



# Angiotensin receptor blocker use is associated with upregulation of the memory-protective angiotensin type 4 receptor (AT<sub>4</sub>R) in the postmortem brains of individuals without cognitive impairment

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**Abstract** The reported primary dementia-protective benefits of angiotensin II type 1 receptor (AT<sub>1</sub>R) blockers (ARB) are believed, at least in part, to arise from systemic effects on blood pressure. However, there is a specific and independently regulated brain renin-angiotensin system (RAS). Brain RAS acts mainly through three receptor subtypes; AT<sub>1</sub>R, AT<sub>2</sub>R, and AT<sub>4</sub>R. The AT<sub>1</sub>R promotes inflammation and mitochondrial reactive oxygen species generation. AT<sub>2</sub>R increases nitric oxide. AT<sub>4</sub>R is essential

for dopamine and acetylcholine release. It is unknown whether ARB use is associated with changes in the brain RAS. Here, we compared the impact of treatment with ARB on not cognitively impaired individuals and individuals with Alzheimer's dementia using postmortem frontal-cortex samples of age- and sex-matched participants (70–90 years old,  $n=30$  in each group). We show that ARB use is associated with higher brain AT<sub>4</sub>R, lower oxidative stress, and amyloid- $\beta$  burden in NCI participants. In AD, ARB use was associated with lower brain AT<sub>1</sub>R but had no impact on inflammation, oxidative stress, or amyloid- $\beta$  burden. Our results may suggest a potential

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role for AT<sub>4</sub>R in the salutary effects for ARB on the brains of not cognitively impaired older adults.

**Keywords** Angiotensin receptor blocker · AT<sub>4</sub>R · Alzheimer's disease · Oxidative stress · Inflammation · Brain

## Introduction

Alzheimer's dementia (AD) is a complex neurodegenerative disease characterized by the accumulation of neuritic plaques and neurofibrillary tangles. It is the most common cause of dementia in older persons [1]. In the USA, AD affects more than 6.1 million people age 65 and older, and this number is projected to increase to 13.8 million by 2060 [2]. Aging is a key risk factor in AD development, partly through chronic inflammation, mitochondrial dysfunction, and oxidative stress [3, 4]. Although multiple potential biosignatures have been proposed, no clear aging-related etiological mechanisms have been identified.

The renin-angiotensin system (RAS), a central regulator of blood pressure and sodium balance, is involved (via activation of the angiotensin II type 1 receptor (AT<sub>1</sub>R)) in several molecular mechanisms that are linked to AD, including chronic inflammation, oxidative stress damage, mitochondrial decline, and amyloid- $\beta$  production [5–8]. Angiotensin (Ang) II type 1 receptor blockers (ARB) have established renal, cardiovascular, and blood pressure benefits and are in widespread clinical use. The primary dementia-protective benefits of ARB are believed to arise, at least partly, from their anti-hypertensive systemic effects. However, there is also a brain-specific RAS [5]. Brain RAS acts mainly through three receptor subtypes; AT<sub>1</sub>R, AT<sub>2</sub>R, and AT<sub>4</sub>R [9]. The AT<sub>1</sub>R promotes inflammation and mitochondrial reactive oxygen species (ROS) generation. AT<sub>2</sub>R increases nitric oxide (NO). AT<sub>4</sub>R, the most recently discovered angiotensin receptor, is essential for dopamine and acetylcholine release and mediates memory consolidation [10–13]. AT<sub>4</sub>R is distributed throughout the brain, but more predominantly in the hippocampus, neocortex, basal ganglia, and amygdala [5, 9, 14]. Recently, higher levels and activity of AT<sub>1</sub>R have been shown in the postmortem brains of older individuals with Alzheimer's dementia [15]. The presence of brain-specific RAS and higher brain AT<sub>1</sub>R levels in AD

prompt the question of whether brain AT<sub>1</sub>R blockade contributes to the salutary effects of ARB. In this study, we compared the impact of treatment with ARB, respectively, on not cognitively impaired individuals and individuals with Alzheimer's dementia using postmortem frontal-cortex samples of age- and sex-matched participants (70–90 years old,  $n=30$  in each group). We examined the effects of ARB on brain RAS and brain health biomarkers, including oxidative stress and inflammation, as well as outcomes such as tangle density, amyloid- $\beta$  burden, and cognitive function.

## Methods

Postmortem frontal cortex brain samples of age- and sex-matched NCI individuals who used ARB ( $n=30$ ) and did not use ARB ( $n=30$ ) and AD participants who used ARB ( $n=30$ ) and did not use ARB ( $n=30$ ) were studied. Protein levels of the brain functional RAS receptors AT<sub>1</sub>R, AT<sub>2</sub>R, and AT<sub>4</sub>R were quantified using western blot technique. Then, to determine the association between ARB and molecular mechanisms linked to AD, brain protein carbonyl (PC) levels as a marker of oxidative stress and serum and brain cytokines (Interleukin 6 (IL-6), IL-1 $\beta$ , Interferon-gamma (IFN- $\gamma$ ), and Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )) as markers of inflammation were measured and compared between the groups. Finally, we assessed the impact of ARB on AD neuropathologies, neurofibrillary tangles, amyloid- $\beta$  burden, as well as cognitive function.

### Human brain samples and protein extraction

Postmortem frontal cortex brain samples came from participants in the Rush Memory and Aging Project [16]. The study was approved by an Institutional Review Board of Rush University Medical Center. All participants signed informed consent, Anatomic Gift Act, and a repository consent to allow their data to be repurposed. Expert neurologists made a clinical AD diagnosis by reviewing all clinical data blinded to all neuroimaging and blood tests results and postmortem data. Participants in the ARB use group were selected if there was a record of taking any ARB in the last follow-up visit. Participants who have never used ARB or angiotensin-converting enzyme inhibitors in any visit were included in the non-ARB group.

Medication was recorded for the previous 2 weeks before each follow-up visits of Rush MAP participants. To obtain brain tissue postmortem, under sterile conditions, gray matter from midfrontal cortices was excised and kept at  $-80\text{ }^{\circ}\text{C}$  without any fixation until analyzed. Methodologies that have been used to obtain postmortem biological specimens were explained in detail [17]. Midfrontal cortex has been selected based on accessibility, availability, and its interconnections with limbic structures, as well as cortical association areas, that are consistently damaged in AD [18]. For protein extraction, 25 mg of the fresh frozen sample was homogenized in a lysis buffer (T-PER, Pierce, MA) using Bullet Blender, a high-throughput homogenizer (Thomas Scientific, NJ). Total protein content was quantified using BCA assay (Pierce, MA).

#### Measurement of angiotensin receptor levels

Protein levels of  $\text{AT}_1\text{R}$ ,  $\text{AT}_2\text{R}$ , and  $\text{AT}_4\text{R}$  were measured by western blot. Fifteen  $\mu\text{g}$  of extracted proteins were resolved by 4% to a 12% acrylamide gradient SDS-PAGE midi gel allowing for protein analysis of 24 participants at a time. Internal control was included with every gel for correction of exposure time differences. Samples were then transferred to a nitrocellulose membrane using standard conditions. Membranes were blocked with 5% skim milk in TBS-T at room temperature for 1 h. Each membrane was then incubated overnight with one of the following rigorously validated, well-characterized primary antibodies at  $4\text{ }^{\circ}\text{C}$ . The following antibodies were used:  $\text{AT}_1\text{R}$  (sc-515884; Santa Cruz, CA) [19],  $\text{AT}_2\text{R}$  (sc-9040; Santa Cruz, CA) [20, 21], and  $\text{AT}_4\text{R}$  (HPA043642; Sigma-Aldrich, MO) [22]. Specificity validation of  $\text{AT}_1\text{R}$  and  $\text{AT}_2\text{R}$  antibodies were made by previous studies studying Chinese hamster ovary cells and naïve HEK-293 T cells lacking  $\text{AT}_1\text{R}$  and  $\text{AT}_2\text{R}$  [20, 23, 24]. Membranes were then washed, incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Normalization was done with Actin. After adding a chemiluminescent substrate, the signal was visualized, digitized, and quantified using a Kodak Gel Logic 2200 image workstation with its associated software. Western blot imaging analysis revealed two distinct proteins with different molecular masses identified by the  $\text{AT}_4\text{R}$  antibody. This result is consistent with other reports of  $\text{AT}_4\text{R}$

quantification [25]. Multiple reasons could have led to this including the presence of 3 isoforms due to alternative splicing as well as posttranslational modifications (glycosylation) of  $\text{AT}_4\text{R}$  protein [25–27]. In our analysis, we have used the most prominent band that was seen around 160 kDa in the majority of our participants.

#### Measurement of brain oxidative stress

Brain protein carbonyl levels were measured by OxiSelect ELISA according to the manufacturer's protocol (Cell Biolabs, CA) [28].

#### Measurement of brain and serum cytokines

Pro-inflammatory cytokines were measured from participants' serum and brain tissue using Mesoscale 4-Plex cytokine assays (K15052D; Meso Scale Diagnostics, MD). These measures were performed in the JHU ICTR Core Laboratory.

#### Brain histopathology

After brain autopsies were performed as previously described [29], aggregated amyloid- $\beta$  proteins and paired helical filaments of tau tangles in slices of brain regions were identified using 10D5 (dilution 1:300; courtesy of Elan Pharmaceuticals, South San Francisco, CA). Pathologic diagnoses of AD were made using NIA-Reagan and modified CERAD criteria. Staging of neurofibrillary pathology was made by Braak Staging in the Rush MAP [29]. Amyloid- $\beta$  and tangle burden were calculated using the average percent density in brain slices for eight relevant brain regions, namely, the entorhinal cortex, calcarine cortex, cingulate cortex, inferior parietal gyrus, inferior temporal gyrus, hippocampus, middle frontal gyrus (MFG), and superior frontal cortex (SFC). Finally, overall amyloid- $\beta$  and tangle scores for the entire brain were obtained using the mean of those from all brain regions.

#### Cognitive performance

The cognitive performance of participants was measured by administering a mini-mental state examination (MMSE) and cognitive performance tests each year, 19 of which assessed five cognitive domains,

including episodic memory, semantic memory, working memory, perceptual speed, and visuospatial ability, as previously described [30]. Raw scores from each test were first standardized to  $z$  scores and averaged to yield a participant's global cognitive function (GCF) score. Then, cognitive score slopes were analyzed with adjustment both demographic (sex, age, and education level) and pathologies of the brain such as amyloid- $\beta$ , PHF tau tangles, gross chronic cerebral infarctions, chronic microinfarctions, Lewy body disease, TDP-43, hippocampal sclerosis, cerebral amyloid angiopathy, cerebral atherosclerosis, and arteriosclerosis as described previously [31, 32].

### Physical function

Gait speed and grip strength were assessed as previously reported [29, 33, 34].

### Statistical analysis

SPSS 23 and GraphPad Prism 9 were used for statistical analysis. Normally distributed values are expressed as a mean  $\pm$  standard deviation (SD), while non-normally distributed values are expressed as a median (interquartile range (IQR)). A  $p$  value

of  $\leq 0.05$  was used as the threshold of statistical significance. An unpaired Student's  $t$  test for normally distributed variables and Mann–Whitney  $U$  tests for non-normally distributed variables were performed to test for the significance of differences. A chi-square test was used to compare binary variables. Correlation analysis was performed with Spearman's correlation test. Four group analysis were done with either one- or two-way ANOVA (with disease state (NCI vs AD) and ARB use as factors).

### Results

Characteristics of study participants are given in Table 1. Body mass index (BMI) and indicators of functional outcomes, such as gait speed and grip strength, were not significantly different between the groups. NCI and AD participants who took ARB had fewer years of education than those who did not take ARB. We also observed a lower number of participants with hypertension diagnosis in the NCI group ( $p < 0.001$ ) compared to the other groups. However, there were no significant difference in average systolic and diastolic blood pressure between the groups ( $p = 0.056$ ,  $p = 0.558$ , respectively).

**Table 1** Characteristics of not cognitively impaired and Alzheimer's dementia participants

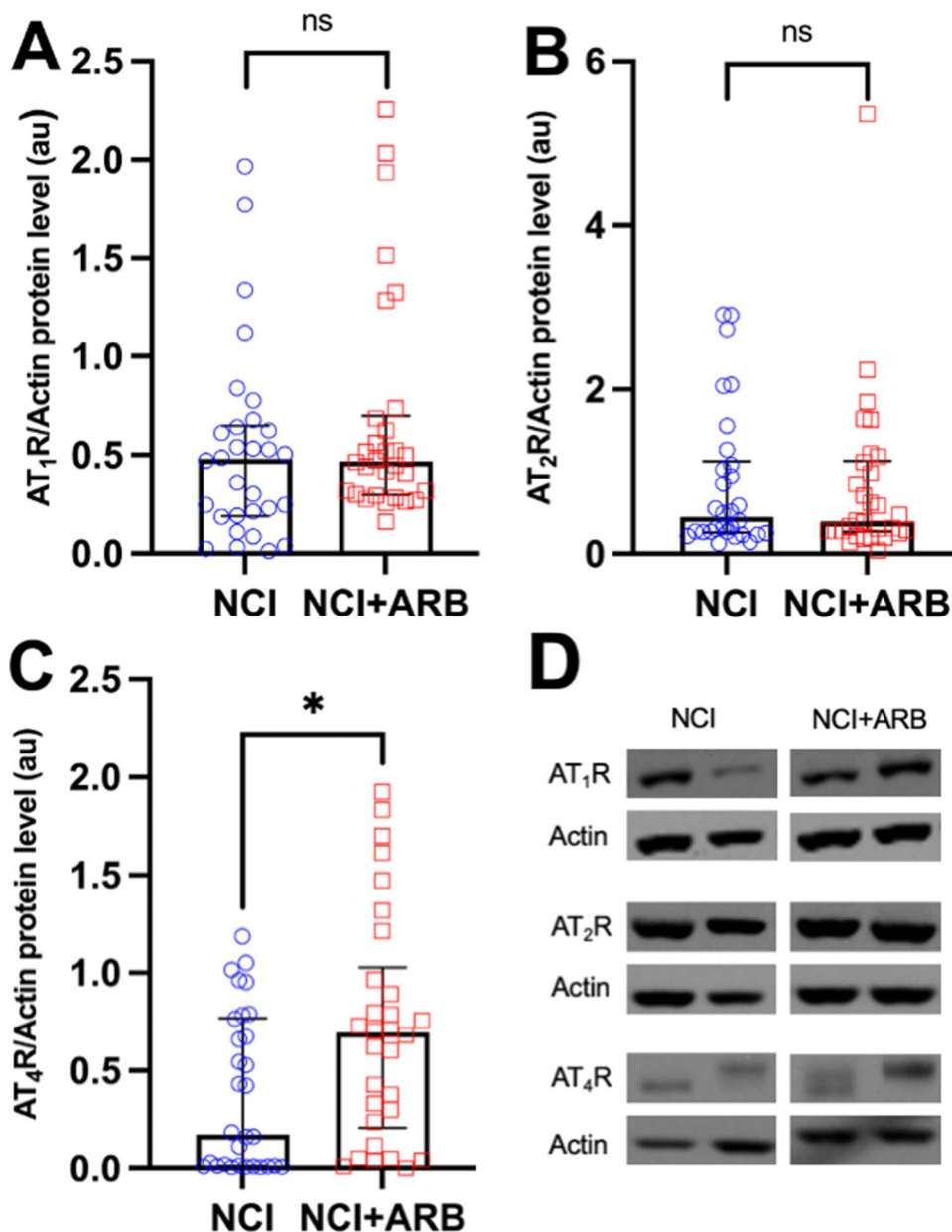
	NCI ( $n = 30$ )	NCI + ARB ( $n = 30$ )	AD ( $n = 30$ )	AD + ARB ( $n = 30$ )	$p$ value
Age, mean (SD)	90.24 (5.1)	90.04 (3.5)	90.11 (5.7)	90.20 (5.5)	0.999
Sex, male, $n$ (%)	5 (16.7)	5 (16.7)	5 (16.7)	5 (16.7)	1
Education, years, median (IQR)	18 (21–16)	15 (17–12)	18 (21–18)	16 (18–12)	<0.001*
BMI, median (IQR)	24.0 (25.2–22.6)	25.6 (28.6–23.0)	24.8 (29.0–23.4)	26.2 (30–24.6)	0.093
Walking speed, m/s, mean (SD)	0.63 (0.24)	0.58 (0.17)	0.56 (0.19)	0.52 (0.20)	0.373
Grip strength, kg, mean (SD)	33.5 (15.1)	36.1 (11.0)	30.1 (10.4)	37.6 (16.2)	0.402
Coronary heart disease, $n$ (%)	7 (23.3)	7 (23.3)	7 (23.3)	10 (33.3)	0.766
Stroke, $n$ (%)	6 (20)	8 (26.7)	6 (20)	6 (20)	0.902
Cancer, $n$ (%)	12 (40)	14 (46.7)	9 (30)	10 (33.3)	0.561
Hypertension, $n$ (%)	16 (53.3)	29 (96.7)	25 (83.3)	24 (80)	<0.001*
Thyroid disease, $n$ (%)	5 (16.7)	12 (40)	11 (36.7)	14 (46.7)	0.086
Claudication, $n$ (%)	5 (16.7)	7 (23.3)	11 (36.7)	7 (23.3)	0.344
Diabetes, $n$ (%)	3 (10)	6 (20)	7 (23.3)	8 (26.7)	0.413
Average systolic BP, mean (SD)	124 (18)	136 (18)	135 (15)	138 (19)	0.056
Average diastolic BP, mean (SD)	69 (10)	71 (10)	73 (11)	72 (10)	0.558
PMI, hours, median (IQR)	5.63 (15.3–4.2)	5.33 (6.5–4.5)	6.93 (10.58–4.25)	5.43 (13.2–4.4)	0.841

AD, Alzheimer's dementia; ARB, angiotensin receptor blockers; BMI, body mass index; BP, blood pressure; IQR, interquartile range; NCI, not cognitively impaired; PMI, post-mortem interval; SD, standard deviation; \* $p \leq 0.05$

Treatment with angiotensin receptor blockers was associated with higher levels of brain AT<sub>4</sub>R only in participants without cognitive impairment

Angiotensin II, which is a bioactive product of

angiotensinogen, acts mainly through two angiotensin receptor subtypes: AT<sub>1</sub>R and AT<sub>2</sub>R. Ang II can also be converted to angiotensin IV by glutamyl aminopeptidase A (AP-A) and alanyl aminopeptidase N (AP-N) [35]. Ang IV acts through AT<sub>4</sub>R. To elucidate



**Fig. 1** Protein levels of angiotensin receptors in not cognitively impaired groups. **(A)** Protein levels of angiotensin II type 1 receptor (AT<sub>1</sub>R). **(B)** Angiotensin II type 2 receptor (AT<sub>2</sub>R). **(C)** Angiotensin type 4 receptor (AT<sub>4</sub>R) in not cognitively impaired (NCI) group treated ( $n=30$ ) and untreated

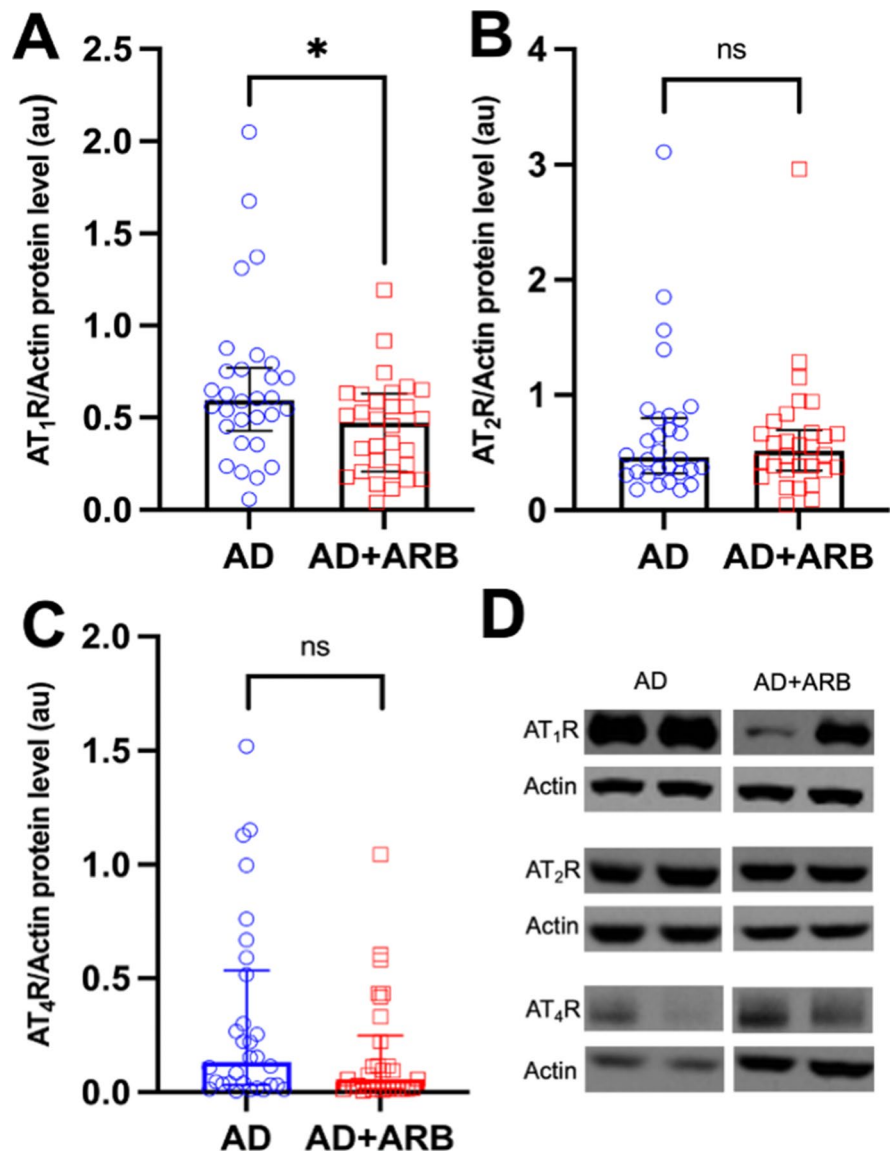
( $n=30$ ) with angiotensin receptor blockers (ARB). Data are presented with median and interquartile range. Comparisons were conducted using Mann–Whitney  $U$  test. ( $*p \leq 0.05$ ). au, arbitrary unit. **(D)** Western blot images of the AT<sub>1</sub>R, AT<sub>2</sub>R, and AT<sub>4</sub>R

the effects of ARB on brain angiotensin receptors, we measured protein levels of AT<sub>1</sub>R, AT<sub>2</sub>R, and AT<sub>4</sub>R and compared them between ARB treated and untreated groups. Our results (Fig. 1) show that in the not cognitively impaired group (without MCI or dementia), there is no significant difference in AT<sub>1</sub>R protein levels (median (IQR) 0.47 (0.63–0.19) vs. 0.46 (0.68–0.30), NCI vs. NCI+ARB,  $p=0.243$ ) (Fig. 1A). Similarly, no significant difference is

observed in AT<sub>2</sub>R protein levels (median (IQR) 0.45 (1.08–0.26) vs. 0.39 (1.11–0.27), NCI vs. NCI+ARB,  $p=0.848$ ) with the ARB treatment (Fig. 1B). Nevertheless, ARB treatment was associated with higher AT<sub>4</sub>R protein levels in NCI participants (median (IQR), 0.17 (0.76–0.01) vs. 0.69 (0.96–0.23), NCI vs. NCI+ARB,  $p=0.018$ ) (Fig. 1C).

In contrast, in the AD group, AT<sub>1</sub>R protein levels were significantly lower in ARB treatment group

**Fig. 2** Protein levels of angiotensin receptors in Alzheimer's dementia groups. (A) Protein levels of angiotensin II type 1 receptor (AT<sub>1</sub>R). (B) Angiotensin II type 2 receptor (AT<sub>2</sub>R). (C) Angiotensin II type 4 receptor (AT<sub>4</sub>R) in Alzheimer's dementia (AD) group treated ( $n=30$ ) and untreated ( $n=30$ ) with angiotensin receptor blockers (ARB). Data are presented with median and interquartile range. Comparisons were conducted using Mann–Whitney  $U$  test ( $*p\leq 0.05$ ). au, arbitrary unit. (D) Western blot images of the AT<sub>1</sub>R, AT<sub>2</sub>R, and AT<sub>4</sub>R



**Fig. 3** Brain protein carbonyl (oxidative stress marker) differences between angiotensin receptor blockers treated and untreated groups. Levels of brain protein carbonyls (PC) in (A) not cognitively impaired (NCI) group treated ( $n=30$ ) and untreated ( $n=30$ ) with angiotensin receptor blockers (ARB) and (B) Alzheimer's dementia (AD) group treated ( $n=30$ ) and untreated ( $n=30$ ) with ARB. Data are presented with median and interquartile range. Comparisons were conducted using Mann–Whitney  $U$  test (ns, non-significant;  $*p \leq 0.05$ )

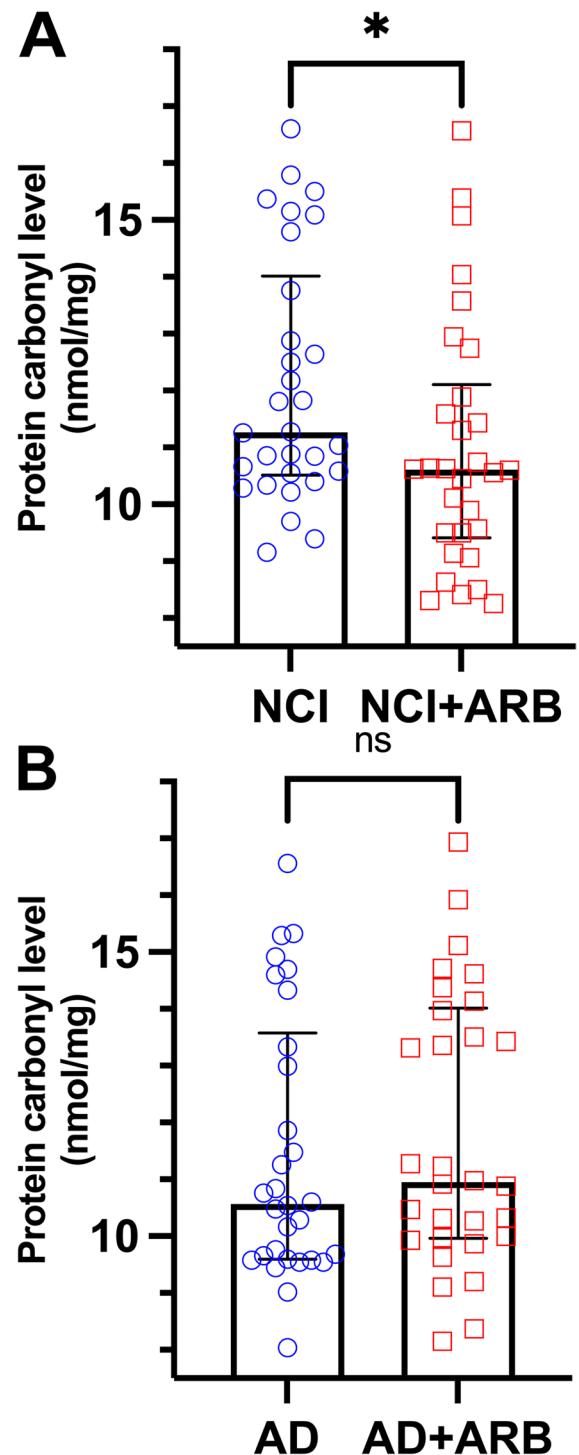
(0.59 (0.76–0.45) vs. 0.47 (0.63–0.21), AD vs. AD+ARB,  $p=0.023$ ) (Fig. 2A). However, there was no significant difference in either  $AT_2R$  (0.46 (0.79–0.32) vs. 0.52 (0.67–0.34), AD vs. AD+ARB,  $p=0.965$ ) or  $AT_4R$  protein levels (0.13 (0.51–0.03) vs. 0.06 (0.22–0.01), AD vs. AD+ARB,  $p=0.117$ ) (Fig. 2B and C). Representative western blot images were added as a supplementary file.

ARB treatment was associated with lower brain oxidative stress levels in the not cognitively impaired participants

Oxidative stress is highlighted as a primary theory of aging and plays a prominent role in cognitive decline in older individuals [36]. While  $AT_1R$  is known for its detrimental pro-oxidative stress effects,  $AT_2R$  and  $AT_4R$  possess anti-inflammatory and anti-oxidative stress downstream signaling effects. The balance among angiotensin receptors is essential for homeostasis [5]. Levels of brain protein carbonyl (PC), a biomarker of oxidative stress, were quantified and compared between the groups.

PC levels were lower in the NCI+ARB group compared to the NCI group (median and (IQR) 10.6 (11.9–9.5) vs. 11.3 (13.8–10.5), NCI+ARB vs. NCI,  $p=0.035$ ) (Fig. 3A). In contrast, no significant difference in brain PC level was observed in the AD group treated with ARB (10.6 (13.3–9.6) vs. 10.9 (13.9–9.9), AD vs. AD+ARB,  $p=0.478$ ) (Fig. 3B).

Given the tight connection between chronic inflammation and cognitive decline, we examined the impact of ARB on the brain and systemic (circulating) inflammatory markers (IL-6, TNF- $\alpha$ , IL-1 $\beta$ , and IFN- $\gamma$ ). Our data show no significant difference in brain and serum cytokine levels between the NCI and AD groups, treated and not treated with ARB. However, brain  $AT_4R$  protein levels were negatively



correlated with brain IL-6 levels ( $r = -0.523$ ,  $p = 0.003$ ) (Fig. 4B) and serum IL-1 $\beta$  ( $r = -0.460$

$p=0.020$ ) in AD+ARB group (Fig. 4D). There was no significant correlation in other groups (Fig. 4A and C).

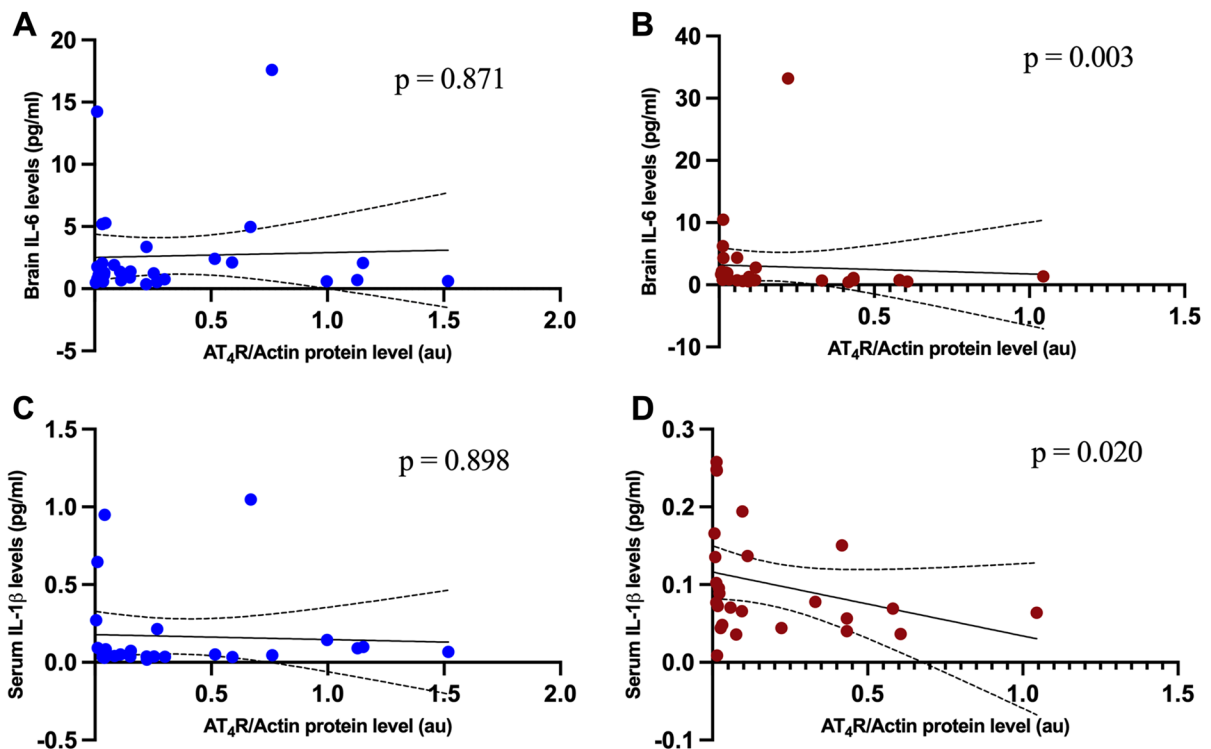
Treatment with angiotensin receptor blockers was associated with lower amyloid- $\beta$  burden in the NCI participants but no effect in AD participants

Next, we examined if the higher brain AT<sub>4</sub>R levels combined with lower oxidative stress in the ARB-treated NCI group or the lower brain AT<sub>1</sub>R levels in the ARB-treated AD group impacted brain amyloid- $\beta$  or tau pathologies. There were significantly lower calcarine cortex amyloid- $\beta$  and inferior temporal amyloid- $\beta$  scores in ARB-treated NCI group compared to the control untreated NCI group (1.92 (4.44–1.17) vs. 0.43 (2.39–0),  $p=0.011$ , 3.47 (8.04–1.61) vs. 1.09 (3.83–0),  $p=0.030$ , NCI vs.

NCI+ARB, respectively) (Fig. 5A and B). There were no significant differences in amyloid- $\beta$  in other regions of the brain, including hippocampus and entorhinal cortex compared to the control untreated NCI group (with 1.15 (2.04–0) vs. 0 (0.56–0),  $p=0.057$ , 3.30 (6.43–1.27) vs. 0.60 (3.58–0),  $p=0.051$ , NCI vs. NCI+ARB, respectively). Additionally, there were no significant difference in amyloid- $\beta$  in the AD groups, treated and untreated.

There were no significant differences in tangle density between treated and untreated participants in any group.

Despite the absence of significant difference in brain inflammation, oxidative stress, or amyloid- $\beta$  burden, our data shows that AD participants treated with ARB had higher semantic memory scores (mean  $\pm$  SD)  $-2.5 \pm 1.8$  vs.  $-1.3 \pm 0.9$ , AD vs. AD+ARB,  $p=0.013$ ) (Fig. 6A). Furthermore, the slope of semantic memory trajectory, adjusted for



**Fig. 4** Illustration of correlation between AT<sub>4</sub>R and cytokine levels. Scatter plot showing the correlation (and 95% confidence interval of the regression line) between levels of (A) AT<sub>4</sub>R and brain IL-6 ( $n=30$ ) ( $p=0.871$ ) in Alzheimer's dementia group. (B) AT<sub>4</sub>R and brain IL-6 ( $n=30$ ) ( $r=-0.523$   $p=0.003$ ) in Alzheimer's dementia group treated with angio-

tensin receptor blockers. (C) AT<sub>4</sub>R and serum IL-1 $\beta$  ( $n=26$ ) ( $p=0.898$ ) in Alzheimer's dementia group. (D) AT<sub>4</sub>R and serum IL-1 $\beta$  ( $n=26$ ) ( $r=-0.460$   $p=0.020$ ) in Alzheimer's dementia group treated with angiotensin receptor blockers. Spearman's correlation analysis was used



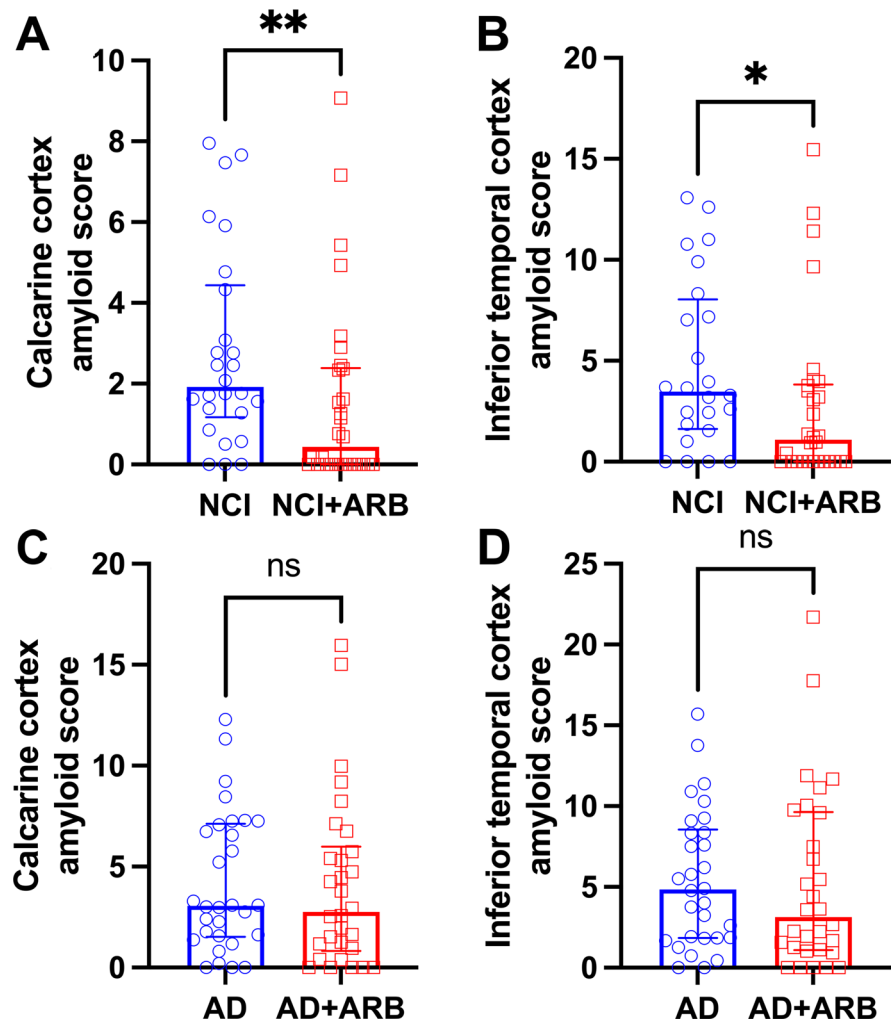
demographics (age, sex, and education level) as well as AD brain pathologies, was higher with ARB treatment ( $-0.06 \pm 0.13$  vs.  $0.007 \pm 0.06$ ), AD vs. AD+ARB,  $p=0.017$ ) (Fig. 6B).

Angiotensin receptor blockers act differently on brain angiotensin receptor levels in NCI and AD participants

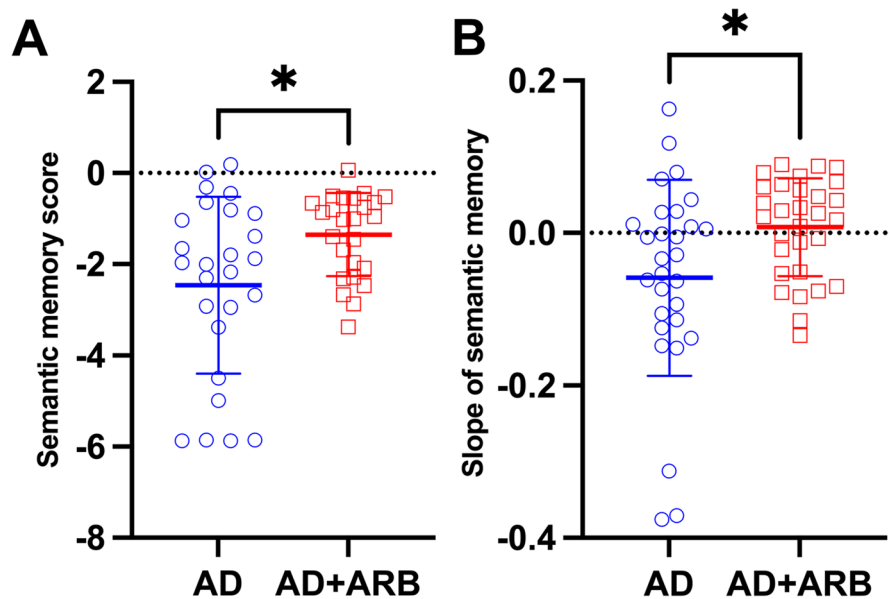
Finally, to determine if ARB treatment had differential effects in NCI group as compared to AD group, we performed a two-way ANOVA analysis for all four groups. There was a significant interaction effect between ARB use and disease status (AD vs. NCI) on angiotensin type 4 receptor level ( $F(1116)=9.349$ ,  $p=0.003$ ,  $\eta^2=0.075$ ). The significance of the

interaction term showed that the effect of ARB use on  $AT_4R$  level is different between the AD and NCI groups. In the follow-up simple effect analysis, while ARB use had a statistically significant effect on  $AT_4R$  level ( $p=0.014$ ) in NCI group, there was no significant effect in AD group ( $p=0.093$ ). Similarly, there was a significant interaction effect between ARB use and disease group on angiotensin type 1 receptor level ( $F(1116)=5.351$ ,  $p=0.022$ ,  $\eta^2=0.044$ ). In the follow-up simple effect analysis, ARB use had a significant effect on  $AT_1R$  level ( $p=0.017$ ) in AD group, with no significant changes in the NCI group ( $p=0.260$ ). There were no interaction or main effect of ARB treatment and disease state (AD vs. NCI) on angiotensin type 2 receptors.

**Fig. 5** Amyloid- $\beta$  scores in not cognitively impaired groups and Alzheimer's dementia groups. (A) Calcarine cortex amyloid- $\beta$  scores in not cognitively impaired (NCI) group treated ( $n=30$ ) and untreated ( $n=26$ ) with angiotensin receptor blockers (ARB). (B) Inferior temporal cortex amyloid- $\beta$  scores in not cognitively impaired (NCI) group treated ( $n=30$ ) and untreated ( $n=24$ ) with angiotensin receptor blockers (ARB). (C) Calcarine cortex amyloid- $\beta$  scores in Alzheimer's dementia (AD) group treated ( $n=30$ ) and untreated ( $n=30$ ) with ARB. (D) Inferior temporal cortex amyloid- $\beta$  scores in Alzheimer's dementia (AD) group treated ( $n=30$ ) and untreated ( $n=30$ ) with ARB. Data are presented with median and interquartile range. Comparisons were conducted using Mann-Whitney  $U$  test (ns, non-significant;  $*p \leq 0.05$ )



**Fig. 6** Semantic memory scores and slope of the semantic memory in Alzheimer's dementia group treated with angiotensin receptor blockers. **(A)** Semantic memory score in Alzheimer's dementia (AD) group treated ( $n=25$ ) and untreated ( $n=26$ ) with ARB. **(B)** Slope of the semantic memory in Alzheimer's dementia (AD) group treated ( $n=30$ ) and untreated ( $n=29$ ) with ARB. Data are presented with mean and standard deviation. Comparisons were conducted using Student's  $t$  test ( $*p < 0.05$ )



## Discussion

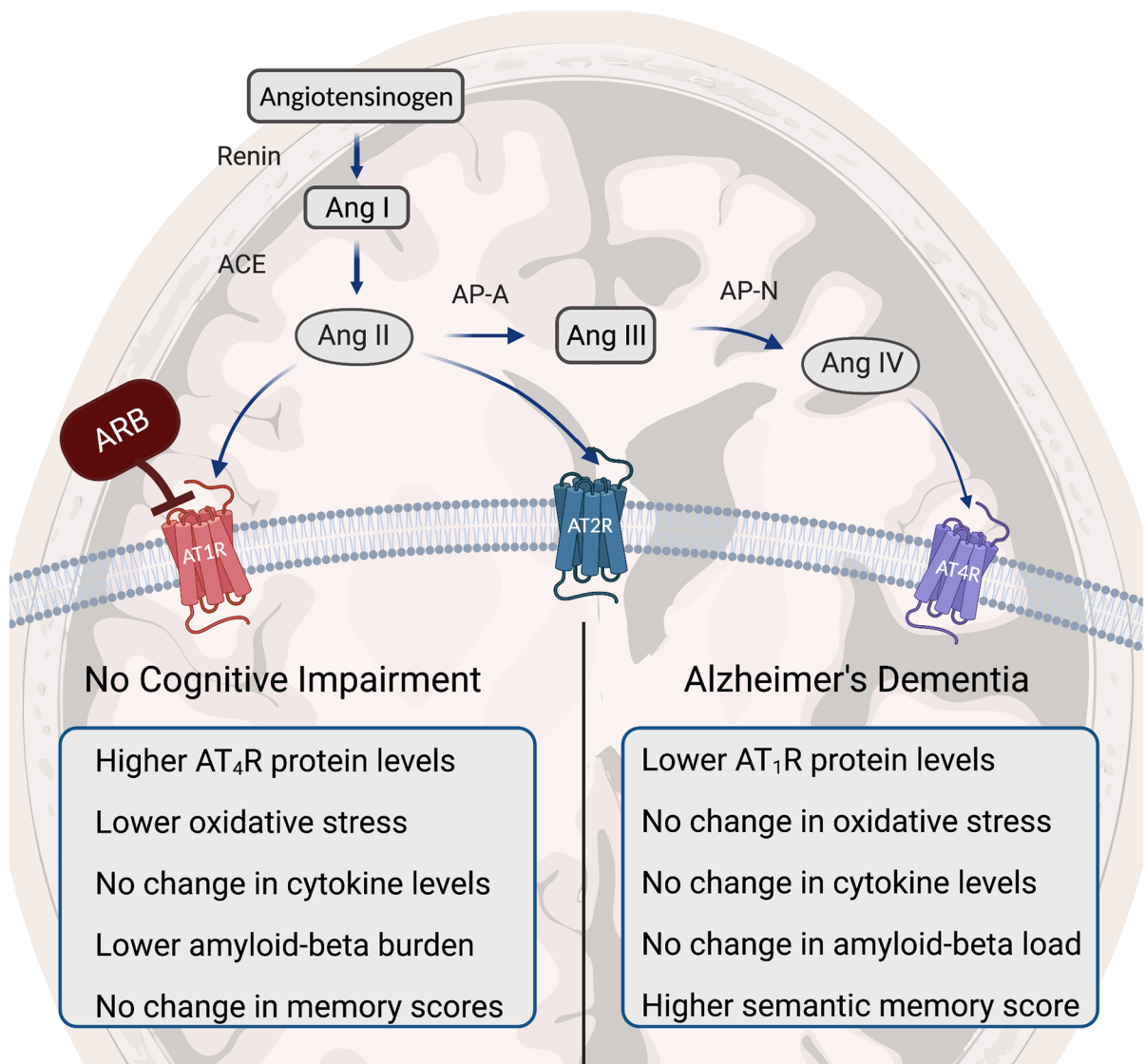
This study investigated b-RAS differences associated with ARB use in the postmortem brains of not cognitively impaired individuals and Alzheimer's dementia participants. In the NCI individuals who had used ARB, higher  $AT_4R$  protein levels, lower oxidative stress, and lower amyloid- $\beta$  scores were seen. However, we did not see any of these differences in comparing AD participants who had used and not used ARB. The only significant difference in angiotensin receptor expression that we saw in AD participants who had used ARB was lower  $AT_1R$  protein levels. Also, semantic memory scores were higher in AD participants using ARB. There was no difference in inflammation between both NCI and AD participants who had used and not used ARB (Fig. 7).

Few human studies examined the association of ARB use with changes in brain renin-angiotensin system receptors to the best of our knowledge. Our results demonstrate discordance between the impact of ARB on the brains of participants with normal cognition as compared to those with AD and a possible role for  $AT_4R$  in the NCI group.  $AT_4R$  is one of the recent members of the functional angiotensin receptor family, primarily found in cholinergic neurons of the cortex and hippocampus [9]. The  $AT_4R$  subtype is known for its beneficial effects on cerebral blood flow, neuroprotection, long-term potentiation,

and memory consolidation and retrieval [14]. Brazsko et al. suggested that Ang II is converted in the brain to Ang IV and that this conversion is responsible for the cognitive protective effects of Ang II in mice [37]. Our results are also buttressed by a prior study reporting that the beneficial effects of losartan, an angiotensin receptor blocker, are abolished by the concomitant blockade of  $AT_4R$  in mice [38].

Furthermore, our finding that ARB use was associated with lower oxidative stress levels in not cognitively impaired individuals is consistent with previous Alzheimer's disease animal models and in vitro studies [39–41]. It was recently shown that higher Ang IV levels are associated with lower oxidative stress [42].

Finally, our result that ARB use is associated with lower amyloid- $\beta$  levels in not cognitively impaired participants is supported by preclinical evidence suggesting the upregulation of enzymes responsible for the catabolism and clearance of amyloid- $\beta$  such as neprilysin and transthyretin with ARB use [43–47]. A direct association between ARB use and reduced amyloid- $\beta$  levels was shown in several animal studies [39, 44, 48, 49] and a human postmortem study [50]. A recent study reported an association between the use of ARB and ACEI and lower amyloid- $\beta$  accumulation, with superiority of ARB compared to ACEI [47]. The lack of beneficial effects in AD in our study is difficult to assess. It is also likely confounded by



**Fig. 7** Summary of the changes with angiotensin receptor blocker (ARB) treatment in both not cognitively impaired and Alzheimer's dementia participants. ACE, angiotensin-converting enzyme; Ang, angiotensin; ARB, angiotensin receptor

blocker; AP-A, aminopeptidase A; AP-N, aminopeptidase N; AT<sub>1</sub>R, angiotensin II type 1 receptor; AT<sub>2</sub>R, angiotensin II type 2 receptor; AT<sub>4</sub>R, angiotensin type 4 receptor

several factors, including the severity of AD, the time between diagnosis and starting ARB medication, and duration of treatment.

In contrast to previous literature, there were no anti-inflammatory effects of ARB in our data, although such anti-inflammatory properties have been shown in *in vitro* and animal studies for both Alzheimer's and non-Alzheimer's models [48, 51–53]. Small sample size, assay sensitivity, and protein

degradation in postmortem brains may have contributed to this negative result. Our data shows a negative correlation between AT<sub>4</sub>R and brain IL-6 and serum IL-1 $\beta$  only in ARB-treated AD participants, in agreement with a recent study that demonstrated lower levels of these cytokines with angiotensin IV infusion and reversal of this effects by divalinal-Ang IV (AT<sub>4</sub>R antagonist) in chronic cerebral hypoperfusion rat models [54].

There are several limitations to our study. First, we do not know the type of ARB used by study participants. This is particularly important since different ARB currently used in clinical setting have different blood–brain penetrability, affinity to the AT<sub>1</sub>R, and any additional non-angiotensin effects (for example activation levels of peroxisome proliferator-activated receptor gamma with losartan treatment) [5, 40, 55]. Second, the duration for which participants have used ARB is unknown, which precludes us from accounting for their time-dependent effects. Another limitation to our study is the low/lack of specificity of commercially available antibodies against AT<sub>1</sub>R and AT<sub>2</sub>R [56–58]. Until the development of more sensitive technology, western blot techniques using validated antibodies remain the cornerstone for measuring angiotensin receptors. As another limitation, in regard to western blot technique, we have not verified linearity of the protein band densities, because of the high variability between different human samples. Another limitation of our study is using protein carbonyl levels as a single marker of oxidative stress. We decided to use protein carbonyl levels as an oxidative stress marker in our study based on their common use, stability, parallelism with protein nitration in AD, and previous studies of the brain angiotensin system and neurodegenerative diseases [59–67]. However, there is a need for more comprehensive analysis of oxidative stress pathways that link to the development of AD. Finally, the small size limits our ability to control important confounding factors such as age, sex, hypertension, education level, and PMI. High blood pressure is a significant contributor to the development and progression of AD. Previous research has shown that higher levels of blood pressure are associated with higher levels of amyloid- $\beta$  [68–70]. Interestingly, our group analysis shows lower amyloid- $\beta$  levels in the NCI group treated with ARBs compared to the untreated NCI group, despite higher percentage of hypertension in the treated group.

As of today, there is no cure or effective prevention strategy for Alzheimer's dementia. AT<sub>1</sub>R-blockers have attracted the attention of the AD research community after epidemiological evidence favoring the use of ARB against AD for prevention and treatment [71, 72] and after the discovery of brain RAS, which has several effects on the central nervous system beyond well-known endocrine RAS hypertensive effects. Our results collectively suggest a role for

AT<sub>4</sub>R in mediating protective effects of ARB in not-cognitively impaired individuals. Further research is needed to understand better the impact of onset and duration of treatment with ARB in AD individuals.

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#### Declarations

**Conflict of interest** The authors declare no competing interests.

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