rank correlation analyses. Because the sample size was insufficient to support a multivariable analysis of these relationships, data reduction was performed via principal

components (PC) analysis with the goal of reducing the dimensionality of the six brain cellular markers to the fewest number of PCs that could explain the majority of its variation. Multivariable PO regression analysis was then used to regress amyloid- $\beta$  load on the selected PCs. A joint test of the association of all PCs with amyloid- $\beta$  was constructed using a multiple degree-of-freedom likelihood ratio  $\chi^2$  test (LRT). Partial tests for association were reported on each individual PC only when the PCs were statistically significant as a group. The PC-based regression analysis was then repeated for secondary outcomes, including white matter hyperintensity, hippocampal volume, and global cognitive score.

## RESULTS

Amyloid PET imaging was available in 67 women from the follow-up KEEPS study conducted three years after completion of the trial; median (IQR) age at time of imaging was 60.4 (58.7, 61.7) years. Phenotypic and clinical characteristics of these women are shown in Table 2. As reported previously for the study sample that included one additional participant, these characteristics were mostly similar among the previous hormone treatment groups. The exceptions were increased presence of the *APOE*  $\epsilon 4$  variant and decreased levels of amyloid- $\beta$  load, after controlling for age, in women assigned to the tE2 group. [22]

The total number of amyloid- $\beta$  associated MV in peripheral blood was significantly different across treatment groups (overall  $p = 0.032$ ), with lower levels in women who were treated with tE2 compared with placebo ( $p = 0.009$ ; Table 3, Fig. 1). There were no differences in the concentration of blood-borne

MV from blood-brain barrier endothelium, neurons, and microglia across hormone treatment groups (Table 3, Fig. 1). Of the six biomarker measures, only the number of astrocyte-derived MV (GFAP) showed a nominally significant correlation with PiB SUV<sub>r</sub> in unadjusted analysis (Spearman  $r_s = 0.27$ ,  $p = 0.026$ ). Overall distributions of amyloid- $\beta$  load, as measured by PiB SUV<sub>r</sub>, and other imaging and cognitive

Table 2  
Baseline phenotypic and clinical characteristics of the study population

Characteristic	<i>N</i>	Total ( <i>n</i> = 67)
Age at follow-up study, y	67	60.4 (58.7, 61.8)
Time since menopause at follow-up study, y	67	8.4 (7.9, 9.2)
Education:	67	
High school or less		7 (10.4%)
Some college/technical/vocational school		27 (40.3%)
College graduate or higher		33 (49.3%)
Smoking status:	67	
Never		46 (68.7%)
Former		19 (28.4%)
Current		2 (3.0%)
Body mass index, kg/m <sup>2</sup>	67	27.4 (24.1, 31.4)
Waist circumference, cm	67	89.0 (80.5, 97.8)
Systolic blood pressure, mm Hg	67	127.3 (116.3, 135.3)
Diastolic blood pressure, mm Hg	67	77.3 (72.3, 82.0)
Fasting glucose, mg/dL	66	94.0 (90.0, 100.0)
Total cholesterol, mg/dL	66	204.5 (189.0, 227.0)
HDL (high density lipoprotein) cholesterol, mg/dL	66	62.0 (54.0, 73.0)
LDL (low density lipoprotein) cholesterol, mg/dL	66	120.6 (106.2, 139.2)
Triglycerides, mg/dL	66	92.5 (71.0, 115.0)
hs-C reactive protein, mg/dL	66	0.14 (0.07, 0.30)
<i>APOE</i> $\epsilon 4$ carrier, n (%)	67	18 (26.9%)

Results are reported as median (25th, 75th percentile), except for education, smoking status and *APOE*  $\epsilon 4$  which are reported as counts (percentages). *N* is the number of non-missing values.

Table 3  
Peripheral blood microvesicles positive for amyloid- $\beta$ , tau, and antigens for cells of neurovascular unit, by prior treatment group

Variable	Comparisons by Treatment			
	PL ( <i>n</i> = 29)	tE2 ( <i>n</i> = 21)	oCEE ( <i>n</i> = 17)	<i>p</i>
A $\beta$ <sub>1-42</sub>	6.06 (2.11, 12.55)	2.49 (0.73, 3.59)*	4.96 (0.83, 10.31)	<b>0.032</b>
Tau	0.42 (0.19, 0.77)	0.38 (0.20, 0.77)	0.66 (0.20, 1.56)	0.696
GFAP	4.87 (3.30, 7.30)	5.95 (4.83, 6.98)	5.54 (3.11, 7.78)	0.370
Tuj1	5.51 (3.75, 10.47)	6.46 (5.03, 11.94)	5.06 (4.07, 6.34)	0.186
LRP1	2.08 (1.60, 6.44)	3.36 (1.34, 6.05)	4.67 (2.88, 9.12)	0.292
Iba1	0.19 (0.10, 0.32)	0.13 (0.07, 0.20)	0.19 (0.10, 0.96)	0.474

Results are reported as median (25th, 75th percentile). Likelihood ratio tests from proportional odds ordinal logistic semiparametric models were used to identify any between-group differences. When significant, pairwise treatment comparisons were additionally assessed using Wald tests on individual parameter estimates. \*Pairwise treatment comparison versus placebo was statistically significant ( $p < 0.05$ ). PL, placebo; tE2, transdermal 17 $\beta$ -estradiol; CEE, oral conjugated equine estrogen; for definitions of the biomarker variables, refer to Table 1.

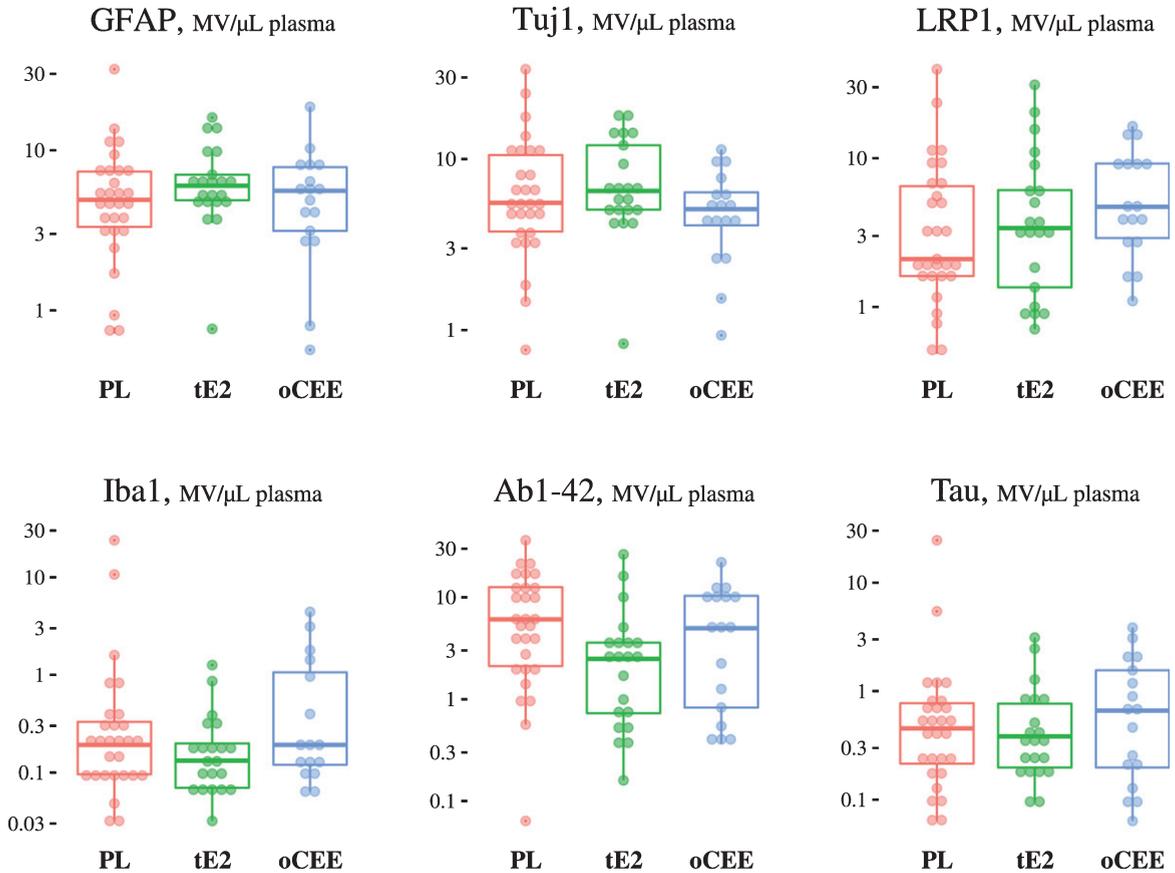


Fig. 1. Distribution of peripheral blood microvesicles positive for markers for cells of the neurovascular unit, and amyloid- $\beta$  and tau by treatment group.

measures are illustrated in Fig. 2. Cognitive assessments were available for 62 of the 67 women, of whom global cognitive scores were comparable across treatment groups (median [IQR]: 0.30 [−2.24, 1.45] in PL; 0.45 [−1.77, 2.29] in tE2 and −0.87 [−2.04, 1.76] in oCEE;  $p=0.908$ ).

Principal components (PC) analysis was used to reduce the dimensionality of the six biomarkers to two PCs: the first PC is a contrast between microglial-positive MV and an average of astrocyte- and neuron-derived MV, while the second PC is a weighted average of all brain cellular markers except astrocyte-derived MV (Table 4). Together these first two principal components explain 58% of the total variation in the six original biomarkers. In a regression analysis, the joint test for association of the two PCs with the level of PiB SUV<sub>r</sub> was statistically significant (Global  $p=0.045$ ; Table 5). Partial tests revealed significant association of the first PC ( $p=0.034$ ; Table 5), but not the second PC ( $p=0.143$ ; Table 5), with PiB SUV<sub>r</sub>. There

was no joint significant effect of the PCs on the secondary outcomes of white matter hyperintensities, hippocampal volume or global cognitive score (Table 5).

## DISCUSSION

This study provides proof of concept that MV-carrying surface amyloid- $\beta$  (a hallmark AD pathology) and MV-derived from cells of the neurovascular unit can be detected in peripheral blood. When analyzed separately, only MV derived from astrocytes was univariately associated with amyloid PET among these postmenopausal women. Collectively, after reducing the six biomarkers to two principal components, both the global and partial tests of these principal components were marginally associated with PiB SUV<sub>r</sub>, suggesting a potential relationship. Although the association of astrocyte-derived MV with amyloid PET was relatively weak ( $r^s=0.27$ ), their contribution to the weighted averages in the PC

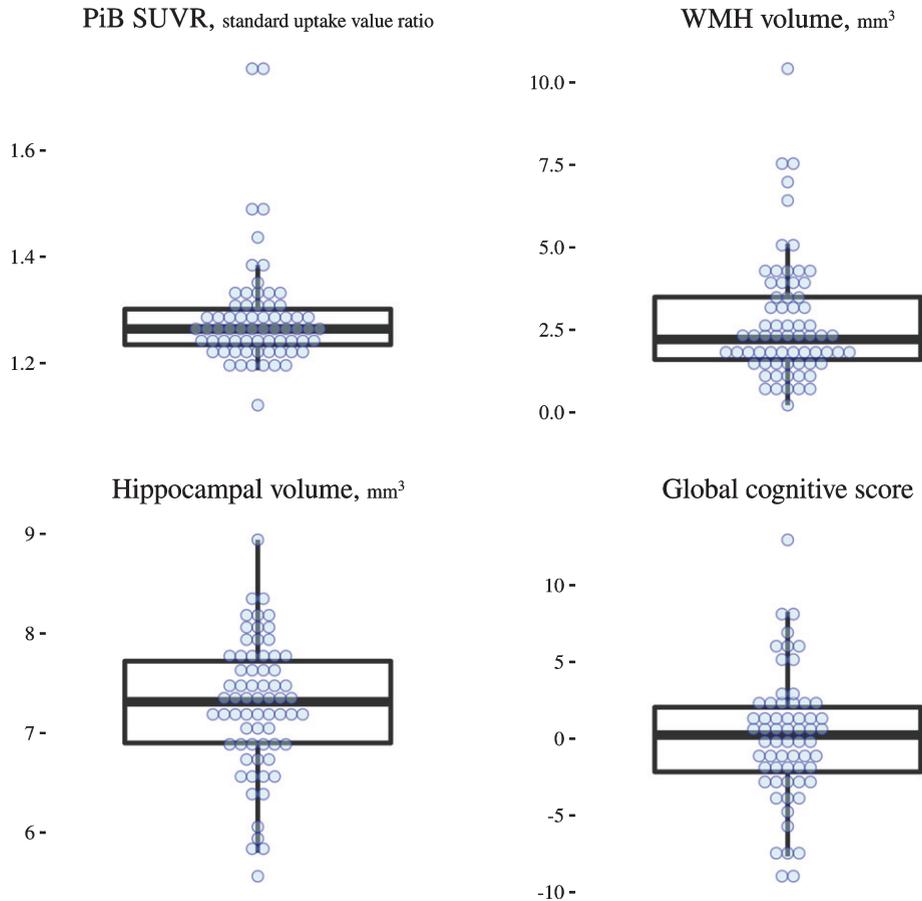


Fig. 2. Data distribution of amyloid- $\beta$  load, white matter hyperintensity, hippocampal volume, and global cognitive score.

Table 4

Principal components of the six markers of the neurovascular unit scoring of individual PCs

Microvesicle Measure	PC#1	PC#2
Astrocyte-derived (GFAP)	0.68	0.07
Neuron-derived (Tuj1)	0.59	0.41
Amyloid- $\beta$ clearance receptor (LRP1)	-0.08	0.42
Microglia-derived (Iba-1)	-0.40	0.41
Amyloid- $\beta$ positive ( $A\beta_{1-42}$ )	-0.14	0.26
Tau positive (tau)	-0.08	0.65
Proportion	<b>31.8%</b>	<b>26.0%</b>
Cumulative	<b>31.8%</b>	<b>57.8%</b>

Principal components analysis was used to reduce the dimensionality of the 6 brain markers to 2 principal components (PC). Based on the loadings above, the first PC is basically a contrast between amyloid- $\beta$  positive and an average of astrocyte- and neuron-derived, while the second PC is a weighted average of all brain markers except astrocyte-derived MV. Together these first 2 PCs explain 58% of the variation in the original 6 markers.

analysis supports their contribution in the neurovascular unit to the overall relationship with amyloid- $\beta$ . It is possible that changes in the neurovascular

unit represent early pathological processes associated with amyloid- $\beta$  pathology of AD that may precede functional changes in cognitively unimpaired women. The global association of the markers of the neurovascular unit with amyloid- $\beta$  on PET load supports the functional contribution of the cells of the neurovascular unit to development of AD pathology.

The detection of markers of activated astroglia and neurons by way of shed MV into peripheral blood is consistent with the “two-hit vascular hypothesis” of AD that proposes that brain microvascular damage is an initial insult through which dysfunction of the blood-brain barrier or hypoperfusion of the brain leads to secondary neuronal injury and primes the brain for accumulation of amyloid- $\beta$  [29]. Indeed, neurovascular injury can initiate a cascade of molecular and cellular events leading to neurodegeneration, cognitive impairment, and AD dementia [30]. The vascular remodeling and inflammatory responses of astrocytes and microglia to the leakage through the blood-brain barrier of neurotoxic

Table 5  
Joint and partial effects of the 2 principal components on amyloid- $\beta$  load

Dependent Variable <sup>‡</sup>	Independent Variable	Test of Association	
Amyloid- $\beta$ load	PCs #1-2	Global	$\chi^2 = 6.2, 2 \text{ df}, p = 0.045$
	PC #1	Partial	$\chi^2 = 4.5, 1 \text{ df}, p = 0.034$
	PC #2	Partial	$\chi^2 = 2.1, 1 \text{ df}, p = 0.143$
White matter hyperintensities	PCs #1-2	Global	$\chi^2 = 1.6, 2 \text{ df}, p = 0.443$
Hippocampal volume	PCs #1-2	Global	$\chi^2 = 3.0, 2 \text{ df}, p = 0.218$
Global cognition score	PCs #1-2	Global	$\chi^2 = 0.5, 2 \text{ df}, p = 0.785$

<sup>‡</sup>Proportional odds ordinal logistic regression was used to separately predict each dependent variable using the 2 principal components. The global test of association for both PCs jointly are from a likelihood ratio  $\chi^2$  test with 2 degrees of freedom (df), while partial tests of association for each PC (reported only when global test was significant) are from Wald  $\chi^2$  tests with 1 df.

proteins could promote neuronal injury by forming or exacerbating neurofibrillary tangles, white matter damage, decreased dendritic spine density, and amyloid- $\beta$  accumulation [29]. This vascular remodeling seems to be specific for amyloid- $\beta$  accumulation, as other components of the neurovascular unit did not associate with white matter hyperintensities, or hippocampal volumes, and seems to precede changes in cognition, as the association of the PC with cognitive tests scores was not significant.

While promising, confirmation of these results using a larger and more diverse cohort or in a longitudinal assessment of women of the KEEPS will be required in order to develop these markers into a simple blood test to identify persons at risk for amyloid- $\beta$  deposition in the brain. The methodology to isolate MV used in this study has been standardized and validated in our laboratory [28]. However, consensus for collection techniques to reduce the impact of preclinical variables for larger studies of diverse groups of men and women is needed [20, 31]. To correct the current data set for other phenotypic variables of participants would not provide useful information as multivariable models require the sample size to be sufficiently large to support the number of predictor variables with accurate and reliable estimates. Furthermore, to understand the contribution of various other phenotypic characteristics on development of MV and the rate of accumulation of amyloid- $\beta$  requires longitudinal assessments as previously noted, for example, the association of thrombogenic MV associated with development of white matter hyperintensities over a period of four years [32]. Unfortunately, assessments of MV derived from the neurovascular unit were not part of the data set collected at baseline in KEEPS. Other future studies could consider other markers of capillary integrity such as mural cells, including pericytes and vascular smooth muscle cells.

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