

# Increased plasma bradykinin level is associated with cognitive impairment in Alzheimer's patients

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## ABSTRACT

Alzheimer's disease (AD) is characterized by the presence of proteinaceous brain deposits, brain atrophy, vascular dysfunction, and chronic inflammation. Along with cerebral inflammation, peripheral inflammation is also evident in many AD patients. Bradykinin, a proinflammatory plasma peptide, is also linked to AD pathology. For example, bradykinin infusion into the hippocampus causes learning and memory deficits in rats, and blockade of the bradykinin receptor lessens cognitive impairment in AD mouse models. Even though it has been hypothesized that plasma bradykinin could contribute to inflammation in AD, the level of plasma bradykinin and its association with beta-amyloid (A $\beta$ ) pathology in AD patients had not been explored. Here, we assessed plasma bradykinin levels in AD patients and age-matched non-demented (ND) control individuals. We found significantly elevated plasma bradykinin levels in AD patients compared to ND subjects. Additionally, changes in plasma bradykinin levels were more profound in many AD patients with severe cognitive impairment, suggesting that peripheral bradykinin could play a role in dementia most likely via inflammation. Bradykinin levels in the cerebrospinal fluid (CSF) were reduced in AD patients and exhibited an inverse correlation with the CSF A $\beta$ 40/A $\beta$ 42 ratio. We also report that bradykinin interacts with the fibrillar form of A $\beta$  and co-localizes with A $\beta$  plaques in the post-mortem human AD brain. These findings connect the peripheral inflammatory pathway to cerebral abnormalities and identify a novel mechanism of inflammatory pathology in AD.

## 1. Introduction

Alzheimer's disease (AD), a severe neurodegenerative disorder, is characterized by progressive loss of cognitive function (Qiu et al., 2009). The mechanisms causing neuronal dysfunction in AD are still not clear, but there is a growing understanding that vascular factors play a crucial role (Strickland, 2018; Sweeney et al., 2019). For example, perturbations in the circulatory system, including the clotting and fibrinolytic pathways, are present in most AD patients and mouse model (Cortes-Canteli et al., 2015; Cortes-Canteli et al., 2012; Cruz Hernandez et al., 2019; de la Torre, 2004; Humpel, 2011; Suidan et al., 2018). Moreover, studies have shown that deposition of fibrin, the main protein component of blood clots, plays a significant role in AD progression in mice (Akassoglou and Strickland, 2002; Cortes-Canteli et al., 2015; Cortes-Canteli et al., 2010; Cortes-Canteli et al., 2012; Davalos and Akassoglou, 2012). Fibrin is a proinflammatory molecule (Davalos and Akassoglou, 2012; Flick et al., 2007), and its deposits in the AD brain can lead to chronic inflammation (Cortes-Canteli et al., 2015).

Dysregulation of the contact activation system and increased plasma

kallikrein have been proposed as inflammatory contributors in AD pathophysiology (Strickland, 2018; Viel and Buck, 2011; Zamolodchikov et al., 2015). Triggering the contact activation system releases bradykinin upon cleavage of high molecular weight kininogen (HK) (Long et al., 2016). Bradykinin and its metabolites, known for their proinflammatory and vasoactive properties (Golias et al., 2007), exert their effects through activation of G-coupled receptors, namely kinin B1 (B1R) and kinin B2 (B2R) (Golias et al., 2007). It has been shown that bradykinin infusion into the hippocampus causes learning and memory deficits in rats (Wang and Wang, 2002). Additionally, pharmacological and genetic blockade of B1R minimizes the beta-amyloid (A $\beta$ )-induced cognitive impairment in AD mice (Prediger et al., 2008). Likewise, B1R blockade protects mice from focal brain injury by reducing inflammation and blood brain barrier (BBB) disruption (Raslan et al., 2010), an abnormality observed in AD (Sweeney et al., 2018). B1R is also upregulated in the hippocampus and in reactive astrocytes surrounding A $\beta$  plaques in the AD mouse brain (Lacoste et al., 2013), and bradykinin levels are increased in the cerebrospinal fluid (CSF) of mice after cerebral injection of A $\beta$  (Iores-Marcas et al., 2006). Bradykinin may also be

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involved in the secretory processing of amyloid precursor protein (APP), the parent molecule of A $\beta$  (Racchi et al., 1998). All these studies suggest that bradykinin could be directly or indirectly responsible for inflammation, as well as other pathologies, in AD. However, this conclusion is based on the results obtained from animal model studies rather than from AD patients. A recent study showed that the contact activation system, is dysregulated in AD patients (Strickland, 2018), yet the plasma bradykinin changes in AD patients have not been reported. Also, whether the bradykinin levels affect the A $\beta$ -induced cognitive impairment in AD patients is not understood. To explore this hypothesis, we analyzed plasma samples from AD patients and age-matched non-demented (ND) individuals. We found that bradykinin levels were indeed significantly higher in AD plasma compared to that of ND cases. Furthermore, plasma bradykinin levels were more abundant in AD patients with severe cognitive impairment, suggesting that plasma bradykinin could contribute to dementia most likely via the inflammatory arm of contact activation system.

We also analyzed the CSF from AD patients and ND controls to determine CSF bradykinin levels. We found that AD CSF bradykinin level correlated with CSF A $\beta$ 40/A $\beta$ 42 ratio, an established biomarker of AD (Anoop et al., 2010). Using NMR spectroscopy and immunohistochemistry, we also examined the interaction of bradykinin with different species of A $\beta$ 42 and the association of bradykinin to A $\beta$  plaques.

We believe that increased plasma bradykinin could enhance cerebral inflammation and contribute to BBB damage observed in AD, all of which could significantly impact the cognitive status of AD patients. Inflammation is now considered a central mechanism in AD, and this study strengthens the connection of the peripheral inflammatory pathway to cerebral inflammatory pathology in AD patients.

## 2. Materials and methods

### 2.1. Human plasma and CSF

Experiments using de-identified human tissue, plasma, and CSF were reviewed and approved by The Rockefeller Institutional Review Board. Plasma and CSF from AD patients and ND controls were purchased from a biobank (PrecisionMed Inc., San Diego, CA) (Suidan et al., 2018). The biobank had obtained medical records for each volunteer, and donors underwent MRI and neurological screening following the criteria established by National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) to classify patients as AD or ND. Table 1 lists characteristics of donors, including scores from mini mental state examinations (MMSE).

### 2.2. Post-mortem AD brain tissue

Human post-mortem brain tissue was obtained from the Harvard Brain Tissue Resource Center. Upon autopsy, the AD cases were classified as Braak stage 5. Blocks of frozen tissue from the superior frontal cortex (3 AD and 3 ND) were sliced by cryostat into 10  $\mu$ m-thick sections and were used for immunofluorescence analysis.

### 2.3. Plasma and CSF bradykinin ELISA

CSF and plasma bradykinin levels were quantified using the ELISA kit (ADI-900-206; Enzo Life Sciences), which detects bradykinin (100%) and Lys-Bradykinin (100%). Cross-reactivity for Les-Des-Arg9-Bradykinin and Bradykinin 1–5 (BK1–5) is negligible (< 1% and < 0.1%, respectively). The ELISA was performed as per manufacturer's instructions. CSF samples (50  $\mu$ l) were diluted in assay buffer (50  $\mu$ l). Plasma samples were normally diluted 5–10-fold in assay buffer for ELISA. Some plasma samples, which showed very high bradykinin level were repeated with higher dilution in assay buffer (30–60-fold). All

**Table 1**  
Characteristics of ND and AD individuals.

Characteristics	ND	AD	p value
<b>Plasma studies</b>			
Individuals, n	32	39	
Ethnicity	Caucasian	Caucasian	
Age (year) at blood draw, mean (SD)	65.25 (4.2)	66.82 (6.0)	p = .22
Disease duration (year), mean (SD)	–	2.81 (2.42)	
Female, n	17	19	
Male, n	15	20	
MMSE score, mean (SD)	29.75 (0.43)	20.8 (3.86)	p < .0001
Vascular risk factor (VRF) status, n	10	27	
<b>CSF studies</b>			
Individuals, n	14	25	
Ethnicity	Caucasian	Caucasian	
Age (year) at CSF draw, mean (SD)	65.64 (5.3)	67.48 (6.3)	p = .36
Disease duration (year), mean (SD)	–	2.84 (2.13)	
Female, n	7	13	
Male, n	7	12	
MMSE score, mean (SD)	29.7 (0.42)	21.2 (3.8)	p < .0001
Vascular risk factor (VRF) status, n	5	16	
APOE status, E4 carrier, n	3	17	
CSF A $\beta$ 40/42 ratio, mean (SD)	12.6 (5.8)	21.7 (7.6)	p = .0005

plasma samples were analyzed in duplicate in ELISA. For the A $\beta$ 42-induced plasma bradykinin release study, blood was drawn from healthy volunteers and plasma was prepared as described in (Yamamoto-Imoto et al., 2018). The plasma samples were incubated with and without 500 nM A $\beta$ 42 oligomers at 37 °C for 2 h prior to performing the ELISA.

### 2.4. A $\beta$ expression, purification, and fibril formation for NMR spectroscopy

For NMR spectroscopy, A $\beta$  was expressed and purified (Finder et al., 2010; Walti et al., 2016). Final A $\beta$  peptide sequence contains the true 42 amino acid residues without containing methionine at the N-terminus. For the monomeric A $\beta$  preparation, the lyophilized peptide was resuspended in phosphate buffer and dissolved by adding sodium hydroxide. The peptide solution was then sonicated on ice, centrifuged and filtered through a 0.22  $\mu$ m syringe filter. Final pH of the peptide solution was adjusted to 7.4. For fibrillar A $\beta$ , monomeric peptide was incubated at a concentration of 100  $\mu$ M at 37 °C with agitation for 3 days. The fibrils formed were centrifuged, and 10% of the fibrils were used as a seed for the 2nd round of fibril generation. Three rounds of seeding were carried out in total for homogeneous fibril formation.

### 2.5. Two-dimensional nuclear magnetic resonance (2D NMR) spectroscopy

The heteronuclear single quantum correlation (HSQC) (Walti et al., 2016) spectra of <sup>15</sup>N-A $\beta$ 42 (25  $\mu$ M) in the presence and absence of equimolar concentration of bradykinin were recorded on a Bruker 700 MHz Avance III HD spectrometer with a cryogenic probe. The number of scans measured was 128 with 256 data point in the indirect dimension for each experiment to achieve a satisfactory signal-to-noise ratio. HSQC experiment was performed in phosphate buffer, pH 7.4 containing 3% D<sub>2</sub>O. The temperature was set to 278 K to prevent possible aggregation of A $\beta$ 42 during the course of the measurement time. All NMR spectra were processed with TopSpin 3.2 (Bruker) and analyzed with Sparky.

### 2.6. Saturation transfer difference (STD)-NMR experiment

The STD experiment is performed in molar excess of ligand molecules (Mayer and Meyer, 2001). A $\beta$ 42 fibrils (25  $\mu$ M) were mixed and incubated with bradykinin (1.25 mM) at room temperature (RT). The reaction mixture was transferred to a 3 mm NMR tube, and the STD experiment was carried out to ascertain the bradykinin-A $\beta$  fibril

interaction. STD experiments were performed on a Bruker 700 MHz spectrometer. The interscan delay was set to 1.5 s with an additional 3 s of irradiation at  $-1$  ppm and at 60 ppm with a train of 50 ms Gaussian pulses at 80 Hz power. The experiments were accumulated over 512 scans and processed in topspin with a quadratic sinus.

## 2.7. Immunofluorescence

Frozen human AD and ND brain sections (10  $\mu$ m) were fixed in 4% paraformaldehyde and rinsed in PBS. Sections were blocked in PBS with 5% BSA, 0.01% triton-X100, rinsed in PBS, and incubated with a rabbit monoclonal anti-bradykinin (1:100) antibody (Biorbyt, orb184822). After overnight incubation, the sections were rinsed in PBS and incubated for 1 h at RT with donkey anti-rabbit Alexa Fluor 488 (Covance) (1:1000). The sections were also stained with Congo Red as described previously (Cortes-Canteli et al., 2015). The tissue was incubated with 0.3% Sudan Black B in 70% ethanol to block lipofuscin autofluorescence (Cortes-Canteli et al., 2015). Imaging was performed using Nikon inverted fluorescence microscope (Eclipse Ti2). Images (20 $\times$ ) were processed using NIH image J software.

## 2.8. Angiotensin converting enzyme (ACE) activity assay

The ACE activity in plasma and CSF samples was measured using ACE activity assay kit (CS0002, Sigma-Aldrich). The assay was performed as per manufacturer's instruction. Briefly, for plasma ACE activity, 10  $\mu$ l plasma was diluted in 40  $\mu$ l assay buffer and 50  $\mu$ l substrate was added. For CSF ACE activity, 20  $\mu$ l CSF was diluted with 30  $\mu$ l assay buffer and 50  $\mu$ l substrate was added in 96-well fluorescence plate. The fluorescence emission was recorded at 405 nm (excitation at 320 nm) using a plate reader (Molecular Devices) at 37  $^{\circ}$ C.

## 2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism 4 software. The statistical comparisons between two groups were performed using unpaired two-tailed student's *t*-test. Comparison between multiple groups was performed using 1-way ANOVA followed by Tukey's multiple comparison test. The correlations were examined using Pearson's correlation coefficient (*r*).

# 3. Results

## 3.1. Plasma bradykinin is significantly elevated in AD

We analyzed bradykinin levels in plasma samples from AD patients and age-matched ND individuals by ELISA. We found that plasma bradykinin level was significantly elevated in AD patients compared to that of ND (2751  $\pm$  458.2 pg/ml vs. 1001  $\pm$  86.69 pg/ml; *p* = .0011; Fig. 1A). Though there was a significant difference between AD and ND groups (Fig. 1A), not all the AD plasma showed high plasma bradykinin. Considering that AD is a multifactorial and complex disorder, it is possible that plasma bradykinin is altered only in a subgroup of AD patients. In fact, many, but not all, AD patients present with vascular risk factors (VRFs), such as hypertension, hyperlipidemia, diabetes, myocardial infarction, stroke, and atrial fibrillation (Helzner et al., 2009; Mielke et al., 2007; Wallin et al., 2012), which could influence plasma protein expression. To minimize the effect of these VRFs, we separately analyzed individuals that did not present with any of these abnormalities (Fig. 1B). Ten of 32 ND controls (31%) and 27 of 39 AD patients (69%) reported with VRFs (Table 1). Even after excluding all samples obtained from persons with VRFs, the bradykinin level in AD remained significantly higher compared to ND (2439  $\pm$  525.9 pg/ml vs. 1035  $\pm$  112.6 pg/ml; *p* = .0018; Fig. 1B). Overall, our results indicate that plasma bradykinin changes could be linked to AD pathology at least in a subgroup of AD and could contribute to cerebral

inflammation and dementia.

## 3.2. Plasma bradykinin changes are associated with extent of memory impairment in AD

We also obtained the cognitive scores of the individuals used in our study (Table 1). The mini-mental state examination (MMSE) is a memory test routinely used to assess cognitive ability in AD patients (Folstein et al., 1975). MMSE score is inversely correlated with memory impairment; the maximum MMSE score (30) represents no cognitive impairment, and lower scores suggest cognitive decline (Kochhann et al., 2010). The mean MMSE scores of our AD and ND patients were 20.8  $\pm$  0.61 and 29.75  $\pm$  0.07, respectively. We separated AD individuals based on their MMSE scores: 1) MMSE score 24 and above (mild dementia; Group 1), and 2) MMSE score below 24 (moderate/severe dementia; Group 2) (Kochhann et al., 2010; Kukull et al., 1994). Group 2 AD patients showed much higher plasma bradykinin levels than Group 1 AD individuals (Fig. 1C; 3395  $\pm$  595.9 pg/ml vs. 1112  $\pm$  118.3 pg/ml; *p* < .01). This result suggests that peripheral bradykinin changes could reflect the extent of memory impairment in AD. We also compared these groups after excluding patients with VRFs. We found that the bradykinin levels in non-VRF Group 2 (moderate/severe dementia) remained significantly higher than non-VRF ND (cognitively normal) or non-VRF Group 1 AD plasmas (mild dementia) (Fig. 1D). These results suggest an association between cognitive impairment and plasma bradykinin level in AD.

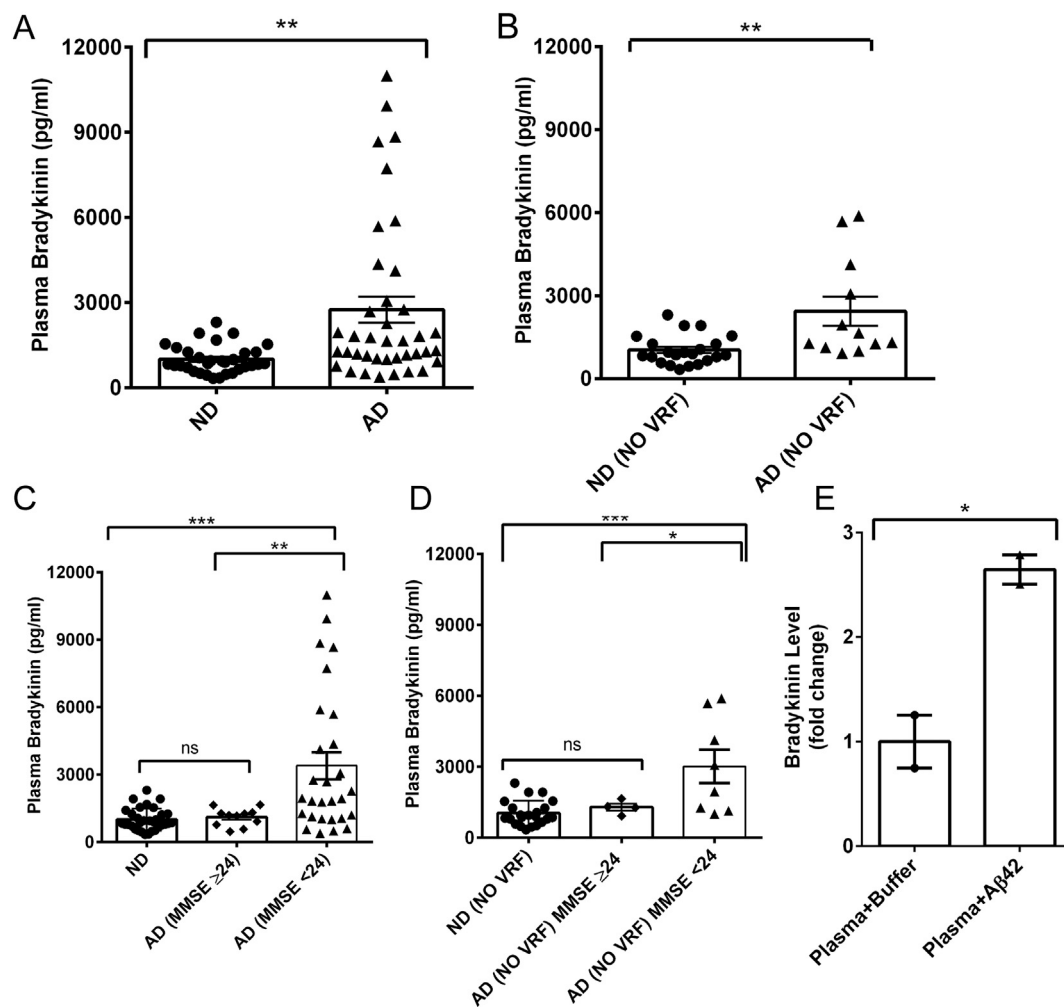
It is possible that A $\beta$  is influencing the release of bradykinin in human plasma. To investigate this possibility, we incubated freshly prepared A $\beta$ 42 oligomers (Yamamoto-Imoto et al., 2018; Zamolodchikov et al., 2015) in normal human plasma and quantified the level of bradykinin in the plasma by ELISA. The plasma bradykinin level was much higher ( $\sim$ 2.6 times) in the presence of A $\beta$ 42 (Fig. 1E), suggesting that bradykinin release in the plasma possibly could be mediated via A $\beta$ 42 in AD.

## 3.3. Bradykinin level is decreased in AD CSF and correlates with CSF A $\beta$ 40/A $\beta$ 42 ratio

Bradykinin levels were determined in CSF from AD patients and ND controls. Contrary to plasma bradykinin, the CSF bradykinin level was significantly lower in AD samples compared to ND controls (19.0  $\pm$  2.4 vs. 33.8  $\pm$  5.9 pg/ml; *p* = .011; Fig. 2A). We also correlated AD CSF bradykinin level with the established AD biomarker, CSF A $\beta$ 40/A $\beta$ 42 ratio, which increases in AD (Anoop et al., 2010). We found that bradykinin level was negatively correlated with the CSF A $\beta$ 40/A $\beta$ 42 ratio (*r* =  $-0.43$ , *p* = .028) (Fig. 2B), indicating a connection between CSF bradykinin and A $\beta$  changes. AD is a complex disease and many AD patients also present with VRFs, such as hypertension, diabetes, myocardial infarction, stroke, and atrial fibrillation (Helzner et al., 2009; Mielke et al., 2007; Wallin et al., 2012). Similar to our plasma analyses, we excluded all CSF samples from patients with comorbid VRFs and repeated our CSF analyses. After exclusion, we still found that AD CSF bradykinin level was significantly lower compared to that of ND (Fig. 2C; 17.3  $\pm$  3.7 vs. 38.2  $\pm$  7.6 pg/ml; *p* = .025).

## 3.4. Bradykinin interacts with fibrillar form of A $\beta$ 42 and shows association with A $\beta$ plaques

Since bradykinin levels are modestly associated with CSF A $\beta$  levels (Fig. 2B), we explored whether bradykinin interacts with different A $\beta$ 42 species using solution state nuclear magnetic resonance (NMR) spectroscopy (Cao et al., 2018). We expressed and purified  $^{15}$ N-labeled A $\beta$ 42 to perform 2D-heteronuclear single quantum correlation (HSQC) experiment (Walti et al., 2016). The HSQC experiment was performed with monomeric A $\beta$ 42 (25  $\mu$ M) in the absence and presence of an



**Fig. 1.** Plasma bradykinin level in ND and AD patients. (A) Plasma bradykinin levels from ND ( $N = 32$ ) and AD ( $N = 39$ ) subjects were quantified by ELISA. Plasma bradykinin was significantly higher in AD than ND samples. (B) Plasma bradykinin levels in ND (22) and AD (12) after excluding individuals with vascular risk factors (VRF). (C) AD samples were grouped according to their MMSE scores (MMSE  $\geq 24$ , mild dementia, Group 1; MMSE  $< 24$ , moderate to severe dementia, Group 2), and their bradykinin levels were compared. Group 2 individuals presented with significantly higher bradykinin compared to ND and Group 1 subjects. (D) Plasma bradykinin levels in Group 2 remained significantly higher than ND and Group 1 after excluding samples from individuals with VRFs. (E) The effect of oligomeric A $\beta$ 42 on plasma bradykinin release was evaluated using normal human plasma. Plasma was incubated with or without oligomeric A $\beta$ 42 (500 nM) for 2 h at 37°C. Bradykinin levels were then quantified by ELISA. The results are presented as mean  $\pm$  SEM. The p value \*  $< 0.05$ , \*\*  $< 0.01$ , and \*\*\*  $< 0.001$ . ns designates not significant.

equimolar ratio of bradykinin. No significant changes in either HSQC peak position or intensity were observed upon addition of bradykinin to  $^{15}\text{N}$ -labeled A $\beta$ 42, which rules out the interaction of bradykinin to monomeric A $\beta$ 42 (Fig. 3A). Because plaques in AD mostly consist of aggregated A $\beta$  species, we also performed an interaction study between fibrillar A $\beta$ 42 and bradykinin using NMR. For this study, a homogenous species of A $\beta$ 42 fibrils was prepared in vitro (unlabelled) by three rounds of seeding (Walti et al., 2016). These fibrils were then used to run one dimensional saturation transfer difference (STD) NMR spectroscopy with bradykinin. STD-NMR is routinely used to detect binding of small molecule ligands to macromolecular receptors (Mayer and Meyer, 2001). This experiment is performed in molar excess of ligand molecules (Mayer and Meyer, 2001). Here, we have used A $\beta$ 42 fibrils and bradykinin in a 1:50 M ratio, and STD data were collected in the presence and absence of bradykinin. The STD signal in the presence of A $\beta$ 42 fibrils was positive (Fig. 3B, green), whereas bradykinin alone (in buffer) showed no STD signal (Fig. 3B, red). For reference, the 1D-NMR spectrum of bradykinin is also shown (Fig. 3B, blue). This result shows that bradykinin interacts with A $\beta$ 42 fibrils transiently.

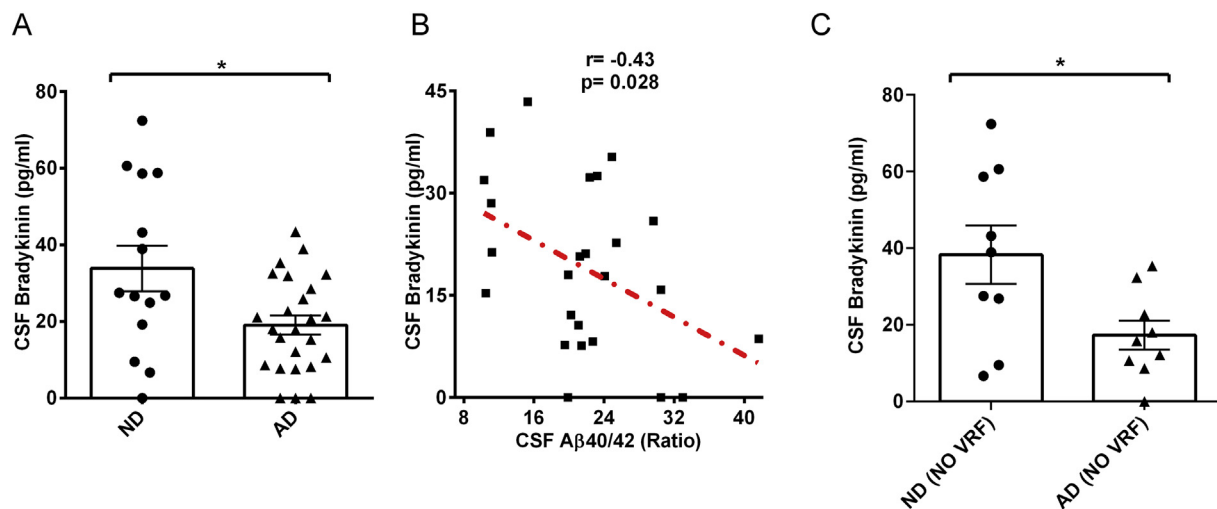
Since we detected an interaction between fibrillar A $\beta$ 42 and bradykinin, we asked whether bradykinin could also be associated with

parenchymal plaques in AD. To investigate this possibility, immunofluorescence analysis was performed using AD human post-mortem brain sections (superior frontal cortex). The sections were stained for bradykinin (green) and A $\beta$  plaques (Congo red staining). Some of the plaques in AD brain sections also showed bradykinin immunoreactivity (yellow), confirming association of bradykinin with A $\beta$  plaques (Fig. 3C, top panel). For control, ND sections (superior frontal cortex) were also immunostained in the same way (Fig. 3C, lower panel).

### 3.5. Plasma and CSF bradykinin changes are not due to altered angiotensin-converting enzyme (ACE) activity in AD patients

We also analyzed angiotensin converting enzyme (ACE) activity in plasma and CSF of AD and ND individuals. ACE is known to degrade vasoactive bradykinin, and therefore, ACE inhibitors are widely used in the treatment of hypertension (Ignjacev-Lazich et al., 2005). In our cohort of samples, the medical information provided indicated that many of the individuals were hypertensive and therefore may have been medicated. However, we were not provided with patients' specific prescription information. We analyzed whether the differences in CSF and plasma bradykinin levels between ND and AD are due to





**Fig. 2.** CSF bradykinin level in AD and ND. (A) CSF bradykinin was quantified by ELISA. CSF from AD patients had significantly lower bradykinin compared to ND CSF ( $N = 14$  ND,  $25$  AD). (B) Correlation graph of CSF bradykinin level and CSF A $\beta$ 40/A $\beta$ 42 ratio in AD. A modest but significant inverse correlation was observed between these two parameters in AD ( $r = -0.43$ ,  $P$  value =  $.028$ ). (C) CSF samples from donors with VRFs were excluded, and bradykinin levels were compared. AD patients' CSF bradykinin was significantly lower than ND individuals' CSF bradykinin. The results are presented with mean  $\pm$  SEM.  $P$  value  $< .05$  (\*).

differences in ACE activity in these samples by measuring ACE activity (Fig. 4). We did not find any difference in plasma ACE activity between ND and AD (Fig. 4A). However, compared to plasma, the CSF ACE activity was very low in both ND and AD CSF, yet there was no significant difference between ND and AD CSF ACE activity (Fig. 4B). This result indicates that the bradykinin changes in ND and AD are not due to any altered ACE activity.

#### 4. Discussion

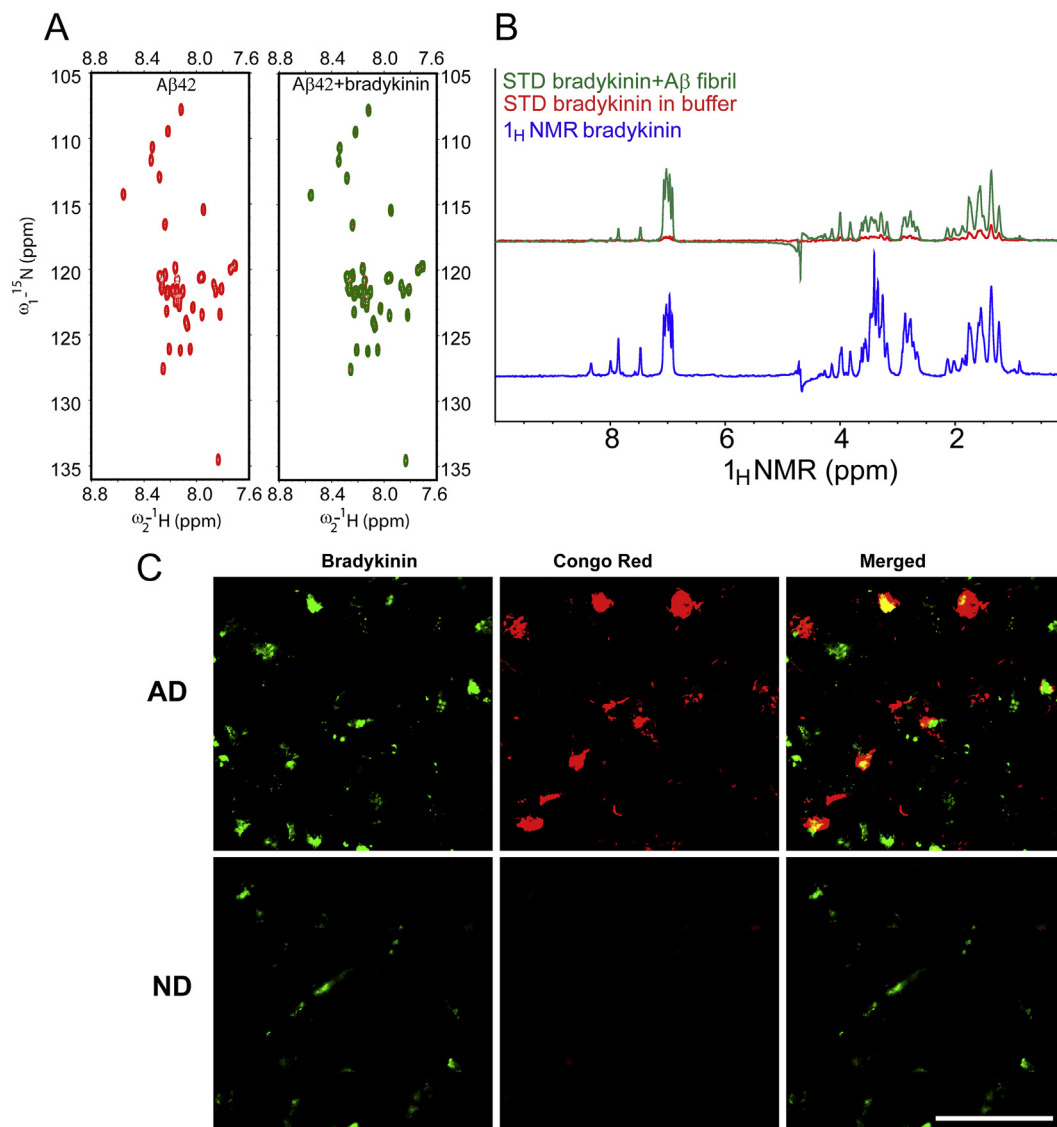
AD is a multifactorial disease, and it is likely that multiple disease mechanisms contribute to AD pathology (Strickland, 2018). Therefore, identifying and targeting various mechanisms in subgroups of AD patients are needed for the advancement of AD therapeutics (Selkoe, 2019). The central pathology in AD is neuronal dysfunction causing cognitive decline (Hardy and Higgins, 1992). However, one pathway that has been generally understudied in AD is vascular dysregulation. Accumulating evidence suggests that vascular dysregulation (Ahn et al., 2014; Baker et al., 2018; Chen et al., 2017; Cruz Hernandez et al., 2019; Strickland, 2018; Sweeney et al., 2019; Zamolodchikov et al., 2015) and inflammation (Akiyama et al., 2000; Ayata et al., 2018; Mandrekar-Colucci and Landreth, 2010; Merlini et al., 2019; Wyss-Coray and Rogers, 2012) play major roles in cognitive decline. The evidence for neuroinflammation in AD also includes data from several epidemiologic studies that generally support an inverse risk relationship between the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and AD (Zhang et al., 2018). Anti-inflammatory compounds are also found to be protective against memory impairment associated with cerebral small vessel disease and cerebral ischemic injury in rats (Chen et al., 2018; Guan et al., 2018; Tian et al., 2019). Recently, several studies have suggested that AD may have systemic manifestations (Wang et al., 2017). Furthermore, cognitive abnormalities correlate with changes in peripheral inflammatory signals such as TNF $\alpha$  and IL-1 $\beta$  (Wang et al., 2017). Here, we demonstrate elevated levels of bradykinin, a proinflammatory molecule, in AD patient plasma (Fig. 1A), which identifies a novel mechanism of inflammatory pathology in AD. Bradykinin is generated by activation of the kallikrein-kinin system, which has been recently reported to be activated in AD (Zamolodchikov et al., 2015). Also, blockade or deletion of bradykinin receptor (B1R) has been shown to improve cognitive function in mice by reducing inflammation and BBB disruption (Raslan et al., 2010). BBB disruption is evident in AD (Montagne et al., 2017; Sweeney et al., 2018) before initiation of

significant cognitive impairment or brain atrophy. It should be noted that bradykinin induces vascular permeability and is linked to BBB damage (Abbott, 2000; Marcos-Contreras et al., 2016). Bradykinin can cause overproduction of nitric oxide (NO), which is believed to be involved in neuroinflammation due to its free radical properties (Bae et al., 2003; Yuste et al., 2015). All these findings suggest that bradykinin could be involved in a pathogenic mechanism of AD. However, most of these conclusions are based on animal model studies, and therefore, a direct quantification of plasma bradykinin in AD patients has been performed in this study.

Another question would be whether these peripheral bradykinin changes affect the pathology and cognition of AD patients. Although MMSE score, a measure of cognitive status, is not specific to AD, it does give an overall view of the cognitive abilities of the patient (Arevalo-Rodriguez et al., 2015). Our data indicate a relationship between bradykinin levels and cognitive state of AD patients. In fact, many of the AD patients with lower MMSE ( $< 24$ ) scores had even higher plasma bradykinin levels (Fig. 1C). Considering the fact that AD is a multifactorial disorder, these data are meaningful even in a subset of AD patients. We found many but not all AD patients showed a high level of bradykinin in their plasma (Fig. 1A). Our data suggest that in the AD population, there could be a subgroup of individuals where elevated plasma bradykinin could significantly contribute to inflammation and AD pathology.

We also believe that there is a link between peripheral bradykinin level and the extent of neuroinflammation in AD. Bradykinin can induce BBB damage and extravasation of plasma proteins into the brain parenchyma. This extravasation causes glial cell activation, and the associated edema can lead to impaired cerebral blood flow. However, additional studies are required to validate our hypothesis. Microglia expresses bradykinin receptors (Ifuku et al., 2007; Noda et al., 2007), and therefore, bradykinin could be associated with activated microglia in the AD patient brain. Moreover, bradykinin distribution was found increased in the AD brain separate from the plaque area (Fig. 3C). This result suggests increased bradykinin extravasation in the AD brain. Furthermore, increased activity of plasma kallikrein, an enzyme required for bradykinin generation, was reported in AD brain parenchyma (Ashby et al., 2012).

Altered bradykinin levels are also linked with other diseases, for example hereditary angioedema (HAE) (Hofman et al., 2016), which is characterized by bradykinin-mediated edema. It should be noted that the contact system is dysregulated in both HAE and AD (Colman and



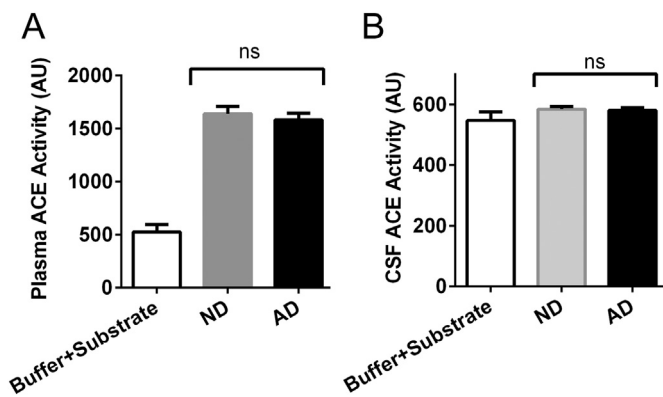
**Fig. 3.** Analysis of bradykinin's interaction with A $\beta$ 42 and association with A $\beta$  plaques. **(A)** Interaction study between bradykinin and different species of A $\beta$ 42 in vitro probed by NMR. 2D [ $^1\text{H}$ - $^{15}\text{N}$ ] HSQC of monomeric/soluble A $\beta$ 42 (25  $\mu\text{M}$ ) in absence (red) and presence (green) of bradykinin. No significant chemical shift and/or peak intensities between these two spectra were observed, indicating that bradykinin does not interact with monomeric A $\beta$ 42. **(B)** 1D STD NMR spectra of fibrillar A $\beta$ -bradykinin mixture (green), STD NMR signal of bradykinin in buffer only (red) and  $^1\text{H}$  NMR spectra of bradykinin (blue). Positive STD signal was observed in fibrillar A $\beta$ -bradykinin mixture, suggesting bradykinin interacts with fibrillar A $\beta$ 42. **(C)** Immunofluorescence analysis of post-mortem human AD and ND brain tissue sections from superior frontal cortex. Sections were immunostained and imaged for bradykinin (green) and amyloid plaques (Congo red). Representative images show the association of bradykinin with amyloid plaques in AD (yellow in overlay, upper panel). The representative ND section immunostaining is shown as a control (Lower panel).  $N = 3$  AD and 3 ND. Scale bar is 100  $\mu\text{m}$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Schmaier, 1997; Strickland, 2018). Some of the approved drugs for treatment of HAE might be useful in treating AD patients with high plasma bradykinin levels in the future (Strickland, 2018).

ACE can degrade vasoactive bradykinin and thus modulates blood pressure (Hofman et al., 2016). Many AD patients suffer from hypertension (Kruger et al., 2015) and are treated with ACE inhibitors among other anti-hypertensives. ACE inhibitors block ACE activity and thus enhance the level of vasoactive plasma bradykinin (Hofman et al., 2016). We found no difference in ACE activity between ND and AD plasma (Fig. 4A), which shows that there was not a significant contribution of drug-mediated elevation of plasma bradykinin in our AD cohort. Moreover, we also excluded all ND and AD patients with hypertension and other vascular risk factors (VRFs) in order to analyze bradykinin changes in ND and AD individuals without any comorbidities or associated medications. In this analysis, we found the same

result in that AD plasma bradykinin level is elevated compared to controls (Fig. 1B) and these bradykinin changes are more pronounced in AD patients with higher memory impairment (Fig. 1D). After exclusion of VRF samples, we had only 12 AD samples. Although this is a small group, we found that many of the AD patients without vascular comorbidities have a high level of bradykinin in the plasma (Fig. 1B). Again, considering the pathogenic complexity in AD, a larger group of ND and AD plasma samples (without vascular comorbidities) should be analyzed.

When the contact system is activated, bradykinin is released upon cleavage of high molecular weight kininogen (HK). It has been reported that HK cleavage is increased in AD patient's plasma and CSF (Bergamaschini et al., 1998; Zamolodchikov et al., 2015). The increased plasma bradykinin in AD patients could be due to the increased HK cleavage observed in AD patient plasma. However, these findings do



**Fig. 4.** Angiotensin converting enzyme (ACE) activity in plasma and CSF of ND and AD patients. **(A)** Plasma from ND ( $N = 32$ ) and AD ( $N = 35$ ) individuals was used to measure ACE activity. The plasma ACE activity between ND and AD were similar, and overall no significant differences were observed. **(B)** ACE activity was analyzed in CSF from ND ( $N = 14$  ND, 25 AD) donors. No differences were found between groups. The results are presented as mean  $\pm$  SEM. ns designates not significant.

not explain the lower CSF bradykinin level in AD patients compared to ND control individuals (Fig. 2A). It is possible that CSF bradykinin is reduced in AD due to its increased degradation by other proteases in the CSF. Bradykinin can be degraded by ACE (Hofman et al., 2016), whose levels are also dysregulated in AD. Plasma ACE activity in AD was reported to be lower than that of control individuals (Vardy et al., 2009), and CSF ACE activity was reported higher in AD (He et al., 2006). However, we did not find any significant difference between ND and AD ACE activity in plasma or CSF (Fig. 4A and B). ACE activity is reported to be higher in AD brain (Miners et al., 2009), but we were unable to measure brain ACE activity in our cohorts.

ACE has been reported to cleave  $A\beta$  in vitro (Hemming and Selkoe, 2005), and there are some reports that ACE inhibitors improve memory in AD patients (Ohrui et al., 2004; Soto et al., 2013). However, this observation is controversial as there are other studies that show that ACE inhibitors could potentially increase the risk of developing AD (Qiu et al., 2013; Qiu et al., 2014; Zou et al., 2007). It should be taken into account that non-ACE enzymes are also capable of degrading ACE substrates (Skidgel, 1992). Therefore, there may be other proteases in the CSF that could degrade and therefore lower the level of CSF bradykinin in AD. It is also possible that bradykinin levels are lower in the CSF because CSF bradykinin is depositing around plaques in the brain (Fig. 3C).

The plasma-CSF inverse correlation is not surprising in AD. For example, glycoprotein CD40, which activates microglia, is elevated in AD patient plasma but is decreased in AD patient CSF (compared to healthy controls) (Buchhave et al., 2009; Ye et al., 2019). Furthermore, plasma  $A\beta_{42}$  levels increase while CSF  $A\beta_{42}$  levels decrease in AD patients (Nakamura et al., 2018; Teunissen et al., 2018). While we did not find an inverse correlation between plasma bradykinin and CSF  $A\beta_{40}/A\beta_{42}$  ratio, we did find one between CSF bradykinin and CSF  $A\beta_{40}/A\beta_{42}$  ratio (Fig. 2B). This finding indicates that CSF bradykinin changes could be influenced by CSF  $A\beta$  changes. We also confirmed that bradykinin interacts with  $A\beta_{42}$  fibrils and is associated with  $A\beta$  plaques in AD (Fig. 3). However, the mechanism for how CSF bradykinin changes are related to AD pathology needs to be explored in detail. Furthermore, we found that adding  $A\beta_{42}$  oligomers to normal human plasma significantly increases bradykinin level (Fig. 1E). All these findings suggest that bradykinin changes could be driven by  $A\beta$ , likely due to the dysregulated contact system in AD.

As mentioned above, VRFs and vascular dysfunction are present in the majority of AD patients and significantly contribute to AD pathology. For example, nearly 70% of our AD patient cohort had VRF

comorbidities (Table 1). In fact, many AD patients with VRF showed very high plasma bradykinin (Fig. 1A and Fig. 1C). Thus, without a detailed analysis of each variable, we cannot fully determine how these comorbidities contribute to bradykinin changes. Therefore, our conclusion is that increased plasma bradykinin level (by any mechanism) could worsen AD pathology and cognition.

#### Authorship contributions

P.K.S. designed and performed the research, analyzed the data, and wrote the manuscript; Z.L.C helped design the research, analyze the data, and prepare the manuscript; D.G performed the research and analyzed the data; S.S helped with study design, data analysis, and manuscript preparation. E.H.N aided in study design, data analysis, and manuscript preparation. All authors edited and approved the final manuscript.

#### Declaration of Competing Interest

None

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