Vascular factors in dementia: prevention and pathology
Richard, E.

Citation for published version (APA):
Cortical capillary density in Alzheimer’s disease

E. Richard
W.A. van Gool
JJ.M. Hoozemans
E.S. van Haastert
P. Eikelenboom
A.J.M. Rozemuller
W.D.J. van de Berg

submitted
Abstract

Introduction: Morphological changes as well as functional changes take place in the capillary network in Alzheimer's disease (AD). It is currently unclear whether the capillary density in AD is different from controls.

Methods: Capillary density was assessed in the temporal and occipital cortex of ten clinically diagnosed and pathologically confirmed Alzheimer patients and ten age-matched controls. Capillaries were immunostained for collagen IV in 40 µm paraffin sections. Using virtual isotropic hemispheres, which were randomly placed within the sections throughout the regions of interest, the capillary length density (CLD) was calculated and compared between Alzheimer and control subjects.

Results: The CLD in the temporal cortex was increased by 33% in Alzheimer patients compared to controls (476 vs. 359 mm/mm³, \( p = 0.04 \)) and the temporal cortical diameter in Alzheimer patients was decreased by 32% compared to controls (1.99 mm vs. 2.94 mm \( p = 0.002 \)). There was a strong negative correlation between temporal CLD and cortical thickness (Pearson's \( r \) -0.62, \( p = 0.003 \)). In the occipital cortex of Alzheimer patients more string vessels were observed compared to controls (\( p = 0.004 \)).

Discussion: The increase in temporal CLD probably results from the decrease in cortical diameter caused by cortical atrophy. The increase of string vessels in AD could be the result of capillary degeneration. Surrounding Aβ could play a role in this degeneration, as a strong co-localization of Aβ and string vessels was found in an exploratory analysis. Although there are morphological and functional changes in the capillary network these data indicate that the density of the capillary network is not altered in AD.
Introduction

Alzheimer’s disease (AD) is a neurodegenerative disease, which is characterized pathologically by intraneuronal accumulation of hyperphosphorylated tau (tangles) and extracellular accumulation of amyloid-β (Aβ; plaques). The importance of vascular factors contributing to the pathogenesis of AD is however increasingly recognized. Vascular risk factors such as hypertension and diabetes mellitus are risk factors for AD and cerebrovascular lesions are common in AD patients. Macrovascular complications including (lacunar) stroke and microhemorrhages probably do not sufficiently explain this ‘vascular component’ of dementia, and microvascular abnormalities may also play a role in the pathogenesis of AD.

Structural changes in the microvascular network in brain tissue of AD patients have been described, including blood-brain barrier alterations and basement membrane thickening. Gross morphological changes of the capillary network in AD include looping, increased tortuosity and the occurrence of string-vessels, probably remnants of degenerated capillaries. Studies on the capillary density in brain tissue of AD patients have so far provided conflicting results, where in some studies a decrease, in some an increase and in some no change in capillary density was described. The reasons for these inconsistent results probably lie in the different methodological approaches used, the selection of patients and the different cerebral regions investigated.

The aim of this study is to compare cortical capillary density and capillary morphological changes in clinically diagnosed and pathologically confirmed AD patients and age-matched control subjects. We studied capillary length densities within the temporal pole and occipital cortex of these cases using the ‘space balls’ method previously described by others.

Methods

Subjects

Post-mortem tissue from ten consecutive AD patients (age 82.6, SD 10.9) fulfilling the clinical criteria ‘probable AD’ and the neuropathological CERAD criteria for AD, and ten age-matched controls (age 84.5, SD 6.9) with no cognitive impairment were obtained from the Netherlands Brain Bank (Amsterdam, The Netherlands). All patients had advanced AD with a clinical dementia rating-score (CDR) of 2 or more. Records including a summary of medical history were available for all patients and controls.

Immunohistochemical staining

All tissue samples were obtained with a short post-mortem interval (4-10 hours) and at autopsy immersed fixed in formalin (10%) for four weeks. Afterwards, serial 700 µm thick coronal slices were cut and regions for evaluation of AD pathology and vascular
changes were dissected. Tissue blocks of the dissected regions were embedded in paraffin and cut using a sliding microtome into 5 mm or 40 µm thick sections. Sections were mounted on superfrost plus tissue slides (Menzel-Gläser, Germany) and depa-raffinized. Immunohistochemical staining was performed as described previously. Neuropathological evaluation for Alzheimer pathology was performed as described previously. For the detection of collagen IV sections were incubated over night at 4°C with mouse anti-collagen IV (1:25 dilution, DAKO, Glostrup, Denmark). After visualization using 3,3’-diaminobenzidine (EnVision Detection system/HRP, 1:50 dilution, DakoCytomation, Glostrup, Denmark, 10 minutes) sections were incubated with 0.5% CuSO₄ solution (Merck, Darmstadt, Germany) for 15 minutes to enhance staining intensity and were finally mounted with DePeX (BDH Laboratories Supplies). To determine co-localization of collagen IV with Aβ, sections were subsequently immunostained using a mouse monoclonal antibody directed at aa 1-17 of the Aβ protein (courtesy of Dr. N. Verwey, VUMC). Aβ was visualized using Liquid Permanent Red (DAKO) as chromogen. Luxol fast blue-PAS staining was performed on 5 µm sections adjacent to the 40 µm sections stained with collagen IV to visualize the boundaries of the cortex.

Quantitative analysis

Capillary length density (CLD) was estimated using the space ball method as previously described by Kreczmanski et al. Briefly, the 40 µm sections stained with the antibody against collagen IV were used for stereological analysis. Regions of interest (ROI’s) in the temporal pole and occipital cortex were delineated at low magnification (10x UplanApo objective) using a computer-assisted morphometry system consisting of a Zeiss Axioplan photomicroscope with a CCD color videocamera and a motorized

Figure 1. 40 µm sections stained with an antibody against collagen IV. a) Low magnification (25x) showing the whole cortical diameter in the occipital cortex. Several regions of interest (ROI) were selected in each section (black lines), and the cortical diameter was measured 3–4 times per ROI and then averaged for the whole section (white lines). b) High magnification (100x) showing the capillary bed and the presence of string vessels (white arrow).
stage controller for automatic sampling equipped with the StereoInvestigator software version 8 (MicroBrightfield, Germany). (fig 1a) The entire cortical diameter was selected for analysis, because of the considerable difference in capillary density between cortical layers.\(^\text{18}\) This way sampling bias within the cortex was limited to a minimum. To take into account variations in cortical diameter resulting from oblique sectioning, ROI’s were selected determined on the best vertical orientation of the cortical ribbon in each section.\(^\text{10,12}\) Cortical diameter was measured 3-4 times per ROI using the quick measure tool in the stereology software. (fig 1a) Mean cortical diameter was calculated by averaging all measurements per section. Measurements of the cortical diameter in the collagen IV sections were compared to measurements in an adjacent Luxol fast blue-Pas section in five subjects, and there was no difference, confirming that cortical diameter could be reliably measured in the collagen IV stained section.

A capillary was defined as any vessel with a diameter of less than 10 µm. Three-dimensional (3-D) counting frames were placed in the ROI using a systematic random design. Virtual isotropic hemispheres with a 27 µm radius were used as a counting probe to overcome the anisotropy of the capillary orientation. Intersections between hemispheres and capillaries were counted until at least 200 profiles were counted in order to make a reliable estimate of the CLD. (fig 2) Section thickness was measured in each section.

To calculate the CLD we used the following formula as previously described by Kreczmanski et al\(^\text{12}\)

\[
CLD = \frac{2 \times \sum is \times (Dx \times Dy \times t)}{2 \times \pi \times r^2} \times \frac{1}{V}
\]

in which \(\Sigma\) is is the total number of counted intersections between capillaries and the virtual isotropic hemispheres, \(Dx\) and \(Dy\) are the distances in x and y direction between the midpoints of the hemispheres (set at 200 µm), \(t\) is the thickness of the tissue in which
we measured and r is the radius of the hemisphere (27 µm). V is the volume of tissue (mm$^3$) in which the counting took place (calculated as n * Dx * Dy * 0.027, in which n is the number of virtual isotropic hemispheres used).

The same counting procedure was followed for string vessels, but due to the low density of these and therefore low number of profiles counted, calculation of the length density can not reliably be performed. Therefore we compared the density of string vessels between AD patients and controls by calculating the number of string-vessels per virtual isotropic hemisphere. As a result of the high number of spheres used per patient (mean 262 temporal and 168 occipital), a sufficiently high number of strings were counted to be able to calculate this density.

**Statistical analysis**

For each group and brain area, mean and standard error of the mean (SEM) were calculated for all investigated variables (i.e. capillary length density, cortical diameter). The difference in CLD, cortical diameter and string-vessels per hemisphere between AD patients and controls was analyzed using the two-tailed Student’s t-test. Correlations were calculated using Pearson correlation coefficient. Statistical significance was established at $p < 0.05$. All calculations were performed with SPSS (version 15.0 for Windows, SPSS, Chicago, IL).

**Results**

Patient characteristics including Braak stage and APOE genotype are described in table 1. All AD patients had Aβ depositions in the investigated regions, i.e. temporal and occipital cortex. Patients and controls were well matched for age (82.6, SD 10.9 vs. 84.5 SD 6.9, $p = 0.65$). The angioarchitecture in AD patients revealed overt differences in pattern and density of capillaries compared to controls in both temporal and occipital regions. The capillaries were more irregularly shaped and a higher degree of branching was seen in AD patients compared to controls. No looping or increased tortuosity was observed in either the AD patients or the controls. The aspect of string vessels in AD patients and controls was similar. The CLD in the temporal cortex of AD patients was 33% higher than in controls (476 mm/mm$^3$, SEM 50 vs. 359 mm/mm$^3$, SEM 17, $p = 0.041$) whereas in the occipital cortex the CLD in AD patients was similar to the controls (677 mm/mm$^3$, SEM 33 vs. 661 mm/mm$^3$, SEM 28, $p = 0.73$). (fig 3a,b). The cortical diameter in the temporal pole in the AD cases was 32% lower than in the controls (1.99 mm, SEM 0.14 vs. 2.94 mm, SEM 0.21, $p = 0.002$), but similar between the two groups in the occipital pole (1.94 mm, SEM 0.13 vs. 1.87 mm, SEM 0.07, $p = 0.63$). (fig 3c,d)

There was an inverse correlation between temporal CLD and cortical diameter (Pearson’s $r$ -0.62, $p = 0.003$) and between occipital CLD and cortical diameter (Pearson’s $r$ -0.55, $p = 0.012$). A strong correlation between increase in temporal CLD and age was
Morphological changes in the capillary network

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>case</th>
<th>age (years)</th>
<th>sex</th>
<th>diagnosis</th>
<th>disease duration (years)</th>
<th>CDR</th>
<th>Braak</th>
<th>cause of death</th>
<th>APOE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86</td>
<td>f</td>
<td>AD</td>
<td>7</td>
<td>3</td>
<td>4 C</td>
<td>pneumonia</td>
<td>4-3</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>f</td>
<td>AD</td>
<td>10</td>
<td>3</td>
<td>5 C</td>
<td>heart failure</td>
<td>3-3</td>
</tr>
<tr>
<td>3</td>
<td>93</td>
<td>m</td>
<td>AD</td>
<td>unknown</td>
<td>3</td>
<td>5 C</td>
<td>unknown</td>
<td>4-3</td>
</tr>
<tr>
<td>4</td>
<td>91</td>
<td>f</td>
<td>AD</td>
<td>6</td>
<td>2</td>
<td>6 C</td>
<td>pulmonary embolism</td>
<td>4-3</td>
</tr>
<tr>
<td>5</td>
<td>89</td>
<td>f</td>
<td>AD</td>
<td>20</td>
<td>3</td>
<td>6 C</td>
<td>pneumonia</td>
<td>4-3</td>
</tr>
<tr>
<td>6</td>
<td>89</td>
<td>f</td>
<td>AD</td>
<td>13</td>
<td>3</td>
<td>5 C</td>
<td>unknown</td>
<td>3-3</td>
</tr>
<tr>
<td>7</td>
<td>81</td>
<td>f</td>
<td>AD</td>
<td>8</td>
<td>3</td>
<td>6 C</td>
<td>pneumonia</td>
<td>3-3</td>
</tr>
<tr>
<td>8</td>
<td>67</td>
<td>m</td>
<td>AD</td>
<td>6</td>
<td>2</td>
<td>5 C</td>
<td>cachexia</td>
<td>3-3</td>
</tr>
<tr>
<td>9</td>
<td>69</td>
<td>m</td>
<td>AD</td>
<td>1</td>
<td>3</td>
<td>6 C</td>
<td>pneumonia</td>
<td>4-3</td>
</tr>
<tr>
<td>10</td>
<td>67</td>
<td>f</td>
<td>AD</td>
<td>9</td>
<td>3</td>
<td>6 C</td>
<td>pneumonia</td>
<td>3-2</td>
</tr>
<tr>
<td>11</td>
<td>77</td>
<td>f</td>
<td>Co</td>
<td>n.a.</td>
<td>0</td>
<td>1 A</td>
<td>cachexia</td>
<td>3-2</td>
</tr>
<tr>
<td>12</td>
<td>85</td>
<td>f</td>
<td>Co</td>
<td>n.a.</td>
<td>0</td>
<td>1 B</td>
<td>heart failure</td>
<td>4-4</td>
</tr>
<tr>
<td>13</td>
<td>89</td>
<td>m</td>
<td>Co</td>
<td>n.a.</td>
<td>0</td>
<td>1 A</td>
<td>pneumonia</td>
<td>3-3</td>
</tr>
<tr>
<td>14</td>
<td>74</td>
<td>m</td>
<td>Co</td>
<td>n.a.</td>
<td>0</td>
<td>3 C</td>
<td>bronchial carcinoma</td>
<td>4-3</td>
</tr>
<tr>
<td>15</td>
<td>79</td>
<td>m</td>
<td>Co</td>
<td>n.a.</td>
<td>0</td>
<td>1 A</td>
<td>hypoglycemia</td>
<td>3-3</td>
</tr>
<tr>
<td>16</td>
<td>80</td>
<td>m</td>
<td>Co</td>
<td>n.a.</td>
<td>0</td>
<td>0</td>
<td>cachexia</td>
<td>3-3</td>
</tr>
<tr>
<td>17</td>
<td>84</td>
<td>m</td>
<td>Co</td>
<td>n.a.</td>
<td>0</td>
<td>1</td>
<td>COPD exacerbation</td>
<td>n.d.</td>
</tr>
<tr>
<td>18</td>
<td>91</td>
<td>m</td>
<td>Co</td>
<td>n.a.</td>
<td>0</td>
<td>1 B</td>
<td>heart failure</td>
<td>n.d.</td>
</tr>
<tr>
<td>19</td>
<td>93</td>
<td>f</td>
<td>Co</td>
<td>n.a.</td>
<td>0</td>
<td>2</td>
<td>cachexia</td>
<td>n.d.</td>
</tr>
<tr>
<td>20</td>
<td>93</td>
<td>f</td>
<td>Co</td>
<td>n.a.</td>
<td>0</td>
<td>1 A</td>
<td>cachexia</td>
<td>n.d.</td>
</tr>
</tbody>
</table>


found (Pearson’s $r = 0.48$, $p = 0.032$), this correlation remained significant when the AD and controls were analyzed separately. In the occipital cortex CLD was not significantly associated with age (Pearson’s $r = 0.14$, $p = 0.54$).

There were more string vessels in AD than in controls in the occipital cortex (0.079, SEM 0.13 vs. 0.031, SEM 0.06 per hemisphere, $p = 0.004$), but in the temporal cortex there was no significant difference (0.058, SEM 0.25 vs. 0.020, SEM 0.05 per hemisphere, $p = 0.15$). ( fig 3e,f)

Double staining of collagen IV and Aβ1-17 showed a close relationship between capillaries and Aβ depositions, especially with the string vessels. ( fig 4)

Discussion

This study contributes to the sparse literature on microvascular abnormalities in AD patients and aged individuals. We used the space ball method to quantify capillary...
length density in the temporal and occipital cortex of well-characterized AD patients and age-matched control subjects. The significant increase in capillary density in AD compared to controls in the temporal cortex is the same order of magnitude as the decrease in cortical diameter. The decrease in cortical diameter of the temporal cortex and correlation between the cortical diameter and CLD in this area suggests that the degeneration of the capillary network keeps pace with the cortical atrophy. The atrophy of the temporal cortex is most likely the results of neuronal loss and profound tangle and Aβ pathology in this area. The extent of the cortical capillary network probably alters to match the number of living neurons. This is in line with an earlier report of

Figure 3. Capillary length density (a,b), cortical diameter (c,d) and string vessels per mm³ (e,f) in the temporal cortex (panels on the left) and the occipital cortex (panels on the right).
increased temporal cortical capillary density in AD patients, which was explained by a decrease of inter-capillary distance.\textsuperscript{9}

This is the first report using quantitative methods considering stereologic principles to investigate CLD in the temporal and occipital cortex of AD patients. Our results suggest that alterations in CLD cannot be considered a general feature of AD, but occur in specific brain regions. Previous autopsy studies have reported conflicting results concerning capillary density in AD.\textsuperscript{5,7,8} The different regions analyzed could be one of the reasons for this discrepancy. As illustrated in our study, there are large regional differences in both atrophy and capillary density. The use of different methods to analyze the capillary density could also contribute to the conflicting results between previous studies and our own study. In previous studies the capillary density was measured using either manual tracing of the capillaries or counting of capillary intersections using the test grid method\textsuperscript{5,7,8}, as opposed to using virtual isotropic hemispheres yielding a capillary length density as measured in a volume of tissue in our study.

The most reliable way to measure the density of a linear structure in tissue is by using thorough stereological techniques based on the Cavalieri principle using serial sections throughout the tissue to be investigated as was done by Bouras et al in the human hippocampus.\textsuperscript{20-22} Measuring the total capillary length in our tissue was not possible, and not required for a comparison between two groups as we made. By using thick sections and a virtual isotropic probe with sufficient measurements in several ROI’s per subject, a fair comparison between the two groups (AD vs. controls) could be made. A possible weakness of the method we used is the sampling bias that could have occurred as a result of using only one thick section per patient.

The increased number of string vessels in the occipital cortex is in line with previous studies reporting on an increase of these structures, which are probably remnants of capillaries, in AD.\textsuperscript{6,23} The reason why this increase is found in the occipital cortex but not in the temporal cortex is unclear. The exploratory analysis of co-localization of collagen IV and Aβ shows a remarkable co-localization of Aβ with string vessels. We
can hypothesize that the presence of Aβ surrounding a capillary can contribute to the capillary degeneration into a string vessel, or even that the presence of Aβ is associated with neuronal loss, leading to less metabolic demand and subsequent degeneration of the capillary. Our qualitative, exploratory analysis however, does not allow for a definitive conclusion on these mechanisms.

To conclude, we have shown that CLD in patients with AD is increased in the temporal cortex and that this increase is strongly correlated with a decrease of cortical diameter, suggesting the increased CLD is only a relative increase. The increased number of string vessels in AD suggests that capillary degeneration takes place in the cortex of AD patients. The proximity of Aβ depositions to the string vessels suggest a relationship between the two, which should be further explored in future studies.
Reference List


