CARDBIOVASCULAR RISK FACTORS AFFECT HIPPOCAMPAL MICROVASCULATURE IN EARLY AD

Abstract
There is growing clinical and neuropathologic evidence suggesting that cognitive decline in early Alzheimer's disease (AD) is aggravated by a synergistic relationship between AD and cerebrovascular disease associated with cardiovascular risk factors such as diabetes and hypertension. Here we used the stereologic "Space Balls" method to investigate the relationships between AD pathology and cardiovascular risk factors in postmortem human brains of patients with hypertension and diabetes in two groups – one consisting of cases with AD diagnosis and one of cases without. Hippocampal CA1 and CA3 microvasculature length density estimates were generated to characterize quantitatively the contribution of cardiovascular risk factors to the severity of neuropathologic changes. Our main finding is that the mean and variance of length density values in the AD group were significantly increased from the non-AD group, regardless of the absence or presence of a cardiovascular risk factor. An additional finding is that in the AD group without a risk factor, dementia severity correlated with amount of length density change in the CA1 field—this correlation did not exist in the AD groups with risk factors. Our findings suggest a role for cardiovascular risk factors in quantifiable change of hippocampal CA1 field microvasculature, as well as suggest a possible role of cardiovascular risk factors in altering microvasculature pathology in the presence of AD.

Key Words
Stereology • Space Balls • Cardiovascular risk factors • CA1 • CA3 • Alzheimer's disease

1. Introduction
Late-onset Alzheimer's disease (AD) is a progressive neurodegenerative disease and the leading cause of dementia in the United States, afflicting up to 20% of individuals 75-84 years of age and nearly 50% of individuals over 85 [1]. The implications are serious considering that the fastest growing population in the United States consists of adults over the age of 65 [2,3]. AD is characterized neuropathologically by aggregates of intraneuronal hyperphosphorylated tau protein and extracellular amyloid beta protein (Aβ) [4,5]. These changes evolve over decades and progress in predictable spatial and temporal patterns—the hippocampus being one of the earliest regions affected [6]. While much attention has been paid to the progression of these diagnostic markers, less attention has been focused on what role cortical microvasculature alterations may play in the development and progression of AD neuropathology. Despite the fact that there is often a coexistence of neurodegenerative and vascular pathology in the aging brain [7], there are few quantitative observations of how the cortical vasculature is affected [8].

Hypertension (HTN) and diabetes mellitus (DM), two common cardiovascular risk factors, both damage cortical microvasculature and both increase risk for developing AD. HTN, currently defined as a systolic blood pressure above 140 mm Hg and/or a diastolic blood pressure above 90 mm Hg [9], is estimated to affect 25% of the general population [10] and over one half of persons over 65 years [11]. HTN is the most significant risk factor for cerebrovascular disease [12]. Several longitudinal studies have suggested that hypertension, particularly during or after midlife, is associated with an increase risk for AD, and several randomized placebo-controlled clinical trials have found that the treatment of HTN with antihypertensive drugs has decreased AD risk [13]. DM is estimated to affect more than 10% of the elderly population in the United States [14]. Type 1 DM is characterized by the progressive inability of pancreatic beta cells to produce insulin, and type 2 DM is characterized by progressive impaired response to insulin [15]. Increased glucose levels in the blood are common to both types, and it is elevation in glucose levels that is thought to damage blood vessels. It is estimated that up to 80% of deaths in diabetic patients are associated with vascular disease [16]. Several longitudinal studies have shown that both types are associated with faster rates of decline in cognition [17,18]. Additionally, several studies point to type 2 DM playing a role in the acceleration of AD progression [19].

Many studies have produced qualitative information about vasculature in the brain in the presence of AD as well as in the presence of a cardiovascular risk factor, however few...
have quantified this information. In this study, we used stereologic approaches to quantify the hippocampal microvasculature in the CA1 and CA3 fields, two areas that are differently affected in AD progression (Figure 1), in AD and non-AD cases. Furthermore, we subdivided these two groups (AD vs non-AD) into further categories according to cardiovascular risk factor (no risk, type 2 DM, or hypertension) to assess whether the presence of a cardiovascular risk factor preferentially affects hippocampal microvasculature length density.

2. Experimental Procedures

2.1 Subjects

All samples were obtained from the Mount Sinai Alzheimer’s Disease Research Center Neuropathology Core and Brain Bank (Table 1). The sample included 15 men and 14 women, for a total of 29 subjects (76.7 ± 10.2 [±SD] years old; age range, 57 to 92). Clinical Dementia Rating score is based on subject interview (or informant interview, in cases of more severe dementia) and characterizes loss in cognitive and functional performance in six domains; memory, orientation, judgment and problem solving, community affairs, home and hobbies, and personal care (CDR score 0 = no dementia, 1 = mild, 2 = moderate, 3 = severe, 4 = profound, 5 = terminal dementia) [20,21]. The Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) scores are based on semiquantitative findings – an observational rating of the frequency of neuritic plaques (NPs) and neurofibrillary tangles (NFTs), which is then related to age to give a score (0 = none, 1 = sparse, 3 = moderate, 5 = severe) [22]. It should be noted that the CERAD protocol does not take into account estimates of soluble amyloid load, aberrant tau accumulation, and synaptic density, nor does it assess vascular pathology [23,24]. In addition, the CERAD sampling does not employ a systematic-random approach and is based on assumption of homogeneous distribution of lesions in the tissue, in part explaining why some of the cases used in this study in the non-AD groups exhibited higher CERAD scores than anticipated. The right hemispheres of the brain from these patients were fixed in 4% paraformaldehyde, and matching hippocampal blocks were prepared immediately prior to the time of this study.

2.2 Tissue processing and Immunohistochemistry

We carried out this study in the hippocampus as it is an area affected early in AD, and where severity of pathology is correlated with severity of early cognitive decline. Blocks approximately 2x3x0.5 cm containing the anterior third of the hippocampus were embedded in agarose and cut into 50 μm-thick coronal serial sections using a Leica VT1000 S vibrating blade microtome (Leica; Wetzlar, Germany). Blocks were matched for location among cases. Every fifth section was then selected for a total of six sections per subject. Sections were free floating during immunohistochemical processing. They were stored in phosphate-buffered saline (PBS) overnight at 4°C, and were also rinsed in PBS after each incubation step. After two initial rinses with PBS, two preincubation steps were performed to block nonspecific staining—the first was done in 0.3% hydrogen peroxide in PBS, and the second in 5% normal goat serum in PBS (MPBio Chemicals, Solon, OH). Next, the
sections were incubated overnight on a shaker at room temperature with mouse monoclonal anti-collagen IV primary antibody (MAB1920; Chemicon International Inc., Temecula, CA). The antibody was diluted 1:2,000 in PBS containing 0.3% Triton X-100 and 5% normal goat serum (MPBio Chemicals). The next day, sections were incubated at room temperature for one hour with a secondary antibody consisting of biotinylated goat anti-mouse IgG (E0433 Dako; Glostrup, Denmark) diluted 1:600 in PBS containing 0.3% Triton X-100. Sections were then incubated for 1 hour with an avidin-biotin peroxidase complex (ABC Elite kit, Vector Laboratories, Burlingame, CA). Nickel (Alfa Aesar, Ward Hill, MA) intensified 3,3’-diaminobenzidine (DAB; Sigma, St. Louis, MO) was used as a chromogen in the glucose oxidase-DAB-nickel method [25]. Sections were then mounted on 2% gelatin-coated slides, dried, and stained for background neuronal architecture with 0.2% cresyl violet (Fluka, Buchs, Switzerland) for approximately 5 minutes. Sections were then dehydrated and coverslipped using DPX (Serva, Heidelberg, Germany). Sections were processed, stained and mounted as soon as possible following tissue cutting to minimize tissue shrinkage in the x-y direction.

2.3 Stereology

All analyses were performed blind to medical history and subject group to reduce bias. Sections were first viewed at low magnification (2.5X) to outline the pyramidal cell layers in the CA1 and CA3 fields using a Zeiss Axioplan 2 microscope and Stereoinvestigator software (MBF Biosciences, Williston, VT). The CA1 and CA3 hippocampal subfields were chosen because these fields show a different susceptibility to AD pathology at different stages of the disease. The length of the microvasculature (which included vessels less than 10 µm in diameter) for both CA1 and CA3 regions were then calculated using the software’s Space Balls probe [26-29]. The dimensions of the random sampling grid were set to place approximately 150 hemispheres throughout the outlined regions. The mean number of sampling sites was 303.17 for CA1 fields and 163.7 for CA3 fields. There were more sampling sites for the CA1 field because we used the same parameters for both fields, however the area of the CA1 field per section in our cases was greater than the area of the CA3 field per section. Once a grid size was determined, hemispheres with a diameter of 11 mm were systematically and randomly placed by the stereology software in the left-hand corner of each grid. A guard zone was also placed at the top and bottom of each sampling site. Microvasculature length was calculated from the number of intersections between vessels and hemispheres. Counts were obtained at higher magnification using a 40X Zeiss PlanApo objective.

The software’s Cavalieri probe was then used to generate estimates of the volume of the CA1 and CA3 fields. The average length density of the microvasculature in the hippocampal CA1 and CA3 fields were thus calculated based on estimates of the length of microvasculature and volume of tissue for each region (CA1 and CA3) for the cases examined in this study.

2.4 Statistics

All analyses were performed separately for the CA1 and CA3 fields. The distribution of length density was examined for non-normality. A two-way analysis of variance (ANOVA) was performed, testing length density differences by group (AD vs non-AD) and cardiovascular risk factor (no risk factor, HTN, DM). If the interaction was not significant, the primary analysis was for group differences regardless of presence or absence of diagnosis of a cardiovascular risk factor was assessed using one way ANOVA. Group differences in the standard deviation (SD) were assessed using Levene’s test of homogeneity of variances. In regards to the CA1 fields, we noticed that the groups had similar minimum values, but that they differed substantially in their maximum values. To reflect this, a Fisher’s exact test was performed, dichotomizing both groups at the lower maximum value, so that only one group was represented in the upper category. To assess possible associations of age or CDR score with length density for group (F = 9.907; df = 1, 23; p = 0.005), but not for risk (F = 0.069; df = 2, 23; p = 0.934) or interaction of group by risk (F = 0.780; df = 2, 23, p = 0.470). Since the interaction was not significant, the primary analysis of group differences was performed without taking account of risk. The mean length density of cortical microvasculature was significantly higher in cases with AD (AD group mean = 302.7 mm/mm³, non-AD group mean = 230.1 mm/mm³; F = 10.558; df = 1,27; p = 0.003). In addition, the variability of length density was significantly higher in cases with AD (AD SD = 82.70 mm/mm³, non-AD SD= 25.35 mm/mm³; F = 14.169; df = 1,27; p = 0.001; Figure 2). Values of length density were most tightly clustered in control cases without a cardiovascular risk factor, and the presence of diabetes or hypertension increased the standard deviation around the mean in groups without AD. The non-AD group had a maximum value of 278.4 mm/mm³, compared with 426.54 mm/mm³ for the AD group. The AD cases had 7 of 14 above 278.4, the maximum for the 15 cases in the non AD group (one-sided Fisher’s exact test, p = 0.002).

The correlations of length density and CDR score (r = 0.369, p = 0.194, n = 14) and age (r = -0.048, p = 0.870, n = 14) were not significant. The pooled within-risk factor correlations were quite similar (CDR score: r = 0.336, p = 0.286, df = 10; age: r = -0.073, p = 0.821, df = 10). The three correlations for separate risk categories did not agree in sign for either
CDR score or age. The sample sizes for the risk categories were only 4 for diabetes and 5 for the no risk category and the HTN category, so extraordinarily strong correlations were required to achieve statistical significance. Nonetheless CDR score was correlated with length density in the no risk factor category ($r = 0.882, p = 0.048, n = 5$) and age was correlated with length density in the DM category ($r = -0.992, p = 0.008, n = 4$).

### 3.2 CA3

The distribution was not markedly non-normal (skewness = 0.894; kurtosis = 0.390). The most extreme observation was only 2.57 standard deviations from the mean. In the analysis of the CA3 field, two-way analysis of variance did not show significant difference in length density for group ($F = 1.531; df = 1, 17; p = 0.233$), risk ($F = 2.182; df = 2, 17; p = 0.143$) or interaction of group by risk ($F = 1.452; df = 2, 17; p = 0.262$). Since the interaction was not significant, the primary analysis of group differences was performed without taking account of risk. The length density did not differ between AD and non-AD cases in either mean (AD group mean = 274.75 mm/mm$^3$, non-AD group mean = 249.66 mm/mm$^3$; $F = 1.279; df = 1, 21; p = 0.271$) or variation (AD SD = 33.871 mm/mm$^3$, non-AD SD = 63.311 mm/mm$^3$; $F = 2.040; df = 1, 21; p = 0.168$). The non-AD group had a maximum value of 396.93 mm/mm$^3$, compared with 331.02 mm/mm$^3$ for the AD group. 2 out of 13 non-AD cases had length densities above 331.02 mm/mm$^3$, the maximum for the ten cases in the AD group (one-sided Fisher’s exact test, $p = 0.308$).

The correlations of length density and CDR score ($r = 0.147; p = 0.685, n = 10$) and age ($r = 0.323; p = 0.362, n = 10$) were not significant. The pooled within-risk factor correlations were quite similar (CDR score: $r = 0.090, p = 0.832, df = 6$; age: $r = 0.372, p = 0.365, df = 6$). The three correlations for separate risk categories did not agree in sign for either CDR score or age. The sample sizes for the risk categories were 2 for DM (so correlation could not be tested) and 4 for the no risk category and HTN category. None of the correlations were significant, not even for age and length density in the no risk category ($r = 0.864, p = 0.136, n = 4$).

### 4. Discussion

Cerebral microvascular pathology is known both to precede and progress along age-related cognitive decline [30,31]. Cerebrovascular pathology is also present in AD. Classic AD microvasculature alterations that have been qualitatively described include a thinning of microvessels (termed “string vessels”) [32], increased tortuosity [33] and vessel fragmentation [34], with alterations most often localized in regions that contain a high amount of AD-related pathology [35]. Vascular changes such as a decrease in density, an increased presence of loop formations, and atrophic vessels can also be seen in other types of dementias [7]. Our own observations of the samples used in this study fit the known descriptions of vasculature change seen in AD cases (Figure 3), however they differ in that we observed in AD...
an increase in microvasculature length density, while many studies support the idea that in AD, vessel number and length is reduced [36]. Although our quantification shows an increase in density, it should be considered that we are measuring local densities independent of global region volume. Due to the global shrinkage and loss of neocortical and hippocampal neurons in AD the microvasculature becomes falsely compacted, making the density measured per unit volume artificially increase, which would not have occurred if we had access to the entire structure of interest to sample from.

It is also possible that the tortuosity of vessels found in AD cases likely increases the length density of the microvasculature by causing the surrounding tissue to aggregate. A recent study which also utilized the Space Balls method to examine capillary length density in AD cases also found an increase in length density of the capillary network in the hippocampus, and postulated that such a change may likely be related to cortical atrophy in regions most heavily affected based on a correlation found between an increase in capillary length density and a decrease in cortical diameter [37]. It is possible that changes in microvasculature is in fact region-specific, and future studies further exploring changes in vascular density in other brain regions under the same conditions of our study would be of value in shedding light on this question.

The main finding in our study is an increase in both mean vessel density and variance in the CA1 field in the AD group, independent of the absence or presence of a cardiovascular risk factor. As the CA1 field is one of the first cortical regions to be affected by AD-type pathology during normal aging and AD [6], our results indicate that hippocampal microvasculature is affected in this area either early in the progression of AD or possibly even prior to AD onset. The CA1 field is sensitive to metabolic stress caused by ischemic injury and hypoxia [38]. Hypoperfusion for 30 minutes has been shown to lead to a typical neuronal response termed “delayed neuronal death” that occurs selectively in CA1 field neurons [39,40]. This apoptotic response, induced by glutamate and calcium, occurs 1-3 days following ischemia and hypoxia [41,42]. Taking into account this CA1-selective vulnerability to local microvasculature damage, the question is raised as to whether or not microvasculature change may be involved in pathological progression of AD pathology itself. If vascular change precedes AD onset, it would indicate that the CA1 region is vulnerable to vascular change, which if true could account for local acceleration or initiation of AD pathological change.

Changes in the microcirculation of the CA1 field likely accompanies the vessel alterations observed in this study, and it is possible that this compromised microcirculation could accelerate AD pathology by promoting the accumulation or preventing the clearance of amyloid plaques and NFT. It has been found that reduced vascular density is associated with reduced cerebral blood flow [43], and this reduction in blood flow can be observed in the early stages of AD in areas where there is an association between the reduction in blood flow and tau accumulation [44,45]. Another possibility is that oxidative stress caused by reduction in cerebral blood flow (which occurs in both aging and AD) [46] renders the microenvironment more susceptible to AD pathological change, and oxidative stress has been shown to cause aggregation of both Aβ [47] and tau [48]. It has also been proposed that angiogenic vascular endothelial growth factor becomes sequestered in AD by binding to Aβ, decreasing its ability to promote vessel growth [49]. In such a scenario one can see how if vascular damage increases the risk for AD, and AD worsens vascular damage, a cycle could be initiated that ultimately would accelerate both pathologies.

We did not observe a comparable increase in mean length density or its variance in the CA3 fields in either the AD or non-AD group. There was a slight, but not statistically significant, increase in length density in the CA3 field in the AD group suggestive of milder vascular alterations. The CA3 region is typically spared until late stage AD [6], and our results indicate a relative parallel sparing of the microvasculature, unlike the situation in the CA1 field, stressing further the coexistence of microvascular damage and formation of the typical lesions of AD.

Cardiovascular risk factors increase the risk of both cognitive decline and AD. Type 2 DM is also associated with an increased rate of cognitive decline [18] and HTN is associated with decreased cognitive function [52]. There are several theories concerning how HTN affects AD and cognitive decline. The first is that HTN causes vascular change that predisposes to an increased rate of lacunar and cortical infarcts [53], and a second is that HTN accelerates the production of Aβ [54]. There are also multiple theories in regards to the mechanism that link DM and dementia – DM predisposes to ischemia induced cerebrovascular disease [55], hyperglycemia can cause neuronal toxicity through oxidative stress and accumulation of glycation end-products [56,57], and insulin can directly affect the metabolism of Aβ [58]. It is possible that vascular pathology seen in AD can be altered by the presence of the comorbidity of a cardiovascular risk factor (such as DM or HTN). Although our study did not find a difference in mean length density or variance between the AD, no risk factor group and either of the AD groups with a cardiovascular risk factor, we did find a correlation between length density in the CA1 field and dementia severity in the AD, no risk group (r = 0.882, p = 0.048, n = 5), which did not exist in the groups with both AD and a risk factor (Figure 4). The existence of a cardiovascular risk factor likely introduces additional pathologic factors that may influence AD progression when the two coexist, and these unknown factors may be what alter the association between vessel density change and CDR score. Schneider and colleagues, in reviewing the findings from two clinicopathologic epidemiological studies, found that macroscopic infarcts often coexist with AD pathology, and also lower the threshold
for cognitive impairment [59]. Multiple studies have in fact shown that the presence of cerebrovascular lesions may participate in determining dementia severity by causing a worsening of clinical symptoms at lower levels of AD pathology [60-63], which could account for the breakdown of the correlation in groups with both AD and a risk factor. It is here also important to note that vascular dementia and AD dementia are often hard to distinguish, and it is also possible that, when they do coexist, cerebrovascular pathology may prevent an accurate diagnosis of AD [59]. Thus it is possible that cases in our study who were placed into the non-AD, risk factor groups may in fact belong in the AD, risk factor groups. It is also important to address the sample size used in this study, which was dictated by the availability of cases that met our criteria. We recognize that this is a limitation, and that our results are in this respect preliminary yet indicative of a pathological process that interacts with the usual course of AD.

Finally, although our sample size for each group is small, it is striking that 3 out of the 4 cases with both AD and DM had the most severe CDR score rating of 5. Thus in the group with both AD and DM, it appears that there is a worsening of dementia in the context of microvascular length density changes similar to cases with AD alone and cases with AD and HTN. A study utilizing autopsy specimens found that type 2 DM is in fact negatively associated with AD neuropathologic changes, and put forth the hypothesis that the cognitive impairments associated with DM likely reflect the impact of metabolic and microvascular change and the interaction that such changes have with NPs and NFTs [64]. This is consistent with the findings of an earlier postmortem study that show that DM is not a risk factor of AD-type pathology, and also suggests that there are other factors at play in AD that worsen cognitive decline [65]. The findings of yet another study show that in non-diabetic subjects, dementia is associated with increased Aβ accumulation, while in cases with diabetes, dementia is associated more with neuroinflammation and microvascular infarct [66]. It is possible that the mechanisms introduced by DM pathology affect microvasculature in ways not identifiable by quantification (such as vessel integrity). Nelson, et al., reviewed the existing data on cerebral neuropathology of type 2 DM, and concluded that although there is some evidence suggesting that changes in brain parenchyma may play a role, more studies are needed as there is in fact a small number of studies investigating vessel pathology in DM, and that an anatomic substrate for cognitive impairment in diabetics remains unknown, yet needed [67]. Such a substrate may not even be related to the microvasculature, and instead interfere with synaptic structure or neuronal function, which could also explain increased severity of clinical symptoms in the absence of increased vascular change.

DM and HTN can and often do exist for decades preceding clinical symptoms of AD. The cognitive decline associated with diabetes has been found to progress slowly over a long time period [68] and it has been observed that HTN in the form of increased systolic blood pressure in midlife increases the risk for developing AD later in life [69,70]. It is also possible that clinical symptoms of AD occur late in disease progression, and that AD-associated neurodegeneration actually begins in midlife [71-73]. It remains to be seen how cortical microvasculature alterations may play a role during the early stages of AD development. Future studies investigating cortical microvasculature changes in middle aged diabetic or hypertensive patients will be crucial in this respect. Independent of the actual onset of AD pathology, it is becoming clear that there is an opportunity in midlife to take advantage of preventative strategies that impact health decades later. A biostatistical study has found that preventative strategies that delay AD onset or dementia severity even modestly have the potential to influence significantly public health [74]. In this context it is important that promoting midlife vascular integrity represents a potential avenue for AD prevention.

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