Platelets in the Alzheimer’s Disease Brain: do they Play a Role in Cerebral Amyloid Angiopathy?

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Abstract: Alzheimer’s disease (AD) is characterized by extracellular beta-amyloid plaques and intracellular tau tangles. AD-related pathology is often accompanied by vascular changes. The predominant vascular lesions in AD are cerebral amyloid angiopathy (CAA) and arteriosclerosis. Platelets circulate along the vessel wall responding immediately to vascular injury. The aim of the present study was to explore the presence and migration of platelets (thrombocytes) to sites of small vascular bleedings and/or to beta-amyloid plaques in the brain. We infused fluorescently labeled red PKH26 mouse platelets into transgenic Alzheimer mice overexpressing APP with Swedish/Dutch/Iowa mutations (APP_SDI) and explored if platelets migrate into the brain. Further we studied whether platelets accumulate in the vicinity of β-amyloid plaques. Our animal data shows that infused platelets are found in the liver and partly in the lung, while in the brain platelets were visible to a minor degree. In mice, we did not observe a significant association of platelets with beta-amyloid plaques or vessels. In the brain of Alzheimer postmortem patients platelets could be detected by immunohistochemistry for CD41 and CD62P, but the majority was found in vessels with or without beta-amyloid load, and only a few single platelets migrated deeper into the brain. Our findings suggest that platelets do not migrate into the brains of Alzheimer disease but are concentrated in brain vessels.

Keywords: Alzheimer, migration, postmortem, platelet, vessel.

INTRODUCTION

Alzheimer’s disease (AD) is the most common cause of dementia in the elderly. The hallmark pathologies include beta-amyloid (βA) depositions in brain (plaques) and vessels (cerebral amyloid angiopathy, CAA), tau pathology, cerebrovascular dysfunction, cholinergic impairment, microglia activation and inflammation. The causes for developing AD are not well known, however vascular risk factors contribute to cognitive decline and vascular damage may play a potent role [1-4]. It is well known that cerebrovascular dysfunction occurs in AD patients leading to alterations in the blood flow that might play an essential role in AD pathology contributing to neuronal loss and memory deficits. Neurotoxic βA plaque formation is seen in the walls of the brain blood vessels (CAA). CAA is a critical factor in the pathogenesis of AD, because the deposition of βA peptides may induce degeneration of vessels subsequently reducing cerebral blood flow. However, the mechanisms how βA modifies hemostasis and thrombosis are not well known.

Platelets are considered as key elements linking βA deposition, peripheral inflammation and endothelial senescence [5]. Further, platelet activation has been observed in diabetes and may contribute to AD development [6]. In fact, platelet activation and adhesion to the vessel wall is the primary step of vascular injury mediating the development and onset of CAA [7]. Thus, besides their normal function in hemostasis, platelets play a central role in pathological thrombus formation, which is a crucial risk factor for AD development [8, 9]. Further, it became increasingly evident that blood platelets contribute to inflammation and cooperate with peripheral immune cells [10] and are altered in neuroinflammatory diseases including AD [11].

Platelets share some properties with neurons, because they store, release and take up the neurotransmitter serotonin, being a therapeutic target [12]. Most importantly, platelets store and process also the amyloid precursor protein (APP) [13], which is cleaved into the toxic βA peptides and accumulates βA in the brain and vessels in AD. Moreover it was seen that altered platelet function such as increased activation [14] or altered membrane fluidity [15, 16] as well as an altered APP ratio [17, 18] are associated with the development of AD. Most importantly, platelets play a central role in pathological thrombus formation and thrombocytopenia, which are fundamental risk factors for AD [9, 10, 19]. Effectively, uncontrolled platelet activation...
can lead to vessel occlusion and subsequently to myocardial infarction or stroke.

The aim of the present study was to investigate to which extent fluorescently labeled platelets are found in the brain of transgenic AD mice and explore if these platelets are associated with βA plaques. The association with βA plaques and vascular pathology is also investigated in AD postmortem human brains.

**METHODS**

**Isolation of Platelets**

Blood was taken from adult anesthetized (50 mg/kg Thiopental) wildtype C57BL/6N mice directly from the heart and collected in EDTA tubes, and was centrifuged at 100xg for 15 min to obtain platelet rich plasma (PRP). All centrifugation steps were performed at room temperature. PGI2 (Prostaglandin, 500 nM, Sigma) was added to prevent platelet activation during processing. Platelets were isolated from PRP by centrifugation at 400xg for 10 min and washed in calcium-free Tyrode buffer (136 mM NaCl, 2.7 mM KCl, 12 mM NaHCO3, 0.42 mM NaH2PO4, 1 mM MgSO4, 5 mM glucose, pH 6.5). After further centrifugation at 400xg for 10 min platelets were finally resuspended in Tyrode buffer (adjusted to pH 7.4) and further processed.

**Labeling of Platelets with PKH26**

Platelets were labeled with the red dye PKH26 Red Fluorescent Cell Linker Kit (Sigma) modified as described previously [20]. Briefly, isolated platelets were resuspended in 100 µl diluent C, then 2 µl of the diluted dye was added and the cells were incubated for 5 min at room temperature. After the incubation, 1 ml of Tyrode buffer (pH 7.4) was added, the cells centrifuged at 400xg for 10 min and resuspended for further use.

**FACS Analysis**

FACS analysis on freshly isolated platelets was performed with a BD FACSs (FACScan; Becton Dickinson) as described recently [21]. Briefly 10 µl of isolated platelets (1:10 diluted) were incubated with antibodies against CD31 (Miltenyi Biotec 1:10) or CD61 (Miltenyi Biotec 1:10) or CD62P (BD 561923) or with IgG1 (Miltenyi Biotec 1:25) or IgG2a (Milteny 130-089-877) as a negative control in 50 µl FACS buffer (2 mM EDTA, 0.5% FCS, in phosphate-buffered saline (PBS), pH 7.1) for 30 min at 4°C washed and analyzed. In order to study cell death, 2 µl 7-aminoactinomycin D (7-AAD, BD 559925) was added just prior to analysis.

**In Vivo Infusion into Alzheimer Mice**

The Alzheimer mouse model (C57Bl/6-Tg(Thy1-APPSwDulowa)Bwevn/Mmjax) was obtained from Jackson Laboratory. These transgenic mice express neurally derived human APP, harboring the Swedish K670N/M6781L, Dutch E693Q and Iowa D694N mutations. This model has been fully characterized [22] and exhibits marked βA plaques in brain and vessels after 6 months of age. Freshly isolated PKH26-labeled platelets (5x10⁸) were resuspended in 0.9% NaCl + 100U/ml heparin and slowly infused via the tail vein using a 30 gauge needle. After 24 hours the mice were anesthetized, blood was collected (and platelets isolated), the brains and organs (lung, liver, spleen) were dissected, one piece was immediately frozen in a CO₂ stream and another piece was put into 4% paraformaldehyde, fixed overnight, then cryoprotected in 20% sucrose overnight and stored in PBS/NaAcide until use.

**Visualization of PKH26 Labeled Platelets and Plaques**

The tissue was cryostat sectioned (40 µm) and thawed onto glass slides, coverslipped in PBS and immediately evaluated under the fluorescence microscope. The red fluorochrome (PKH26) displays excitation at 551 nm and emission at 567 nm and is seen under the red channel (EX 535/50, EM 610/75) and is not seen in the green channel (EX 480/40 nm, EM 527/30 nm). As a control 1 µl of labeled platelets was pipetted onto control brains (spiking). Plaques in the Alzheimer mouse model were visualized using the green Thioflavine S dye (Sigma, 1.6 µg/ml, overnight) and 4,6-diamidino-2-phenylindole,dilacatete (DAPI, Sigma, 0.1 µg/ml, 60 min) was used to stain nuclei.

**Postmortem Tissue**

Brain sections of two age-matched control human brains (Sez. 253/08 and Sez.836/09) and 3 Alzheimer cases (Sez. 350/08, Sez. 937/07 and Sez. 232/10) were obtained from Klagenfurt Hospital (see Table 1).

**Immunohistochemistry of Postmortem Brains**

Immunocytochemistry was performed on paraffinized sections (40 µm) using antigen retrieval. Briefly, sections were rehydrated in acid-n-butylester, (3x 5 min), and then in

### Table 1. Postmortem cases.

| Case     | Diagnosis | Age Died | Cause Death | postM Delay [hr] |
|----------|-----------|----------|-------------|-----------------|
| Sez. 253/08 | control  | 81       | Pneumonia   | 16              |
| Sez.836/09 | control  | 40       | Adenocarcinoma | 14             |
| Sez. 350/08 | AD       | 78       | Peritonitis  | 16              |
| Sez. 937/07 | AD       | 82       | Pneumonia   | 20              |
| Sez. 232/10 | AD       | 78       | Bronchopneumonia | 18            |
a series of ethanol (3x3 min 100% EtOH; 2x3 min 96% EtOH; 1x 3min 80% EtOH), then in a.d. (5 min) and finally cooked in 10 mM sodiumcitrate (pH 6.0) for 20 min at 100°C. Slides were then cooled down to room temperature, rinsed in PBS, incubated 30 min in PBS+0.1% Triton (T-PBS), blocked with 20% horse serum/0.2% bovine serum albumin/T-PBS and further incubated with the primary antibody mouse anti-cluster of differentiation 41 (CD41, integrin alpha 2b) (Serotec, 1:2000), rabbit anti-cluster of differentiation 62P (CD62P, P-selectin) (Abcam, 1:1000) or beta-amyloid (monoclonal anti-β-amyloid [13-28], clone BAM90.1, Sigma) in 0.2% bovine serum albumin/T-PBS at 4°C for 2 days. Sections were washed and incubated with secondary (anti-mouse for βA and CD41; anti-rabbit for CD62P) fluorescently labeled antibodies (Alexa 350 or Alexa 488 or Alexa 546, Invitrogen) for 1hr (1:400), washed and the sections coverslipped with Vectashield. Some organ sections were stained using the ABC dianibenzidine (DAB) method. Controls included omitting the primary antibody.

Quantification of Fluorescence Staining

The number of platelets was counted under the fluorescence microscope (Olympus, BX61) at a 40x magnification. Positive FITC staining was always verified by investigation in the other channel, where no autofluorescence is seen. Alexa-488 and Thioflavin S were visualized under the green filter L5 (EX 480/40 nm, EM 527/30 nm), PKH26 under the red filter Y3 (EX 535/50, EM 610/75) and Alexa-350 and DAPI under the blue filter (EX 360/40; EM 470/40).

RESULTS

Characterization of Mouse Platelets

Mouse platelets were isolated and characterized by FACS. A single population of platelets was observed (Fig. 1A) and only <1% was necrotic (Fig. 1B). Freshly isolated mouse platelets expressed CD31 (Fig. 1D), CD61 (Fig. 1E) and CD62P (Fig. 1F), compared to IgG controls (Fig. 1C). Platelets were labeled with the red PKH26 dye as seen in FACS (Fig. 2A) or fluorescence microscopy (Fig. 2B). The labeling was specific because red labeled platelets were not seen in the green channel (EX 480/40 nm, EM 527/30 nm) (Fig. 2C).

In wild type mice, no βA plaques were seen (Fig. 3A), while 12 month old APP_SDI mice contained a very high number of green Thioflavin S+ plaques (Fig. 3B). As a control freshly isolated red platelets were pipetted onto control brains, and a high number of red platelets was seen under the fluorescence microscope (Fig. 3C), however, it was not seen in the green channel (EX 480/40 nm, EM 527/30 nm) (Fig. 3D). Co-staining with DAPI+ nuclei revealed the small size of the platelets (Fig. 3E). In order to test the migration into the AD mouse model, PKH26 labeled platelets were infused via the tail vein and the brain and organs analyzed after 24 hour. Only very few platelets migrated into the brain (Fig. 3F) and were poorly seen close to Thioflavin S+ plaques (Fig. 3G&H). Quantitative analysis showed that only 2±0.5 platelets were found in a whole 40 μm thick brain section (n=5 brains; 22 sections analyzed each). In order to test the capture of red PKH26 platelets in the periphery, different organs were analyzed 24hr after infusion. Most of the platelets were found in the liver (10-20%; Fig. 2D&E) and in the lung (<1%; Fig. 2F&G), but not in the spleen and only a few platelets were seen in the brain (Fig. 2H&I) in the ventricle (Fig. 2I) or associated with a vessel (Fig. 2J). In the blood only <5% of the PKH26 labeled platelets was found (data not shown).

Platelets in Postmortem Human Brain

In order to investigate the presence of platelets in the human brain, 2 controls and 3 Alzheimer brains (Table 1) were stained for platelet marker CD41 and CD62P. Again visualization under the green fluorescence filter (EX 480/40 nm, EM 527/30 nm) compared to the red fluorescence filter (EX 535/50, EM 610/75) showed the specificity of the staining and not only autofluorescence (Fig. 4). In control brains only very few CD41+ or CD62P+ platelets were found outside of vessels in the brain tissue, while the majority was seen within vessels (Fig. 4). In Alzheimer brains, the characteristic neuropathological features were found with intensely stained βA plaques and βA depositions in vessels (CAA) (Fig. 5). Only very few CD62P+ platelets were seen in the tissue not associated with βA plaques (Fig.; Fig. 6). Many CD62P+ platelets were associated directly with a βA plaque or with a vessel close to a βA plaque (Fig. 5). The majority of CD62P+ platelets was seen in vessels without βA plaques or associated with cerebral amyloid angiopathy (Fig. 6). The platelets in association with βA plaques also displayed a discoid shape and were not different from platelets found in the vessels.

DISCUSSION

In the present study we show that platelets do not migrate deep into the AD brain but concentrate in vessels contributing to CAA. Besides we observed that infused platelets are captured in the liver and in the lung.

Characterization of Mouse Platelets & Labeling

Platelets are about 3 μm small cells and are processed from megakaryocytes and do not have a nucleus. For platelets characterization we used FACS analysis for CD31, CD61 and CD62P, antigens that are expressed on the surface of resting platelets. Platelets express CD31 (cluster of differentiation 31), a member of the immunoglobulin superfamily that is expressed on the surface of circulating platelets, monocytes, neutrophils, and particular T-cell subsets [23]. CD61 is also known as integrin β3 and is well established as a platelet marker. It is present as a subunit for the fibrinogen-receptor, playing a role in blood clotting [24]. CD62P is stored in alpha-granules of platelets, but normally is not expressed on the surface. This calcium dependent protein migrates during platelet activation to the plasma membrane and mediates platelets interaction with endothelial cells or leukocytes and CD62P serves as a marker for activated platelets. [25]. Thus, the isolation of the platelets
Fig. (1). Characterization of mouse platelets. Platelets from wild type mice C57BL/6N were isolated and characterized by FACS. FACS analysis revealed a common population of freshly isolated mouse platelets (A) with very low dead 7-amino-actinomycin (7-AAD+) cells (0.5±0.2%; B). Platelets stained positive for CD31 (34±15%, D green), CD61 (40±12%, E green) and CD62P (78±4%, F green) compared against an IgG1 control (red C) or IgG2a control (green C). (The color version of the figure is available in the electronic copy of the article).
Fig. (2). Labeling of mouse platelets with the red dye PKH26 and infusion into mice. Freshly isolated mouse platelets were stained with PKH26 and characterized by FACS (A) or by fluorescence microscopy (B&C). The fluorochrome PKH26 is seen in the red channel (EX 535/50, EM 610/75) and is not seen in the green channel (EX 480/40 nm, EM 527/30 nm) (C). Freshly isolated red platelets (5x10^8 in 100 μl) were injected via the tail vein and analyzed in the liver (D&E), lung (F&G) or brain (H&I) after 24 hr. The platelets were either stained by CD61+ immunohistochemistry using DAB chromogen (D, F, H) or directly by their fluorescence (E, G, I). In the liver strong CD61+ platelets were seen in hepatic vessels (* in D), but not in the liver tissue (Lt). In the lung CD61+ platelets were seen in the lung vessels (* in F) and in alveoli tissue (Al) but not in bronchioles (Br). In the brain, platelets were found in the plexus choroideus (* in H). Red platelets were poorly seen in the brain and associated with vessels (I). Scale bar = 6 μm (B&C) and 50 μm (D-I). (The color version of the figure is available in the electronic copy of the article).
Fig. (3). Infusion of PKH26 labeled platelets into the APP_SDI 12 month old mouse. Twelve month old APP_SDI mice show many Thioflavin S+ (green) beta-amyloid plaques in the cortex (B), while no staining is seen in age-matched C57BL/6N wildtype mice (A). As a control PKH26 red platelets were pipetted (1 μl spiking) onto control sections and visualized under the fluorescence microscope (C-E). Note several red platelets in the red channel (EX 535/50, EM 610/75) (C) but not in the green channel (EX 480/40 nm, EM 527/30 nm) (D). Co-staining with nuclear DAPI (blue in E) shows the small size of the platelets (E). When freshly isolated red platelets (5x10⁶ in 100 μl) were injected via the tail vein into 12 month old APP_SDI mice only very few platelets were found in the brain (F), and poorly associated with Thioflavin S (G, green) beta-amyloid plaques (H). Scale bar = 150 μm (A&B), 10 μm (C-H). (The color version of the figure is available in the electronic copy of the article).
Fig. (4). Platelets in a human postmortem control brain. Paraffin embedded brain sections were stained for CD41 (A&B) and CD62P (C-F) using fluorescence (Alexa 488, green, EX 480/40 nm, EM 527/30 nm) immunohistochemistry. Very few CD41⁺ (A) and CD62P⁺ (C) platelets were seen in the brain tissue, while the majority of CD62P⁺ platelets was associated with vessels (E). As a control for autofluorescence the same picture is shown also in the red channel (EX 535/50, EM 610/75) (B, D, F). Scale bar = 50 μm. (The color version of the figure is available in the electronic copy of the article).
Platelets in a human postmortem Alzheimer brain. Paraffin embedded brain sections were stained for beta-amyloid (Alexa 350, blue, EX 260/40, EM 470/40, ADGJ) and CD62P (Alexa 488, green, EX 480/40 nm, EM 527/30 nm, BEHK) by fluorescence immunohistochemistry. Fig. CFIL show the merged pictures. Example 1 (ABC) shows platelets (arrows) not associated with a beta-amyloid plaque (*). Example 2 (DEF) shows platelets (arrows) associated directly with a plaque (*) or in a vessel close to a plaque. Example 3 (GHI) shows platelets (arrow) in a beta-amyloid (*) vessel (c) representing cerebral amyloid angiopathy. Example 4 (JKL) shows platelets (arrow) in a vessel wall adjacent to the brain tissue probly entering the brain. Scale bar = 35 μm. (The color version of the figure is available in the electronic copy of the article).

Platelets are non-nuclear cell fragments and are derived from bone marrow megakaryocytes. They usually survive in the bloodstream of humans for 7-10 days [26]. Fresh isolated platelets were infused directly into the blood stream of mice via the tail vein. We used this vein, because it allows an easy fast and reproducible infusion into the mice without anesthesia. In order to study if these exogenous infused platelets are captured in the periphery, we found that a majority was captured in the liver, and also partly in the lung but not spleen, and a minority was seen in the blood. While we only observed this capture after 24hr, we cannot exclude that the exogenous platelets were degraded at earlier time points and we also cannot exclude capture in other organs. Anyhow, we found that only a few exogenous red labeled platelets entered the brain.

Infusion In Vivo, Capture and Migration Through the BBB

The blood-brain barrier (BBB) provides a tight barrier and protects the brain from entry of toxic molecules into the brain. In the intact brain, so far very few cells can enter the brain, however, it has been demonstrated that blood cells can enter the brain during normal immune patrol [27, 28]. However, in the diseased brain the BBB is damaged and small bleeds are found in the brain and thus blood cells can more easily enter the degenerating brain, including AD [29-31]. Indeed it has been shown that T-cells, monocytes, mast
cells or microglia can migrate into the brain [27,28, 32]. In fact, recent papers show that monocytes can migrate into the brain and are found close to βA plaques [33-38]. To our knowledge, we are the first to study if platelets can migrate into the brain. Platelets express a number of surface antigens and can directly interact with receptors at the BBB. Normally, platelets do not adhere to healthy non activated vessels, however, the interaction of platelets with the inflamed damaged endothelium of vessels is a complex process involving several different surface markers [39]. Platelets adhesion at sites of vascular injury involves tethering and rolling along the endothelium, however, stable adhesion requires additional complex inflammatory responses [39].

Migration into the Brain of an AD Mouse Model

AD is characterized by βA plaques and tau pathology, including inflammation and cerebrovascular dysfunction. Transgenic animal models allow to study some pathological hallmarks of this disease, but do not fully display an AD model. The APP_SDI model is a powerful model, βA plaques are markedly produced and in a 12 month old mouse the brain is nearly fully with βA depositions. The advantage of this model is also that it shows βA depositions in vessels, the CAA. The disadvantage of this model is that it does not have any tau pathology. Anyhow, we think that this model is very useful to study if platelets may enter the brain. Thus, red platelets were infused into the APP_SDI mice and the brains analyzed after 24hr. However, although the APP_SDI mice display a severe degenerative pathology, we could not find a marked migration of platelets into the AD mouse brain. Only here and there a few exogenous red platelets were seen. Technically it is not easy to visualize exogenous platelets, because they are very small (around 3-4 μm) and close to the detection limit by conventional fluorescence microscopy. Thus in order to verify fluorescence analysis we spiked a normal brain with red fluorescent platelets and observed a high number close to DAPI+ nuclei to directly compare their size. Although we verified that PKH26 is very specific, autofluorescence could not be excluded due to degradation dependent alteration after vein infusions. Thus, it was very important at any step to prove the specific fluorescence in the red channel (EX 535/50, EM 610/75) without any fluorescence in the green channel (EX 480/40 nm, EM 527/30 nm). However, although we show that platelets do not invade the AD mouse brain, there is clear indication that platelets may mediate and enhance the adhesion and possibly migration of leukocytes [40].

Platelets in Human Postmortem Brains

In the present study we performed postmortem analysis using antigen retrieval. In control brains only very few CD41+ or CD62P+ platelets could be seen in the brain. Again platelets could only be visualized in vessels in control human brains. The diagnosis in postmortem Alzheimer brains was verified by βA plaque histochemistry and tau pathology (not shown). Several βA plaques were seen throughout the brain section. Using immunofluorescence we found the majority of the CD62P+ platelets in brain vessels, either with or without βA depositions. Only a few CD62P+ platelets were visualized in the brain tissue but poorly associated with plaques. However, since we did not stain for brain vessels, we cannot exclude that the platelets seen in the tissue are associated with a small brain vessel. As initially stated, the adhesion of platelets to the inflamed tissue is very complex [10, 39], thus it is very unlikely that platelets can cross the BBB, which may be in line with our data in the mouse AD model. However, on the other hand, in severe AD massive vascular impairments, including microbleeds, and vascular lesions have been observed [41-45] and it is very likely that platelets can enter the brain via such lesion sites. In fact, our data may represent such a severe vascular impairment in the human postmortem brains.

Platelets and Cerebral Amyloid Angiopathy

Beta-amyloid deposition is a major hallmark in AD, not only in brain tissue but also in vessels, defined as cerebral amyloid angiopathy [46, 47]. It well known now that in the AD brain MRI+ microbleeds and small vascular bleeding are seen [44, 48-50]. However, it is unclear, if these vascular lesions occur prior or during the AD pathology, that means do lesions occur before or after βA depositions. We and others have already hypothesized [2, 8, 11, 51-54] that
vascular lesions may be a primary event in the development of AD. It seems likely that long lasting vascular impairment occurs over decades, as a cause of chronic exposure to vascular risk factors, such as e.g. cholesterol or homocysteine [55-57]. Similar as in the periphery, these small vascular bleeds activate blood platelets close to the rupture. As AD is a chronic disease, many such small vascular lesions may be induced during decades, however, the capacity of the platelets to repair these lesions may be exhausted in the elderly. Once platelets are activated at sites of vascular inflammation, they release bioologically active molecules which can modify the function of the vascular wall [58]. It is an interesting fact, that platelets contain very high amounts of APP, which generates the βA peptides. Platelets mainly express the smaller 40 amino acid form of βA, and although not fully proven, it has been suggested that this peptide may contribute to thrombus formation. It is now hypothesized that these βA deposits in the damaged vessel wall of beginning AD may be the inducer of CAA. Further, it needs to be proven if these CAA lesions are the origin of the brain plaques.

Taken together, platelets are an important key blood cell linking vascular pathology and AD. Platelets are altered in AD and could serve as extremely useful biomarkers for dementia [59] or as therapeutic targets [19]. Our present data show that freshly isolated platelets do not migrate deep into the Alzheimer mouse brain, but a majority is seen in the Alzheimer human postmortem brain. These platelets are mainly located in vessels and not close to βA plaques and we conclude that platelets may contribute to cerebral amyloid angiopathy in AD.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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