



# Amyloid- $\beta$ is found in drusen from some age-related macular degeneration retinas, but not in drusen from normal retinas

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**Purpose:** Age-related macular degeneration (AMD) is the most common cause of irreversible vision loss in the elderly. Increased understanding of the pathogenesis is necessary. Amyloid-beta ( $A\beta$ ), a major extracellular deposit in Alzheimer's disease plaques, has recently been found in drusen, the hallmark extracellular deposit in AMD. The goal of this study was to characterize the distribution and frequency of  $A\beta$  deposits in drusen from AMD and normal post mortem human retinas to gain additional insight about the potential role of  $A\beta$  in AMD pathogenesis.

**Methods:** Immunocytochemistry was performed with three  $A\beta$  antibodies on sections from 9 normal and 9 AMD (3 early, 3 geographic atrophy, 3 exudative AMD) retinas. Five sections from each eye were evaluated.  $A\beta$  positive deposits in drusen were identified using epifluorescence and confocal microscopy. Antibodies were pre-adsorbed with  $A\beta$  peptide to verify specificity. Some sections were stained with PAS-hematoxylin to aid in evaluation of morphology.

**Results:** To test and optimize immunocytochemistry,  $A\beta$  was detected in amyloid plaques from Alzheimer's brains.  $A\beta$  label was blocked by pre-adsorption of antibody with  $A\beta$  peptide, verifying specificity. Four of the 9 AMD retinas and none of the 9 normal retinas had  $A\beta$  positive drusen. Two of the early AMD eyes had a few  $A\beta$  positive drusen, each with a few  $A\beta$ -containing vesicles, and 2 of the geographic atrophy (GA) eyes had many  $A\beta$  positive drusen with many  $A\beta$  containing vesicles.

**Conclusions:**  $A\beta$  was present in 4 of 9 AMD eyes. Within these eyes,  $A\beta$  localized to a subset of drusen. None of the 9 normal eyes surveyed, some of which had small drusen, were  $A$  beta positive.  $A\beta$  positive vesicles were most numerous in GA eyes at the edges of atrophy, the region at risk for further degeneration. These results suggest that  $A\beta$  in drusen correlates with the location of degenerating photoreceptors and retinal pigment epithelium (RPE) cells. Further work will be necessary to determine whether  $A\beta$  deposition in drusen may contribute to or result from retinal degeneration.

Age-related macular degeneration (AMD) is the most common cause of irreversible vision loss in the elderly [1], yet its pathogenesis remains poorly understood. Current treatments are inadequate and would be improved by a better understanding of the molecular events causing the degeneration. It may be possible to gain insight into AMD pathogenesis by exploring similarities to another age-related disease of the central nervous system: Alzheimer's disease (AD). Extracellular amyloid beta ( $A\beta$ ) deposition, oxidative stress, and inflammation are important molecular mediators of Alzheimer's disease [2]. Similarly, recent evidence implicates  $A\beta$ , oxidative stress, and inflammatory processes in the pathogenesis of AMD. A role for  $A\beta$  in AMD has been suggested by the recent finding of  $A\beta$  in drusen, the extracellular deposits that are the earliest sign of AMD [3]. This  $A\beta$  in drusen might increase oxidative stress and inflammation in AMD, as it does in AD plaques [2,3].

Cell loss in AMD can occur by apoptosis of retinal pigment epithelial cells followed by apoptosis of photoreceptors [4]. The apoptotic stimulus for retinal pigment epithelium (RPE) death may involve drusen, located just beneath the RPE.

It is possible that  $A\beta$  deposition in drusen is a cause or a consequence of RPE and photoreceptor degeneration. While solid evidence has been presented that vesicular  $A\beta$  deposits are present in drusen [3], we sought to learn more about the prevalence of  $A\beta$  deposits in drusen and the association of these deposits with AMD pathology. We report immunocytochemical analysis of  $A\beta$  in 9 normal and 9 AMD post mortem retinas.

## METHODS

**Source of tissue and population profile:** Most post mortem eyes were obtained through the Foundation Fighting Blindness (FFB) eye donor program. The eye pathology reports provided the patient age, gender, brief ocular and medical history, cause of death, and post mortem interval. A few eyes were obtained directly from eye banks. One donor had a potentially relevant co-morbid condition; patient 99-35 had insulin-dependent diabetes mellitus (but no  $A\beta$ -positive vesicles). The histopathologic studies followed the tenets of the Declaration of Helsinki, and informed consent was obtained from all eye donors ante mortem. Approval for research on human post mortem donor eyes was obtained from the University of Pennsylvania.

Brain samples from AD patients followed clinically by the National Institute on Aging-funded Penn Alzheimer's Dis-

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ease Center Core (ADCC) were obtained following similar consent procedures and AD was confirmed by ADCC investigators according to established consensus criteria (National Institute on Aging and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease).

**Tissue processing and histology:** Upon enucleation, a small incision was made in the pars plana and the eyes were fixed in 4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer for several days. The fixed eyes were transferred to 2% paraformaldehyde for storage. A block of tissue containing the optic disc and macula approximately 1 cm in length and 0.5 cm in width was processed as serial 10 mm thick cryosections as previously described [5].

**Immunocytochemistry and pre-adsorption:** Immunocytochemistry (ICC) was performed using secondary antibody conjugated to Cy3 (red fluorescence emission) as described previously [4]. Five sections from each retina spaced at approximately 400  $\mu$ m intervals were labeled with monoclonal anti-A $\beta$  antibodies 4G8 and 6E10 (Chemicon, Temecula, CA; 1:1000) and polyclonal antibody pAb 2332 that recognizes multiple species of human A $\beta$  as described by Uryu et al. and additional references therein [6]. Anti-rhodopsin mAb 4D2 (provided by R. Molday; 1:80) was also used. Peptide blocking experiments were performed with a five-fold molar excess of A $\beta$  peptide 1-42 (BioSource International, Camarillo, CA), pre-adsorbing with the primary antibody at working dilution for 1 h with gentle agitation at room temperature. Images were obtained with a Nikon TE300 epifluorescence microscope equipped with a Spot RT Slider camera and ImagePro software. Images were also obtained with a Zeiss LSM 510 confocal microscope.

## RESULTS

To learn more about the prevalence of A $\beta$  deposits in drusen and the association of these deposits with AMD pathology we used ICC to detect A $\beta$  in normal and AMD post mortem retinas. To test and optimize ICC, we first detected A $\beta$  in amyloid plaques from Alzheimer's brains. A $\beta$  label in plaques (Figure

1A, arrows) was blocked by pre-adsorption of antibody with A $\beta$  peptide, verifying specificity (Figure 1B). The pink color remaining in Figure 1B is from tissue autofluorescence. Plaques were detected with our polyclonal antibody pAb 2332 as well as commercially available antibodies mAb 6E10 and 4G8 (not shown).

Sections from each of 18 retinas were then immunostained with A $\beta$  antibodies. Nine of the retinas were from donors with normal eyes and 9 with AMD as defined by histologic detection of drusen and at least some RPE and/or photoreceptor loss. Three retinas had drusen and minimal RPE and photoreceptor loss (early AMD), 3 had geographic atrophy (GA), and 3 had exudative AMD (Table 1). Four of the 9 AMD retinas and none of the normal retinas had A $\beta$  positive drusen. Two early AMD retinas had a few A $\beta$  positive vesicles in a few

**TABLE 1. NUMBER OF A $\beta$  POSITIVE VESICLES IN AMD AND CONTROL RETINAS**

Case	Age/Sex	COD	PMI	Drusen	A $\beta$ vesicles	Other pathology
AMD (drusen and photoreceptor or RPE atrophy)						
99-19	74	CA	8.25 h	few	none	small exudate
99-30	85/M	ICB	6.5 h	many large	>100	large GA
99-35	76	RF	8 h	few large	none	mild atrophy
00-11	94	CHF	19.5 h	many large	>100	large GA
00-17	89	PNEU	NA	few	none	large exudate
00-19	71/F	MI	13 h	few	none	large exudate
00-32	66/M	COPD	5 h	few large	4	mild atrophy
00-48	76/F	CA	17 h	few	none	large GA
00-1676	81/F	CA	3.5 h	many large	2	mild atrophy
Normals (no photoreceptor or RPE atrophy)						
95-10	79/F	COPD	6.5 h	none	none	
96-30	65/M	MI	6 h	few small	none	
6-23-00	74/F	CHF	4.5 h	few small	none	
99-11-23	NA	SURG	NA	none	none	
00-0165	NA	NA	NA	few small	none	
00-0164	75/M	CA	6.25 h	none	none	
00-12A151	73/M	CA	NA	1 large	none	
00-46	78/F	PE	20 h	none	none	
1-41	82/M	CA	2.5 h	none	none	

Number of A $\beta$  positive vesicles in AMD and control retinas per 5 cm combined length of sections analyzed. COD: cause of death, PMI: post mortem interval, CA: cancer, ICB: intracranial bleed, RF: respiratory failure, CHF: congestive heart failure, PNEU: pneumonia, MI: myocardial infarction, COPD: chronic obstructive pulmonary disease, SURG: this eye was surgically removed for squamous cell tumor in the orbit, PE: pulmonary embolus, NA: not available. Small drusen were <63  $\mu$ m, Large drusen were >125  $\mu$ m.

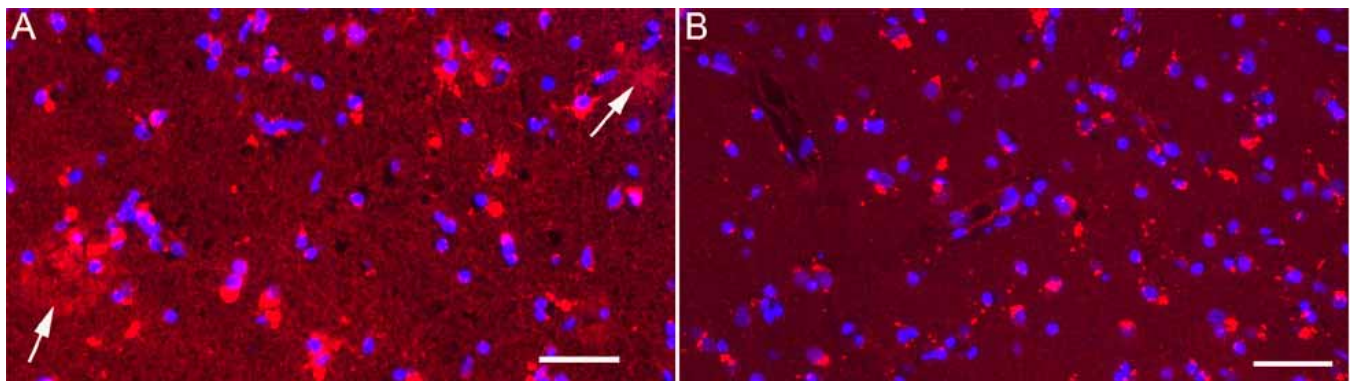


Figure 1. A $\beta$  in brain amyloid plaques. Fluorescent photomicrographs showing A $\beta$  in amyloid plaques (red fluorescence, arrows) in section from human Alzheimer's brain A $\beta$  (A). Plaque label was blocked by pre-adsorption of antibody with A $\beta$  peptide, demonstrating specificity (B). Nuclei are stained with DAPI (blue). Scale bar represents 50  $\mu$ m.

drusen, and 2 GA retinas had many A $\beta$  positive vesicles in many drusen. All retinas with drusen contained at least some A $\beta$ -negative drusen, as determined by immunolabeling serial sections through entire drusen.

Retina 00-11, from a donor with GA throughout much of the macula, had multiple large drusen at the temporal edge of the macula, where photoreceptors and RPE cells were still present, although there was some loss of photoreceptor nu-

clei. A $\beta$  positive vesicles were detected in these drusen with all three anti-A $\beta$  antibodies, but not with negative control anti-rhodopsin (red label-Figure 2A,B,D). Vesicles were not detected by the secondary antibody alone (Figure 2E). Pre-adsorption of the anti-A $\beta$  antibody with A $\beta$  peptide greatly diminished vesicular labeling (Figure 2C,G).

Retina 99-30, also from a donor with GA throughout much of the macula, had many vesicles in a region of sub-RPE de-

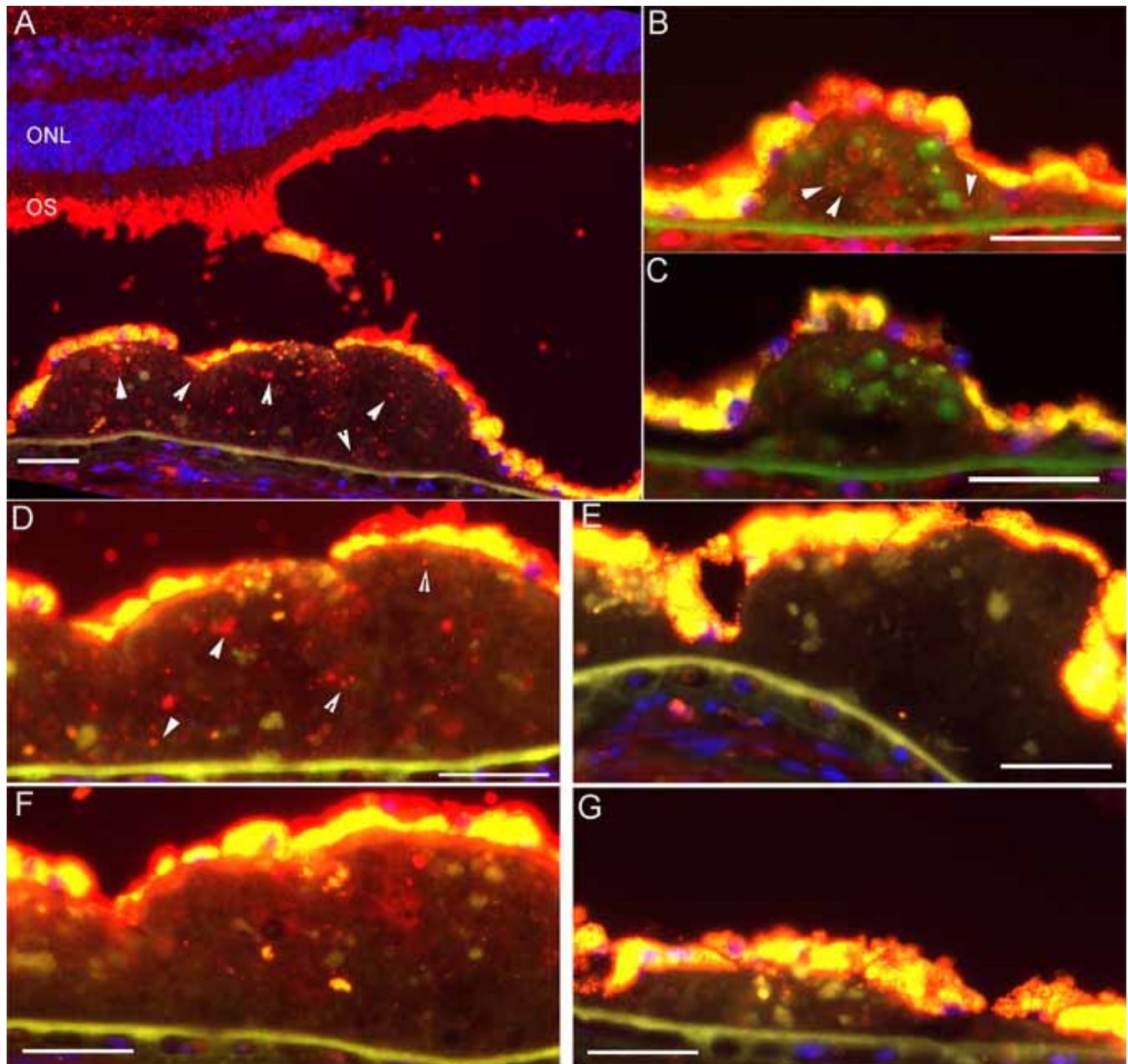


Figure 2. A $\beta$  in drusen in GA retina 00-11. Fluorescent photomicrographs showing A $\beta$  in drusen in GA retina 00-11. This donor had GA throughout much of the macula. **A:** Anti-A $\beta$  mAb 4G8 label (red) is present in vesicles (arrowheads) within a large druse and photoreceptor outer segments at the temporal edge of atrophy. Nuclei are labeled with DAPI (blue). RPE cytoplasm appears gold due to lipofuscin autofluorescence. ONL: outer nuclear layer; OS: outer segments. **B:** Anti-A $\beta$  pAb 2332 labels vesicles (arrowheads) within another large druse. **C:** Pre-adsorption with A $\beta$  peptide eliminates label. **D-G:** are closely spaced sections labeled with anti-A $\beta$  mAb 4G8 (**D**), secondary antibody only (**E**), anti-rhodopsin antibody mAb 4D2 (**F**), and 4G8 pre-adsorbed with A $\beta$  peptide (**G**). Of these four images, the only one with significant A $\beta$ -positive vesicles is (**D**), verifying labeling specificity. Scale bar represents 50  $\mu$ m.



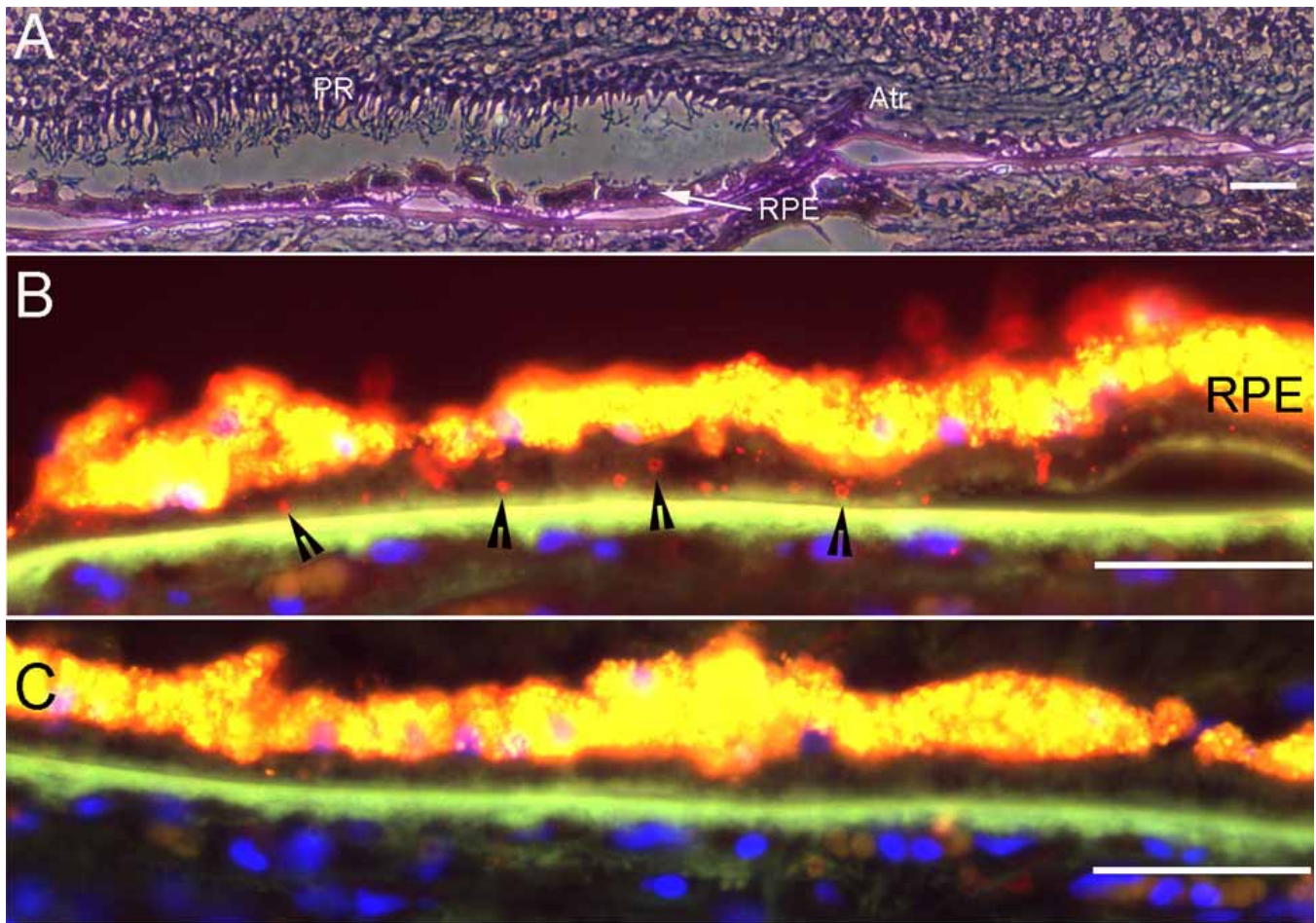


Figure 3. A $\beta$  in drusen in GA retina 99-30 near atrophic area. Fluorescent photomicrographs showing A $\beta$  in drusen in GA retina 99-30. **A:** PAS-hematoxylin-stained low power view of edge of atrophy (atr). **B:** Anti-A $\beta$  antibody 4G8 detects vesicles (arrowheads) in sub-RPE deposit. **C:** Pre-adsorption with A $\beta$  peptide eliminates label. Scale bar represents 50  $\mu$ m.

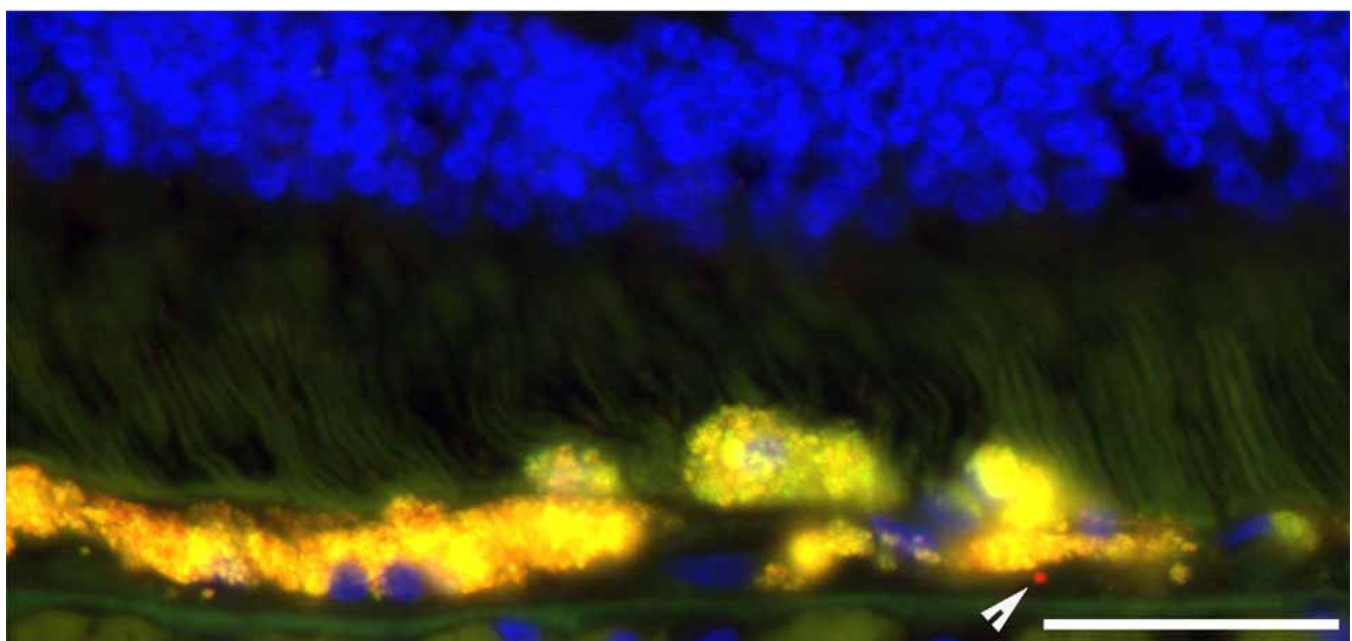


Figure 4. Single A $\beta$  positive vesicle in early AMD retina 00-32. Fluorescent photomicrograph showing A $\beta$  in drusen in early AMD retina 00-32. Arrowhead indicates single A $\beta$ -positive vesicle underlying RPE atrophy and displacement into the subretinal space. Scale bar represents 50  $\mu$ m.



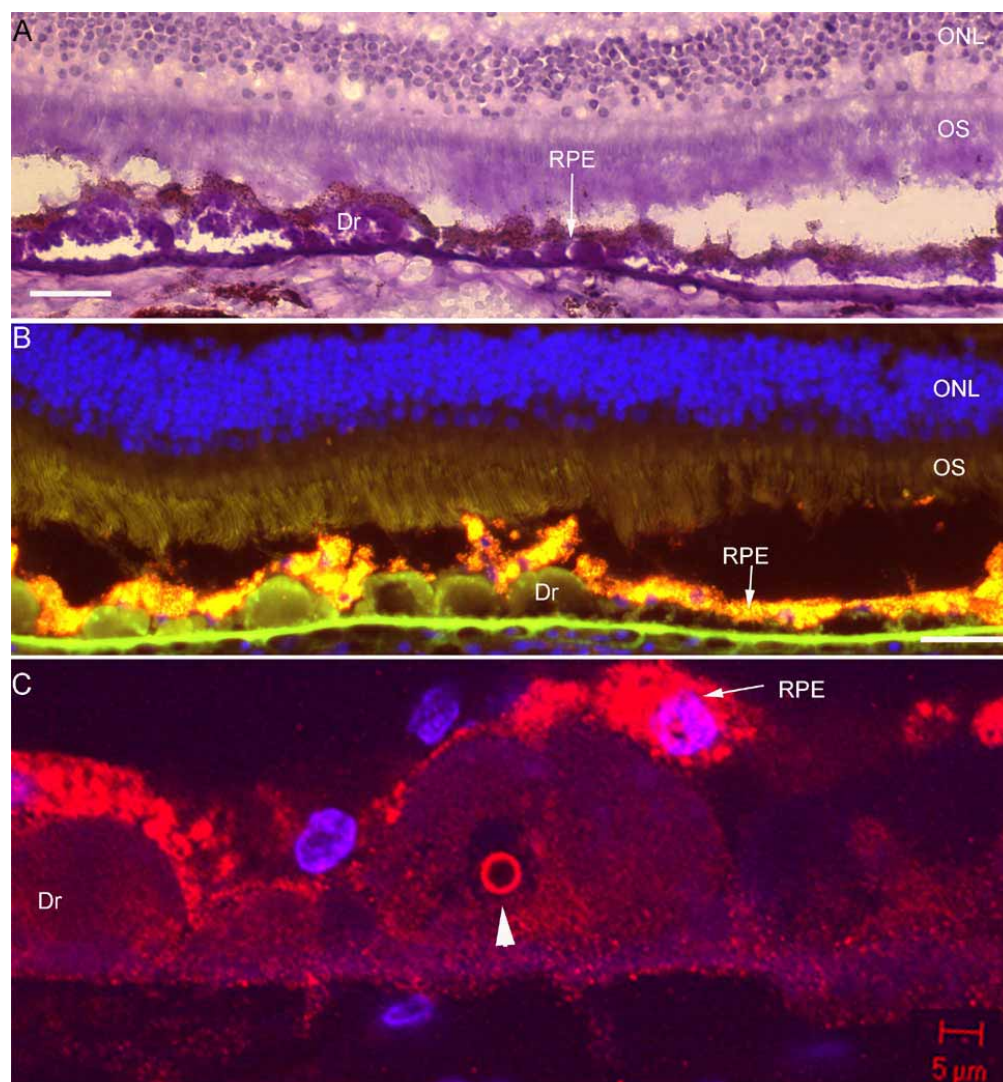


Figure 5. Few A $\beta$  positive drusen in early AMD retina 00-1676. Photomicrographs showing A $\beta$  in only a few areas in early AMD retina 00-1676. **A:** PAS-hematoxylin-stained low power image with soft drusen and minimal photoreceptor atrophy. **B:** Anti-A $\beta$  antibody 6E10 is negative in this section. **C:** In another area, anti-A $\beta$  antibody 2332 detects a single vesicle and punctate granular deposits within drusen. Image was acquired by confocal microscopy. Scale bar represents 50  $\mu$ m.

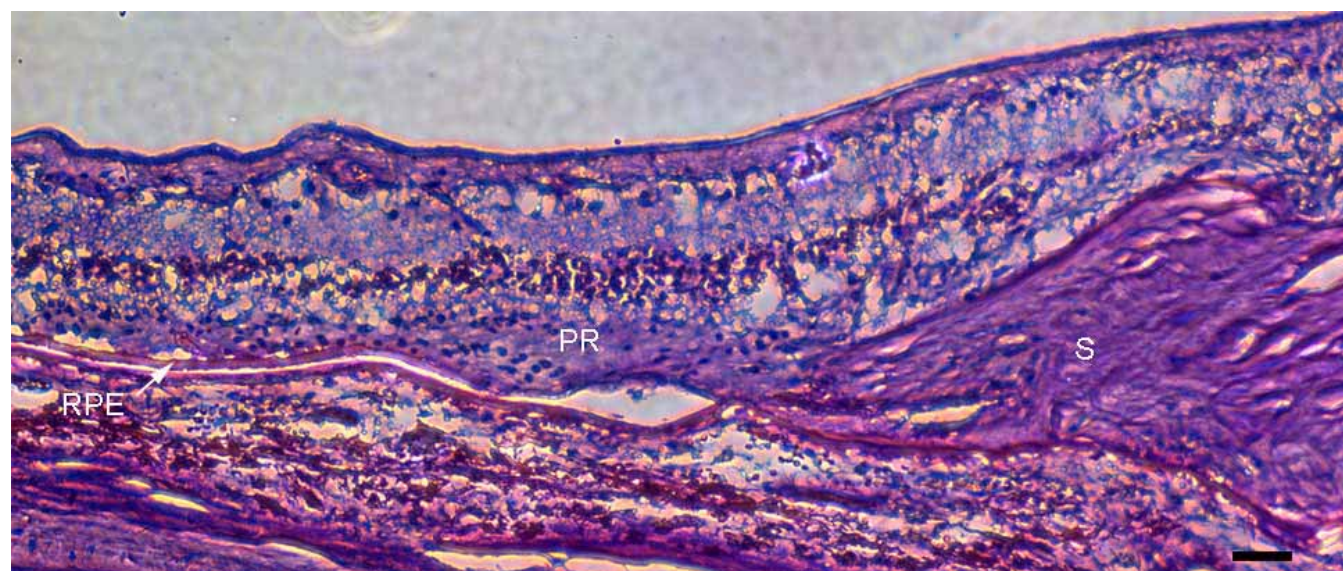


Figure 6. Exudative AMD retina 00-17. Photomicrograph of exudative AMD retina 00-17 stained with PAS-hematoxylin. This retina had no A $\beta$ -positive vesicles on ICC (not shown). Thick exudative scar (S) is present on the right. Photoreceptors (PR) are present just temporal (to the left) to scar. Further temporal, the RPE monolayer is present, with minimal sub-RPE deposit. Scale bar represents 50  $\mu$ m.

posit beginning at the margin of atrophy, 1 mm temporal to the foveola and continuing another 2 mm temporal. The concentration of vesicles was highest near the margin of atrophy (Figure 3A). Pre-adsorption of antibody with A $\beta$  peptide blocked vesicular labeling, demonstrating specificity (Figure 3B).

Two retinas with minimal photoreceptor and RPE atrophy had a few A $\beta$ -positive vesicles. Retina 00-32 had a few large drusen and focal areas of free RPE cells in the subretinal space. Associated with these RPE abnormalities were a few A $\beta$ -positive vesicles (Figure 4). This retina had minimal photoreceptor atrophy. Retina 00-1676 had many small and large drusen, and mild RPE atrophy (Figure 5A). While most of these drusen had no A $\beta$  immunoreactivity (Figure 5B), a few had both A $\beta$ -positive vesicles and smaller granular A $\beta$  particles visible on confocal microscopy (Figure 5C).

None of the three exudative AMD retinas had A $\beta$ -positive vesicles, but they also lacked large drusen. Retina 00-17 had a large exudative scar (Figure 6). In areas where RPE and photoreceptors were present (left side of image), the sub-RPE deposits were thin and A $\beta$  negative. Similarly, none of the normal eyes, some of which had small drusen, had A $\beta$ -positive vesicles (not shown).

## DISCUSSION

The pathogenesis of AD and AMD may be related. A $\beta$ , a major component of AD plaques, is also present in some drusen. Some of the A $\beta$  in drusen is in vesicles and co-localizes with complement suggesting an association with inflammation [3]. We sampled retina sections from 18 eyes, 9 with AMD and 9 normal, to determine the frequency and distribution of A $\beta$  deposits in post mortem human eyes. We found A $\beta$ -positive vesicles in drusen in 4 of 9 AMD eyes and none in normal eyes. The A $\beta$ -positive vesicles were most numerous in GA eyes, near edges of atrophy. A few A $\beta$ -positive vesicles were also present in drusen two AMD retinas with minimal RPE and photoreceptor atrophy.

The vesicles were detected with 3 different anti-A $\beta$  antibodies. The label was blocked by pre-adsorption of each antibody with A $\beta$  peptide, and was not present when A $\beta$  antibody was omitted. These results strongly suggest that the vesicles contain A $\beta$ .

The highest quantity of A $\beta$ -containing vesicles occurred near edges of atrophy in GA eyes, the area at risk for expansion of GA [4,7,8]. The vesicles may either contribute to the pathogenesis of cell death in these RPE and photoreceptors at risk, or result from ongoing photoreceptor or RPE dysfunction or death. The vesicles are neither present in areas of severe atrophy, where RPE cells and photoreceptors are absent, nor in association with exudative scars lacking RPE cells and photoreceptors. These findings suggest that the vesicles may be cleared following RPE and photoreceptor apoptosis.

The most likely source of A $\beta$  in drusen is the overlying RPE cells, which are known to deposit material into drusen. Like most other cell types, RPE cells express amyloid precursor protein [3] (APP), a membrane glycoprotein cleaved by proteases to produce the A $\beta$  peptide. The biological functions

of A $\beta$  are unclear, but both A $\beta$  multimers and fibrils can be neurotoxic [9]. The quantity of A $\beta$  present in drusen may depend upon alterations in protease activity producing A $\beta$  and upon its clearance rate.

The finding of a few A $\beta$ -positive vesicles associated with RPE abnormalities in early AMD suggests that A $\beta$  may play a role in the early phases of AMD pathogenesis. However, as in the GA eyes, it is not clear whether the A $\beta$ -positive vesicles are a cause or result of RPE and photoreceptor degeneration.

Many drusen in normal and AMD eyes lacked A $\beta$  positive vesicles, so the vesicles are not a consistent component of drusen. Within AMD eyes, vesicles were apparent in both small (<63  $\mu$ m) and large drusen.

Confocal imaging revealed that A $\beta$  signal within drusen consisted not just of vesicles, but also of granular particles. It is possible that the granular particles assemble into vesicular structures, as A $\beta$  peptides can self-assemble into spherical configurations [10].

All three A $\beta$  antibodies labeled drusen from 4 of 9 AMD retinas. In contrast, these antibodies gave an inconsistent pattern of photoreceptor outer segment labeling. Future studies will employ additional A $\beta$  antibodies and ELISA on retinal extracts to further investigate this issue.

Our data reveal that A $\beta$  in drusen is associated with degenerating RPE and photoreceptors, suggesting that the A $\beta$  may contribute to or result from degeneration. The finding of similarities in the pathogenesis of AMD and AD suggests that much can be learned about one disease from the other. Anti-A $\beta$  therapies currently under development for AD may also prove useful for AMD. Thus, further investigation of the role of A $\beta$  in AMD pathogenesis is warranted.

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