

1 **Higher Angiotensin I Converting Enzyme 2 (ACE2) levels in**  
2 **the brain of individuals with Alzheimer’s disease.**

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26 **Abstract**

27 The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a major cause of death in  
28 the elderly. Cognitive decline due to Alzheimer's disease (AD) is frequent in the geriatric  
29 population disproportionately affected by the COVID-19 pandemic. Interestingly, central nervous  
30 system (CNS) manifestations have been reported in SARS-CoV-2-infected patients. In this study,  
31 we investigated the levels of Angiotensin I Converting Enzyme 2 (ACE2), the main entry receptor  
32 of SARS-COV-2 in cells, in *postmortem* parietal cortex samples from two independent AD cohorts,  
33 totalling 142 persons. Higher concentrations of ACE2 protein and mRNA were found in individuals  
34 with a neuropathological diagnosis of AD compared to age-matched healthy control subjects. Brain  
35 levels of soluble ACE2 were inversely associated with cognitive scores ( $p = 0.02$ ), markers of  
36 pericytes (PDGFR $\beta$ ,  $p=0.02$  and ANPEP,  $p = 0.007$ ) and caveolin1 ( $p = 0.03$ ), but positively  
37 correlated with soluble amyloid- $\beta$  peptides (A $\beta$ ) concentrations ( $p = 0.01$ ) and insoluble phospho-  
38 tau (S396/404,  $p = 0.002$ ). No significant differences in ACE2 were observed in the 3xTgAD  
39 mouse model of tau and A $\beta$  neuropathology. Results from immunofluorescence and Western blots  
40 showed that ACE2 protein is mainly localized in neurons in the human brain but predominantly in  
41 microvessels in the mouse brain. The present data show that an AD diagnosis is associated with

42 higher levels of soluble ACE2 in the human brain, which might contribute to a higher risk of CNS  
43 SARS-CoV-2 infection.

44 **Key Words:** ACE2; Alzheimer's disease; Cognitive dysfunction.

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## 46 **Introduction.**

47 SARS-CoV-2 (Severe Acute Respiratory Syndrome CoronaVirus 2), the cause of Coronavirus  
48 disease 2019 (COVID-19), remains a significant public health concern. Epidemiological studies  
49 have shown that fatality rates increase with age, particularly after 65 years of age [40]. In terms of  
50 death and other clinical complications, people with dementia are disproportionately impacted by  
51 COVID-19 [14, 44, 62, 66, 86]. Whether this is due to age *per se* or other factors associated with  
52 cognitive decline is currently unknown.

53 Although the SARS-CoV-2 virus mainly infects lower respiratory and nasopharyngeal tracts,  
54 causing respiratory failure, CNS manifestations have also been described in more than one third of  
55 hospitalized patients, especially those with severe condition [14, 46, 49, 52, 61, 85, 86]. Non-  
56 specific neurological symptoms like headache and dizziness are also commonly observed in  
57 different cohorts [33, 49] but other neurological complications reported include ischaemic stroke  
58 [8], encephalopathy [34], meningo-encephalitis [34], demyelination [87], infarcts and  
59 microhaemorrhages [26, 48].

60 An association between COVID-19 infection and cognitive decline due to Alzheimer's disease  
61 (AD) or other causes is emerging. Risk factors for COVID-19 complications are often the same as  
62 those for dementia - age, obesity, cardiovascular disease, hypertension, and diabetes mellitus [44,  
63 63, 79]. Notably, dementia *per se* is a strong predictor of COVID-19 mortality [50]. Using de-

64 identified population-level electronic health records (EHR) from over 60 million individuals, a  
65 retrospective study showed that patients with dementia and COVID-19 had significantly worse  
66 outcomes (6-month hospitalization risk and mortality risk) than patients with dementia and no  
67 COVID-19 or patients with COVID-19 but no dementia [79]. This association remained significant  
68 after adjusting for age, sex and known COVID-19 risk factors (including, for example type 2  
69 diabetes, cardiovascular diseases, pulmonary diseases, asthma and others). Concerns have also  
70 been raised that COVID-19 increases the risk of developing cognitive impairments, possibly  
71 secondary to cerebral ischemia in brain areas linked to cognition [3, 21, 39, 54].

72 Angiotensin I Converting Enzyme 2 (ACE2) is a membrane carboxypeptidase considered the main  
73 site of entry of SARS-CoV-2 into cells [38, 45, 64]. ACE2 is highly expressed in the lung,  
74 consistent with respiratory dysfunction being the first clinical consequence of the infection [15,  
75 28]. Interestingly, ACE2 is expressed in other tissues such as the kidney, intestine, liver, testis and  
76 brain [36, 67]. The distribution of ACE2 in the brain is controversial, and original reports failed to  
77 identify the protein in the human CNS [19, 73]. Still, low levels of ACE2 mRNA were detected in  
78 the human brain using quantitative real-time RT-PCR [30]. Cerebral immunostaining was reported  
79 in endothelial and arterial smooth muscle cells [29], as well as in neurons [20]. More recently,  
80 single-cell RNA sequencing data have brought new insights on the cellular distribution of ACE2  
81 transcripts in the brain vasculature. According to the Betsholtz mouse database, the expression of  
82 ACE2 is very high in microvascular mural cells (such as pericytes and venous vascular smooth  
83 muscle cells), but not in endothelial cells [32, 57, 77]. However, other databases report ACE2  
84 mRNA expression in endothelial cells of mice [15, 88, 89]. So far, the available data suggest that  
85 the expression of ACE2 is lower in both endothelial cells and pericytes in the human brain  
86 compared to the mouse brain, albeit with important interregional variability [15, 31, 51, 84, 85]. In

87 previous outbreaks of SARS-CoV, which also use ACE2 as an entry point, the virus was detected  
88 in the brains of infected patients but reported almost exclusively in neurons [18, 29, 58, 71]. Based  
89 on the above evidence, ACE2 expression in cells forming the neurovascular unit could provide a  
90 means by which SARS-CoV-2 enters the CNS.

91 Here, to investigate whether ACE2 levels in the brain could be associated with cognitive  
92 dysfunction, we compared mRNA and protein levels of ACE2 in *postmortem* brain samples from  
93 individuals of two different cohorts, including subjects diagnosed with AD. In the first cohort from  
94 the Religious Order Study (n=60), ACE2 protein levels were evaluated according to (i) the clinical  
95 diagnosis of no cognitive impairment (NCI), mild cognitive impairment (MCI), or AD, (ii) the  
96 neuropathological diagnosis of AD (ABC scoring) and the *antemortem* assessment of cognitive  
97 function. Associations between ACE2 and neurovascular markers were also examined. In the  
98 second cohort from other US sources (n=82), the relationships between brain ACE2 and mRNA  
99 concentrations were investigated in individuals with a Braak-based neuropathological AD  
100 diagnosis. Finally, we compared the cellular localization of ACE2 between human and mouse  
101 brains and assessed ACE2 levels in a triple transgenic mouse model of AD neuropathology, the  
102 3xTg-AD mouse.

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## 109 **Materials and methods**

### 110 **Human samples**

#### 111 **Cohort #1**

112 Gray matter samples from the Brodmann area 7 (BA7) corresponding to the posterior parietal  
113 cortex were obtained from participants in the Religious Orders Study (*Rush Alzheimer's Disease*  
114 *Center*), an extensive longitudinal clinical and pathological study of aging and dementia [5, 6].  
115 Each participant enrolled without known dementia and underwent annual structured clinical  
116 evaluations until death. A total of 21 cognitive performance tests were performed for each subject.  
117 At the time of death, a neurologist, blinded to all *postmortem* data, reviewed clinical data and  
118 rendered a summary diagnostic opinion regarding the clinical diagnosis proximate to death.  
119 Participants thus received a clinical diagnosis of NCI ( $n = 20$ ) or MCI ( $n = 20$ ) or AD ( $n = 20$ ), as  
120 previously described [5-7]. The neuropathological assessment for the subjects included in the  
121 present study was performed using the ABC scoring method found in the revised National Institute  
122 of Aging – Alzheimer's Association (NIA-AA) guidelines for the neuropathological diagnosis of  
123 AD [56]. Three different neuropathological parameters were evaluated for each subject: (A) the  
124 Thal score assessing phases of A $\beta$  plaque accumulation [72], (B) the Braak score assessing  
125 neurofibrillary tangle pathology [13] and (C) the CERAD score assessing neuritic plaque pathology  
126 [55]. These scores were then combined to obtain an ABC score, reported as AX, BX and CX with  
127 X ranging from 0 to 3 for each parameter [56]. To determine a dichotomic neuropathological  
128 diagnosis in accordance with the revised NIA-AA guidelines [56], participants with intermediate  
129 or high levels of AD neuropathological markers were classified as AD, whereas participants with  
130 no or a low level of AD neuropathological changes were classified as Controls. Relevant data from  
131 the ROS samples used here are summarized in Table 1.

132 **Cohort #2**

133 Gray matter samples from the parietal cortex were obtained from 3 different institutions in the  
134 United States: 1- *Harvard Brain Tissue Resource Center*, Boston, Massachusetts, 2- *Brain*  
135 *Endowment Bank*, Miami, Florida. 3- *Human Brain and Spinal Fluid Resource Center*, Los  
136 Angeles, California [23]. All 82 parietal cortex samples were from the Brodmann area 39 (BA39),  
137 corresponding to the inferior region of the parietal cortex. Neuropathological diagnoses were based  
138 on Braak scores that were available for all cases. Braak scores of I or II were classified as Controls  
139 while Braak scores of III, IV, V and VI were considered as AD (Table 1).

140 **Immunostaining**

141 To demonstrate ACE2 localization in *postmortem* human brain tissue, we tested a number of  
142 commercially available antibodies using a wide range of immunostaining protocols.  
143 Immunostaining was performed on formalin-fixed, paraffin-embedded (FFPE) tissue sections (6  
144  $\mu\text{m}$ ) of human parietal cortex, on fresh-frozen (FF) tissue sections (12  $\mu\text{m}$ ) from human and mouse  
145 hippocampus, as well as on human and murine isolated brain microvessels (see below). Briefly,  
146 FFPE sections were deparaffinized in CitriSolv hybrid and rehydrated with decreasing  
147 concentrations of ethanol in water. Antigen retrieval was then performed by boiling slides in Tris  
148 buffer (10 mM, pH 9.0) with 1 mM EDTA and 0.05% (v/v) Tween-20 in a microwave for 15  
149 minutes and letting them cool for 30 minutes at room temperature. Sections were quenched with  
150 50 mM  $\text{NH}_4\text{Cl}$ , digested with trypsin 0.1% (w/v, Sigma-Aldrich) at 37°C for 15 min, and incubated  
151 in Tris-buffered saline (TBS) with 0.3 M glycine for 15 minutes. Sections were then blocked  
152 sequentially with Bloxall, avidin/biotin blocking kit (Vector Laboratories, CA) and Superblock  
153 (Thermo) with 0.2% Triton-X100, and used for immunohistochemistry. FF sections and brain  
154 microvascular fractions were kept at -80°C until use, then vacuum-dried at 4°C and fixed in 4%

155 (w/v) paraformaldehyde (pH 7.4) for 20 min at room temperature. All sections were then blocked  
156 and permeabilized for 1h with Superblock containing 0.2% (v/v) Triton X-100. Incubation with  
157 primary antibodies (various rabbit anti-ACE2, mouse anti-NeuN MAB377, goat anti-collagen IV  
158 AB789) was performed overnight at 4°C in Superblock with 0.05% Tween-20. For  
159 immunohistochemistry, after multiple washing in PBS, sections were incubated with biotinylated  
160 secondary antibodies (Jackson Immunoresearch) and then with streptavidin-HRP (ABC Elite kit).  
161 ACE2 localization was revealed using the ImmPACT AMEC red substrate and nuclei were  
162 counterstained with Mayer's hematoxylin. For immunofluorescence, after washes, secondary  
163 antibodies (conjugated to Alexa Fluor 555, 647 and 750, which use channels with less  
164 autofluorescence) were added to sections for 1h. Slides were then sequentially incubated with 4',6-  
165 diamidino-2-phenylindole (DAPI) and TrueBlack Plus (Biotium, CA). Photomicrographs were  
166 recorded with a Cytation 5 or EVOS fl Auto Imaging System (Thermo Fisher).

### 167 **Protein fractionation from human parietal cortex homogenates**

168 Each inferior parietal cortex sample (~100 mg) from the Cohort #1 was sequentially sonicated and  
169 centrifuged to generate two protein fractions: a Tris-buffered saline (TBS)-soluble fraction  
170 containing soluble intracellular, nuclear and extracellular proteins and a detergent-soluble protein  
171 fraction containing membrane-bound proteins extracted with a mix of detergents (0.5% Sodium  
172 dodecyl sulfate (SDS), 0.5% deoxycholate, 1% Triton), as previously reported [74, 75]. For  
173 samples of the Cohort #2, frozen extracts from the parietal cortex were ground into a fine powder  
174 on dry ice with a mortar and pestle. Approximately 50 mg of this fine powder was used for total  
175 protein extraction. Then, a lysis buffer (50 mM Tris-HCL to pH 7.4, 150 mM NaCl, 1% triton and  
176 0.5% sodium deoxycholate) containing protease (complete 25 X and Pepstatin A) and phosphatase  
177 inhibitors (sodium fluoride and sodium vanadate) was added in the proportion of 4 times the sample



178 weight (400  $\mu$ L for 50 mg). The sample solution was homogenized on ice by sonication using a  
179 Sonic Dismembrator (Fisher, Pittsburgh, PA) with two 10-second pulses and a 30-second stop  
180 between steps. Samples were centrifuged 20 minutes at 10,000 g at 4 °C. Protein contents of  
181 supernatants were quantified using a bicinchoninic acid assay (ThermoFisher cat: P123227). Protein  
182 homogenates in Laemmli were prepared as described below.

### 183 **Isolation of human brain microvessels**

184 The method used to generate microvessel-enriched extracts from frozen human parietal cortex  
185 samples has been described in our previous publications [10-12]. Briefly, this method consists of  
186 a series of centrifugation steps, including one density gradient centrifugation with dextran, after  
187 which the tissue is filtered through a 20- $\mu$ m nylon filter. Two fractions were generated: one  
188 enriched in cerebral microvessels (isolated microvessel-enriched fraction) and the other consisting  
189 of microvessel-depleted parenchymal cell populations. Cerebral fractions enriched and depleted in  
190 endothelial cells were evaluated using immunoblotting of vascular and neuronal markers, as shown  
191 previously [11]. Isolation of brain microvessels was performed on human samples of Cohort #1  
192 (final n = 57) and the fractions were used for immunostaining and Western blot analysis.

### 193 **RNA extraction and RT-qPCR analysis**

194 As mentioned above, powderized parietal cortex tissues of Cohort #2 were kept at -80°C.  
195 Approximately 100 mg of this fine powder was used for total RNA extraction with TRIzol  
196 (Ambion). Unless otherwise noted, all steps were performed on ice or at 4°C. Samples were  
197 homogenized by sonication with a Sonic Dismembrator (Fisher, Pittsburgh, PA) with 4-sec pulses  
198 in 500  $\mu$ L of TRIzol. Chloroform (100  $\mu$ L) was added to the solution, mixed and incubated for 2  
199 minutes before centrifugation at 12,000 g for 15 minutes. Supernatants were kept, 250  $\mu$ L of

200 isopropanol were added followed by a 10-min incubation at  $-80^{\circ}\text{C}$  and a 10-min centrifugation, at  
201 12,000 g. The pellet was resuspended in 500  $\mu\text{L}$  of ethanol 75 % and centrifuged 5 minutes at 7500  
202 g. The dried pellet was resuspended in 80  $\mu\text{L}$  RNase-free water and incubated 10 minutes at 57  
203  $^{\circ}\text{C}$ . The RNA concentration was measured with an Infinite F200 (Tecan). The reverse transcription  
204 (RT) was performed with 1  $\mu\text{g}$  of RNA. As a first step, genomic DNA was removed following the  
205 AccuRT Genomic DNA removal protocol (Applied Biological Materials, ABM, Vancouver,  
206 Canada). Then the RT master mix (ABM) was added to RNA samples and incubated (10 min at  
207  $25^{\circ}\text{C}$ , 50 min at  $42^{\circ}\text{C}$  and 5 min at  $85^{\circ}\text{C}$ ) as per the manufacturer's protocol. All qPCR experiments  
208 were performed on the LightCycler 480 (Roche) with the BrightGreen mix (ABM) and primers at  
209 10  $\mu\text{M}$ . After enzyme activation for 10 min at  $95^{\circ}\text{C}$ , 50 cycles were performed (15 sec at  $95^{\circ}\text{C}$  and  
210 1 min at  $60^{\circ}\text{C}$ ), followed by 1 sec at  $95^{\circ}\text{C}$  and 1 min at  $45^{\circ}\text{C}$ . Reference gene GAPDH (primers  
211 Forward: TCTCCTCTGACTTCAACAGCGAC and Reverse  
212 :CCCTGTTGCTGTAGCCAAATTC) was used to normalize the mRNA expression. The relative  
213 amounts of each transcript were calculated using the comparative Ct ( $2^{-\Delta\Delta\text{Ct}}$ ) method. For *Ace2*  
214 qPCR (primers Forward: GTGCACAAAGGTGACAATGG and Reverse:  
215 GGCTGCAGAAAGTGACATGA), 12 controls and 19 AD individuals were used. We used a cut-  
216 off of  $\geq 35$  cycles for these samples.

### 217 **Isolation of murine brain microvessels and protein fractionation**

218 Four (4) or six (6)-, 12- and 18-month-old 3xTg-AD (APP<sup>swe</sup>, PS1M146V, tauP301L) mice  
219 produced at our animal facility were used in equal numbers of males and females in each group.  
220 These mice show progressive accumulation of A $\beta$  plaques and neurofibrillary tangles which are  
221 detectable at 12 months and are widespread after 18 months [9, 16]. Mice from our colony were  
222 fed a standard chow (Teklad 2018, Harlan Laboratories, Canada) from breeding to 5 months of

223 age. For microvessels extraction, mice were then fed a control diet (CD; 20%kcal from fat) or a  
224 high-fat diet (HFD; 60%kcal from fat) from 6 to 18 months of age, in order to worsen  
225 neuropathology, memory performance and also induce metabolic impairments [4, 41, 68, 76],  
226 which are associated with a higher risk of developing severe SARS-CoV-2 infections [1, 40].

227 The protein extraction method results in a TBS-soluble fraction (intracellular and extracellular  
228 fraction), a detergent-soluble fraction (membrane fraction) as previously described [70].

229 Brain microvessels from 3xTg-AD mice were generated with a protocol similar to the one used for  
230 frozen human brain samples, as reported previously [10-12] (see Supplementary material). All  
231 experiments were performed in accordance with the Canadian Council on Animal Care and were  
232 approved by the Institutional Committee at the Centre Hospitalier de l'Université Laval (CHUL).

### 233 **Western blot analysis**

234 Protein homogenates from human parietal cortex and murine whole brain extracts were added to  
235 Laemmli's loading buffer and heated 10 min at 70°C. TBS- and detergent-soluble fractions from  
236 homogenates of human parietal cortex were also added to Laemmli's loading buffer and heated 5  
237 min at 95°C. Equal amounts of proteins per sample (8 µg for both human and murine brain  
238 microvessel extracts and 12 µg for protein homogenates of human parietal cortex, 15 µg for protein  
239 homogenates of mouse brain) were resolved by sodium dodecyl sulphate-polyacrylamide gel  
240 electrophoresis (SDS-PAGE). All samples, loaded in a random order, were run on the same  
241 immunoblot experiment for quantification. Proteins were electroblotted on PVDF membranes,  
242 which were then blocked during 1h with a PBS solution containing 5% non-fat dry milk, 0.5%  
243 BSA and 0.1% Tween-20. Membranes were then incubated overnight at 4°C with primary  
244 antibodies (rabbit anti-ACE2, #ab108252, 1:1000, rabbit anti-TMPRSS2 #ab109131, 1:1000).

245 Membranes were then washed three times with PBS containing 0.1% Tween-20 and incubated  
246 during 1h at room temperature with the secondary antibody (goat/donkey anti-rabbit HRP Jackson  
247 ImmunoResearch Laboratories, West Grove, PA; 1:60,000 or 1:10,000 in PBS containing 0.1%  
248 Tween-20 and 1% BSA). Densitometric analysis was performed using ImageLab (Bio-Rad).  
249 Uncropped gels of human samples immunoblot assays are shown in the Supplementary Material  
250 (Figure S5 and Figure S6).

### 251 **Data and statistical analysis**

252 An unpaired Student's t-test was performed when only two groups were compared, with a Welch  
253 correction when variances were not equal. If the data distribution of either one or both groups failed  
254 to pass the normality tests (Shapiro-Wilk test or Kolmogorov-Smirnov test), groups were compared  
255 using a non-parametric Mann-Whitney test. When more than two groups were compared,  
256 parametric one-way ANOVA followed by Tukey's multiple comparison tests or two-way ANOVA  
257 were used. If criteria for variance (Bartlett's) or normality were not met, non-parametric Kruskal-  
258 Wallis ANOVA followed by Dunn's multiple comparison tests were used. If needed, data were log  
259 transformed to normalize distributions. For all data, statistical significance was set at  $P < 0.05$ .  
260 Individual data were excluded for technical reasons or if determined as an outlier using the ROUT  
261 (1%) method in GraphPad Prism. Statistical analysis was done by Pearson correlation to analyze  
262 the correlation between ACE2, *antemortem* evaluation and different proteins. All statistical  
263 analyses were performed with Prism 9 (GraphPad, San Diego, CA, USA) or JMP (version 16; SAS  
264 Institute Inc., Cary, IL) software.

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266

## 267 **Results**

### 268 **Association between ACE2 in the parietal cortex, the neuropathological diagnosis of AD and** 269 **cognitive scores.**

270 Table 1 summarizes the clinical and biochemical data from Cohorts #1 (Religious Order Study,  
271 ROS) and #2 (Other US sources). ACE2 proteins from parietal cortex samples of each subject were  
272 fractionated into TBS-soluble (cytosolic, extracellular, nuclear and secreted proteins), detergent-  
273 soluble (membrane-bound proteins) and microvessel-enriched fractions (vascular proteins).  
274 Representative Western immunoblots of ACE2 and analyses are shown in Figure 1 for Cohort #1  
275 and Figure 2 for Cohort #2. A band migrating at approximately 100 kDa, corresponding to full-  
276 length ACE2, was observed in each fraction (Figure 1A,E,I, Figure 2A).

277 We first evaluated whether ACE2 protein levels in extracts from the parietal cortex from 60  
278 individuals from the Cohort #1, ROS. When the subjects were classified according to the  
279 neuropathological ABC diagnosis, higher levels of ACE2 protein were found in TBS-soluble  
280 fractions from AD subjects compared to non-AD participants ( $p = 0.0087$ ) (Figure 1C). When the  
281 subjects were classified according to the clinical diagnosis, only a non-significant trend towards  
282 higher ACE2 concentrations was observed in the TBS-soluble fraction, using a non-parametric  
283 Kruskal-Wallis ANOVA ( $p = 0.1471$ ) (Figure 1B). However, the difference between AD and  
284 Controls was statistically significant when this comparison was performed only in individuals with  
285 parenchymal cerebral amyloid angiopathy ( $p = 0.0022$ ) (pCAA; Figure S1). On the other hand,  
286 ACE2 levels assessed in the detergent-soluble fraction, enriched for membrane-associated proteins,  
287 remained similar between groups (Figure 1E-G). We next measured ACE2 protein levels in  
288 microvessel extracts. Given the high interindividual variability, only a non-significant trend

289 towards increased ACE2 protein levels was observed in individuals with an AD clinical diagnosis  
290 ( $p = 0.1712$ ) (Figure 1J, -K).

291 Interestingly, the levels of ACE2 found in TBS-soluble and microvessel-enriched fractions were  
292 inversely associated with *antemortem* global cognitive scores (Figure 1D, L and Figure 3). This  
293 association remained significant after adjustment for age at death and sex.

294 To corroborate these results, we performed Western immunoblots on a second series of human  
295 brain samples from the Cohort #2 [23] (Figure 2A). Consistently, higher levels of ACE2 protein  
296 were detected in individuals with a neuropathological diagnosis of AD (Figure 2B). In addition,  
297 *Ace2* mRNA levels were significantly higher in individuals with a Braak-based diagnosis of AD  
298 compared to Controls (Figure 2B), suggesting a regulation at the transcriptional level. No  
299 difference was observed in the levels of transmembrane protease serine type 2 (TMRPSS2) (Figure  
300 S2), a protein that plays a key role in SARS-CoV-2 infection by activating the spike protein,  
301 facilitating entry into target cells using ACE2 [38].

302 **TBS-soluble ACE2 is positively associated with clinical, neuropathological, and vascular**  
303 **markers of AD, while detergent-soluble ACE2 displays opposite trends**

304 Hierarchical clustering of correlation coefficients (strength of association) was performed to  
305 identify variables associated with differences in ACE2 in Cohort #1. Although the leading risk  
306 factor for AD is age, no significant correlation was found between ACE2 levels in all fractions  
307 tested and the ages of death (Figure 3, Figure S3), which were equivalent between groups (Table  
308 1). These observations suggest that the greater soluble ACE2 in individuals with AD in the ROS  
309 cohort was not driven by age. However, the age interval (74-98 years) was too small to detect an  
310 effect of aging *per se* on cerebral ACE2. Beside the inverse association with global *antemortem*

311 cognitive scores of participants ( $r^2 = -0.09$ ,  $P < 0.05$ , Figure 1D and Figure 3), higher *postmortem*  
312 TBS-soluble concentrations of ACE2 were also significantly correlated with failing episodic  
313 memory, a domain predominantly affected in AD (Figure 3).

314 Associations were then examined with neuropathological markers of AD, previously assessed in  
315 the parietal cortex from the sample series (Figure 3). TBS-Soluble ACE2 levels were positively  
316 associated with markers that are greater in AD like diffuse plaque counts, soluble A $\beta$  levels and  
317 insoluble phospho-tau (pS396/404 epitope) (Figure 3). In contrast, levels of detergent-soluble  
318 ACE2 were negatively associated with the insoluble phosphorylated form of TDP-43 (which is  
319 higher in AD [12]) but positively with soluble phospho-TDP-43 C-terminal fragment migrating at  
320 approximately ~35 kDa (which is lower in AD [12]) and soluble tau (Figure 3). Similarly,  
321 neurovascular proteins such as platelet-derived growth factor receptor  $\beta$  (PDGFR $\beta$ ), ABCB1 and  
322 Caveolin1 correlated positively with membrane-bound ACE2 but inversely with TBS-soluble and  
323 vascular forms of ACE2, which in turn correlate with  $\beta$ -secretase 1 (BACE1) and Advanced  
324 glycosylation end product-specific receptor (RAGE), proteins involved in the formation and  
325 accumulation of A $\beta$  (Figure 3).

326 Single-cell RNA sequencing data in the mouse and human brain show that ACE2 mRNA  
327 expression is enriched in pericytes [31, 57], and PDGFR $\beta$ , a pericyte marker, is reduced in AD [12,  
328 53]. Here, we found that microvascular PDGFR $\beta$  and Aminopeptidase N (ANPEP) levels were  
329 negatively correlated with TBS-soluble ACE2 levels but were positively associated with detergent-  
330 soluble ACE2 levels (Figure 3, Figure S3), suggesting a possible release of ACE2 from membranes  
331 linked with pericyte-related dysfunctions at the blood-brain barrier (BBB).

332 Together, these results suggest that the elevation of ACE2 in TBS-soluble and, to a lesser extent,  
333 in microvessel fractions are associated with more advanced A $\beta$  and tau pathologies and with a

334 pattern of changes in vascular proteins consistent with AD progression. By contrast, membrane-  
335 bound ACE2 exhibited opposite trends and was strongly associated with reduced TDP-43  
336 proteinopathy and consolidated BBB markers.

### 337 **ACE2 is observed in human and murine neurons and cerebral vessels**

338 Localizing ACE2 within the neurovascular unit at the interface between the blood and the brain  
339 provides basic information about SARS-CoV-2 penetration into the CNS. Therefore, we sought to  
340 determine whether ACE2 protein was enriched in brain microvessel extracts compared to post-  
341 vascular parenchymal fractions and unfractionated homogenates from human (parietal cortex) and  
342 mouse (whole brain) samples (Figure 4). We found a strong enrichment of ACE2 in murine  
343 microvessels, along with endothelial (Claudin5) and pericyte (PDGFR $\beta$ ) markers (Figure 4F).  
344 However, in human brain samples, ACE2 protein levels were more comparable between  
345 microvessel and parenchymal fractions, the latter being enriched in the neuronal marker  
346 (synaptophysin) (Figure 4A). Thus, these Western blot results suggest that the localization of ACE2  
347 in the brain differs between both species, with a cerebrovascular predominance in the mouse not  
348 observed in humans.

349 To confirm the cellular localization of ACE2, immunostaining was also performed on fractionated  
350 brain homogenates (Figure 4B-E, G, H). A moderate immunofluorescent signal was detected inside  
351 microvessels isolated from human brains (collagen IV-positive, Figure 4B, C) and NeuN-positive  
352 neurons (Figure 4C, E). By contrast in the mouse, ACE2 immunosignal was intense in microvessels  
353 and colocalized well with collagen IV (Figure 4G, H). To validate immunostaining in human tissue  
354 sections, nine anti-ACE2 antibodies were used in human testis samples where ACE2 is highly  
355 expressed in Leydig and Sertoli cells (Figure S4). All antibodies showed a clear signal in this tissue,  
356 but detection of ACE2 in the human brain, where levels are at least 20 times lower (results not



357 shown), was challenging. In human hippocampal sections, ACE2 was detected in NeuN-positive  
358 neurons, particularly in large ones staining weakly for DAPI and in small ones with a strong DAPI  
359 signal (Figure 5A, B). In sections of human parietal cortex, ACE2 detection was also more  
360 prominent in neuron-like cells (Figure 5C, D, E). On the other hand, in mouse sections, ACE2  
361 staining was more intense in the cerebrovasculature and colocalized neatly with PDGFR $\beta$ ,  
362 indicating an expression in mouse pericytes (Figure 5F-H). These results are consistent with  
363 Western blot data, showing that ACE2 can be detected in neuron-like cells in the human brain,  
364 whereas it is concentrated in cerebrovascular cells in the mouse.

### 365 **Microvascular and whole-brain ACE2 protein levels are not altered in a mouse model of AD**

366 To probe whether changes in ACE2 could be a consequence of classical tau and A $\beta$   
367 neuropathology, we used the triple transgenic mouse model of AD (3xTg-AD) [59], which  
368 develops A $\beta$  plaques and neurofibrillary tangles by 12 months of age. We quantified ACE2 protein  
369 levels in both 3xTg-AD and non-transgenic mice from two different cohorts: (i) mice of 4 or 6, 12,  
370 and 18 months of age, (ii) and 18-month-old mice fed either a control or a HFD that exacerbates  
371 neuropathology [76] (Figure 6). No significant change was observed in protein levels of ACE2 in  
372 TBS-soluble or detergent-soluble fractions according to genotype and age (Figure 6A). Similarly,  
373 ACE2 in cerebrovascular fractions did not vary according to genotype, age, and diet (Figure 6B).  
374 These results suggest that the development of human tau and A $\beta$  neuropathology in mice is  
375 insufficient to increase murine ACE2 levels, even when combined with aging and HFD, two risk  
376 factors for AD and COVID-19 infection.

### 377 **Discussion**

378 This present *postmortem* study investigated ACE2 concentrations in the brain of individuals with  
379 AD from two different cohorts. We assessed ACE2 protein levels in all subjects and mRNA  
380 expression in a subset. We observed a significant relationship between ACE2 levels, the  
381 neuropathological diagnosis of AD, and *antemortem* cognitive evaluation. Overall, our data  
382 indicate that (1) levels of TBS-soluble ACE2 in the parietal cortex were higher in persons with AD  
383 when compared to control subjects, accompanied by an elevation in ACE2 mRNA transcripts; (2)  
384 lower cognitive scores were associated with higher levels of ACE2 in TBS-soluble and  
385 cerebrovascular fractions; (3) an apparent transfer of ACE2 from membranes to soluble  
386 compartment was associated with pericyte loss and other markers of AD progression; (4) ACE2  
387 levels remained unchanged in an animal model of AD-like neuropathology; (5) whereas ACE2 was  
388 concentrated in microvessels in the mouse brain, it was predominantly located in neurons in the  
389 human brain. Such a series of observations highlight that an AD diagnosis is associated with higher  
390 levels of specific forms of ACE2 in the brain, which might contribute to the higher risk of SARS-  
391 CoV-2 CNS infection in cognitively impaired individuals.

392

393 **Higher levels of soluble ACE2 are associated with AD and cognitive decline.**

394 The present observation of higher levels of soluble ACE2 in AD is in agreement with a previous  
395 report using a limited number of hippocampal samples of AD subjects (n = 13) compared to  
396 Controls (n = 5) [17]. Furthermore, preliminary human brain microarray data mentioned in a letter  
397 to the Editor also cite higher ACE2 expression levels in AD patient [47]. Although an association  
398 between SARS-CoV-2 infection and cognitive impairment has been previously evidenced at the  
399 population level [79] and hinted by genetic studies [25, 42], a significant correlation between ACE2  
400 in the brain and cognitive scores has not been reported previously.

401 Several mechanisms could explain the higher levels of ACE2 in AD. Since old age increases the  
402 risk of infection with SARS-CoV-2 and developing cognitive decline and AD, we could have  
403 expected an association between cerebral ACE2 levels and advanced age. However, no correlation  
404 between age and ACE2 could be evidenced here in both human and mouse samples, suggesting  
405 that changes in TBS-soluble ACE2 are not directly related to age but rather to AD pathology and  
406 cognitive decline. This is consistent with health records data showing that dementia is associated  
407 with a higher risk for COVID-19, independently of age [79]. Indeed, a recent network analysis  
408 suggest that AD and COVID-19 share defects in neuroinflammation and microvascular injury  
409 pathways [90]. Second, the increase in ACE2 could be a consequence of the AD neurodegenerative  
410 process and/or of potential compensatory mechanisms in response to AD neuropathology, which  
411 could include an increase in gene transcription, which is consistent with the higher mRNA  
412 expression measured in AD samples. However, data gathered in 3xTg-AD mice do not fully  
413 support that hypothesis. Indeed, the accumulation of human A $\beta$  peptides and hyperphosphorylated  
414 tau in this mouse model did not lead to changes in murine ACE2 protein levels, suggesting that an  
415 ACE2 increase is not a consequence of classical AD neuropathology. Nevertheless, it should be  
416 reminded that such a mouse model displays an amount of A $\beta$  and tau 1 to 3 logs lower than what  
417 is typically found in an AD brain. Moreover, ACE2 pathways may simply be regulated differently  
418 in the mouse, as suggested by the different localization of the protein in the murine brain.

419 ACE2 is part of the renin angiotensin system (RAS), which regulates the vascular system. An  
420 increase of cerebral ACE2 may impact the brain RAS, thereby affecting blood flow, arterial  
421 pressure, neuroinflammation and, consequently, brain function. Such a dysregulation of the RAS-  
422 equilibrium in the brain could contribute to the aetiology of several neurodegenerative diseases,  
423 including AD [2, 80, 81]. For example, in a cohort study including community-dwelling older

424 adults with mild to moderate AD, the use of ACE inhibitors (ACEi) was associated with a slower  
425 cognitive decline, independent from their antihypertensive effects [69]. ACEi and angiotensin II  
426 receptor blockers (ARBs) are also under investigation to improve cognitive impairment associated  
427 with AD [24, 27, 37, 65]. Moreover, twice higher soluble ACE2 has been reported in the  
428 cerebrospinal fluid of hypertensive patients [83]. In our study, we did not detect any association  
429 with the use of drugs acting on ACE, such as ARBS or ACEi, and brain levels of ACE2, but the  
430 study was not designed for that purpose. However, it is important to note that the levels of ACE2  
431 detected by immunoblotting may not directly inform on ACE2 activity. Indeed, a *postmortem*  
432 assessment of ACE2 enzymatic activity with a fluorogenic assay instead showed a reduction in AD  
433 [43]. Studies in animals indicate that pharmacological activation of ACE2 rather reduces  
434 hippocampal soluble A $\beta$  and reverses cognitive impairment in the Tg2576 model of A $\beta$   
435 neuropathology [22].

436 Another peculiar observation is the difference between ACE2 found in soluble fractions containing  
437 cytoplasmic/extracellular proteins *versus* ACE2 retrieved in detergent-soluble fractions containing  
438 membrane-bound proteins. Overall, ACE2 in TBS-soluble fractions was higher in subjects with  
439 AD, while no such trend was observed with ACE2 located in membranes. Moreover, the correlation  
440 between ACE2 and AD-relevant markers, most notably the pericyte markers PDGFR $\beta$  and others  
441 like ANPEP, differed significantly between the two fractions. The strong inverse association with  
442 TDP-43 pathology was also limited to detergent-soluble ACE2. Previous studies did not distinguish  
443 TBS-soluble *versus* detergent-soluble ACE2 [17, 47]. Although ACE2 is generally considered a  
444 membrane protein, its actual attachment to the cytoplasm membrane is relatively weak. For  
445 example, the ACE2 ectodomain can be cleaved by ADAM17 or TMPRSS2 and released in the  
446 cytoplasm [35, 91]. Recent studies report that a decrease in active membrane-bound ACE2 due to

447 ADAM17 and TMPRSS2 overactivation could be deleterious for SARS-CoV-2-infected patients  
448 [35, 60, 82]. However, we did not observe differences in mRNA and protein levels of TMPRSS2.  
449 Vascular ACE2 was also specifically measured in this study. Despite associations with cognitive  
450 scores and PDGFR $\beta$  levels, no significant difference was detected between groups, possibly due to  
451 the interindividual variability induced by the separation process. An intriguing possibility  
452 explaining the higher content in ACE2 specific in the TBS fraction, as detected with an antibody  
453 targeting the N-terminal extracellular domain, could be an enhanced release of ACE2 from the  
454 membrane to the cytosol or the extracellular parenchyma in AD, also termed ACE2 shedding [35,  
455 78]. Such a detachment of ACE2 from cell membranes may be a pathological phenomenon  
456 associated with AD, warranting further study.

457 The present work also unveils additional information on the cellular localization of ACE2 in the  
458 human brain. While we observed an enrichment of ACE2 in mouse microvessels, such was not the  
459 case in human samples. ACE2 was detected in human samples using Western blot and RT-qPCR,  
460 but a clear immunofluorescence signal was more challenging to achieve. Unlike in the mouse,  
461 where the enrichment in microvessels was evident, the detection of ACE2 in human brain  
462 capillaries became apparent only after microvascular fractionation. However, ACE2 was clearly  
463 present in neurons in human brain sections, corroborating Western blot results. Nonetheless, it  
464 should be noted that brains from mice were harvested quickly after transcardial perfusion. On the  
465 other hand, human brain tissue underwent *premortem* and *postmortem* events, which may have  
466 affected ACE2 distribution and detection. In sum, the present data obtained using several different  
467 antibodies indicate that the cerebral distribution of ACE2 is less strictly vascular, more neuronal in  
468 humans compared to the mouse. At the very least, work related to human ACE2 but performed in

469 mouse models should be interpreted with caution regarding their possible application to the brain  
470 RAS, AD and other neuropathologies, as well as central SARS-CoV-2 infection in humans.

## 471 **Conclusions**

472 In summary, the present data show an accumulation of the soluble form of ACE2 associated with  
473 cognitive decline in individuals with a neuropathological diagnosis of AD. ACE2 levels were not  
474 influenced by age or biological sex. We also observed a strong association between soluble ACE2  
475 levels and AD neuropathology, as well as pericyte loss. The search for molecular cues that cause a  
476 rise of TBS-soluble ACE2 and regulate the brain RAS in AD subjects may ultimately lead to the  
477 discovery of new therapeutics to prevent cognitive decline and AD.

## 478 **List of Abbreviations**

479 ACEi = Angiotension I Converting Enzyme Inhibitors; ACE2 = Angiotensin I Converting Enzyme  
480 2; AD = Alzheimer's disease; ARBs = Angiotensin II receptors blockers; BA = Brodmann area;  
481 BBB = Blood brain barrier; CD = control diet; CNS = Central Nervous System; COVID-19  
482 =Coronavirus disease 2019; DAPI = 4',6-diamidino-2-phenylindole; EHR = electronic health  
483 records; FFPE = formalin-fixed, paraffin-embedded; GWAS = genome wide associations study;  
484 HRP = horseradish peroxidase; HFD = High fat diet; MCI = Mild-cognitive impairment; NIA-AA  
485 = National Institute of Aging – Alzheimer's Association NCI = No cognitive impairment; O.D. =  
486 Optical density; RBD = Receptor Binding Domain; NHS = Normal Horse Serum; PBS =  
487 phosphate-buffered saline; pCAA = parenchymal cerebral angiopathy amyloid; PDGFR $\beta$  =  
488 Platelet-derived growth factor receptor  $\beta$ ; ROS = Religious Order Study; SARS-CoV-2 =Severe  
489 Acute Respiratory Syndrome CoronaVirus 2; SDS-PAGE = sodium dodecyl sulphate-

490 polyacrylamide gel electrophoresis; TBS = Tris-Buffered Saline; Tmprss2 = Transmembrane  
491 protease serine 2.

## 492 **Declarations**

### 493 **Ethics approval and consent to participate**

494 All procedures performed with volunteers included in this study were in accordance with the ethical  
495 standards of the institutional ethics committees and with the 1964 Helsinki Declaration. Written  
496 informed consent was obtained from all individual participants included in this study. All  
497 procedures relating to mouse care and experimental treatments were approved by the Laval  
498 University animal research committee (CPAUL) in accordance with the standards of the Canadian  
499 Council on Animal Care.

### 500 **Consent for publication**

501 Not applicable.

### 502 **Availability of data and material**

503 The datasets analysed during the current study available from the corresponding author on  
504 reasonable request. Data from the ROS can be requested at <https://www.radc.rush.edu>.

### 505 **Competing interests**

506 The authors report no competing interests.

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## 512 **Authors' contributions**

513 LR, VE, SH, and FC designed the study. LR, ML, VE, AL, PB, and CT performed experiments.  
514 DAB provided ROS samples. LR and FC analyzed data. LR, VE and FC wrote the first drafts of  
515 the paper.

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## 520 **Supplementary material**

521 Supplementary material is available online.

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## 818 Legends

819 **Figure 1: Levels of TBS-Soluble ACE2 protein are higher in AD individuals and are**  
820 **negatively correlated with global cognitive score.** Parietal cortex levels of ACE2 protein from  
821 Cohort #1 were determined by Western Blot in three fractions: a TBS-soluble fraction (A-D), a  
822 detergent-soluble fraction (E-H) and a microvessel-enriched fraction (I-L). No statistical difference  
823 was detected for ACE2 in the three fractions when subjects were classified according to  
824 *antemortem* clinical diagnosis (B, F, J). Levels of the ACE2 protein were higher in the TBS-soluble  
825 fraction in individuals with a neuropathological diagnosis of AD based on ABC scoring (C, G, K).  
826 TBS-soluble ACE2 and microvascular ACE2 levels were negatively correlated with the global  
827 cognitive score (D, L). An equal amount (12 µg) of proteins per sample for both TBS-soluble and  
828 detergent soluble fractions was loaded and 8 µg of proteins per sample was loaded for microvessel-  
829 enriched fractions. All samples, loaded in a random order, were run on the same gel and transferred  
830 on the same membrane before immunoblotting for quantification. Examples were taken from the  
831 same experiment, and consecutive bands loaded in random order are shown. Actin and cyclophilin  
832 B are shown as loading controls. Data are represented as a scatterplot. Horizontal lines indicate  
833 mean ± SEM. Statistical analysis: Mann-Whitney test \*\*p < 0.01, Coefficient of determination &p  
834 < 0.05. *Abbreviations: ACE2, Angiotensin-Converting Enzyme 2; A/AD, Alzheimer's disease; C,*  
835 *control; Clin Dx, clinical diagnosis; ABC Dx, ABC neuropathological diagnosis; CyB, cyclophilin*  
836 *B; M/MCI, mild cognitive impairment; N/NCI, healthy controls with no cognitive impairment;*  
837 *O.D., optical density; TBS, Tris-Buffered Saline.*

838 **Figure 2: Higher ACE2 protein and mRNA levels in parietal cortex of AD participants from**  
839 **the second cohort.** AD subjects from Cohort #2 had higher levels of ACE2 protein and mRNA  
840 compared to controls. Diagnosis was determined using Braak staging. ACE2 levels were  
841 determined by Western-Blot and qPCR analysis (A, B). Statistical analysis: Mann-Whitney test \*p  
842 < 0.05, Unpaired t-test \*\*p < 0.01. All samples, loaded in a random order, were run on the same  
843 immunoblot experiment for quantification. Examples were taken from the same immunoblot  
844 experiment, and consecutive bands loaded in random order are shown. GAPDH is shown as a  
845 loading control. Data are represented as a scatterplot. Horizontal lines indicate mean ± SEM.  
846 *Abbreviations: A, Alzheimer's disease (Braak scores III-VI); ACE2, Angiotensin-Converting*  
847 *Enzyme 2; C, control (Braak scores I or II); Dx, diagnostic; O.D., optical density.*

848 **Figure 3: ACE2 protein in TBS-soluble/microvascular protein fractions show opposite**  
849 **relationships with detergent-soluble ACE2 when correlating with AD markers.** Heat-map of  
850 hierarchical clustering analysis of correlation coefficients from partial correlation analyses with  
851 *antemortem* evaluation, neuropathological markers (soluble and insoluble proteins of the parietal  
852 cortex) and BBB markers (microvascular fractions of the parietal cortex). The significance of the  
853 correlation (inverse in blue, positive in red) between two elements is entered in the associated box.  
854 Statistical analysis: Coefficient of determination \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001. All  
855 proteins presented in these heat-maps were determined by Western-blot analysis except Aβ  
856 peptides which were quantified using ELISA. Total soluble and insoluble Tau was detected using  
857 Tau (640-680) antibody. AD2 antibody recognized Tau phosphorylated at S396. Phosphorylated  
858 TDP43 antibody recognized at pSer409/410. *Abbreviations: ABCB1, ATP Binding Cassette*

859 *Subfamily B Member 1; ACE2, Angiotensin-Converting Enzyme 2; ANPEP, Aminopeptidase N;*  
860 *BBB, blood-brain barrier; BACE1, Beta-Secretase 1; CD31 or PECAM1, Platelet endothelial cell*  
861 *adhesion molecule; LRP1, Low density lipoprotein receptor-related protein 1; NS, non*  
862 *significant; PDGFR $\beta$ , Platelet Derived Growth Factor Receptor Beta; RAGE, Receptor for*  
863 *Advanced Glycation Endproducts;  $\alpha$ SMA, alpha smooth muscle actin; TDP-43, TAR DNA binding*  
864 *protein 43; TBS, Tris-Buffered Saline.*

865 **Figure 4: In fractionated brain homogenates, ACE2 immunosignal is predominantly**  
866 **observed in neurons in human samples and in the vasculature in mice.** (A, F) Immunoblotting  
867 detection of ACE2 in human (parietal cortex) (A) and mouse (whole brain)(F) vascular fractions  
868 “Va”, compared to postvascular parenchymal samples depleted in vascular cells “P” and total  
869 unfractionated homogenates “T”. For comparison purposes, synaptophysin (a synaptic/neuronal  
870 marker), Claudin5 (endothelial marker), and PDGFR $\beta$  (pericyte marker), are also shown. In the  
871 mouse brain, ACE2 is highly enriched in microvessels compared to the postvascular fraction,  
872 different to what is observed in the human (A, F). (B-E, G, H) Representative immunostaining of  
873 ACE2 (green) in human (B-E) and murine cerebrovascular fractions (G, H), with collagen IV  
874 (endothelial marker) in red (B, D, G, H) or blue (C, E), as well as NeuN (neuronal marker) in red  
875 (C, E) and DAPI (nuclei) in blue (B-H). In human samples, moderate ACE2 staining is observed  
876 in neurons, whereas vascular ACE2 staining is strong in mice. Red arrows point to ACE2+/NeuN+  
877 cells, blue arrows to ACE2+/NeuN- cells, and green arrows to erythrocytes. ACE2 antibodies: rb  
878 mAb #ab108252 (A, F), rb pAb #HPA000288 (B, D, G) and #35-1875 (C, E, H). Scale bar: 10  $\mu$ m.  
879 *Abbreviations: ACE2, Angiotensin-Converting Enzyme 2; Coll IV, Collagen IV; Coloc,*  
880 *Colocalization; PDGFR $\beta$ , Platelet Derived Growth Factor Receptor Beta. mAb, monoclonal*  
881 *antibody; pAb, polyclonal antibody.*

882 **Figure 5: In tissue sections, ACE2 immunostaining is predominantly observed in neurons in**  
883 **the human brain and in the cerebrovasculature in mice.** (A-E) Representative immunostaining  
884 of ACE2 (green or red) in fresh frozen human hippocampus (A, B), formalin-fixed paraffin-  
885 embedded parietal cortex (C-E), and in murine fresh frozen hippocampal and cerebellar sections  
886 (F-H). For immunofluorescence, NeuN (neuronal marker)(A, B), collagen IV (endothelial  
887 marker)(F, G) or PDGFR $\beta$  (pericyte marker) (H) are in red, and DAPI (nuclei) is in blue (A-C, F-  
888 H). For immunohistochemistry, sections were counterstained with hematoxylin (nuclei)(D, E), and  
889 negative control is inserted in (E). In human samples, strong ACE2 staining is observed in large  
890 and small neurons, whereas in mice, neuronal signal is moderate and vascular ACE2 staining is  
891 strong and colocalizes well with PDGFR $\beta$ . Red arrows point to ACE2+/NeuN+ cells and green  
892 arrows to erythrocytes; dashed white line highlights small ACE2+/NeuN+ cells that stain strongly  
893 for DAPI. ACE2 antibodies: rabbit mAb #ab108252 (C, G), rabbit pAb #HPA000288 (A, B, E, F)  
894 and #35-1875 (D) or goat pAb #AF3437. Scale bars: 200  $\mu$ m (A, F, G, H), 20  $\mu$ m (B, D, E) and 10  
895  $\mu$ m (C). *Abbreviations: ACE2, Angiotensin-Converting Enzyme 2; Coll IV, Collagen IV; Coloc,*  
896 *Colocalization; PDGFR $\beta$ , Platelet Derived Growth Factor Receptor Beta; mAb, monoclonal*  
897 *antibody; pAb, polyclonal antibody.*

898 **Figure 6: ACE2 levels are not altered in a model of AD, the 3xTg-AD mouse.** (A)  
899 Determination of ACE2 levels by Western immunoblotting in brain homogenates from NonTg and  
900 3xTg-AD mice aged 4, 12, and 18 months. No difference was observed in TBS-soluble and



901 detergent-soluble ACE2. (B) In brain microvessel-enriched fractions from NonTg and 3xTg-AD  
902 mice aged 6, 12, 18 months, and in 18-month-old animals fed either a control diet or a high fat diet,  
903 no difference was observed in microvascular ACE2 levels. ACE2 levels in the mouse brain are not  
904 influenced by age or a diet that exacerbates AD-like neuropathology. Examples were taken from  
905 the same immunoblot experiment, and consecutive bands loaded in random order are shown. Actin  
906 and cyclophilin B are shown as loading controls. Data are represented as mean  $\pm$  SEM. Statistical  
907 analysis: Kruskal-Wallis, ns, non-significant. *Abbreviations: Angiotensin-Converting Enzyme 2;*  
908 *Non-Tg/NT, non-transgenic mice; 3x, 3xTg-AD mice; CD/C, control diet; CyB, cyclophilin B;*  
909 *HFD/H, high-fat diet; O.D., optical density.*

910 **Table 1: Characteristics of Cohort #1 (Religious Order Study) and Cohort #2 (US other**  
911 **sources).**

912 **Cohort #1 characteristics (Religious Order Study):** Participants were assigned to the “Control”  
913 or “AD” group based on the level of AD neuropathological changes associated with their ABC  
914 scores [70]. ABC scores were converted into one of the four levels of AD neuropathological  
915 changes (not, low, intermediate, or high) using the chart described in the revised NIA-AA  
916 guidelines [70]. Intermediate or high levels of AD neuropathological changes were assigned to the  
917 “AD” group, while those with no or a low level of AD neuropathological changes were rather  
918 assigned to the “Control” group [70]. Parenchymal CAA stages in parietal cortex were determined  
919 in the angular gyrus. Brain pH was measured in cerebellum extracts. Soluble A $\beta$  peptide  
920 concentrations were determined by ELISA in whole homogenates of inferior parietal cortex.  
921 Values are expressed as means (SD) unless specified otherwise. Statistical analysis (compared to  
922 controls): Mann Whitney test: #p < 0.01; ¶p < 0.001; &p < 0.0001; Pearson test: £p < 0.01.  
923 Claudin5 and CD31 data in microvessel extracts were normalized with cyclophilin B as a loading  
924 control. **Cohort #2 characteristics (Other US Sources):** Brain samples of this cohort were  
925 provided by Harvard Brain Tissue Resource Center (Boston), Miller School of Medicine (Miami)  
926 and Human Brain and Spinal Fluid Resource Center (Los Angeles). Participants were assigned to  
927 the “Control” or “AD” group based on the Braak score. Values are expressed as means (SD).  
928 Statistical analysis (compared to controls): Unpaired t test, Pearson test. *Abbreviations: AD,*  
929 *Alzheimer’s disease; C, contingency; CAA, cerebral amyloid angiopathy; CERAD, Consortium to*  
930 *Establish a Registry for Alzheimer’s Disease; MCI, mild cognitive impairment; MMSE, Mini-*  
931 *Mental State Examination; NCI, healthy controls with no cognitive impairment; ROD, relative*  
932 *optical density.*

933 **Figure S1: AD subjects with parenchymal CAA have higher soluble ACE2.** Individuals were  
934 grouped based on their ABC neuropathological diagnosis or clinical diagnosis and subdivided  
935 based on the presence of parenchymal CAA (pCAA). (A) Levels of soluble ACE2 were higher in  
936 subjects with an AD neuropathological and clinical diagnosis with the presence of pCAA. (B)  
937 Levels of detergent-soluble ACE2 did not change depending on the presence or absence of pCAA.  
938 All samples, loaded in a random order, were run on the same immunoblot experiment for  
939 quantification. Examples were taken from the same immunoblot experiment, and consecutive  
940 bands loaded in random order are shown. Data are represented as a scatterplot. Horizontal lines  
941 indicate mean  $\pm$  SEM. Statistical analysis: two groups: unpaired t-test \*p < 0.05 or three groups:  
942 Tukey's multiple comparisons test \*p < 0.05, \*\*p < 0.01. *Abbreviations: A/AD, Alzheimer’s disease;;*

943 *C, control; Clinical Dx, clinical diagnosis; M/MCI, mild cognitive impairment; N/NCI, healthy*  
944 *controls with no cognitive impairment; O.D., Optical density; pCAA, parenchymal cerebral*  
945 *amyloid angiopathy.*

946 **Figure S2: Levels of TMPRSS2 protein, which is employed by SARS-CoV-2 for Spike protein**  
947 **priming, are unchanged in AD individuals.** (A) Levels of the TMPRSS2 protein were measured  
948 in Cohort #1: no difference was identified according to clinical or neuropathological (ABC)  
949 diagnosis. (B) In Cohort#2, TMRPSS protein and mRNA quantification did not reveal difference  
950 between control and AD. All samples, loaded in a random order, were run on the same immunoblot  
951 experiment for quantification. Examples were taken from the same immunoblot experiment, and  
952 consecutive bands loaded in random order are shown. Data are represented as a scatterplot.  
953 Horizontal lines indicate mean  $\pm$  SEM. Statistical analysis: Ordinary on-way ANOVA or Mann-  
954 Whitney test, non-significant. *Abbreviations: A/AD, Alzheimer's disease; ABC Dx, ABC*  
955 *neuropathological diagnosis; Braak Dx, Braak staging diagnosis; C, control; Clinical Dx, clinical*  
956 *diagnosis; M/MCI, mild cognitive impairment; N/NCI, healthy controls with no cognitive*  
957 *impairment; ROD, relative optical density; TMRPSS2, Transmembrane protease serine 2.*

958 **Figure S3: Examples of correlation plots between ACE2 in the three protein fractions and**  
959 **age or AD-related proteins are shown.** No significant correlation was established between ACE2  
960 in all fractions tested and the age of death. TBS-soluble ACE2 was positively correlated with  
961 soluble A $\beta$ 42 peptides and phospho-tau AD2. Both TBS-soluble and microvessel ACE2 were  
962 positively associated with microvascular RAGE but negatively with microvascular PDGFR $\beta$ .  
963 Significant correlations are shown with the sign & in red. All proteins presented in these  
964 correlations were determined by Western-blot analysis except A $\beta$  peptides, which were determined  
965 using ELISA. Coefficient of determination &  $p < 0.05$ , &  $p < 0.01$ , &  $p < 0.001$ . *Abbreviations:*  
966 *ACE2, Angiotensin-Converting Enzyme 2; AD, Alzheimer's disease; MCI, mild cognitive*  
967 *impairment; NCI, healthy controls with no cognitive impairment; PDGFR $\beta$ , Platelet Derived*  
968 *Growth Factor Receptor Beta; RAGE, Receptor for Advanced Glycation Endproducts; Relative*  
969 *OD, relative optical density.*

970 **Figure S4: Human formalin-fixed paraffin-embedded testis sections were used as positive**  
971 **controls for ACE2 immunostaining, which is strong in this tissue.** Antibodies used: (A) goat  
972 pAb #PA5-4788 from ThermoFisher, (B) goat pAb #AF933 from BioTechne, (C) goat pAb  
973 #AF3437 from BioTechne, (D) mouse mAb #sc-73668 from Santa Cruz, (E) rabbit pAb #35-1875  
974 from Abeomics, (F) rb pAb #ab15348 from Abcam, (G) mouse mAb #sc-390851 from Santa Cruz,  
975 (H) rabbit mAb #ab108252 from Abcam and (I) rabbit pAb #HPA000288 from Atlas. Scale bars:  
976 400  $\mu$ m (A-H) and 200  $\mu$ m (I). *Abbreviations: ACE2, Angiotensin-Converting Enzyme 2; Coll IV,*  
977 *Collagen IV; mAb, monoclonal antibody; pAb, polyclonal antibody.*

978 **Figure S5: Western blots in human TBS-soluble, detergent-soluble and microvessel-enriched**  
979 **extracts from Cohort#1.** The clinical and neuropathological diagnoses are given above each  
980 sample. *Abbreviations: A, Alzheimer's Disease; ABC Dx, ABC neuropathological diagnosis;*  
981 *ACE2, Angiotensin-Converting Enzyme 2; C, Control; ClinDx, Clinical diagnosis; M, mild*  
982 *cognitive impairment; N, healthy controls with no cognitive impairment; TMRPSS2,*  
983 *Transmembrane protease serine 2.*

984 **Figure S6: Western blots in brain homogenates from Cohort#2.** Diagnosis was determined  
985 using Braak staging. *Abbreviations: A, Alzheimer's Disease; ACE2, Angiotensin-Converting*  
986 *Enzyme 2; Braak Dx, Braak staging diagnosis; Cal, Calibrator; C, Control; GAPDH,*  
987 *Glyceraldehyde 3-phosphate dehydrogenase; TMRPSS2, Transmembrane protease serine 2.*

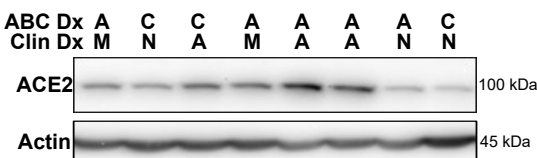
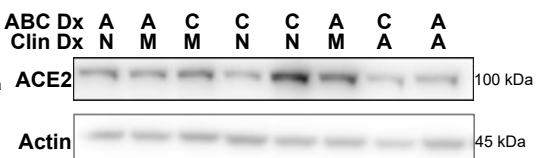
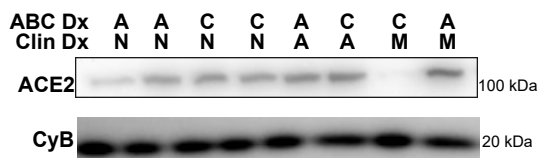
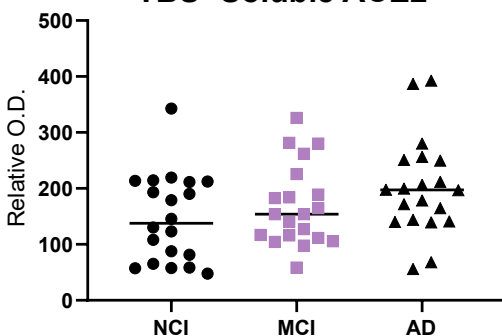
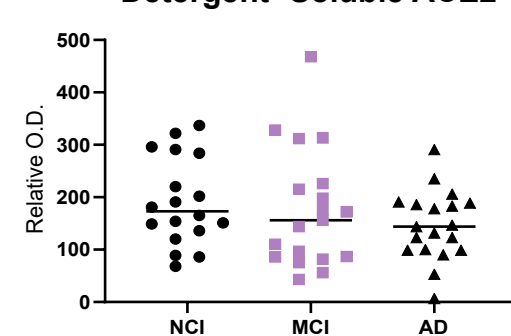
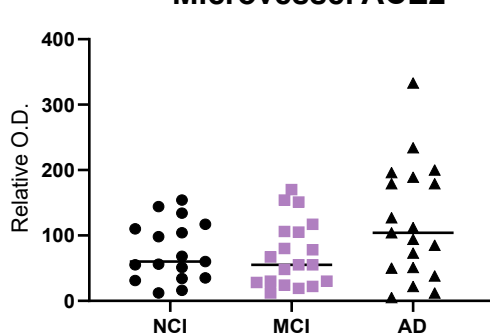
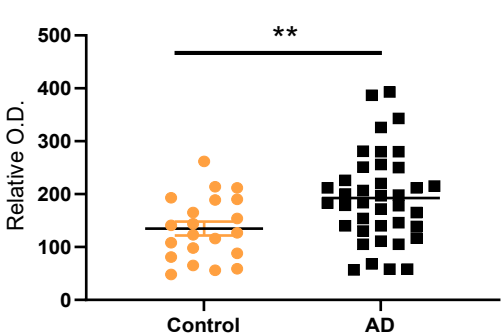
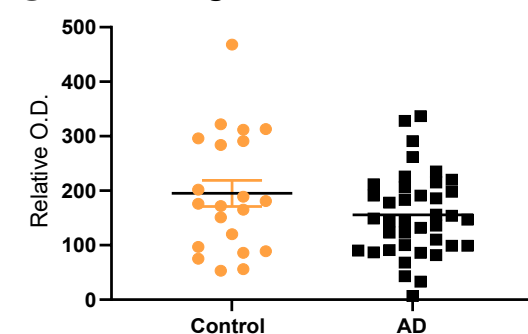
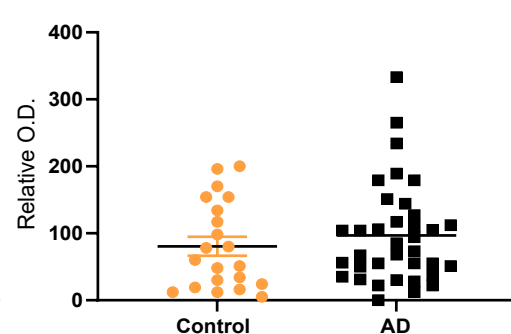
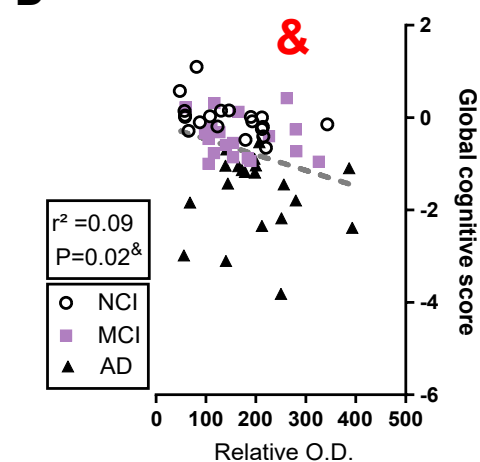
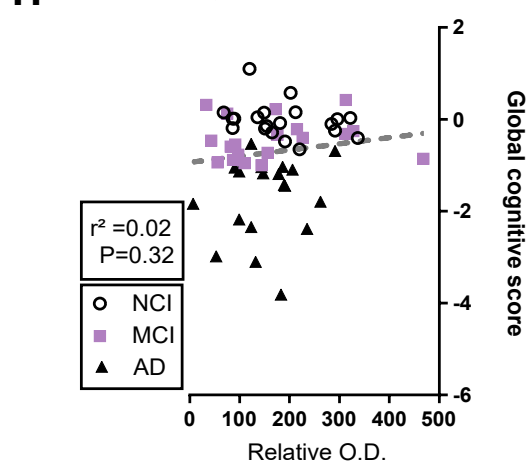
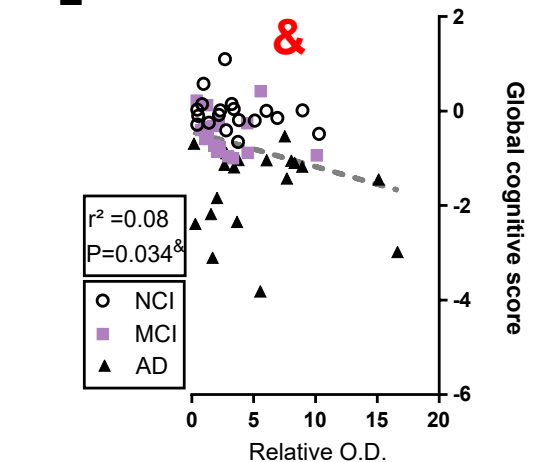
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989 **Supplementary method:**

990 *Isolation of murine brain microvessels*

991 The procedure used for isolation of murine brain microvessels has been reported in our previous  
992 work (Bourassa *et al.*, 2019a). Nontransgenic and 3xTg-AD mice aged 6, 12 and 18 months were  
993 sacrificed with an intracardiac perfusion of ice-cold PBS containing 0.32 M sucrose and protease  
994 (SIGMAFAST Protease Inhibitor tablets, Sigma-Aldrich) and phosphatase (1 mM sodium  
995 pyrophosphate and 50 mM sodium fluoride) inhibitors, under deep anesthesia with  
996 ketamine/xylazine. The brains were immediately collected, and brainstem, cerebellum and  
997 meninges were removed. Murine brain samples were then chopped and frozen in 0.5 mL of MIB  
998 containing 0.32 M sucrose and protease and phosphatase inhibitors (Bimake). For a milder freezing  
999 we used Mr. Frosty™ Freezing Container (Thermo Scientific). The microvessel enrichment  
1000 procedure was then conducted as described for human samples. To validate the enrichment of  
1001 mural cell markers, the microvessel-enriched and the microvessel-depleted fractions were  
1002 compared to a total brain homogenate obtained from the homogenization of a whole hemisphere  
1003 of a control mouse in the lysis buffer. Protein concentrations in all fractions were determined using  
1004 the bicinchoninic acid assay (Thermo Fisher Scientific)

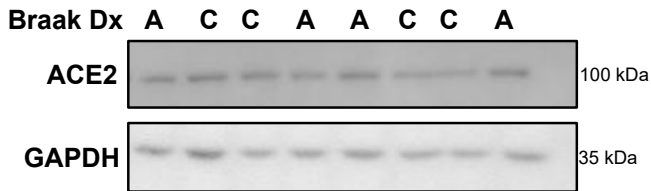
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**Figure 1****A TBS-Soluble proteins from the parietal cortex****E Detergent-Soluble proteins from the parietal cortex****I Isolated microvessel proteins from the parietal cortex****Clinical diagnosis****B TBS- Soluble ACE2****F Detergent- Soluble ACE2****J Microvessel ACE2****Neuropathological diagnosis****C TBS- Soluble ACE2****G Detergent- Soluble ACE2****K Microvessel ACE2****Association with global cognitive score****D TBS- Soluble ACE2****H Detergent- Soluble ACE2****L Microvessel ACE2**

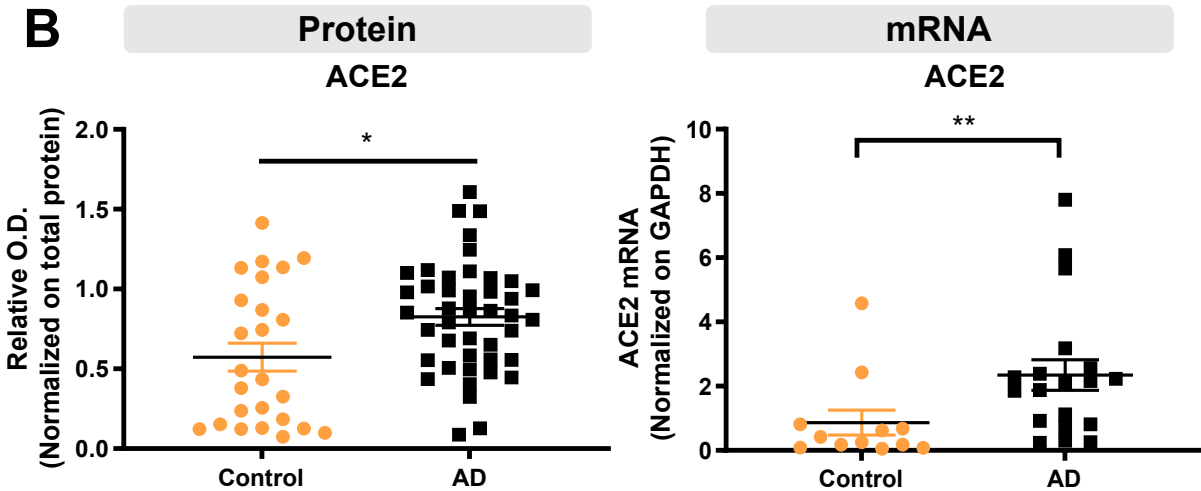
**Figure 2**

**Quantification of ACE2 in the parietal cortex from Cohort #2**

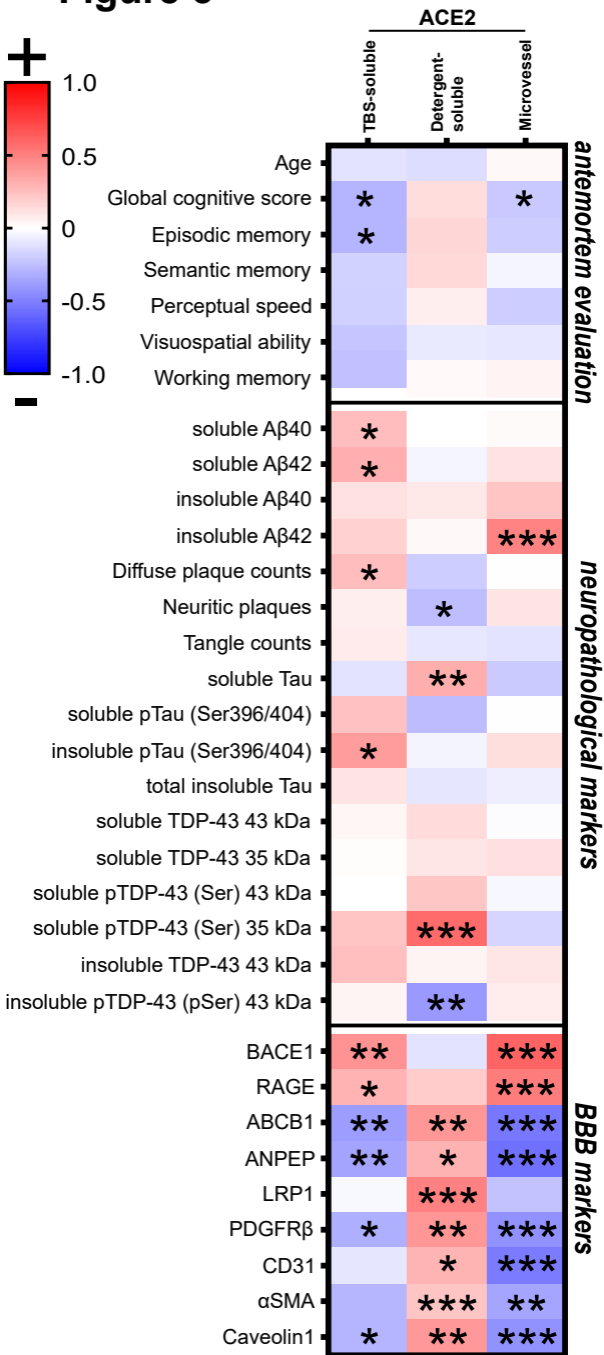
**A**

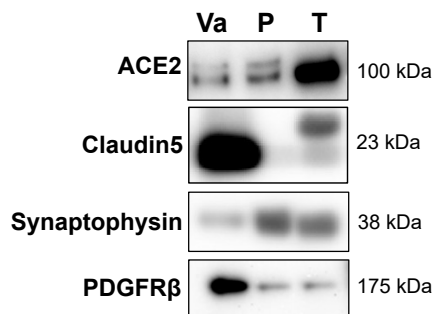
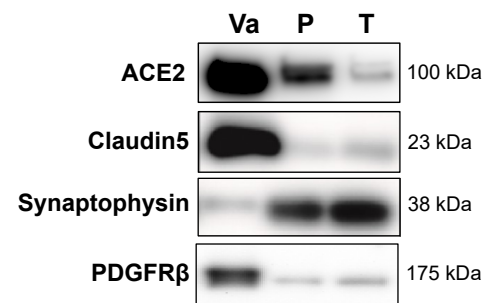


**B**



# Figure 3



**Figure 4****ACE2 in isolated microvessel-enriched fraction****A Human brain****F Mouse brain****Human****Mouse**

ACE2 Coll IV Coloc DAPI

ACE2 Coll IV Coloc DAPI

ACE2 Coll IV Coloc DAPI

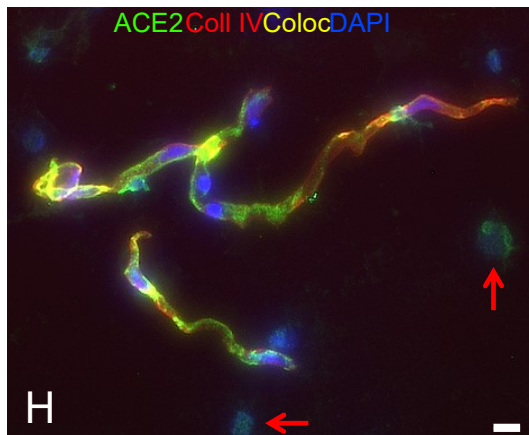
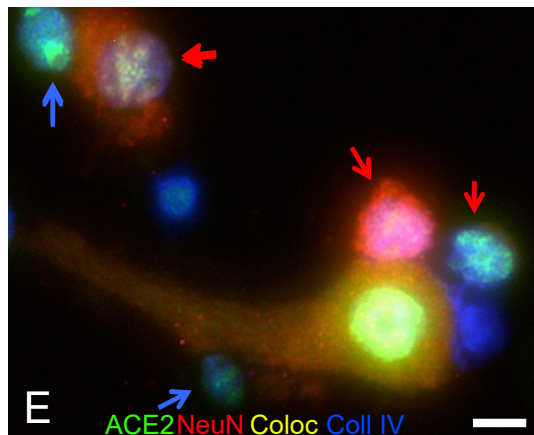
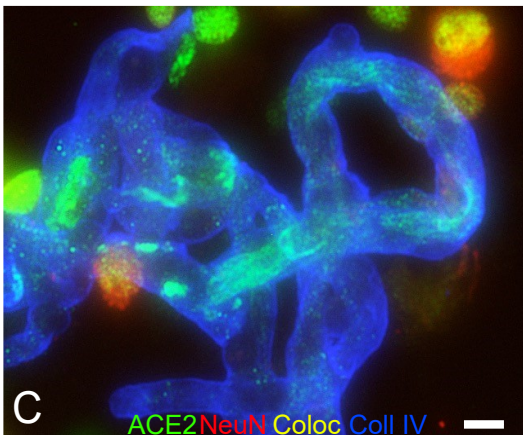
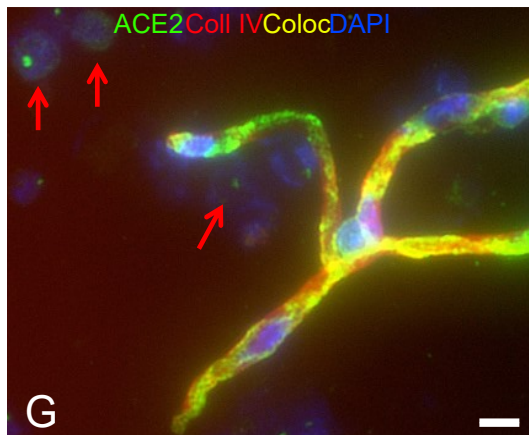
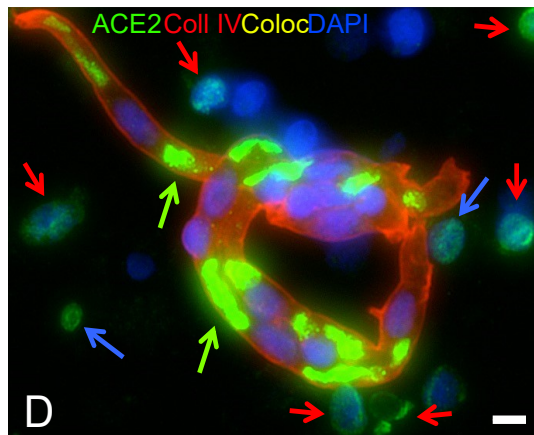
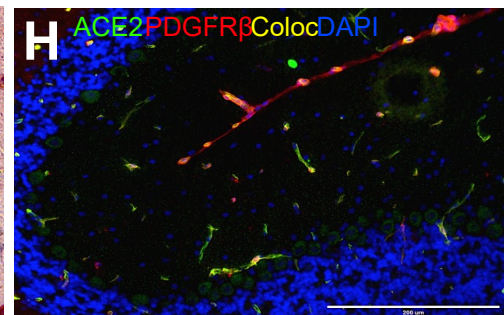
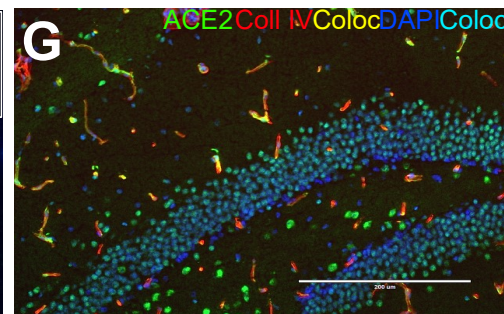
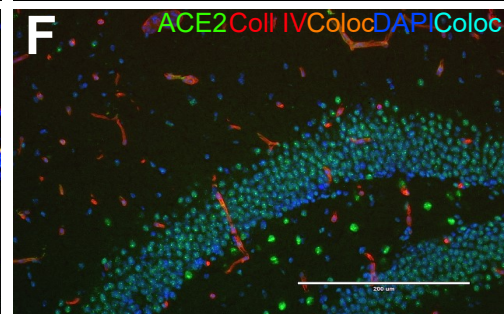
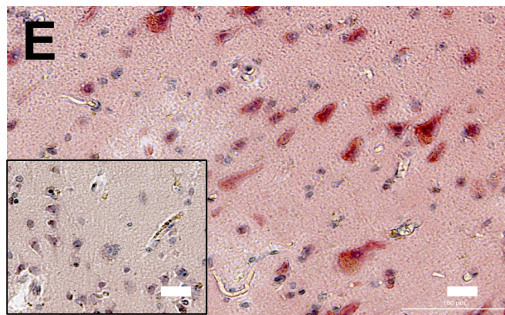
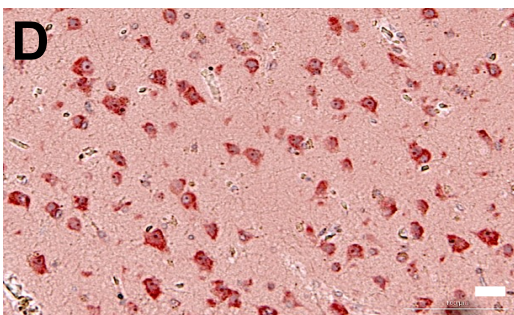
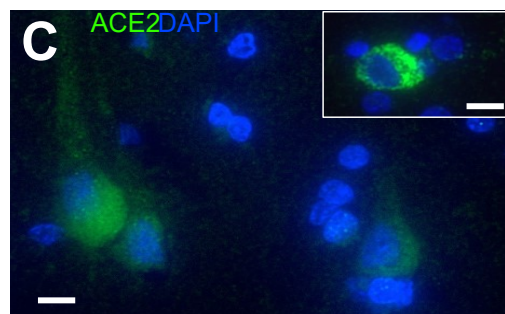
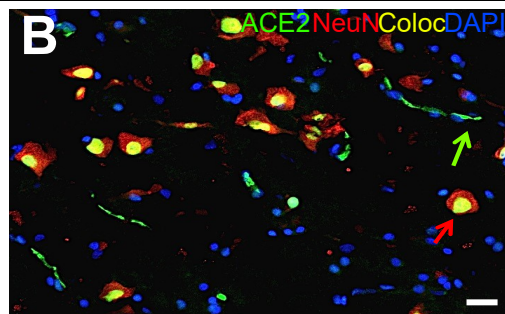
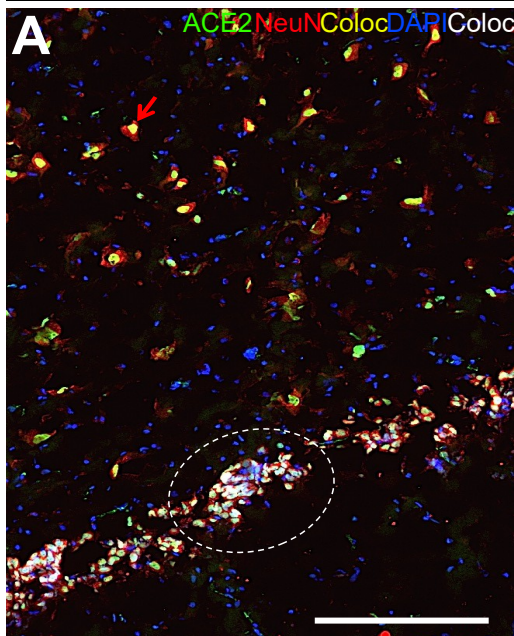


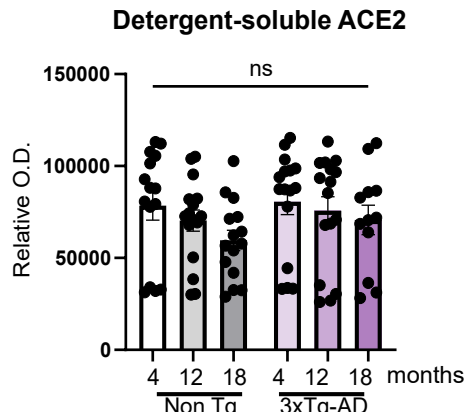
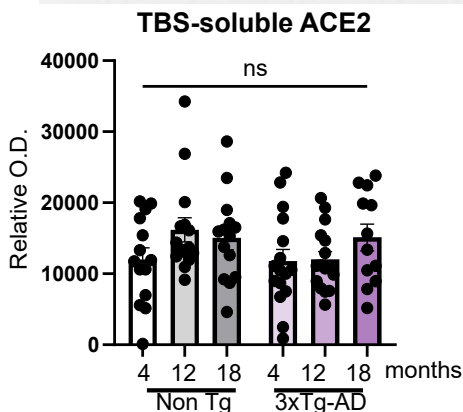
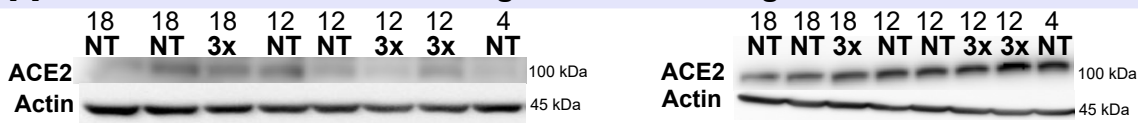
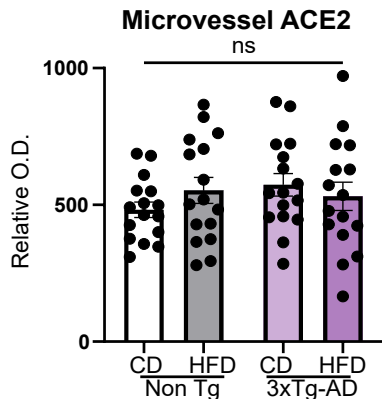
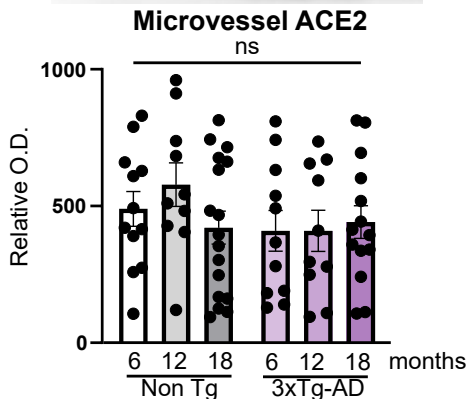
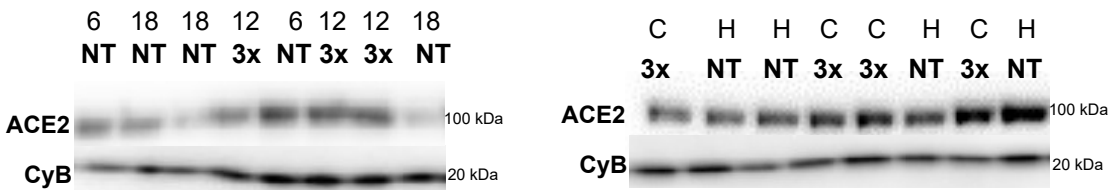
Figure 5

## Humain brain

## Mouse brain





**Figure 6****A Brain cortex homogenates from 3xTg-AD mice****B Isolated brain microvessels from 3xTg-AD mice**

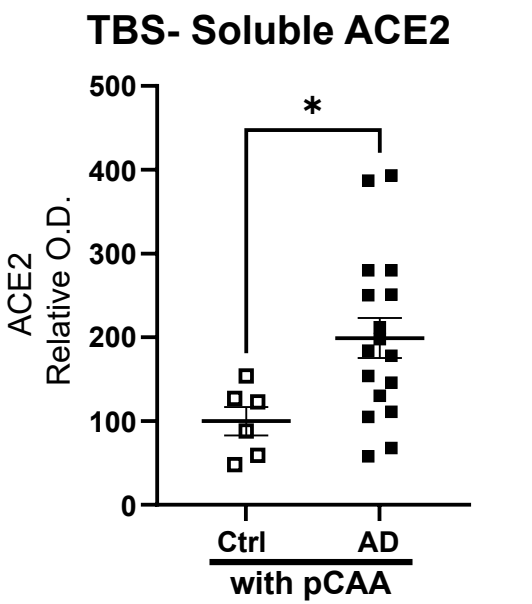
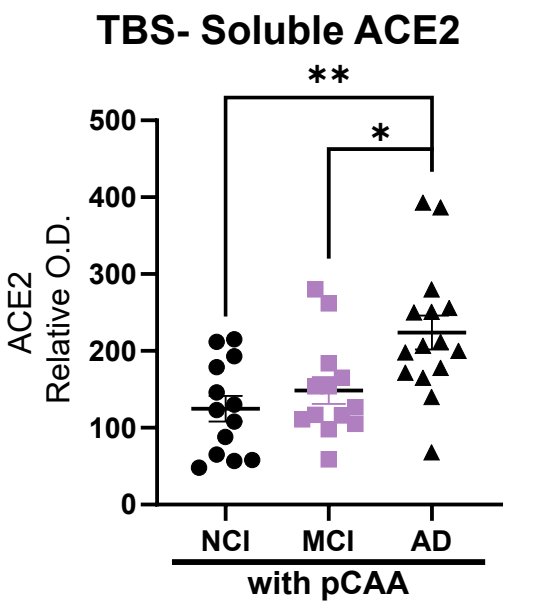
<b>Religious Order Study (Cohort #1)</b>			
<b>Characteristics</b>	<b>Control</b>	<b>AD</b>	<b>Statistical Analysis</b>
<i>N</i>	22	38	-----
Men, %	41	29	C; Pearson test, $\chi^2 = 0.897$ ; $p = 0.3436$
Mean age at death	86.7 (4.3)	87.5 (5.7)	Mann Whitney test, $p = 0.5548$
post-mortem delay (h)	7.9 (5.1)	7.5 (5.1)	Mann Whitney test, $p = 0.6648$
Mean education, years	18.3 (3.5)	18.1 (3.1)	Mann Whitney test, $p = 0.5236$
Mean MMSE	25.0 (4.5)	21.6 (7.9)	Mann Whitney test, $p = 0.0791$
Global cognition score	-0.32 (0.8)	-0.94 (0.9) <sup>#</sup>	Mann Whitney test, $p = 0.0044$
apoE $\epsilon$ 4 allele carriage (%)	9	45 \$	C; Pearson test, $\chi^2 = 8.182$ ; $p = 0.0042$
Clinical diagnosis NCI/MCI/AD (n)	11/8/3	9/12/17	-----
Thal amyloid score 0/1/2/3 (n)	7/13/2/0	0/3/15/20	-----
Braak score 0/1/2/3 (n)	0/7/15/0	0/0/27/11	-----
CERAD score 0/1/2/3 (n)	14/4/4/0	1/3/16/18	-----
Parenchymal CAA stage in parietal cortex 0/1/2/3/4 (n)	15/4/1/1/0	18/7/5/2/3	C; Pearson test, $\chi^2 = 3.830$ ; $p = 0.4295$
Presence of chronic cortical macroinfarcts 0/1 (n)	20/2	33/5	C; Pearson test, $\chi^2 = 0.224$ ; $p = 0.6363$
Presence of chronic cortical microinfarcts 0/1 (n)	17/5	34/4	C; Pearson test, $\chi^2 = 1.627$ ; $p = 0.2021$
Usage of antihypertensive medications 0/1 (n)	2/20	5/33	C; Pearson test, $\chi^2 = 0.224$ ; $p = 0.6363$
Usage of diabetes medications 0/1 (n)	15/7	33/5	C; Pearson test, $\chi^2 = 3.032$ ; $p = 0.0816$
Cerebellar pH	6.4 (0.37)	6.3 (0.36)	Mann Whitney test, $p = 0.2933$
Diffuse Plaque Counts in parietal cortex	3.8 (8.0)	20.3 (16.8) <sup>&amp;</sup>	Mann Whitney test, $p < 0.0001$
Neuritic Plaque Counts in parietal cortex	1.3 (3.2)	15.7 (12.5) <sup>&amp;</sup>	Mann Whitney test, $p < 0.0001$
Neurofibrillary Tangle Counts	0.09 (0.43)	2.92 (8.35) <sup>#</sup>	Mann Whitney test, $p = 0.0096$
Soluble A $\beta$ 40 concentration, pmol/L	125.4 (245.9)	363.2 (695.2) <sup>¶</sup>	Mann Whitney test, $p = 0.0009$
Soluble A $\beta$ 42 concentration, pmol/L	299.6 (475.0)	1173.6 (503.9) <sup>&amp;</sup>	Mann Whitney test, $p < 0.0001$
Soluble A $\beta$ 40/A $\beta$ 42 ratio	0.99 (1.09)	0.34 (0.59)	Mann Whitney test, $p < 0.0001$
Cyclophilin B in microvessel extracts (loading control)	2.74 (0.77)	2.66 (0.79)	Mann Whitney test, $p = 0.7309$
Claudin5 levels in microvessel extracts (normalized ROD)	1.17 (0.50)	1.16 (0.41)	Mann Whitney test, $p = 0.6613$
CD31 levels in microvessel extracts (normalized ROD)	0.45 (0.40)	0.41 (0.36)	Mann Whitney test, $p = 0.7366$
<b>US other sources (Cohort #2)</b>			
<b>Characteristics</b>	<b>Control</b>	<b>AD</b>	<b>Statistical Analysis</b>
<i>N</i>	30	52	-----
Men, %	70	52	C; Pearson test, $\chi^2 = 2.561$ ; $p = 0.1095$
Mean age at death	74.9 (9.7)	78.9 (14.0)	Unpaired t test, $p = 0.0784$
post-mortem delay (h)	16.3 (4.9)	17.6 (4.8)	Unpaired t test, $p = 0.2185$
Braak stages 0-2/3-6 (n)	30/0	0/52	-----
Atherosclerosis (%)	n.d.	61,5	-----

Figure S1

# A TBS-soluble proteins from the parietal cortex

Clinical Diagnosis

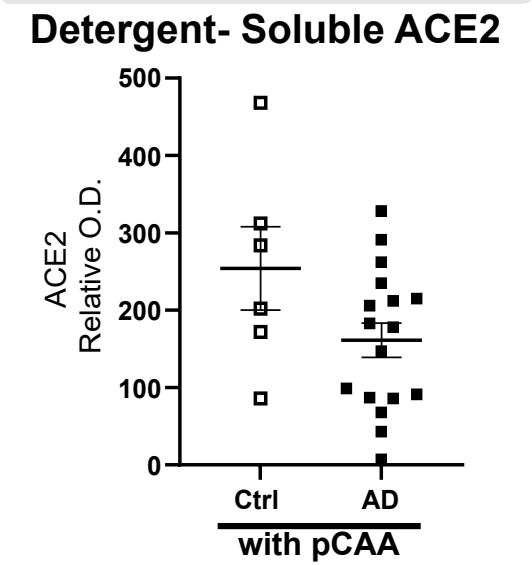
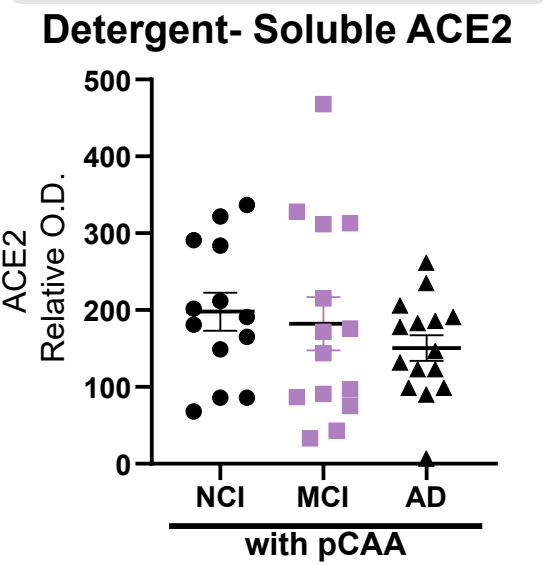
Neuropathological diagnosis

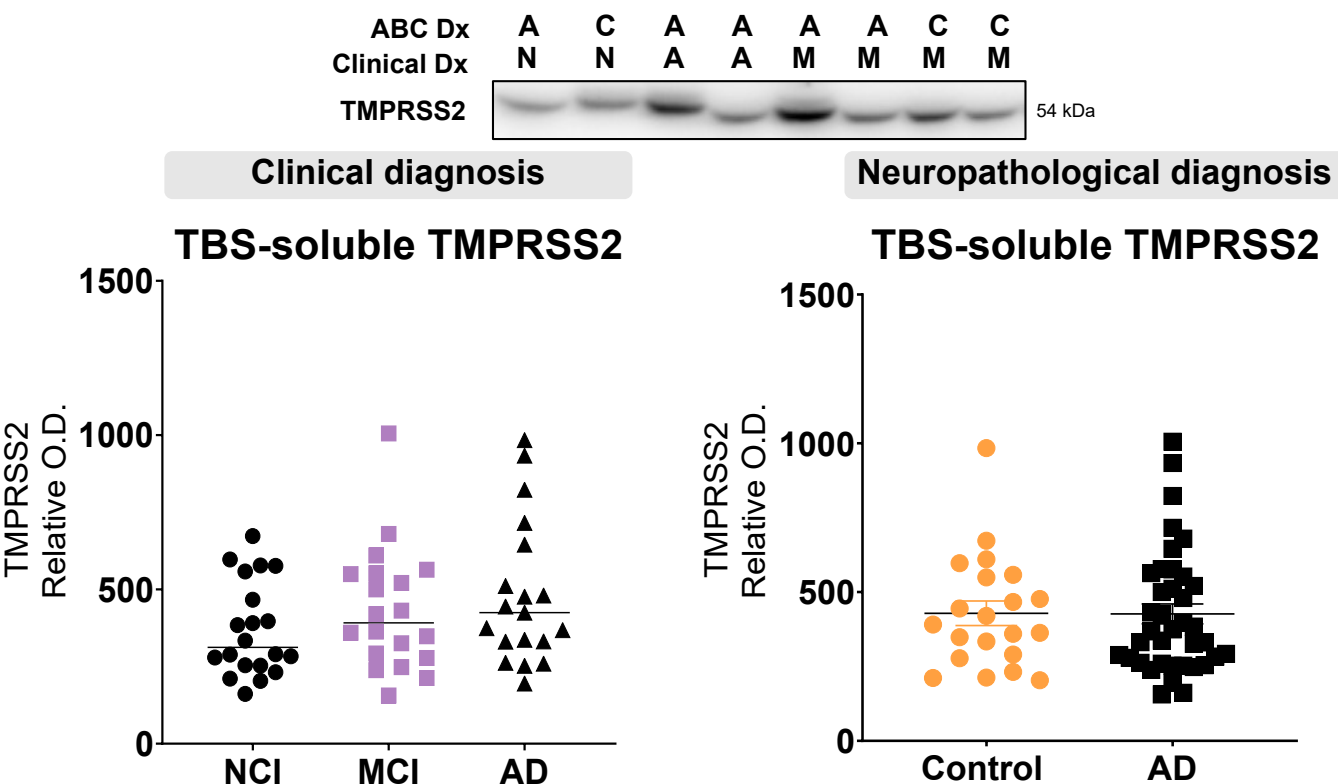
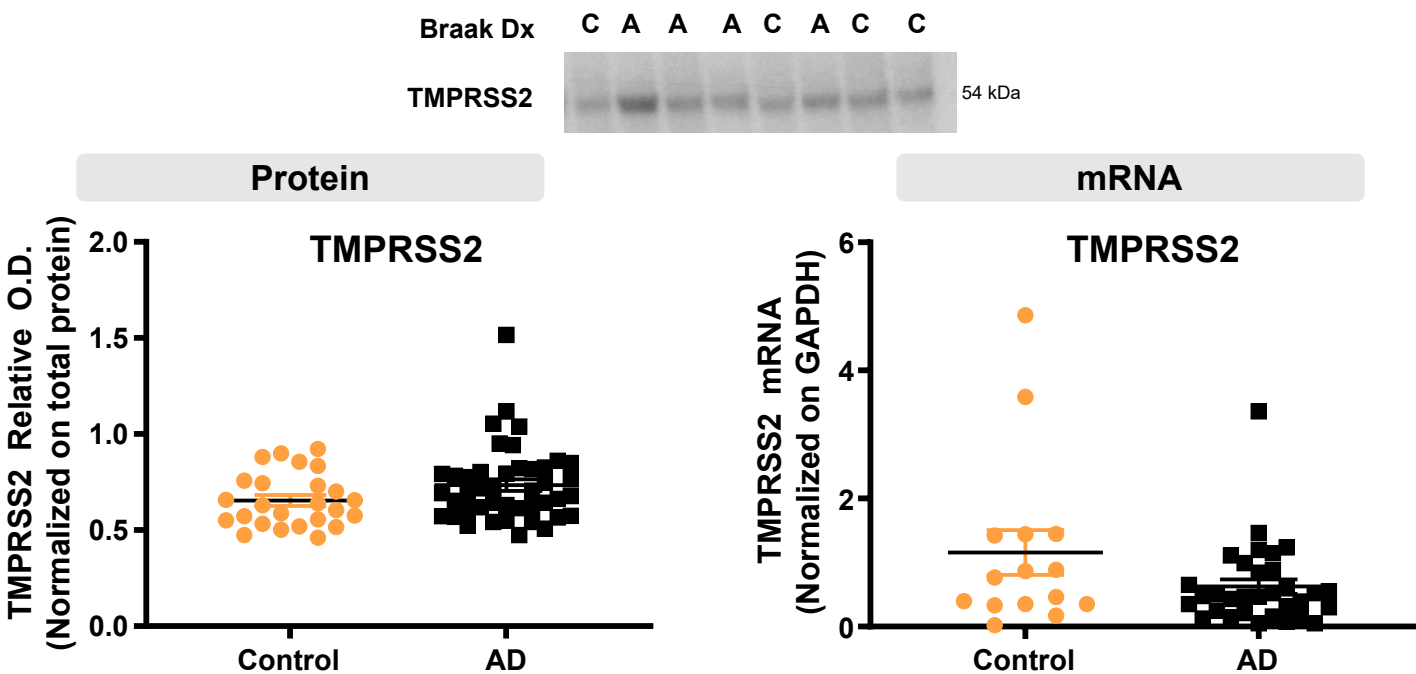


# B Detergent-soluble proteins from the parietal cortex

Clinical Diagnosis

Neuropathological diagnosis



**Figure S2****A** Quantification of TBS-soluble TMPRSS2 in the parietal cortex from Cohort #1**B** Quantification of TMPRSS2 in the parietal cortex from Cohort #2

**TBS-Soluble proteins from the parietal cortex**

**Detergent-Soluble proteins from the parietal cortex**

**Isolated microvessels proteins from the parietal cortex**

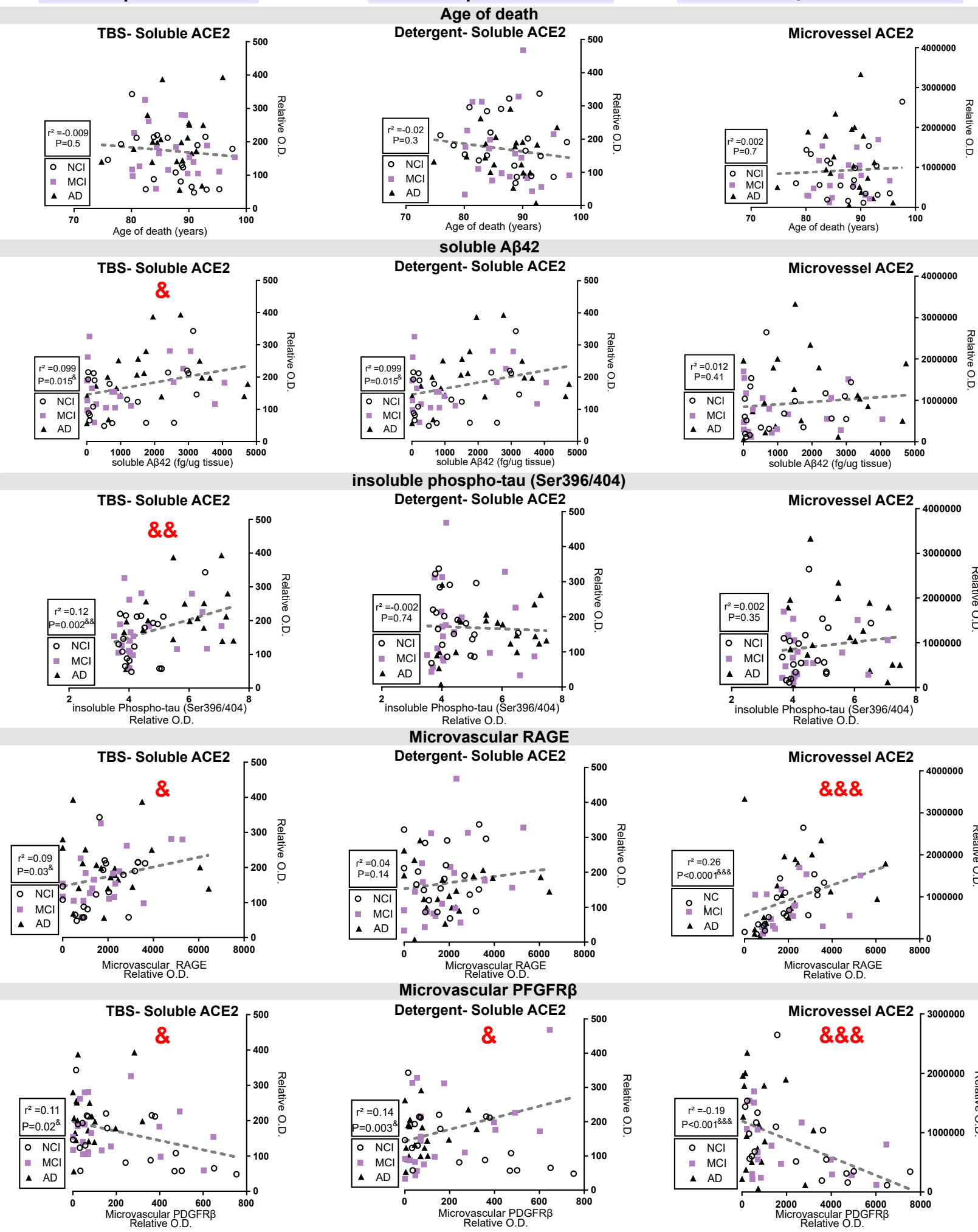
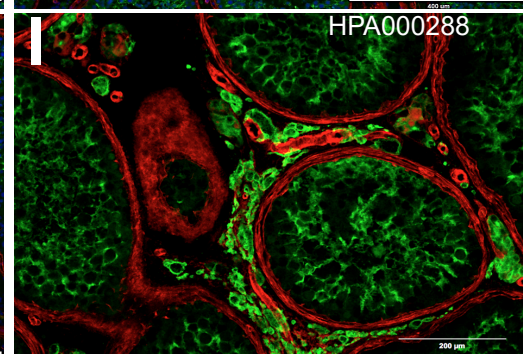
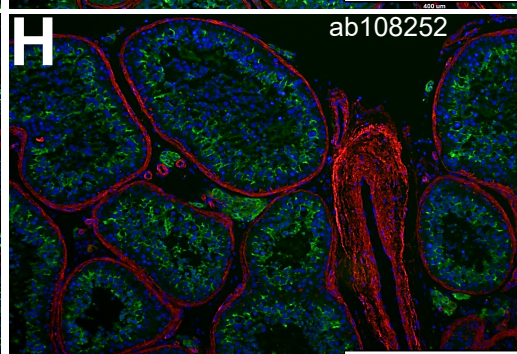
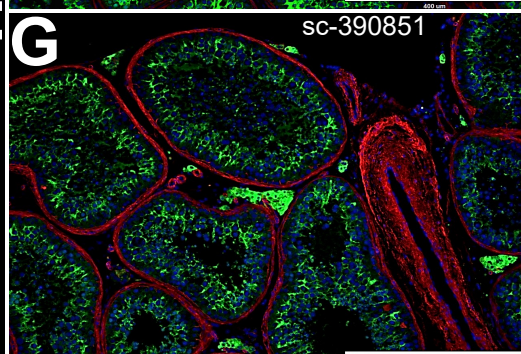
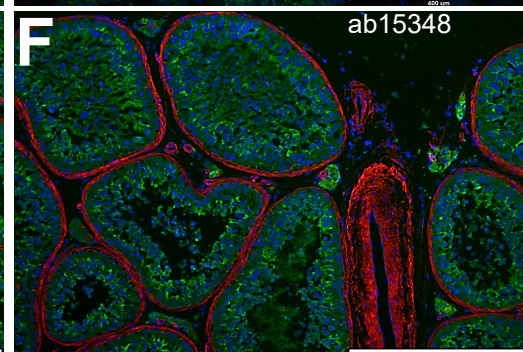
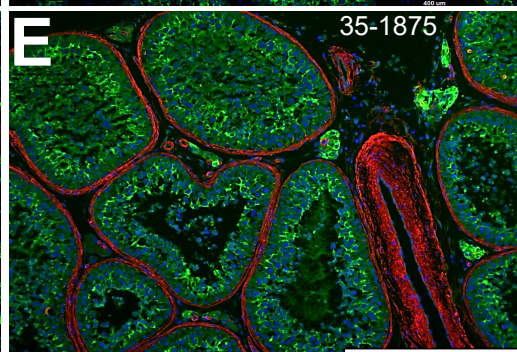
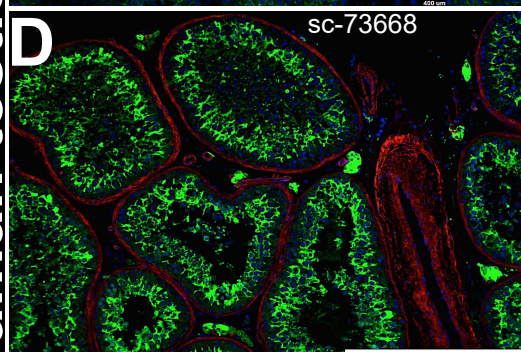
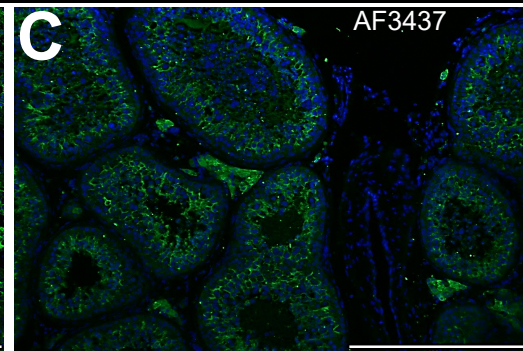
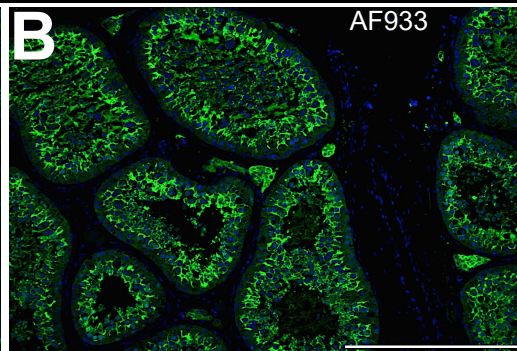
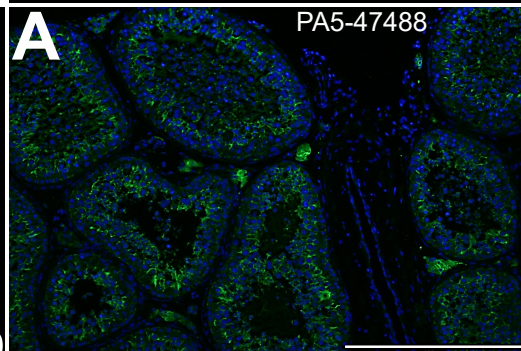


Figure S4

ACE2 CoII IVD DAPI

Human testis





**Brain homogenates from parietal cortex**

