

**Investigating Long-term, Progressive Cerebrovascular
Alterations Using Optical Coherence Tomography in
Alzheimer's Disease**

by

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Chapter 1

Introduction

1.1 Alzheimer's disease (AD)

1.1.1 Epidemiology and current challenges

Alzheimer's Disease (AD) is the most common form of dementia among elderly and the fifth leading cause of death in the United States [1]. It is an irreversible neurodegenerative disorder with severe cognitive impairment that can ultimately affect daily activities of many peoples' lives. As the aging population grows, the number of AD cases is expected to double every 5 years for people beyond age 65 and to nearly triple to 14 million by 2050 in the United States [2]. Globally, over 36.5 million people are clinically diagnosed with AD and related dementia, and an estimated 5 to 7 million new cases of AD are recorded every year [3–7]. Total healthcare costs is estimated to be \$355 billion in 2021 [1], and expected to rise which could place a significant economic and social burden on the families and caregivers. Unfortunately, the etiology of AD is still unknown due to itself being a multifactorial disease. Aging is considered as the primary risk factor for AD, however, pre-existing conditions such as sex, genetic variants, and also environmental factors in-

cluding diet and general lifestyle could all play their parts in AD pathogenesis. Moreover, the progression of AD is believed to start decades earlier than clinical symptoms [8], suggesting early detection of the disease is crucial and could benefit not only AD patients for better treatment plans but also their caregivers for making all the necessary arrangements in advance.

AD can be categorized into two types: early-onset or familial AD (FAD), sporadic or late-onset AD (LOAD). FAD affects people early in their life (the age of 65 or younger), but accounts for a very small portion ($\sim 3\%$) of all AD cases [9]. The vast majority ($\sim 97\%$) of cases are commonly referred to as LOAD which affects people over age of 65, with multiple genetic and environmental factors possibly involved in its pathogenesis. While the pathogenic cause of FAD is known to be inherited genetic mutations in amyloid precursor protein (APP), presenilin 1 (PSEN1), presenilin 2 (PSEN2) [10], the molecular pathogenic cause of LOAD still remains elusive. With many other genes possibly contributing to LOAD pathogenesis, genome-wide association studies have identified that the $\epsilon 4$ allele of the apolipoprotein E (APOE4) gene is the highest genetic risk factor for LOAD [11, 12], and also shows its association with FAD [13, 14]. The APOE gene exists as three different polymorphic alleles ($\epsilon 2$, $\epsilon 3$, and $\epsilon 4$) [15]. Among three APOE isoforms, the frequency of APOE4 is $\sim 15\%$ in general populations but $\sim 40\%$ in patients with LOAD. Several studies have confirmed that individuals carrying APOE4 genes have three to four times more risk of being diagnosed with AD than noncarriers [16, 17], and with lower age of onset [18].

Before an individual is diagnosed with AD (FAD or LOAD), there are three different stages within the AD continuum: preclinical, mild cognitive impairment (MCI), AD with mild, moderate, severe dementia. First, the preclinical AD stage is considered as a long asymptomatic phase, in which individuals show evidence of AD pathological changes but no sign of cognitive decline that would affect their daily activities. The duration of the preclinical AD stage, although varying between individuals, typically lasts 6 to 10 years de-

pending on the age of onset [19, 20]. The risk of preclinical stage AD patients progressing to either MCI or AD with severe dementia can be influenced by different factors including age, sex, and genetic predisposition such as APOE status [20, 21]. It should be noted that not all individuals with signs of AD pathological changes will progress to MCI or AD [22, 23]. In fact, from a recent meta-analysis study with six longitudinal cohorts and average follow-up rate of 3.8 years, only 20% of patients in preclinical AD stage show a progression to MCI due to AD-related factors [20]. Similarly, another study with an average follow-up rate of 4 years reports 29.1% of patients in preclinical AD stage progressing to MCI due to AD [21]. Patients in MCI stage display initial clinical symptoms such as short-term memory impairment with marginal deficits in cognitive function. When progressed from MCI to AD, patients show severe cognitive deficits and increasingly abnormal behavioral symptoms that can start to affect not only themselves but also their caregivers in daily lives [24]. Due to this AD continuum (shown in Figure 1.1) and long asymptomatic phase in preclinical stage, an early and accurate detection of the disease is crucial yet challenging. Detecting AD in early clinical stages is often hindered by barriers such as technical limitations of diagnosing neuropathological changes, and also individuals dismissing symptoms as part of normal aging process. For accurate detection and fast diagnosis, a deeper understanding of how multiple etiologies and age-related prodromal processes contribute during the AD continuum is necessary. To do so, the current AD research needs to evolve “to develop new programs on systems biology and integrative physiology to gain a deeper understanding of the complex biology of the disease.”, stated as one of the overarching transformative concepts at the AD Research Summit [25].

1.1.2 ATN framework: definition of disease

The National Institute on Aging and Alzheimer's Association (NIA-AA) defines dementia as a ‘syndrome’ composed of signs and symptoms caused by multiple diseases (eti-

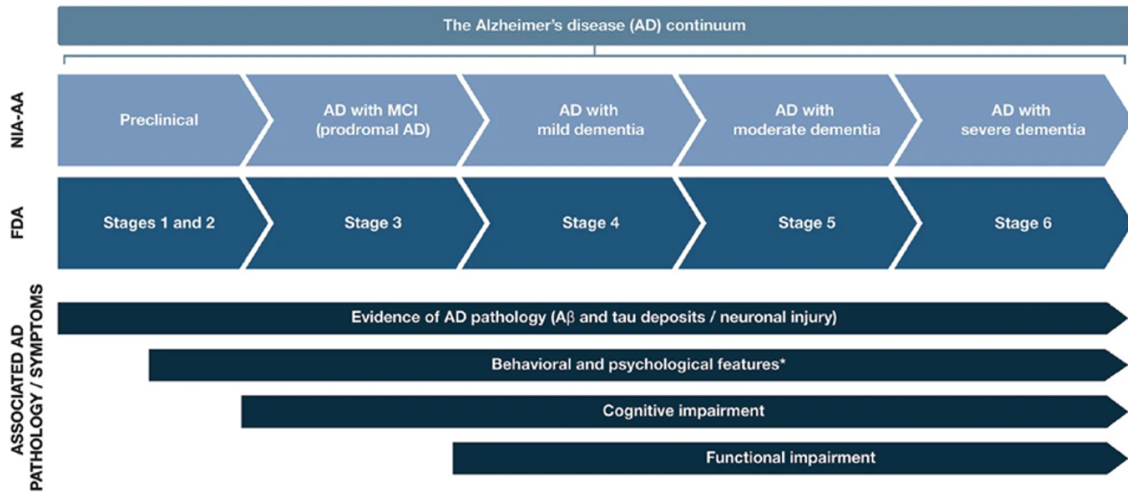


Figure 1.1: Stages within the Alzheimer's disease continuum. Reproduced from [26].

ologies). According to NIA-AA, using the same analogy toward AD can be problematic due to two major reasons: it lacks neither sensitivity nor specificity for neuropathological changes that defines AD, and it cannot identify individuals displaying biological evidence of AD but not yet manifesting signs or clinical symptoms [27, 28]. Moreover, neuropathological changes in AD are present in 30 to 40% of cognitively unimpaired elderly persons at autopsy [24]. The opposite finding has been reported also where nearly 30% of individuals clinically diagnosed with AD display no neuropathological changes at autopsy [29]. These conflicting results suggest, rather than viewing AD as a clinical-pathologic construct, it needs to be defined as a biological construct which can be identified by biomarkers in living person, enabling the concepts of symptoms and disease interchangeable. In other words, an approach that incorporates more of AD biomarkers over patients' clinical symptoms, which appear relatively late in the AD continuum, is needed. Among many biomarkers currently available, NIA-AA outlines a descriptive classification scheme which categorizes AD biomarkers into three major groups based on their pathologic processes [24]: beta amyloid ($A\beta$) plaques (labeled "A"), fibrillar tau (labeled "T"), neurodegeneration or neuronal injury (labeled "(N)") in Table 1.1. Adding cognitive symptoms (labeled "(C)") to the table, NIA-AA creates the AT(N)(C) framework which can be used as a guide for investigators

not only to study AD as a biological construct but also in developing novel biomarkers for detecting subtle changes during preclinical AD stage with long asymptomatic phase.

Table 1.1: AT(N)(C) biomarker grouping.

A: Aggregated A β or associated pathologic state
CSF A β 42
CSF A β 42/40 ratio
T: Aggregated tau (neurofibrillary tangles) or associated pathologic state
CSF Phosphorylated tau
Tau PET
(N): Neurodegeneration or neuronal injury
Anatomic MRI
FDG PET
CSF total tau
(C): Cognitive symptoms
A and T indicate specific neuropathologic changes that define AD, whereas (N) and (C) are not specific to AD and are therefore placed in parentheses. Abbreviations: A β , beta amyloid; CSF, cerebrospinal fluid; PET, positron emission tomography; MRI, magnetic resonance imaging; FDG, fluorodeoxyglucose.

1.2 Current AD biomarkers for early detection

1.2.1 Neuroimaging biomarkers: PET

Positron emission tomography (PET) is an advanced imaging technology that uses radioactive agents to visualize molecules of interest. Amyloid-PET imaging ($A\beta$ -PET) measures the accumulation of $A\beta$ plaques and also enables monitoring of disease progression in the brain regions of AD patients. The current clinically-approved radioactive diagnostic agents in $A\beta$ -PET are florbetapir, flutemetamol, and florbetaben [30]. A good correlation between florbetapir-PET image scores and cortical $A\beta$ pathology at autopsy has been reported from the phase 3 study (a cohort of 29 patients) [31]. Another cohort study (59 primary analysis participants) use florbetapir as diagnostic agents and has shown a sensitivity of 92 to 96% in detecting moderate to frequent $A\beta$ plaques [32], distinguishing patients with no or sparse $A\beta$ plaques from the ones with higher $A\beta$ deposition in the brain. Also, phase 3 studies using flutemetamol [33] and florbetaben [34] have shown similar sensitivity and specificity to florbetapir-PET in detecting $A\beta$ plaques. In addition to these three diagnostic agents, Pittsburgh compound B is another PET diagnostic agent which binds to $A\beta$ plaques in the brain, and has been used in demonstrating reduction of fibrillar $A\beta$ accumulation in AD patients when treated with bapineuzumab [35].

Tau PET imaging (tau-PET), on the other hand, uses radiotracers which bind to hyperphosphorylated tau forming neurofibrillary tangles (NFTs) that are known to be closely associated with synaptic loss, brain atrophy, and cognitive impairment. Currently, however, there is a limited choice of tracer options, mainly due to tau proteins forming intracellular aggregates (NFTs) which require the radiotracers to cross blood-brain barrier (BBB) [36]. Also, post-translational modification of tau proteins makes the effort to develop specific radiotracers for tau-PET challenging [37]. Nevertheless, in preclinical AD studies, tau-PET shows earlier detection of cognitive changes than $A\beta$ -PET [38, 39], suggesting it being

a potential biomarker for early AD diagnosis. A cross-sectional study which used [18F] flortaucipir, the most validated tau PET radiotracer currently, has found that this radiotracer distinguishes AD from all non-AD neurodegenerative disorders in the medial-basal and lateral temporal cortex with about 90% sensitivity and specificity [40]. A case study reports that *in vivo* retention of [18F] flortaucipir is correlated with postmortem tau pathology (tau-positive density, NFTs, total tau burden) in the AD brain [41]. Moreover, tau-PET outperforms A β -PET in predicting cognitive changes [42, 43], suggesting the possibility of tau-PET being an effective early diagnostic tool.

In addition to A β -PET and tau-PET, Fluorodeoxyglucose PET (FDG-PET) is a robust biomarker for predicting cognitive decline in FAD. By measuring glucose use in the brain, FDG-PET can detect signs of neuronal dysfunctions such as synaptic loss and BBB breakdown in AD [44], and has also shown high sensitivity and specificity (>90%) distinguishing between dementia with Lewy bodies and healthy controls [45, 46]. Moreover, FDG-PET performs better than structural magnetic resonance imaging (MRI) in predicting patients with MCI converting to AD in future [47].

1.2.2 Neuroimaging biomarkers: MRI

Structural MRI is considered as one of the widely used imaging modalities in AD diagnosis which provides a comprehensive overview of pre-mortem, AD-associated structural changes such as brain atrophy [48], significant loss of brain volume that is pathologically proven to be a positive predictive measure for AD [49]. Using T2-weighted MRI, a study has demonstrated the detection of cerebral atrophy patterns [49], while susceptibility-weighted MRI shows its capability in identifying microbleeds [50], which may reflect A β burden in the brain [51]. In addition, functional MRI (fMRI) is another rising area that can detect early functional changes in AD based on blood oxygenation level-dependent (BOLD) signals [52]. A recent study (20 AD patients and 20 healthy controls) using

resting-state-fMRI, diffusion-MRI, and T1-weighted, has found AD-associated brain connectivity changes by assessing multiple regions of the brain (white matter, gray matter, cortical gray matter, hippocampus) [53]. Also, with the recent advances in machine learning technologies, AD-resemblance atrophy index based on MRI scans has been developed and outperformed conventional MRI markers with high sensitivity (92%) and good specificity (81%) in detecting preclinical and prodromal AD [54].

1.2.3 Fluid biomarkers: CSF

CSF, surrounding the extracellular spaces of the brain and given its direct interaction with the interstitial fluid, can adequately reflect the metabolism of proteins ($A\beta$ and tau) in the brain. As a diagnostic tool for AD, there are three core biomarkers in CSF: $A\beta_{42}$, total tau (T-tau), threonine-181-phosphorylated tau (P-tau181) [55]. First, CSF $A\beta_{42}$ is a widely validated biomarker where reduced CSF $A\beta_{42}$ can be indicative of both preclinical and severe AD [56]. Several studies have shown a strong correlation between CSF $A\beta_{42}$ and $A\beta$ -PET [28, 57], suggesting this biomarker being a viable alternative to PET. Additionally, rather than CSF $A\beta_{42}$ alone, the CSF $A\beta_{42}/A\beta_{40}$ (denoted as $A\beta_{42/40}$) ratio performs better not only in identifying AD patients but also in predicting AD conversion of patients with MCI [55, 56]. Second, T-tau is a biomarker which reflects the intensity of neurodegeneration and neuronal loss. A significant increase in CSF T-tau level has been reported from multiple AD studies [58–60], suggesting this biomarker is predictive of rapid progression during AD [61]. In discriminating between AD and healthy control groups, T-tau shows a sensitivity above 80% and a specificity of 90% [62]. Even in early stages of AD, several studies have found a significant elevation in CSF T-tau level [63–65]. In contrast, a recent study suggests that T-tau may not be associated with neurodegeneration but with $A\beta$ -mediated tau metabolism [66]. Lastly, P-tau181 is a validated biomarker reflecting abnormalities in tau metabolism. Similar to CSF T-tau, the elevated P-tau181 levels

have been found in AD and prodromal AD [56], and another cohort study reporting significant correlation with levels of CSF P-tau181 even in normal and MCI subjects [67]. Additionally, there are other emerging CSF markers such as neurofilament light protein (NfL) and threonine-217-phosphorylated tau (P-tau217). CSF NfL is a marker of neuroaxonal damage and neurodegeneration which occurs not only in AD but also in many other neurological disorders [68]. It has been reported that elevated CSF NfL level is observed in early AD and MCI patients with structural changes in the brain, and also associated with cognitive decline [69]. A recent study has demonstrated P-tau217 has a better performance than CSF P-tau181 in distinguishing AD from other neurodegenerative disorders [70].

1.2.4 Fluid biomarkers: Blood-based

Blood-based (and plasma) biomarkers have an advantage over CSF due to their relatively easy accessibility and less invasiveness involved when extracting samples. However, due to the presence of many different proteins and other substances in the bloodstream [56], it needs to be carefully validated by high-sensitivity assays before clinical use. Four biomarkers that are closest to clinical implementation for AD diagnosis are A β , P-tau, NfL, and Glial fibrillary acidic protein (GFAP). The meta-analysis study in 2016 has reported that plasma concentrations of A β 42 and A β 40 show no difference between AD patients and healthy individuals [71]. Since then, there have been growing efforts of comparing plasma A β with CSF A β levels and A β -PET, and with cognitive decline in longitudinal manner [71, 72]. Several studies using mass spectrometry assays have found the ratio plasma A β 42/40 identifies A β abnormalities in CSF or PET status from individuals across AD continuum, with accuracies of 82-97% [72–76]. By taking APOE4 status into account, the better performance is observed when classifying AD patients from individuals with MCI [76]. However, the difference in blood biomarkers was smaller than the CSF biomarker, for instance 20% versus 50% lower values in plasma A β 42/40 and CSF

A β 42/40 against healthy controls, respectively [72, 74]. Many factors might contribute to this difference, for instance, the inclusion of peripheral sources in the plasma samples leads to binding of peripheral blood proteins (much higher concentration in plasma than in CSF), which may directly affect the result. Nevertheless, many studies have shown that low plasma A β 42/40 or presence of misfolded A β 42 are associated with AD risk among not only individuals with cognitive decline or MCI but also cognitively unimpaired individuals (preclinical stage), showing the potential of plasma A β 42 for early AD detection [77–81].

Similar to plasma A β , many methods such as mass spectrometry assays and other immune-based approaches have been used to detect tau pathology [70, 82]. The increased concentration of plasma phosphorylated tau have been reported at different sites (pTau181, pTau217, or pTau231) in AD when compared to people with non-AD dementia [83] or without any cognitive impairment [84]. Plasma pTau181, correlated with increased tau-PET [85], is elevated in people with AD but not with non-AD dementia [70, 86]. A cohort study with healthy and cognitively impaired individuals has found plasma pTau18 (both baseline and longitudinal changes) is associated with tau aggregation 6 years later [87]. Also, increased plasma pTau181 also shows its association with grey matter atrophy observed only in individuals with A β pathology [88], suggesting pTau181's specificity in detecting AD-associated neuropathological changes. Moreover, a better performance from pTau181 has been reported when compared to plasma A β 42/40 [89]. Plasma pTau217 has recently gained some attention with a high accuracy (96%) in differentiating AD from non-AD dementias reported from a large cohort study [83], similar to accuracy scores from CSF or tau-PET biomarkers. A study has shown the abnormality in plasma pTau217 observed before tau-PET, and plasma pTau217 level starts to increase about 20 years before the onset of MCI in FAD, suggesting its potential as an early AD biomarker [90]. In comparison with pTau181, plasma pTau217 shows a better performance in detecting A β -PET positivity [91]. A similar performance has been reported from Plasma pTau231 in a recent cohort study [92], although another study has not replicated the same result when all three

elements (pTau181, pTau217, or pTau231) put into test [93]. Interestingly, the best performing model for AD prediction is a combination of multiple biomarkers and test (plasma pTau217 or pTau181, ApoE status, and cognitive tests) [94], emphasizing the necessity of a multi-metric approach when monitoring the disease progression for early AD diagnosis.

NfL is a core neurofilament protein which plays a crucial role not only in axonal growth and stability but also in synaptic organization and function in the brain [95]. This protein has been evaluated and established itself as a potential biomarker for neurodegeneration in various neurological disorders including AD [96, 97]. The NfL concentrations are observed highest in frontotemporal, vascular dementias, amyotrophic lateral sclerosis and Parkinson's disease [98]. In relation to AD (more specifically LOAD), increased concentrations of NfL in plasma and CSF show association with longitudinal neurodegeneration determined by MRI [99]. Although the concentration of NfL increases even in normal aging which can lead to misinterpretation of the results, it can still serve as a valuable tool in monitoring treatment effects by checking the severity of AD progression in clinical practice.

Reactive astrogliosis is considered as one of the factors that drives AD pathological changes [100]. GFAP, as a major cytoskeletal constituent of astrocytes, has higher concentration near dense A β plaque regions and also with respect to tau accumulation reported from a population-based cohort study [101]. Moreover, using ultrasensitive immunoassays, several studies have found the elevation of plasma GFAP concentrations observed in both FAD and LOAD patients [89, 102, 103]. Plasma GFAP shows a strong linear association with A β -PET positivity, accuracy of about 81% [80], however, the GFAP levels differ and this linear association is missing when in high A β -PET burden [104]. The increase in plasma GFAP is correlated with neuropsychological test performance and brain atrophy measured by MRI [102, 103, 105]. Plasma GFAP detects A β pathology in patients with MCI but more importantly predicts future conversion of these patients to AD [106],

suggesting its value as a monitoring tool during AD progression.

1.2.5 Retinal biomarkers

Several studies have explored possibilities of developing retinal biomarkers using eye scans as a non-invasive, inexpensive regular check-up tool to predict AD. The retina in the eye, considered as a part of the central nervous system (CNS), manifests similar abnormalities (either $A\beta$ or tau) found in the brain of AD patients [107–109]. From a retinal amyloid imaging study using a modified scanning laser ophthalmoscope with solid-lipid curcumin, increased retinal $A\beta_{42}$ plaques observed in AD patients versus healthy controls [110]. $A\beta$ plaque burden is significantly greater in the proximal mid-periphery of individuals with MCI and AD compared to that in cognitively unimpaired individuals [111]. The levels of $A\beta$ in retina shows correlation with hippocampal $A\beta$ level [112], strengthening the notion of the retina as a window of the brain and reflecting AD-related pathological changes. Post-mortem studies have shown that not only $A\beta$ -plaque and NFTs are present but also other responses such as ganglion neurodegeneration, astrogliosis, and microglial activation observed in the retina of AD patients [57, 113]. Moreover, in preclinical studies using transgenic AD mice, $A\beta$ -plaques and oligomers in the retina are exhibited at the presymptomatic stage before pathological change occurring in the brain [114, 115]. In relation to tauopathy, the detection of pTau has been demonstrated from several studies [113, 116], with one study showing a diffuse immunoreactive signal for pTau increases while absence of $A\beta$ -plaques in the AD retina [117]. In addition to neuropathological change detections, other metrics such as light-induced pupillary responses and macular ganglion cell-inner plexiform layer thickness have been suggested as candidates for early AD biomarkers [118], although more thorough validation seems necessary.

1.3 Thesis goal

1.3.1 Microvascular alterations in AD

Cerebrovascular alterations, evidently showing a strong association with progressive neurodegeneration, are observed in AD. Three vascular factors are considered as major contributors in progressive neurodegeneration: hypoperfusion, dysregulated functional hyperemia, and reduced clearance of toxic metabolites. First, severe hypoperfusion causes homeostatic changes in neuronal network by altering electrolyte balances and water gradients, leading to edema and white matter damage [119–121]. A chronic hypoxic state caused by prolonged hypoperfusion leads to neuronal damage, and ultimately extensive neuronal death [122]. At the microvascular scale, disturbances in capillary flow patterns are directly related to the extraction efficacy of oxygen in the brain parenchyma [123, 124], even in the absence of physical flow-limiting vascular pathology [125]. Cerebral hypoperfusion promotes cortical microinfarcts, accelerates cerebral amyloid angiopathy (CAA) [126], $A\beta$ deposits building up on the walls of the arteries [127]. CAA facilitates $A\beta$ generation [128] and soluble $A\beta$ is found to constrict the cerebral vasculature; $A\beta_{40}$ resulted in decreased blood flow and increased vascular resistance specifically in the cerebral cortex, but not in the heart or kidneys [129]. $A\beta$ deposition occurs both in the parenchyma and in vessel walls, causing vascular attrition, abnormal alteration of the cerebral microvasculature. Interestingly, even without $A\beta$ plaque present, studies have found both structural alterations in microvasculature and cerebral hypoperfusion exhibited at young ages [130, 131]. However, it is still important to understand the faulty clearance of $A\beta$ across the blood-brain barrier, aberrant angiogenesis, and disruption of the cerebrovascular system could initiate vessel regression, hypoperfusion, neurovascular inflammation and uncoupling, eventually leading to blood-brain barrier compromise, accelerated $A\beta$ accumulation, chemical imbalance in the neuronal environment, and synaptic and neuronal dysfunction [132–137]. Other

vascular alterations linked to hypoperfusion in AD are increased vessel tortuosity, more string vessels, thickened and irregular basement membranes, reduced capillary density, increased capillary transit time heterogeneity, and increased number of capillary stalling due to neutrophils adhered in capillary segments [138–142].

Many imaging AD studies, although the extent and degree of hypoperfusion differing by brain regions and measurement techniques, display consistent results of significant reduction in blood flow in the brain regions such as hippocampal, parietal, and temporal cortices [143–151]. A population-based study has found a high association between cerebral hypoperfusion and accelerated cognitive decline with increased risk of dementia [152]. Reduced regional CBF, particularly in frontal lobes, showed a close association with severity of dementia [153, 154]. The cerebral vasomotor reactivity, the basis for blood flow regulation in the brain in accordance with its metabolic status, is impaired not only in cerebrovascular diseases but also in AD [155].

Second, functional hyperemia (also known as neurovascular coupling) is a neuronal activity-dependent cerebrovascular dilation, mediated primarily by the vasoactive metabolites of arachidonic acid by nitric oxide and potassium [156]. Dysregulated functional hyperemia is reported from hypertension, ischemic stroke, and AD [157]. A recent study shows that a reduction in functional hyperemia independently of the resting-state hypoperfusion leads to a lowering of brain oxygen level and neuronal loss in older pericyte-deficient mice [158]. Moreover, the excessive blood flow increase may be needed to ensure sufficient oxygen delivery to neuronal structures distant farthest from capillaries [159], suggesting the importance of functional hyperemia in maintaining neuronal health and preventing progressive neurodegeneration. The attenuation of functional hyperemia is found in AD patients during memory-related tasks [160, 161], verbal tasks [162, 163], and in response to visual stimuli [164, 165]. From preclinical studies, early homogenization of capillary blood flow in response to neural activation is absent in aged (18-month-old) AD mice, suggesting

profound disturbances of both neurovascular coupling and capillary function [166]. Third, dysfunctions in either the intramural peri-arterial drainage pathway of interstitial fluid or the glymphatic system pathway of CSF leads to ineffective removal of metabolic wastes, which may contribute to neurodegeneration [167].

Additionally, BBB, although not within the vascular system, plays an important role as a mediator between the periphery and the CNS. When BBB breakdown occurs, it initiates multiple pathways of neurodegeneration [168], followed by leakage of neurotoxic blood-derived products to neurons such as thrombin and reactive oxygen species [169, 170], leading to hypoperfusion through albumin-promoted vasogenic edema [171]. An interesting clinical study has recently shown that the carriers of APOE4, a strongest known genetic risk factor for sporadic AD, exhibit higher BBB breakdown in medial temporal lobe and hippocampus than non APOE4 carriers. More importantly, the observed BBB breakdown is not related to A β and tau pathology measured in CSF [172].

1.3.2 Toward retinal microvasculature-based novel biomarker

Despite increasing evidence of cerebrovascular alterations reported in many AD studies, there remains some important knowledge gap where the temporal relationships of various cerebrovascular alterations during AD progression are not clear. This knowledge gap, in fact, was clarified in a comprehensive review [132]: “The time course of these vascular alterations and how they relate to dementia and Alzheimer’s disease pathology remain unclear, as no protocol that allows the development of the diverse brain vascular pathology to be scored, and hence to be tracked with aging, has so far been developed and widely validated.” Since then, Iturria-Medina *et al.* has conducted a multifactorial data-driven analysis study combining diverse biomarkers which includes brain images, CSF/blood-based biomarkers and cognitive tests, and has generated a hypothetical model as seen in Figure 1.2, showing the biomarker abnormality projected in relation to AD progression with

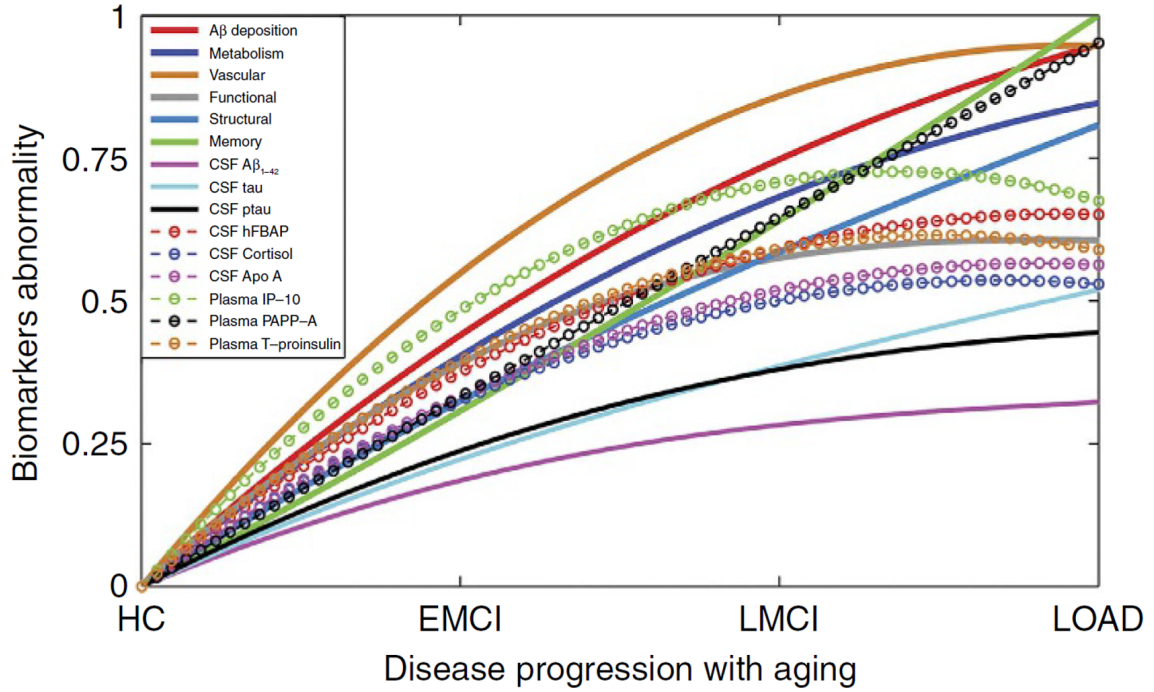


Figure 1.2: Multifactorial temporal ordering in disease progression, based on the factor-specific abnormality trajectories (temporal abnormalities averaged across all brain regions), memory deficit and three CSF biomarkers ($A\beta_{1-42}$, tau and ptau). All of the results were calculated for the HC to LOAD clinical transition. Abbreviations: HC, healthy controls; EMCI, early mild cognitive impairment; LMCI, late mild cognitive impairment; LOAD, late-onset Alzheimer's disease. Reproduced from [134].

aging. Among all the biomarkers, the intra-brain vascular dysregulation is projected to be the earliest pathological event among all biomarkers during LOAD development [134]. However, there is no study conducted yet to support this projected model due to the challenging nature of longitudinal tracking where AD pathologies slowly develop over a fraction of the lifespan, decades in humans [173] or many months in mouse models [174]. Essentially, a more rigorous and systematic approach is required for tracking cerebrovascular alterations in a longitudinal manner.

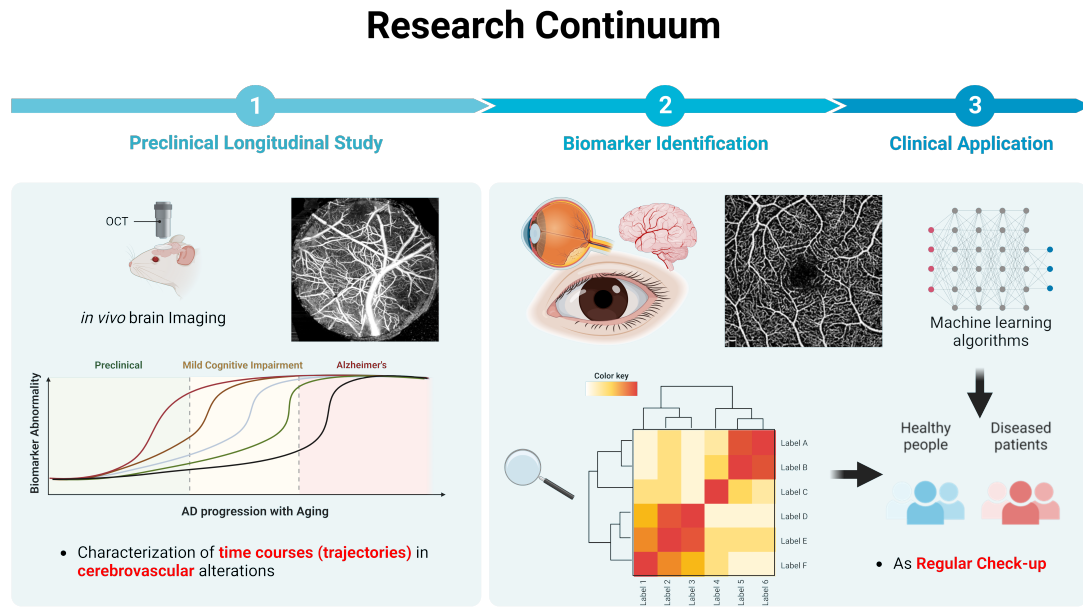


Figure 1.3: Research continuum toward retinal microvasculature-based novel AD biomarker. Created with BioRender.

Here, the goal of this thesis is to obtain time courses of various microvascular alterations in the cortex of AD model mice, for the first time to our knowledge. Based on the scientific premise that microvascular alterations in the brain also appear in the retina, the findings from this thesis will facilitate further studies toward the retinal microvasculature-based novel biomarker development, represented in Figure 1.3. As a complementary or potentially more sensitive biomarker, our long-term goal is to develop an image processing algorithm to sensitively and specifically predict AD from eye scan images so that subjects with risks (e.g., APOE4 carriers) can use it as a regular check-up.

To meet this goal, the present thesis consists of three studies. First, since the technical feasibility of longitudinally imaging brain microvasculature in mice as long as a year was not proven, we conducted a 1-year longitudinal imaging experiment in the 3xTg model (**Chapter 2**). We chose this model because it manifests AD pathologies the earliest than other models, while it was uncertain how long the longitudinal imaging would be possible through a cranial window installed on the mouse brain.

Second, we addressed two major limitations in the first technical feasibility study, which were the use of anesthesia for imaging and the unknown sensitivity of our approach. Since the isoflurane we used for anesthesia is well known to affect microvasculature, this anesthesia effect may distort the time courses of microvascular alterations we obtain in the third study. Also, from the first study alone, it was unclear how subtle changes in microvasculature our approach can detect. Therefore, we characterized this anesthesia effect and established an awake imaging technique, as well as determining the sensitivity of our measurement (**Chapter 3**).

Finally, based on the technical feasibility and awake imaging technique, we conducted another 1-year longitudinal *awake* imaging experiment with a clinically more relevant model, APOE4 model, while having sex as a key biological variable (**Chapter 4**). As discussed in 1.1.1, APOE4 gene is the major risk of late-onset AD, but a non-invasive and inexpensive regular check-up approach to detecting this APOE4-related AD onset has not been established. Also, APOE4 is known to be related to various microvascular dysfunctions, including BBB integrity [172]. Therefore, the time courses of microvascular alterations obtained from this study, separately for each sex, will provide novel insights to our long-term effort of developing effective biomarkers based on simple eye scans which could be useful not only for detecting early vascular changes in AD but also for monitoring the disease progression and response to treatment.

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Chapter 2

Validating the technical feasibility of longitudinally imaging brain microvasculature in AD mice (3xTg)

2.1 Introduction

Vascular dysfunction has recently gained some attention with its high prevalence in AD [1], for example, rapid change in vasculature, BBB breakdown, increased tortuosity, reduced capillary density, and regional hypoperfusion [2–6]. In particular, these abnormalities in cerebrovasculature are often observed earlier than cognitive decline or even earlier than neuropathology, suggesting the role of vascular dysfunction as a possible etiology. For example, at the preclinical stages of AD, several studies found cerebral blood flow decreases observed in the brain regions such as hippocampus, entorhinal cortex and amygdala, that are closely linked to cognition [7, 8]. From population-based studies, it has been reported that this cerebral hypoperfusion is associated with accelerated cognitive decline and an increased risk of dementia [9, 10].

To investigate vascular alterations, there are several imaging modalities such as laser speckle contrast imaging (LSCI), fluorescence microscopy, optical coherence tomography, and so on. LSCI can measure blood flow changes in cortical surface but is limited in depth-resolved volumetric imaging capability, and only provides relative change values [11, 12]. For a deeper volumetric and target-specific imaging, fluorescence microscopy such as two-photon microscopy (TPM) can be a great option with the high resolution for visualizing vasculature [13, 14]. However, TPM generally requires scanning over all three axes, limiting its speed for blood flow imaging. Also, having to use fluorescence dyes makes TPM not suitable for longitudinal and intact imaging in general. In this regard, spectral-domain optical coherence tomography (OCT) can be a suitable option with its label-free, in-depth volumetric imaging [15], which enables us to visualize the changes in cerebral vasculature in a longitudinal manner [16]. Plus, for microcirculation, doppler optical coherence tomography (DOCT) enables the quantification of absolute blood flows in penetrating arterioles and venules based on phase shifts of blood cells flowing through vessels [17]. With these advantages of OCT, it is capable of providing the long-term stability *in vivo* imaging of vascular alterations in the brain.

The aim of this study is to validate the technical feasibility of longitudinally imaging brain microvasculature in AD mice as long as a year using OCT with its techniques such as OCT angiography (OCTA) and DOCT to extract and quantify vascular properties in pial and penetrating vessels. Due to uncertainty in how long the longitudinal imaging would be possible with a cranial window installation, we choose one of the most widely used FAD mouse models, 3xTg which have the well-established expression of the major manifestations in human AD in relatively early manner compared to other AD mice models: A β plaques by 6-12 months of age, NFTs by 12 months of age, neuronal loss, congophilic amyloid angiopathy [18–21], and spatial memory loss at 6-9 months of age [22, 23].

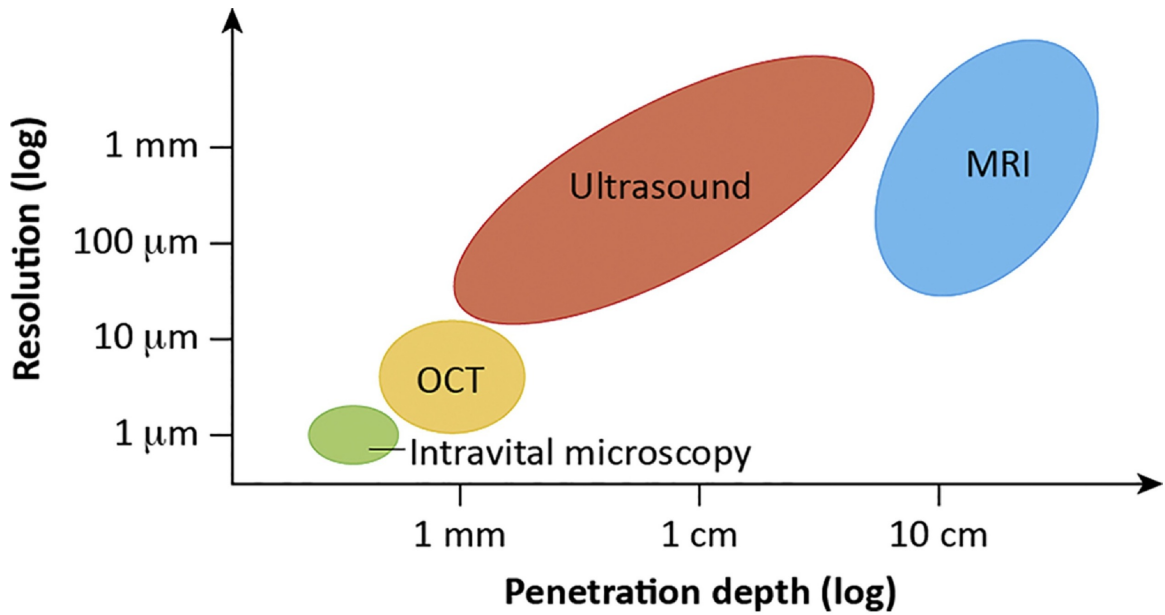


Figure 2.1: Schematic comparison of spatial resolution versus imaging depth between Intravital Microscopy, OCT, and standard clinical imaging modalities. Reproduced from [24]

2.2 Methods

2.2.1 Optical Coherence Tomography (OCT)

OCT is a non-invasive, label-free, micro-level resolution imaging which provides 3D volumetric imaging of microvasculature, with the axial resolution ranging from 1 to 15 μm and the lateral resolution dependent on the objective lens we use. Based on the backscattering of light (low-coherence interferometry), the penetrating depth of OCT ranges from 1 to 3 mm in most biological samples. Compared to other imaging modalities, OCT fills up the gap nicely for both resolution and imaging depth shown in Figure 2.1.

By replacing a point detector with a spectrometer from the predecessor (time-domain OCT), it allows much faster imaging acquisition by collecting backscattered light from all depths at once. Applying Fourier transform, from the raw OCT signal (frequency domain, wavenumber) to the spatial domain, we can obtain depth information. As shown in Fig-

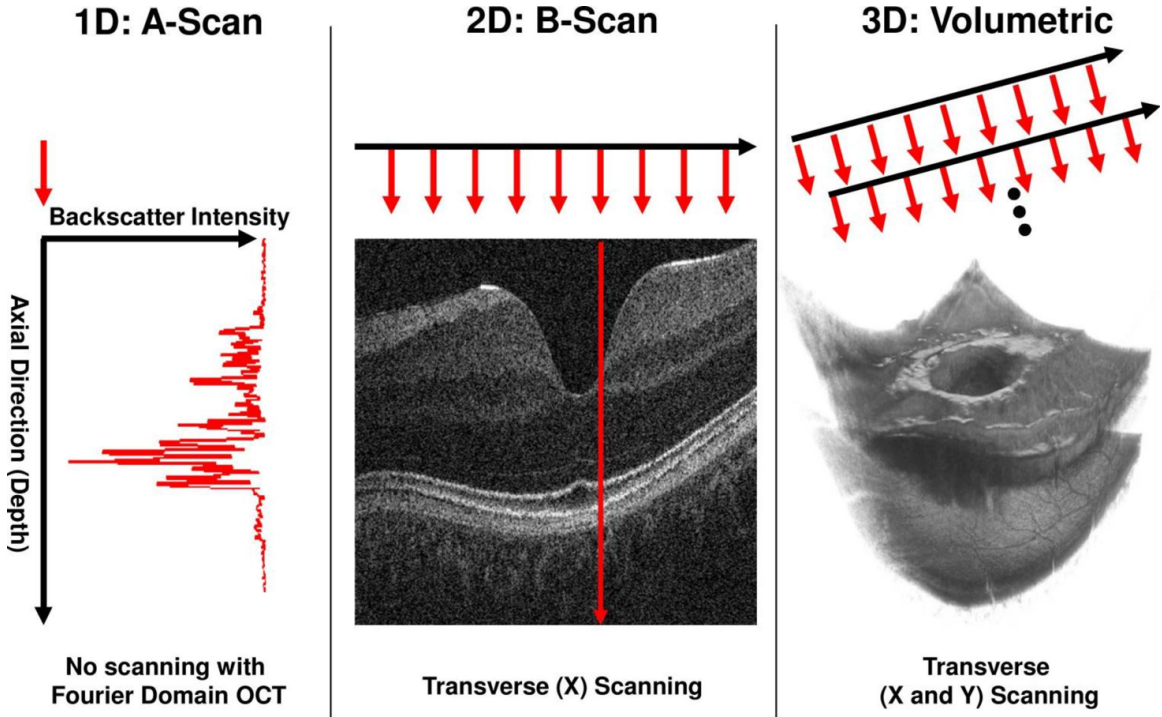


Figure 2.2: OCT scanning and scanner coordinate system. Left: 1D acquisition (A-Scan). A single depth profile is acquired which measures backscattered intensity vs. axial dimension (depth). Middle: 2D imaging (B-Scan). The OCT beam is scanned in a transverse direction while A-Scans (red arrows) are acquired. Right: 3D acquisition. Multiple B-Scans are acquired such that A-scans are sampled on a 2D grid in the transverse plane. Reproduced from [25].

Figure 2.2, A single A-scan represents sample reflectivity over (x,z) position. By scanning along one dimension (many A-scans), the 2D information can be obtained and is termed B-scan. Merging multiple B-scans from different y -positions will generate a 3D volumetric imaging.

For technical specification, Spectral domain OCT system (Telesto III, Thorlabs, USA) was used to collect all experimental data in this thesis. It uses a near-infrared (NIR) laser source with a center wavelength of 1310 nm and wavelength bandwidth of 170 nm which leads to an axial resolution of $3.5 \mu\text{m}$ in air (calculated). The system uses a high-speed 2048-pixel line-scan camera to achieve 147,000 A-scan/s providing a relatively large imaging depth (2.5 mm maximum). The $5\times$ NIR objective lens ($\text{NA} = 0.14$, Mitutoyo, Japan) was used and produced a lateral resolution of $7 \mu\text{m}$ in air (measured with a USAF target).

The field of view (FOV) had $1024 \times 1024 \times 1024$ voxels corresponding to $3.08 \times 3.08 \times 3.58 \text{ mm}^3$, and transverse and axial sampling intervals of $3 \text{ }\mu\text{m}$ and $3.5 \text{ }\mu\text{m}$, respectively.

2.2.2 OCT-Angiography (OCTA)

OCTA is a contrast-based concept in which red blood cells (RBCs) continuously pass through vessels while the surrounding tissues remain static. By scanning the same region twice with a small time delay, OCTA can capture the dynamic passage of RBCs in vessels for obtaining the vasculature map. In this study, to minimize surface reflections, the focal plane was located at approximately $100 \text{ }\mu\text{m}$ below the cortical surface when acquiring OCTA. It should be noted that this focal depth may cause slight defocus on the imaged pial vessels that are located at the cortical surface, but its effect on the major outcome of our study would be minimal as the study focuses on differences in the diameter between ages and groups rather than the absolute value. The volumetric scan was repeated 5 times to reduce speckle noise. Acquired data was reconstructed to 3D images using the widely used method which involves numerical dispersion compensation, k-space interpolation, and inverse Fourier transformation [26]. After volume averaging, median and gaussian filtering, we obtained maximum intensity projection (MIP) images and calculated full width half maximum (FWHM) values for vessel diameter measurement. In detail, when the user selected a point near a vessel, our software user interface identified the centerline of the vessel, extracted 10 cross-sectional intensity profiles from 10 adjacent pixels along the centerline, fitted the average profile to a Gaussian fitting, and measured the full-width half-maximum as the diameter of the selected vessel. This averaging along the vessel centerline and Gaussian fitting-based sub-pixel measurement allows for more robust measurements than a simple counting of pixel numbers along the vessel width.

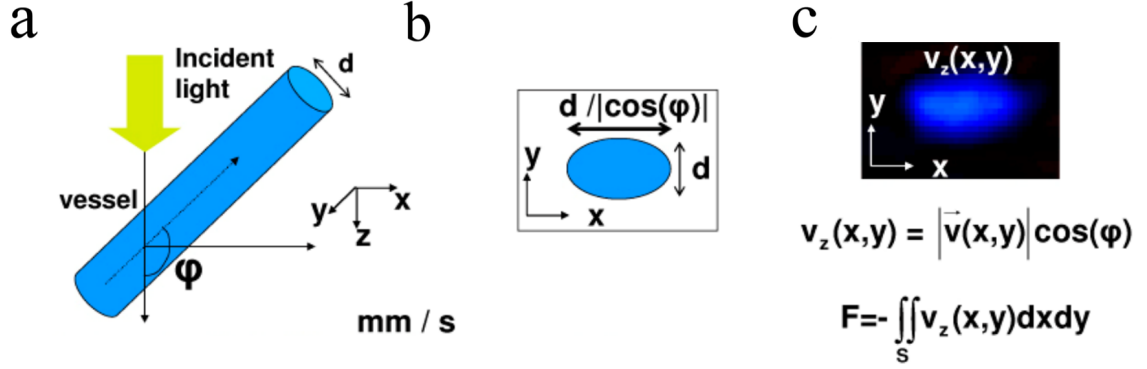


Figure 2.3: Absolute blood flow quantification without explicit calculation of vessel angle. (a) Blood vessels can be oriented at any angle φ relative to the incident light. (b) The area of the vessel in the *en face* (xy) transverse plane is inversely proportional to $\cos(\varphi)$. (c) Zoom of a penetrating venule showing the z projection of velocity over the vessel cross-section. While the area of the vessel in the xy plane is inversely proportional to $\cos(\varphi)$, the z-projection of the velocity is proportional to $\cos(\varphi)$. Reproduced from [15].

2.2.3 Doppler-OCT (DOCT)

DOCT is based on the concept of Doppler shift where the phase changes occur from reflected light (axial translation of scatterers). This can be explained as the rotation of OCT signals in the complex plane, and by calculating this rate of rotation (slope of the phase in time), we obtain velocity information in a vessel. However, since this velocity information only reflects on the axial component of blood flow in a vessel, the angle of the vessel to the imaging beam needs to be considered for calculating total velocity of the vessel as shown in Figure 2.3a. By summing up the axial velocity information across the *en face* cross-section of the vessel, absolute blood flow of selected vessel area can be quantified using the equation displayed in Figure 2.3c.

We acquired DOCT images by repeating 8 A-scans at each (x,y) position with the temporal sampling interval of $6.8 \mu s$, and the FOV was $512 \times 512 \times 1024$ voxels corresponding to $1.54 \times 1.54 \times 3.58 \text{ mm}^3$. To minimize surface reflections, the focal plane was located at approximately $100 \mu m$ below the cortical surface. After 3D reconstruction, we obtained blood flow maps using improved clutter rejection algorithm with Kasai autocorrelation

method previously published [27, 28] and the equations in Figure 2.3c. Median and low-pass filtering were also applied. To extract the information, we drew a circle around the selected vessel for finding the center of mass, then set the region of interest (ROI) with around $60 \times 60 \mu\text{m}^2$. A line profile was drawn at each of four angles, passing the center of ROI, where the four angles represent the horizontal, vertical, 45 deg, and 135 deg. We then applied a gaussian fit to the average of the four profiles for obtaining FWHM as diameter for penetrating arterioles and venules. When we selected penetrating arterioles and venules from an *en face* Doppler OCT image, we distinguished between the two types by considering that the anatomy of cerebral vasculature in the cortex has arterioles and venules result in positive and negative velocity values in Doppler data, respectively. To select the same vessels across different time points within each animal, we registered the *en face* images to a reference time point image and considered spatial distribution of the penetrating vessels and relative positions between each other as appeared in the registered images. We also considered the corresponding OCTA image of the same animal and age point when helpful. This selection approach was consistently applied across data of all animals and ages.

2.2.4 Novel Object Location test (NOL)

Mice were placed into an open-field apparatus consisted of a square arena ($40 \times 40 \times 49 \text{ cm}^3$). The arena is digitally divided into 4 quadrants, with the center of the northeast (NE), northwest (NW), southeast (SE), and southwest (SW) corners which serve as placement locations for two identical objects (rods, radius = 1 cm, height = 5 cm) as shown in Figure 2.4. For 5 min, each mouse's behavior was recorded by the automated tracking software (EthoVision XT, Noldus) to calculate the exploration time determined by a circular zone ($<5 \text{ cm}$) from the objects' center. Two different sessions were carried out with the delay of 20 min in between: a habituation stage for 5 min (objects placed on NE and NW) and a testing stage for 5 min (objects placed on SE and NW). The arena is cleaned

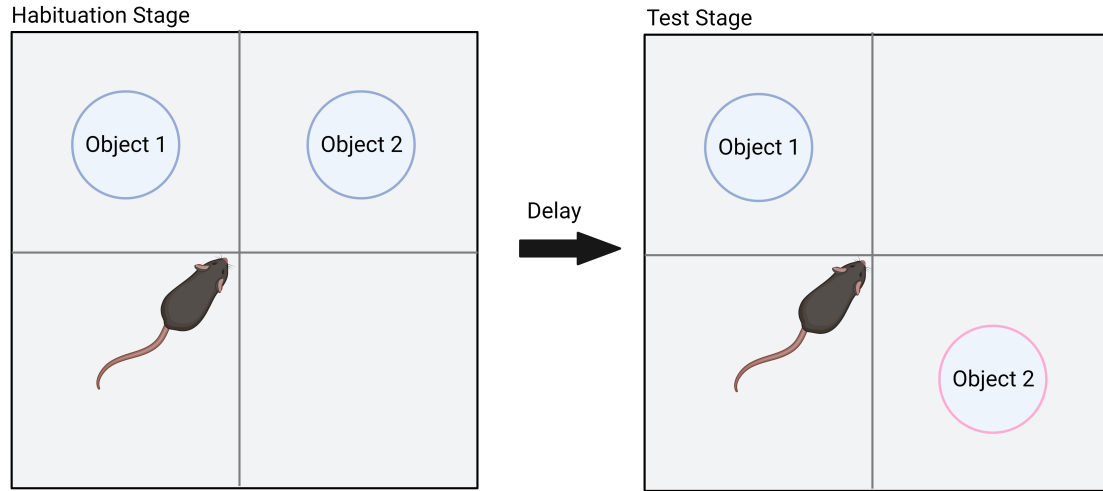


Figure 2.4: Schematic diagram of the novel object location (NOL) test. The arena is digitally divided into 4 quadrants, with the center of the northeast (NE), northwest (NW), southeast (SE), and southwest (SW) corners which serve as placement locations for two identical objects (rods, radius = 1 cm, height = 5 cm). Created with BioRender.

with 70% ethanol and dried in between sessions to eliminate any potential odor cues left beforehand. To determine the sign of cognitive decline, we calculated Z-score using the following equation:

$$Z_{score} = \frac{T_{SE}}{T_{SE} + T_{NW}}$$

where T_{SE} is the amount of time spent exploring the object in the novel position, T_{NW} is the amount of time spent exploring the object in the same position. The z-score of 0.5 or below, indicating the mouse not remembering one object being moved to a novel location, thus was considered as the sign for cognitive decline [29].

2.2.5 Animal preparation

All animal-based experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Brown University (IACUC-1612000239), according to the guidelines and policies of office of laboratory animal welfare and public health service, National Institutes of Health. We conducted a power calculation (α of 0.05 and 80% power), with the intra-group mean difference in vessel diameter of about 6 μm between young and old AD mice and the inter-group standard deviation of about 2 μm from the literature [2], which suggested 2 mice per group. We used 4 mice per group by considering unexpected risks associated with the longitudinal study. Our design has two groups: wild-type (WT) and 3xTg-AD (AD) mice. Following this design, male WT mice ($n = 4$, strain C57BL/6, Jackson Lab) and AD mice ($n = 4$, strain 3xTg-AD, B6;129-Tg(APP^{Swe},tauP301L)1Lfa Psen1^{TM1Mpm}/Mmjax, Jackson Lab) [18, 30], both groups with 9 weeks of age (WOA), underwent craniotomy surgeries following the widely used protocol [31]. The animal was kept under isoflurane anesthesia (1.5%, delivered with 1 L/min O₂) during imaging. Oxygen saturation, pulse rate, and temperature were continuously monitored with pulse oximetry and a rectal probe throughout the surgical procedure and imaging experiments. The body temperature was maintained at 37 °C and the pulse rate remained within the normal range of 250 - 350 pulse/min. The mouse weight and administered drug amount were checked every week. We performed the longitudinal experiment involving the described OCT imaging, NOL test, and continuous drug treatment from 11 to 57 WOA, but we had to pause data acquisition for 3 months in the middle of this 1-year long experiment for a logistic reason.

2.2.6 Bielschowsky Silver Staining (BSS)

The coronal sections were deparaffinized and rehydrated, then incubated in preheated (37 °C) 20 % silver nitrate solution for 25 min. While the slides (N = 4 sections per animal) were held in warm distilled water, ammonium hydroxide 28-30 % was added drop by drop in the silver nitrate solution until precipitate disappeared. The slides were placed back into the silver nitrate solution with added developer for 5-15 min until the sections became dark brown. They were then washed in distilled water for 5 min, placed in 5 % sodium thiosulfate solution for 5 min, dehydrated, and mounted [32]. For quantitative analysis, we used ImageJ to binarize two coronal sections for each animal based on a threshold value by which the amyloid plaques were well segmented. We applied this threshold (80 when the max pixel intensity was 255 [8-bit image]) consistently to all the slides, then quantified area fraction of amyloid plaque burden in the hippocampal region.

2.2.7 Statistical analysis

The statistical results are denoted as mean value $\pm$ standard error (SE) measured after each experiment. For the statistical comparison between young and old ages Figure 2.8, the Kolmogorov-Smirnov test was used for testing normality, and then Student t-test was performed since data were normally distributed. The p-value lower than 0.05 was considered statistically significant.

2.3 Results

2.3.1 Evaluation of cognitive impairment using NOL and BSS

To confirm cognitive impairment in 3xTg-AD mice known to occur around 36-44 WOA [30], we conducted the NOL test every 4 weeks for all mice groups. We estimated all of the NOL Z-scores in which the score of 0.5 (along with linear regressions) were used to determine the sign of cognitive decline in our mice groups. As shown in Figure 2.5a, blue dots represent the mean of Z-scores with standard errors (SE) for WT group, displaying a slight decrease with the slope of -0.00123 estimated by linear fit (black dotted line). The average of Z-scores changed from 0.62 to 0.56 for 11 and 57 WOA, and there was no crossing below the 0.5 threshold, implying no sign of cognitive decline. As a confirmation for AD neuropathological change, we performed BSS as shown in Figure 2.5a inset and the mean (and SE) of regional A β plaque burden (fractional area) was $0.15 \pm 0.02\%$ for WT group. For AD group, the fitted line had the slope of -0.00476 (about 4-fold decrease compared to WT) and did cross the 0.5 threshold at around 40 WOA (denoted as red shaded region with the 5 weeks width), and the mean of Z-scores for 11 and 49 WOA decreased from 0.64 to 0.46, as shown in Figure 2.5b. The mean (and SE) of regional plaque burden for the AD group was significantly higher ($1.52 \pm 0.30\%$, $p=0.0013$) than that of the WT group.

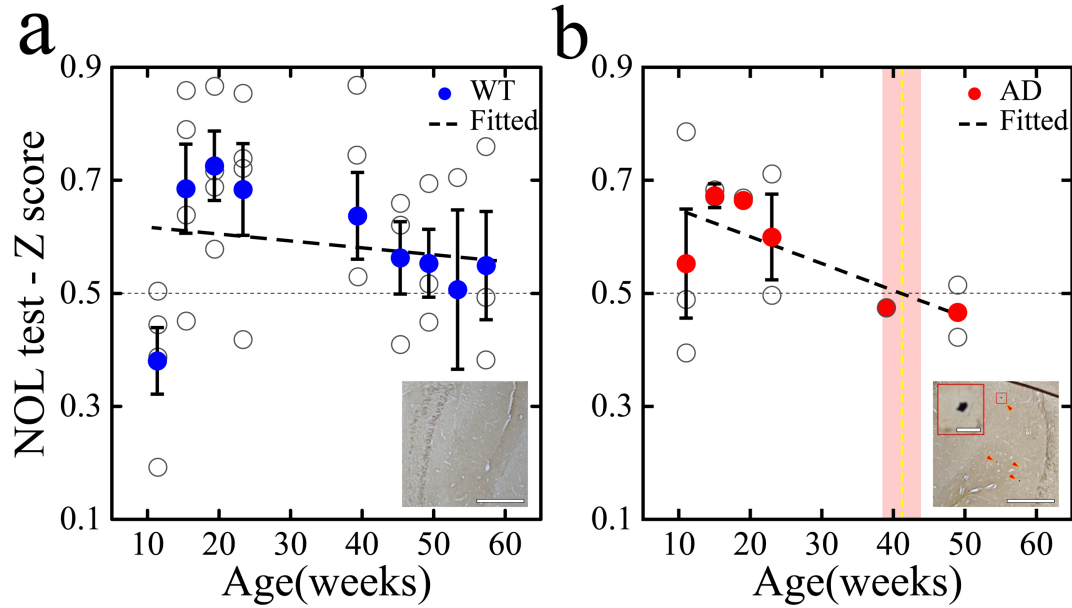


Figure 2.5: Cognitive impairment determined by NOL Z-scores and the quantification of amyloid deposits by BSS images. (a) NOL Z-score result with the fitted line (black dotted line) for (a) WT group (individual animal data points denoted as gray circle). (b) AD group and the sign of cognitive decline denoted as red shaded region. Inset represents BSS image with amyloid plaque (red arrow heads). Scale bars: 500 μm and inset 50 μm .

2.3.2 Temporal dynamics of early vascular alterations in AD

We investigated the WT group for pial vessel diameter change using 4 mice from 11 to 57 WOA with a total of 16 vessels (see color lines in Figure 2.6a for an example of selected vessels, with one of them shown in detail in Figure 2.6b). As exemplified in Figure 2.6b, we measured the diameter of each vessel at each age point by fitting the averaged cross-sectional profile of the vessel to a Gaussian function and taking the FWHM as the diameter. The WT group ($N = 16$ vessels) showed a slight increase in pial vessel diameter (from $30.8 \pm 2.3 \mu\text{m}$ to $37.4 \pm 3.6 \mu\text{m}$ between 11 and 57 WOA), with a fitted slope of 0.078 as shown in Figure 2.6c. In contrast, the AD group ($N = 11$ vessels) showed a 21% decrease in pial vessel diameter from $31.6 \pm 1.7 \mu\text{m}$ to $25.0 \pm 4.5 \mu\text{m}$ between 11 and 57 WOA shown in Figure 2.6f. Interestingly, the tendency of decreasing was noticeably different between early and late stages with the turning point around 20 WOA, consistent with the

early alterations of brain vascular volume and morphology observed in the 3xTg-AD mice [33, 34]. The slopes were -0.25 and -0.08, for the early and late stage, respectively (green and magenta lines in Figure 2.6f). The absolute value of these two slopes differs by about 3-fold. Considering this tendency, we applied the exponentially decaying function: $y = A + Be^{(-x/\tau)}$ resulting in the decay rate of 0.052 ($R^2 = 0.74$). The decay time constant (τ : corresponding to $1/e$) was 19.4 (denoted as a red shaded region with yellow dotted line in Figure 2.6f, implying that the 36.7% of the total diameter changes already occurred at 19.4 WOA. As a result, the change in pial vessel diameter occurred around 20 WOA, much earlier than that of cognitive decline observed at 40 WOA.

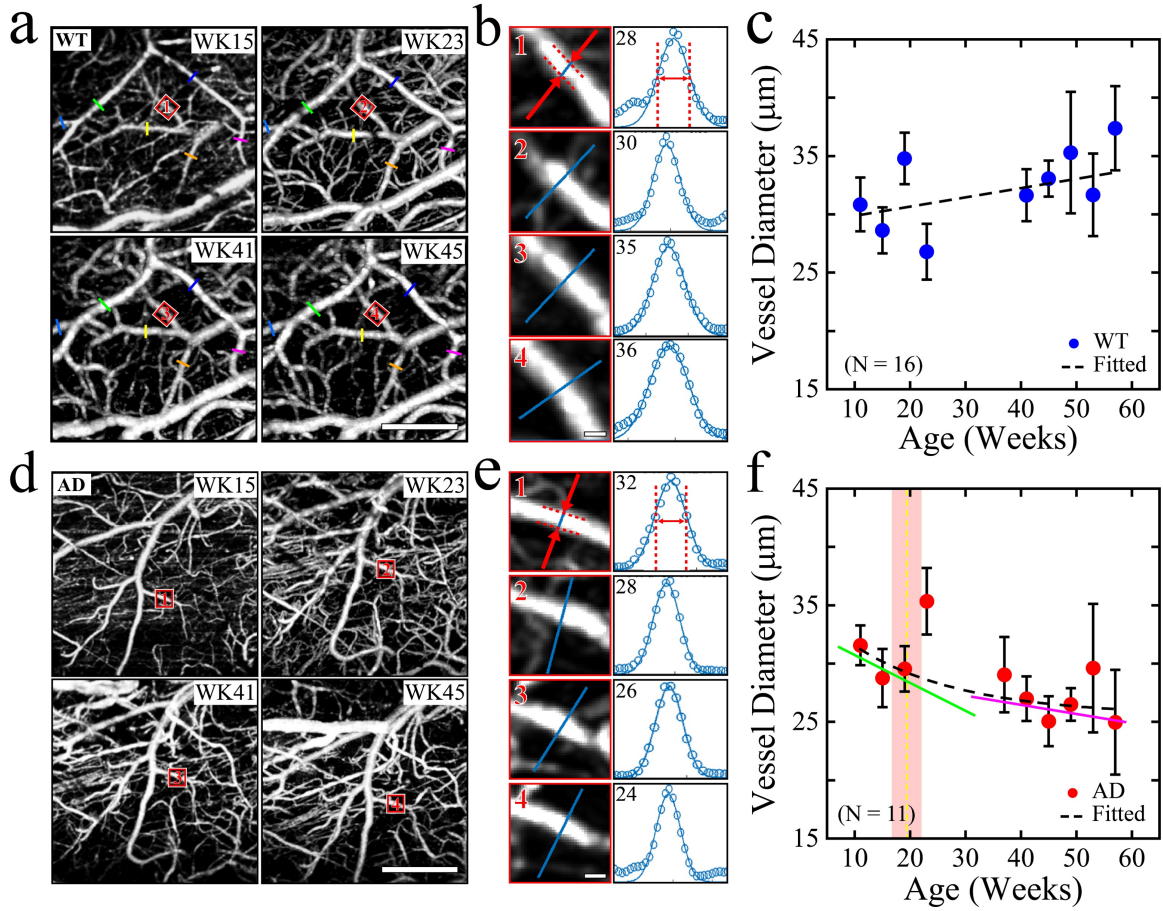


Figure 2.6: Quantification of pial vessel diameter using OCTA images. (a) Example MIP images of a selected set of age points (15, 23, 41, and 45 WOA) for one of the WT mice. Color lines indicate the selected set of same vessels over which the diameter was measured and tracked. The red boxes indicate the representative vessel with which the vessel diameter measurement process is shown in detail in (b). (b) Left: enlarged views of the representative vessel at each age point. The blue lines represent the line along which the cross-sectional profile was extracted. We extracted ten adjacent cross-sectional profiles and used their average (see Materials and Methods for details). Right: the intensity profile (circles) with its Gaussian fit (line). Red line illustrates an example of the measured FWHM as diameter. (c) Pial vessel diameter change of WT group (N = 16 vessels) with mean (blue dots) and standard error from 11 to 57 WOA. (d) MIP images for AD group. (e) Left: diameter tracking of the same vessel (numbered red boxes) for AD group. Right: intensity profile of the vessel with gaussian fit and estimated FWHM values as diameter. (f) Pial vessel diameter change of AD group (N = 11 vessels) with mean (red dots) and standard error. Red shaded region with the yellow dotted vertical line represents the decay time constant τ (around 20 WOA). Scale bars: 500 μm in (a) and (d), 50 μm in (b) and (e).

To further investigate vascular alterations with respect to dynamics, we acquired the 3D blood flow maps using DOCT which can distinguish between penetrating arterioles and venules. We focused the depth between 250 μm and 350 μm (to which the visualization of arterioles and venules were optimized) and extracted the flow and diameter information of the same individual vessels (arterioles and venules) over time. Figure 2.7a (upper) represents the merged *en face* image of DOCT and corresponding OCTA images from two different timepoints (15 and 45 WOA). A typical arteriole and venule (denoted as cyan and a magenta boxes) for WT group are displayed in Figure 2.7a (lower). For the selected arteriole, the values for diameter and blood flow were 40 μm and 1.3 $\mu\text{L}/\text{min}$ for both 15 and 45 WOA. The selected venule had the values of 25 μm and 24 μm for diameter and blood flow of 0.3 $\mu\text{L}/\text{min}$ at 15 and 45 WOA in WT group. Further, we calculated the relative change in diameter and confirmed no significant change over time with the slopes of 0.0010 and 0.0009 for arterioles and venules, approximately (Figure 2.7b and 2.7c). For blood flow, we also confirmed no significant change with the slopes of 0.0004 and -0.0010 in arterioles and venules, approximately (Figure 2.7d and 2.7e).

On the other hand, the diameter in the AD group for the selected arteriole (cyan boxes) decreased from 36 μm to 32 μm as shown in Figure 2.7f(lower). Based on the overall tendency found in pial vessel diameter change from OCTA analysis, we applied the exponential fit. For the relative change in diameter for all arterioles, we obtained the decay rate of 0.07 and τ of 12.9 WOA (denoted as a red shaded region with a yellow dotted line) as shown in Figure 2.7g. In addition, the blood flow values for the selected arteriole were 1.3 and 0.9 $\mu\text{L}/\text{min}$ shown in Figure 2.7f (lower). The overall tendency showed the decay rate of 0.06 and τ of 17.9 WOA (denoted as a red shaded region with a yellow dotted line, Figure 2.7i. For venules, the values for diameter and blood flow of the selected venule (magenta boxes) were 29 and 25 μm , 0.4 and 0.2 $\mu\text{L}/\text{min}$ for 15 and 45 WOA, as shown in Figure 2.7f (lower). The relative change of the diameter in venules did not show exponential decay tendency, however, still a slight decrease with the slope of -0.0007 using a linear

fit as shown in Figure 2.7h. Despite this linearly decreasing tendency, the relative change of blood flow showed exponentially decaying tendency with the obtained decay rate of 0.08 and τ of 11.8 WOA (denoted as yellow dotted line in a red shaded region, Figure 2.7j). When analyzing the DOCT result by vessel types, penetrating arterioles seemed to be more influenced where both diameter and flow exhibited exponentially decaying trends starting in early ages. For penetrating venules, only the flow showed such a trend. This might be possibly due to the different nature of alterations such as increased tortuosity for arteries and excessive collagen depositions in veins [35], although many other underlying mechanisms could be involved in explaining this discrepancy between arterioles and venules.

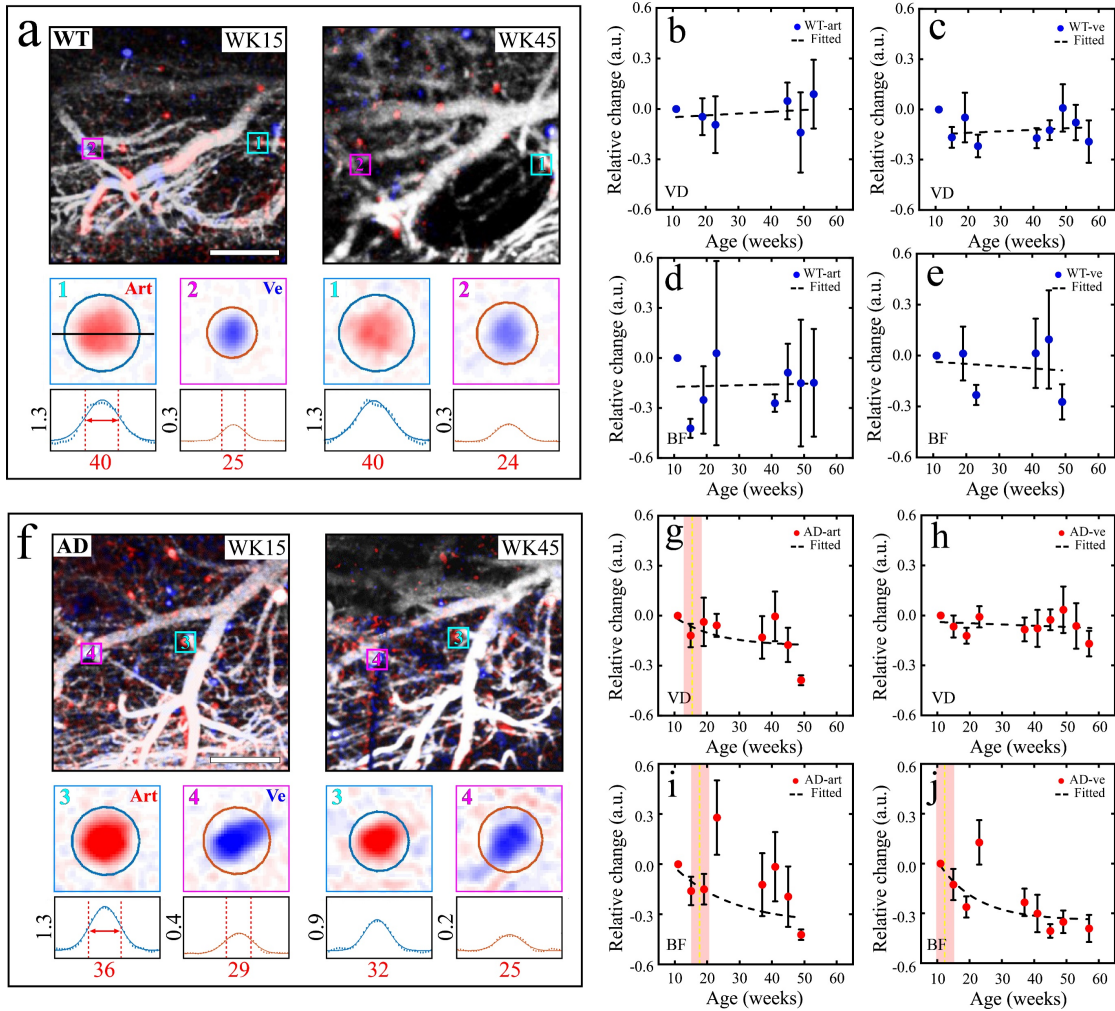


Figure 2.7: Measurement of vessel diameter and blood flow using DOCT. (a) Upper: merged images of DOCT and OCTA *en face* image for 15 and 45 WOA of WT group at the depth of 320 μm beneath the cortical surface. Lower: enlarged views of numbered boxes, arteriole (cyan), venule (magenta). Intensity profile (dotted curve) with gaussian fit (solid curve) and estimated FWHM values as vessel diameter (dotted red lines). Relative change for vessel diameter of WT group (b) for arterioles and (c) for venules. Also, for blood flow (d) in arterioles and (e) in venules. (f) Upper: merged images of DOCT and OCTA *en face* image for 15 and 45 WOA of AD group at the depth of 290 μm beneath the cortical surface. Lower: enlarged views of numbered boxes, arteriole (cyan), venule (magenta). Intensity profile with gaussian fit and estimated FWHM values as vessel diameter. Relative change for vessel diameter of AD group (g) for arterioles and (h) for venules. Also, for blood flow (i) in arterioles and (j) in venules. Red shaded region with the yellow dotted line represents the decay time constant. Scale bar: 500 μm .

2.4 Discussion and Conclusion

We have successfully validated the technical feasibility of longitudinal OCT imaging using OCTA and DOCT techniques over a 1-year timespan. We investigated the temporal dynamics of vascular alterations such as vessel diameter and blood flow change in relation to the result from cognitive test (NOL test) in 3xTg mice. As a result, AD group showed the sign of cognitive decline at around 40 WOA, consistent with previous studies using the same mouse (3xtg) [30]. Interestingly, the pial vessel diameter change from OCTA analysis had two different tendencies where the slope for early stage (before 20 WOA) was -0.25 (estimated by linear fit) and that for late stage (after 30 WOA) was -0.08, about 3-fold high in magnitude for the early stage. To better interpret, this trend was well fitted by an exponential function with a decay time constant τ (19.4), implying that about 37 % of total change in pial vessel diameter occurred at 20 WOA in AD group, approximately. This result shows not only early pial vessel diameter change preceding cognitive decline (40 WOA) but also the majority of total diameter change occurring during the early stage. Regarding early vascular alterations in AD, Meyer et al. reported structural alterations in microvasculature such as vessel deformation and elimination are found at young ages (~ 3 months) of APP23tg model using fluorescence TPM imaging, even when amyloid beta plaques are not yet present [34]. Thus, our finding shows its consistency with previous studies but also provides a better understanding of temporal dynamics in vasculature change.

DOCT shows its capacity of distinguishing penetrating arterioles and venules based on phase-shifts of red blood cells scattering within the vessels. It provides the absolute blood flow and diameter information from arterioles and venules. AD group showed the exponentially decaying tendency (τ of 12.9 WOA) in the relative change of diameter over time for arterioles, shown in Figure 2.7g. The amount of decrease in mean diameter for arterioles from early (before 20 WOA) to late (after 40 WOA) stage was 18.3% ($p < 0.05$)

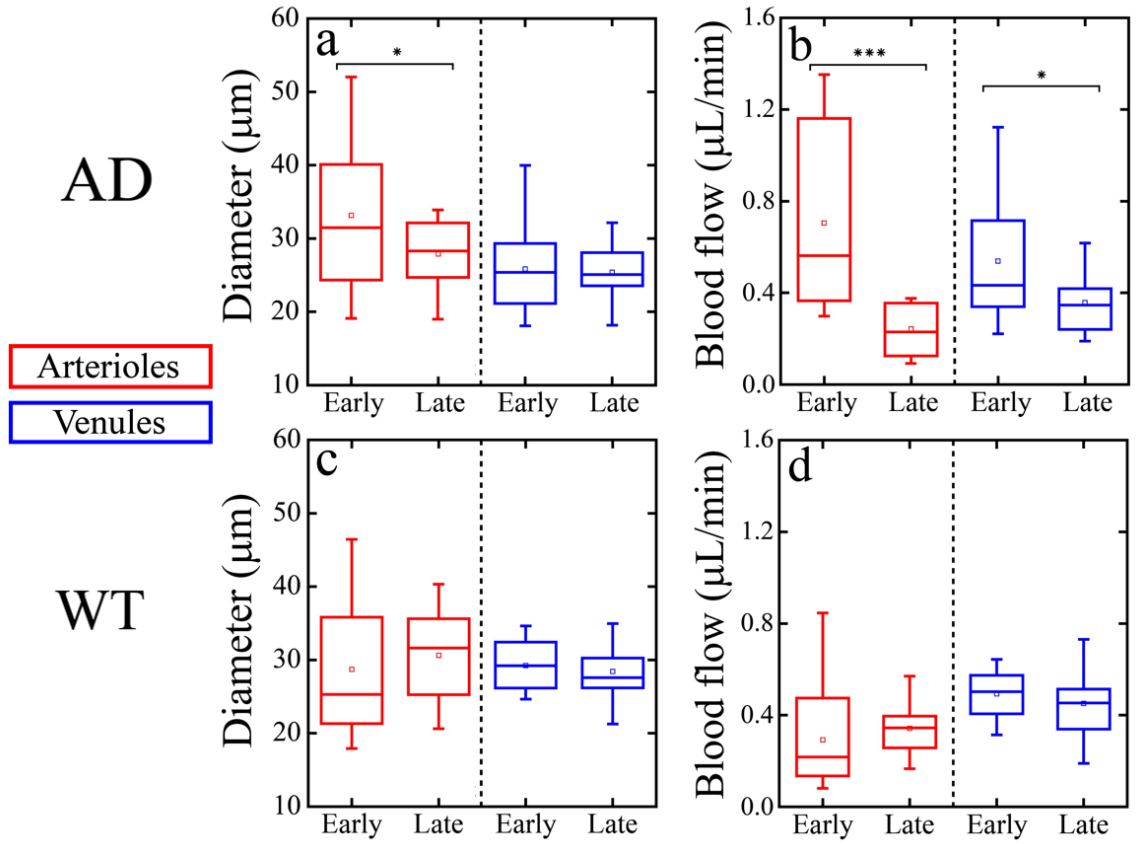


Figure 2.8: Comparison between early and late values of vessel diameter and blood flow for AD and WT group. (a) and (c) distributions of diameters for penetrating arterioles and venules in AD and WT. (b) and (d) distributions of blood flow for penetrating arterioles and venules in AD and WT. The chart shows the mean value (square inside the box), median value (line inside the box), the interquartile range (box), and the minimum and maximum values within 1.5 times the interquartile range (whiskers).

as shown in Figure 2.8a (left). On the other hand, for venules, only a slight linear decrease was observed over time, seen in Figure 2.7h. The decreasing amount in mean diameter from early to late stage was 1.7% for venules, shown in Figure 2.8a (right). Interestingly, the change in mean diameter for arterioles was about 10-fold high to that of venules. Such tendencies could be possibly due to increased tortuosity in arterioles [35, 36], leading to more influence of the diameter change observed from arterioles in our result. With respect to amyloid deposition in AD pathology, this result could also be explained by majority of amyloid accumulation occurring in arterioles whereas less involvement in venules [37].

Furthermore, we measured absolute blood flow for penetrating arterioles and venules in AD mice. For the relative change in arterioles, τ was 17.9 WOA, implying early alterations are highly correlated with both diameter and blood flow changes. For venules, the relative change of blood flow showed τ of 11.8 WOA whereas the diameter change shows a relatively slow decreasing tendency. The amount of decrease in mean blood flow from early to late stage for arterioles was 61.3% ($p < 0.001$) as shown in Figure 2.8b (left). For venules, the amount of change was 33.3% ($p < 0.05$) as shown in Figure 2.8b (right). Blood flow and diameter change for venules show a low correlation in overall tendency of alteration. However, it still presents that the blood flow change in venules show an exponentially decaying trend similar to arterioles in AD group. Regarding early blood flow change in AD mice, Guo *et al.* recently reported that age- and brain region-associated alterations of cerebral blood flow in early stages (at around 2 and 3.5 months) of A β PPSWE/PS1 Δ E9 transgenic mice [38]. WT group showed no significant change in diameter and blood flow for penetrating arterioles and venules over time shown in Figure 2.8c and 2.8d, and consistent with OCTA analysis shown in Figure 2.6c. Additionally, it should be noted that due to logistic reasons, we had to pause data acquisition for 3 months in the middle of the 1-year long experiment, leading to missing data points as can be seen in Figure 2.5 - 2.7. However, this missing data at the middle would not significantly weaken our major conclusion of Figure 2.8 which compares the vascular properties between young and old ages. Also, the missing data may not make the fitting of the whole-period data to a linear line untrustworthy, unless a vascular property should exhibit a severely non-monotonic trace like a Gaussian function. Regarding the specific model used in this paper, studies reported that 3xTg-AD mice have sex differences in terms of their pathology [39, 40] and treatment response [41, 42]. Yet, the present study only used male mice to focus more on the vasodilation effect and cognitive decline. Extending the described approach to both sexes and considering the sex as a key biological variable will be important in future work.

In summary, we have demonstrated and validated the technical feasibility of OCT for label-free *in vivo* studies to investigate cerebrovascular alterations over 1-year timespan in 3xTg mice. With a 3D volumetric imaging, OCTA can measure the diameter change of individual pial vessels over time, and the DOCT technique can distinguish penetrating vessels into arterioles and venules and provide diameter and absolute blood flow measurements. Moreover, we found that vascular alterations such as vessel diameter and absolute blood flow change (20 WOA) precede cognitive decline (40 WOA) in 3xTg mice. Interestingly, the amount of mean diameter change over age for penetrating arterioles was about 10-fold high to that of penetrating venules in 3xTg mice, suggesting the importance of possibly monitoring the arteriolar alterations in early stages of AD.

2.5 Additional study: the effects of early vasodilation

2.5.1 Method

To determine whether vascular alteration is correlated with cognitive decline in 3xTg mice, we used 3 different drugs with vasodilating effects [43–45]: nicorandil, memantine, and donepezil (N3539, PHR1886, PHR1584, Sigma-Aldrich). The drugs were dissolved by aqueous buffer, phosphate-buffered saline directly, then administered via drinking water starting after 15 WOA, with the dose of 15, 30, 4 mg/kg/day for nicorandil, memantine, and donepezil, respectively. We used the same AD mouse model as before ($n = 4$ each group, 9 WOA, 20 - 25 g in-weight, strain 3xTg-AD, B6;129-Tg(APP^{Swe},tauP301L)1Lfa Psen1^{TM1Mpm}/Mmjax, Jackson Lab). Our design has three drug-treated groups: nicorandil-treated (NC), memantine-treated (MM), and donepezil-treated (DN) groups. We have used the same experimental protocols for animal preparation, imaging, and NOL tests (see section 2.2.5 for details).

2.5.2 Results

As shown in Figure 2.9, for NOL score, the three drug-treated groups did not show neither a decreasing trend nor a crossing below 0.5 threshold, with the slopes of 0.00229, 0.00253, 0.0004 for NC, MM, and DN, respectively. From BSS analysis, the means of regional plaque burden were significantly less in three drug-treated groups than in the AD group: 0.48 ± 0.09 % ($p=0.013$), 0.89 ± 0.03 % ($p=0.039$), 0.78 ± 0.06 % ($p=0.024$), for NC, MM, and DN groups, respectively.

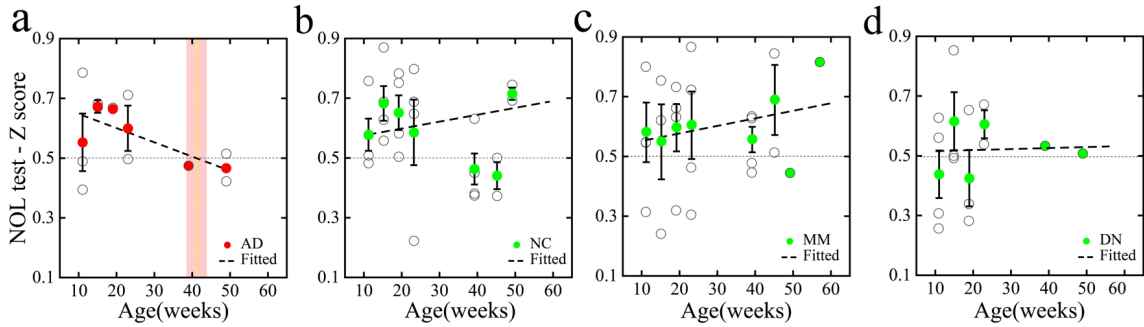


Figure 2.9: Cognitive impairment determined by NOL Z-scores in drug-treated mice. NOL result with the fitted line (black dotted line) for (a) AD group, (b) NC group, (c) MM group, (d) DN group.

Using OCTA images, the total number of selected pial vessels (color lines) were 19, 19, and 38 for NC, MM, and DN group using 4 mice, respectively, as displayed in Figure 2.10. Unlike the AD sham group, the overall tendency of pial vessel diameter change in the NC group showed a slightly increasing tendency with the slope of 0.065, shown in Figure 2.10b, implying vasodilating effect may have contributed to prevent the decrease in pial vessel diameter found in AD group. The similar tendency was found for MM and DN groups, with the slopes of 0.042 and 0.105, respectively, seen in Figure 2.10c and 2.10d. This result from MM and DN group seemed to similarly represent the vasodilating effects observed from NC group.

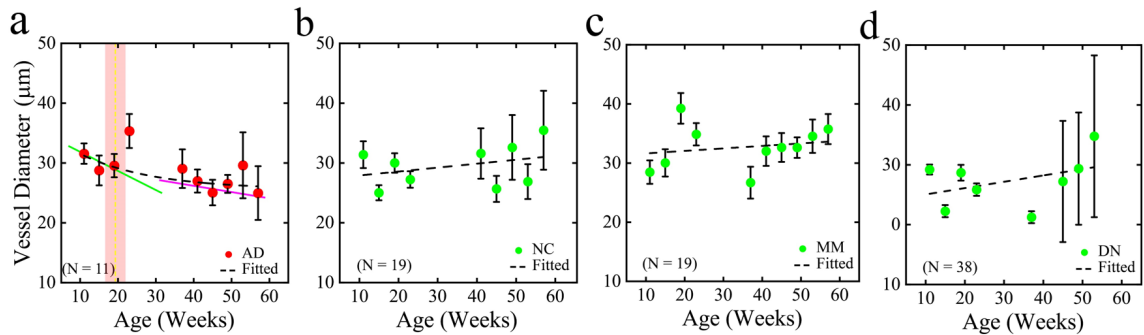


Figure 2.10: Pial vessel diameter quantification using OCTA for 3 drug-treated groups compared to AD sham group. (a) AD sham group (N = 11 vessels), (b) NC group (N = 19 vessels), (c) MM group (N = 19 vessels), (d) DN group (N = 38 vessels).

From DOCT analysis, all three drug-treated groups showed no decreasing tendency observed in the AD group, as displayed from Figure 2.11a to 2.11d. Interestingly, the slope for arteriolar diameter of DN group was 0.0034, about 6-fold high compared to NC and MM group. This could be possibly due to DN specifically dilating parenchymal arterioles [45], thus leading to the higher slope than other two drug groups. For arteriolar flow, the relative changes of fitted lines remained near zero rate and did not exhibit exponentially decaying tendency observed in the AD group, as shown in Figure 2.11e to 2.11h. In penetrating venules, all three drug-treated groups exhibited slightly increasing slope for diameter change also similar to that in the AD group (2.12). For venular flow, the DN group had increasing slope (0.0035) whereas both NC and MM groups showed slight decreasing tendency with age (the fitted slopes listed in Table 2.1).

Table 2.1: Slopes of relative change in diameter and blood flow for penetrating arterioles and venules for drug-treated mice. The numbers (N) represent vessel numbers.

	Diameter			Blood Flow		
	NC	MM	DN	NC	MM	DN
Arterioles	0.0007 (N = 12)	0.0005 (N = 6)	0.0034 (N = 17)	-0.0011 (N = 12)	0.0010 (N = 6)	-0.0027 (N = 17)
Venules	0.0009 (N = 11)	0.0011 (N = 7)	0.0009 (N = 19)	-0.0004 (N = 11)	0.0020 (N = 7)	0.0035 (N = 19)

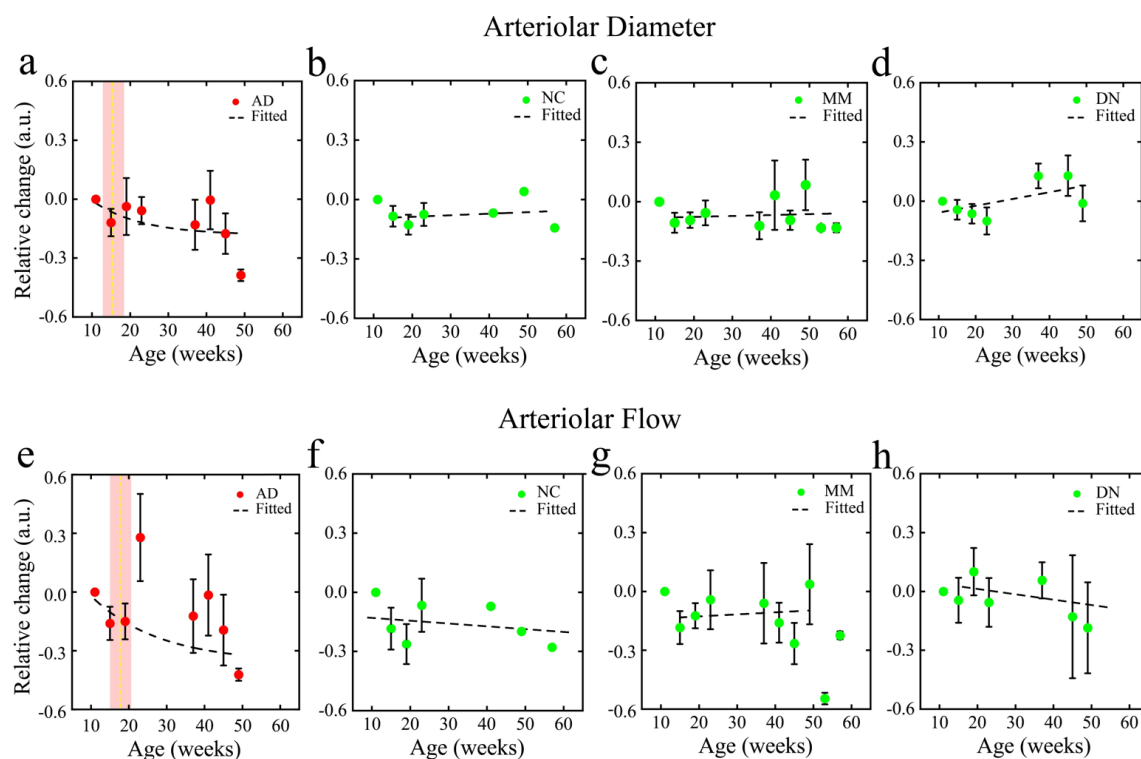


Figure 2.11: Relative change of penetrating arterioles from DOCT analysis for diameter and flow. (a-d) diameter change, (e-h) flow change for AD, NC, MM, DN groups.

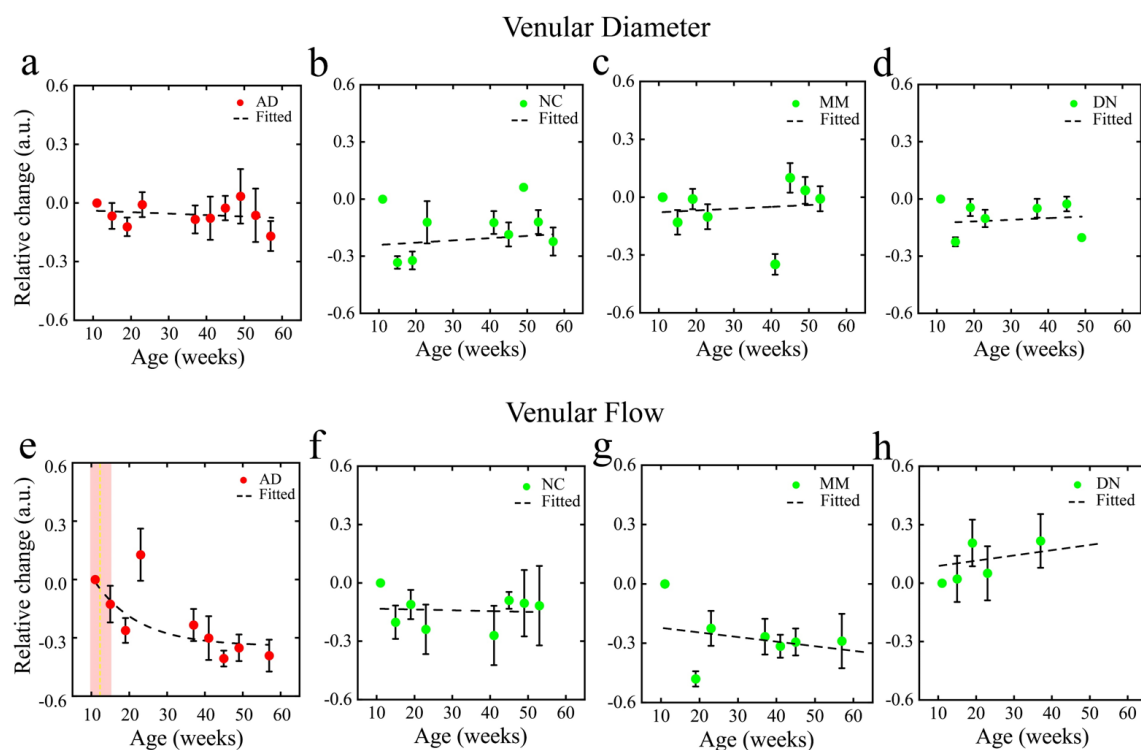


Figure 2.12: Relative change of penetrating venules from DOCT analysis for diameter and flow. (a-d) diameter change, (e-h) flow change for AD, NC, MM, DN groups.

2.5.3 Discussion

In this additional study, we used three drugs that are known to have vasodilating effects and administered (via drinking water) to the same mouse model (3xTg) to investigate how early vascular intervention affect vascular properties and also cognitive decline (shown at around 40 WOA). As a result, three drug-treated mice groups did not show any sign of cognitive decline. NC is a typical balanced vasodilator which acts as both arterial K^+ ATP channel opener and NO donor. It improves spatial learning and memory in chronic cerebral hypoperfusion-induced vascular dementia [43]. For two FDA-approved drugs, MM improved spatial learning and memory in a rodent model of chronic cerebral hypoperfusion [44]. DN dilates cerebral parenchymal arterioles via the selective activation of neuronal nitric oxide synthase [45]. Based on the literature and the NOL test result (shown in Figure 2.9), these vasodilating drugs may play some roles in mitigating cognitive impairment in 3xTg mice when intervened early. Also, for both vasculature and vasodynamics, there was no exponentially decaying tendency observed from the drug-treated groups. Within the drug-treated groups, the slope for arteriolar diameter change in the DN group was about 6-fold high compared to the NC and MM group as shown in Figure 2.11. This could be possibly due to DN specifically dilating parenchymal arterioles [45], resulting in the higher slope than others. However, due to the small sample size used in this study, there were relatively high variations especially in some of DOCT analysis. Thus for more thorough investigation, future studies with higher statistical power (larger sample size) seem necessary to better understand the effect of early vasodilation on cognitive decline. Nevertheless, our finding from this study still emphasizes the importance of vascular alterations in relation to AD and also suggests the vasodilatory agents could help mitigate cognitive impairment when intervened early.

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Chapter 3

Investigating anesthesia effect on and sensitivity of OCT measurements of microvasculature

3.1 Introduction

The cerebral vasculature is a crucial part of the brain with its primary function as maintaining constant blood flow in supplying nutrients to neurons and glial cells. To investigate the role of cerebrovascular properties in brain functions, many *in vivo* studies have been conducted using different imaging modalities such as functional magnetic resonance imaging, positron emission tomography, and laser doppler flowmetry [1–3]. Regardless of imaging modalities, the use of general anesthesia is often involved in animal experiments to suppress motion artifacts by immobilizing animals during image acquisition. However, the anesthetic use is known to have negative influence not only on cerebral metabolisms and blood flow but also on neurophysiological states [4]. As a consequence, these effects can make experimental results from anesthetized animals more vulnerable for misinterpre-

tation. Therefore, it is important to study the effect of anesthetic agents on cerebral vasculature as a whole to accurately interpret experimental data which then can better transition toward human physiology in awake.

Isoflurane, one of the most widely used vasodilatory anesthetic agents in imaging studies, has proven to disrupt the baseline cerebral blood flow (CBF) and dilate both large vessels and capillaries [5]. It also alters neurovascular coupling by attenuating and delaying hemodynamic response to local neural activity [6–8]. This anesthetic agent affects neurophysiological state by decreasing brain metabolism and reducing neuronal activity [9]. A study with two-photon phosphorescence microscopy shows twice as high oxygenation level under isoflurane anesthesia compared to awake [10]. Cerebral metabolic rate of oxygen is significantly reduced in the anesthetized brain during hypoxia [11]. In contrast, a smaller infarct size was observed from a photothrombotic stroke study [12] suggesting neuroprotective effects of isoflurane. Although the isoflurane effect has been investigated in many areas within cerebral vasculature, there is still a knowledge gap on which vascular properties are affected by this anesthetic agent in an age-related manner, and which are not.

The aim of this study is to address two major limitations found in chapter 2 (validation of technical feasibility), which were the involvement of anesthetic agent (isoflurane) during imaging and the unknown sensitivity of our OCT imaging system. Since isoflurane is well known to affect microvasculature, this effect might distort the time courses of vascular alterations we obtain in the third study. Since the measured slopes are essential when interpreting the longitudinal data to understand temporal dynamics of vascular alterations, we use two mice groups, young (15 WOA) and old (55 WOA), to determine vasodilatory effect of isoflurane in age-related manner that no previous study has done it before. Also using the same OCT techniques (OCTA and DOCT), we estimate the sensitivity of how much change our imaging system is capable of quantifying which would be important for the longitudinal data we obtain in the third study.

3.2 Methods

3.2.1 OCT-Angiography (OCTA)

We have used the same OCT imaging protocol as Chapter 2 (see 2.2.2 for details).

3.2.2 Doppler-OCT (DOCT)

We have used the same OCT imaging protocol as Chapter 2 (see 2.2.3 for details).

3.2.3 Animal preparation

All animal-based experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Brown University (IACUC-1612000239), according to the guidelines and policies of the office of laboratory animal welfare and public health service, National Institutes of Health. Male young and old WT mice ($n = 4$ each, 15 and 55 WOA, strain C57BL/6, Jackson Lab) underwent craniotomy surgeries following the widely used protocol [13] before the first imaging session.

3.2.4 Anesthesia effect measurement experiment

The animal was kept under isoflurane anesthesia (1.5%, delivered with 1 L/min O_2) during imaging. Oxygen saturation, pulse rate, and temperature were continuously monitored with pulse oximetry and a rectal probe throughout the surgical procedure and imaging experiments. The body temperature was maintained at 37°C and the pulse rate remained within the normal range of 250 - 350 pulse/min. The mouse weight and administered drug amount were checked every week.

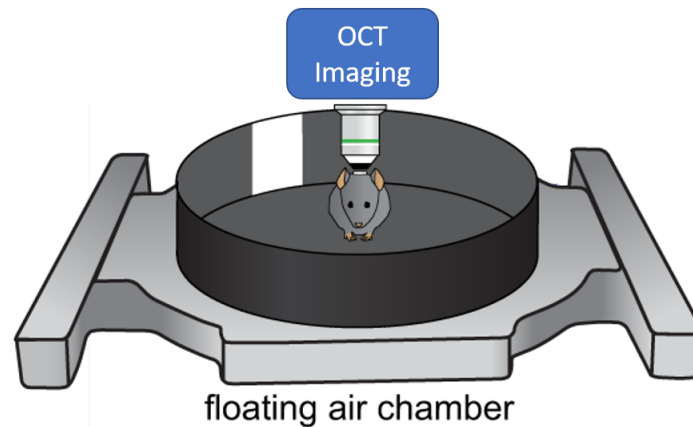


Figure 3.1: Schematic of the awake imaging set-up. Mice are head fixed in a floating chamber and allowed to voluntarily move the chamber floor using their paws, allowing control of their position and orientation within the chamber. Reproduced from [14].

3.2.5 Sensitivity measurement experiment

Seven days after installing a chronic cranial window on a mouse as described in the section 3.2.3, we placed the mouse on an air-floating platform (Mobile Homecage, Neurotar, Figure 3.1) for a head-fixed, freely-walking awake imaging condition, while connecting the mouse nose to the isoflurane vaporizer. The brief (<1 min) anesthesia was induced during head-fixation procedure to minimize handling stress [15]. We acquired nine sets of OCTA and DOCT images, following the exact protocol described in section 3.2.4 for anesthetized imaging (timepoint #2 and #3), at the following time point using young WT mice:

1. Awake as a baseline
2. 5 minutes after turning ON isoflurane supply
3. 25 minutes after turning ON isoflurane supply
4. Right after turning OFF isoflurane supply
5. 7 minutes after turning OFF isoflurane supply
6. 15 minutes after turning OFF isoflurane supply
7. 30 minutes after turning OFF isoflurane supply
8. 45 minutes after turning OFF isoflurane supply
9. 60 minutes after turning OFF isoflurane supply

3.2.6 Statistical analysis

We used the linear-mixed effects model (LME) method which involves animal-specific random effects, excluding any outliers when a residual was more than three scaled median absolute deviations from the median. When this LME method resulted in non-normally distributed residuals, we used a bootstrapped LME method with a bootstrapping number of 2,000. Since this bootstrapped LME analysis, like other bootstrap methods, only provides confidence intervals (CIs), we considered it statistically significant ($p < 0.05$) when the bootstrapped CI with $\alpha = 0.05$ does not involve zero.

3.3 Results

3.3.1 Isoflurane effect in age-related manner

We acquired OCTA data during awake and anesthetized state for young and old WT mice ($n = 4$ each group) and reconstructed the MIP images of these datasets as shown in Figure 3.2 and Figure 3.3. To quantify the structural change of pial vessels from the acquired images, we selected a set of the same individual vessels identified from both awake and anesthetized states (Figure 3.4a, colored lines), and then measured the diameter of each vessel by fitting its cross-sectional profile to a Gaussian function (Figure 3.4b). This allowed us to compare a total of 61 and 59 vessels for young and old mice, respectively. As shown in Figure 3.4c, the pial vessel diameter was significantly larger under anesthesia by 29.5% (95% CI, 23.5 to 35.5; $p = 5.0 \times 10^{-15}$). In comparison between young and old mice, the isoflurane-induced increase in pial vessel diameter was significantly smaller in old mice ($p < 0.05$) as shown in Figure 3.4d.

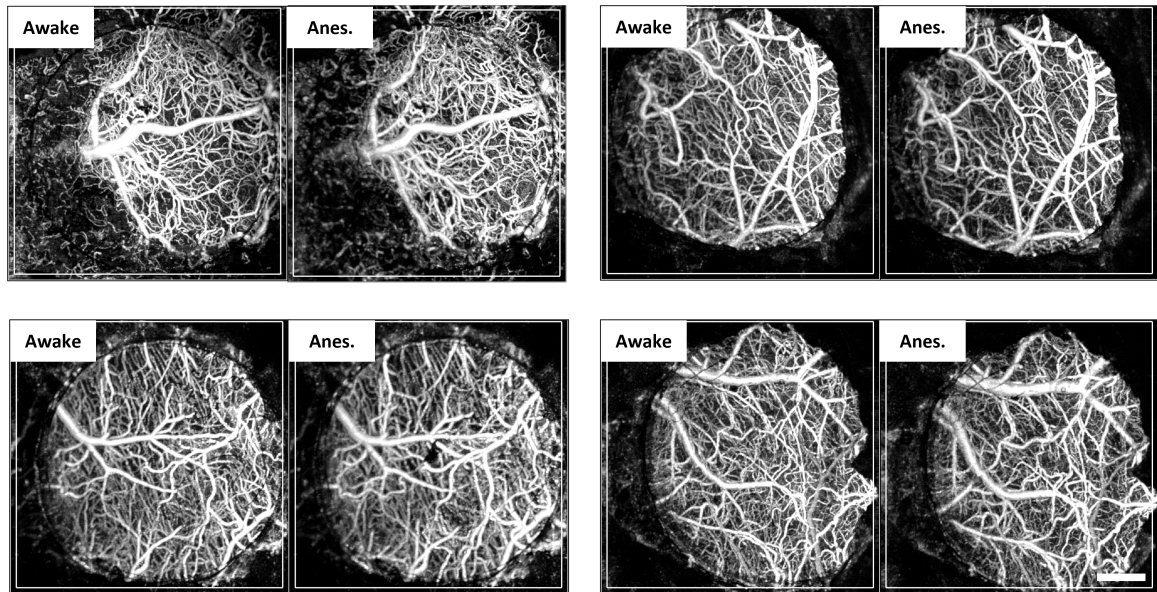


Figure 3.2: Maximum intensity projection images of acquired OCTA data for both awake and anesthetized state in young WT mice (n=4). Scale bar, 500 μm .

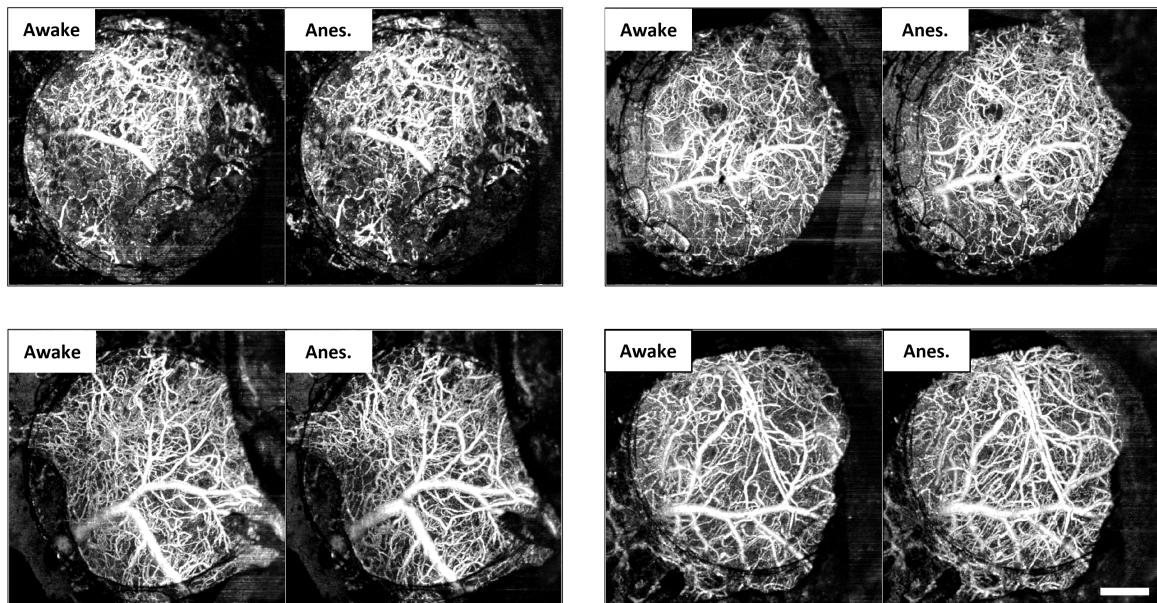


Figure 3.3: Maximum intensity projection images of acquired OCTA data for both awake and anesthetized state in old WT mice (n=4). Scale bar, 500 μm .

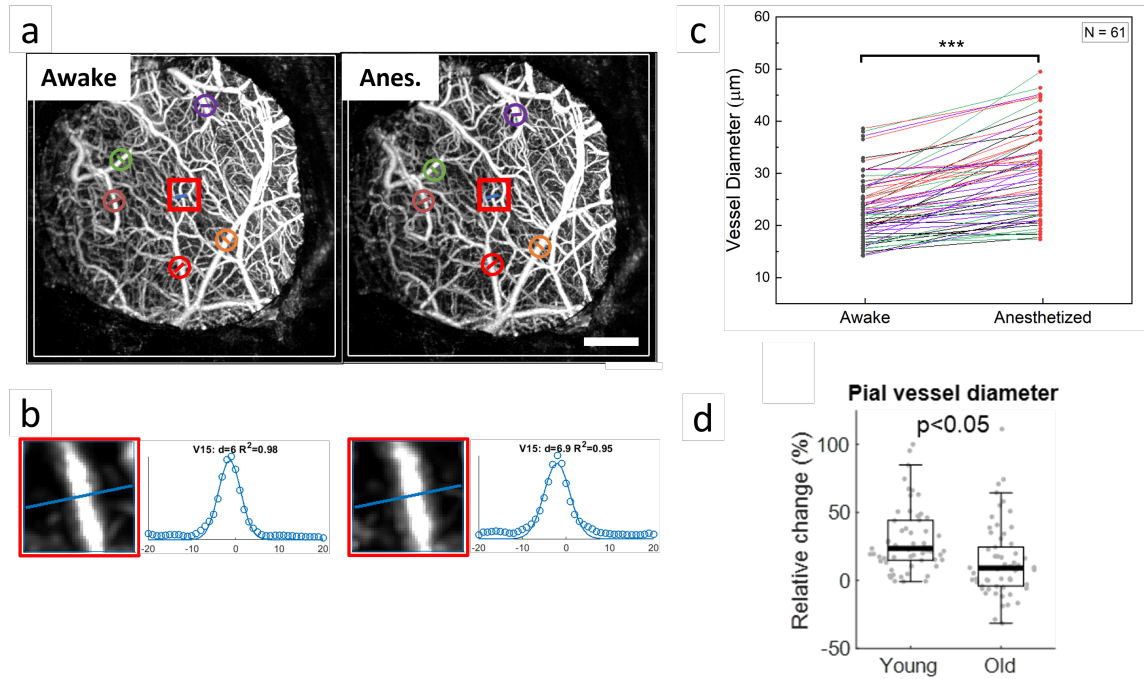


Figure 3.4: The OCTA analysis of Pial vessel diameter change in awake vs. anesthetized state. (a) Comparing a set of same vessels between awake and anesthetized state. Scale bar, $500 \mu\text{m}$. (b) An example of the cross-sectional profile of the same vessel (red box in (a)), and its fitting to a Gaussian function to measure the vessel diameter as the full width at half maximum (FWHM). (c) Pial vessel diameter measurement between the awake and anesthetized state for young mice ($p < 0.001$). (d) Effects of isoflurane on pial vessel diameter. 61 vessels (young) and 59 vessels (old) were measured and analyzed by bootstrapped LME, as traditional LME analysis produced non-normally distributed residuals.

To measure structural and functional changes in penetrating arterioles and venules, we also acquired DOCT images for both awake and anesthetized state in young and old mice. Following the ‘same-vessel tracking’ approach applied in OCTA data, we measured changes in the diameter and blood flow of individual penetrating vessels (Figure 3.5a). The diameter of each vessel was measured by fitting its en-face cross-section to a Gaussian function, while the area-integral method [16] followed by noise reduction [17] was applied for measuring the blood flow. This enabled us to track the same set of 26 and 33 penetrating vessels (11 and 15, 16 and 17 for arteries and veins) in young and old mice, respectively. The isoflurane-induced increase in penetrating vessels (arteriolar diameter, arteriolar flow, venular diameter, venular flow) did not show statistically significant difference between young and old mice shown in Figure 3.5b. All the vascular properties with the results are listed in Table 3.1.

Table 3.1: Slopes of relative change in diameter and blood flow for penetrating arterioles and venules. The numbers (N) represent vessel numbers.

Properties	Relative change (%, 95% CI) in young mice	Effect size of the older age (percent points, 95% CI)	Sample size (young and old) and the used method
Pial vessel diameter	24 to 35	-24 to -6 (p<0.05)	61 and 59 vessels bootstrapped LME
Arteriolar diameter	7 to 27	-12 to 14 (p=0.878)	11 and 16 vessels LME
Venular diameter	3 to 50	-41 to 25 (p=0.608)	15 and 17 vessels LME
Arteriolar flow	24 to 108	-86 to 31 (p=0.335)	11 and 16 vessels LME
Venular flow	-5 to 60	-47 to 43 (p=0.923)	15 and 17 vessels LME

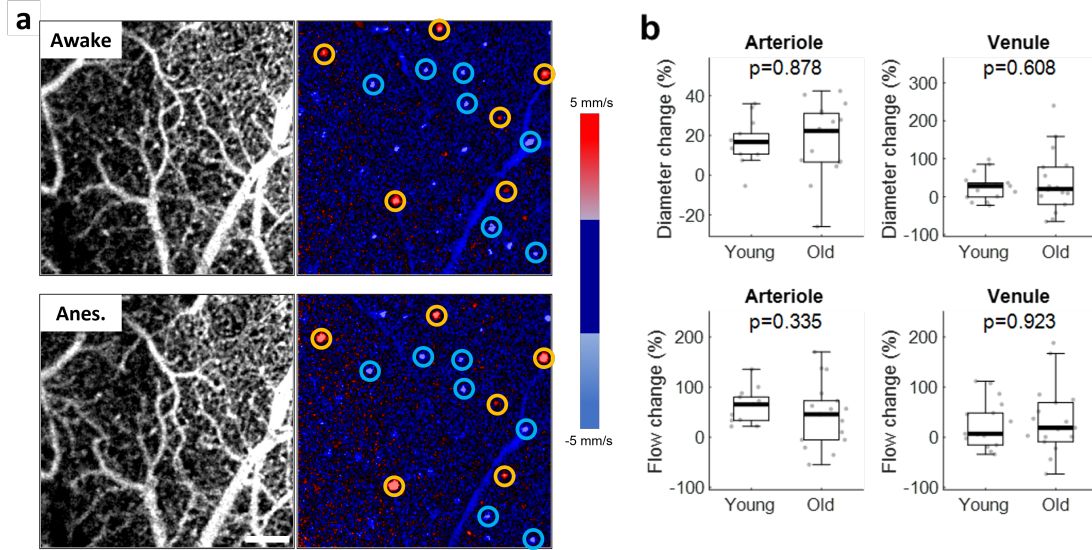


Figure 3.5: The DOCT analysis of penetrating vessel diameter and flow change. (a) *en face* images of DOCT (right) and corresponding OCTA data (left) for awake and anesthetized state in young mouse. Scale bar, 250 μm . (b) Effects of isoflurane on arteriolar and venular diameter and flow. 11 arterioles and 15 venules (young) and 16 arterioles and 17 venules (old) were measured and analyzed by LME. Residuals were normally distributed.

3.3.2 Sensitivity of OCT measurement

We have conducted another experiment to directly quantify the sensitivity of OCT measurement in detecting changes in vessel diameter and blood flow. We used isoflurane anesthesia, which is known to induce vasodilation in cerebral vasculature. As expected, the pial vessel diameter increased with isoflurane and returned to the baseline after recovery (Figure 3.6a). Our approach was sensitive enough to detect a detectable 7% change in the pial vessel diameter between the first awake state and the state 5 minutes after turning on the isoflurane supply. The average pial vessel diameter in the awake state was 19.7 μm , and the increased diameter in the second state was 1.4 μm ($p < 0.05$, $n = 59$ vessels from four animals; Figure 3.6b). Our approach was also robust enough to produce statistically insignificant results between the first and the final states as physiologically expected ($p = 0.932$, Figure 3.6c) while still detecting the 1.4 μm change.

Similarly, the arteriolar diameter and flow increased with isoflurane and returned to the

baseline after recovery, while the venular diameter and flow did not (Figure 3.6d). We found subtle fluctuations during the recovery phase, the degree of which our approach was able to detect. The average arteriolar diameter in the first of the final two states (45 minutes after turning off isoflurane supply) was $27.5 \mu\text{m}$, and the increased diameter in the final state was $1.6 \mu\text{m}$, a 6% change ($p < 0.05$, $n = 7$ arterioles from four animals; Figure 3.6e, top). The average arteriolar flow changed by $0.098 \mu\text{L}/\text{min}$ from $0.409 \mu\text{L}/\text{min}$, a 24% change ($p = 0.052$, Figure 3.6e, bottom). Our approach was also robust in producing statistically insignificant results between the first and the final states as biologically expected ($p = 0.942$ and 0.436 for diameter and flow, respectively; Figure 3.6f).

Overall, the results showed that our approach is sensitive enough to detect changes in vessel diameter and flow, with a conservative detection limit of about 7% for pial vessel diameter, about 6% for arteriolar blood flow, and about 24% for arteriolar blood flow. Although the statistical results presented in the original manuscript support that our approach was sensitive enough to reveal subtle differences in those vascular changes with age between AD and WT mice, this data of directly quantifying the measurement sensitivity can further strengthen the rigor of our approach in the following chapter (Chapter 4).

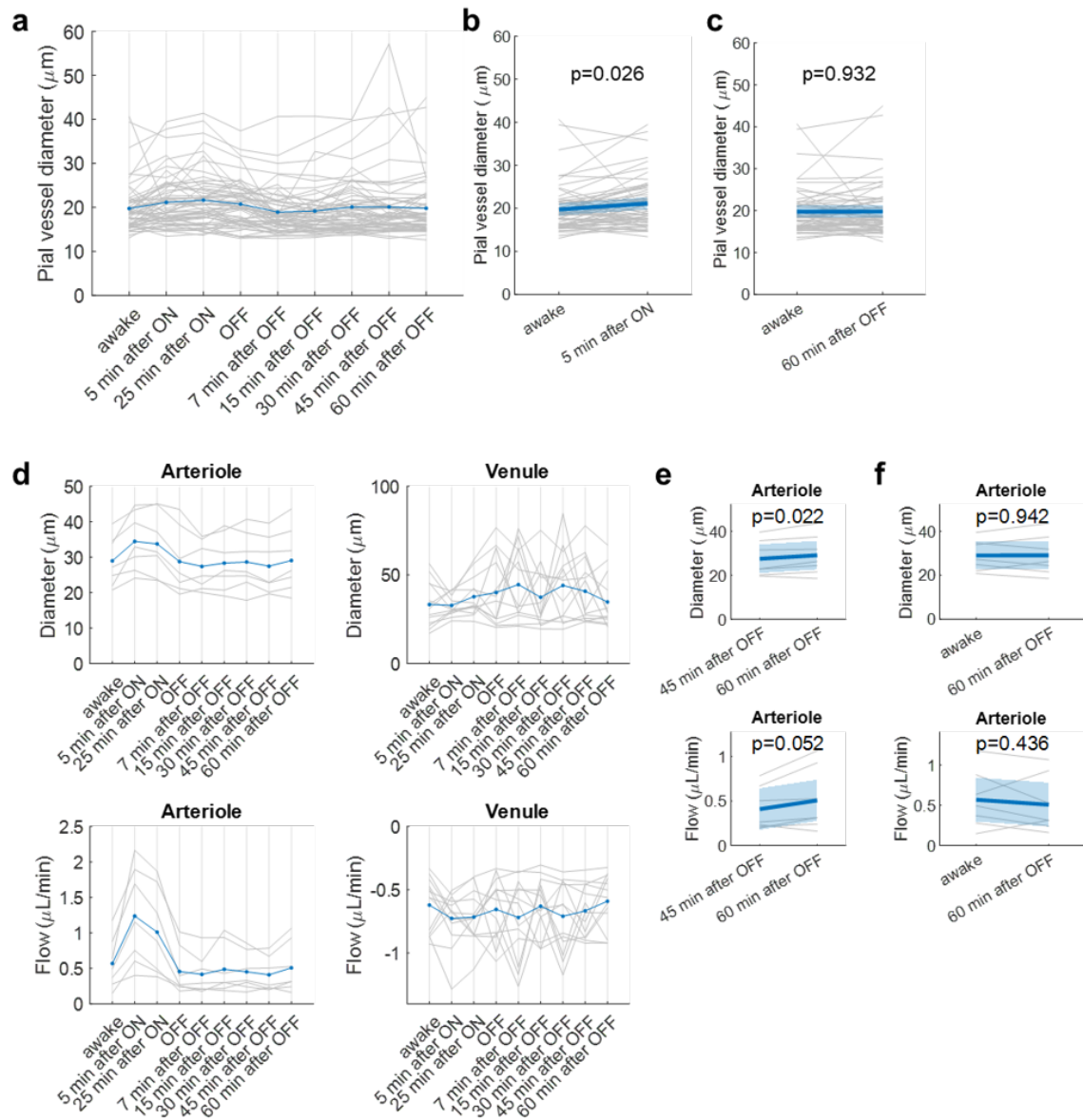


Figure 3.6: OCT-measured changes in vascular diameter and flow during vasodilation and recovery. a) Tracked vessel diameter over 59 same pial vessels in four animals, with the blue line indicating the simple average of the diameters for each time point. b) Pial vessel diameter changes between the awake state and the state after 5 minutes of turning on isoflurane supply. The selected time points produced a p value close to 0.05. c) Pial vessel diameter changes between the first state (awake) and the final state (after 60 minutes of turning off isoflurane supply). d) Tracked vessel diameter and flow over seven same arterioles and 13 same venules in four animals. e) Arteriolar diameter and flow changes between the state before the final state (45 minutes after turning off isoflurane supply) and the final state, with the selected time points producing p values close to 0.05. f) Arteriolar diameter and flow changes between the first and final states.

3.4 Discussion and Conclusion

The sensitivity measurement experiment provides a quantitative measure of the sensitivity of our approach for detecting changes in vascular diameter and blood flow. We found the sensitivity of detecting changes in pial vessel diameter, arteriolar diameter, and arteriolar blood flow to be $1.4\ \mu\text{m}$ (7% change), $1.6\ \mu\text{m}$ (6% change), and $0.098\ \mu\text{L}/\text{min}$ (24% change), respectively. It is important to note that these sensitivity values serve as a conservative estimate of the actual sensitivity of the measurements we obtain in the third study (Chapter 4). It is because this experiment used fewer animals (four, against 10 per group in the third study) and compared only two time points (for anesthetized imaging), whereas the third study employs twelve time points to determine the slope (rate of change with age, RCA). It is likely that the RCA measurement described in the third study has higher statistical power and thereby higher sensitivity.

In the isoflurane effect experiment, both diameter and blood flow showed significant increase during anesthetized state compared to awake, confirming the vasodilating effect of isoflurane. When compared between young (15 WOA) and old (55 WOA) mice, the aging effect was significant only in pial vessel diameter change. This result strongly suggested us to conduct awake imaging in the third study (Chapter 4) using APOE4 mice model for investigating temporal dynamics of vascular alterations in AD progression.

Although this experiment does not aim to investigate the biological mechanisms underlying the observed age-related isoflurane effect, here we discuss several relevant points. First, endothelial dysfunction and oxidative stress may play a role in age-associated vascular dysfunction. It is known that the capacity of vascular endothelium to generate nitric oxide (NO) declines even during normal aging [18]. NO, formed by endothelial NO synthase, plays an important role in cerebral autoregulation such as vasodilation, increased blood flow, decreased vascular resistance, a decrease in smooth muscle cell proliferation,

and inhibitions of platelet aggregation and adhesion [19]. It also protects endothelial cells from apoptosis [20]. Endothelial dysfunction resulting from attenuated NO production due to aging causes vascular dysfunction especially in the brain and heart [18]. Preclinical and clinical studies agree impaired vasodilation and vasoreactivity with aging found in the peripheral and cerebral circulation [21, 22]. In addition to impaired NO synthesis and actions, oxidative stress is considered as one of the main reasons for endothelial dysfunction not only in normal aging[23] but also in neurodegenerative events such as AD [24]. The different state of oxidative stress in endothelium between young and old mice may explain less change in specifically for pial vessel diameter during the isoflurane effect experiment.

Second, the morphological and physiological differences between pial and penetrating vessels may explain our finding. Pial vessels, located on the surface of the brain, have a thicker vascular wall and are surrounded by connective tissue and glial cells, while penetrating vessels have thinner walls (with penetrating arterioles having thicker walls containing smooth muscle cells) [25]. Pial vessels are highly interconnected, and strongly related to the neural activities in the cerebral cortex [26]. In contrast, penetrating vessels are not interconnected, thus in a disruptive event such as stroke, the pial vessel (arterioles) is the main player for regulating blood supply to the vital pathways to the penumbra [27]. Penetrating vessels (arterioles) are more responsible for supplying blood to the deeper tissues of the brain, including the white matter and subcortical structures. In relation to aging, several studies have reported that CBF decreases by about 70-80 % in mice [28, 29], accompanied by morphological and molecular changes in vessels such as increased tortuosity and collagen concentrations [28, 30, 31], possibly leading to dysfunction specifically in pial vessels.

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Chapter 4

Investigating cerebrovascular alterations in late-onset AD: The effect of APOE4

4.1 Introduction

While the pathogenic cause of FAD is an inherited genetic mutation (APP, PSEN1, PSEN2), the pathogenic cause of LOAD, which accounts for the vast majority ($\sim 97\%$) of all AD cases, still remains unknown [1]. With many other genes possibly contributing to LOAD pathogenesis, genome-wide association studies have identified that APOE gene, specifically APOE4 gene, is the strongest genetic risk factor for LOAD [2, 3], and also showing its association with FAD [4, 5]. There are three common alleles of the APOE gene ($\epsilon 2$, $\epsilon 3$, and $\epsilon 4$) [6]. Each person carries two copies of the APOE gene, thus six possible genotypes: $\epsilon 2/\epsilon 2$, $\epsilon 2/\epsilon 3$, $\epsilon 2/\epsilon 4$, $\epsilon 3/\epsilon 3$, $\epsilon 3/\epsilon 4$, $\epsilon 4/\epsilon 4$. Compared to non-carriers of APOE4, several studies have reported that the risk of AD increases by 3-4 fold in heterozygotes and 9-15 fold in homozygotes [7–9], and also with lower age of onset for APOE4 carriers [7, 10]. A population-based cohort study has found that APOE4 homozygote individuals have 30-35% lifetime risk of MCI or LOAD, whereas a slightly lower risk (20-25%) for

APOE4 heterozygote individuals [11]. In addition, APOE4 is a risk factor for other diseases such as atherosclerosis [6], CAA-associated cerebral hemorrhage [12], tauopathies and dementia with Lewy bodies [13], Parkinson's disease [14] and multiple sclerosis [15]. The APOE4 gene negatively affects memory performance [16], and shows association with the increased risk of progression from MCI to AD [17–19]. In MCI patients carrying APOE4 gene, the adverse effects of APOE4 on cognitive functions are correlated with the severity of neuropathological indicators such as lower CSF A β 42 levels, higher tau levels and greater brain atrophy observed when compared to those of noncarriers [20].

Key pathological changes in AD include A β accumulation/aggregation associated with an imbalance between production and clearance of A β peptides in the brain; NFTs made up of hyperphosphorylated tau protein, CAA, and BBB breakdown further impeding A β clearance [21]. Aggregates of toxic A β (come in three different forms: A β oligomers, intraneuronal A β , and amyloid plaques) deteriorate synapses, and ultimately cause neurodegeneration in AD [22]. Vascular dysfunction is thought to be an important prodromal process in AD, along with or often independently of the A β deposition [23]. Despite the growing evidence supporting the APOE4 effect in cerebral vasculature (in the presence [24] and absence [25–27] of A β -dependent mechanisms), there are still significant knowledge gaps such as temporal dynamics of vascular alterations in APOE4 gene and in relation to cognitive decline that show up later stages in AD continuum. More importantly, there has been no *in vivo* longitudinal imaging study conducted yet which investigates the alterations in the vascular system at microvascular scale.

The aim of this study is to conduct a 1-year longitudinal awake imaging experiment with a clinically more relevant model, APOE4 model, while having sex as a key biological variable. Despite APOE4 being the strongest known genetic risk factor in LOAD and is strongly related to various microvascular dysfunctions, including BBB integrity [28], a non-invasive and inexpensive regular check-up approach to detecting this APOE4-related

AD onset has not been established. Also, females are known to be more susceptible to AD [29], but yet no study has compared the effect of sex in relation to APOE4-related vascular alterations in a longitudinal manner. Therefore, the time courses of microvascular alterations obtained from this study, separately for each sex, will provide novel insight to our long-term effort of developing effective biomarkers based on simple eye scans.

4.2 Methods

4.2.1 OCT-Angiography (OCTA)

We have used the same OCT imaging protocol as Chapter 2 (see 2.2.2 for details).

4.2.2 Doppler-OCT (DOCT)

We have used the same OCT imaging protocol as Chapter 2 (see 2.2.3 for details).

4.2.3 Study design and animal preparation

The literature shows that the difference in the mean of either vascular metric or cognitive test score ranged between 1.4 and 4.6 of standard deviation between APOE4 and controls [30, 31]. Based on this literature, and with α of 0.05 and 80% power, it suggests the sample size per group to be between 2 and 8. Considering the survival rate with cranial windows was 70-80% in the pilot study (7-month), thus assuming 60-70% for this 12-month longitudinal study, we will need 3-11 animals per group. Ten animals per group have been chosen as our sample size. We longitudinally repeated the measurements every 4 weeks for 48 weeks (12 time points).

We had APOE3 male mice as a shared control. To investigate the effect of APOE4, we added APOE4 male mice as an experimental group. To further investigate the combined effect of APOE4 and female, we added APOE4 female mice as another experimental group. Our selected mouse models are: APOE4-targeted replacement (TR) mice (male and female, n=10 each, 11-12 WOA, 1549-M and 1549-F, B6.129P2-Apoe^{tm3(APOE*4)Mae}>, Taconic) and APOE3-TR mice (male, n=10, 11-12 WOA, 1548-M, B6.129P2-Apoe^{tm2(APOE*3)Mae}>, Taconic). All mice underwent craniotomy surgeries following the widely used protocol [32]. All animal-based experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Brown University (IACUC-1612000239), according to the guidelines and policies of office of laboratory animal welfare and public health service, National Institutes of Health.

4.2.4 Novel Object Location test (NOL)

Mice were placed into an open-field apparatus consisting of a square arena (40 × 40 × 40 cm³), customly built following the protocol [33]. The arena is digitally divided into 4 quadrants, with the center of the northeast (NE), northwest (NW), southeast (SE), and southwest (SW) corners which serve as placement locations for two identical objects (rods, radius = 1 cm, height = 5 cm). For 5 min, each mouse's behavior was recorded by the automated tracking software (ANY-maze) to calculate the exploration time determined by a circular zone (< 5 cm) from the objects' center. Two different sessions were carried out with the delay of 25 min in between: a habituation stage for 5 min (objects placed on NE and NW) and a testing stage for 5 min (objects placed on SE and NW). The arena is cleaned with 70 % ethanol and dried in between sessions to eliminate any potential odor cues left beforehand. To quantify how the animal memorizes the object locations and discriminates

between the staying and relocated objects, we defined a discrimination index based on [34]:

$$Z = \frac{(T_{reloc} - T_{stay})^2}{(T_{reloc} + T_{stay})^2}$$

where T_{reloc} and T_{stay} are the duration the animal spent near the relocated object or the staying object, respectively. This index becomes zero when the animal has no discrimination and becomes one when the animal shows perfect discrimination. We used the square for the following analysis of generalized linear mixed effect (GLME) with the logit link function to fit data to a logistic trajectory. We then smoothened the individual trajectories by averaging the discrimination indices over adjacent three age points. During the GLME fitting, subject-specific random effects were allowed for the intercept.

4.2.5 Statistical analysis

We used the linear mixed-effects (LME) method to fit each set of measured time courses to either a linear model or a nonlinear model following a sigmoidal curve $y = c + \frac{1}{\exp(k(b-age))}$ and then selected the model with the lowest Akaike Information Criterion (AIC) following the widely used method [35, 36]. The AIC also informed our choice of random effects based on the intercept, slope and/or time-group interaction. We normalized each metric for all mice groups by the intercept as determined by the fitted model. Outliers were excluded based on Cook's distance: any value with a Cook's distance greater than 3 times the mean was considered an outlier, as practiced in many studies[37, 38].

The rate of change was simply defined by the slope for the linear model. For the sigmoid model, we defined the rate of change by measuring 90% of the total changes observed during the ages of measurement (11-57 WOA; from the bottom 5% to the top 5%) and then by dividing the 90% change by the age duration corresponding to the 90% change. The same was done on the 95% confidence intervals of the nonlinear fitting to obtain the

confidence intervals for the nonlinear slope. When comparing the rate of change between individual groups, for linear models, we recorded the p-value of the interaction term to determine significant differences between slopes. For nonlinear models, we determined the p-value by conducting a t-test between the estimated parameters L for each nonlinear model. We selected vascular properties for further analysis which were associated with significant p-values at an overall level of $\alpha = 0.05$ after Benjamini-Hochberg correction to control false discoveries. For these metrics, we determined the age at which APOE3-m and APOE4-m/f groups differed by assessing the degree of overlap between two CIs. To account for multiple hypothesis testing using Bonferroni correction, we aimed to find the age at which the groups were significantly different at a rate of $\alpha = 0.05/7$, which in turn leads to the age at which the 93% CIs just touch. In detail, we computed the distance between the lower CI bound of the group with higher values and the upper CI bound of the other group. The age from which this distance becomes larger than zero was selected as the age at which two compared groups started to differ.

4.3 Results

4.3.1 Pial vessels

We acquired OCTA datasets over a 1-year timespan, composed of 12 time points total for each animal in awake state (see Methods for details). Whereas the ‘same-vessel tracking’ method was used in the previous chapters, we found the same method not applicable to the dataset in this study due to high degree of vascular deformation over time as seen in several animals per group (demonstrated in Figure 4.1). Alternatively, we selected as many vessels as possible for each time point for each animal, with the same quantification method 2.2.2 used for measuring the diameter of each selected vessel. This allowed us to measure a minimum of 15 vessels per time point for each animal.

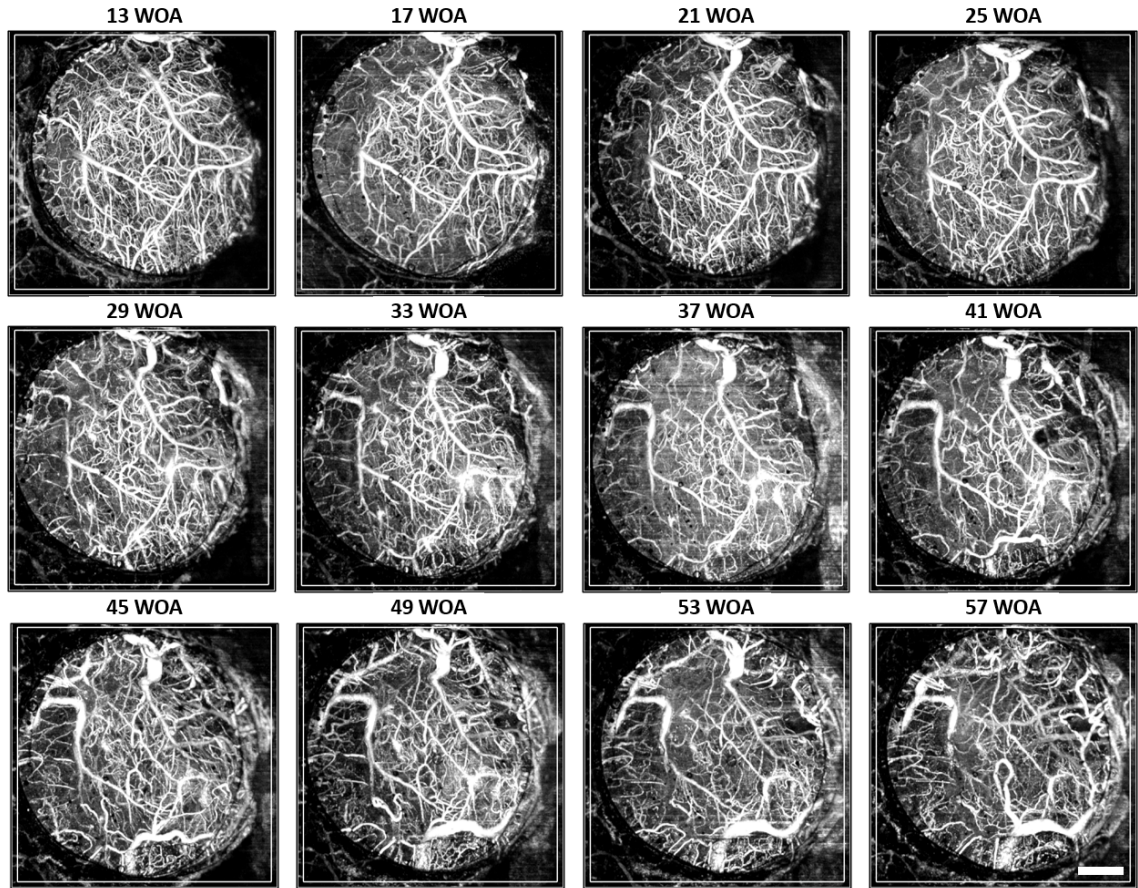


Figure 4.1: An example set of the maximum intensity projection images of OCTA data from the same animal over a 1-year timespan (from 13 to 57 WOA). Scale bar, 500 μm .

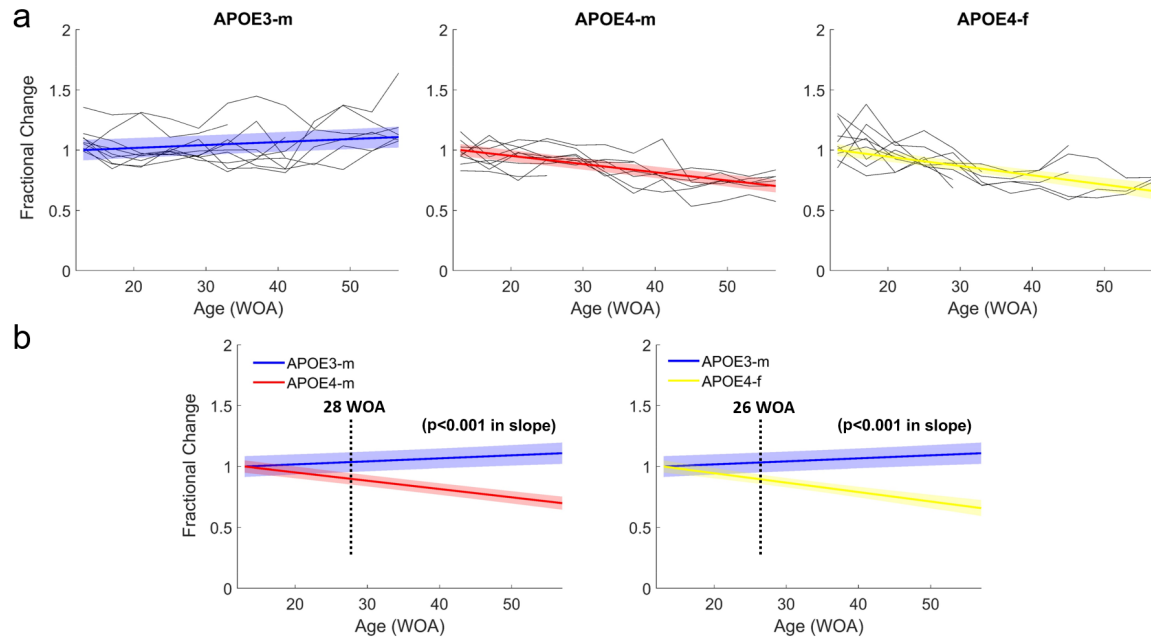


Figure 4.2: The result of fractional change in pial vessel diameter for (a) Individual groups, each gray line represents the trajectory of the mean values for each animal. (b) RCA comparison of pial vessel diameter by the effect of APOE4 and female versus control (APOE3-m). Dotted lines represent AOS between two groups compared.

We calculated the mean diameter of each timepoint of each animal and used those values for the LME fitting and statistical analysis. Figure 4.2a displays the fractional change of the mean pial vessel diameter for individual animals (gray lines). Based on LME analysis, both APOE4-m and APOE4-f groups showed vessel diameter decreasing with age at a rate of 2.7 and 3.1% per month (95% confidence interval [CI], -3.1 to -2.3 and -3.9 to -2.3), respectively. In contrast, the control group (APOE3-m) exhibited a slight increase in vessel diameter at a rate of 1.0% per month ([CI], 0.4 to 1.6, Figure 4.2 and 4.1).

Table 4.1: Statistical analysis of RCAs in pial vessel diameter.

	Rate of change [95% CI] (%/month)	P-value (against zero rate)
APOE3-m	1.0 [0.4 to 1.6]	0.00092
APOE4-m	-2.7 [-3.1 to -2.3]	1.4×10^{-21}
APOE4-f	-3.1 [-3.9 to -2.3]	3.3×10^{-11}

Then we compared the RCA values between the groups to find the effects of APOE4 and female (see Table 4.2). When comparing APOE4-m to APOE3-m, the negative RCA of APOE4 male mice was significantly different from that of APOE3 male mice ($p < 0.001$), with the age of significance (AOS) for the effect of APOE4 being 28 WOA. Adding the effect of female with APOE4, the result showed an earlier AOS of 26 WOA (Figure 4.2b and Table 4.2).

Table 4.2: Statistical analysis of RCA between groups by genotype and sex in pial vessel diameter.

	p-value (RCA between groups)	AOS (WOA)
Effect of APOE4	1.0×10^{-21}	28
Effect of APOE4 + female	2.6×10^{-16}	26

4.3.2 Penetrating arterioles and venules

We acquired DOCT datasets over the 1-year timespan, a total of 12 timepoints for each animal in awake state as shown in Figure 4.3 (see Methods for details). One issue related to DOCT images was the increased noise level for datasets in later timepoints for several animals possibly due to cranial window degradation (See Figure 4.10 in Discussion). Thus, instead of tracking the same individual vessels over time, we used the approach of ‘selecting as many vessels as possible’ (as used in OCTA pial vessel diameter analysis). We extracted diameter and flow information from the selected penetrating arterioles and venules. This allowed us to collect a total of 244 and 229 datapoints of penetrating arterioles and venules from all animals, respectively.

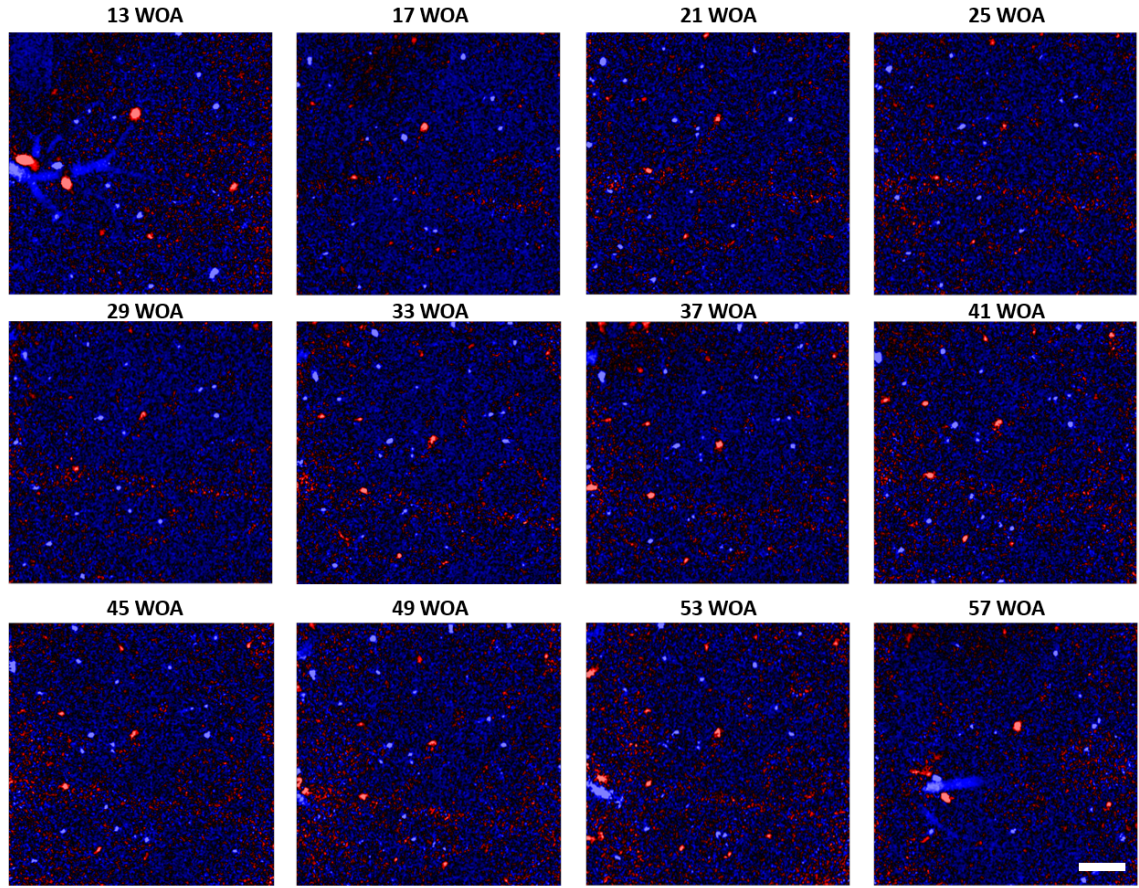


Figure 4.3: The *en face* images of DOCT data from the same animal over a 1-year timespan (from 13 to 57 WOA). Scale bar, 250 μm .

The trajectories of fractional change in the mean diameter of penetrating arterioles for all three mice groups are displayed in Figure 4.4a. The RCA was 1.0%, -0.4%, and -5.2% per month ($p=0.0498$, $p=0.43$, $p<0.001$) in APOE3-m, APOE4-m, and APOE4-f mice, respectively. When comparing APOE4-m to the control to investigate the effects of APOE4, the RCA was significantly different ($p<0.05$) but no AOS was found until the final age of the experiment (Figure 4.4b, left). Adding the female effect, the RCA showed a greater difference ($p<0.001$), and we found the AOS of 27 WOA (Figure 4.4b, right; Table 4.4).

The RCAs of the fractional changes in the mean arteriolar flow were -1.2, -5.6, and -12.8% per month, for APOE3-m, APOE4-m, and APOE4-f, respectively (Figure 4.5a). In the control group (APOE3-m), the RCA did not show statistical significance against the zero rate. However, both male and female APOE4 mice exhibited significantly lower RCAs ($p<0.001$; Table 4.3), implying a significant decreasing trend in arteriolar flow. As for group comparisons, similar to the result from arteriolar diameter, no AOS was found when comparing the RCAs between APOE3 and APOE4 male groups. In contrast, adding the female effect, we observed the AOS of 28 WOA (Figure 4.5b, Table 4.4), about one week later to the AOS found from the arteriolar diameter comparison.

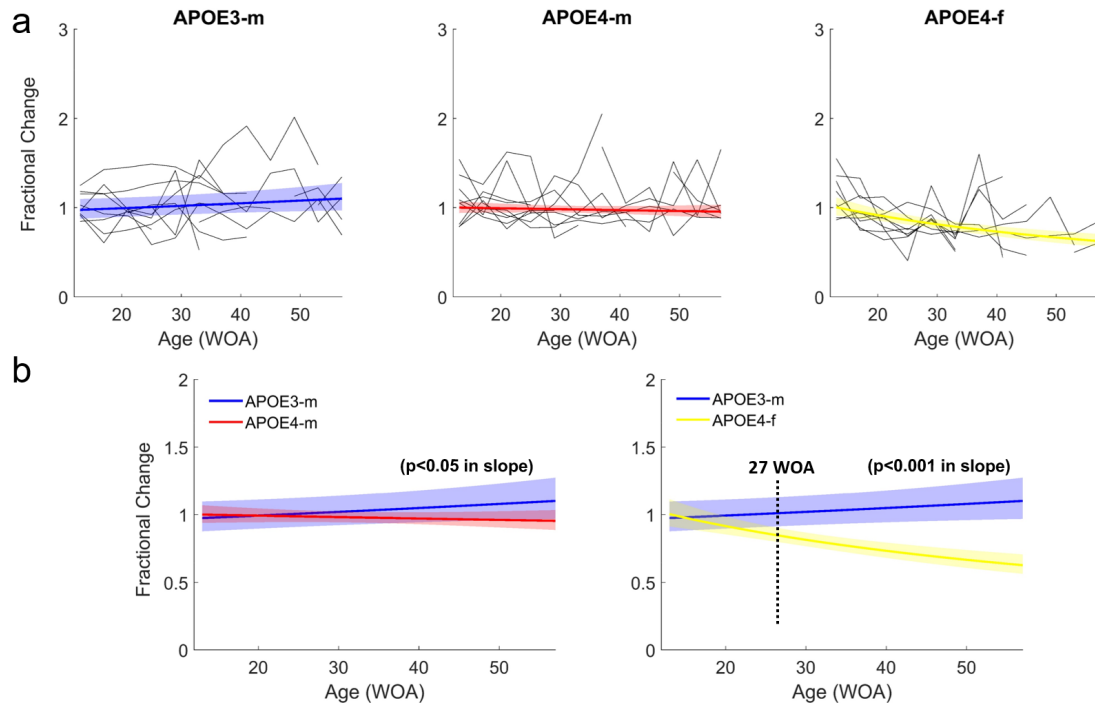


Figure 4.4: The plots of fractional change in Arteriolar diameter for (a) individual groups, (b) in group comparison by the effect of APOE4 (left) and APOE4+female (right). Age of significance is denoted as dotted vertical lines.

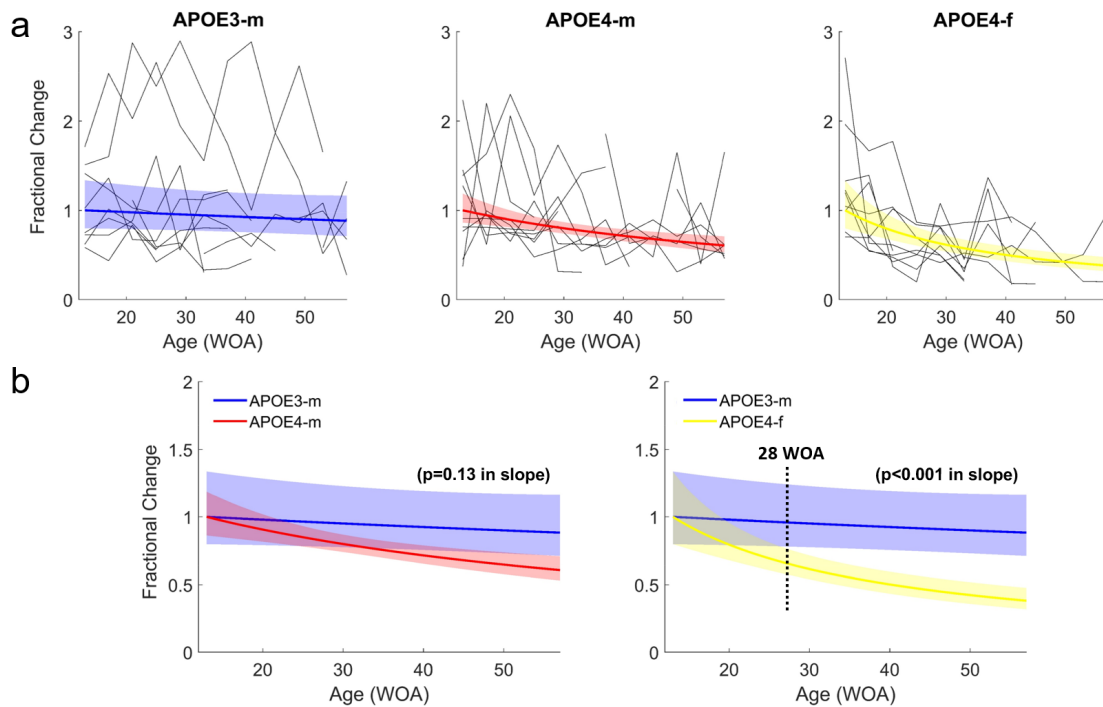


Figure 4.5: The plots of fractional change in Arteriolar flow for (a) individual groups, (b) in group comparison by the effect of APOE4 (left) and APOE4+female (right). Age of significance is denoted as dotted vertical lines.

For penetrating venules, the RCAs of fractional change in mean diameter and flow for APOE3-m were -0.1 and 0.3% per month ($p>0.05$, Figure 4.6a and Figure 4.7a, Table 4.3). In contrast, both male and female groups of APOE4 exhibited more rapidly decreasing trends in diameter and flow for penetrating venules. For venular diameter, the RCAs were -4.0 and -2.7% per month for APOE4-m and APOE4-f, respectively, with the male group only showing statistical significance against zero rate ($p=0.001$, Table 4.3). In venular flow, the RCAs were -7.0 and -6.2% per month for APOE4-m and APOE4-f, respectively, with both male and female groups showing statistical significance in the slopes ($p<0.001$, Table 4.3).

When comparing between APOE3 and APOE4 male groups in venular diameter, we found the AOS of 34 WOA. in contrast, no AOS was observed when the female effect was taken into account (Figure 4.6b, Table 4.4). For venular flow, we found both the effect of APOE4 and the effect of female from the RCA comparisons with the AOS of 30 and 35 WOA in APOE4-m and APOE4-f (compared to APOE3-m), shown in Figure 4.7b and Table 4.4. In relation to the AOS findings, there may be sex-dependent vascular alterations in penetrating vessels, where the male APOE4 group showed vulnerability in venular diameter and flow and the female APOE4 group exhibiting higher and earlier risk of vascular alterations occurring from arteriolar diameter and flow side.

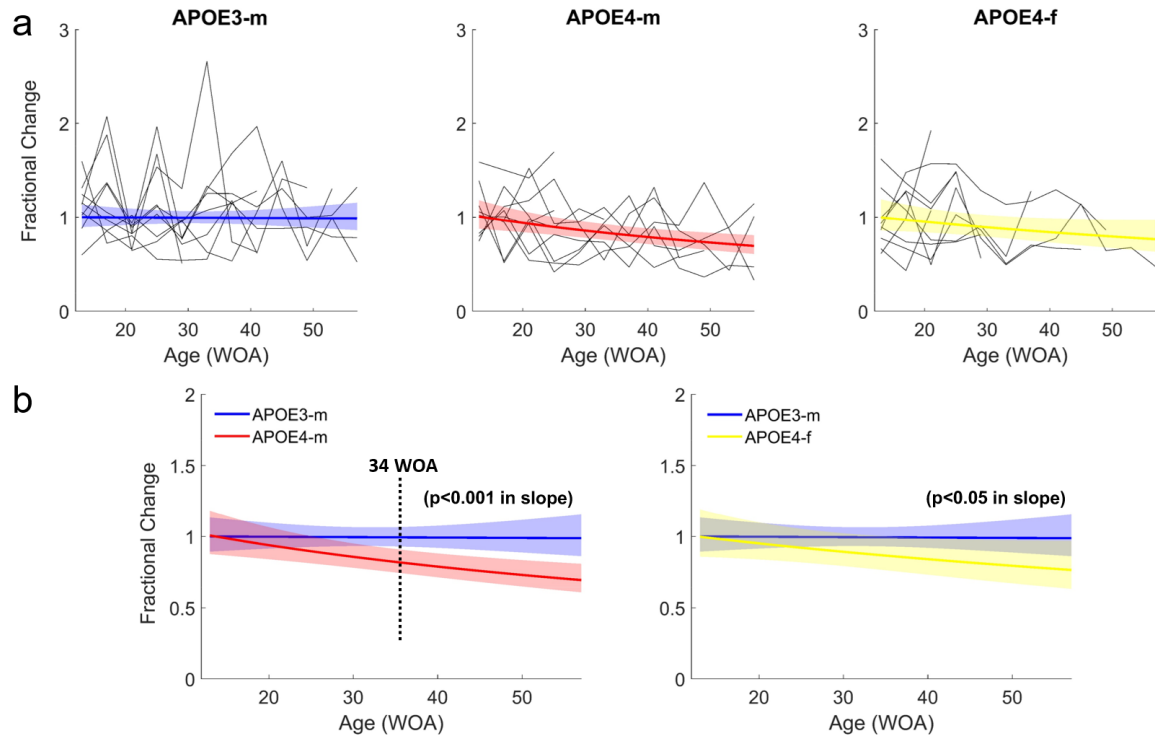


Figure 4.6: The plots of fractional change in Venular diameter for (a) individual groups, (b) in group comparison by the effect of APOE4 (left) and APOE4+female (right).

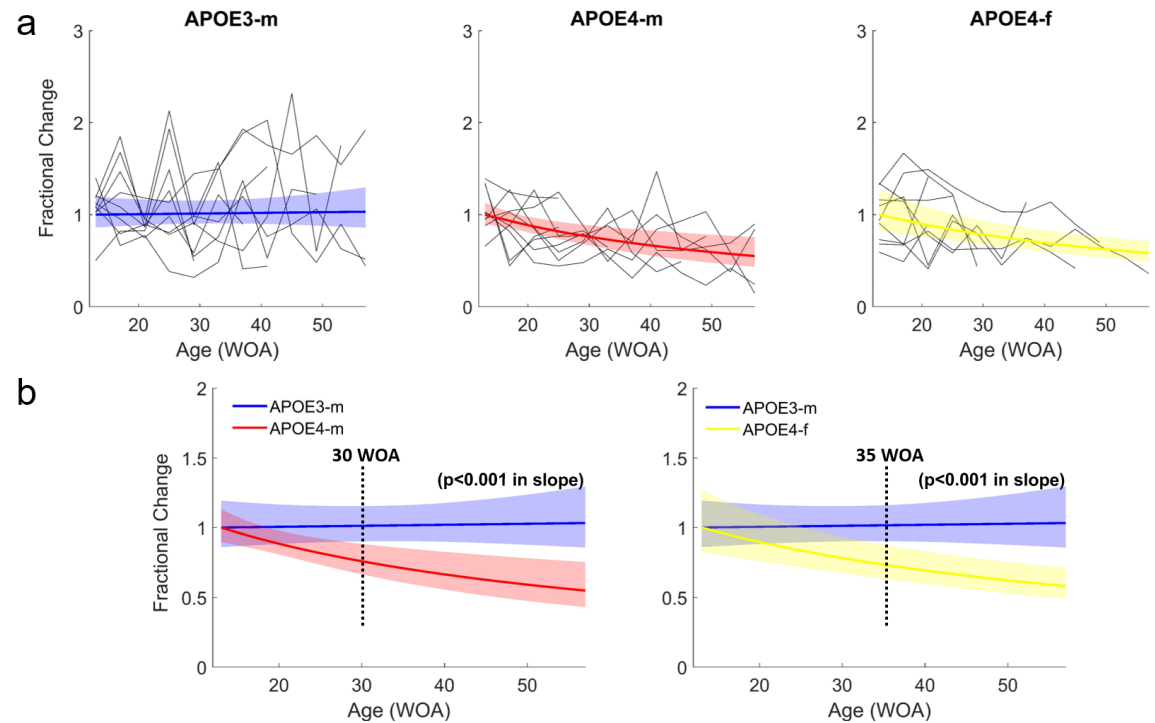


Figure 4.7: The plots of fractional change in Venular flow for (a) individual groups, (b) in group comparison by the effect of APOE4 (left) and APOE4+female (right). Age of significance is denoted as dotted vertical lines.

Table 4.3: Statistical analysis of RCA in diameter and flow for penetrating arterioles and venules.

Properties	Group	Rate of change [95% CI] (%/month)	p-value (against zero rate)
Arteriolar Diameter	APOE3-m	1.0 [0.01 to 2.1]	0.05
	APOE4-m	-0.4 [-1.5 to 0.7]	0.43
	APOE4-f	-5.2 [-7.1 to -3.2]	2.7×10^{-6}
Arteriolar Flow	APOE3-m	-1.2 [-3.1 to 0.8]	0.24
	APOE4-m	-5.6 [-8.2 to -2.7]	0.0003
	APOE4-f	-12.8 [-16.9 to -8.3]	1.7×10^{-6}
Venular Diameter	APOE3-m	-0.1 [-2.2 to 2.0]	0.91
	APOE4-m	-4.0 [-6.1 to -1.7]	0.001
	APOE4-f	-2.7 [-5.4 to 0.2]	0.07
Venular Flow	APOE3-m	0.3 [-2.1 to 2.8]	0.82
	APOE4-m	-7.0 [-11.0 to -2.5]	0.003
	APOE4-f	-6.2 [-8.6 to -3.6]	2.7×10^{-4}

Table 4.4: Statistical analysis of RCA comparison between groups by genotype and sex in venular diameter and flow

Properties	Comparison by	P-value (RCA between groups)	AOS (weeks of age)
Arteriolar Diameter	Effect of APOE4	0.043	-
	Effect of APOE4+female	1.8×10^{-8}	27
Arteriolar Flow	Effect of APOE4	0.13	-
	Effect of APOE4+female	0.0003	28
Venular Diameter	Effect of APOE4	9.0×10^{-5}	34
	Effect of APOE4+female	0.034	-
Venular Flow	Effect of APOE4	0.0004	30
	Effect of APOE4+female	0.007	35

4.3.3 Cognitive function: NOL test

In addition to vascular properties analyzed in this longitudinal study, we conducted the NOL test to determine cognitive performance in APOE3-m and APOE4-m/f groups. To obtain the time courses of cognitive impairment, we plotted the discrimination index (see 4.2.4 for details) for each mouse (gray lines in Figure 4.8a). As a result, only the APOE4-f showed a negative RCA (-0.02%/month, $p < 0.001$, Table 4.5). When comparing against the

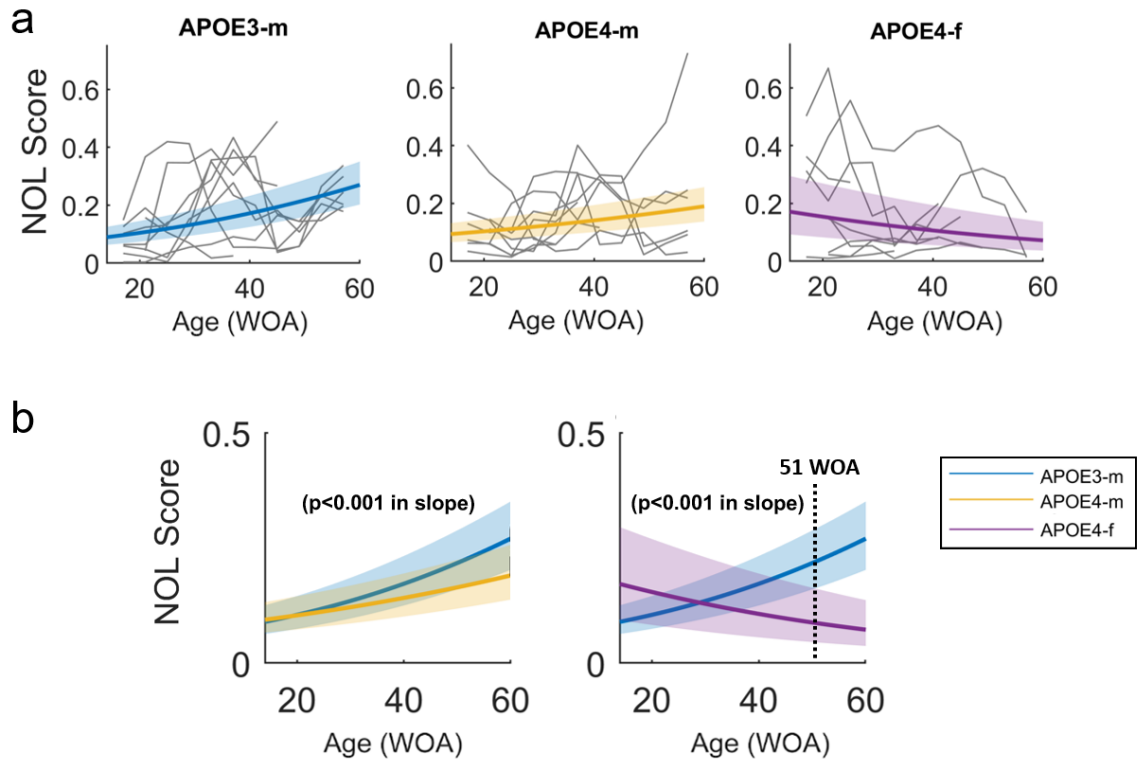


Figure 4.8: The NOL test result for (a) individual groups, (b) group comparisons by genotype in male and female mice.

control group, the APOE4 effect on the NOL score was significant in both sexes (different RCAs, $p < 0.001$) but was greater when combined with the female effect, resulting in AOS before the final age of the experiment (51 WOA), as shown in Figure 4.8b and Table 4.6.

Table 4.5: Statistical analysis of RCA in all mice groups for NOL z-score.

	Rate of change (%/month)	p-value (against zero rate)
APOE3-m	0.029	1.4×10^{-47}
APOE4-m	0.018	1.4×10^{-29}
APOE4-f	-0.021	7.8×10^{-26}

Table 4.6: Statistical analysis of RCA in all mice groups for NOL z-score.

	Effect on RCA [95% CI] (% per month)	P-value (RCA between groups)	AOS (WOA)
Effect of APOE4	-0.013 [-0.016 to -0.011]	1.2×10^{-18}	-
Effect of APOE4+female	-0.049 [-0.052 to -0.046]	7.1×10^{-68}	51

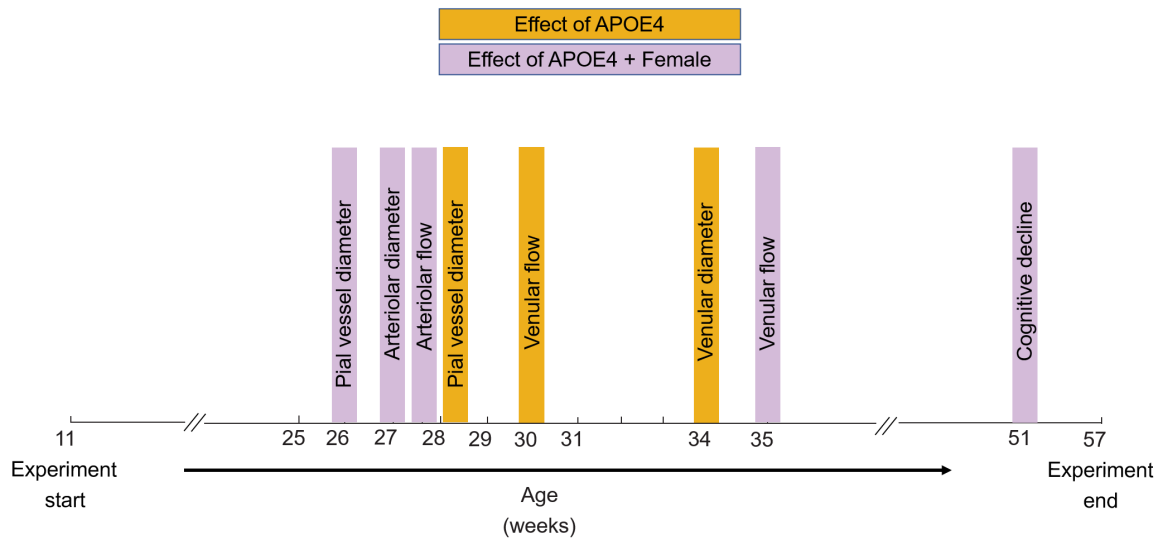
4.4 Discussion and Conclusion

In this study, for the first time, we conducted a 1-year longitudinal OCT imaging to investigate temporal dynamics of vascular alterations at microvascular scale in human APOE4 gene carrying mice. As a result, we found vascular alterations in both pial and penetrating vessels occurred earlier than cognitive decline, being generally greater and earlier in female APOE4 mice than APOE4 male mice. This result was also consistent with the hypothetical projected model introduced in Chapter 1 (Figure 1.2), suggesting the vascular dysfunction in APOE4 being one of the earliest events in LOAD pathogenesis.

In detail, the effect of APOE4 on the slope of trajectory (RCA) was significant ($p < 0.05$) in the NOL score and all vascular properties except for the arteriolar flow, while the effect of combined APOE4 and female was significant in all NOL and vascular properties. But the effect of APOE4 and female was generally greater than that of APOE4 alone as shown in Table 4.7, making the trajectories become separate enough from the control trajectory before the final age of our experiment (57 WOA). Therefore, we identified AOS in more properties in APOE4 female mice than APOE4 male mice against the shared control of APOE3 male mice, as shown in Figure 4.9. For cognitive function (NOL test), a previous study has shown that the cognitive decline occur late (15-18 months of age) in the same APOE4 mouse model [39], but still, the sign of cognitive decline (the AOS of 51 WOA) observed in the female APOE4 before the final age of our experiment. In relation to AOS, when comparing between the male and female APOE4 group, earlier alterations (about 2 weeks) were observed from the female group in pial vessel diameter as illustrated in Figure 4.9. This female effect in APOE4, although the underlying mechanisms behind such finding is beyond the scope of this study, could still provide valuable insights on understanding vascular dysfunction in sex-dependent manner.

Table 4.7: A statistical analysis of APOE4 and female effect on RCA in all vascular properties and NOL test score.

	APOE4 effect on RCA (% per month)	APOE4 and female effect on RCA (% per month)
Pial vessel diameter	-3.7 ($p=1.0 \times 10^{-21}$)	-4.7 ($p=2.6 \times 10^{-16}$)
Arteriolar diameter	-1.4 ($p=0.043$)	-6.2 ($p=1.8 \times 10^{-8}$)
Arteriolar flow	-6.8 ($p=0.13$)	-14.0 ($p=0.0003$)
Venular diameter	-3.9 ($p=9.0 \times 10^{-5}$)	-2.6 ($p=0.034$)
Venular flow	-7.3 ($p=0.0004$)	-6.5 ($p=0.007$)
NOL score	-0.01 ($p=1.2 \times 10^{-18}$)	-0.05 ($p=7.1 \times 10^{-68}$)

**Figure 4.9:** The timeline of age of significance found in vascular properties and NOL test result with respect to the effect of APOE4 and female.

There are several limitations found from this longitudinal study which can be addressed and improved for future studies. First, the degradation of the cranial window affects the imaging quality in the later time points of our data, which make the quantification of vascular properties such as diameter and blood flow challenging. For example, DOCT images as shown in Figure 4.10 demonstrates how the *en face* DOCT images of one animal from similar depth in later imaging sessions have become much more noisy, which directly have affected the number of vessels that we could select to extract diameter and flow information from. Several studies suggest alternative methods could increase longevity of good quality imaging [40, 41], although the validation of those methods for longitudinal studies (over 1-year) still remains to be seen. Second, there is a lack of measurements at the capillary vessel level. Our group has developed OCT imaging techniques to measure various morphological and topological properties of capillary networks [42]. Future works will involve applying these techniques to a similar study design as the third study, revealing the time courses of various capillary properties in APOE4 carrying mice and compared to APOE3. Lastly, the measurements of the neurovascular coupling is absent in this study. Our group has established simultaneous somatosensory stimulation and functional hyperemia imaging in awake mice using air puff stimuli of whiskers and intrinsic optical signal and laser speckle contrast imaging. Since some studies suggested the neurovascular coupling also undergoes early pathological alterations in AD, future works will include utilizing the established measurements within a similar study design as the third study.

Although this study does not aim to investigate the biological mechanisms underlying the observed APOE4 and female-related effect in cerebrovasculature, here we discuss several relevant points. First, the APOE protein is expressed and secreted by various cell types in CNS including neurons, astrocytes, oligodendrocytes, pericytes, and choroid plexus cells [43, 44]. The major types of lipids found in CNS particles that contain APOE are free cholesterol, phospholipids, and triglycerides [45]. The proper lipidation of APOE is critical for its function not only in lipid (cholesterol) transport but also in metabolisms linked

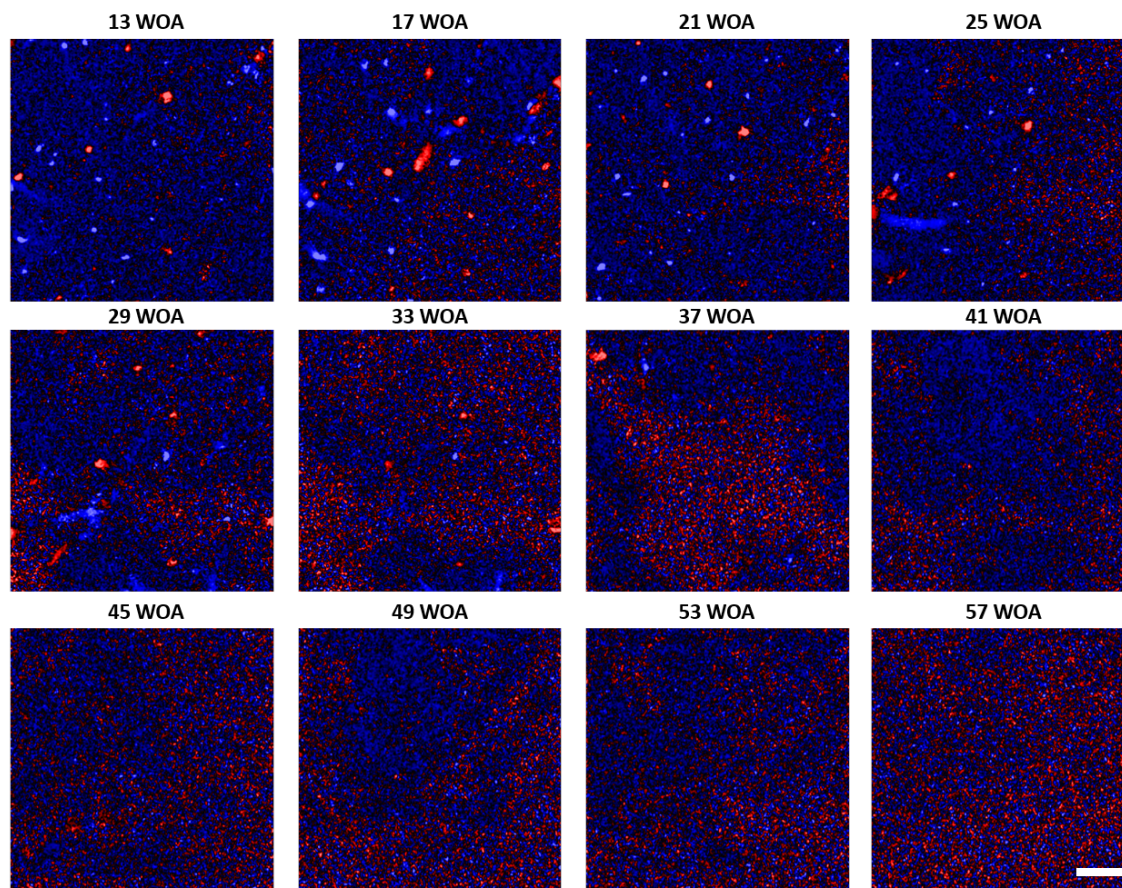


Figure 4.10: The degradation of DOCT image quality from the same animal over a 1-year timespan (from 13 to 57 WOA). Scale bar, 250 μm .

to A β clearance/deposition, synapse regeneration, and immune modulation [46]. Among APOE genotypes, APOE4 exhibits a relatively poor lipidation, resulting in a greater proportion of APOE protein left in the brain and forming aggregates that are more toxic to neurons [47]. Also, APOE4-expressing cells are less effective in transporting brain cholesterol than APOE2 and APOE3, as reported by a study using cultured neurons[48]. More recent study shows a consistent result with higher cholesterol accumulation inside APOE4-expressing astrocytes than APOE3-expressing astrocytes [49], suggesting the lower efflux of cholesterol and phospholipids by APOE4. The decreased ability of APOE4 to remove cholesterol and phospholipids might be linked to the increased risk of LOAD in APOE4 carriers. More importantly, this dysfunctional lipidation of APOE4 can lead to the accumulation of lipids forming foam cells (lipid droplets) causing inflammation and damage to the vessel wall [50], and ultimately result in vessel thinning. Our findings suggest this vessel thinning may differ in APOE isoform-dependent manner and in vessel types where pial vessels show earlier alterations (starting 26 WOA) than penetrating arterioles and venules in APOE4 mice.

Second, APOE4, the strongest known genetic risk factor for LOAD [2, 3], is known to affect pericytes, predominantly found in capillaries and post-capillary venules, which regulate key neurovascular functions such as maintaining BBB integrity and vascular stability, capillary blood flow regulation for oxygen supply and clearance of toxic wastes [51]. Several studies have shown that APOE4 leads to pericyte degeneration in capillaries and accelerated BBB breakdown [24, 25, 52–54], resulting in neurovascular uncoupling causing oxygen deficiency in the brain [55]. This loss of pericytes weakens vascular walls and vessel integrity, and may also have negative effects on the neighboring cells within the neurovascular unit such as endothelial cells, astrocytes and neurons [51]. A recent in vitro model study has shown that APOE4 expression causes toxic gain of cellular dysfunction in endothelial cells, with significant effects on signaling pathways involving blood coagulation and BBB [56]. Our finding of vascular alterations in APOE4 mice may suggest

a possible association between pericyte degeneration in capillaries and other vessel types such as pial and penetrating vessels, although which preceding the other remains elusive.

Third, females have an increased risk of developing LOAD than males [57]. In relation to APOE4, when a female carries a single APOE4 isoform, the risk of AD is significantly higher to a male carrying two APOE4 isoforms [7]. The LOAD risk for females with one APOE4 copy is about 4-fold when compared to males with one APOE4 copy [29], and several studies have reported that this risk may be associated with tau pathology [58–61]. A recent study has shown a significant three-way interaction among APOE4, sex, and cardiovascular disease risk on tau deposition in multiple brain regions including the entorhinal cortex, inferior temporal cortex, and meta-region [62]. APOE4 gene may also contribute to gene regulation that are linked to microtubule disassembly, synaptic function, programmed cell death and aging in sex-dependent manner [63]. Moreover, differences in gene expression patterns associated with inflammation, oxidative stress, and aging have been observed between male and female APOE4 positive individuals [64]. The cerebrovascular effects of APOE4 such as pericyte degeneration and BBB breakdown show high correlation with cognitive decline [28]. This high correlation might be due to the great influence of APOE4 on not only A β accretion and clearance but also in tau pathogenesis leading to neuroinflammation and neurodegeneration in LOAD [25, 65–67]. Also, venular dysfunctions such as occlusion of penetrating venules leads to microinfarcts [68], and is correlated with cognitive dysfunction such as spatial memory loss [69]. Penetrating venules not being able to effectively drain out metabolic waste causes a decline in cognitive function shown from both preclinical and clinical studies [70, 71]. Thus, the use of both arteriolar and venular alterations may enable earlier detection of LOAD, at least as suggested from the APOE4 mouse model in this study.

Lastly, in recent years, there has been a growing number of research investigating structural and functional changes in the retina associated with AD pathology. The retina is

considered as a window to the brain due to its structural, cellular, and functional similarities [72, 73], and provides more accessibility to a non-invasive repeated imaging [74]. Two major hallmarks, $A\beta$ plaques and NFTs, have been identified in the retina of AD patients [75], with $A\beta$ deposits found inside blood vessel walls, perivascular and along blood vessels [76, 77]. This $A\beta$ accumulation in the retina is thought to cause microvascular network attenuation such as lower venular fractal dimension, arteriolar tortuosity, and reduced venous blood flow observed from AD patients compared to cognitively normal controls [78, 79]. Also, a significant reduction in retinal nerve fiber layer (NFL) observed in MCI and AD (mild, moderate, severe) patients compared to healthy controls [80], and retinal ganglion cell layer (GCL) being associated with the severity of AD reported from several studies [81, 82]. The increased blood oxygen saturation levels have been observed in retinal arterioles and venules in MCI patients compared to healthy individuals [83]. $A\beta$ -positive subjects showed a higher mean tortuosity in retinal venules compared with that of $A\beta$ -negative subjects [84]. A more recent study has found early and progressive loss of retinal vascular platelet-derived growth factor receptor-beta and pericytes associated with $A\beta$ deposition in retinal vessels from postmortem tissues of MCI and AD patients [85]. APOE4 is thought to cause pericyte degeneration and BBB breakdown in the brain [28], although its effect on retinal vasculature remains elusive. Nevertheless, many of these findings from the AD retinal vasculature are consistent with our findings from the AD brain vasculature (vessel thinning and blood flow reduction), therefore suggesting a close association between retina and brain in AD. Moreover, O'Bryhim et al. has conducted a OCTA imaging study and found cognitively healthy individuals with preclinical AD have retinal microvascular abnormalities and structural alterations (retinal NFL thickness, GCL thickness, inner and outer foveal thickness, foveal avascular zone, macular volume, vascular density) [86], suggesting retinal microvascular biomarkers as a potential candidate for detecting early AD-related vascular changes in preclinical stages where individuals showing no cognitive symptoms.

4.5 References

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Chapter 5

Conclusion and Future works

This thesis establishes the technical feasibility for and conducts a study with a 1-year longitudinal imaging experiment to investigate microvascular alterations in APOE4 carrying mice, the strongest known genetic risk factor for LOAD which accounts for $\sim 97\%$ of all AD cases.

In the first study (Chapter 2), we have validated the technical feasibility of our OCT imaging system in 1-year timespan using one of FAD mouse models (3xTg) as our animal model since not only the selected model exhibits early AD neuropathological changes reported from the literature [1] but also due to the uncertainty on how long our OCT imaging is capable of acquiring the data. We have acquired the almost 1-year data using OCT techniques such as OCTA and DOCT to investigate both structural and functional alterations in pial and penetrating arterioles and venules. Based on linear and exponential fitting, we detect early vascular alterations (around 20 WOA) compared to late cognitive decline (40 WOA) observed from 3xTg mice. More importantly, we successfully validate our OCT imaging protocol for data acquisition in a 1-year timespan, which can help us characterize the time courses (trajectories) of vascular alterations planned in the third study (Chapter 4) using APOE4 carrying mice for investigating early vascular alterations in relation to

LOAD.

In the second study (Chapter 3), we have addressed the major limitations from the first study (Chapter 2) that are the use of anesthesia (isoflurane) during imaging and the unknown sensitivity of our OCT measurements. We first investigated the isoflurane-induced effect in an age-related manner and have confirmed the vasodilating effect of isoflurane. The effect of aging is significant only in pial vessel diameter change (but not in penetrating vessels), suggesting the aging effect taking into account when one conducts imaging studies using anesthesia. Although no aging effect shows up in penetrating vessels, this finding gives enough motivation for us to conduct awake imaging in the third study (Chapter 4) to avoid any misinterpretation of the data due to the age-dependent effect of anesthesia. In addition to the isoflurane effect experiment, the sensitivity of OCT measurement experiment provides us a quantitative measure of the sensitivity of our approach in detecting changes in diameter and blood flow of pial and penetrating vessels.

For the third study (Chapter 4), we have conducted the longitudinal 1-year awake imaging study using APOE4 mice and acquired OCTA and DOCT data to investigate temporal dynamics of microvascular alterations along with the behavioral test for determining cognitive decline. Since several studies have shown that females are more vulnerable to LOAD [2, 3], we have included both male and female APOE4 mice to better understand the effect of not only genotype but also sex. As a result, early vascular alterations (starting from 26 WOA) are observed in pial and penetrating vessels when compared to cognitive decline (51 WOA observed only in the female APOE4 group) as illustrated in Figure 4.9. More thorough investigations on underlying mechanisms of vascular alterations in APOE4 seem necessary to better interpret the biological meaning of the findings in this study. Nevertheless, the characterization of time courses of these vascular alterations can still provide good insights on which vascular properties would be crucial when developing novel retinal-based vascular biomarkers to detect AD earlier.

Future works include more in-depth OCT measurements for longitudinal OCT awake imaging. It is known that APOE4 causes pericyte degeneration and BBB breakdown which also show high correlation with cognitive decline [4]. Application of OCT imaging techniques to measure various morphological and topological properties of capillary networks [5] would enable more thorough investigation in capillary levels of AD brain by generating similar time courses from capillary properties as demonstrated in this thesis. Deeper understanding of trajectories in various vascular alterations including capillaries can better guide investigators interested in developing retina microvascular novel biomarkers for early detection of AD. Additionally, BBB imaging can be another area that OCT might be applicable with its high sensitivity in measuring subtle changes in the brain tissues, and also demonstrated by a recent study [6]. Also, extending this knowledge to the blood-retinal barrier [7] may help identifying retinal microvascular biomarkers in AD. Also, neurovascular coupling is an area which lacks knowledge for APOE4 effect exhibited in longitudinal manner. Some studies have suggested that the neurovascular coupling also undergoes early pathological changes in AD [8, 9]. We have established simultaneous somatosensory stimulation and functional hyperemia imaging in awake mice using air puff stimuli of whiskers and intrinsic optical signal and laser speckle contrast imaging. A similar study design as the third study may unveil the neurovascular coupling alterations in LOAD.

Overall, this thesis has filled the knowledge gap with longitudinal OCT brain imaging in developing early novel retinal microvascular AD biomarkers. By conducting the 1-year study, the time courses of vascular properties have been characterized from APOE4 models. Longitudinal OCT imaging has shown that it can provide valuable information in detecting early vascular changes compared to late cognitive decline in APOE4 gene carrying mice. We believe the findings from longitudinal brain imaging using OCT can ultimately lead to the translation and development toward novel retinal-based microvascular biomarkers that could be implemented as a cost-efficient, regular check-up tool in clinical practice for detecting early AD-related vascular changes.

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