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Fibrillin-1 impairment enhances blood-brain barrier permeability and xanthoma formation in brains of apolipoprotein E deficient mice

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Abstract

We recently reported that apolipoprotein E (ApoE)-deficient mice with a mutation in the fibrillin-1 gene (ApoE^{-/-}Fbn1^{C1039G+/-}) develop accelerated atherosclerosis with enhanced inflammation, atherosclerotic plaque rupture, myocardial infarction and sudden death. In the brain, fibrillin-1 functions as an attachment protein in the basement membrane, providing structural support to the blood-brain barrier (BBB). Here, we investigated whether fibrillin-1 impairment affects the permeability of the BBB proper and the blood cerebrospinal fluid barrier (BCSFB), and whether this leads to the accelerated accumulation of lipids (xanthomas) in the brain. ApoE^{-/-} (n=61) and ApoE^{-/-}Fbn1^{C1039G+/-} (n=73) mice were fed a Western-type diet (WD). After 14 weeks WD, a significantly higher permeability of the BBB was observed in ApoE^{-/-}Fbn1^{C1039G+/-} mice compared to age-matched ApoE^{-/-} mice. This was accompanied by leukocyte infiltration, enhanced expression of pro-inflammatory cytokines, matrix metalloproteinases and transforming growth factor- β , and by decreased expression of tight junction proteins claudin-5 and occludin. After 20 weeks WD, 83% of ApoE^{-/-}Fbn1^{C1039G+/-} mice showed xanthomas in the brain, compared to 23% of their ApoE^{-/-} littermates. Xanthomas were mainly located in fibrillin-1 rich regions, such as the choroid plexus and the neocortex. Our findings demonstrate that dysfunctional fibrillin-1 impairs BBB/BCSFB integrity, facilitating peripheral leukocyte infiltration, which further degrades the BBB/BCSFB. As a consequence, lipoproteins can enter the brain, resulting in accelerated formation of xanthomas.

Keywords

Xanthomas; fibrillin-1; blood-brain barrier; choroid plexus

1 Introduction

2 The blood brain barrier (BBB) is a dynamic interface that separates the brain from the
3 circulating blood, ensuring maintenance of the brain microenvironment and protecting it from
4 intrusion of toxic and inflammatory substances.¹ The BBB generally restricts entry of polar
5 and/or large molecules into the brain. However, nutrients (glucose, amino acids and vitamins)
6 and many regulatory neuroactive peptides can cross the BBB using specific transporters
7 expressed in the brain endothelium.²⁻⁶ The BBB exists at two main interfaces, i.e. between the
8 blood and the brain (the BBB proper), created by endothelial cells that form the walls of the
9 capillaries in the brain parenchyma, and between the blood and the cerebrospinal fluid (CSF)
10 at the choroid plexus in the brain ventricles (blood-CSF barrier, BCSFB), created by epithelial
11 and fenestrated capillary endothelial cells.¹ Both barriers are characterized by the presence of
12 tight junction (TJ) proteins such as occludin and claudins, which firmly hold the endothelial
13 cells of the BBB proper and epithelial cells of the BCSFB together and make them
14 impermeable for macromolecules and leukocytes.⁷ These endothelial and epithelial cell layers
15 are attached to an underlying basement membrane, which is mainly composed of large
16 glycoprotein extracellular matrix (ECM) molecules such as laminin, collagen IV,
17 proteoglycans (e.g. perlecan) and microfibrils (predominantly fibrillin-1), and provides
18 structural support.^{8,9}

19 When the integrity of the BBB is compromised, such as during inflammation, plasma proteins
20 can enter the brain, causing neuronal damage.^{10, 11} In the context of atherosclerosis, ApoE
21 deficiency (ApoE^{-/-}) has been shown to impair the BBB in mice, a condition that is
22 exacerbated by age and/or a cholesterol-rich diet.^{12, 13} Brain xanthomas have been described in
23 ApoE^{-/-} mice, with a prevalence that increased when the mice were fed a Western-type diet
24 (WD).^{14, 15} Brain xanthomas have also been observed in humans, in which case they were

often seen in the brain ventricles.¹⁶ Although brain xanthomas are considered as benign lesions, they can result in disabling symptoms or even death if left untreated.¹⁷ The glycoprotein fibrillin-1 is an essential component of elastic fibers. In humans, mutations in the gene encoding fibrillin-1 cause Marfan syndrome, which is predominantly characterized by aortic aneurysms.¹⁸ Furthermore, it can be responsible for ageing of arteries in mice.¹⁹ We recently reported that a mutation in the fibrillin-1 gene in ApoE^{-/-} mice (ApoE^{-/-} Fbn1^{C1039G+/-}) on WD leads to accelerated atherogenesis and the development of large and unstable atherosclerotic plaques.²⁰ Compared to those in regular ApoE^{-/-} mice, atherosclerotic plaques in ApoE^{-/-} Fbn1^{C1039G+/-} mice are characterized by increased expression of the pro-inflammatory cytokines interleukine-1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α) and the matrix metalloproteinases (MMP) -2 and -9, which are known to affect BBB integrity.²¹ In the brain, fibrillin-1 is an element of the capillary basement membrane, where it acts as an attachment protein between the two basal laminas.²² Therefore, we examined in the present study the effect of fibrillin-1 impairment in ApoE^{-/-} mice on BBB and BCSFB permeability and the development of xanthomas in the brain.

Methods

Mice

Female ApoE^{-/-} Fbn1^{C1039G+/-} mice (n=73) and their ApoE^{-/-} littermates (n=61, 6 weeks of age) were fed a WD (TD88137, Harlan Teklad, Madison, WI) for 20 weeks, unless otherwise described. ApoE^{-/-} Fbn1^{C1039G+/-} mice fed a normal diet (ND; n=20) were used as controls. Female mice were chosen because in contrast to male mice, the Fbn1 mutation did not result in aortic dissection in the time frame of the experiments. The animals were housed in a temperature-controlled room with a 12-hour light/dark cycle with free access to water and food. At the end of the experiment, mice were anesthetized with sodium pentobarbital (75

mg/kg, i.p.) and blood samples were taken from the retro-orbital plexus for plasma cholesterol analysis by means of a commercial kit (Randox, Crumlin, UK). Subsequently, animals were euthanized with an overdose of sodium pentobarbital (250 mg/kg, i.p.). All experiments were approved by the ethical committee of the University of Antwerp.

Blood-brain barrier permeability

Evans blue dye

ApoE^{-/-} (n=10) and ApoE^{-/-}Fbn1^{C1039G+/-} mice (n=10) on WD were injected intraperitoneally with a 2% Evans blue solution (4 ml/kg body weight; n=4 per group at 14 and 21 weeks on WD; n=2 per group at 30 weeks on WD). Three hours later, mice were sacrificed, transcardially perfused with ice-cold saline and brains were collected and weighted. Evans blue was extracted with formamide (Sigma, St Louis, MO) for 72 hours and the optical density of the extracted dye was measured at 620 nm.¹²

Gadolinium-gadoterate meglumine acid (Gd-DOTA) enhanced magnetic resonance imaging (MRI)

ApoE^{-/-} (n=7) and ApoE^{-/-}Fbn1^{C1039G+/-} mice (n=4) on WD for 21 weeks were cannulated with a 26-gauge needle (BD Vasculon Plus, Helsingborg, Sweden) via the tail vein for subsequent contrast agent injection (gadolinium (Gd); Dotarem®, Guerbet). T₁ measurements were acquired with a 7 Tesla Pharmascan (Bruker, Karlsruhe, Germany) using a multiple inversion-recovery echo-planar imaging sequence, with the following parameters: varying TIs: 28; 800; 1000; 1400; 4000; 8000 ms; BW: 300 kHz; TR/TE: 10 s/20.4 ms; acquisition matrix 128 x 128 (zero-filled read and partial FT; acquired matrix: 98 x 84), FOV 20 x 20 mm², NEX: 2; slice thickness: 1 mm. Several sets of T₁ maps were obtained to investigate the effect of BBB disruption, before Gd administration (baseline T₁, pre-Gd) and every 2 min. up to 30 min.

after (post-Gd) administration of Gd (0.2 mmol/kg body weight). T₁ maps were calculated for all the different data sets acquired in time using a mono-exponential fit. Calculations were done using Bruker software. Regions of interest (ROIs) in the cortex, hippocampus, thalamus, and hypothalamus were manually defined based on a standard mouse brain atlas.²³

Post mortem and in vivo MRI of the brain

Post mortem- After euthanasia, mice were perfused transcardially with 4% paraformaldehyde (PFA) for 1 hour and the heads were stored in 4% PFA. High resolution *post mortem* 3D images of the total brain (in the skull) were acquired with a 7 Tesla Pharmascan using a T₂-weighted RARE sequence (TE/TR=32/4000 ms, voxel size = (78x78x156) μm³, scan time=14 hours). Images were visually inspected for the presence of xanthomas²⁴ using AMIRA software (Mercury Computer Systems, Chelmsford, MA) in the coronal, axial and sagittal planes. The mean lesion volume was calculated by manually delineating affected regions. Localization of lesions in the brain was examined by means of a mouse brain atlas.²³ Presence, size and localization of xanthomas were analyzed by two independent and blinded observers.

In vivo- ApoE^{-/-}Fbn1^{C1039G^{+/+}} mice (n=5) were scanned longitudinally after 13, 16, 19, 22, 25, and 28 weeks on WD. Mice were anesthetized with isoflurane (IsoFlo, Abbott, Illinois, USA) (3% for induction and 1.5% for maintenance) in a gaseous mixture of 70% N₂ and 30% O₂. Respiration rate was measured using a small animal pressure pad (MR compatible Small Animal Monitoring and Gating System, SA Instruments, Inc.). Body temperature was maintained at 37°C using a feedback-coupled hot air heating system (MR compatible Small Animal Heating System, SA Instruments, Inc.). At each time point, high resolution 3D horizontal images of the total brain were acquired using a T₂-weighted RARE sequence (TE/TR=44/2500 ms, voxel size = (78x156x187) μm³, scan time= 1 hour). To assess possible

anomalies in cerebral flow, horizontal and coronal MR angiograms were acquired with a 2D Time-of-Flight sequence in coronal and axial directions: TE/TR=3.2/15 ms, Flip Angle 90°, FOV= (20x20) mm² within a 256x256 image matrix, 40 slices with thickness of 0.4 mm, 4 averages. After the experiment, the mice rapidly woke up in an infrared heated recovery box. Maximum intensity projections in the coronal and horizontal planes were calculated to show the brain vessel anatomy and blood supply. At week 30, mice were sacrificed and perfusion fixed with 4% PFA for one hour via a catheter in the left ventricle. High resolution *post mortem* 3D images of the total brain (in the skull) were acquired and inspected as described above.

Histological analysis

PFA-fixed brains were dissected out of the skull, processed and embedded in paraffin. Histological analysis was performed on hematoxylin and eosin (H&E)-stained sections. Fibrillin-1 expression was assessed by immunohistochemistry using an anti-fibrillin-1 antibody (ab53076, Abcam, Cambridge, UK). The antibody was checked for specificity for fibrillin-1 (no cross-reactivity with fibrillin-3) in the NCBI protein bank. Basement membranes were visualized by means of a periodic acid-Schiff (PAS) stain.

Assessment of brain hypoxia

Brains of ApoE^{-/-}Fbn1^{C1039G^{+/+}} mice (n=3) diagnosed with a xanthoma were freshly isolated, sliced into 1.5 mm sections and incubated for 20 minutes with 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, 2% in PBS), a mitochondrial marker indicating cellular respiration. Thereafter, tissues were fixed overnight in 4% PFA at 4°C.

Transmission electron microcopy (TEM)

Choroid plexus tissue of ApoE^{-/-}Fbn1^{C1039G+/-} and ApoE^{-/-} mice (16 weeks on WD) was dissected under a microscope,²⁵ fixed overnight in 0.1 M sodium cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde solution, and washed three times in 0.1 M sodium cacodylate-buffered (pH 7.4) 7.5% saccharose solution. Postfixation was performed by incubating cells with 1% OsO₄ solution for 2 h, followed by 1 hour in 1% tannic acid. After dehydration in an ethanol gradient, samples were embedded in EM-bed812. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Tecnai G2 Spirit Bio TWIN microscope (Fei, Europe BV, Zaventem, Belgium) at 120 kV.

Western blotting

The choroid plexus was isolated from brains of ApoE^{-/-} and ApoE^{-/-}Fbn1^{C1039G+/-} mice (14 or 21 weeks on WD; n=3 per group, plus two ApoE^{-/-}Fbn1^{C1039G+/-} mice with macroscopically visible xanthomas), lysed in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and denatured for 4 min. A 20 µg sample was loaded on Bolt 4-12% or 12% (Bis)-Tris gels (Invitrogen, Carlsbad, CA). After electrophoresis and blotting, membranes were probed with the following primary antibodies: anti-ICAM-1 (ab27536, Abcam), anti-VCAM-1 (01811D, Pharmingen), anti-IL-1β (ab9722, Abcam), anti-TNF-α (1221-00, Genzyme), anti-MMP-2 (ab86607, Abcam), anti-MMP-9 (ab38898, Abcam), anti-TGF-β (3711, Cell Signaling Technology), anti-claudin-5 (ab15106, Abcam), anti-occludin (ab31721, Abcam), anti-collagen IV (LS-C79587, Novus), anti-laminin (NB300-144, Novus), anti-β-actin (A5441, Sigma). Secondary antibodies were species-appropriate horseradish peroxidase conjugated (Dako, Glostrup, Denmark; ICAM-1, TGF-β, claudin-5, occludin, laminin and β-actin) or avidin/biotin conjugated (Vectastain ABC kit, Burlingame, CA; VCAM-1, IL-1β, TNF-α, MMP-2, MMP-9, collagen IV). Immunodetection was performed with SuperSignal West Pico

or Femto Substrate (Thermo Fisher Scientific, Rockford, IL) using a Lumi-Imager (Roche Diagnostics, Mannheim, Germany).

Statistical analysis

Data are presented as mean±S.E.M., unless otherwise stated. Statistical analyses were performed with the SPSS software (version 20, IBM Corporation, Armonk, NY) or in GraphPad Prism (version 5.0, La Jolla, CA). Differences were considered significant at $P < 0.05$. The tests used are mentioned in the table and figure legends.

Results

ApoE^{-/-}Fbn1^{C1039G+/-} mice show increased blood-brain barrier breakdown

Total plasma cholesterol was similar in ApoE^{-/-} and ApoE^{-/-}Fbn1^{C1039G+/-} mice after 20 weeks on WD (577±23 and 509±41 mg/dl, respectively), whereas ApoE^{-/-}Fbn1^{C1039G+/-} mice on ND had significantly lower levels (153±43 mg/dl). Assessment of BBB permeability by means of Evans blue revealed a significantly higher leakage in ApoE^{-/-}Fbn1^{C1039G+/-} mice compared to ApoE^{-/-} mice after 14 weeks on WD (Figure 1A). After 30 weeks on WD, ApoE^{-/-}Fbn1^{C1039G+/-} mice showed lesions in the brain, which occupied large areas of the brain ventricles and (neo)cortex. The lesions were associated with extensive Evans blue leakage, pointing toward a massive BBB and BCSFB breakdown (Figure 1B). Interestingly, some areas in the lesions appeared to be more intensely colored than others. Due to the massive presence of lesions (lipids) at 30 weeks, Evans blue could not be accurately measured by spectrophotometry. Gd-enhanced MRI at 21 weeks confirmed the enhanced BBB permeability in the presence of lesions in ApoE^{-/-}Fbn1^{C1039G+/-} mice, which was observed by a drop in delta T₁ values and Gd leakage around the lesion in the neocortex (Figure 1C).

ApoE^{-/-}Fbn1^{C1039G+/-} mice show increased presence of xanthomas in the brain

MRI images showed lesions in the brain (Figure 2A-C). Histological evaluation of these lesions on H&E- stained sections revealed cholesterol clefts and foam cells, indicative of xanthomas (Figure 2D-F). At 20 weeks on WD, 83% of the ApoE^{-/-}Fbn1^{C1039G+/-} mice showed xanthomas in the brain, compared to 23% of their ApoE^{-/-} littermates (n=54 and n=40, respectively, Table 1). The number of xanthomas varied from 0 to 10, with a median of 1 and 0 xanthomas per mouse in ApoE^{-/-}Fbn1^{C1039G+/-} and ApoE^{-/-} mice, respectively. Moreover, xanthoma size was more than doubled in ApoE^{-/-}Fbn1^{C1039G+/-} compared to ApoE^{-/-} mice (Table 1). Brain xanthomas were not observed on MRI in age-matched ApoE^{-/-}Fbn1^{C1039G+/-} mice on ND.

Xanthomas are mainly located in brain ventricles and neocortex

MRI demonstrated that the majority of the xanthomas were located in the neocortex and the brain ventricles (Table 2, Figure 3). Xanthomas were less frequently found in the olfactory bulb, brain stem and cerebellum (Table 2).

Xanthomas increase in size over time

In order to study xanthoma formation and development over time, brains of ApoE^{-/-}Fbn1^{C1039G+/-} mice (n=5) were scanned longitudinally *in vivo*. Three ApoE^{-/-}Fbn1^{C1039G+/-} mice developed one or more xanthomas in the brain, which could be detected from 16 weeks WD onward and whose size increased exponentially in time (Figure 4A, B). A TTC stain of the brain did not show signs of hypoxia in the proximity of the xanthomas (Figure 4C).

Xanthomas are observed in fibrillin-1 rich regions

Immunohistochemistry showed high expression of fibrillin-1 in the brain vasculature, especially in the choroid plexus and capillaries in the neocortex, corresponding with the

basement membrane (Figure 5A) and the major sites of xanthoma formation (Figure 5B). When increasing in size, xanthomas further damaged the epithelial cells of the choroid plexus, allowing the lesion to grow into the brain parenchyma (Figure 5B). Xanthomas developed around or adjacent to blood capillaries in the choroid plexus or neocortex. Advanced lesions frequently showed presence of small blood vessels and hemorrhage (Figure 5C, D). The choroid plexus in ApoE^{-/-}Fbn1^{C1039G+/-} mice on WD showed flattened and non-adherent epithelial cells and a thicker basement membrane, compared to the cubic and adherent epithelial cells seen in ApoE^{-/-} mice (Figure 5E). TEM confirmed these observations (Figure 6A, B). Moreover, the microvilli at the surface of the epithelial cells of ApoE^{-/-}Fbn1^{C1039G+/-} mice appeared elongated and less dense compared to those of ApoE^{-/-} mice, indicating epithelial activation (Figure 6C, D). Leukocytes were frequently observed adherent to the endothelium in ApoE^{-/-}Fbn1^{C1039G+/-} mice or even across the fenestrated endothelium and underlying basement membrane (Figure 6B). The TEM images suggest a ‘loosened’ (less compact) basement membrane in ApoE^{-/-}Fbn1^{C1039G+/-} compared to ApoE^{-/-} mice (Figure 6E, F).

Enhanced BBB permeability is associated with increased levels of IL-1 β , TNF- α , MMP-2 and -9, TGF- β , and with decreased expression of occludin and claudin-5

The finding that numerous leukocytes adhered to the endothelial cell layer in ApoE^{-/-}Fbn1^{C1039G+/-} mice let us to investigate whether the expression of adhesion molecules was augmented. We found that VCAM-1 and ICAM-1 expression were significantly increased in the choroid plexus of ApoE^{-/-}Fbn1^{C1039G+/-} mice after 14 weeks on WD, compared to their ApoE^{-/-} littermates. After 21 weeks on WD, VCAM-1 expression remained elevated, whereas ICAM-1 expression was decreased to the levels seen in the choroid plexus of ApoE^{-/-} mice

(Figure 7A). The presence of xanthomas did not further increase VCAM-1 or ICAM-1 expression.

The expression levels of IL-1 β , TNF- α , MMP-2 and -9 were significantly increased in the choroid plexus of ApoE^{-/-}Fbn1^{C1039G^{+/+}} mice when compared to their ApoE^{-/-} littermates, particularly in the presence of xanthomas. Importantly, this increase could already be observed in ApoE^{-/-}Fbn1^{C1039G^{+/+}} mice after 14 weeks on WD (Figure 7A), i.e. coinciding with BBB and/or BCSFB degradation (Figure 1A, B). Similarly, we also observed a rise in the inflammatory mediator TGF- β , which peaked in the presence of a xanthoma (Figure 7A). Furthermore, a significant decrease in occludin and claudin-5 expression was observed in ApoE^{-/-}Fbn1^{C1039G^{+/+}} mice, whereas collagen IV remained unchanged and laminin was slightly increased (Figure 7B).

Discussion

The present study shows that fibrillin-1 impairment, resulting from a mutation (C1039G^{+/+}) in the fibrillin-1 gene, leads to an accelerated degradation of the BBB and BCSFB and consequent development of xanthomas in brains of ApoE^{-/-} mice. Degradation of the BBB coincided with enhanced expression of various pro-inflammatory cytokines (IL-1 β , TNF- α), matrix-degrading proteases (MMP-2 and -9) and TGF- β . Importantly, in ApoE^{-/-}Fbn1^{C1039G^{+/+}} mice, BBB permeability as well as inflammation markers were significantly increased after 14 weeks on WD, i.e., before xanthomas were detected on MRI. This indicates an earlier onset of inflammation and BBB/BCSFB breakdown as compared to their ApoE^{-/-} littermates. Moreover, the BBB permeability increased with age, suggestive of a progressive BBB breakdown that has to reach a certain threshold before large molecules such as lipoproteins are able to enter the brain.

Xanthomas were mostly observed in brain regions where fibrillin-1 was highly expressed (choroid plexus and neocortex), implying a local effect of fibrillin-1 impairment. Fibrillin-1 is the major component of microfibrils, which are present in many extracellular matrices to provide elasticity to the connective tissue. In the brain, microfibrils act as attachment proteins between the two main layers of the basement membrane (i.e., the basal lamina and lamina reticularis) and strongly interact with the basement membrane component perlecan.^{8, 22} Mutations in the fibrillin-1 gene give rise to misfolding and decreased secretion of the corresponding protein, leading to a defective formation of the microfibrils,^{26, 27} which in turn results in a basement membrane that is loosened and more permeable for macromolecules.

Apart from providing structural support, fibrillin-1 also serves as a reservoir for the protein TGF- β . Deficiency in fibrillin-1 leads to an increase in unsequestered TGF- β , which may trigger inflammation, accompanied by the release of proteases that degrade ECM components.²⁸ In the present study, we observed that TGF- β levels were elevated in the choroid plexus of ApoE^{-/-}Fbn1^{C1039G+/-} mice compared to ApoE^{-/-} mice. This increase was accompanied by enhanced expression of IL-1 β and TNF- α , MMP-2 and -9, which lends further support to the hypothesis that TGF- β acts as a mediator in inflammation.²⁹ This rise in TGF- β was already apparent in ApoE^{-/-}Fbn1^{C1039G+/-} mice after 14 weeks on WD and peaked in the presence of xanthomas. High TGF- β levels have been linked to BBB breakdown in inflammatory central nervous system diseases (e.g. multiple sclerosis and Alzheimer's disease).³⁰ Yet, high local expression of TGF- β alone does not seem to induce inflammation and consequent BBB degradation, but can potentiate existing/ongoing inflammation, for example from a peripherally triggered immune response.²⁹ Thus, the local elevated TGF- β expression observed in ApoE^{-/-}Fbn1^{C1039G+/-} mice does not initiate inflammation and BBB breakdown, but starts to play a role later in the disease process.²⁹ We demonstrated significantly higher expression levels of the adhesion molecules ICAM-1 and VCAM-1 in the

1 choroid plexus of ApoE^{-/-}Fbn1^{C1039G+/-} mice after 14 weeks on WD, indicating an activated
2 endothelium. Because peripheral inflammation (e.g. atherosclerosis) is able to cause BBB
3 damage by activated leukocytes (T-cells, monocytes), which in turn will activate adhesion
4 molecules on the BBB endothelium,⁷ it is conceivable that the increased atherogenesis in
5 ApoE^{-/-}Fbn1^{C1039G+/-} mice contributes to the increased inflammation in the brain. The absence
6 of xanthomas in ApoE^{-/-}Fbn1^{C1039G+/-} mice on ND at the same time point, which is presumably
7 due to less pronounced atherosclerosis and inflammation, supports the latter hypothesis.

8 Besides from being found in the choroid plexus, xanthomas were also frequently encountered
9 in the parenchyma of the neocortex, indicating a damaged BBB proper. Interestingly, some
10 lesions in the neocortex were also observed at the surface of the brain, suggesting that they
11 were formed at the BBB-BCSFB interface at the pia mater. Xanthomas were also present in
12 the olfactory bulb, brain stem and cerebellum, i.e., which are all circulated by CSF,³¹
13 supporting the latter observation. Overall, our observations suggest that the ApoE^{-/-}
14 background together with a WD, initiates BBB and BCSFB degradation in ApoE^{-/-}
15 Fbn1^{C1039G+/-} mice, and that this degradation is potentiated by fibrillin-1 impairment leading to
16 a decreased assembly of the microfibrils. As a result, the capillary basement membrane is
17 loosened and the (local) expression of the inflammation regulator TGF-β is increased,
18 promoting local inflammation and matrix degradation. Moreover, ECM breakdown might be
19 responsible for the detachment of pericytes from the basal lamina,³² which can result in BBB
20 disruption and an additional increase in inflammatory cytokines.³³ The ongoing peripheral
21 inflammation (atherosclerosis), which is accelerated by the fibrillin-1 mutation, stimulates the
22 entry of leukocytes in the brain, crossing and breaking down the BBB/BCSFB (Figure 8).

23 Based on our findings at the level of the choroid plexus, we hypothesize that xanthoma
24 formation has similarities with atherosclerotic plaque development, as also proposed by
25 others.¹⁶ In this regard, the accelerated development of xanthomas in ApoE^{-/-}Fbn1^{C1039G+/-}

1 mice might occur as follows: fenestrated endothelial cells of the choroid plexus are stimulated
2 to express adhesion molecules, such as ICAM-1 and VCAM-1, due to inflammation in the
3 periphery (atherosclerosis). Leukocytes adhere and subsequently cross the endothelial layer
4 and underlying basement membrane, which is weakened by the deficient microfibrillar
5 association. The activated leukocytes secrete pro-inflammatory cytokines and matrix-
6 degrading enzymes IL-1 β , TNF- α and MMP-2 and -9, progressively attacking the TJ proteins
7 occludin and claudin-5 of the epithelial layer, an effect that is potentiated by the elevated
8 TGF- β levels. Furthermore, a slight increase in laminin was observed, which could be the
9 result of a reparative response.³⁴ The damaged BCSFB becomes permeable for lipoproteins,
10 which are now able to enter the choroid plexus where they will be ‘cleared’ by residual
11 phagocytes and eventually turn into foam cells. Eventually, small foam cell lesions may
12 progress to advanced lesions, which can completely fenestrate the choroid plexus epithelium,
13 invade the brain ventricles and grow into the brain parenchyma. This was illustrated by the
14 progressive increase in xanthoma size over time. We observed a rather small increase in size
15 during the first weeks after development but an exponential increase during the last weeks
16 (week 25 up to 30). The Evans blue gradient in the xanthomas suggests growth from the more
17 intensively colored center, where the BBB/BCSFB is most leaky. In addition, hemorrhages
18 were often observed in the xanthomas, which might accelerate xanthoma growth due to the
19 release of cholesterol-rich erythrocytes.¹⁶ These hemorrhages likely arise from the leakage
20 and/or rupture of small vessels in the xanthoma (some of them presumably neovessels).
21 In conclusion, our results demonstrate that dysfunctional fibrillin-1 impairs BBB/BCSFB
22 integrity, where it facilitates the entry of peripheral leukocytes, which further degrades the
23 BBB/BCSFB. As a consequence, lipoproteins can enter the brain, accumulate and form
24 xanthomas.

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6 **Disclosures**

7 None.

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Figure Legends

Figure 1. Increased BBB/BCSFB permeability in ApoE^{-/-}Fbn1^{C1039G+/-} mice. (A) After 14 weeks on WD, a significant Evans blue leakage is observed in brains of ApoE^{-/-}Fbn1^{C1039G+/-} compared to ApoE^{-/-} mice (spectrophotometric quantification in the right hand panel). (B) After 30 weeks on WD, ApoE^{-/-}Fbn1^{C1039G+/-} mice show impressive lesions in large areas of (neo)cortex (Co) and brain ventricles (V), which are associated with extensive Evans blue leakage, pointing toward a breakdown of the BBB/BCSFB. Different gradients of Evans blue are observed in the lesions (n=4 per group, P=0.018, unpaired Student's t-test). Spectrophotometric quantification of Evans blue at 30 weeks could not be performed due to the large presence of lesions (lipids), interfering with the measurement. (C) Gadolinium (Gd)-enhanced MRI at 21 weeks on WD showed an increased drop in delta T₁ values in ApoE^{-/-}Fbn1^{C1039G+/-} mice (red) compared to ApoE^{-/-} (blue) and WT control mice (black). Data from two different mice, representative of each group, were shown. Gd leakage was more pronounced in the neocortex of ApoE^{-/-}Fbn1^{C1039G+/-} mice and peaked in the presence of a lesion (white circle). Right hand panel: upper T₂ and lower T₁-weighted image.

Figure 2. Xanthomas in the brain of ApoE^{-/-}Fbn1^{C1039G+/-} mice. (A-C) MRI of the brain of an ApoE^{-/-}Fbn1^{C1039G+/-} mouse on WD for 17 weeks (axial (A), sagittal (B) and coronal (C) plane). Lesions are encircled in white. (D-F) Histological evaluation of the lesion detected by MRI. Brain lesions are composed of cholesterol clefts (asterisks) and foam cells (arrowheads), typical of xanthomas. (E) Magnification of box in (D); (F) magnification of box in (E). Scale bar=1mm (D) and 50μm (E, F).

Figure 3. Xanthomas in ApoE^{-/-}Fbn1^{C1039G+/-} mice are mainly observed in the brain ventricles and in the neocortex, as shown by MRI. (A) lateral ventricle (B) third ventricle (C) fourth ventricle and (D) neocortex.

Figure 4. Xanthomas increase in size over time and do not induce brain hypoxia. (A) Longitudinal MRI study of the brain of an ApoE^{-/-}Fbn1^{C1039G+/-} mouse on WD for up to 30 weeks. Lesions were detected after 16 weeks on WD and increased exponentially in size over time (R²=0.98). (B) Quantification of xanthoma growth in the brain of mouse in (A). Weeks 16-28 on WD *in vivo* and high resolution *post mortem* MRI at 30 weeks on WD. (C) Two examples of a TTC stain of brains of ApoE^{-/-}Fbn1^{C1039G+/-} mice showing a xanthoma (boxed area) in the neocortex. Hypoxia was absent in the proximity of the xanthomas. Scale bar=200µm.

Figure 5. Fibrillin-1 is expressed in the choroid plexus and capillaries of the neocortex (20 weeks on WD), corresponding with the capillary basement membrane. (A) Brain sections of ApoE^{-/-}Fbn1^{C1039G+/-} mice showing presence of fibrillin-1 in the choroid plexus of the lateral and third ventricles (left hand panel) and capillaries of the neocortex (right hand panel). (B) Fibrillin-1 stain showing that in the presence of a xanthoma, the choroid plexus appears damaged and fenestrated (arrowheads in the high magnification image) or even completely disappears (dashed line), allowing the growth of the lesion into the parenchyma (xanthomas are indicated with dotted lines, foam cells and cholesterol clefts with asterisks). (C) H&E stain showing hemorrhage (arrowheads) and small blood vessels in a xanthoma in the lateral ventricle (left hand and middle panel) and neocortex (right hand panel), respectively. (D) Early development of a xanthoma in the choroid plexus. Foam cells (asterisks) are observed around a (capillary) vessel (V) in the choroid plexus, damaging the epithelium (arrowheads, H&E stain, left hand panel). The right hand panel shows the extravasation of foam cells adjacent to a blood vessel in the neocortex (PAS stain). (E) PAS stain showing an organized (ApoE^{-/-}, left hand panel) or damaged (ApoE^{-/-}Fbn1^{C1039G+/-}, right hand panel) structure of the choroid plexus. The basement membrane (arrowheads) appears to be thicker in ApoE^{-/-}

Fbn1^{C1039G+/-} compared to ApoE^{-/-} mice. Scale bar=50μm (high magnification images in A and C, D, E), 200μm (B, C) and 1mm (low magnification brain images in A, B).

Figure 6. Morphological changes in the choroid plexus of ApoE^{-/-}Fbn1^{C1039G+/-} mice (20 weeks on WD) on TEM. In contrast to the adjacent and cubic epithelial cells of ApoE^{-/-} mice (A), the epithelial cells of ApoE^{-/-}Fbn1^{C1039G+/-} mice appeared flattened and detached and showed ‘stretched’ microvilli at their surface (B). In ApoE^{-/-}Fbn1^{C1039G+/-} mice, leukocytes (L) were often observed adherent to the choroid plexus endothelium or even migrated into the choroid plexus parenchyma, having crossed the fenestrated endothelium and basement membrane (B). (C, D) Detail of the microvilli on the epithelial cells in (A, B). The microvilli on the epithelial cells of ApoE^{-/-}Fbn1^{C1039G+/-} mice appeared less dense and stretched compared to those of ApoE^{-/-} mice. (E, F) Detail of the basement membrane (arrowheads, BM) in an ApoE^{-/-} (E) and an ApoE^{-/-}Fbn1^{C1039G+/-} mouse (F). Ep=epithelial cell, Mv=microvilli, Lu=lumen of blood capillary, L=leukocyte, EC=endothelium, BM=basement membrane. Scale bar=1μm.

Figure 7. Expression of adhesion molecules, cytokines, matrix degrading enzymes and tight junction proteins in the choroid plexus. (A) Expression of the adhesion molecules VCAM-1 and ICAM-1 is enhanced in the choroid plexus of ApoE^{-/-}Fbn1^{C1039G+/-} mice as compared to ApoE^{-/-} mice after 14 weeks on WD, indicating activated endothelium (A= ApoE^{-/-}, F= ApoE^{-/-}Fbn1^{C1039G+/-}, X= choroid plexus with xanthomas). Furthermore, expression of IL-1β, TNF-α, TGF-β, active MMP-2 and -9 is significantly increased in the choroid plexus of ApoE^{-/-}Fbn1^{C1039G+/-} mice compared to their ApoE^{-/-} littermates, particularly in the presence of a xanthoma (X). Importantly, these cytokines (with the exception of TGF-β) and matrix-degrading enzymes were already higher in ApoE^{-/-}Fbn1^{C1039G+/-} after 14 weeks on WD as compared to age-matched ApoE^{-/-} mice, pointing toward an earlier onset of inflammation. (B) Expression of the tight junction proteins occludin and claudin-5 is diminished in ApoE^{-/-}

Fbn1^{C1039G+/-} compared to ApoE^{-/-} mice, confirming BBB breakdown. As far as basement membrane components are concerned, collagen IV remained unchanged and laminin was slightly increased. ****P*<0.001, ***P*<0.01 vs. ApoE^{-/-}Fbn1^{C1039G+/-} with xanthoma (One-way ANOVA followed by Bonferroni's post-hoc test) and \$\$\$*P*<0.001; \$\$*P*<0.01; \$*P*<0.05 ApoE^{-/-}Fbn1^{C1039G+/-} vs. ApoE^{-/-} at the same age (Two-way ANOVA followed by Tukey's post-hoc test). X= choroid plexus with xanthomas.

Figure 8. Mechanism of accelerated xanthoma formation in the brain of ApoE^{-/-}Fbn1^{C1039G+/-} mice. The ApoE^{-/-} background together with a WD initiates BBB and BCSFB degradation. The BBB damage at the level of the capillaries and the BCSFB damage at the choroid plexus facilitate the entry of leukocytes and lipoproteins in the brain, promoting the development of xanthomas in the brain parenchyma and brain ventricles, respectively. The fibrillin-1 mutation potentiates xanthoma formation at three different levels: (1) by causing a decreased assembly of the microfibrils in the basement membrane, making it more loose, (2) by accelerating peripheral inflammation (atherosclerosis), stimulating the entry of leukocytes in the brain and (3) by increasing the (local) expression of the inflammation regulator TGF-β, promoting local inflammation and matrix degradation. In addition, due to the severe damage of the choroid plexus in ApoE^{-/-}Fbn1^{C1039G+/-} mice, CSF may be enriched with lipoproteins and activated leukocytes, leading to xanthoma formation in the parenchyma of e.g. the olfactory bulb, brain stem and cerebellum (dashed line).

TABLES

Table 1. Characteristics of xanthomas in the brain of ApoE^{-/-} and ApoE^{-/-}Fbn1^{C1039G+/-} mice after 20 weeks on Western-type (WD) or normal diet (ND), as evaluated by MRI.

Xanthomas in the brain	ApoE ^{-/-} WD, n=40	ApoE ^{-/-} Fbn1 ^{C1039G+/-} WD, n=54	ApoE ^{-/-} Fbn1 ^{C1039G+/-} ND, n=20	<i>P</i> value
Percentage of mice	23	83	0	<i>P</i> <0.001
Number per mouse	0 [0-7]	1 [0-10]	n.a.	0.0048
Xanthoma size (μm ³)	21±5	43±7	n.a.	0.016

The percentage of mice with xanthomas in the brain was analyzed with the Chi square test. The number of xanthomas per mouse (median [min.-max.]) was evaluated with the Mann-Whitney test. Xanthoma size was compared between groups using an unpaired Student's t-test. n.a.= not applicable.

Table 2. Localization of xanthomas in the brain of ApoE^{-/-} and ApoE^{-/-}Fbn1^{C1039G+/-} mice on a Western-type diet for 20 weeks, as detected by MRI. n=number of xanthomas evaluated.

Localization (%)	ApoE ^{-/-} (n=22)	ApoE ^{-/-} Fbn1 ^{C1039G+/-} (n=84)
Neocortex	70	51
Brain ventricles lateral	9	16
third	0	8
fourth	2	4
Olfactory bulb	9	5
Brain stem	0	6
Cerebellum	4	3
Other	6	7

Figure 1
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