Imaging the Retina in Alzheimer's Disease

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Thesis aim and research questions

The aim of this thesis was to assess if retinal features can serve as non-invasive diagnostic biomarkers for AD. Using different study designs we investigated whether different pathophysiological pathways of AD are involved in the retina and could serve as biomarkers. We addressed the following research questions:

- Does literature support retinal layer thinning in AD and MCI compared to controls?
- Is retinal (layer) thickness decreased in amyloid proven AD?
- Is retinal (layer) thickness decreased in posterior cortical atrophy (PCA), relative to typical AD and controls?
- Is retinal (layer) thickness decreased in preclinical AD?
- How does retinal thickness relate to cortical atrophy in AD and controls?
- Is choroidal thickness decreased in AD compared to controls?
- Are retinal vascular parameters altered in AD compared to controls?
- Are macular vascular density and foveal avascular zone altered in AD compared to controls?
- Does curcumin bind to amyloid-beta (Aβ) and to other protein deposits in neurodegenerative disease?
- Do different curcumin forms shows comparable binding characteristics?
- Are Aβ and hyperphosphorylated tau (pTau) deposited in post mortem AD retinas?
- Can retinal amyloid be visualized using amyloid-PET?
- Can retinal amyloid be visualized in-vivo using target fluorescence with curcumin as labeling fluorophore?

Part I Retinal Neurodegeneration

In Chapter 1 we performed a meta-analysis of 25 diagnostic studies to assess retinal thinning in AD and MCI compared to controls. We found that the peripapillary retinal nerve fiber layer (RNFL) and macular thickness are significantly decreased in AD patients compared to HC (SMD-0.98 [CI -1.30, -0.66], p<.01, and -0.88 [CI -1.12, -0.65], p=<.01) respectively). In addition, we associated the effect size of group differences with mean age, mean MMSE and glaucoma using meta-regression. Concomitant glaucoma possibly overestimates the effect of AD on retinal thickness in the described studies. Recent literature also supports the notion that glaucoma and age-related macular degeneration are more prevalent in AD[1]. In addition, funnel plot asymmetry was indicative of publication bias and could have possibly added to overestimation of findings. Taken together, our findings therefore query the use of OCT as a diagnostic biomarker for AD in clinical practice and needs confirmation in well described cohorts of preclinical, prodromal (i.e. MCI) and demented AD patients with information on amyloid status, considering ophthalmological confounders[2]. Correlating OCT measurements with other biomarkers of neuronal injury (i.e. hippocampal atrophy on MRI, hypo-metabolism on FDG-PET, or increased CSF tau and pTau levels) may give increased insight in the role of OCT measurements as biomarker for neurodegeneration in AD.

In Chapter 2, 3, 4 and 5 we assessed retinal (layer) thickness in different cohorts using well characterized AD and PCA patients, preclinical AD cases and controls, taking ophthalmological confounders into account. In our pilot study in Chapter 2 we showed that in our cohort of amyloid proven AD cases and amyloid negative controls without glaucoma, retinal layer thickness did not differ between AD and control cases. In the total cohort, and in separate disease groups, macular thickness was correlated to parietal cortical atrophy in both EOAD (Spearman Rho -0.623, p<0.01) and controls (Spearman Rho -0.587, p=0.035). This suggests parallel changes in cortical and retinal thickness. We concluded that longitudinal studies including a larger number of AD patients correlating retinal thickness with biomarkers for amyloid/neuronal injury as well as disease specific biomarkers (e.g. retinal amyloid) are needed to elucidate the possible role of the retina as a source of diagnostic and/or prognostic biomarkers in AD.

In Chapter 3 we expanded our retinal imaging cohort with EOAD as well as LOAD participants. In addition, we enriched our sample with cognitively normal, amyloid negative monozygotic twins from the EMIF PreclinAD study[3]. One sibling of each twin pair was selected to avoid genetic dependency between twins. Comparing 57 AD cases with 85 controls, confirmed by amyloid biomarkers, and exclusion of ophthalmological confounders, we confirmed the findings of our pilot cohort. We showed no significant

differences in macular (layer) thickness and peripapillary RNFL between AD and control participants, despite unequivocal group differences on clinical, neuro-imaging, CSF and PET measures. Stratifying our analysis for EOAD versus LOAD did not alter results. In secondary analyses we found significant associations between macular thickness and global cortical atrophy (β -0.314; p<0.01) and parietal cortical atrophy (β -0.276; p<0.01). These findings imply that the effect size of structural changes in the retina might be smaller than in the brain and/or that changes emerge later in the disease course. Taken together, our results do not support the notion that retinal thickness measurements with conventional OCT might serve as a diagnostic biomarker for AD .

In Chapter 4 we tested the hypothesis of retinal thinning in AD neurodegeneration in a unique cohort of PCA cases from the UCL Dementia Research Center in London. PCA is also described as the visual variant of Alzheimer's disease or Benson's disease, as first described by neurologist Dr. Frank Benson in 1988[4]. As PCA predominantly affects parietal-occipital cortices, cortices involved in visual processing with projections to the retina, we hypothesized to find retinal thinning in PCA cases relative to typical AD and controls. Surprisingly, retinal (layer) thickness did not discriminate between well characterized cases of PCA, typical AD, or control participants despite apparent group differences on standard clinical, neuro-imaging and CSF measures. The use of cross-sectional retinal (layer) thickness measurements with conventional OCT as AD biomarker in PCA therefore appears to be doubtful.

Lastly, in Chapter 5 we assessed retinal layer thickness in preclinical AD using participants from the EMIF PreclinAD Twin study, stratified for amyloid status[3]. Confirming our findings in AD and PCA cases, no differences were found in retinal (layer thickness) between amyloid positive participants (preclinical AD) and amyloid negative subjects, while retinal measures showed high intra-twin correlations, suggesting a high contribution of genes or shared environment to these traits.

Taken together, despite the suggestion of retinal thinning in AD in our meta-analysis, findings from our different observational studies in Part I of this thesis do not support the role of cross-sectional measurements of retinal (layer) thickness using conventional OCT as non-invasive biomarker in AD in the dementia stage, either with a typical amnestic phenotype, nor PCA phenotype, nor in the preclinical phase of AD.

Part II Retinal Vasculature

In Chapter 6 we investigated whether changes in retinal vasculature can serve as biomarker in AD. Using established and new techniques we assessed retinal vascular caliber using Singapore I Vessel Analysis (SIVA) software on fundus photographs, choroidal thickness measurements using enhanced depth imaging (EDI) OCT and macular microvasculature using OCT angiography (OCTA). We found no disease effects on retinal vascular measures, adjusted for confounders. Venular tortuosity was inversely associated with Fazekas-score in controls [β -0.56, $p<0.01$], while vessel density in the outer ring of the macula was inversely associated with Fazekas-score in AD cases [β -0.64, $p<0.01$]. Taken together, findings from our observational study in Part II of this thesis do not support the role of cross-sectional measurements of retinal microvasculature with three different imaging modalities as biomarker in the dementia stage of AD. Our findings do not support intrinsic involvement of retinal microvasculature in AD pathophysiology. As our cohort consisted of cases with relatively little vascular comorbidity, studies in VCI and mixed pathology are warranted to assess the use of retinal vascular parameters to detect vascular (co)-pathology in these populations.

Part III Amyloid and Tau pathology

In Part III we assessed the binding of curcumin to amyloid and tau in post mortem AD brain tissue, the presence of AD neuropathological hallmarks (amyloid-beta(Aβ) and Tau) in post mortem AD retinas, and the possibility of imaging retinal amyloid in vivo using PIB-PET and targeted fluorescence using curcumin as labeling fluorophore.

Curcumin, the active substance in turmeric, is reported to bind Aβ[5-8] and is fluorescent by nature[9], and could therefore be an interesting label for in-vivo diagnostics[10]. Orally administered curcumin is however poorly absorbed in the intestine hampering bio-availability[11]. This has led to development of bio-available forms of curcumin increasing bio-availability[12-14]. Curcumin reaching the systemic circulation is conjugated by the liver to increase water solubility and renal clearance and represents the majority of circulating curcuminoïds[11]. In Chapter 7 we assessed the binding properties of curcumin, its conjugates and bio-available forms. Using a post-mortem study design in which post mortem brains of AD, FTD (tau, TDP-43, Pick's disease), PART, PD, DLB, and control cases were assessed, we showed that curcumin, its isoforms, conjugates and bio-available forms bind to fibrillar Aβ plaques and CAA, and faintly stain neurofibrillary tangles in post mortem AD brain tissue, confirming earlier reports[5-8,15]. We are the first to show that different curcumin forms do not show binding in control brain or to

specific structures observed in other neurodegenerative disease like FTLD, PART, PD and DLB. As conjugates and bio-available curcumin forms showed comparable binding properties, curcumin, its isoforms and conjugates might be interesting candidates for in-vivo diagnostics in AD using a targeted fluorescence approach.

In Chapter 8 we assessed the presence of neuropathological hallmarks of AD in post mortem retinas of AD patients. Before doing so we optimized several steps in the methodology that are briefly summarized below.

Methodology: tissue processing and producing retinal cross-sections

In the first phase of this project we addressed several methodological questions concerning storage, processing, dissection and staining of retinal tissue using retinal cross-sections. We found that directly freezing post mortem eyes on PBS and storage at -80°C, is to be preferred as it enables analysis using immunohistochemistry (IHC) as well as with biochemical methods (e.g. Western Blot, ELISA). We found that thawing snap frozen eyes in formalin yields similar morphology as eyes that were directly formalin fixed, so snap frozen eyes can be thawed in formalin if IHC analysis methods are to be used. Formalin fixation showed improved morphological integrity compared to frozen sections, and improves interpretation of IHC on retinal cross-sections as it substantially increases the retinal surface that can be studied. Assessing morphology, pretreatment with citrate was superior compared to acetone, and PFA (all unpublished data).

Amyloid and tau in post-mortem AD retinas

In Chapter 8 we compared retinas of 6 AD patients and 6 control donors who donated brains and eyes to the Netherlands Brain Bank. Formalin fixed retinas were dissected in quadrants and cross sections of medial and superior retinas were made. Immunohistochemical stainings were performed for Aβ, amyloid precursor protein (APP) and pTau. We found that diffuse phosphorylated tau in the retina separated AD cases from controls. In contrast, immunoreactivity for APP and Aβ in the retina did not differ between groups. We did observe increased immunoreactivity for anti-Aβ/APP antibodies intracellularly and in small extracellular deposits in cases and controls that were negative for 4G8 and curcumin. A subset of these deposits could be characterized as corpora amylacea. In conclusion, we found that retinal manifestations of AD pathology appear to be different from cerebral AD pathology. Tau related changes were found to be present in cases with cerebral AD pathology, suggesting retinal tau as a potential molecular biomarker in the retina for AD. Translation of these findings might yield a non-invasive molecular retinal biomarker for AD diagnosis and therapeutic read-out.

In vivo imaging of retinal amyloid

We assessed the possibility of in-vivo imaging of retinal amyloid with an amyloid tracer using a retrospective cohort of amyloid-PET (^{11}C-Pittsburgh Compound (PIB)) scans from AD and control participants. In addition we prospectively enrolled AD cases and controls aiming to visualize retinal amyloid using retinal fluorescence imaging.

In vivo imaging of retinal amyloid using ^{11}C-PIB-PET

The aim of this pilot study was to assess the possibility of quantifying retinal amyloid deposition using amyloid-PET (for detailed methods is referred to the Appendix of Summary and Discussion, p227). While both AD and PCA patients (Table 1, Appendix of Summary and Discussion) demonstrated widespread neocortical PIB-binding by visual assessment, no tracer binding was observed in the retina (Figure 1, Appendix of Summary and Discussion). Moreover, quantification of [^{11}C] PIB in the retina showed negative binding potential (BP_{ND}) values, without group differences between AD (mean BP_{ND}: -0.53±0.17), PCA (-0.53±0.08) and control (-0.52±0.06) participants, consistent with major partial volume effects. Considering the retinal thickness to be between 80 μm in the far periphery and 320 μm in the macula and the surface of the macula spanning 1095 mm^2, the total retinal volume is in the order of 200-300 mm^3, while the macular volume is a modest 8-9 mm^3. Compared to the spatial resolution of the Siemens HR+ camera; approximately 7mm, the PET-signal from the retina could have therefore been sub threshold for the Siemens HR+ camera. In conclusion, we could not detect specific [^{11}C] PIB binding in the retina. This could well be due to the limited spatial resolution of the Siemens HR+ camera, impaired tracer distribution over the blood-retinal barrier, partial volume effects and/or absence of amyloid in the retina in AD patients. Future research should focus on different techniques to study the retina with better spatial resolution (e.g. using optical techniques) for example by using fluorescent or label free techniques to detect retinal amyloid.

In-vivo imaging of retinal amyloid using curcumin as labeling fluorophore

In Chapter 9 we aimed to replicate findings from Koronyo and colleagues[16], who claimed to have visualized retinal amyloid in AD cases using curcumin as labeling fluorophore. In our in-vivo study design using two bio-available forms of curcumin as labeling fluorophore we did not visualize increased fluorescence after curcumin intake in amyloid proven AD-cases. Caution should be taken however in interpreting these findings, as possibly insufficient amounts of curcumin might have reached the retina to successfully label amyloid pathology. This could be due to a relatively lower bio-availability of Theracurmin and Novasol versus Longvida curcumin as reflected in lower plasma levels or insufficient dosing. These findings thus stress the need of independent replication of Koronyo's study design[16] using a similar curcumin formulation (Longvida).

In addition, findings from our ex-vivo study did not support presence of fibrillary Aβ in post mortem AD retinas, similar to the findings in three previous studies from other groups[17-19]. This raises the question whether findings of Koronyo et al. might represent drusen, deposits associated with age, also containing Aβ[20,21]. Further research using retinal flatmounts to complement our study using retinal cross-sections is needed to rule out or confirm presence of fibrillar amyloid in the retina and assess the role of drusen in AD.

Methodological considerations

In the following sections limitations and methodological considerations of our in-vivo and ex-vivo designs will be discussed together with suggestions for future research.

In vivo cohorts

Our cohorts described in Part I and Part II of this thesis included well-characterized cases fulfilling recently published NIA-AA criteria[22] from two international respected tertiary memory clinics; the Amsterdam Dementia Cohort (ADC) of the Amsterdam Alzheimer Center and the University College (UCL) London Dementia Research Center (DRC). All participants were clinically assessed by expert cognitive neurologists. Biomarkers for neurodegeneration, neuronal injury and amyloid were used to support clinical diagnosis (MRI, CSF, and PET). In our retinal imaging cohort of the ADC we performed a thorough ophthalmic baseline measurement to rule out confounding ocular pathology such as age-related macular degeneration (AMD), glaucoma and diabetes mellitus. In the UCL-DRC cohort no ophthalmological baseline was performed. Participants' ocular history was assessed and OCT and fundus photographs were reviewed by an expert ophthalmologist for confounding pathology. Therefore, subtle, pre-clinical changes of glaucoma and diabetic retinopathy cannot completely be ruled out in the UCL-DRC cohort and might have influenced the findings in our study.

In addition, the use of tertiary memory clinic samples could have caused selection bias. Compared to other memory clinics our AD patients are typically younger (mean 65 years) and a large proportion of cases are defined as early onset AD (EOAD). We deliberately studied this population to assess downstream effects of AD pathology without large contributions of age related factors. In the course of our study we hypothesized that the retina might be differently involved in EOAD compared to LOAD, possibly explaining negative findings in our pilot cohort. In Chapter 3, we therefore deliberately expanded our cohort and enrolled late onset AD (LOAD) cases to assess retinal measures in this population. These analyses yielded similar results, rejecting the hypothesis of different involvement of retinal thinning in EOAD versus LOAD.

A second limitation is that in our studies we only assessed the central retina and not the peripheral retina. In our clinical studies we used different retinal imaging methods available, including OCT, fundus photography, OCTA and targeted fluorescence imaging. All these techniques use lenses with a field of view of 30° angle and therefore selectively visualize the central retina. There is however an increasing interest in the peripheral retina in ophthalmology using wide-field images with a 200° field of view[23]. In our post-mortem study we did observe hyperphosphorylation of tau to be more prominent in the peripheral retina. In addition a similar peripheral gradient was observed both post-mortem and in-vivo by Koronyo and colleagues[16]. As changes of the peripheral retina were not assessed in this thesis, future research should focus on imaging the peripheral retina using wide-field imaging.

Ex-vivo cohorts

In our post-mortem studies we used well-characterized cases of the Netherlands Brain Bank with a confirmed pathological diagnosis based on NIA-AA criteria for post-mortem AD diagnosis[24]. Our analysis of Aβ and tau consisted of qualitative methods using retinal cross-sections. Using a wide panel of antibodies we were able to assess conformational changes of AD hallmarks. The most important limitation is the limited number of retinal cross-sections that can be analyzed, significantly under sampling the whole retinal surface, which might cause sampling bias. In order to increase the retinal surface studied, and overcome sampling bias of retinal cross sections, retinal flatmounts can be prepared for fluorescent or IHC stainings. By dissecting the neuro-retina from the eyecup and placing radial incisions, the neuro-retina can be prepared flat on a slide and subsequently processed and stained. In our cohort we performed experiments in veil and human eyes to test extraction and staining of the neuro-retina as a whole. Despite enzymatic digestion protocols, some aqueous humor still remained on the retinal surface and hampered penetration of antibody, thereby preventing reliable interpretation of stainings. Future studies in collaboration with a vitreoretinal surgeon will be performed to completely remove aqueous humor and overcome these technical difficulties. By doing so we will be able to assess the retina as a whole and assess the complete central and peripheral distribution of pathology that might yield in-vivo imaging.

In a broader perspective, independent studies using comparable protocols for tissue processing and staining are needed to resolve discrepancies between different labs concerning amyloid presence in the retina[16-19]. These studies could help determine which of the observed retinal changes are age-related and which are disease related and could serve as a target for in-vivo imaging. Optical techniques can then be tailored to the observed retinal changes. Future research in post-mortem retinas should focus on replicating earlier findings of retinal amyloid and phosphorylated tau using a flat mount approach, expanding assessment of phosphorylated tau in AD and other

neurodegenerative diseases and quantification of findings. To further establish retinal tau as a target for in vivo retinal imaging we suggest expansion of the cohort with AD and control cases with a wide range of tau and amyloid stages, taking age as confounding factor into account. In addition, assessing other tauopathies, such as frontotemporal dementia (FTD), progressive supra-nuclear palsy (PSP), corticobasal degeneration (CBD) and glaucoma is needed to assess specificity of retinal tau hyperphosphorylation for AD. Different methods could be used to quantify findings. Quantification of IHC stainings, ELISA/Simoa analysis on retinal lysates, (imaging) mass-spectrometry and/or proteomics or RNA analysis can be used to detect and quantify molecular changes in the retina. Correlation analysis with cortical pathology can eventually be used to assess relationships between molecular changes in the retina and cortex.

Eyes on the future: molecular imaging in the eye in neurodegeneration?

The findings of this thesis suggest that molecular changes, and not so much structural (retinal thinning) or vascular retinal changes, have the most potential as retinal imaging biomarkers. We showed that hyperphosphorylated tau can be observed in post mortem AD retinas. With emerging techniques such as targeted fluorescence (in the retina) on the horizon, this could open the door to molecular imaging in the retina for neurodegenerative diseases such as AD. Given the non-invasive nature of retinal imaging, and its high resolution, this approach could have large benefits for early diagnostics of neurological disease. A recent proof-of-concept study from Cordeiro et al. shows promising validation of targeted fluorescence as method, showing in-vivo cell apoptosis in glaucoma patients using targeted fluorescence[25]. Imaging molecular changes in ophthalmological disease in the retina can potentially be translated to neurodegeneration as a whole, as the greatest common divisor in neurodegeneration is accumulation of misfolded proteins[26]. Assessing the presence of disease specific proteins, e.g. *α-synuclein* in Parkinson's disease, Parkinson's dementia, dementia with Lewy bodies, and multiple system atrophy; *tau* in frontotemporal dementia, AD, progressive supranuclear palsy and corticobasal degeneration; and *amyloid* in AD in post-mortem retinas could help identify targets for in-vivo molecular retinal imaging as diagnostic biomarker and as therapeutic read-out. Emerging (label free) techniques such as two-photon microscopy[27], (stimulated) Raman[28] and hyperspectral imaging[29] might be tools to image these molecular changes in the retina in-vivo in the future.

Retinal imaging and machine learning?

Not a single ophthalmologist will be able to determine a patient's sex based on their color fundus photographs only. Computer algorithms trained with large datasets of fundus photographs are however able to do so[30,31]. Recently, deep learning has been applied in ophthalmology for example to detect glaucoma, AMD and diabetic retinopathy[31-33]. Applying similar methods on retinal imaging data of AD patients and controls could

pave the road for non-invasive diagnostic-or screening tools for AD. A project using hyperspectral imaging data of the retina to predict cortical amyloid will be started in the ADC in 2019 using such an approach.

As Voltaire said: *'Judge a man by his questions rather than by his answers'*. As in most research, this PhD-trajectory resulted in interesting answers, yet in even more interesting new questions. New projects tailored to these questions will give both new answers and will result in new questions. Who knows, in the future a visit to the optician could be a first screening test for neurodegenerative disease.

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Appendix of Summary and Discussion

Methods

Dynamic 90 minute ^{11}C-PIB-PET scans were performed in 9 AD patients and 2 patients with Posterior Cortical Atrophy (PCA) due to AD from the Amsterdam Dementia Cohort and 5 amyloid negative healthy controls. Parametric non-displaceable binding potential (BP_{ND}) images were generated using Receptor Parametric Mapping (RPM2) with cerebellar gray matter as the reference region, using the Siemens HR+ PET-MRI camera . T1-weighted structural MRI was used to delineate the retina as a region-of-interest using a locally available imaging analysis tool. Visual assessment of the PET scans was performed to determine the presence or absence of global cortical Aβ pathology, and group differences in retinal BP_{ND} were examined using one-way ANOVA. Cohort characteristics are shown in Table 1.

	AD	PCA	Controls	p-value
Number	9	2	5	
Sex (m/f)	5/4	2/0	3/2	
Age	65.2±7.0	62.2±4.3	64.2±5.6	0.779*
MMSE	22.6±3.9	24.5±2.1	29.4±0.9	0.017*
[11C]PIB PET (n positive)	9	2	0	
Global RPM2 BP_{ND}[1]	0.86±0.32	0.63±0	0.1±0.1	0.001*
Retinal RPM2 BP_{ND}	-0.53±0.17	-0.53±0.08	-0.52±0.06	0.993*

Table 1

Abbreviations: AD= Alzheimer's disease, PCA= Posterior Cortical Atrophy, m=male, f=female, MMSE= Mini-Mental State Examination, RPM2 BP_{ND}= Parametric non-displaceable binding potential.
*One-Way ANOVA. [1]For one subject global RPM2 BP_{ND} was missing.

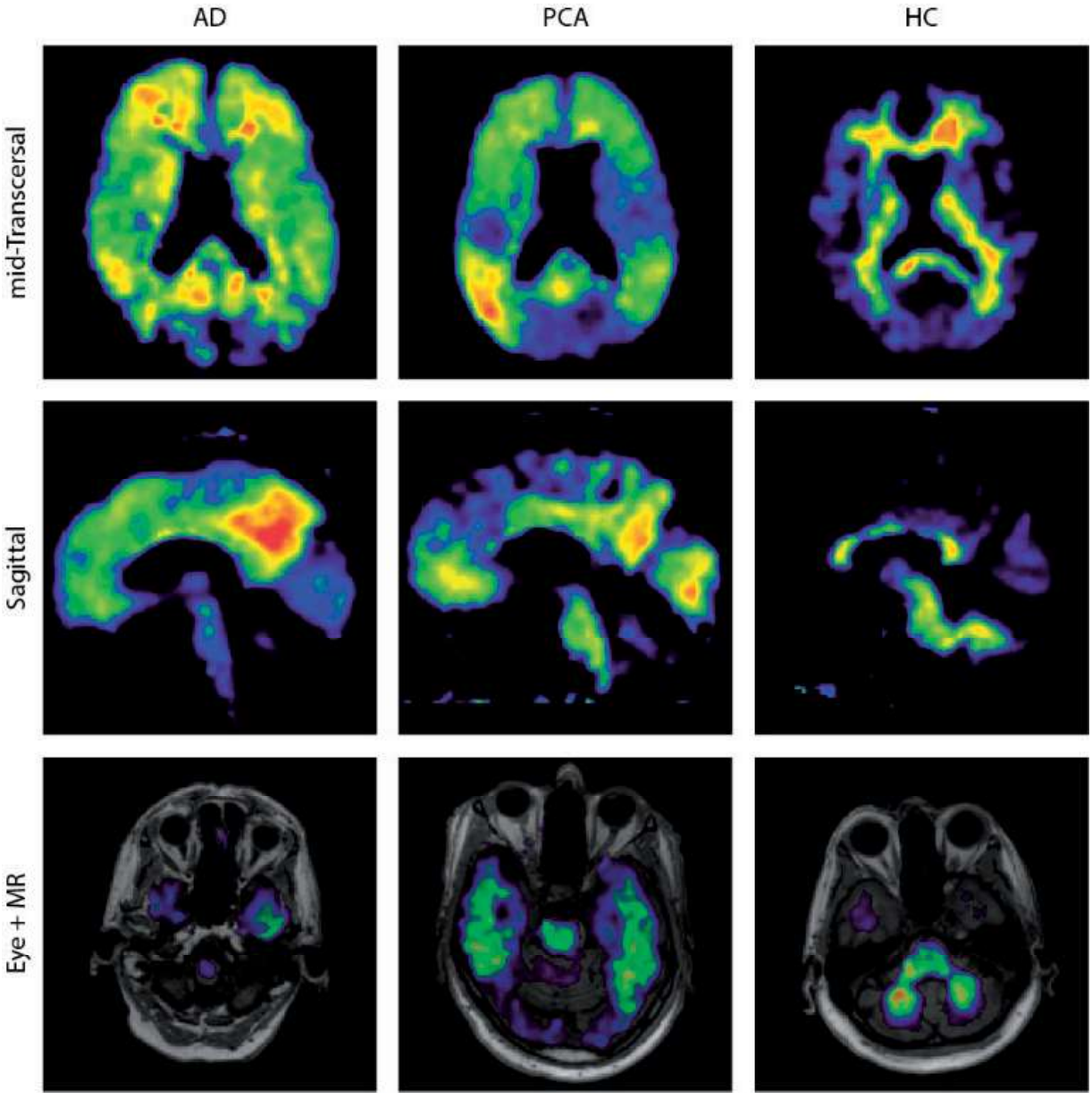

Figure 1 Cortical and retinal [^{11}C] PIB-PET

RPM2 BP_{ND} images of three representative subjects showing cortical binding in a mid-transversal slide, in the posterior cingulate in a mid-sagittal slide, and absence of retinal binding in a low transversal slide with corresponding T1-MRI imaging.
Abbreviations: AD=Alzheimer's disease, HC=Healthy Controls, PCA= Posterior Cortical Atrophy.