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Bink, D.I.

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Neuropathology in mouse models of atherosclerosis

Diewertje I. Bink, Katja Ritz, Claire Mackaaij, Artem Khmelinskii, Onno J. de Boer, Lobke M. Gierman, Judith C. Sluimer, Louise van der Weerd, Mat J.A.P. Daemen

Submitted
Abstract

Atherosclerosis is associated with white matter lesions and brain atrophy. While atherosclerosis has been studied extensively in mice, there is only little information on the neuropathological changes. It is also unknown whether atherosclerosis plays a role in the development of neuropathological changes. We therefore compared the volume of the whole brain and six anatomical brain structures in one year old ApoE<sup>−/−</sup> versus C57Bl/6 mice and in one year old ApoE<sup>∗3L</sup>.CETP mice on high-cholesterol diet (HCD) versus chow diet using MRI and performed histological analysis of the brains. Aged ApoE<sup>−/−</sup> mice showed an increased cerebellar volume and decreased volume of the (hypo)thalamus compared to C57Bl/6 mice. Tight junction marker claudin-5 was decreased in the cortex and hippocampus, coinciding with increased blood-brain barrier leakage and decreased white matter myelin basic protein. Microglial activation was absent. Although ApoE<sup>∗3L</sup>.CETP mice on HCD had severe aortic atherosclerotic lesions, brain volumes, tight junctions, blood-brain barrier leakage and Iba-1 expression were not different from ApoE<sup>∗3L</sup>.CETP mice on chow. The discrepancy in the presence of microvascular changes between the severe atherosclerotic ApoE<sup>−/−</sup> and ApoE<sup>∗3L</sup>.CETP models could indicate a more significant role for the lack of the ApoE gene on cerebral microvascular changes in ApoE<sup>−/−</sup> mice compared to the effect of atherosclerosis.
Introduction

Peripheral atherosclerosis, vascular calcification and increased intima-media thickness have been related to smaller brain volumes, white matter lesions and lower cognition scores.\textsuperscript{1-10} Suitable animal models may importantly contribute to unravel the mechanisms of neuropathological changes induced by peripheral atherosclerosis. Whereas the extent, severity and molecular regulation of atherosclerosis in large extracranial arteries has been studied in great detail in a wide array of animal models, the data on the association of atherosclerosis with neuropathological changes in mice are scarce. Apolipoprotein E knockout (ApoE\textsuperscript{-/-}) mice show increased cerebral inflammation, increased blood-brain barrier (BBB) permeability, microvessel degeneration, reduction of neurogenesis and memory impairment compared to C57Bl/6 mice.\textsuperscript{11} These effects are stronger and in some cases only seen when mice were fed a high-fat diet (HFD). At the age of 2-5 months the volumes of the dorsal hippocampus, forebrain and ventricles measured with MRI do not differ between ApoE\textsuperscript{-/-} mice and C57Bl/6 mice.\textsuperscript{12} It is unknown if volume differences appear at a later age, when the atherosclerotic burden increases. ApoE\textsuperscript{-/-} mice show extensive extracranial atherosclerosis, yet the presence or subtype of the ApoE gene may have a bigger influence on these brain changes than the effect of the extracranial atherosclerotic lesions, as it has been shown that differences in ApoE isoforms affect BBB permeability.\textsuperscript{13-15}

Another widely used atherogenic mouse model, the LDLr\textsuperscript{-/-} mouse, also shows increased cerebral inflammation, microvascular changes and memory impairment.\textsuperscript{11} In addition, several ApoE-isotype specific or crossbred transgenic mouse models have been created, which are thought to be more representative of human atherosclerotic disease. Apolipoprotein E*3Leiden (ApoE*3L) mice are such a model and these mice contain a construct of the human ApoE3 gene with the ApoE*3-Leiden mutation and is associated with a dominantly inherited form of familial dysbetalipoproteinemia.\textsuperscript{16} In contrast to ApoE\textsuperscript{-/-} mice, ApoE*3L mice on a HFD show normal BBB function at 10.5 months of age, although they have a similar degree of extracranial atherosclerosis compared to ApoE\textsuperscript{-/-} mice.\textsuperscript{14} This ApoE*3L mouse has been crossbred with a human cholesteryl ester transfer protein (CETP) knockin mouse.\textsuperscript{17} CETP is a protein that is part of high density lipoprotein and normally not present in mice. ApoE*3L.CETP mice have increased cholesterol and triglyceride levels and more advanced atherosclerosis compared to ApoE*3L mice.\textsuperscript{17} No data on cerebral pathology of ApoE*3L.CETP mice have been published so far. Although the absence of the ApoE protein in itself is known to cause neuropathological changes, we hypothesized that
severe extracranial atherosclerosis will also contribute to the development of these neuropathological changes. To test this hypothesis we investigated the brain volumes and the neuropathological changes with high field MRI and histological analysis in aged ApoE<sup>−/−</sup> and ApoE*3L.CETP mice.

**Materials and Methods**

**Animals**

Heads of fifty-two weeks old female ApoE<sup>−/−</sup> (n=13) and C57Bl/6 mice (n=10) receiving a standard chow diet were included in the study. In addition, heads of 53–59 weeks old female ApoE*3L.CETP mice (n=24), originally bred on a C57Bl/6 background, undergoing an *in vivo* experimental procedure as previously described by Gierman et al. (TNO Metabolic Health Research) were included. In short, mice were fed standard lab chow (V1534 Sniff Spezialdiäten GmbH, Soest, Germany) until the start of the study at the age of 10–16 weeks. Twelve mice received a semisynthetic Western-type diet (AB diets, Woerden, the Netherlands) supplemented with 0.1% (w/w) cholesterol for an initial 4 weeks and were then switched to a Western-type diet containing 0.3% (w/w) cholesterol (high-cholesterol diet, HCD group). Twelve mice remained on chow. Female mice were chosen because they have higher cholesterol levels compared to male mice undergoing the same *in vivo* experimental procedure (unpublished data) and female ApoE*3L mice develop larger atherosclerotic lesions with less cholesterol exposure compared to male mice.

At the end of the study all mice were perfused transcardially and the heads were stripped from the skin. They were placed in 1:40 Gd-DOTA in 4% PFA for 6 months (ApoE*3L.CETP) or a week in 4% PFA and subsequently in 1:40 Gd-DOTA in PBS for 6 weeks (ApoE<sup>−/−</sup> and C57Bl/6) to increase MRI contrast. Results of the ApoE*3L.CETP groups cannot be directly compared to the ApoE<sup>−/−</sup> or C57Bl/6 mice, due to differences in formalin fixation time and due to that differences in incubation time of antibodies. Experiments were approved by the institutional Animal Care and Use Committee of TNO, LUMC and Maastricht University and were performed in accordance with national and European regulations.

**MRI**

MRI experiments were conducted on a 7T MRI system (Pharmascan, Bruker, Ettlingen, Germany) with a circular polarized MRI transceiver coil for 1H with an inner diameter of 23 mm and Paravision 5.1 software (Bruker, Ettlingen, Germany).
Ex vivo MRI on mouse skulls was performed after 6 weeks or 6 months incubation with Dotarem (1: 40, 0.5 mmol/ml, Guerbet, Nederland). The skulls were placed in Fomblin (perfluoro polyether, Solvay Solexis, Italy) during the scan. A T1 3D Flash sequence was used to image the brain with the following parameters: TR 15 ms, TE 5.3 ms, NA 12, matrix 256x186x186, FOV 18x18x13 cm, hermite FA 30°, resolution 70 µm/pixel, total scan time 58min48s.

Image registration and VOIs analysis
The registration scheme included registration of each subject brain to a template brain compiled of n=18 C57Bl/6 mice. Volumes of interest (VOIs) for the whole brain and 22 selected anatomical structures were manually segmented based on the Waxholm mouse brain atlas, the Franklin and Paxinos atlas and the Allen Brain Atlas using AMIRA (v5, FEI Software, Oregon, USA). The whole brain volume was defined as the brain tissue limited caudally by the cerebellum and rostrally by the rhinal fissure, as demonstrated in the sagittal section in figure 1A. Using the information provided by the inverse deformation field for each subject-to-template registration, the template VOIs were propagated to the individual datasets, enabling quantitative comparison of corresponding areas (figure 1B-C). The VOIs were evaluated quantitatively for volume change without manual adjustments. The quality and success of the registration was verified by two independent observers by visual inspection using a custom-made graphic user interface built with MeVisLab (v2.5.1, MeVis Medical Solutions AG, Bremen, Germany).

The registration was implemented using the open source image registration toolbox Elastix. Registration was performed in a coarse-to-fine process. Initially, rigid registration was performed to compensate for translation and rotation. Afterwards, an affine registration was conducted to compensate for differences in brain size, followed by a non-rigid B-spline registration to compensate for local changes. A Gaussian image pyramid was employed in all registration steps, applying four resolutions for the rigid and B-spline and two for the affine registration. Mutual information was used as a similarity metric. Detailed information on the used registration parameters can be found at the Elastix website (http://elastix.bigr.nl/wiki/index.php/Par0033). To take into account imprecisions in the segmentation of certain structures due to low contrast differences, some structures were merged based on their functional connectivity. Structures with acceptable segmentation alone or as functional units were statistically tested for significant differences in absolute volumes. These included whole brain, basal ganglia (globus pallidus, caudate putamen and substantia nigra), cerebellum,
colliculus (superior and inferior colliculus), (hypo)thalamus (combined thalamus and hypothalamus), cortex, and hippocampus (figure 1C).

**Histology and immunohistochemistry**

After the *ex vivo* MRI scan the brains (n=10 per group) were split coronally in three parts, processed and embedded in paraffin. The dorsal hippocampal area was cut into serial 5 µm sections. Dorsal hippocampal sections of 4 bregma levels were used for each staining between bregma -1.3 mm and -2.4 mm. Brain structures present on the slides include the cortex, corpus callosum, dorsal hippocampus, caudal amygdala, thalamus, hypothalamus, optical nerve, fimbria, caudal caudate putamen and internal capsule. Haematoxylin & Eosin (HE) staining was used for identification of microinfarcts and –bleedings and scored blinded. Luxol fast blue staining was used to identify white matter damage. Congo red staining was used to identify amyloid beta (Aβ) fibrillary depositions. Immunohistochemistry was performed with the antibodies listed in table 1. Whole brain slides were screened for microgliosis (Ionized calcium-binding adapter molecule 1, Iba-1) or astrogliosis (Glial fibrillary acidic protein, GFAP).

**Image analysis**

Areas used for image analysis are listed in table 1. Ki67 positive cells were counted in the subgranular zone of the dentate gyrus (DG) and expressed as number of Ki67 positive cells per section. Photomicrographs (20x objective) from the left hippocampus and dorsal medial cortex were taken of GFAP, Iba-1, CD31 and claudin-5 stainings using a Leica DM5000B microscope equipped with a DFC500 camera (Leica, Germany) and LAS v4.5 software. Aβ positivity was screened throughout the entire brain section and the number of plaques per section was counted. Immunoglobulin G (IgG), Intercellular Adhesion Molecule 1 (ICAM-1) and myelin basic protein (MBP) expression was analyzed on whole slide images which were scanned with an Olympus scanning microscope (Olympus dotSlide, Tokyo, Japan). For ICAM-1 analysis, a region of interest (ROI) of the medial dorsal hippocampus including the border between the hippocampus and thalamus was selected, because this area is important for memory and showed the most differences on visual inspection. Image analysis was performed with Image Pro Premier 9 (Media Cybernetics, Rockville, USA). Immunopositive areas were segmented using the ‘smart segmentation’ option, and used to determine the positive staining area (%) and the mean optical density (OD).
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Statistical analysis

Data are presented as mean ± standard deviation. SPSS 21 (SPSS, Inc., an IBM Company; Chicago, IL) was used to test normality with the Independent-Samples Kolmogorov-Smirnov test. Because of the low animal number and absence of normality in some measurements, statistical differences (p<0.05) between variables were calculated using a Mann-Whitney U test with Graphpad Prism 5. Ex vivo MRI analyses were adjusted for multiple comparisons using the Bonferroni-correction (p=0.05/7=0.007). Outliers were detected with the Grubbs test (p<0.05).

Results

Brain volumes by 7T MRI

Total brain volume of the one year old ApoE−/− mice was comparable to that of the wild-type C57Bl/6 mice (see also table 2). Also the volume of the hippocampus,
cortex, colliculus and basal ganglia was not different. However, the volume of the cerebellum of ApoE-/- mice was 14% larger, while the volume of the (hypo)thalamus was 5% smaller compared to C57Bl/6 mice (table 2). This decrease could not be explained by increased neuronal apoptosis, since staining for caspase-3 was nearly negative in all mice (data not shown).

No differences in total brain volume or volumes of any of the brain structures were found in one year old HCD fed ApoE*3L.CETP mice compared to ApoE*3L.CETP mice on chow (table 2). In addition to volume changes, brain scans were screened for gross abnormalities. No gross abnormalities or hyperintensities were seen in any of the brains with the T1-weighted images.

**Histological cerebral changes**

For histology, the dorsal hippocampal area was investigated because of the known role of the hippocampus in memory formation, and the memory impairment and hippocampal changes reported in ApoE-/- mice.\textsuperscript{13, 26-29} Four coronal sections per animal were entirely analyzed for the presence of gross brain abnormalities, (micro)infarcts and -bleedings encompassing the corpus callosum, optical nerve, caudal caudate putamen and internal capsule, hippocampus, cortex, thalamus and hypothalamus. No
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such lesions were found, except for a cortical microinfarct with scarring in the dorsal cortex of one ApoE−/− mouse (figure 2).

White matter integrity was measured by MBP positive area and intensity throughout the whole coronal section, including the corpus callosum, optical nerve, caudate putamen and fimbria. The MBP positive area was significantly reduced in ApoE−/− compared to C57Bl/6 mice, while the intensity measured with the OD remained similar between the two groups (percent area 8.53±2.70% vs 13.78±4.74%, p=0.017, OD 0.123±0.004 vs 0.129±0.005, p=0.055, figure 3A-C). HCD fed ApoE*3L.CETP mice showed a significant increased amount of MBP both in percent area and intensity compared to chow fed animals (percent area HCD 14.59±2.49% vs Chow}

<table>
<thead>
<tr>
<th>Regions of interest (mm³)</th>
<th>ApoE−/−</th>
<th>C57Bl/6</th>
<th>p-value</th>
<th>ApoE*3L.CETP HCD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total brain</td>
<td>513±14</td>
<td>506±12</td>
<td>0.256</td>
<td>491±13</td>
<td>0.751</td>
</tr>
<tr>
<td>Basal ganglia</td>
<td>28±1</td>
<td>29±1</td>
<td>0.019</td>
<td>26±1</td>
<td>0.751</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>63±2</td>
<td>55±2</td>
<td>0.000*</td>
<td>53±2</td>
<td>0.215</td>
</tr>
<tr>
<td>Cortex</td>
<td>150±5</td>
<td>148±3</td>
<td>0.463</td>
<td>144±6</td>
<td>0.686</td>
</tr>
<tr>
<td>Colliculus</td>
<td>16±1</td>
<td>16±1</td>
<td>0.062</td>
<td>16±1</td>
<td>0.708</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>26±2</td>
<td>26±1</td>
<td>1.000</td>
<td>25±1</td>
<td>0.751</td>
</tr>
<tr>
<td>(Hypothalamus)</td>
<td>53±1</td>
<td>56±2</td>
<td>0.001*</td>
<td>55±2</td>
<td>0.471</td>
</tr>
</tbody>
</table>

HCD: High-cholesterol diet. Values are presented as mean ± standard deviation. * p<0.0071 is significant (p<0.05 with Bonferroni-correction for multiple comparisons).

Table 2. Volumes of the different brain structures as measured with MRI

Figure 2. A cortical infarct was identified in one of the ApoE−/− mice. An overview of the HE staining of the cortex is shown in (A)(2.5x) and in more detail (10x) the HE (B), GFAP (C) and Iba-1 (D) stainings are shown.
9.65±2.58%, p=0.001; OD 0.153±0.008 vs 0.141±0.003, p=0.004, figure 4A-C). Despite the different intensity of MBP staining between the ApoE<sup>−/−</sup> and C57Bl/6 mice and between the ApoE*3L.CETP mice on HCD and chow, the morphology of the myelinated areas and individual myelinated fibres was not different in the MBP and Luxol fast blue staining. This indicates that although changes occurred at the molecular level, the white matter integrity was still intact.

**Vascular leakage and activation**

The vascular density in CD31 stained sections was measured in the hippocampus and cortex, areas which are well-known for their importance in spatial and working memory, and was similar in ApoE<sup>−/−</sup> mice compared to C57Bl/6 mice (data not shown). The BBB appeared to be compromised in ApoE<sup>−/−</sup> mice based on a significant decrease in the tight junction marker claudin-5 (OD Cortex: 0.134±0.006 vs 0.147±0.006, p<0.001; DG: 0.137±0.005 vs 0.150±0.006, p<0.001, figure 3G-I). Additionally, an almost 300% increase in IgG leakage was observed throughout the whole coronal brain section (percent area 0.56±0.56 vs 1.66±1.06%, p=0.035, figure 3D-F). Semi-quantitative grading of the IgG staining performed by two blinded observers, to test the accuracy of the automated measurements, showed similar results (data not shown). IgG leakage was most pronounced in the fimbria, optical nerve and hippocampus.

ApoE<sup>−/−</sup> mice showed a significant increase in the intensity of ICAM-1 staining in the hippocampal area, while the positive area remained similar, indicating elevated endothelial activation compared to C57Bl/6 mice (OD 0.166±0.007 vs 0.155±0.005, p=0.001, figure 3J-L). ICAM-1 positivity was most pronounced in the arterioles in the hippocampal fissure and the border between the hippocampus and thalamus.

In contrast to ApoE<sup>−/−</sup> mice, HCD fed ApoE*3L.CETP mice did not show evidence of a compromised BBB. Vascular density (CD31) and claudin-5 positive tight junctions were not decreased in ApoE*3L.CETP mice receiving HCD compared to chow diet (claudin-5 OD cortex: 0.134±0.006 vs 0.131±0.005, p=0.315; DG: 0.149±0.020 vs 0.138±0.006, p=0.089, figure 4G-I), which corresponded with the presence of an intact barrier function as IgG positive areas were not different (percent area 1.28±1.09 vs 0.79±0.98%, p=0.218, figure 4D-F). However, ICAM-1 density in the vessels in and around the hippocampus was increased in HCD fed mice compared to chow fed mice (OD 0.179±0.006 vs 0.160±0.008, p<0.001, figure 4J-L), which is suggestive for increased endothelial activation.
Figure 3. Histological cerebral changes in ApoE−/− mice. Representative images and statistics are shown of white matter marker MBP (A-C), BBB leakage marker IgG (D-F), tight junction marker claudin-5 (G-I) and endothelial activation marker ICAM-1 (J-L) in ApoE−/− mice (B,E,H,K) compared to C57Bl/6 mice (A,D,G,J). * p<0.05.

Figure 4. Histological cerebral changes in ApoE*3L.CETP mice on HCD. Representative images and statistics are shown of white matter marker MBP (A-C), BBB leakage marker IgG (D-F), tight junction marker claudin-5 (G-I) and endothelial activation marker ICAM-1 (J-L) analyses in ApoE*3L.CETP chow fed mice (A, D, G, J) compared to HCD mice (B, E, H, K). * p<0.05.
Astrogliosis and microglial activation in the hippocampus and cortex

The astrocyte marker GFAP and the microglia marker Iba-1 were used to investigate astrogliosis and microglial activation. No evidence for microgliosis or astrogliosis was found in the dorsal hippocampal area, including the corpus callosum, optic nerve, caudal caudate putamen and internal capsule, hippocampus, cortex, thalamus and hypothalamus, as observed by the absence of activated amoeboid microglia and astrocytes in any of the animals. As mentioned above, only one ApoE−/− mouse exhibited a cortical lesion, which contained activated microglia and reactive astrocytes (figure 2C-D). In addition, activation of astrocytes and microglia in the gray matter was studied in the cortex and hippocampus, important areas for memory formation. There was no difference in GFAP between ApoE−/− and C57Bl/6 mice (OD Cortex: 0.116±0.003 vs 0.114±0.005, p=0.353; DG: 0.132±0.003 vs 0.133±0.005, p=0.631), nor between HCD and chow fed ApoE*3L.CETP mice (OD Cortex: 0.108±0.003 vs 0.109±0.002, p=0.604; DG: 0.125±0.002 vs 0.127±0.003, p=0.156). A subtle, though significant increase in Iba-1 intensity was found in the cortex of ApoE−/− mice compared to C57Bl/6 mice (OD Cortex: 0.174±0.05 vs 0.165±0.014, p=0.003), but not in the hippocampus (DG: 0.134±0.006 vs 0.141±0.003, p=0.436). No differences in Iba-1 staining intensity were measured between the HCD and chow fed ApoE*3L.CETP mice (Iba-1: Cortex: 0.117±0.004 vs 0.119±0.006, p=0.315; DG: 0.114±0.006 vs 0.115±0.003, p=0.579).

Alzheimer pathology and neurogenesis

No Aβ plaques were detected in any of the mouse brains using Congo red or Aβ antibody staining.

The number of proliferating neurons in the subgranular zone of the DG as measured with a Ki67 staining did not differ between ApoE−/− and C57Bl/6 mice or between ApoE*3L.CETP HCD fed mice compared to chow fed mice (data not shown).

Discussion

There is increasing evidence in human disease for a link between peripheral atherosclerosis and neuropathological changes,1, 5, 6, 8, 9 and an increased risk of cognitive decline.1-4, 32-37 However, there is limited information on such a link in animal models of atherosclerosis, while animal models are crucial to study the mechanisms behind the observed associations in humans. In this pilot study we
show that aged female ApoE⁻/⁻ mice on a chow diet showed several neuropathological changes, like increased BBB leakage, increased endothelial activation, white matter changes, an increased cerebellar volume and a decrease in the volume of the (hypo)thalamus. In contrast to our hypothesis, ApoE*3L.CETP mice on HCD showed only very limited neuropathological changes when compared to chow fed ApoE*3L.CETP mice, such as an increase in the endothelial activation marker ICAM-1 and an increase in MBP, without differences in white matter morphology. Microglial activation and Aβ plaques were absent in all investigated mice, the latter indicating a lack of increased Alzheimer’s disease pathology.

The ApoE⁻/⁻ mouse model is one of the most extensively used animal models for atherosclerotic research⁵⁸, ³⁹ and shows extensive extracranial atherosclerosis, but no intracranial atherosclerosis.⁴⁰⁻⁴² However, the presence of the extracranial atherosclerotic lesions might not be causing the microvascular neuropathological changes in ApoE⁻/⁻ mice. The subtype of the ApoE gene might have a bigger influence on these changes.¹³⁻¹⁵ The ApoE protein is involved in lipid transport and in the brain it is produced by glial cells.⁴³ The protein is also involved in Aβ accumulation in the brain, an important hallmark of Alzheimer’s disease.⁴³ The importance of the ApoE gene on brain function is frequently shown by differences between ApoE-isoform carriers. The different isoforms of the ApoE gene are associated with differences in the risk for developing Alzheimer’s disease and can influence BBB permeability, cerebral blood flow (CBF), synaptic function, dendritic spine density, neuronal integrity, oxidative stress levels, glial activation, anxiety and cognition.¹³, ⁴³⁻⁵⁰ The ApoE protein is thought to be important in the maintenance of cerebrovascular integrity, which is necessary for normal neuronal function, in an isoform-specific manner by regulating the CypA–NF-κB–MMP9 pathway.¹³ The cerebral changes observed in atherosclerotic LDLr⁻/⁻ mice might also be influenced by ApoE protein function instead of atherosclerotic load, since the absence of the LDLr gene significantly changes cerebral ApoE mRNA and protein levels.⁵¹⁻⁵³ This may explain the very limited neuropathological changes observed in the ApoE*3L.CETP mice, despite their high cholesterol levels and severe atherosclerotic lesions. The ApoE*3L.CETP mice on HCD used in this study had a 5.6 times higher cholesterol exposure, a 91.8 times larger total atherosclerotic lesion area and more severe lesions in the aortic root area compared to chow fed mice.¹⁸ Despite the high cholesterol levels, large atherosclerotic lesions and an increase in the expression of plasma inflammation markers,¹⁸ we observed no increase in activated astrocytes and
microglia or BBB leakage in these mice. We however did find increased BBB leakage in ApoE\textsuperscript{−/−} mice as shown with IgG in combination with reduced tight junctions as shown with claudin-5, confirming literature\textsuperscript{13, 14, 54-58}.

It is unlikely that the differences in these microvascular neuropathological findings between the ApoE\textsuperscript{−/−} versus C57Bl/6 mice and ApoE*3L.CETP HCD versus chow mice can be explained by serum cholesterol level dissimilarities. The cholesterol levels in the HCD fed ApoE*3L.CETP were similar to cholesterol levels reported in literature in ApoE\textsuperscript{−/−} mice on chow, and chow fed ApoE*3L.CETP mice had comparable levels to C57Bl/6 mice\textsuperscript{18, 59} ApoE\textsuperscript{−/−} mice on chow and ApoE*3.CETP mice on HCD both have advanced atherosclerotic lesions in the aortic roots at the age of 12-13 months\textsuperscript{18, 60}, indicating that there is no link between the extent of extracranial atherosclerosis and the observed cerebrovascular pathology in the ApoE\textsuperscript{−/−} mice. The neuropathological changes in ApoE\textsuperscript{−/−} mice are also not likely the effect of cerebral macrovascular changes, since ApoE\textsuperscript{−/−} mice do not show intracranial atherosclerosis\textsuperscript{40-42}.

The neuropathological changes could be a result of detrimental effects of reduced cerebral perfusion due to the presence of extracranial atherosclerosis. Regional CBF reductions determined by \textsuperscript{14}C-iodoantipyrine autoradiography in ApoE\textsuperscript{−/−} mice on chow have been reported\textsuperscript{13}. These CBF reductions were however correlated with reductions in microvascular length and are therefore more likely to result from microvascular changes than from systemic effects. All things considered, it is very likely that observed microvascular changes in ApoE\textsuperscript{−/−} mice are due to the absence of the ApoE protein instead of intracranial or extracranial atherosclerotic burden.

We did not find evidence for microgliosis or increases in GFAP expression in our 12 month old ApoE\textsuperscript{−/−} mice, which is in contrast to the observations by Badaut et al. who observed a significant increase in GFAP expression in the cortex of 14 months old ApoE\textsuperscript{−/−} compared to C57Bl/6 mice\textsuperscript{58}. The type of diet is known to effect inflammation load and endothelial activation\textsuperscript{61} and probably contributes to the observed differences between the two studies. The ApoE\textsuperscript{−/−} mice in our study were fed a chow diet, while the ApoE\textsuperscript{−/−} mice in the Badaut et al. study were fed a Western-type diet. In addition, the two months age difference may account for some of the discrepancy. Gender may also influence plaque load and endothelial function\textsuperscript{62-66}. However, the gender of the mice used in the Badaut et al. study has not been specified.

Although in humans the presence of peripheral atherosclerosis is associated with a decrease in total brain volume\textsuperscript{1, 2} we did not find a difference in total brain volume in ApoE\textsuperscript{−/−} mice compared to C57Bl/6 mice at the age of one year. The MRI data in our study show a 14% increase in cerebellar volume and a small yet significant decrease
of 5% in the volume of the (hypo)thalamus in ApoE−/− mice at the age of 12 months. The thalamus is thought to be a relay between the cortex and subcortical structures. Degeneration of this structure might lead to a distortion in neuronal information processing and cognitive functioning.67-70 Brain volume changes were absent in the ApoE*3L.CETP mice on HCD.

CETP polymorphisms are linked to the risk of Alzheimer’s disease, vascular dementia and white matter lesions development.71-77 However, in contrast to humans, the CETP knockin mouse model lacks CETP expression in the brain.17, 78 Therefore, mouse brains in our study were probably unaffected by direct cerebral effects of CETP.

In conclusion, aged female ApoE−/− mice on normal chow showed microvascular neuropathological changes, while ApoE*3L.CETP mice lack neuropathological changes. We did not find any evidence in this study for a role of extracranial atherosclerosis in the development of neuropathological changes in atherosclerotic mouse models. The most commonly used atherosclerotic model, the ApoE−/− model, is not a good model to explore the link between atherosclerosis and neuropathy due to the direct effect of the ApoE protein on for example the BBB. On the other hand, we cannot exclude that the ApoE*3L.CETP or other human ApoE expressing models may still be useful models to study the observed associations in humans between atherosclerosis and neuropathy. However, the present study suggests that at the age of 12 months the model is still a too mild atherosclerotic model to induce neuropathy. Further research in humanized mouse models with severe atherosclerosis is needed with larger numbers of mice and correlations between plaque severity, CBF and neuropathology to establish whether there is a link between the amount of peripheral atherosclerosis and pathological changes in the brain.

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Conflict of Interest
The authors declare no conflict of interest.
CHAPTER 5

References


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