

1 **Neuroprotection in early stages of Alzheimer’s Disease is promoted by Transthyretin angiogenic properties**

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37 **Abstract**

38 While still controversial, it has been demonstrated that vascular defects can precede the onset of the other AD
39 hallmarks features, making it an important therapeutic target. Given that the protein transthyretin (TTR) has been
40 established as neuroprotective in AD, here we investigated the influence of TTR in the vasculature. AD transgenic
41 mice with TTR genetic reduction, AD/TTR+/-, exhibited a thicker BM in brain microvessels and decreased vessel
42 length than animals with normal TTR levels, AD/TTR+/+. Further *in vivo* investigation, using the chick
43 chorioallantoic membrane (CAM) assay, revealed that TTR is a pro-angiogenic molecule. Also, TTR increased
44 the expression of key angiogenic molecules, by endothelial cells under tube formation conditions. We showed
45 that TTR reduction leads to a thicker BM in AD mice than in NT animals, strengthening the idea that TTR is a
46 neuroprotective protein. We also studied the effect of TTR tetrameric stabilization on BM thickness, showing that
47 AD mice treated with iododiflunisal (IDIF) displayed a significant reduction of BM thickness and increased vessel
48 length when compared to non-treated littermates. Our *in vivo* results show the involvement of TTR in
49 angiogenesis, particularly as a modulator of vascular alterations occurring in AD. Since TTR is decreased early
50 in AD, its tetrameric stabilization can represent a therapeutic avenue for the early treatment of AD through the
51 maintenance of the vascular structure.

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54 **Keywords:** Transthyretin; Alzheimer's Disease; Basement Membrane; Angiogenesis; Neuroprotection; Chick
55 Chorioallantoic Membrane Assay; TTR tetramer stabilizers.

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77 **Introduction**

78 Alzheimer's Disease (AD) patients undergo several neurovascular changes at different levels. Brain vascular
79 dysregulation is the earliest and strongest factor during the disease progression and is followed by amyloid- β (A β)
80 peptide deposition, glucose metabolism dysregulation, functional impairment and gray matter atrophy, in this
81 order [1]. Decreased expression of the low-density lipoprotein receptor-related protein 1 (LRP-1) and P-
82 glycoprotein (P-gp), as well as up-regulation of the receptor for advanced glycation end products (RAGE), are
83 mechanisms reported to be changed in AD patients, leading to A β accumulation in the brain [2,3]. In addition to
84 defective clearance mechanisms, increased endothelial pinocytosis, decreased number of mitochondria, decreased
85 glucose transporter (GLUT)-1 and loss of tight/adherents junctions are features detected in AD [4]. The reduction
86 of the capillary density is also characteristic of the AD brains [5]. This is due to an aberrant angiogenesis with
87 premature pruning of capillary networks. This defective angiogenesis may be caused by a lack of angiogenic
88 stimuli and unresponsive endothelium [6]. Although other authors describe increased vascular density in AD [7],
89 the underlying angiogenic process has pathological characteristics. Some studies suggest that promotion of
90 angiogenesis results in concomitant BBB disruption and vessel leakiness [7]. Other studies defend that the
91 vascular damage is a consequence of poor blood perfusion of the brain, leading to hypoperfusion/hypoxia causing
92 the BBB dysfunction [8]. Other authors argue that the accumulation of A β in the walls of the capillaries can
93 contribute to the reduced brain capillary density in AD via anti-angiogenic activity [9,10]. Another observed
94 alteration in AD is the increased thickness of the vascular BM in AD [11]. Since the increase in BM thickness
95 occurs before A β deposition, it is speculated that it functions as a physical barrier to the A β clearance across the
96 BBB [12]. Some studies have related this BM thickening with increased collagen IV content, in AD and ageing
97 [13,14].

98 Transthyretin (TTR), a 55 kDa homotetrameric plasma and cerebrospinal fluid (CSF) protein, transports retinol
99 through binding to the retinol-binding protein (RBP), which binds at the surface of TTR, and thyroxine (T4),
100 which binds at a central hydrophobic channel formed at the dimer-dimer interface [15]. In the CSF, TTR is the
101 main A β binding protein [16], providing neuroprotection by avoiding A β aggregation [17–24] and toxicity
102 [17,25], and by participating in A β brain efflux at the BBB [26]. TTR is early decreased in AD, both in plasma
103 [27–29] and in the CSF [30], probably due to its tetrameric instability [27,31], hypothesized to result in accelerated
104 clearance and lower levels. TTR instability is also a key feature in familial amyloid polyneuropathy (FAP), a
105 systemic amyloidosis that is usually caused by mutations in TTR. The amyloidogenic potential of the TTR variants
106 is inversely correlated with its tetrameric stability [32], and the dissociation of the tetramer into monomers is at
107 the basis of the events that culminate with TTR amyloid formation [33,34]. TTR stabilization, used as a therapy
108 in FAP [35,36], can be achieved through the use of small-molecule compounds sharing molecular structural
109 similarities with T4 and binding in the T4 central binding channel [37–39]. Although no TTR mutations have
110 been found in AD patients [22], TTR stabilization has also been proposed as a therapeutic strategy to recover its
111 ability to protect in AD [19,40], and shown beneficial in a mouse model of AD [40,41]. Iododiflunisal (IDIF), a
112 potent TTR stabilizer, was administered to AD mice and bound plasma TTR displacing T4, resulting in decreased
113 A β amyloid burden and total A β brain levels, and improved cognition [41]. Interestingly, TTR stabilization by
114 IDIF improves TTR-assisted A β brain efflux in vitro and enhanced the expression of LRP-1 in vivo [31]. The
115 formation of TTR-IDIF complexes enhances BBB permeability of both IDIF and TTR, in vivo [42].

116 TTR has also been implicated in angiogenesis and the first reports of its involvement have been described in
117 diseases such as FAP [43]; in diabetic retinopathy [44,45], and lately, in cancer [46]. As reported, a study
118 investigated the effect of TTR in angiogenesis by treating human umbilical vein endothelial cells (HUVECs) with
119 wild-type (WT) TTR or a common FAP TTR mutant, V30M. The authors concluded that the TTR mutant inhibited
120 cell migration and decreased survival relative to the WT TTR, by down-regulating several pro-angiogenic genes
121 for angiopoietin-2 (Ang-2), VEGF receptors 1 and 2, basic fibroblast growth factor (bFGF) and transforming
122 growth factor-beta 2 (TGF- β 2) [43]. In another study, to investigate how TTR affects the development of new
123 vessels in diabetic retinopathy (DR), human retinal microvascular endothelial cells (hRECs) were cultured with
124 TTR in natural and simulated DR environments (hyperglycemia and hypoxia). In the DR environment, TTR
125 inhibited cell proliferation, migration and tube formation, by repressing the expression of the pro-angiogenic genes
126 Ang-2 and VEGF receptors 1 and 2 [44]. Conversely, in a low glucose environment, these angiogenesis-related
127 features were improved by TTR. Recently, it was reported that TTR levels were increased in human serum of lung
128 cancer patients. Additionally, TTR was shown able to promote tumour growth by enhancing several lung ECs
129 functions as permeability, migration and tube formation [46]. However, TTR potential in angiogenesis has never
130 been addressed *in vivo* and the possible participation of TTR in brain angiogenesis and vascular alterations has
131 never been elucidated.

132 Taking these evidences into account, this work aimed at investigating the angiogenic potential of TTR and at
133 assessing its involvement in the vascular impairment that occurs in AD.

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141 **Material and Methods**

142 **Animals**

143 Two mouse models were used in this work, an AD transgenic and a non-transgenic (NT) mouse models, both
144 established in different TTR genetic backgrounds.

145 The AD mouse model A β PPswe/PS1A246E/TTR was generated by crossing the AD mouse model
146 A β PPswe/PS1A246E [47] (B6/C3H background) purchased from The Jackson laboratory with TTR-null mice
147 (TTR^{-/-}) (SV129 background) [48] as previously described [49]. F1 animals A β PPswe/TTR^{+/-} and
148 PS1A246E/TTR^{+/-} were crossed to obtain A β PPswe/PS1A246E/ TTR^{+/+}, A β PPswe/PS1A246E/TTR^{+/-},
149 A β PPswe/ PS1A246E/TTR^{-/-} and NT controls NT/TTR^{+/+}, NT/TTR^{+/-} and NT^{-/-}. The colony was maintained
150 on a B6/C3H/SV129 genetic background. Hereafter, the A β PPswe/PS1A246E/TTR colony will be referred to as
151 AD/TTR, and the different genotypes A β PPswe/PS1A246E/TTR^{+/+}, A β PPswe/PS1A246E/TTR^{+/-}, and
152 A β PPswe/PS1A 246E/TTR^{-/-} referred to as AD/TTR^{+/+}, AD/ TTR^{+/-}, and AD/TTR^{-/-}, respectively. Animals
153 were housed in a controlled environment (12-hour light/dark cycles, temperature between 22-24°C, humidity
154 between 45–65% and 15-20 air changes/hour), with freely available food and water. All the above experiments
155 were approved by the Institute for Research and Innovation in Health Sciences (i3S) Animal Ethics Committee
156 and in agreement with the animal ethics regulation from Directive 2010/63/EU.

157 In order to study the role of TTR in collagen IV deposition or in vessel density, cohorts of littermates 7-month-
158 old female mice AD/TTR^{+/+} (n=7) and AD/TTR^{+/-} (n=7), cohorts of littermates 3-month- old female mice
159 NT/TTR^{+/+} (n=4) and NT/TTR^{+/-} (n=4) and one cohort of 3-month-old female mice AD/TTR^{+/-} were used.
160 AD/TTR^{+/-} female control mice (n=6) or treated with IDIF (n=6) [41] for two months (from 5 to 7-month-old),
161 were used to investigate the relevance of TTR stabilization in collagen type IV levels, in AD.

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163 **Collagen IV Immunohistochemistry**

164 Free-floating 30 μ m-thick coronal brain sections of mice were permeabilized with 0.25% Triton X-100 in
165 phosphate-buffered saline (PBS) for 10 min at room temperature (RT), blocked with 5% bovine serum albumin
166 (BSA) in PBS for 1 hour at RT and incubated with primary rabbit anti-collagen IV antibody (1:100) (Abcam) in
167 1% BSA in PBS overnight at 4°C. Next, sections were washed with PBS and incubated with Alexa Fluor-568
168 goat anti-rabbit IgG antibody (1:2000) for 1 hour at RT. All steps were performed with agitation. To remove tissue
169 autofluorescence, sections were covered with Sudan black B solution (0.3% Sudan black B in 70% ethanol)
170 applied for 5 minutes at RT, followed by multiple washing steps with PBS at RT with agitation. The brain sections
171 were dried for 20 minutes at RT and mounted on 0.1% gelatin-coated slides with Fluoroshield™ with DAPI
172 (Sigma-Aldrich). Sections were visualized and photographed using a Zeiss Axio Imager Z1 microscope equipped
173 with an Axiocam MR3.0 camera and Axivision 4.9.1 software. A total of twenty randomly selected vessels in the
174 hippocampus and/or cortex of each mouse was photographed at 100x magnification, and the ratio intensity/area
175 was measured using the ImageJ software.

176 To assess the vascular density of mice brains, 30 μ m-thick coronal brain sections were boiled at 90 ° C in citrate
177 buffer for 15 minutes for antigenic recovery and then washed with 0.3% Triton X-100 in PBS for 10 minutes at
178 RT. Tissues were blocked/permeabilized with a solution of 1% BSA and 0.5% Triton X-100 in PBS, overnight at
179 4°C. The coronal sections were then incubated for 72 hours at 4°C with primary rabbit anti-collagen IV antibody
180 (1:200) (Abcam) in a solution with 1% BSA, 0.5% Triton X-100 and 2% fetal bovine serum (FBS) in PBS. After,

181 tissues were washed with 0.3% Triton X-100 in PBS at 4°C. Next, sections were incubated with Alexa Fluor-568
182 goat anti-rabbit IgG antibody (1:500) overnight at 4°C, followed by washing with 0.3% Triton X-100 in PBS and
183 then dried for 20 minutes at RT and mounted on silane pre-coated slides with Fluoroshield™ with DAPI (Sigma-
184 Aldrich). Sections were visualized and photographed using a Zeiss Axio Imager Z1 microscope equipped with an
185 Axiocam MR3.0 camera (Carl Zeiss) and Axiovision SE64 Rel. 4.9.1 software. A total of twenty-twenty five
186 fields of view were randomly selected from each brain section and photographed at 20x magnification. The total
187 length of the blood vessels per field were measured using the ImageJ software.

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189 **Production and purification of human recombinant TTR**

190 Human recombinant WT TTR (rec TTR) was produced in a bacterial expression system using *Escherichia coli*
191 BL21 [50] and purified as previously described [51]. Briefly, after growing the bacteria, the protein was isolated
192 and purified by preparative gel electrophoresis after ion exchange chromatography.

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194 **Purification of human TTR from sera**

195 Human plasma from donors who were informed of the purpose of the study and gave their written consent, were
196 collected in accordance with the approved guidelines. Samples were subjected to affinity chromatography to
197 isolate human TTR (hTTR); for this we used 1 mL column of NHS-activated Sepharose coupled to rabbit anti-
198 human TTR (Dako). The column was washed with PBS and then incubated with 500 µL of human plasma for 2
199 hours at RT. To elute TTR from the column, 5 mL of Gentle Ag/Aβ elution buffer (Thermo Scientific) were
200 applied, and 1 mL-aliquots were collected and OD 280 nm was registered.

201

202 **Cell culture**

203 The immortalized human cerebral microvascular endothelial cell line, hCMEC/D3 (Tebu-Bio) is a well-
204 characterized *in vitro* model of BBB. The hCMEC/D3 cells were used between passage 25 and 35 and cultured
205 following the available data sheet. All culture flasks were coated with rat tail collagen type I solution (Sigma) at
206 a concentration of 150 µL/mL and were incubated for 2 hours at 37°C. Cells were cultured in EBM-2 medium
207 (Lonza) containing 5% FBS (Gibco), 1% of penicillin-streptomycin (Lonza), 1.4 µM of hydrocortisone (Sigma-
208 Aldrich), 5 µg/mL of ascorbic acid (Sigma-Aldrich), 1% of chemically defined lipid concentrate (Gibco), 10 mM
209 of 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid (HEPES) (Gibco) and 1 ng/mL of human bFGF (Sigma-
210 Aldrich). Cells were incubated at 37 °C in a humidified atmosphere with 5% of CO₂. Cell culture medium was
211 changed every 2–3 days.

212

213 **Tube formation assay**

214 hCMEC/D3 cells, grown in 25 cm² flasks, at a confluence of 80-90% were incubated for 24 hours with EBM-2
215 medium (Lonza) containing 1% FBS (Gibco) (negative control), with bFGF (35 ng/mL) (positive control), or with
216 rec TTR at different concentrations (10, 25, 250, 500 nM and 1 µM) or with hTTR 250 nM. Then, cells were
217 transferred into 96-well plates, previously coated with Matrigel (Corning), and grown in the same conditions of
218 media, bFGF or TTR for another 9 hours. Then, cells were photographed using the In Cell Analyzer 2000 (GE
219 Healthcare) (magnification ×10). The supernatants were collected, centrifuged at 14.000 rpm for 10 minutes and
220 stored at -20°C. Each condition was performed in triplicate and experiments were repeated three times.

221 **Quantification of angiogenesis-related proteins**

222 The angiogenesis-related proteins interleukins 6 and 8 (IL-6, IL-8), angiopoietin 1 and 2 (Ang-1, Ang-2),
223 epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet endothelial cell adhesion molecule
224 (PECAM-1), placental growth factor (PlGF), VEGF and tumor necrosis factor α (TNF- α) were quantified in the
225 supernatants collected from hCMEC/D3 grown under conditions of tube formation in the presence of media alone
226 or with 1 μ M rec TTR, using the LEGENDplex Human Angiogenesis Panel (BioLegend) bead-based
227 immunoassay. The assay was performed according to the manufacturer's recommendations. Analysis was
228 performed using a BD Accuri C6 (BD Biosciences) and LEGENDplex™ Data Analysis software v8.0
229 (BioLegend).

230

231 **ELISA analysis for IL-6**

232 IL-6 was also quantified in the supernatants collected from hCMEC/D3 cells used for the tube formation, in the
233 presence of media alone or with rec TTR at different concentrations (10, 25, 250 nM and 1 μ M), using a LEGEND
234 MAX™ Human IL-6 Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) Kit (BioLegend) with pre-
235 coated plates. The assay was performed according to the manufacturer's recommendations. Analysis was
236 performed using Synergy Mx and by measuring absorbance at 450 and 570 nm. A standard curve was generated
237 for IL-6 from 7.8 pg/mL to 500 pg/mL.

238

239 **Angiogenesis chick chorioallantoic membrane (CAM) assay**

240 Commercially available fertilized chick (*Gallus gallus*) eggs were horizontally incubated at 37 °C, in a humidified
241 atmosphere. On embryonic development day (EDD) 3, a square window was opened in the shell after removal of
242 1.5–2 mL of albumen, to allow detachment of the developing CAM. The window was sealed with a transparent
243 adhesive tape and eggs re-incubated. On EDD10, rec TTR (1 μ M), hTTR (1 μ M), PBS (vehicle, negative control)
244 and bFGF (50 ng/ μ L, positive control) were placed on top of the CAM, into 3 mm silicone rings, under sterile
245 conditions (1 condition per egg). Eggs were re-sealed and returned to the incubator for an additional 72 hours. On
246 EDD13, rings were removed, the CAM was excised from embryos and photographed *ex-ovo* under a stereoscope,
247 using a 20 \times magnification (Olympus, SZX16 coupled with a DP71 camera). The number of new vessels (< 20 μ m
248 diameter) growing radially towards the inoculation area was counted in a blind fashion manner.

249

250 **In vivo analysis of vascular permeability**

251 The CAM model was also used to evaluate vascular permeability or vessel leakage, as measure of TTR induced
252 neo-vessels functionality. Embryos were cultured *ex ovo*. To prepare shell-less CAM, eggs were incubated as
253 described above and on EDD3, the content of the egg was transferred to sterile weigh boats, covered with square
254 petri dishes and returned to the incubator for additional 7 days. At EDD10, 10 μ l of PBS, rec TTR (1 μ M) and
255 VEGF (4 ng/ μ L) were inoculated on distinct sites of the same egg, twice each, into 3mm silicone rings under
256 sterile conditions. Three independent experiments were performed summing a total 16 CAM sites/condition (8
257 eggs). After 3 days (EDD13), chicken embryos were injected intravenously with 100 μ l of Even's Blue Dye (EBD,
258 Sigma) solution (0.5% EBD, 5% BSA in PBS) and further incubated for 60 minutes. After incubation, embryos
259 were perfused with saline. The tissue underlying the rings (inoculation sites) was removed, cleaned in saline,
260 blotted dry, weight, homogenized and incubated in 200 μ l of formamide (Sigma), at 38^a for 48h, to release the

261 extravasated dye. The samples were centrifuged and 175 μ l of supernatant was quantified spectrophotometrically
262 at 620 nm. The amount of EBD in the experimental samples was calculated by interpolating to a standard curve
263 and the concentration of EBD per g of tissue was determined.

264

265 **Statistical Analysis**

266 All quantitative data were expressed as mean \pm standard error of the mean (SEM). Initially, data was assessed
267 whether it followed a Gaussian distribution. In the cases of non-Gaussian distribution comparisons between two
268 groups were made by non-parametric Kruskal-Wallis test and comparisons between two groups were made by
269 Student t-test with a Mann Whitney test.

270 When found to follow a Gaussian distribution, differences among conditions or groups were analyzed by one-way
271 ANOVA with the appropriate post hoc pairwise tests for multiple comparisons tests. Differences in CAM assay
272 and IL-6 Elisa kit were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. P-values
273 lower than 0.05 were considered statistically significant. Statistical analyses were carried out using GraphPad
274 Prism 8 software for Windows.

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284 **Results**

285 **TTR influences vascular features in the mouse brain**

286 For this work we have used AD/TTR^{+/+}, AD/TTR^{+/-}, NT/TTR^{+/+} and NT/TTR^{+/-} animals. We did not analyze
287 the respective TTR^{-/-} animals, although we obtained them in the course of breeding, to avoid indirect effects
288 of TTR-deficiency including compensatory processes, that could confound our interpretations. Additionally, the
289 TTR^{+/-} animals, in particular the AD/TTR^{+/-}, are a better representation of the behavior of TTR in AD, since
290 TTR is decreased in this pathology but not absent.

291

292 Reduction of TTR increases the thickness of the collagen IV layer in brain microvessels of AD mice

293 To investigate a possible relation of TTR reduction with the thickening of the BM and with the structural vascular
294 alterations reported in AD, we evaluated collagen IV levels in brain microvessels, in the AD/TTR mouse model.
295 This model established in different TTR genetic backgrounds [49], bears two AD-related transgenes (APP and
296 PSEN1) and A β deposition starts at around 6 months [52]. In comparison to males, females present a more severe
297 form of AD-like disease, thus in this study we used 7-month-old female animals. AD/TTR^{+/-} females were
298 compared to littermates with normal TTR expression, AD/TTR^{+/+}. Our results revealed a significantly thicker
299 collagen IV layer in 7-months old AD/TTR^{+/-} as compared to AD/TTR^{+/+} animals, as analysed in cortex
300 microvessels (Figure 1.A1). Altogether, our results implicate TTR in vascular processes, which are known to be
301 early dysregulated in AD.

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303 Reduction of TTR decreases the length of brain microvessels in AD mice

304 To understand if TTR affects other cerebrovascular features and if the effect observed at the level of the BM is
305 related to angiogenesis, we measured brain vascular density in the same animals.

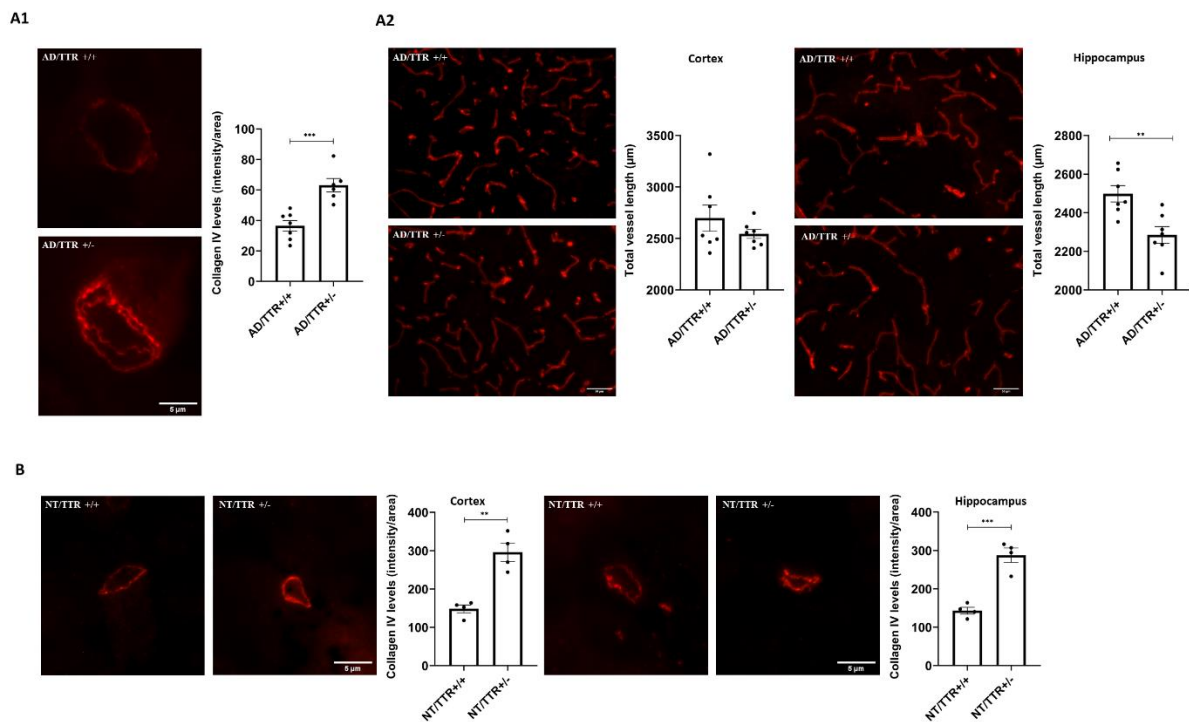
306 Both cortex and hippocampus were analyzed and our results show that, in hippocampus, reduction of TTR resulted
307 in decreased vessel length in AD/TTR^{+/-} mice as compared to AD/TTR^{+/+} (Figure 1.A2, right panels). In the
308 cortex, the differences were not statistically significant (Figure 1.A2, left panels), although there was always a
309 pattern of reduction of the length, as TTR is reduced. These observations support the results obtained for the BM
310 thickness and further implicate TTR in angiogenesis, especially in the hippocampus, a particularly relevant brain
311 area in the initial stages of AD.

312

313 Reduction of TTR increases the thickness of the collagen IV layer in brain microvessels of young non-transgenic 314 mice

315 Although our results suggest that TTR influences the thickness of the BM, in particular the collagen IV layer, we
316 could not determine if the effect was direct or indirect. One hypothesis is that high levels of A β , as it happens in
317 AD, either due to increased production, reduced elimination or both, could be responsible for the increase in
318 collagen IV. It is possible that AD/TTR^{+/-} mice show increased amount of collagen IV because less TTR is
319 available to interact with and to eliminate A β . Thus, in order to unravel this question, we compared collagen IV
320 levels in non-transgenic (NT) littermate mice with two different TTR backgrounds, NT/TTR^{+/+} and NT/TTR^{+/-}
321 allowing to understand if TTR is directly involved. Furthermore, this evaluation was performed at the age of 3
322 months, which in the AD background is prior to amyloid deposition [52]. Results presented in Figure 1.B clearly
323 show that NT/TTR^{+/-} mice presented significantly higher levels of collagen IV in brain microvessels of both the

324 cortex and hippocampus, as compared to NT/TTR+/+ animals, thus suggesting that it is, in fact, a direct effect of
 325 TTR.
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 328 **Figure 1- Effect of TTR reduction in vascular features of brain microvessels**
 329 (A1) Representative images and quantification plots of collagen IV levels in the BM of cortex vessels derived
 330 from 7-month-old AD mice with different TTR genetic backgrounds, AD/TTR+/+ (n=7) and AD/TTR+/- (n=6),
 331 showing significantly increased levels in microvessels from AD/TTR+/- compared to AD/TTR+/+ mice. Scale
 332 bar = 5 μm. (A2) Representative images and quantification plots of length and area of brain vessels,
 333 as evaluated by collagen IV staining, from 7-month-old AD mice with different TTR genetic backgrounds,
 334 AD/TTR+/+ (n=7) and AD/TTR+/- (n=7), showing significantly decreased vessel length in the hippocampus of
 335 AD TTR+/- compared to AD TTR+/+. Scale bar = 50 μm. (B) Representative images of the cortex and
 336 hippocampus and quantification plots of collagen IV immunostaining in microvessels of NT/TTR+/+ and
 337 NT/TTR+/- 3-month-old mice. An increase in collagen IV content in NT/TTR+/- mice (n=4) relative to
 338 NT/TTR+/+ littermates (n=4) is observed. Scale bar = 5 μm. Data are expressed as mean ± SEM. ** p<0.01; ***p<0.001.

339
 340 **TTR possesses angiogenic activity**

341 TTR promotes tube formation by hCMEC/D3 cells

342 The tube formation assay is a powerful *in vitro* test encompassing EC adhesion, migration, protease activity and
 343 tube formation (capillary-like structures). Thus, and to explore the angiogenic activity of TTR, endothelial cells
 344 of human brain origin, hCMEC/D3 cells, grown under tube formation-conditions, on Matrigel, were incubated
 345 with different concentrations of rec TTR. The results are displayed in Figure 2.A, and reveal that TTR affects the
 346 tube formation processes in a dose dependent-manner. Concentrations of TTR equal or above 250 nM result in a
 347 significantly higher area covered by the capillary-like structures, as compared to the negative control. These TTR
 348 concentrations are well below its physiologic concentration in plasma, and are similar to its concentration in the

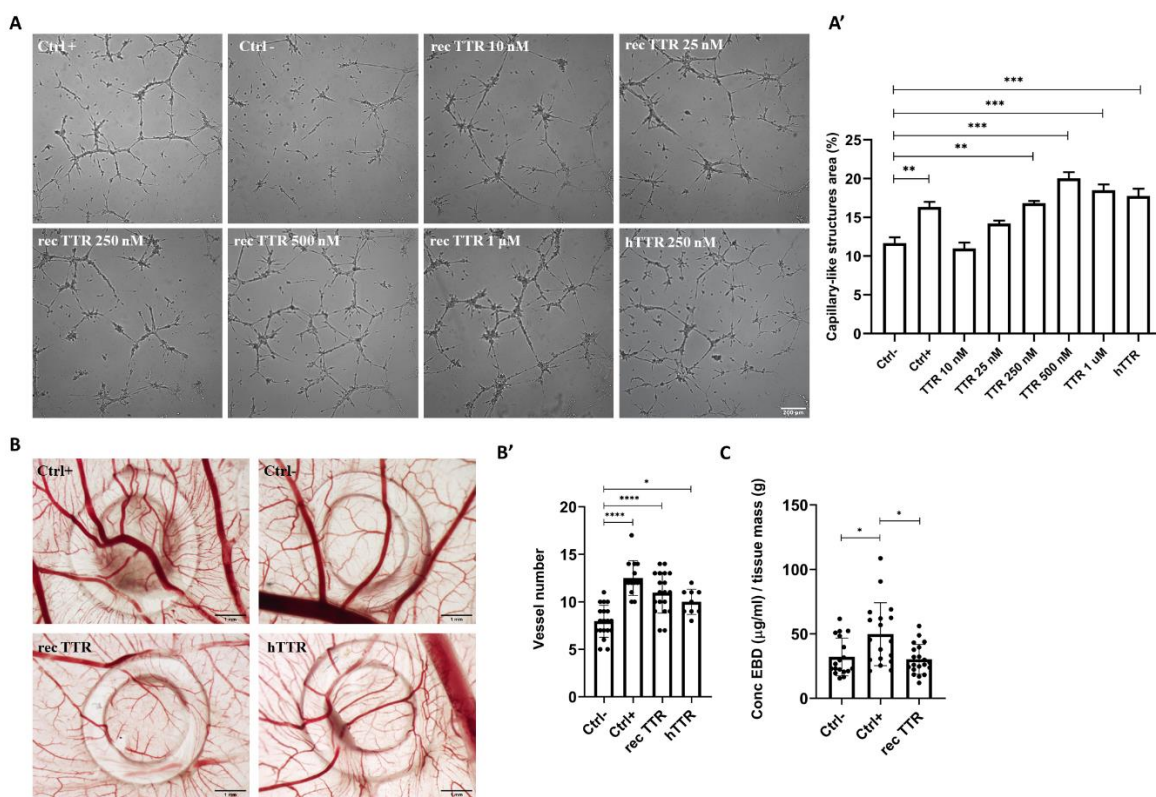
349 CSF. To confirm that the angiogenic effect was indeed provided by TTR, human TTR isolated from serum (hTTR)
 350 was also evaluated, corroborating the angiogenic activity of TTR (Figure 2.A').

351

352 TTR is angiogenic *in vivo* and the neovessels formed are functional

353 To further confirm the angiogenic activity of TTR, we used the CAM assay. Both rec TTR and hTTR were tested
 354 and at 1 μM induced a significantly higher angiogenic response than the negative control, as deduced by the higher
 355 number of detected new vessels (vessels with a diameter under 20 μm) (Figure 2.B and 2.B'). TTR angiogenic
 356 response was comparable to the positive control (bFGF), in particular for the rec TTR.

357 Also using the CAM *in vivo* model, we studied the permeability of the TTR-induced vessels, by quantifying the
 358 leakage of EBD. This assay indicated that the permeability of TTR-induced vessels is comparable to the negative
 359 control (PBS), and significantly different from the positive control (VEGF) (Figure 2.C). It can be inferred that
 360 TTR induced neo vessels are functional (in contrast with leakier vessels induced by VEGF).



361

362 **Figure 2- TTR angiogenic activity.**

363 (A) Representative images of tube formation by hCMEC/D3 cells. Cells were plated on Matrigel in the absence
 364 (negative control, Ctrl-) or presence (positive control, Ctrl+) of bFGF (35 ng/mL) or with TTR at different
 365 concentrations (10 nM-1 μM). Scale bar = 200 μm . (A') The quantification plot shows that TTR concentrations
 366 equal or above 250 nM result in a significantly higher area covered by the capillary-like structures, than in the
 367 negative control. (B) Representative images of the chick chorioallantoic membrane (CAM) assay. (B')
 368 Quantification plot of the number of new vessels (< 20 μm) growing towards the inoculation site, delimited by the
 369 ring mark, induced by PBS), (Ctrl-, n=18), basic fibroblast growth factor (bFGF, 50 ng/ μL) (Ctrl+, n=14), human
 370 recombinant TTR (rec TTR, 1 μM , n=19) or TTR isolated from human plasma (hTTR, 1 μM , n=9). TTR, both
 371 rec TTR and hTTR, had a significantly higher angiogenic response than the negative control. Scale bar = 1 mm.

372 (C) *In vivo* vascular permeability was measured in CAM model by quantification of leaked EBD. The permeability
373 of the new vessels induced by TTR (n=20) was similar to the negative control (PBS, n=18), in contrast to the
374 significantly higher permeability of vessels induced by VEGF (n=18). Data are expressed as mean \pm SEM. *
375 $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

376

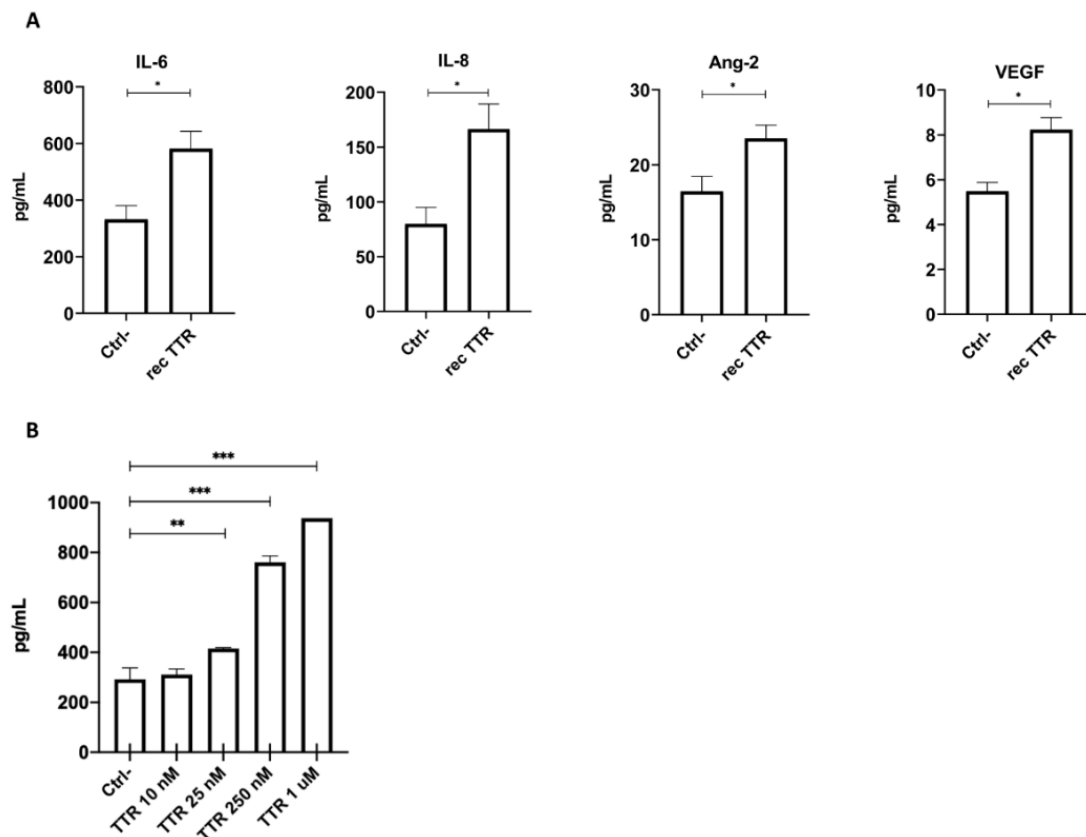
377 TTR regulates angiogenic molecules

378 To further explore the molecular mechanisms underlying the angiogenic activity of TTR, supernatants of
379 hCMEC/D3 cells grown under tube-formation conditions, in the presence of rec TTR (1 μ M) or with media alone,
380 were used to identify key targets involved in angiogenesis which could be affected by TTR.

381 Among the ten molecules analyzed, IL-6, IL-8, Ang-2 and VEGF were differentially overexpressed in the
382 presence of TTR, whereas the remaining six presented concentrations below the detection limit. As shown in
383 Figure 3.A, the expression of detected molecules was significantly increased relative to the negative control when
384 stimulated with rec TTR (1 μ M) indicating that TTR acts as a pro-angiogenic molecule by increasing the
385 expression of those molecules. It is possible that TTR affects other angiogenic molecules, possibly even those that
386 were undetected by the current approach.

387 We also confirmed the effect of IL-6 using an ELISA approach and as can be appreciated in Figure 3.B it is clear
388 a dose-response effect as TTR concentration is increased. While at 10 nM the differences are not significant,
389 concentrations between 25 nM and 1 μ M lead to significantly increased expression of IL-6, as compared to the
390 negative control. These results are also in line with those of the tube formation assay (Figure 2.A').

391



392

393 **Figure 3- Quantification of angiogenesis-related proteins.**

394 Supernatants from hCMEC/D3 cells grown under conditions of tube formation were collected 9h after incubation
395 with media alone or with rec TTR (1 μ M). (A) Ten of the most common angiogenesis-related proteins were
396 quantified using bead-based LEGENDplex assay by flow cytometry. Rec TTR 1 μ M revealed ability to
397 significantly increase the levels of IL-6, IL-8, Ang-2 and VEGF. Comparisons are relative to the negative control.
398 (B) IL-6 levels measured by ELISA showed that while TTR 10 nM did not affect IL-6, TTR concentrations 25
399 nM-1 μ M increased, in a concentration-dependent manner, the levels of IL-6. Data are expressed as mean \pm SEM.
400 * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

401

402 **The impact of TTR reduction on BM thickening is greater in AD than in NT mice**

403 To understand if TTR reduction impacts differently in an AD and in a non-AD environment, we analyzed the
404 effect of the same TTR reduction on the collagen IV layer, in the AD and in the NT backgrounds (NT/TTR+/-
405 versus AD/TTR+/-). Figure 4 depicts the results obtained and shows that the impact of TTR reduction is greater
406 in an AD-like environment, adding relevance to TTR neuroprotection in AD. Given that these are 3-month-old
407 animals and that, in this model, deposition begins at around 6 months, our results support other findings that
408 suggest brain vascular dysregulation as the earliest factor during the disease progression.

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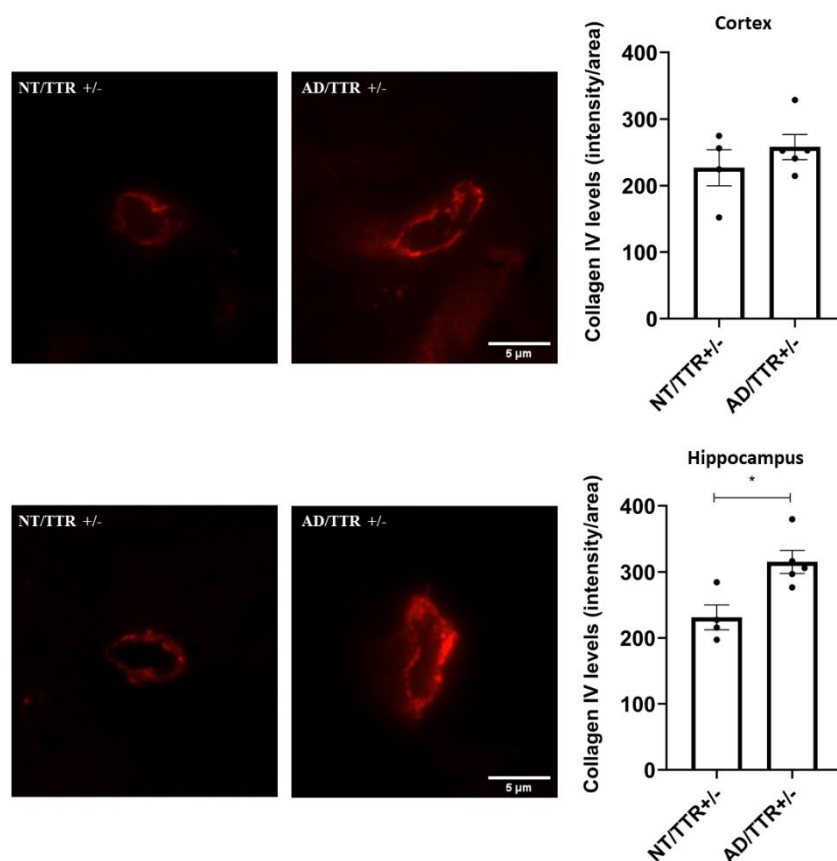
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429 **Figure 4- Effect of TTR reduction on collagen IV levels, in NT and AD mice**

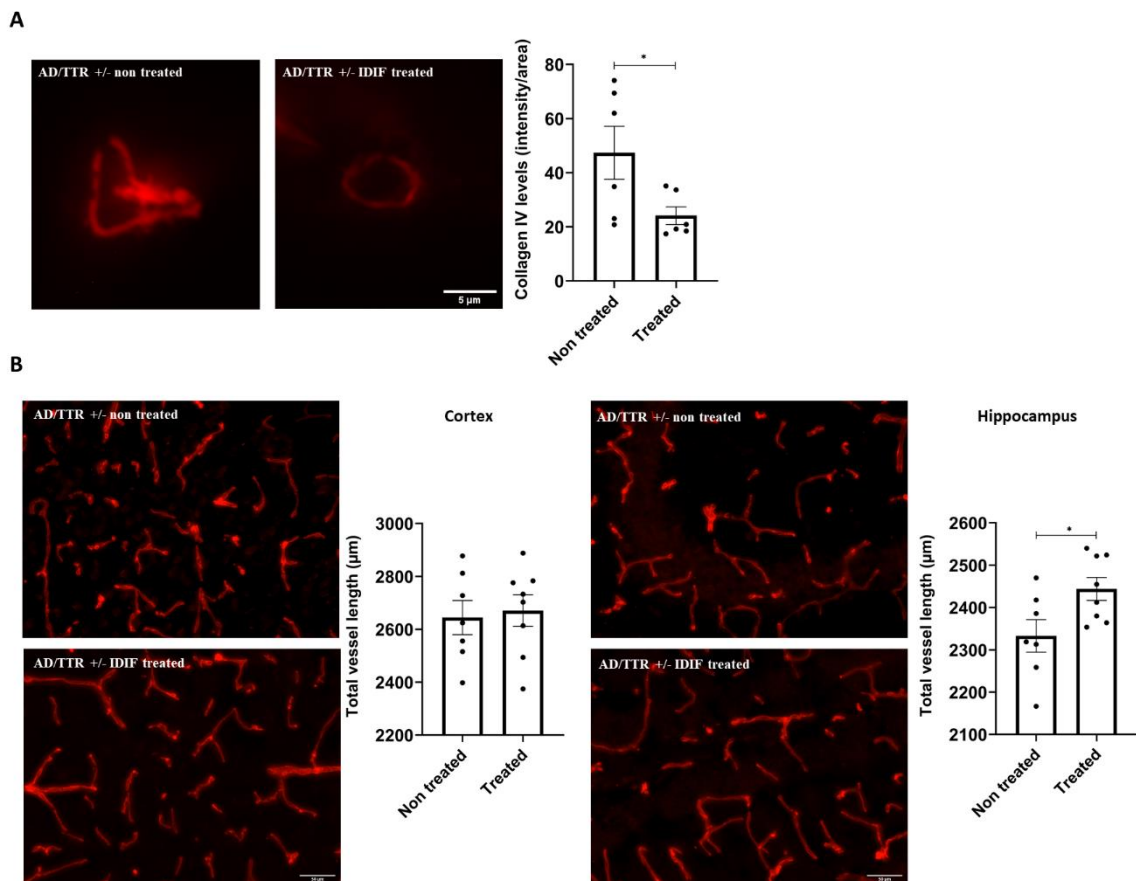
430 Representative images and quantification plots of collagen IV (red) immunostaining depicting microvessels of
431 NT/TTR+/- (n=4) and AD/TTR+/- (n=5) 3-month-old mice in cortex and hippocampus, showing an increase in
432 collagen IV expression in AD/TTR+/- mice relatively to NT/TTR+/- littermates, in the hippocampus. Data are
433 expressed as mean \pm SEM. * $p < 0.05$. Scale bar = 5 μ m.

434 **TTR stabilization results in decreased thickness of the collagen IV layer in brain microvessels of AD mice**

435 So far, in this work, we have shown that TTR reduction worsens AD features in mice, as BM thickening. To
436 further investigate a possible neuroprotective effect of TTR in the vascular context, and given that TTR
437 stabilization is used to improve its activity, we analysed the BM thickness in brain microvessels in AD mice
438 treated with one TTR stabilizer, IDIF.

439 Administration of IDIF to AD mice from the age of 5 to 7 month-old, resulted in amelioration of some AD features,
440 such as the cognitive function and decreased A β brain levels [41].

441 In this work, we used brain slides obtained in our previous study, above mentioned [41] and performed collagen
442 IV staining to assess BM thickness and vessels length in AD/TTR \pm animals, non-treated versus IDIF-treated.
443 As depicted in Figure 5, AD/TTR \pm mice treated with IDIF presented a significant reduction in the BM thickness
444 and increase in vessel length, as compared to non-treated animals. Altogether, these results indicate that TTR
445 stabilization might be an avenue for early treatment in AD.



446
447 **Figure 5 – Effect of TTR stabilization by IDIF in the thickness of the collagen IV layer and vessel length of**
448 **AD/TTR \pm mice.** Representative images and quantification plots of vessels derived from AD/TTR \pm mice non-
449 treated (n=6), or treated with IDIF (IDIF treated) (n=6) evidencing (A) a significantly decreased collagen IV layer
450 in treated mice. Scale bar = 5 µm, and (B) a significantly increased vessel length in the hippocampus of IDIF
451 treated compared to non-treated mice. Scale bar = 50 µm. Data are expressed as mean \pm SEM. * p<0.05.

452

453 **Discussion**

454 TTR is a homotetrameric protein typically known as a carrier of T4 and retinol in plasma and CSF. During the
455 last years, several functions have been attributed to this protein, in particular, as a neuroprotector protein in
456 physiologic and in disease contexts. In ischemia models, induced by permanent middle cerebral artery occlusion,
457 TTR has been shown to be protective, as evidenced by the significant increase in cortical infarction, cerebral
458 edema and the microglial-leukocyte response in mice with TTR deficiency compared with normal TTR levels
459 [53]. Also, TTR deficiency results in spatial reference memory impairment [54]. Other works showed that TTR
460 promotes nerve regeneration and axonal growth [55,56].

461 In AD, TTR binds to A β avoiding its aggregation, accumulation and toxicity, and facilitating its efflux across the
462 BBB [26]. This barrier is essential to maintain brain homeostasis, however, during normal ageing and AD, BBB
463 becomes dysfunctional contributing to disease progression. Molecules known to be important for A β brain
464 homeostasis, such as LRP-1 and P-gp are reduced, and TTR was previously shown to increase LRP-1 expression
465 in brain ECs and liver [26]. Thus, it is possible that TTR can regulate the neurovasculature in other ways, namely
466 by influencing angiogenesis.

467 BM thickening through increased collagen IV levels is one such features observed in ageing and, more severely,
468 in AD. Previous work by González-Marrero and co-workers described concomitant reduced TTR expression and
469 thickening of the BM at the choroid plexus, in a triple transgenic mouse model of AD [57]. In addition, the authors
470 reported increased A β 42 in epithelial cytosol and in stroma surrounding choroidal capillaries [57]. Here, we
471 showed that reduction of TTR expression in an AD mouse model influenced not only the BM, resulting in a thicker
472 collagen IV layer, but also the vessel density, resulting in decreased vessel length. To ascertain if the differences
473 were due to a direct or indirect effect of TTR, collagen IV levels were evaluated also in NT 3-month-old mice.
474 NT/TTR \pm mice exhibited more collagen IV around brain microvessels than NT/TTR $+/+$ littermates, suggesting
475 a direct effect of TTR.

476 In the AD model at the age of 3 months, A β deposition has not started. However, at the same age AD/TTR \pm
477 mice show decreased TTR compared to NT/TTR $+/+$ [49]. In addition, mild cognitive impaired patients show TTR
478 decrease which further continues as AD progresses. Therefore, it is possible that TTR is early involved in the
479 vascular alterations that occur in AD. Understanding the causes for TTR decrease and finding ways to restore its
480 normal values and activity may be key in the development of a TTR-based therapy for AD.

481 It is not yet clear what leads to increased collagen IV levels in neurovasculature, but these changes are also found
482 in rats suffering from chronic cerebral hypoperfusion [58,59], suggesting that decreased blood flow in the brain
483 leads to high collagen IV content around the vessels. Indeed, diminished cerebral blood flow is an early impact
484 event during AD development [1]. The thickened and rigid vascular wall may slow down nutrient supply and
485 waste elimination, and possibly disturb perivascular drainage. This event along with the formed barrier will
486 potentially contribute to progressive endothelial dysfunction and to an increasing accumulation of A β in the brain.
487 We questioned if the effect of decreased TTR in the BM and vessel density could be related to TTR participation
488 in angiogenesis, since a number of works implicated TTR in this process. These works suggest that TTR
489 contributes to disease development by modulation ECs function. It is interesting to note that TTR levels are
490 increased in those situations, such as diabetes type II, and in some reported situations of cancer, diseases where
491 abnormal angiogenesis is an established hallmark [46,60,61]. TTR is decreased in AD [27–30] but its angiogenic

492 potential was never evaluated *in vivo*. Using the *in vivo* CAM assay, we demonstrate that WT TTR, both produced
493 recombinantly and purified from human plasma, influences angiogenesis by promoting the formation of new
494 functional vessels.

495 As for the relation with brain angiogenesis, we showed that TTR promotes the formation of capillary-like
496 structures by hCMEC/D3 cells and found that VEGF, Ang-2, IL-6 and IL-8 were significantly up-regulated in the
497 presence of the protein. VEGF is a major driver of angiogenesis, playing a role in most of the steps of the process.
498 Previous works have suggested a link between TTR and VEGF, and for example, elevated VEGF was found in
499 the vitreous of patients with TTR amyloidosis [62]. Another work, proposed an interaction between the two
500 molecules, although reporting that inhibition of VEGF in branch retinal vein occlusion (BRVO) upregulated TTR
501 [63]. This can be interpreted as an attempt of the cells to restore VEGF levels by increasing TTR, thus
502 corroborating our observations in hCMEC/D3 cells.

503 Ang-2 was previously found to be up-regulated in retinal ECs after treatment with TTR [44] and plays a
504 controversial role in angiogenesis. If, on one hand, it increases migration capacity and tube formation in brain
505 ECs [64], on the other hand, *in vivo* retinal studies showed that Ang-2 promotes EC death and vessel regression
506 if VEGF is absent. However, when in the presence of VEGF it stimulates an increase in capillary diameter,
507 remodeling of basal lamina, proliferation and migration of EC [65]. These studies support our findings where
508 TTR promotes an increase of both VEGF and Ang-2, which should result in the promotion of angiogenesis.

509 The observed upregulation of IL-8 and IL-6 is also consistent with an increase in angiogenesis given that IL-8
510 enhances proliferation, survival, migration and tube formation [66,67]; and IL-6 was shown to induce an increase
511 in EC proliferation, migration and tube formation [68,69].

512 The importance of TTR in the AD pathogenesis is also patent when comparing AD with NT 3 months-old mice
513 with the same TTR reduction, evidencing a thicker BM in the AD animals. Although this happens prior to
514 amyloid- β deposition, we cannot exclude the presence of other A β species that can contribute to this increase, and
515 add to the direct effect of TTR observed in the NT mice.

516 It is not known if the reduction of TTR in AD is a cause or effect of the disease but it is well known that TTR
517 stability is key for its activity. Mutations in TTR, associated with amyloidosis, create tetrameric instability leading
518 to dissociation into monomers. TTR stabilization seems also important to prevent pathological changes to the
519 brain vasculature, and for example, heterozygous individuals with TTR T119M allele, which renders a more stable
520 tetramer, have a reduced risk of cerebrovascular disease compared to homozygotes for WT TTR [70]. In AD, TTR
521 stability is decreased, leading to accelerated clearance and consequently, to lower levels. We previously showed
522 that TTR stabilization, achieved through the use of small-molecule compounds, sharing structural similarities with
523 T4 and binding in the TTR central channel, results in improved TTR binding to A β [19]. One of those small-
524 molecule stabilizers, IDIF administrated to our AD/TTR \pm mouse model resulted in the amelioration of AD
525 features [40,41]. In this work we showed that IDIF reverted, at least partially, the vascular alterations induced by
526 TTR decrease.

527 Our work provides positive and new results but it also reveals some limitations that should be mentioned.
528 Concerning the studies with animals, only females were used, while a final conclusion on the effect of TTR
529 decrease regarding the vascular alterations in AD may require the use of both genders. The animal model used in
530 this study shows gender-associated modulation of brain A β levels by TTR, and females present a more severe

531 AD-like neuropathology [49], which results in a more favourable scenario to assess the involvement of TTR in
532 AD, explaining why we carried out our experiments in females.

533 Our results uncover angiogenesis as a mechanism in which TTR participates and importantly, it shows that TTR
534 reduction has an impact in the vascular alterations that occur early in AD with the possibility of recovery upon
535 TTR stabilization.

536

537 **Conclusions**

538 In summary, this work shows that TTR has pro-angiogenic properties, up-regulating molecules such as IL-6, IL-
539 8, Ang-2 and VEGF. TTR is also involved in the early vascular alterations occurring in AD, which may be used
540 as a target for therapeutic intervention in AD.

541

542 **Abbreviations**

543 AD: Alzheimer's Disease; BM: basement membrane; TTR: Transthyretin; NT: non-transgenic; CAM: chick
544 chorioallantoic membrane assay; IL: interleukin; Ang-2: angiopoietin-2; VEGF: vascular endothelial growth
545 factor; IDIF: iododiflunisal; A β : amyloid- β peptide; LRP-1: low density lipoprotein receptor-related protein 1;
546 BBB: blood-brain barrier; EC: endothelial cell; CSF: cerebrospinal fluid; FAP: Familial Amyloid Polyneuropathy;
547 T4: thyroxine; bFGF: basic fibroblast growth factor; hCMEC/D3: human cerebral microvascular endothelial cell
548 line; bEnd.3: mouse brain endothelial cell line.

549

550 **Declarations**

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557 **Conflicts of Interest/Competing interests:** The authors declare no conflict of interest.

558 **Ethics approval:** All the above experiments were approved by the Institute for Research and Innovation in Health
559 Sciences (i3s) Animal Ethics Committee and in agreement with the animal ethics regulation from Directive
560 2010/63/EU.

561 **Consent to participate:** Not applicable

562 **Availability of data and material:** All data and material present in this study available upon reasonable request
563 to the corresponding author.

564 **Code availability:** Not applicable

565 **Author Contributions:** TG, JS and JRV performed the experiments; MP was responsible for the CAM assays,
566 and respective analysis and data interpretation; IC was responsible for conception and supervision of the work.
567 IC, TG, JS and GA discussed the results and wrote the manuscript. All authors reviewed the article and have read
568 and agreed to the published version of the manuscript.

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