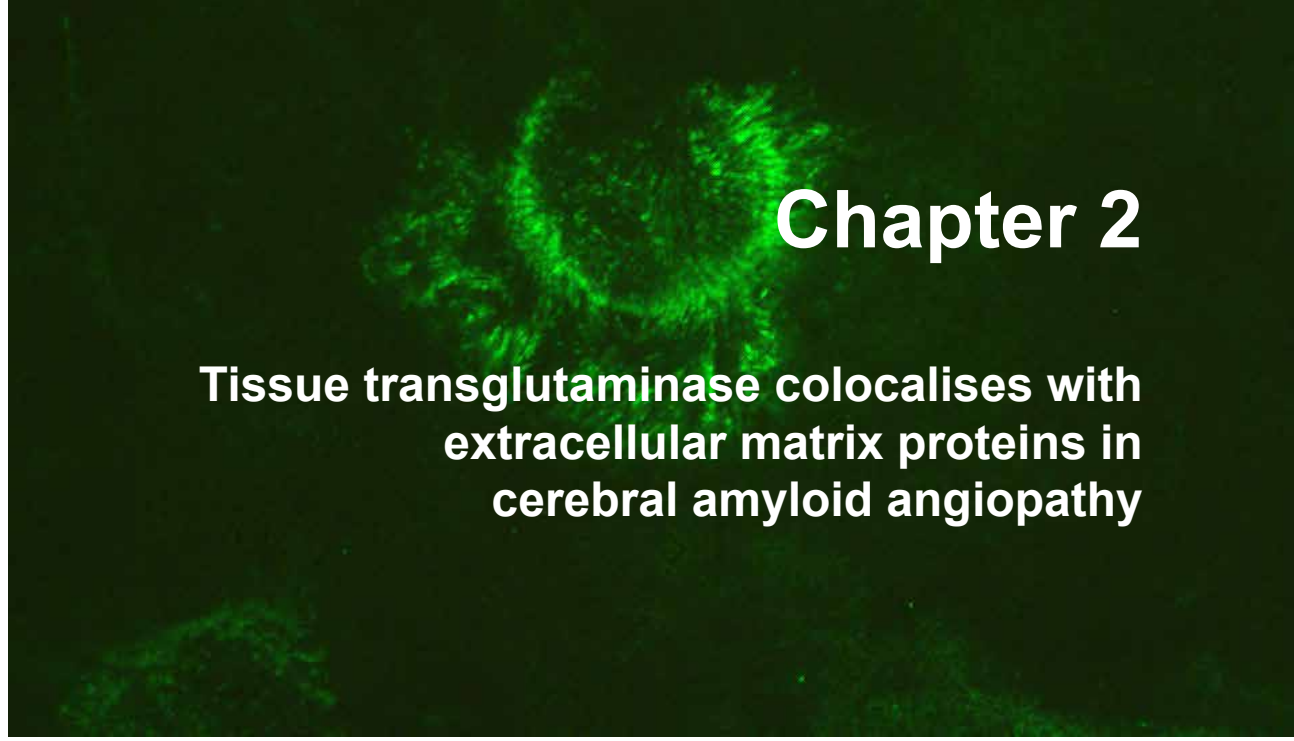

Chapter 2

Tissue transglutaminase colocalises with extracellular matrix proteins in cerebral amyloid angiopathy

Mieke de Jager[1],
Berend van der Wildt[2],
Emma Schul[1],
John G.J.M. Bol[1],
Sjoerd G. van Duinen[3],
Benjamin Drukarch[1],
Micha M.M. Wilhelmus[1]

1 Department of Anatomy and Neurosciences, Neuroscience Campus Amsterdam, VU University Medical Center, Amsterdam, The Netherlands
2 Department of Nuclear Medicine and PET research, VU University Medical Center, Amsterdam, the Netherlands
3 Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands

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Abstract

Cerebral amyloid angiopathy (CAA) is a key histopathological hallmark of Alzheimer's disease (AD) and hereditary cerebral haemorrhage with amyloidosis of the Dutch type (HCWHA-D). CAA is characterised by amyloid-beta (Aβ) depositions and remodelling of the extracellular matrix (ECM) in brain vessels and plays an important role in the development and progression of both AD and HCHWA-D. Tissue transglutaminase (tTG) modulates the ECM by molecular cross-linking of ECM proteins. Here, we investigated the distribution pattern, cellular source and activity of tTG in CAA in control, AD and HCHWA-D cases. We observed increased tTG immunoreactivity and colocalisation with Aβ in the vessel wall in early stage CAA, whereas in later CAA stages, tTG and its cross-links were present in halos enclosing the Aβ deposition. In CAA, tTG and its cross-links at the abluminal side of the vessel were demonstrated to be either of astrocytic origin in parenchymal vessels, of fibroblastic origin in leptomeningeal vessels, and of endothelial origin at the luminal side of the deposited Aβ. Furthermore, the ECM proteins fibronectin and laminin colocalised with the tTG-positive halos surrounding the deposited Aβ in CAA. However, we observed that in situ tTG activity was present throughout the vessel wall in late stage CAA. Together, our data suggest that tTG and its activity might play a differential role in the development and progression of CAA, possibly evolving from direct modulation of Aβ aggregation to cross-linking of ECM proteins resulting in ECM restructuring.

Keywords: Alzheimer's disease, HCHWA-D, cerebral amyloid angiopathy, tissue transglutaminase, extracellular matrix proteins

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterised by deposition of amyloid-beta (Aβ) peptide aggregates in the brain. In the parenchyma, Aβ aggregates in the form of senile plaques (SPs), whereas Aβ deposition in the vasculature results in cerebral amyloid angiopathy (CAA) [1]. The Aβ protein is a proteolytic cleavage product of the amyloid precursor protein (APP) and is produced in two major forms ($Aβ_{1-40}$ and $Aβ_{1-42}$), both capable of interacting with themselves which results in toxic Aβ aggregates [2]. SPs mainly consist of $Aβ_{1-42}$, whereas $Aβ_{1-40}$ is the major component of CAA [3]. CAA is present in more than 90% of all AD patients [4] and is also the major pathological hallmark in patients with hereditary cerebral haemorrhage with amyloidosis of the Dutch type (HCHWA-D). HCHWA-D is characterized by the E22Q mutation in the APP gene that results in a highly toxic form of Aβ (i.e. D-$Aβ_{40}$) which accumulates preferentially in brain vessel walls and leads to severe cerebral haemorrhaging [5]. Importantly, the presence of CAA does correlate with cognitive decline of CAA-affected patients [3, 6]. In general, two types of CAA have been identified: type I CAA includes capillary Aβ depositions in addition to CAA in other cortical and leptomeningeal vessels, whereas in type II CAA, capillary Aβ depositions are not present [7]. CAA is characterized by degeneration of smooth muscle cells, disruption of the basement membrane and remodelling of extracellular matrix (ECM) [3, 8, 9]. Together, these alterations lead to weakening of the vessel wall, impaired vascular functioning and, ultimately, haemorrhages [3, 9]. Despite the strong connection between cognitive decline, reduced functioning of brain vasculature and CAA, the factors and mechanisms underlying accumulation and deposition of Aβ in the vessel wall remain largely unknown.

Tissue transglutaminase (tTG) is a member of the transglutaminase family (TGs, EC 2.3.2.13) and is ubiquitously expressed in the human brain [10, 11]. tTG is a calcium-dependent enzyme involved in covalent posttranslational modifications of proteins such as amine incorporation and molecular cross-linking [12]. The latter is formed by the formation of a γ-glutamyl-ε-lysine bond between a glutamine residue and a lysine residue of a peptide chain, which can be either intra- or intermolecular [12–14]. This cross-linking activity provides tTG with the powerful capacity to stably alter conformation of proteins and induce formation of protein complexes [12]. An important biological role of this cross-linking activity is formation of ECM protein complexes, which results in remodelling of the ECM in response to e.g. cell stress and tissue injury [14–16]. Evidence is mounting that tTG and its transamidation activity play an important role in AD pathogenesis [17]. In AD brain, tTG levels and its cross-links are elevated [18–20] and correlate with the cognitive decline observed in these patients [20, 21]. Furthermore, tTG binds to Aβ, and tTG-mediated cross-linking modulates the Aβ aggregation pathway [22–25]. In fact, a recent study demonstrated that tTG activity is able to induce formation of neurotoxic and protease

resistant Aβ complexes at low, i.e. nanomolar, concentrations of Aβ [26].
We recently demonstrated for the first time that not only tTG, but also tTG-mediated cross-links are associated with the pathological hallmarks of AD [11]. Interestingly, in this study we found that tTG and its cross-links are associated with both SPs and CAA; however, the distribution pattern of tTG and its activity in both lesions appeared different [11]. In SPs, immunoreactivity of both tTG and its cross-links colocalised with the deposited Aβ, in contrast to CAA where no spatial overlay was observed. Instead, in CAA, tTG and its cross-links were present as a luminal and abluminal halo which enclosed the deposited Aβ in middle-sized parenchymal vessels [11]. Remarkably, the tTG-immunoreactive halos that surround the deposited Aβ, resembled that of earlier immunohistochemical stainings of major ECM proteins (i.e. fibronectin [FN] and laminin [LN]), and other known tTG substrates reported by others [27–29]. These results suggest a role for tTG-mediated ECM remodelling in CAA which might be one of the unknown factors that affect Aβ deposition in the vessel wall and CAA development [30].

In order to gain more insight into the role of tTG and its transamidation activity in CAA, we studied the distribution pattern and cellular source of tTG and its cross-links in different brain vessels affected by CAA by use of immunofluorescence on snap-frozen brain tissue of AD, HCHWA-D and control cases. In addition, we investigated colocalisation of tTG and its cross-links with ECM proteins, in particular the tTG substrates fibronectin and laminin [14, 31], in both AD and HCHWA-D brains. Finally, we also investigated the distribution pattern and overlay of in situ TG activity with CAA in control, AD and HCHWA-D cases.

Materials and methods

Brain tissue

Human neocortex tissue samples from 5 AD patients with CAA, including two patients with capillary CAA (capCAA) (age 83.8 ± 11.8 years; post-mortem interval 8.0 ± 2.2 hr) and 7 non-demented control subjects without neurological disease including two controls with CAA (age 81.4 ± 4.7 years; post-mortem interval 6.6 ± 1.5 hr) were obtained from The Netherlands Brain Bank (Amsterdam, The Netherlands). Brain tissue of the neocortex from 5 HCHWA-D patients was obtained (age 55.2 ± 4.0 years; post-mortem interval 4.4 ± 1.1 hr) from Dr. S van Duinen (Department of Pathology, Leiden University Medical Centre, Leiden, The Netherlands). After autopsy, samples were immediately frozen in liquid nitrogen. Table 1 shows an overview of patient details including gender, age, post-mortem interval, diagnosis, cause of death, Braak grading (both for neurofibrillary tangles and SPs) and CAA score. The diagnosis of AD was based on neuropathological and clinical criteria [32, 33]. CAA scoring was performed as described previously [11]: the number of CAA-affected vessels of at least 4 microscopic fields (magnification 4x, 16 mm2 per microscopic field) were counted and classified as follows: 0 (-, no CAA), 1-10 (+, sparse CAA), 11-20 (++, moderate CAA) and >21 (+++, severe CAA).

Double immunofluorescence

Experiments were performed as described previously [11, 34, 35]. Serial sections of temporal neocortex (6 µm) were fixed with acetone (100%) for 10 minutes and blocked with 3% bovine serum albumin (BSA; PAA Laboratories, Pasching, Austria) in Tris buffered saline (TBS) with 0.5% TritonX-100 (TBS-T). Negative controls were incubated in this solution without the primary antibodies. Primary antibodies (Table 2) were diluted in 3% BSA/TBS-T and incubated overnight at 4°C. After washing with TBS, sections were incubated with the appropriate secondary antibody. Secondary antibodies used were donkey anti-mouse, donkey anti-goat and donkey anti-rabbit, all coupled to Alexa 488 or Alexa 594, (dilution 1:400, Invitrogen, Camarillo, CA, USA). Biotin-labelled donkey anti-mouse-IgM (dilution 1:800, Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) was used for the tTG crosslink antibody (81D4) and staining was enhanced with the Vectastain avidin-biotin kit (dilution 1:800 in TBS-T, Vector laboratories Inc., Burlingame, CA, USA). As a second enhancement step for the 81D4 antibody, biotinylated tyramide in 0.005% H2O2-TBS was used (generous gift from Dr. I. Huitinga, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands). The conjugate streptavidin Alexa 488 (Invitrogen) was used as a fluorchrome for the 81D4 antibody; streptavidin Alexa 594 was used as a secondary antibody for biotin-labelled mouse anti-human Aβ antibody. In between incubation steps, sections were extensively washed with TBS. Sections were mounted with Vectashield (Vector Laboratories Inc). The specificity of the antibodies directed against tTG in human brain was demonstrated in our previous reports [11, 34]. The specificity of the anti-TG-catalysed cross-link antibody was demonstrated by preadsorption with H-Glu(H-Lys-OH)-OH (Bachem AG, Bubendorf, Switserland) in human brain tissue [11]. To visualise the fluorescence stainings, a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Rijswijk, The Netherlands) was used. To exclude false positive fluorescence signals for each channel, a series of images was obtained separately in both channels through a 40x lens (zoom factor 1 to 4x, Z-increment 0.15 µm, approximately 20 images of 512×512 pixels).

Table 1 Patient characteristics

Patient number	Diagnosis	Gender	Age	PMI (hr)	Grade (Braak, NFTs)	Grade (Braak, Aβ)	Grade CAA	Cause of death
1	Control	M	79	8	1	A	-	Pneumonia
2	Control	F	84	7	1	O	-	Myelodysplasia
3	Control	M	80	8	1	O	-	Not known
4	Control	F	82	5	1	O	-	Not known
5	Control	F	84	5	1	O	-	Heart failure
6	Control/ CAA	F	73	8	1	B	++	Palliative sedation
7	Control/ CAA	M	88	5	2	A	+++	Gastro-intestinal bleeding
8	AD/CAA	F	65	6	6	C	++	Pneumonia
9	AD/CAA	F	90	3	5	C	+++	Cachexia
10	AD/cap-CAA	F	81	3	6	C	+++	Stroke
11	AD/cap-CAA	F	96	4	5	C	+++	Cachexia and dehydration
12	AD/CAA	F	87	8	6	C	+++	Heart failure
13	HCHWA-D	F	60	5	1	-	+++	Cerebral hemorrhage
14	HCHWA-D	M	59	4	1	-	+++	Cerebral hemorrhage
15	HCHWA-D	F	51	4	0	-	+++	Cerebral hemorrhage
16	HCHWA-D	F	53	3	0	-	+++	Cerebral hemorrhage
17	HCHWA-D	F	53	6	1	-	+++	Cerebral hemorrhage

Abreviations: Aβ = amyloid-beta, AD = Alzheimer's disease, CAA = cerebral amyloid angiopathy, cap-CAA = capillary CAA, F = Female, M = Male, ND = not determined, NFT = neurofibrillary tangles, PMI = Post Mortem interval (hr = hours). Grading of AD (Braak) and of CAA was performed as described in the materials and methods section.

In situ TG activity

In situ TG activity detection was performed as described previously [36], with some minor changes. In short, unfixed 6 µm thick tissue sections of neocortex of AD, HCHWA-D and control patients were pre-incubated for 30 minutes at room temperature in a 100 mM Tris-HCl, pH 7.4, 5mM CaCl2, 1 mM dithiothreitol (DTT, Promega, Leiden, The Netherlands) buffer with or without 100 µM of the tTG activity inhibitor Z-DON-Val-Pro-Leu-OMe (Z-006) [37], purchased from Zedira GmbH, Darmstadt, Germany. Then, incubation was continued for 40 minutes at 37°C with the same incubation buffer with or without inhibitor to which 0.05 mM biotinylated 5-(biotinamido)-pentylamine (BAP; Thermo Fisher Scientific, Fremont, CA, USA), a substrate for TGs [38, 39], was added. Thereafter, sections were air dried, fixed for 10 minutes with 100% acetone and subsequently incubated with a primary antibody directed against tTG (Ab1, Neomarkers) in 3% BSA/TBS-T for 1 hour at room temperature followed by 1 hour incubation at room temperature with secondary antibodies donkey anti-rabbit coupled to Alexa 594 to detect tTG (dilution 1:400) and streptavidin coupled to Alexa 488 to detect BAP incorporation (dilution 1:400). Sections were washed with TBS in between and after antibody incubation and mounted with Vectashield. The Leica TCS SP2 AOBS confocal laser scanning microscope was used to visualise the staining as described above.

Table 2 Primary antibodies

Antigen	Primary antibody	Species raised in	Dilution	Company
tTG	Guinea pig tTG, Ab-1	Mouse	1:300	Thermo Fisher Scientific, Fremont, CA, USA
	Guinea pig tTG (06471)	Goat	1:4000	Millipore, Temecula, CA, USA
Aβ	Human Aβ biotinylated, clone 4G8	Mouse	1:400	Covance, Emeryville, CA, USA
	Human Aβ1-16 AB10	Mouse	1:200	Millipore, Temecula, CA, USA
	Human Aβ (715800)	Rabbit	1:100	Invitrogen, Camarillo, CA, USA
tTG cross-link	H-Glu(H-Lys-OH)-OH, 81D4	Mouse	1:100	Covalab, Villeurbanne, France
Astrocytes	Bovine GFAP	Rabbit	1:2000	Dako Cytomation, Glostrup, Denmark
Endothelial cells	Human Von Willebrand Factor	Rabbit	1:500	Dako Cytomation, Glostrup, Denmark
Fibroblasts	Vimentin	Mouse	1:2000	Dako Cytomation, Glostrup, Denmark
Smooth muscle cells	Human smooth muscle actin	Mouse	1:200	Dako Cytomation, Glostrup, Denmark
Fibronectin	Mouse fibronectin	Rabbit	1:400	Millipore, Temecula, CA, USA
Laminin	Mouse laminin	Rabbit	1:100	Cappel, Solon, Ohio, USA

Abbreviations: tTG = tissue transglutaminase; Aβ = amyloid-β; GFAP = glial fibrillary acidic protein

Results

Distribution pattern of tTG and its cross-links in CAA of control, AD and HCHWA-D cases

The general staining pattern of tTG and TG-catalysed cross-links observed in the neocortex of control and AD cases are in line with our previous findings [11]. Thus, in vessels of control cases and non-CAA affected vessels in AD and HCHWA-D cases, tTG staining was present in all layers of the vessel wall in both leptomeningeal and parenchymal vessels (Figure 1A-C, arrow), and in capillaries. In addition, weak anti-tTG cross-link immunoreactivity was observed in all layers of the vessel wall in both leptomeningeal and parenchymal vessels, predominantly at the endothelial side of the vessel wall (not shown). In CAA of AD cases, no spatial overlay of anti-tTG immunoreactivity with anti-Aβ staining was observed (Figure 1A-C). However, tTG staining was present as a halo at both the luminal and abluminal side of deposited Aβ, in parenchymal vessels (Figure 1A-C), leptomeningeal vessels (Figure 1D-F) and in capillaries (Figure 1G-I). Similar to tTG immunoreactivity in CAA, tTG cross-link staining was present in an abluminal and/or luminal halo surrounding Aβ deposition in leptomeningeal and parenchymal vessels (Figure 1J-L), but only in an abluminal halo in capillary CAA (Figure 1M-O). In CAA present in non-demented control cases, a similar staining pattern for both tTG and its cross-links was observed (not shown). Interestingly, in probable CAA precursors, observed in the form of local Aβ deposition in a large parenchymal vessel (Figure 2A-C) and a capillary vessel (Figure 2D-F) of AD cases, anti-tTG immunoreactivity was elevated compared to the unaffected part of the vessel, and, in contrast to the fully developed CAA, spatially colocalised with the deposited Aβ. To investigate whether the appearance of tTG and its cross-links as halos surrounding CAA might be a general phenomenon in CAA, we performed the above-described stainings in HCHWA-D patients. The distribution of tTG (Figure 3) and cross-links (not shown) in CAA in HCHWA-D cases was similar to CAA in AD cases, although tTG staining was more intense as compared with AD.

Colocalisation of tTG with cellular markers in CAA of AD cases

In order to investigate the cellular origin of the observed tTG staining enclosing the deposited Aβ in CAA, double immunofluorescence staining of tTG with cell specific markers (glial fibrillary acidic protein [GFAP] for astrocytes [40], Von Willebrand Factor [VWF] for endothelial cells [41, 42], vimentin for fibroblasts [43] and smooth muscle actin [SMA] for smooth muscle cells [44]) was performed. In control cases, weak tTG staining was observed in astrocytes associated with brain vessels (Figure 4A-C). In CAA of AD cases however, strong anti-tTG immunoreactivity was observed in GFAP-positive astrocytes at the abluminal side of CAA in parenchymal vessels (Figure 4D-F). Especially the astrocytic end feet demonstrated strong tTG staining in CAA (Figure 4D-F). In CAA of

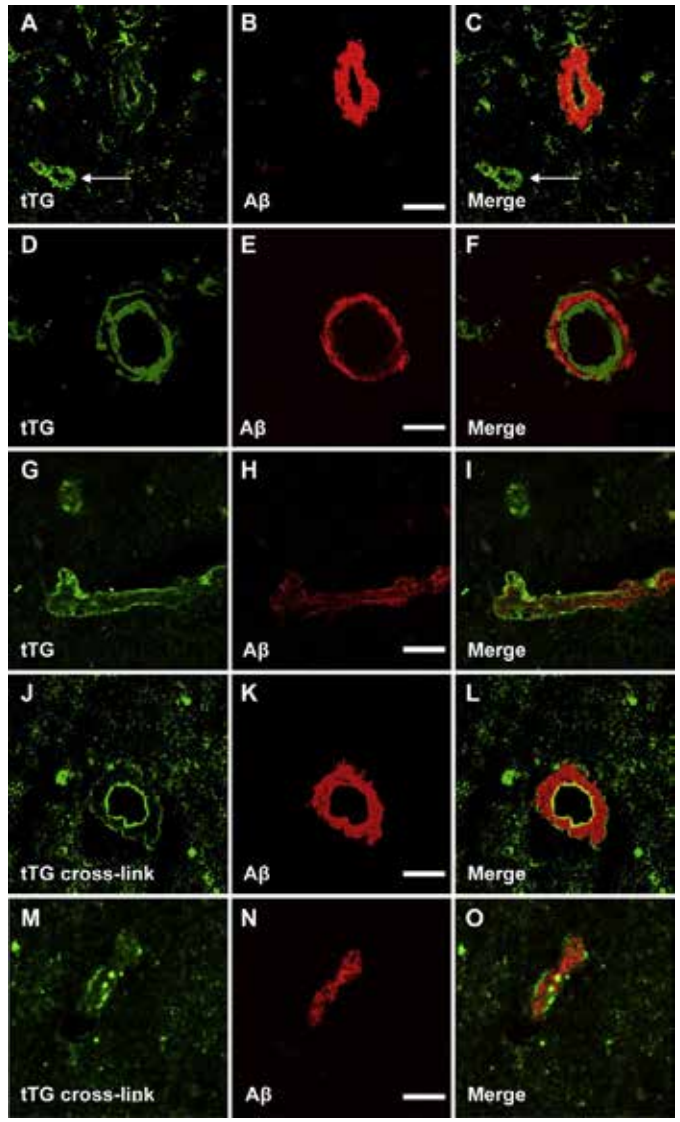

Figure 1 Double immunofluorescence staining of tTG and tTG cross-links in CAA in neocortex of AD cases. The anti-Aβ antibody (715800) stained Aβ deposition in CAA-affected vessels (B, E, H, K, N). Double immunofluorescence staining of the anti-Aβ antibody with either the anti-tTG (Ab1) or anti-TG-catalysed cross-link antibody was performed. In AD cases, tTG staining was observed in control vessels (A, arrow). In CAA however, tTG staining was observed in an abluminal and luminal halo enclosing the Aβ deposition in both parenchymal (A-C) and leptomeningeal (D-F) vessels. In capillary CAA, tTG (Ab1 antibody) was present in an abluminal halo (G-I). Anti-tTG cross-link immunoreactivity was present at the abluminal and luminal side of the Aβ deposition in larger cortical vessels (J-L) and at the abluminal side of capillary CAA (M-O). Scale bars: B, E, H, K, N: 30 μm. Abbreviations: tTG = tissue transglutaminase, Aβ = amyloid-beta, CAA = cerebral amyloid angiopathy.

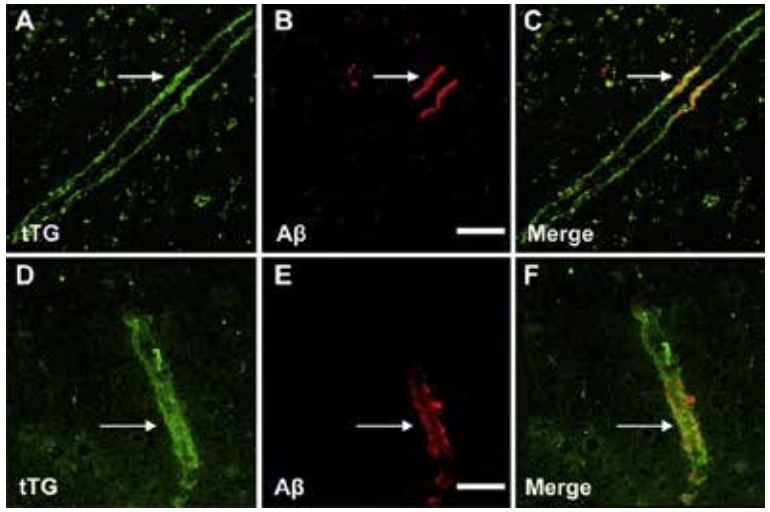

Figure 2 Double immunofluorescence staining of tTG with Aβ in vessels partly affected by Aβ deposition. In a large parenchymal vessel (A-C) and capillary vessel (D-F), the anti-Aβ antibody (AB10 and 715800, respectively) stained the Aβ deposition (B, E). The anti-tTG antibody (06471 and Ab-1, respectively) showed a strong immunoreactivity in the Aβ-affected part of the vessel compared to the non-affected part of the blood vessel. In addition, tTG staining colocalised with the Aβ staining (A-F, arrows). Scale bars: B: 30 μm, E: 15 μm. Abbreviations: tTG = tissue transglutaminase, Aβ = amyloid-beta.

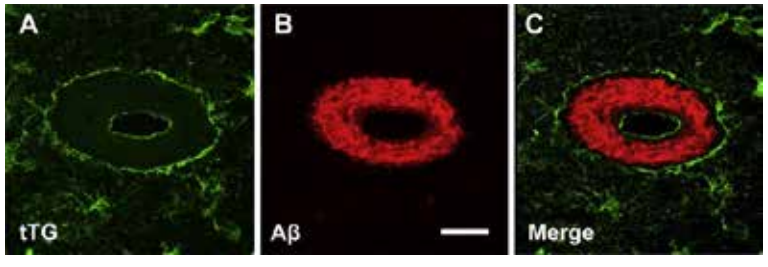

Figure 3 Double immunofluorescence staining of tTG in CAA in neocortex of HCHWA-D cases
The anti-Aβ antibody (4G8) stained Aβ deposition in CAA (B). Double immunofluorescence staining of the anti-Aβ antibody with the anti-tTG (Ab1) antibody was performed. In CAA, tTG staining was observed in an abluminal and luminal halo enclosing the Aβ deposition (A-C). Scale bar B: 30 μm. Abbreviations: tTG = tissue transglutaminase, Aβ = amyloid-beta.

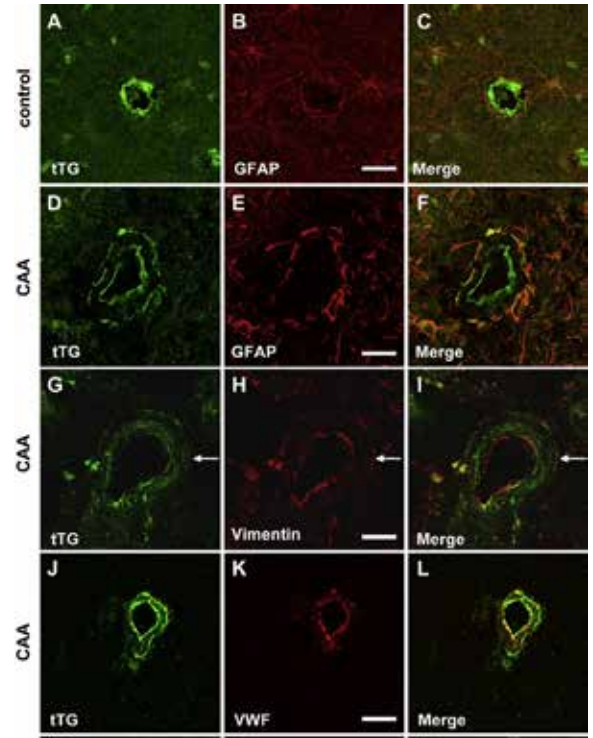

Figure 4 Double immunofluorescence staining of tTG with cellular markers in CAA in control and AD cases. The anti-GFAP antibody stained astrocytes in parenchymal vessels (B, E), whereas the anti-vimentin antibody stained fibroblasts in the adventitia of the vessel wall of leptomeningeal vessels (H). The anti-VWF antibody stained endothelial cells in blood vessels (K) and the anti-SMA antibody stained smooth muscle cells in the media of the vessel wall (N). Double immunofluorescence staining of the anti-tTG (Ab1) antibody with either the anti-GFAP or anti-VWF antibody and double immunofluorescence of the anti-tTG (06471) antibody with either the anti-vimentin or anti-SMA antibody was performed. In control vessels, sporadic colocalisation of tTG staining with GFAP staining was observed (A-C). In parenchymal CAA vessels, tTG staining in the abluminal halo (D) colocalised with the GFAP staining in astrocytic endfeet (E, F). In leptomeningeal CAA vessels, tTG in the abluminal halo (G) colocalised with the vimentin staining (H, I, arrow). The tTG staining in the luminal halo J) in CAA vessels colocalised with the anti-VWF antibody (K, L). tTG in the abluminal and luminal halo surrounding CAA did not spatially colocalise with SMA staining (M-O). Scale bars B, H, K, N: 30 μm, e: 15 μm. Abbreviations: tTG = tissue transglutaminase, GFAP = glial fibrillary acidic protein, VWF = Von Willebrand Factor, SMA = smooth muscle actin, CAA = cerebral amyloid angiopathy.

leptomeningeal vessels tTG colocalised partly with vimentin, a marker for fibroblasts, in the abluminal halo (Figure 4G-I). Double immunofluorescence staining of tTG immunoreactivity with the anti-VWF antibody in control cases and in non-CAA affected vessels of AD cases, demonstrated tTG staining in all layers of the vessel wall, which at the luminal side colocalised with endothelial cells (not shown). In CAA of AD cases the luminal halo of tTG colocalised with endothelial cells, in both parenchymal (Figure 4J-L) and leptomeningeal vessels (not shown). SMA staining was present in all layers of the vessel wall in control vessels where it colocalised with tTG (not shown). However, although SMA staining was present in all layers of vessels affected by CAA, no colocalisation with tTG was observed (Figure 4M-O).

In HCHWA-D cases, like the AD staining, tTG and its cross-links colocalised with the astrocyte marker GFAP in astrocytic endfeet at the abluminal side of Aβ in parenchymal vessels, and with the endothelial marker VWF at the luminal side of Aβ (not shown).

Colocalisation of tTG and its cross-links with ECM proteins in CAA of AD cases

tTG plays an important role in modulation of the ECM by cross-linking of ECM proteins [14, 31]. As demonstrated previously, several ECM proteins are associated with CAA, but do not spatially colocalise with the actual Aβ deposition itself [27–29]. Interestingly, the staining pattern of these ECM proteins resembles that of tTG and cross-links in CAA, as described in this study. Together, these data suggested that tTG might cross-link the ECM in CAA. We therefore performed double immunofluorescence staining of tTG and cross-links with antibodies directed against the ECM proteins FN and LN which are well-known tTG substrates [14, 31]. In vessels of control cases and non-affected vessels in AD cases, FN and LN were present in all layers of the vessel wall of leptomeningeal and parenchymal blood vessels, and in capillaries (not shown). These data are in line with previous reports [45, 46]. In CAA in AD cases, elevated anti-FN and anti-LN immunoreactivity was found as an abluminal and/or luminal halo enclosing the deposited Aβ (Figure 5B, E). In addition, both the anti-FN and anti-LN immunoreactivity colocalised with tTG (Figure 5A-C, D-F respectively) and tTG cross-link staining (Figure 6A-C, D-F). In CAA of HCHWA-D cases, the distribution of FN and LN staining was similar to the AD cases, showing an abluminal and/or luminal halo that spatially colocalised with the anti-tTG antibody (Figure 5G-I and J-L respectively).

In situ TG activity in CAA in AD and HCHWA-D cases

We observed that tTG staining was present in the Aβ part of CAA precursors, whereas in later stages of CAA, tTG was not present in the Aβ deposition. Epitope masking of tTG may have caused this lack of staining. In addition, the conformation of tTG, which is an important property of the enzyme that influences its function [47], may affect the recognition by the antibodies we used. Therefore, to investigate whether tTG is still present and active in the Aβ part of CAA, we investigated the in situ activity of TGs by the incorporation

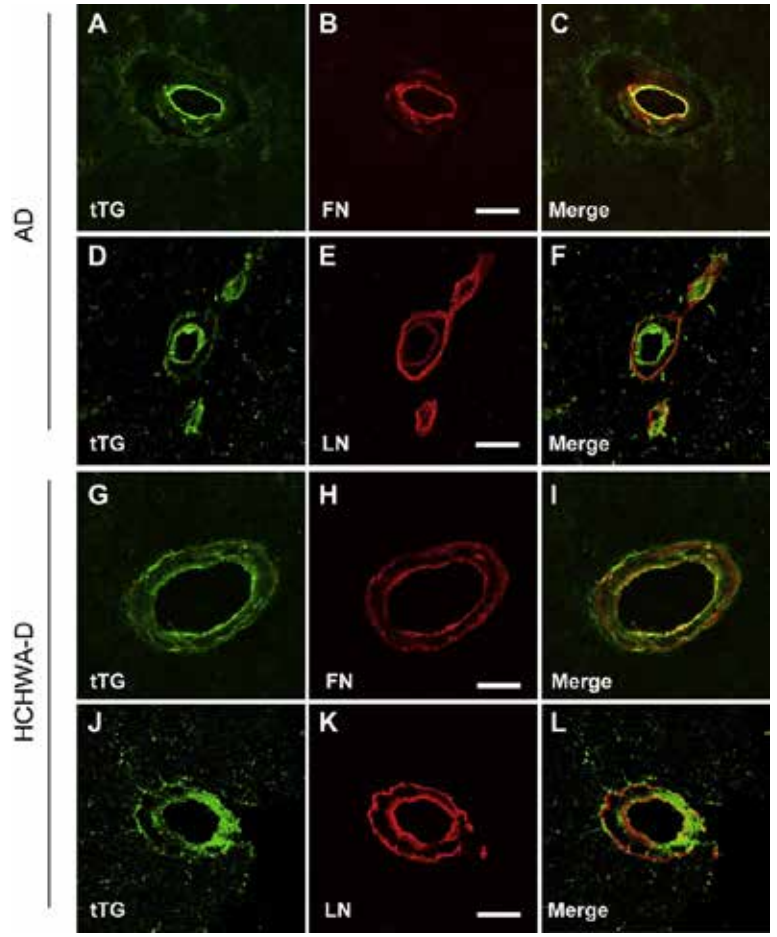

Figure 5 Double immunofluorescence staining of tTG with ECM proteins in CAA in AD and HCHWA-D cases. Double immunofluorescence staining was performed with either the anti-FN or anti-LN antibody with the anti-tTG antibody (Ab1). In CAA vessels of AD patients, FN and LN staining colocalised with the tTG staining (FN: A-C, LN: D-F) in an abluminal and/or luminal halo enclosing the Aβ deposition. The same staining pattern was observed in HCHWA-D cases. FN staining was observed in both an abluminal and luminal halo of the vessel, colocalising with the tTG staining (G-I). Also anti-LN immunoreactivity was present in an abluminal and luminal halo enclosing the Aβ deposition, and colocalised with the tTG staining (J-L). Scale bars B, E, H, K: 30 μm. Abbreviations: tTG = tissue transglutaminase, ECM = extracellular matrix, FN = fibronectin, LN = laminin, CAA = cerebral amyloid angiopathy.

of the TG substrate BAP. Snap frozen tissue sections of the neocortex of AD, HCHWA-D and control cases were incubated with the TG substrate BAP and stained for the presence of tTG and BAP. In vessels of control cases and non-CAA affected vessels in AD and HCHWA-D cases, weak BAP staining was observed in all layers of the vessel wall which colocalised with tTG staining (Figure 7A-C). After co-incubation with the specific tTG inhibitor Z-006, no BAP staining was detectable, in contrast to the tTG staining, which remained unaffected (Figure 7D-F). In CAA in AD and HCHWA-D cases, however, BAP staining was remarkably increased compared to control vessels, and was present in all layers of the vessel wall (Figure 7G). In addition, BAP staining also colocalised with the typical tTG halos enclosing the Aβ part of CAA (Figure 7G-I, arrow). After co-incubation with the specific tTG inhibitor Z-006, no BAP staining was detectable in CAA, whereas tTG staining was still present in the two halos surrounding the deposited Aβ (Figure 7J-L).

Discussion

In this study, we describe for the first time that immunoreactivity of tTG in a vessel wall partly affected by Aβ deposition, which may indicate an early stage, is increased and colocalises with the deposited Aβ. In contrast, in later stages of CAA, both tTG and its cross-links do not colocalise with the deposited Aβ anymore, but are present in an abluminal and luminal halo enclosing the Aβ deposition. We observed that the abluminal halo of parenchymal vessels in CAA are of astrocytic origin and that tTG might derive from fibroblasts in leptomeningeal vessels, whereas tTG in the luminal halo in all vessel types is of endothelial origin. Furthermore, tTG substrates and important ECM components FN and LN colocalise with tTG and its cross-links in the halos that enclose Aβ deposition in CAA. We observed this distribution pattern of tTG and its cross-links in CAA in non-demented controls, AD and HCHWA-D cases, which suggests that this pattern is a general phenomenon of CAA. Surprisingly however, although we did not observe tTG and tTG cross-link staining in the actual Aβ deposition in CAA, we found that tTG is still present and could be activated in situ in the deposited Aβ. Together our data suggest that tTG plays a unique role in CAA development and progression. Initially, tTG levels are elevated in early stages of CAA, and may even precede Aβ deposition, whereas in later stages tTG is likely to be involved in alteration and/or remodelling of the ECM in CAA.

Previously, we noticed an important discrepancy between the distribution pattern of tTG and its activity in SPs and CAA. tTG and its cross-links colocalised with the deposited Aβ in both diffuse and classic SPs, whereas in CAA, tTG and its cross-links did not colocalise with the Aβ aggregate itself [11]. Instead, in CAA, both tTG and its cross-links were present in an abluminal and luminal halo enclosing the Aβ deposition in middle-sized parenchymal vessels with CAA in AD patients [11]. In the present study, we found that apart from middle-sized parenchymal vessels affected by CAA in AD cases, tTG and its

cross-links enclose the Aβ deposition in all parenchymal vessels and leptomeningeal vessels, as well as in capillaries affected by CAA. In addition, tTG and its cross-links were present in a similar distribution pattern in CAA in HCHWA-D cases and in CAA-affected vessels of non-demented controls. Together, these findings point out that the presence of tTG and cross-links in halos that enclose Aβ is a common phenomenon found in both type I and type II CAA (with or without capillary CAA respectively), in different diseases that are characterised by CAA, and even in CAA in non-demented controls.

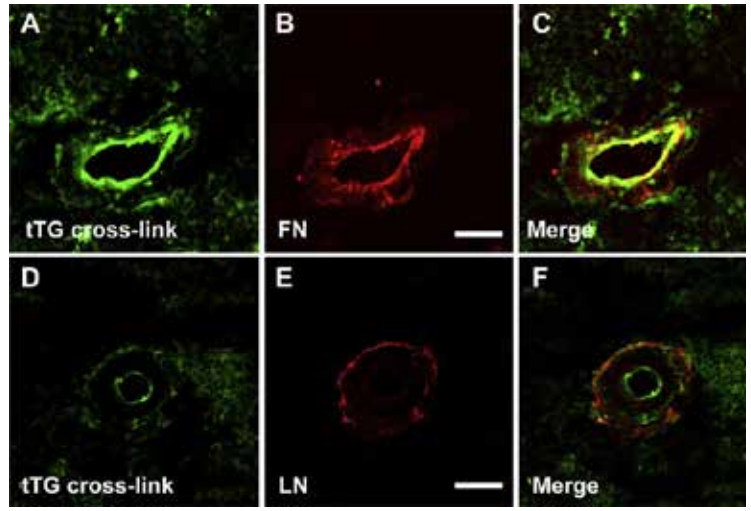

Figure 6 Double immunofluorescence staining of tTG cross-links with ECM proteins in CAA in AD cases. Double immunofluorescence staining was performed with either the anti-FN or anti-LN antibody with the anti-tTG cross-link antibody. In CAA vessels of AD cases, FN and LN staining colocalised with the tTG cross-link staining (FN: A-C, LN: D-F) in an abluminal and/or luminal halo enclosing the Aβ deposition. Scale bars B, E: 30 μm. Abbreviations: tTG = tissue transglutaminase, ECM = extracellular matrix, FN = fibronectin, LN = laminin, CAA = cerebral amyloid angiopathy

Interestingly, in a parenchymal and capillary vessel only partly affected by CAA, which may indicate an early stage of CAA development, tTG was present in the Aβ deposition itself. In later stages of CAA, tTG and its cross-links did not colocalise with Aβ, although tTG could still be activated with our in situ assay in both the Aβ part of CAA and the tTG-immunoreactive halos enclosing the Aβ. However, it should be noted that although it is highly likely that our in situ approach demonstrates tTG activity, this technique might also activate other TGs. However, although both TG1 and TG3 are present in the human brain, there is no association of these TGs with cerebral vessels or CAA [10, 11]. In addition, the potent tTG inhibitor Z-006 that was used in our experiments could inhibit other TGs, yet it has a significantly higher affinity for tTG compared to other TGs [37]. Together we therefore conclude that tTG is present in CAA in early and later stages and that its activity may have a unique role in Aβ deposition and development of CAA.

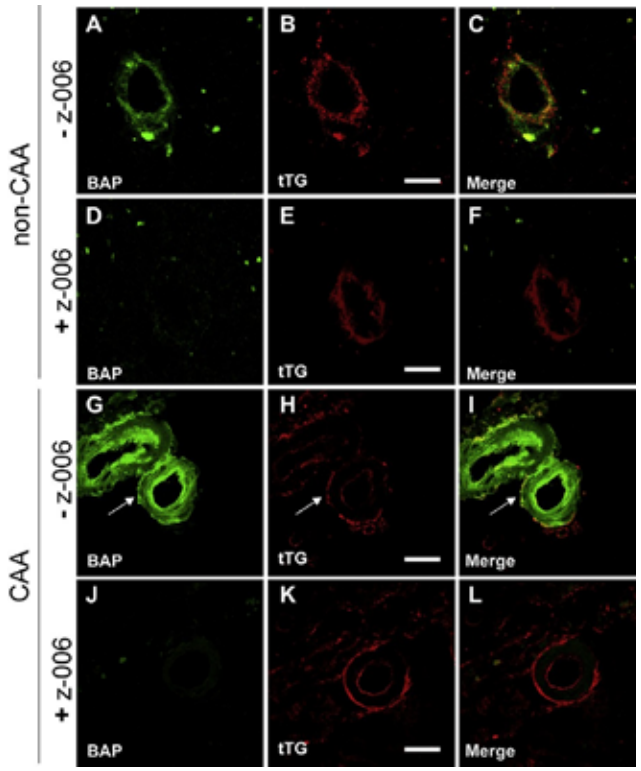

Figure 7 *In situ* transglutaminase activity. Double immunofluorescence was performed with the anti-tTG antibody (Ab1) and streptavidin coupled to Alexa 488 directed against the biotin-label of BAP. In non-CAA vessels of AD cases, weak staining of both BAP and tTG was observed in all layers of the vessel wall (A-C). In addition, BAP and tTG staining colocalised in all layers of the vessel wall (A-C). Co-incubation of the specific tTG inhibitor Z-006 (100 μM) with BAP resulted in absence of BAP staining in the control vessel (D). In CAA, strong BAP staining was observed in all layers of the vessel wall (G), whereas no BAP staining was present after co-incubation with Z-006 (100 μM) (J). tTG was again present in an abluminal and luminal halo surrounding the Aβ deposition in CAA (H, K). Colocalisation of BAP with tTG staining was observed in CAA (G-I arrow). Scale bar B, E: 15 μm, H, K: 30 μm. Abbreviations: tTG = tissue transglutaminase, CAA = cerebral amyloid angiopathy, BAP = biotinylated 5-(biotinamido)-pentylamine.

In early stages of CAA development, Aβ accumulates in the media around the vascular smooth muscle cells (SMCs) [3]. We observed tTG immunostaining in the Aβ deposition itself in vessels partly affected by CAA. In this stage of CAA, tTG might originate from SMCs, as these cells are known to produce and secrete tTG [48, 49]. A similar tTG staining was present in capillaries suggesting that pericytes are the cellular source in capillaries. Hence, endothelial cells are also known to produce tTG [50]. Although the cellular origin of tTG in capillaries and larger vessels might differ, tTG staining in the Aβ

deposition was increased compared to the non CAA-affected part of the vessel wall. This suggests that either tTG levels are elevated in response to the Aβ deposition in the vessel wall, or that induced tTG levels and activity result in Aβ deposition in the vessel wall. An in vitro study has shown that tTG is upregulated in a monocytic cell line upon treatment with $Aβ_{1-42}$ [51]. Furthermore, tTG can cross-link Aβ and affect its aggregation pathway [22–26]. Therefore, in vivo tTG expression might be increased in response to Aβ deposition in the vessel wall, which may lead to Aβ cross-linking in the initial stages of CAA. On the contrary, increased levels of tTG may also precede Aβ deposition. tTG is normally present in the vessel wall, where it is important in cross-linking of ECM proteins leading to remodelling of the vessel wall [15]. Levels of tTG in the vasculature increase with age, which leads to enhanced ECM cross-linking and subsequent vascular stiffness [52]. These changes in the vessel wall might influence Aβ deposition. In addition, several proteins that are known to affect the Aβ cascade, in particular heparan sulphate proteoglycans (HSPGs) and small heat shock protein B2 [53, 54], are also substrates for tTG [55, 56]. The fact that these tTG substrates are present in CAA as well suggests that tTG activity might affect Aβ aggregation and deposition, either directly or via cross-linking of Aβ chaperones. However, the role of tTG in initial stages of CAA remains to be investigated, and the use of in vivo models, in particular animal models of CAA, will be instrumental for this purpose.

In late stages of CAA, we found that tTG staining in the abluminal halo was of astrocytic origin in parenchymal vessel and derived from fibroblasts in leptomeningeal vessels, whereas tTG was of endothelial origin in the luminal halo. All these cell types are known to produce tTG [31, 57–59], and tTG is upregulated in response to cell stress and inflammation [60]. Aβ deposition is suggested to precede inflammation [61], thus expression of tTG may be increased especially in the cells surrounding the Aβ deposition in CAA. In parenchymal vessels, fibroblasts may produce tTG as well, although we could not distinguish astrocytes from fibroblasts, as vimentin stains mesenchymal cells including astrocytes and fibroblasts. Taken together, although the tTG in CAA is likely to be derived from astrocytes, fibroblasts and endothelial cells, a role in the production of tTG by smooth muscle cells, as in early stage CAA, cannot be excluded.

The presence and activity of tTG in the abluminal and luminal halo points towards a role in remodelling of the ECM. Altered expression of ECM proteins has already been observed in CAA and the brain microvasculature in AD [8, 27–29, 62, 63]. In our study we observed, in line with previous studies [27–29], altered expression of the ECM proteins FN and LN. Both FN and LN were present in an abluminal and/or luminal halo that surrounded the Aβ deposition. We here found that FN and LN colocalised with tTG and its cross-links in CAA of AD and HCHWA-D patients, suggesting that tTG might cross-link these ECM proteins in CAA. The putative cross-linking of FN and LN by tTG might have detrimental effects, since excess production and cross-linking of ECM proteins in diabetic nephropathy is associated with renal fibrosis and scarring [64]. Furthermore, the formation of cross-linked proteins at the endothelial side of the vessel could impair transport of solutes and

molecules across the blood brain barrier leading to impaired supply of nutrients in the brain thus compromising brain functioning. In addition, tTG-mediated cross-linking of ECM proteins may stiffen the vessel wall, resulting in impaired clearance of Aβ from the brain via the interstitial fluid (ISF) drainage [30, 65, 66]. Furthermore, impaired clearance of Aβ via this route might be not only be the underlying cause of Aβ accumulation in the vessel wall leading to CAA, but also contributes to the accumulation of Aβ in the brain parenchyma [66].

On the other hand, the cross-linked ECM proteins might have a protective effect in CAA, because this cross-linked barrier may prevent the weakening of the vessel wall [9] and leakage of the blood-brain barrier, possibly reducing the risk of haemorrhages which can be the result of CAA [3, 9, 67]. In addition, at the abluminal side, the cross-linked ECM proteins may serve as a barrier to prevent spreading of toxic Aβ into the brain parenchyma. Clearly, further investigations are required to investigate these options.

In conclusion, we found that tTG immunoreactivity is increased in early stages of CAA where it colocalised with Aβ deposition in the vessel wall, whereas tTG and its cross-links in more mature CAA lesions colocalised with ECM proteins in an abluminal and luminal halo enclosing the Aβ deposition. The role of tTG in early stage CAA might therefore be different from end stage CAA, when tTG-mediated ECM remodelling might be more important than its effect on Aβ aggregation per se. However, since we observed an increased tTG transamidation activity in the Aβ deposition also in end stage CAA lesions, this may also be the consequence of epitope masking in these densely packed Aβ depositions. Chronological evaluation of CAA lesion development in animal models of CAA will hopefully provide answers to the outstanding questions on the role of tTG in vascular pathology in AD and related disorders.

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Disclosure Statement

The authors declare that they have no conflict of interest.

References

1. Selkoe D (1994) Cell biology of the amyloid beta-protein precursor and the mechanism of Alzheimer's disease. Annu Rev Cell Biol 10:373–403
2. Walsh D, Hartley D, Kusumoto Y (1999) Amyloid β-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. J Biol Chem 274:25945–25952
3. Attems J, Jellinger K, Thal DR, Van Nostrand W (2011) Review: sporadic cerebral amyloid angiopathy. Neuropathol Appl Neurobiol 37:75–93
4. Jellinger KA, Attems J (2005) Prevalence and pathogenic role of cerebrovascular lesions in Alzheimer disease. J Neurol Sci 229-230:37–41
5. Maat-Schieman M, Roos R, Duinen S Van (2005) Hereditary cerebral hemorrhage with amyloidosisâ€□Dutch type. Neuropathology 25:288–297
6. Greenberg SM, Gurol ME, Rosand J, Smith EE (2004) Amyloid angiopathy-related vascular cognitive impairment. Stroke 35:2616–9
7. Thal DR, Ghebremedhin E, Rüb U, Yamaguchi H, Del Tredici K, Braak H (2002) Two types of sporadic cerebral amyloid angiopathy. J Neuropathol Exp Neurol 61:282–93
8. Perlmutter LS (1990) Microangiopathy, the vascular basement membrane and Alzheimer's disease: a review. Brain Res Bull 24:677–686
9. Zipfel GJ, Han H, Ford AL, Lee J-M (2009) Cerebral amyloid angiopathy: progressive disruption of the neurovascular unit. Stroke 40:S16–9
10. Kim S, Grant P, Lee J (1999) Differential expression of multiple transglutaminases in human brain. J Biol Chem 274:30715–30721
11. Wilhelmus MMM, Grunberg SCS, Bol JGJM, van Dam A-M, Hoozemans JJM, Rozemuller AJM, Drukarch B (2009) Transglutaminases and transglutaminase-catalyzed cross-links colocalize with the pathological lesions in Alzheimer's disease brain. Brain Pathol 19:612–22
12. Lorand L, Graham RM (2003) Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat Rev Mol Cell Biol 4:140–56
13. Fesus L, Piacentini M (2002) Transglutaminase 2: an enigmatic enzyme with diverse functions. Trends Biochem Sci 27:534–539
14. Griffin M, Casadio R, Bergamini C (2002) Transglutaminases: nature's biological glues. Biochem J 396:377–396
15. Bakker ENTP, Buus CL, Spaan J a E, Perree J, Ganga A, Rolf TM, Sorop O, Bramsen LH, Mulvany MJ, Vanbavel E (2005) Small artery remodeling depends on tissue-type transglutaminase. Circ Res 96:119–26
16. Ientile R, Caccamo D, Griffin M (2007) Tissue transglutaminase and the stress response. Amino Acids 33:385–94
17. Wilhelmus MMM, van Dam A-M, Drukarch B (2008) Tissue transglutaminase: a novel pharmacological target in preventing toxic protein aggregation in neurodegenerative diseases. Eur J Pharmacol 585:464–72
18. Appelt DM, Kopen GC, Boyne LJ, Balin BJ (1996) Localization of transglutaminase in hippocampal neurons: implications for Alzheimer's disease. J Histochem Cytochem 44:1421–1427
19. Johnson GV., Cox TM, Lockhart JP, Zinnerman MD, Miller ML, Powers RE (1997) Transglutaminase activity is increased in Alzheimer's disease brain. Brain Res 751:323–329
20. Wang D, Uchikado H, Bennett D a, Schneider J a, Mufson EJ, Wu J, Dickson DW (2008b) Cognitive performance correlates with cortical isopeptide immunoreactivity as well as Alzheimer type pathology. J Alzheimers Dis 13:53–66
21. Sárvári M, Fésüs L, Nemes Z (2002) Transglutaminase-mediated crosslinking of neural proteins in Alzheimer's disease and other primary dementias. Drug Dev Res 56:458–472
22. Dudek SM, Johnson G V (1994) Transglutaminase facilitates the formation of polymers of the beta-amyloid peptide. Brain Res 651:129–33
23. Ikura K, Takahata K, Sasaki R (1993) Cross-linking of a synthetic partial-length (1-28) peptide of the Alzheimer beta/A4 amyloid protein by transglutaminase. FEBS Lett 326:109–11
24. Rasmussen LK, Sørensen ES, Petersen TE, Gliemann J, Jensen PH (1994) Identification of glutamine and lysine residues in Alzheimer amyloid beta A4 peptide responsible for transglutaminase-catalysed homopolymerization and cross-linking to alpha 2M receptor. FEBS Lett 338:161–6
25. Schmid AW, Condemi E, Tuchscherer G, Chiappe D, Mutter M, Vogel H, Moniatte M, Tsybin YO (2011) Tissue transglutaminase-mediated glutamine deamidation of beta-amyloid peptide increases peptide solubility, whereas enzymatic cross-linking and peptide fragmentation may serve as molecular triggers for rapid peptide aggregation. J Biol Chem 286:12172–88
26. Hartley DM, Zhao C, Speier AC, Woodard G a, Li S, Li Z, Walz T (2008) Transglutaminase induces protofibril-like amyloid beta-protein assemblies that are protease-resistant and inhibit long-term potentiation. J Biol Chem 283:16790–800

27. Miners JS, Ashby E, Van Helmond Z, Chalmers K a, Palmer LE, Love S, Kehoe PG (2008) Angiotensin-converting enzyme (ACE) levels and activity in Alzheimer's disease, and relationship of perivascular ACE-1 to cerebral amyloid angiopathy. Neuropathol Appl Neurobiol 34:181–93
28. Van Duinen SG, Maat-Schieman ML, Bruijn JA, Haan J, Roos RA (1995) Cortical tissue of patients with hereditary cerebral hemorrhage with amyloidosis (Dutch) contains various extracellular matrix deposits. Lab Invest 73:183–9
29. Zhang WW, Lempessi H, Olsson Y (1998) Amyloid angiopathy of the human brain: immunohistochemical studies using markers for components of extracellular matrix, smooth muscle actin and endothelial cells. Acta Neuropathol 96:558–63
30. Wilhelmus MMM, de Jager M, Drukarch B (2012) Tissue transglutaminase: a novel therapeutic target in cerebral amyloid angiopathy. Neurodegener Dis 10:317–9
31. Zemskov E, Janiak A, Hang J, Waghray A, Belkin A (2006) The role of tissue transglutaminase in cell-matrix interactions. Front Biosci 11:1057–1076
32. Braak H, Alafuzoff I, Arzberger T, Kretzschmar H, Del Tredici K (2006) Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. Acta Neuropathol 112:389–404
33. Thal DR, Rüb U, Orantes M, Braak H (2002) Phases of A beta-deposition in the human brain and its relevance for the development of AD. Neurology 58:1791–800
34. Wilhelmus M, Verhaar R (2011) Novel role of transglutaminase 1 in corpora amylacea formation? Neurobiol Aging 32:845–856
35. Wilhelmus MMM, Verhaar R, Andringa G, Bol JGJM, Cras P, Shan L, Hoozemans JJM, Drukarch B (2011) Presence of tissue transglutaminase in granular endoplasmic reticulum is characteristic of melanized neurons in Parkinson's disease brain. Brain Pathol 21:130–9
36. Jin X, Stamnaes J, Klöck C, DiRaimondo TR, Sollid LM, Khosla C (2011) Activation of extracellular transglutaminase 2 by thioredoxin. J Biol Chem 286:37866–73
37. Schaertl S, Prime M, Wityak J, Dominguez C, Munoz-Sanjuan I, Pacifici RE, Courtney S, Scheel A, Macdonald D (2010) A profiling platform for the characterization of transglutaminase 2 (TG2) inhibitors. J Biomol Screen 15:478–87
38. Jeon WM, Lee KN, Birckbichler PJ, Conway E, Patterson MK (1989) Colorimetric assay for cellular transglutaminase. Anal Biochem 182:170–5
39. Lee KN, Birckbichler PJ, Patterson MK (1988) Colorimetric assay of blood coagulation factor XIII in plasma. Clin Chem 34:906–10
40. Sofroniew M V, Vinters H V (2010) Astrocytes: biology and pathology. Acta Neuropathol 119:7–35
41. Girma JP, Meyer D, Verweij CL, Pannekoek H, Sixma JJ (1987) Structure-function relationship of human von Willebrand factor. Blood 70:605–11
42. Jaffe E a, Hoyer LW, Nachman RL (1974) Synthesis of von Willebrand factor by cultured human endothelial cells. Proc Natl Acad Sci U S A 71:1906–9
43. Franke WW, Schmid E, Osborn M, Weber K (1978) Different intermediate-sized filaments distinguished by immunofluorescence microscopy. Proc Natl Acad Sci U S A 75:5034–8
44. Skalli O, Vandekerckhove J, Gabbiani G (1987) Actin-isoform pattern as a marker of normal or pathological smooth-muscle and fibroblastic tissues. Differentiation 33:232–238
45. Colognato H, Yurchenco PD (2000) Form and Function : The Laminin Family of Heterotrimers. 218:213–234
46. Stenman S, Vaheri A (1978) Distribution of a major connective tissue protein, fibronectin, in normal human tissues. J Exp Med 1:1054–1064
47. Pinkas DM, Strop P, Brunger AT, Khosla C (2007) Transglutaminase 2 undergoes a large conformational change upon activation. PLoS Biol 5:e327
48. Greenberg C, Birckbichler P, Rice R (1991) Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. FASEB J 5:3071–3077
49. Van den Akker J, van Weert A, Afink G, Bakker ENTP, van der Pol E, Böing AN, Nieuwland R, VanBavel E (2012) Transglutaminase 2 is secreted from smooth muscle cells by transamidation-dependent microparticle formation. Amino Acids 42:961–73
50. Thomázy V, Fésüs L (1989) Differential expression of tissue transglutaminase in human cells. An immunohistochemical study. Cell Tissue Res 255:215–24
51. Currò M, Ferlazzo N, Condello S, Caccamo D, Ientile R (2010) Transglutaminase 2 silencing reduced the beta-amyloid-effects on the activation of human THP-1 cells. Amino Acids 39:1427–33
52. Santhanam L, Tuday EC, Webb AK, et al (2010) Decreased S-nitrosylation of tissue transglutaminase contributes to age-related increases in vascular stiffness. Circ Res 107:117–25
53. Wilhelmus MMM, Otte-Höller I, Wesseling P, de Waal RMW, Boelens WC, Verbeek MM (2006) Specific association of small heat shock proteins with the pathological hallmarks of Alzheimer's disease brains. Neuropathol Appl Neurobiol 32:119–30
54. Wilhelmus MMM, Waal RMW, Verbeek MM (2007) Heat Shock Proteins and Amateur Chaperones in Amyloid-Beta Accumulation and Clearance in Alzheimer's Disease. Mol Neurobiol 35:203–216
55. Boros S, Ahrman E, Wunderink L, Kamps B, de Jong WW, Boelens WC, Emanuelsson CS (2006) Site-specific transamidation and deamidation of the small heat-shock protein Hsp20 by tissue transglutaminase. Proteins 62:1044–52
56. Verderio EAM, Scarpellini A, Johnson TS (2009) Novel interactions of TG2 with heparan sulfate proteoglycans: reflection on physiological implications. Amino Acids 36:671–7
57. Bakker ENTP, Pistea A, VanBavel E (2008) Transglutaminases in vascular biology: relevance for vascular remodeling and atherosclerosis. J Vasc Res 45:271–8
58. Nurminskaya M V, Belkin AM (2012) Cellular functions of tissue transglutaminase., 1st ed. Int Rev Cell Mol Biol. doi: 10.1016/B978-0-12-394305-7.00001-X
59. Yamada T, Yoshiyama Y, Kawaguchi N, Ichinose A, Iwaki T, Hirose S, Jefferies W (1998) Possible roles of transglutaminases in Alzheimer's disease. Dement Geriatr Cogn Disord 9:103–110
60. Wang D, Dickson D, Malter J (2008a) Tissue transglutaminase, protein cross-linking and Alzheimer's disease: review and views. Int J Clin Exp Pathol 1:5–18
61. Heneka MT, O'Banion MK, Terwel D, Kummer MP (2010) Neuroinflammatory processes in Alzheimer's disease. J Neural Transm 117:919–47
62. Kalaria R (1996) Cerebral vessels in ageing and Alzheimer's disease. Pharmacol Ther 72:193–214
63. Perlmutter LS (1994) Microvascular pathology and vascular basement membrane components in Alzheimer's disease. Mol Neurobiol 9:33–40
64. Skill NJ, Johnson TS, Coutts IGC, Saint RE, Fisher M, Huang L, El Nahas a M, Collighan RJ, Griffin M (2004) Inhibition of transglutaminase activity reduces extracellular matrix accumulation induced by high glucose levels in proximal tubular epithelial cells. J Biol Chem 279:47754–62
65. Schley D, Carare-Nnadi R, Please CP, Perry VH, Weller RO (2006) Mechanisms to explain the reverse perivascular transport of solutes out of the brain. J Theor Biol 238:962–74
66. Weller RO, Preston SD, Subash M, Carare RO (2009) Cerebral amyloid angiopathy in the aetiology and immunotherapy of Alzheimer disease. Alzheimers Res Ther 1:6
67. Wisniewski H, Vorbrodt AW, Wegiel J (1997) Amyloid Angiopathy and Blood–Brain Barrier Changes in Alzheimer's Disease. Ann N Y Acad Sci 26:161–172