Developing retinal biomarkers for the earliest stages of Alzheimer's disease: What we know, what we don't, and how to move forward

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Abstract

The last decade has seen a substantial increase in research focused on the identification, development, and validation of diagnostic and prognostic retinal biomarkers for Alzheimer's disease (AD). Sensitive retinal biomarkers may be advantageous because they are cost and time efficient, non-invasive, and present a minimal degree of patient risk and a high degree of accessibility. Much of the work in this area thus far has focused on distinguishing between symptomatic AD and/or mild cognitive impairment (MCI) and cognitively normal older adults. Minimal work has been done on the detection of preclinical AD, the earliest stage of AD pathogenesis characterized by the accumulation of cerebral amyloid, absent clinical symptoms of MCI or dementia. The following review examines retinal structural changes, proteinopathies, and vascular alterations that have been proposed as potential AD biomarkers, with a focus on studies examining the earliest stages of disease pathogenesis. In addition, we present recommendations for future research to move beyond the discovery phase and towards validation of AD risk biomarkers that could potentially be used as a first step in a multi-step screening process for AD risk detection.

Introduction

Alzheimer's disease (AD) is a gradually progressive neurodegenerative disorder characterized pathologically by a cascade of events and marked by the abnormal cerebral aggregation of fibrillar beta-amyloid (Aβ) and hyperphosphorylated *tau* proteins. The disease slowly degrades self-identity and, ultimately, all cognitive function. With the aging baby boomer generation, the number of AD cases in the United States is projected to triple by 2050, and the total cost burden for American tax payers is expected to escalate during this same period [1], reflecting global population trends.

The past two decades have seen a refocusing of AD research strategy towards secondary prevention in clinical trials. The advent and validation of several disease-specific biomarkers over the last two decades has led to research diagnostic criteria for the preclinical stage of Alzheimer's disease [2–5], a 'silent' period, during which Aβ accumulates at an irregular rate in the cerebral cortex but patients remain clinically asymptomatic. A recent revision of these criteria has led to a new research definition of AD based only on biomarker profiles [3]. Moreover, recent clinical trials have focused on this preclinical AD population in an attempt to modify AD risk by reducing amyloid accumulation prior to onset of clinical symptoms (cf. [6]).

The current gold standard biomarkers for detection of preclinical AD are Aβ positron emission tomography (PET) imaging and cerebrospinal fluid (CSF) assays. The Aβ PET [7,8] scan was approved by the FDA in 2004, and uses one of four validated amyloid ligands for *in vivo* visualization of cerebral amyloidosis. Analogous tracers are under various stages of development and validation for *tau* PET imaging. Although PET imaging has transformed our ability to identify early disease burden, access to this technology is limited due to high cost, invasiveness, and lack of availability for large parts of the world. CSF biomarkers for Aβ and phosphorylated and total *tau* have been widely available and commonly used in clinical research and clinical practice for decades. However, these assays are subject to substantial instrumental error across laboratories, and obtaining a CSF sample requires lumbar puncture, which is an invasive

procedure requiring a trained specialist. Hence, widely available, accessible and cost-effective screening biomarkers, that can be obtained by point-of-care clinicians for the early detection of high-risk individuals, remains a global public health imperative.

The retina in Alzheimer's disease

The cortex and the retina share many characteristics, including embryologic origin, precise neuronal cell layers, complex neurochemistry and neurotransmitter systems, microglia, astroglia, blood-barriers, and microvasculature. Axons from the optic nerve form a direct connection between the retina and the brain and facilitate the transport of amyloid precursor protein (APP) created in retinal ganglion cells. However, unlike the rest of the central nervous system, the retina can be visualized directly using standard ophthalmology techniques. Direct visual access to the retina and shared neurobiology with the brain make retinal imaging a promising target for the development of a point-of-care test to detect disease-related biomarkers in the earliest stages of preclinical AD.

Additionally, there have been vast improvements in ophthalmologic imaging techniques in terms of both resolution and accessibility, over the past decade. Application of novel, high-resolution retinal imaging techniques such as optical coherence tomography (OCT), blue light autofluorescence (BAF), and scanning laser ophthalmoscopy (SLO) have enabled researchers to explore AD-related pathology of the retina as an extension of the central nervous system (CNS).

Research on retinal AD biomarkers began as early as 1991, and early work relied on techniques such as optical coherence tomography (OCT) to examine volumetric changes in segmented retinal layers, [9–13] and scanning laser polarimetry to examine the structure of the optic nerve head [14,15]. Very early studies applied electroretinography to approximate group differences in retinal ganglion cell degeneration between AD patients and health controls (cf. [11]). Vascular factors (e.g. blood velocity, blood flow rate, density of the vascular bed) were measured using laser Doppler and fundus photography (cf. [16]). Many recent studies utilizing advanced imaging techniques have demonstrated retinal abnormalities in patients with MCI and AD,

including changes in retinal nerve fiber layer (RNFL) thickness and/or volume, retinal ganglion cell (RGC) degeneration, and reduction of retinal blood flow accompanied by other vascular changes [17–22]. Importantly, independent researchers have confirmed the presence of Aβ1-42 and phosphorylated tau (*ptau*) in retinal tissues of AD patients [23–25].

Taken together, this body of work supports a clear link between retinal and cerebral changes in AD. Although some studies have identified structural, proteomic, metabolic, and/or vascular changes in the retina in symptomatic AD patients, others have found no significant differences in retinal pathology between AD patients and healthy controls. There are several possible reasons for these discrepancies, not the least of which is that equipment, methods of analysis, and study populations (both in terms of age range, stage of disease, and diagnostic criteria for MCI/AD) differ across studies. Moreover, the vast majority of research to date has focused on cross-sectional comparisons of symptomatic AD vs. healthy older adults, and there is a paucity of published longitudinal observational studies. Finally, even less is known with respect to retinal changes in the preclinical stage of the disease. The purpose of this report is to review what is currently known with respect to structural, protein aggregation, and vascular retinal biomarkers in preclinical AD.

Structural retinal changes in Alzheimer's disease

Over the past decade, there have been numerous publications reporting retinal nerve fiber layer (RNFL) thinning in association with AD using OCT (for recent meta-analyses, see [26,27]). Yet the characterization and progression of any such structural changes in the retina, particularly during earlier stages of the disease (i.e., preclinical AD and MCI), remains unclear in part due to a near absence of within-subjects longitudinal research, as well as variability in how study groups are defined (i.e., MCI versus early AD), often in the absence of biomarker (e.g., amyloid PET) confirmed disease staging. Additionally, a majority of studies have focused primarily on participants with substantial disease burden. There are relatively few reports describing structural changes between MCI, mild AD, and controls, and even fewer have focused on the preclinical

stage of the disease. The following sections provide an overview of what we know so far about AD-related structural changes in the retina.

RNFL Thinning.

Several studies reported evidence of macular RNFL (mRNFL) thinning in AD, with the greatest degree of thinning generally observed in the outer 6mm ring of the Early Treatment Diabetic Retinopathy Study (EDTRS) grid, centered over the fovea. Given that the macular region contains more than 50% of total retinal ganglion cells (RGCs), it has been predicted to be more sensitive to neurodegenerative processes than the peripapillary RNFL region, which would make it an ideal target for early detection of AD related change [28–30]. This thinning may reflect RNFL loss in the periphery [22,31–33], but at this point, only a few studies have examined macular volume and/or thickness in MCI patients. Gao and colleagues [20] found reduced macular volumes in both MCI and AD groups relative to HCs; however, they found no significant difference in volumes between the MCI and AD groups. In contrast, another study found greater macular volumes in their MCI group compared to HCs and AD patients [34]. Overall, the variability in these findings suggests that the timing of macular changes in the retina over the course of AD requires further elucidation.

Research suggests progressive thinning of the RNFL with AD progression. Garcia-Martin and colleagues [35] have shown that the thickness in the RNFL is negatively correlated with disease duration in patients with mild AD. Consistent with these findings, two recent meta-analyses that measured peripapillary RNFL (pRNFL) thickness in MCI and AD patients versus healthy controls (HC) found that the average RNFL thickness in the MCI group fell between the thickness measurements for HC and AD patients [26,27]. There has, however, been some inconsistency between studies with regard to identification of the actual location of where mRNFL thinning occurs (cf. [36]). Several studies of AD patients report that the greatest degree of thinning occurs in the superior quadrant [16,37], or superior and inferior quadrants [32,38]. In their meta-analysis, den Haan and colleagues [26] found that mRNFL thickness was lower in the superior

and inferior quadrants compared to nasal and temporal quadrants in patients with AD. The superior and inferior quadrants contain the highest concentrations of axonal bundles in the RNFL, coursing towards the optic nerve head, so early degenerative changes may be first noticeable in these quadrants. Moreover, prominent thinning of the RNFL superior quadrant may e related to reported observations of inferior visual field loss in AD [35,39].

Findings have been less consistent when both MCI and AD groups are examined. Kesler and colleagues [40] found a significant decrease in RNFL thickness in both AD and MCI groups compared to healthy older adults (HCs), particularly in the inferior quadrants of the optic nerve head; however, the superior quadrants were significantly thinner only in AD, and overall no significant difference in RNFL thickness was found between MCI and AD patients. Others have presented evidence of thinning occurring in other quadrants first. Gao and colleagues [41] found reduced RNFL thickness in both MCI and AD groups relative to controls in the superior and temporal quadrants. The AD group had significantly reduced mean RNFL thickness in the inferior quadrant in comparison to the MCI group, suggesting that inferior quadrant thinning may occur earlier in the disease. Finally, others have suggested that thinning occurs and progresses with similar timing in all four quadrants [42], and such degenerative changes may start in the preclinical stage of the disease [37]. Taken together, these findings suggest that more research is needed in order to carefully stage and compare RNFL thickness and/or volume changes over the progression of AD [43].

Segmentation of Other Retinal Neuronal Layers.

Ganglion Cell Complex: Several studies have recently reported changes in various aspects of the macular ganglion cell complex (GC-IPL) using high-definition (i.e., spectral-domain) OCT technology [22,35,44]. Studies comparing GC-IPL thicknesses in large samples of patients with MCI or AD and HCs found reduced GC-IPL thickness in all quadrants in both MCI and AD patients relative to HCs [45,46]. By comparison, RNFL thickness was significantly reduced between AD and HCs, but only in the superior quadrant, and RNFL thickness in the MCI group

was not significantly changed from HCs [45]. These findings provide support for the possibility that neurodegenerative changes in the GC-IPL may be easier to detect at earlier disease stages relative to those in the RNFL.

Choroidal thickness: Choroidal thickness has been another target of possible disease-related change, and Bulut and colleagues [47] have shown significantly reduced choroidal thickness in both MCI and AD participants compared to HCs. Thus far, other studies examining choroidal thinning have been limited to individuals with at least mild AD, but findings have been consistent with a pattern of retinal structural thinning in association with AD pathology [44,48,49]. In a recent prospective, observational study of choroidal volume in patients with mild to moderate AD and HCs, Trebbastoni and colleagues [50] found that from baseline to 12-month follow-up, AD patients showed significant thinning compared to HCs. One notable problem with studying choroidal changes, however, is that it has proven to be particularly challenging to distinguish AD and glaucoma related changes in choroidal volume using OCT methodology [49,51], an indication that this biomarker may not be specific to AD.

Associations with Other Biomarkers of AD: Only a limited number of studies have compared OCT retinal measures with MRI, CSF or PET biomarkers, in order to benchmark their sensitivity as potential screening markers for AD. Our lab (Snyder, Alber and colleagues) has compared cortical amyloid deposition (using florbetapir PET amyloid imaging) and potential OCT markers of AD in cognitively normal adults with subjective memory complaints and a positive family history of AD, 10 of whom were Aβ+ [52]. A trend was observed toward a selective volume increase in the inner plexiform layer (IPL) in Aβ+ versus Aβ- participants. We suggest the possibility that this IPL volume increase could reflect early inflammatory processes associated with cholinergic disruption and possibly concurrent Aβ protein accumulation within the inner retina (as in the cerebral cortex, for these same individuals). This interpretation has recently found additional support from preliminary research showing increased thickness in the superior macula associated with brain and retinal Aβ deposition in HCs, which the author's posit reflects early

inflammation in this area in pre-clinical AD [53]. Retinal thickness in this area was also positively associated with retinal Aβ burden in a subgroup of MCI participants with high brain Aβ burden, whereas a pattern of thinning in the superior macula was found in AD relative to HCs, consistent with the literature.

Golzan and colleagues [54] have also recently investigated associations between retinal structural changes and amyloid burden in the elderly in a comparison study of patients with AD, preclinical AD (SUVR >1.4), and HCs who underwent MRI and 18F-florbetaben (FBB)-PET amyloid imaging. In contrast to the positive associations between macular volume and Aβ deposition in HCs reported by Asanad and colleagues [52], they found no association between RNFL or RGCL thickness and Aβ SUVR in any of their groups. Nonetheless, GCL thickness, but not RNFL thickness, was found to be significantly lower in the clinical AD group compared to HCs and preclinical AD patients, but was not significantly different between the preclinical AD and HC groups [54].

Finally, den Haan and colleagues [26] have reported findings from a small sample investigation of RNFL thickness in 15 patients with early onset AD (EOAD) who were amyloid positive for either CSF or PET imaging biomarkers. In contrast to previous research in primarily late onset AD, macular and RNFL thickness was not significantly decreased in EOAD patients compared to controls. Additionally, total macular thickness, but not RNFL thickness, correlated with global and parietal cortical atrophy in both the EOAD and HC groups; however, these atrophy scores were determined only by visual ratings of MRI. In a recent follow-up, den Haan and colleagues [43] found a correlation between macular RNFL thickness and general cortical as well as parietal atrophy in a sample of biomarker confirmed AD patients. To our knowledge, no other study has assessed the relationship between cortical atrophy and retinal changes specifically in a group of AD patients; however, one previous study has reported an association between medial temporal lobe volume, but not memory performance, and RNFL thickness in cognitively healthy individuals [55].

In the only longitudinal within-subjects studies of structural changes in preclinical AD that we have been able to identify, Santos et al [30], found that preclinical AD patients experienced accelerated mRNFL thinning over a 27-month period compared to healthy older adults, which remained statistically significant after accounting for age. Moreover, the mRNFL thinning had a significant linear relationship with amyloid PET SUVr, such that increased mRNFL thinning corresponded to increased cerebral amyloidosis. This was an initial exploratory study with a sample size of 65 participants, and additional larger studies in preclinical AD samples are needed to confirm whether mRNFL thinning is a sensitive and reliable biomarker for disease progression in pre-clinical AD. In a recent large, population-based study (N=32,038; ages 40-69 yrs.), Ko et al [56] found that RNFL thinning was associated with poor cognitive performance at baseline and was predictive of cognitive decline at 3-year follow-up. Furthermore, Mutlu et al [57] followed 3,289 cognitively normal adults and found that RNFL thinning at baseline was associated with increased risk of dementia onset after 3-8-year follow-up.

Ultimately, further research is needed to examine the associations between structural changes in the retina and other neurological, genetic, and cognitive features of AD. Furthermore, our knowledge of how retinal changes occur over the course of the disease will be greatly improved by research that utilizes biomarker confirmed groups of low-risk HC, preclinical AD, MCI, and AD participants that are followed over time.

Retinal proteinopathies in Alzheimer's disease

There is abundant interest in identifying retinal Aβ and tau in animal models and humans with the ultimate goal of developing a sensitive, *in vivo*, cost-effective and noninvasive biomarker for the early detection of AD. Studies have been published by several groups focusing on early identification of retinal Aβ, its relation to cerebral Aβ, the spatiotemporal relationship to other AD pathologic features and the mechanism by which Aβ becomes detrimental to the retina. After numerous published studies, the results nonetheless remain somewhat inconclusive. Research

has thus far focused on retinal imaging findings in symptomatic Alzheimer's disease patients, and studies describing findings in preclinical stages are lacking.

Beta-Amyloid Protein Deposits in the Retina.

Though animal models show promise in identifying retinal Aβ *in-vivo*, research on identification of retinal Aβ in humans is limited and inconsistent. Both *in vivo* and *ex vivo* transgenic mouse models have demonstrated considerably more promising results, with respect to the identification of retinal Aβ deposits, than have human studies. Jiang et al. [58] reviewed the diagnostic accuracy of Aβ detection in Alzheimer's patients, and they only identified 5 of 493 published studies to be of adequate quality [24,25,59–61] for inclusion in their meta-analysis. Within those five studies, they found significant heterogeneity in staining, tissue mounting techniques and other methodologic parameters, making it impossible to draw any definitive conclusions. Moreover, these studies differed considerably with respect to participant inclusion/exclusion criteria, diagnostic criteria for AD, median ages of the samples, allowance for comorbid diagnoses, and sample sizes.

Whereas most prior work has not led to reliable detection of retinal Aβ in human tissue, Koronyo-Hamaoui [59] and colleagues showed positive results, which are described below. Jiang et al [58] point out an important factor why Koronyo-Hamaoui's group has had greater success in the histological identification of Aβ deposits, in that this group used five Aβ antibody clones (against the N-terminus, the mid-portion, or the C-terminus of the amino acid), while the other studies used only one clone (against either the N-terminus or the mid portion). Koronyo-Hamaoui et al. [59] identified retinal Aβ in postmortem eyes of 8 AD patients and 5 suspected early stage AD cases. Of note, it is not entirely clear how these latter cases were determined to be suspected early stage AD. They note clinical diagnosis was determined through review of clinical and cognitive assessments. In the same study, the authors demonstrated a non-invasive, in vivo approach to identify retinal Aβ plaques in transgenic mouse models, and they notably found that retinal Aβ in AD-transgenic mice preceded cerebral Aβ deposition. This is the first known study

to identify *in vivo* retinal Aβ in animal models. Aβ was detected through the administration of curcumin, a safe fluorochrome label, as the contrast agent. Using the same method, the authors identified Aβ in whole mount retinas of AD human subjects, which were undetectable in non-AD human retinas. They performed double staining with curcumin and several anti Aβ antibodies to identify different epitopes. The authors found multiple intracellular and extracellular Aβ deposits in AD patients.

Building on the Koronyo-Hamaoui findings, Koronyo et al. [62] reported the development of an *in vivo* technique to detect Aβ in human AD patients. Based on data from 23 AD patients, with clinical and autopsy confirmed AD and 14 age- and sex-matched controls, retinal flat mounts and cross sections were stained with various anti-Aβ compounds (12F4, 6E10, 4G8, 11A5-B10) using peroxidase- and fluorescent-based techniques. In all AD cases, the authors demonstrated increased retinal Aβ immunoreactivity and deposits compared with controls, particularly in the superior quadrants, and these deposits were morphologically similar though smaller than their cerebral counterparts. Subsequently, the Koronyo group used a proprietary curcumin supplement to identify autofluorescence patterns. They found that retinas of definite AD patients contained multiple extracellular Aβ deposits including classical plaques and deposits associated with blood vessels, especially in the ganglion cell layer which has been previously found to have a marked degeneration.

It is important to note that retinal Aβ deposition is characteristically found in acute macular degeneration (AMD), located in retinal drusen. This is a disease that has a prevalence rate of between 9 and 12 percent in older adults, depending on the study cited, and AMD often co-occurs with AD. Retinal drusen are typically located in the outer retina, at the level of the retinal pigment epithelium (RPE) and Bruch's membrane [63,64]. It is important to ensure that the Aβ deposition in AD can be differentiated from the retinal drusen of AMD that may also contain amyloid. With this consideration in mind, Snyder and colleagues [52] recently reported a significant increase in both the number and surface area of retinal "inclusion bodies", which are thought to contain fibrillar

Aβ deposits, similar to cerebral Aβ in AD [65–67] in individuals with preclinical AD, compared to age- and sex-matched healthy controls, using BAF imaging. The reported inclusion bodies were distinctive from the drusen in AMD (see [52] for details), and both the number and size of these inclusion bodies were moderately correlated with neocortical amyloid aggregation (amyloid PET imaging), and they tended to be localized to the inner plexiform layer (IPL), which is known to be the "cholinergic-rich" region within the vertebrate retina [52]. Snyder et al. also reported modestly increased thickness of the IPL in their preclinical AD sample, possibly reflecting an early disease-related inflammatory process [52].

Chidlow et al. [68] were unable to replicate Koronyo-Hamaoui's [59] findings despite performing various histologic, immunochemical and molecular tests to characterize retinas of double transgenic mice (APP/PS1) in the early stages of disease progression.   APP/PS1 mice are genetically modified to overexpress mutated human APP. Four antibodies were utilized for immunolabeling. They found diffuse deposits and fibrillary plaques in the brains of 4-month old Tg-mice, there was no retinal Aβ found in any Tg or wild type (WT) mice from 3 to 12 months of age, and this finding was consistent among the four antibodies. They mention a possible limiting factor was the lack of whole mount specimens studied; however, the authors point out that Koronyo-Hamaoui et al. [59] found Aβ in both whole mount and transverse sections. The authors found robust expression of amyloid precursor protein (APP) in Tg mice compared to WT mice in all neuronal classes but more so in RGCs. One particular inconsistency in this type of animal research is significant variability in the strains of AD mice, and even between different populations of the same original strain where genetic backgrounds and housing/breeding conditions differ.

Shah et al. [69] performed a review of the literature on Aβ in the aging eye in animal models and in humans noting inconsistent findings in human studies of post mortem eyes. The authors note more promising findings in animal models of AD, which described Aβ deposition in age dependent fashion in AD-transgenic mice compared to wild type controls [70,71]. Shah and colleagues [69] also note their own positive results using the same proprietary curcumin

supplement as described by Koronyo-Hamaoui's lab, which showed high correlation with cerebral Aβ plaques. Other studies have provided suggestive but not definitive evidence of retinal Aβ in AD human cases. Tsai et al. [25] reported "plaque-like structures" identified in whole mount retina of 2/6 autopsy confirmed human AD cases by anti-human Aβ plaque-specific antibody 6E10. Ho and colleagues [60] performed immunostaining for Aβ, phosphorylated-tau and α-synuclein and Congo red stains in both brains and eyes from 11 cases of AD, 6 cases of PD or PD with dementia, and 6 age-matched controls. The authors were unable to identify amyloid deposits nor abnormal tau in the retinas or other parts of the eyes of AD patients.

Tau Protein Deposits in the Retina.

Another pathologic feature of AD involves intranuclear inclusions of hyperphosphorylated *tau*. Furthermore, *tau* burden has been shown to be more consistent with level of cognitive impairment in AD than cerebral Aβ accumulation. The ability to detect retinal *tau* and correlate to cerebral *tau* levels could provide a minimally invasive, cost-effective approach to diagnosis and treatment monitoring, particularly for patients in the early to moderate stages of the disease (and less so in the pre-clinical and MCI stages). Similar to the pattern of results described above, with regard to the detection of Aβ deposits, at this point the detection of *tau* in the retinas of animal models of AD has yielded more consistently promising results than for human cases of AD (for which there remains a paucity of data). As mentioned above, Ho and colleagues [60] did not detect *tau* in the eyes of 11 AD human cases with immunostaining techniques. Of note, the Ho paper was considered sufficient quality to be included in the Jiang et al [58] meta-analysis. Other studies have looked for retinal *tau* in normal aging and have reported variable findings. One study [72] of 24 eyes of cases with retinitis pigmentosa or age-related macular degeneration examined non-phosphorylated *tau*, APP and Aβ in 24 eyes of varying ages, and found increased APP but not Aβ immunoreactivity in the retinas of older individuals. The retinal *tau* staining pattern was different than that for the brains of the same subjects, and there was no association with age. Leger et al. [73] studied protein aggregation of the aging retina, examining 19 eyes of patients

49–87 years of age, two of whom had clinical dementia. They found age-dependent increases in *tau*-positive retinal ganglion cells but found no retinal phosphorylated-*tau* or Aβ in the retina.

Schon et al. [24] demonstrated the first long term in vivo imaging technique, using scanning laser ophthalmoscopy, in a human P301S tau transgenic mouse line, a well-established model of frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), which develops severe *tau*-pathology. They utilized this approach to examine postmortem retinas of six patients with clinical AD (n=6) and progressive supranuclear palsy (n=2) along with healthy controls (n=4). They were able to detect hyperphosphorylated *tau*, but no fibrillar *tau* or Aβ aggregates were identified. Recently, den Haan et al. [74] performed an autopsy study examining amyloid and *tau* in 6 pathologically confirmed AD patients and 6 age-matched healthy older adults. Using immunohistochemical stainings, they found a diffuse signal for phosphorylated *tau* in the inner and outer plexiform layers, but no significantly increased presence of Aβ plaques or APP in the AD group. Interestingly, the authors used cross-sectional sections rather than whole mounts, which is a known methodological discrepancy in assessing retinal amyloid thus far (see above).

Using a triple transgenic mice, Chiasseu et al [75] performed western blots, qPCR, and immunohistochemistry in the mouse retina and visual pathways. They found age-dependent retinal *tau* accumulation, as early as three months, notably in the retinal ganglion cell (RGC) soma and dendrites. The retinal *tau* build-up preceded cerebral *tau* accumulation. Furthermore, the authors demonstrated significant impairment in anterograde axonal transport which preceded cell death in the AD retina, and, therefore, may be an early marker of neuronal dysfunction.

Macular Pigment Optical Density in Alzheimer's disease

The xanthophylls, lutein (L), zeaxanthin (Z), and meso-zeaxanthin (MZ), collectively known as macular pigment (MP), are oxygenated carotenoids that accumulate in the macula, functioning as short-wavelength (blue) light-absorbers and localized antioxidants in a region at high-risk for light-induced oxidative stress [76]. These carotenoids cannot be synthesized *in vivo*

by vertebrates and invertebrates and must be obtained from dietary consumption. Low carotenoid status is associated with various deleterious health effects including degenerative retinal diseases, such as AMD [77], and has an inverse relationship to cognitive function [78].

Macular pigment optical density (MPOD) is a non-invasive measure of retinal carotenoids and surrogate measure of brain carotenoid status through psychophysical and physical approaches. Psychophysical techniques include color matching, motion photometry, heterochromatic flicker photometry and customized heterochromatic flicker photometry. Physical techniques available for measuring macular pigment include resonance Raman spectroscopy, fundus autofluorescence, and fundus reflectance [76]. MPOD has been found to correlate with brain levels of L and Z xanthophylls in primates [79] and humans [80].

The mechanistic relationship between MPOD and cognition is unclear. Renzi and Hammond posit that MP's effect on improving processing speed may be due to direct effects on brain connectivity, possibly by enhancing gap junctions between neurons [81]. Multiple studies have shown cognitive improvement and subsequent higher MPOD following L and Z supplementation, using purified supplements and whole foods in community-dwelling older adults [78,80,82,83], and individuals with MCI [84]. When comparing MCI cases with cognitively normal controls, Renzi and colleagues found that MP status more strongly correlated with cognition in those with established cognitive impairment [84]. MPOD shows promise as a biomarker of early cognitive decline in neurodegenerative disease; however, larger, systematic trials are needed to determine whether or not it is specific to AD, or a general marker of neurodegeneration. Indeed, the only clinical trial of carotenoid supplementation in AD to date [85] failed to meet cognitive endpoints. Of note is that the mean age of the participants in that trial was 80 years, and the mean MMSE score was 19. Whether supplemental carotenoids would improve cognitive and visual outcomes in earlier stages of the AD disease severity spectrum remains unstudied.

Retinal Vascular Changes in Alzheimer's disease

Decades of research has shown that AD, cardiovascular disease, and cerebrovascular disease are frequently co-morbid and share common risk factors and pathophysiology (see [86] for a review). Retinal vascular changes may be reflective of AD cerebrovascular pathology due to their shared pathophysiology. Vascular factors and cerebral small vessel disease are implicated in AD, but due to the challenge of visualizing cerebral microvasculature, data are somewhat limited. Just as structural similarities exist between cerebral and retinal vasculature, emerging evidence has identified similarities in hemodynamic properties between cerebral and retinal circulation. Retinal imaging is a promising approach to more easily characterize blood vessels *in vivo* and to provide surrogate markers of the integrity and function of cerebral microvasculature in AD. To that end, several papers have described retinal microvascular changes in AD [19,87,88]. Here, we discuss current data on retinal imaging to measure retinal vascular alterations across the AD spectrum, of which most published studies assess retinal changes in MCI and symptomatic AD, while fewer studies target the preclinical AD population.

A recent study by O'Bryhim and colleagues examined the relationship between retinal vascular changes and amyloid biomarkers in preclinical AD [89]. The authors examined the retinas of 32 cognitively normal older adults, 14 of whom were positive for cerebral Aβ biomarkers (CSF or PET testing) using OCT-angiography (OCT-A), and found an increased area of foveal avascular zone (FAZ) in the biomarker positive vs. the biomarker negative group. The authors conducted a receiver operating curve (ROC) analysis and found that FAZ area reliably discriminated amyloid negative from amyloid positive cognitively normal older adults (AUC = 0.80). However, the authors point out that their study was cross-sectional in design, and longitudinal follow-up of these participants would be required to rule out confounding factors of foveal avascular zone enlargement or whether the observed change was specific to preclinical AD. To our knowledge, this is the only study with biomarker confirmed preclinical AD subjects at this time. Other studies have examined the retinal vasculature of MCI and AD patients.

Blood Flow Measures

Structural retinal abnormalities identified in AD are often found in other diseases affecting the retina, such as diabetes or hypertension. As well, static retinal images may vary depending on vascular diameter changes related to the cardiac cycle [90]. Therefore, retinal imaging techniques that capture pulse driven variability may be more specific to AD [91]; however, this remains unclear due to limited data. Bulut and colleagues [92] used OCTA to measure retinal choroidal vascular structures and choroidal thickness in 26 AD patients and 26 healthy controls and found that the retinal vascular density was significantly lower and the foveal avascular zone (FAZ) was significantly enlarged compared to the control group. Furthermore, a significant correlation was found between mini-mental status exam (MMSE) scores and all vascular density parameters, CT parameters and FAZ delineated by OCTA imaging.

Feke and colleagues [93] found decreased flow velocities in the retinal central veins in both MCI and AD. Compared to controls, retinal central vein blood flow was decreased by 38.6% in AD patients and by 19.4% in MCI. In a small prospective cross-sectional study of 9 patients with mild to moderate probable AD, Berisha and colleagues [16] found a specific pattern of RNFL loss, narrow veins, and decreased retinal blood flow in those veins. Both of these studies relied on retinal laser Doppler flowmetry to evaluate retinal blood flow, and it is important to note that this technology does not accurately reflect the flow of deoxygenated blood through larger vessels [94]. Therefore, other techniques are necessary to evaluate retinal microcirculation, such as a "Retinal Function Imager system" (Optical Imaging Ltd., Rehovot, Israel), which applies a stroboscopic light source and a high-resolution digital camera to rapidly take a series of retinal images to measure retinal blood flow rate (BFR). Using this latter technology to measure the velocity of arterioles and venules in the macular region, Jiang and colleagues found decreased macular BFR, but no difference in velocities, in patients with AD and MCI compared to cognitively normal controls [95]. Gameiro et al [46] used a similar imaging system to evaluate macular blood flow in arterioles and venules and found reduced macular tissue perfusion and flow volume in AD patients compared to age-matched healthy older adults. Golzan et al. [54] identified a significant

correlation between degree of retinal vascular pulsatility and cerebral amyloid burden in older adults with subjective cognitive impairment (N=73), compared to individuals with AD (N=28), independently of other risk factors. Retinal vascular pulsation and dynamic vascular response were measured with SD-OCT, using a technique where the retina was stimulated by three 30 second sets of flicker-induced light, allowing for the identification of arteries, veins, and their dilatory response along with the amplitude of vessel pulsatility [54]. Systemic vascular parameters (pulse pressure and pulse wave variability) were also assessed along with 18F-florbetaben PET measurement of neocortical Aβ burden, to characterize a 'preclinical' group (SUVR >1.4, N= 23). The authors found a negative correlation between amplitude of retinal venous pulsations and Aβ SUVR in the preclinical cohort, but a positive correlation was found between retinal arterial pulsation amplitude and Aβ SUVR. Of note, there was no significant association between retinal venous and arterial diameters with Aβ SUVR. The authors point out the discrepant findings of other studies with regards to venular caliber association with risk of cognitive decline and posit that this is further evidence that focusing on dynamic retinal factors may be more applicable in the preclinical population [54]. To this end, Querques et al. [96] found group differences between both AD and MCI patients versus healthy controls in terms of arterial dilation and reaction amplitude using a dynamic vessel analysis technique, but no significant group differences in OCT-A measures.

Vessel Morphometry, Caliber, Branching, and Tortuosity.

In addition to blood flow, physical changes in retinal vessel structure, and in the complexity of the vascular bed, have been examined in context of AD. There have been multiple recent publications showing reduced retinal vascular complexity and fractal dimension (D_f; an index relying on fractal geometry to summarize vessel branching complexity and vascular density) in subjects with MCI and AD [90,92,97–100]. Of note, McGrory and colleagues reported an opposite effect in their study of 683 community-dwelling older adults, but this sample was not selected based on concerns for pathological aging [101].

Very few studies have sought to focus on such changes in the pre-clinical stage of AD. In one cross-sectional study to explore retinal vascular changes in preclinical AD, Frost et al. [19] identified a relationship between elevated cortical Aβ on PiB PET imaging, and increased vessel branching asymmetry and length-diameter ratios, in Aβ+ healthy older adults (N=15) vs. Aβ- healthy older adults (N=30). For this work, Frost and colleagues relied on the Singapore I Vessel Assessment (SIVA) technology to evaluate vessel caliber, branching symmetry, tortuosity, and length-diameter ratio. SIVA software is a common methodology in the assessment of fractal dimension and other vascular parameters, and is applied to fundus photography [102].

Broadly, retinal vessel caliber refers to the diameter of vessels, while retinopathy is defined by damage to the retina due to vascular change. Components of retinopathy include arteriovenular (AV) nicking, microhemorrhages, soft exudates or cotton wool spots, hard exudates, focal necrosis, and other damage [103,104]. Changes in retinal vascular caliber – specifically arteriolar narrowing and venular widening – have been commonly associated with aging, hypertension, diabetes, atherosclerosis, stroke/cerebral small vessel disease, renal disease, diabetic retinopathy, age-related macular degeneration, and more [103]. More recently, researchers have proposed a link between vessel caliber/retinopathy and Alzheimer's Disease; however, the extent of this relationship requires further exploration. An in-depth systematic review published by Heringa et al. [104] summarized 29 cross-sectional studies examining AD patients and healthy controls, and found they found a consistent association between retinal microvascular changes (vessel caliber, retinopathy) and dementia. However, only 9 longitudinal studies existed at this time, and this association was weaker in longitudinal cohorts. Interestingly, there was an association in these longitudinal studies between retinopathy and volume loss on MRI.

Most publications on this topic have included a combination of OCT and digital fundus photography imaging to assess vessel caliber and retinopathy in MCI and AD patients. Studies with AD patients confirmed by disease-specific biomarkers are lacking, with most MCI and AD subject groups defined solely by clinical diagnoses and cognitive outcomes. Retinal vascular

abnormalities have been examined in several large cohort studies. The Rotterdam study cohort (N=5,553; N=655 with dementia at 10 year follow-up) found an association between increased venular caliber at baseline and risk of vascular dementia, but not AD [105]. The Atherosclerosis Risk in Communities (ARIC) cohort (N=8,734) showed retinopathy (microhemorrhages, soft exudates) was associated with impaired performance on cognitive testing [106] at baseline, but this diminished when within-subjects longitudinal data were examined. An association between retinopathy has been replicated in other cohort studies in cross-sectional designs [107,108]. Berisha and colleages [16] used a small cohort (N=9) of AD patients and age-matched cognitively normal older adults to demonstrate vessel narrowing in AD retinae, however this was not replicated in larger cohorts [108–110].

Overwhelmingly, studies of potential retinal vascular biomarkers of AD are cross-sectional in design (with a few notable exceptions, as noted above), and do not include biomarker confirmed diagnostic criteria, probably due to invasiveness and added research expense. Moreover, the neuropsychological tools used to assess dementia in the majority of these studies (e.g., MMSE) are not sensitive in terms of detecting cognitive impairment, nor change over time, in the earliest stages of the disease. Additionally, there have been no serious attempts to standardize any of these procedures across labs in terms of methodology, techniques, or data processing software. Therefore, presently we cannot yet draw firm conclusions with respect to the reliability or clinical utility of any potential retinal vascular biomarkers in AD, and we certainly cannot draw such conclusions in preclinical AD, for which there has been a serious paucity of published peer-reviewed research. Future studies require longitudinal, repeated assessments relying on standardized methodologies, software, and featuring gold standard biomarkers for pathologic comparison. Ideally, autopsy studies will help to validate whether retinal vascular biomarkers are sensitive and/or specific to Alzheimer's disease vascular pathology.

Discussion: How to Move Forward

The literature reviewed above, in combination with the shared pathophysiology and neurochemistry between the retina and the cerebral cortex, indicates that the retina holds exciting potential as a target for the development of low-cost, non-invasive AD risk biomarkers. Importantly, this research is in its earliest stages. Similar to the earliest stages of any brain imaging technology (e.g., MRI, PET), the engineering advances that support exquisitely detailed retinal imaging have been both rapid and have outpaced our ability to develop commensurate signal processing and data interpretation approaches.

Ultimately, retinal imaging may lead to a widely available *screening* tool, to identify persons at high-risk during prodromal stages of the disease, and perhaps in conjunction with other appropriate screening markers (e.g., potentially a blood test) and in combination with known risk markers (e.g., genetic risk markers, age, family history) that are all embedded in a diagnostic algorithm. One attractive feature of retinal imaging, and one that would often lead to fairly regular repeat testing, is that instruments like OCT systems are increasingly being incorporated into general practices of optometry and ophthalmology. The vast majority of adults over the age of 50 experience presbyopia, and hence, most adults require visits to eye care specialists for corrective lenses and periodic adjustments to their prescriptions. Moreover, OCT imaging systems are far lower in cost than, say, a PET imaging system. Hence, retinal imaging is more available to the general public in developing countries than cutting-edge neuroimaging technologies.

However, this body of research remains in its pathfinding stages, and several unanswered questions remain. We do not yet have consensus on which retinal imaging modalities, methodologies, and measures will be most likely to lead to reliable, validated biomarker development. Technical parameters for image acquisition must be standardized in order to reliably compare data across laboratories and cross-culturally. We need to assess sensitivity and specificity of these biomarkers to determine diagnostic and prognostic value. Additionally, as a field we should be taking a systems biology approach to understand the effect of AD on the retina, as it is likely the case that biomarkers that are useful to detect the earliest pathologic stages of

the disease may not be the same markers that are best for staging progression with increased severity of disease. Indeed, inverted U-shaped associations have recently been found between cognitive endpoints and GC-IPL degeneration in cognitively normal, community dwelling adults [111], indicating dynamic retinal biomarker changes. Comprehensive, longitudinal studies of AD pathophysiology in the retina across the cognitive aging spectrum, with gold-standard biomarker and cognitive measures, are the next step in the process of retinal AD biomarker validation. Study design should incorporate successful strategies for standardization employed by previous large cohort biomarker validation studies in AD [112–115] and data should be made public so that other researchers are able to conduct analyses and combine this cohort with existing data. Moving forward, these successful AD biomarker validation efforts provide a guide map for the validation of retinal biomarkers in a multi-step diagnostic process that is cost-efficient, minimally invasive, and eventually identifies those ideal for AD prevention therapeutics.

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