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Visualizing retinal pigment epithelium phenotypes in the transition to atrophy in neovascular age-related macular degeneration

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Abstract

Purpose—To enable future studies of retinal pigment epithelium (RPE) fate in the macular atrophy occurring in eyes with neovascular AMD (nvAMD), we determined how RPE morphology changes across the transition from health to atrophy in donor eyes with nvAMD.

Method—In RPE-Bruch’s membrane flat mounts of 5 nvAMD eyes the terminations of organized RPE cytoskeleton and autofluorescent material were compared. In high-resolution histological sections of 27 nvAMD eyes, RPE phenotypes were assessed at ± 500 and ± 100 μ m from the descent of the external limiting membrane (ELM) towards Bruch’s membrane. Thicknesses of RPE, basal laminar deposit (BLamD), and RPE+BLamD were determined. Shapes of the ELM descent were recorded.

Results—Approaching the ELM descent, the percentage of different RPE phenotypes (Zanzottera, IOVS 2015) and the thickness of RPE, BLamD, and RPE + BLamD each stayed roughly constant. When compared to a separately described cohort of eyes with geographic atrophy, eyes with nvAMD were more likely to have RPE dysmorphia that did not worsen towards

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the atrophy border, thinner BLamD overall ($3.25 \pm 3.46 \mu\text{m}$ vs $7.99 \pm 7.49 \mu\text{m}$ for geographic atrophy), and a higher proportion of oblique ELM descents (47.9% vs 31.9%).

Conclusion—The distribution of RPE phenotypes at the transition to macular atrophy in eyes with nvAMD differs from that in primary geographic atrophy, likely reflecting greater photoreceptor loss and the effects of exudation in nvAMD. This distribution, the shape of ELM descents, and thickness profiles may be useful metrics in clinical studies of macular atrophy utilizing optical coherence tomography and fundus autofluorescence.

Précis

Morphological phenotypes of the retinal pigment epithelium (RPE) distribute across the transition to atrophy in neovascular age-related macular degeneration in a manner different from primary geographic atrophy, as described separately. Thickness of RPE-basal laminar deposit may be useful as a metric to distinguish macular atrophy from geographic atrophy by optical coherence tomography.

Keywords

Age-related macular degeneration; retinal pigment epithelium; neovascularization; histology; optical coherence tomography; autofluorescence; macular atrophy

Introduction

Intravitreally injected anti-vascular endothelial growth factor (VEGF) agents help preserve vision in many eyes with neovascular complications of age-related macular degeneration (nvAMD).^{1, 2} Ten years of clinical anti-VEGF experience has shown that atrophy occurring in eyes receiving long-term therapy is associated with worse visual outcomes.³ The term “macular atrophy” has been proposed to describe the degeneration of the retinal pigment epithelium (RPE) and outer retina seen in the context of nvAMD, and to distinguish this form of atrophy from the primary degeneration of non-neovascular AMD known as “geographic atrophy” (GA).

In early trials of anti-VEGF agents, visual acuity often declined even as the effects of neovascularization were blunted⁴. Analyses of trial data suggested that continuous monthly treatment (vs *pro re nata*) was associated with a higher risk of incident atrophy and faster atrophy progression over 1–2 years^{5–8}. This effect varies with the neovascularization subtype^{8–12} and may depend on the specific anti-VEGF agent used⁸. In contrast, when followed 5 years or more, vision loss may progress more slowly in eyes receiving a continuous vs *pro re nata* anti-VEGF regimen². Interestingly, in some continuously dosed eyes with type 1 neovascularization, atrophy grows slower than that occurring in a fellow eye with non-neovascular AMD^{10, 13}. Concern regarding a possible deleterious effect of anti-VEGF treatment on atrophy progression is further heightened because neutralization or deletion of RPE-derived VEGF in mouse models results in choriocapillary endothelium dedifferentiation and atrophy^{14–16}. Because good vision in nvAMD is highly dependent on RPE survival,^{9, 17–21} a current question of clinical importance is how to best monitor RPE health in these patients.

The advent of intravitreal anti-VEGF therapy coincided with the availability of spectral domain optical coherence tomography (SDOCT) to guide treatment decisions based on cross-sectional chorioretinal anatomy. In addition, fundus autofluorescence (FAF) is informative for monitoring RPE health and metabolism by enabling detection of autofluorescence attributable to lipofuscin and melanolipofuscin, long-lasting intracellular residual bodies rich in bisretinoid derivatives of vitamin A. As assessed with FAF, RPE atrophy is inferred in nvAMD from the loss of homogenous and contiguous signal, in concert with vision loss^{9, 17–21}. Further, discontinuities in the RPE layer of treated nvAMD eyes are visible via polarization sensitive OCT, which reveals RPE cell bodies specifically.²² A challenge to understanding macular atrophy is that standardized definitions for RPE atrophy after anti-VEGF therapy are not yet available.²³ Thus opinions vary about whether macular atrophy in nvAMD eyes undergoing anti-VEGF therapy is the same⁸ or different^{3–5, 21} from primary GA, with evidence tipping towards different²⁴. The most detailed descriptions^{3, 19, 21} focus on clinical features such as earlier age of onset, more central macular involvement, outward direction of spread, and lack of sharply demarcated borders in nvAMD relative to GA.

The interpretation of macular atrophy in clinical imaging would benefit from a detailed histopathologic description of RPE in human eyes with nvAMD. Previous clinicopathologic correlations for nvAMD (reviewed by Curcio et al²⁵) focused on which aspect of RPE contacted neovascular membranes²⁶ with little attention to cells at atrophy margins. A notable exception is Sarks and associates¹⁹ who reported that fibrovascular scars lack a hyperpigmented border clinically and have alternating RPE attenuation and hyperpigmentation histologically. These findings contrasted with the hyperpigmented border and RPE cells of irregular shape and pigmentation seen in GA²⁷. Using high-resolution histology, we recently developed a system of RPE morphologic phenotypes and determined that many are visible by SDOCT^{28, 29}. In eyes with GA³⁰, we found that the RPE layer thickens near the border of atrophy, due to a progressive dysmorphia that also helps explain variable FAF at the margin of atrophy³¹. Here we use these same methods to investigate RPE morphology and thickness in the transition to atrophy in eyes with nvAMD. We find distinct differences between macular atrophy occurring with nvAMD and GA that may provide a basis for new metrics in future clinical studies utilizing SDOCT and FAF as core imaging technologies.

Methods

RPE flat mounts of nvAMD eyes were studied to assess the relationship of histological autofluorescence (AF) and cytoskeleton at the termination of the intact RPE layer, as done for GA.^{30, 32} In other nvAMD eyes, sub-micrometer histological cross-sections through the fovea and superior perifovea were studied to assess phenotypes of RPE morphology, as done for GA.^{28–30} Tissues came from a bio-repository of short post-mortem (<6 hr) eyes assembled for AMD research from donors to the Alabama Eye Bank 1995–2009. More information on all eyes is available (Supplementary material²⁸). Studies were approved by institutional review at the University of Alabama at Birmingham and adhered to the Tenets of the Declaration of Helsinki.

Clinical histories were not available. AMD maculopathy status was determined at the time of accession using stereo color photographs and internal examination using a dissecting microscope.³³ Eyes used in this study had atrophic areas detectable by inspection of the post-mortem fundus. Maculopathy was further checked at the time of histologic preparation (2011–2013) using *ex vivo* multimodal imaging including SDOCT and FAF (Spectralis, Heidelberg Engineering, Heidelberg, Germany).³⁴ Atrophy in one nvAMD eye used for cross-sectional analysis, revealed by *ex vivo* color photography and FAF imaging, is shown in Figure 1A–C.

Flat-mounts

Of 35 RPE-Bruch's membrane flat-mounts previously prepared from 35 Caucasian donors³², 5 nvAMD tissues were revisited. To enable definition of RPE cytoskeleton, F-actin was labeled with Alexa 647 conjugated phalloidin (Life Technologies, Grand Island, NY, USA). To illustrate RPE cell melanosome content and to illustrate F-actin and lipofuscin/melanolipofuscin distribution, bright field and fluorescence imaging, respectively, was performed using a confocal microscope (BX51, Olympus, Center Valley PA USA) at predefined locations and settings, as described³⁵, plus additional images in areas affected by atrophy.

Cross-sectional histology and analysis

From 82 AMD eyes prepared for sub-micrometer epoxy sections stained with toluidine blue as described,^{36, 37} 39 nvAMD eyes of 39 donors were identified by fibrovascular scar in the sub-RPE or sub-retinal space in the presence of basal linear deposit or drusen, as determined by histology (Figure 1D–E). These eyes also had histologically confirmed atrophy, often visible by *ex vivo* SDOCT (Figure 1D–E). In areas of RPE atrophy, a boundary between subretinal and sub-RPE compartments was defined by basal laminar deposit (BLamD) (Figure 2), which persisted across the atrophic area in continuity with BLamD at the atrophy margin.^{37, 38} If BLamD was absent, then the neurosensory retina contacted Bruch's membrane directly and for the purpose of analysis was considered equivalent to the subretinal compartment. The sub-RPE compartment was located between BLamD and inner collagenous layer of Bruch's membrane. If BLamD was absent, then there was no sub-RPE compartment. Because we did not have clinical histories, and we did not exhaustively section entire maculas, we cannot state definitively that these eyes lacked variations and/or sequelae of neovascularization such as vessels that originated in one compartment and penetrated another, quiescent sub-RPE vessels, chorioretinal anastomoses, multilayered pigment epithelial detachments, or RPE tears.

As recently done for eyes with GA³⁰, we annotated RPE morphology and measured RPE and BLamD thickness at atrophy borders in two sections, one through the foveal center and another at 2 mm superior to the foveal center. The border of atrophy was defined as the descent of the external limiting membrane (ELM) towards Bruch's membrane.³⁰ As described^{30, 39}, the descent of the ELM is comprised of reactive Müller cell processes extending outward to approach Bruch's membrane in areas of photoreceptor loss. An ELM descent was analyzed if it was at least 500 μ m from the termination of subretinal fibrovascular scar. Sub-RPE scar in these locations was not an exclusion criterion. At 100

μm and 500 μm on either side of the ELM descent, we annotated RPE morphology and measured RPE and basal laminar deposit (BLamD) thickness, for a total of 4 assessments per transition, 2 in the atrophic area, with positive distances (+100 and +500 μm) and 2 in the non-atrophic area, with negative distances (−500 and −100 μm), so that progression moves like a time-line from left (non-atrophic) to right (atrophic) in all figures. This sampling pattern, centered around a defined point, is the same as our independent analysis of eyes with GA³⁰ and differs from our previous publications in which samples were spaced systematically across the whole macula.^{28, 29} Annotations were recorded in a custom database (Filemaker, Adobe, San Jose CA) while using a 60× numerical aperture = 1.4 objective and CCD camera to display tissue images at 1900× on a monitor. We used the nomenclature for RPE morphology as defined by Zanzottera et al^{28, 29} and applied in analyzing GA³⁰. In brief, cells containing spindle-shaped melanosomes and lipofuscin apposed to a basal lamina or BLamD were considered RPE. Cells in other layers or spaces containing those organelles or plausibly derived from such cells, were considered RPE-derived. These included two phenotypes found only in nvAMD eyes²⁹: ‘entombed’ within disciform scars thought to arise from ‘bilaminar’ RPE and ‘melanotic’, with spherical melanosomes visible fundusoscopically as black pigment inside scars. The percentage of each RPE phenotype was referenced to the total number of samples with RPE (also differing from our previous studies^{28, 29}). The shape of ELM descent was recorded as curved, reflected, oblique, or indeterminate, as described³⁰. The percentage of (curved + reflected) / (curved + reflected + oblique – indeterminate) was calculated for nvAMD and also for the separately reported GA eyes, so that they could be compared.

We compared mean RPE thickness for each phenotype at −500 μm and −100 μm and mean BLamD thickness at $\pm 500 \mu\text{m}$ and $\pm 100 \mu\text{m}$. Per previous convention^{28, 29}, only cells in the RPE layer (and not pigmented cells out of the layer) were measured. RPE thicknesses were available for all RPE morphologies except ‘atrophy with BLamD’, ‘atrophy without BLamD’, and ‘dissociated’ RPE. Generalized estimating equations were used to test if the mean thickness of RPE (or BLamD) at each location was different from zero. A pooled variance was used so that a *p*-value could be generated even if only one observation was included in one of the groups. A *p*-value <0.05 was considered statistically significant.

Results

In flat-mounts we compared the termination of intact RPE layer, signified by orderly cytoskeleton delimiting polygonal cells, to the distribution of AF attributable to lipofuscin and melanolipofuscin (Figure 3). A sharp co-termination of cytoskeleton and RPE AF is apparent in some locations (Figure 3, **row A1–4**), but not all. AF material does not necessarily respect the termination of RPE cytoskeleton and can extend beyond it (Figure 3, **row B1–4**). This AF material represents RPE cells and cellular fragments, as supported by simultaneous appearance of melanosomes (Figure 3, **column 4**). Thus the atrophy border in nvAMD is not sharply defined in the RPE layer itself, just like in GA³⁰, justifying the use of the ELM descent as a reference point for analyzing the sequence of RPE degeneration.

We analyzed cross-sectional histology in 27 nv-AMD eyes of 27 donors meeting our criteria (16 women, 11 men, age 84.8 ± 7.3 years). Of these 27 eyes, 21 had subretinal fibrovascular

scar, 23 had sub-RPE fibrovascular scar, 1 had subretinal hemorrhage, 2 had subretinal fibrin, and 1 had a serous pigment epithelium detachment³⁴. We analyzed 39 sections (18 central, 21 superior), 70 transitions, and 194 assessment locations (45 at $-500\ \mu\text{m}$, 51 at $-100\ \mu\text{m}$, 50 at $+100\ \mu\text{m}$, 48 at $+500\ \mu\text{m}$). The number and pattern of assessment locations differed among eyes, because atrophic areas extended off section edges or were less than $1000\ \mu\text{m}$ wide.

The distribution of RPE morphologies with respect to the ELM descent³⁰ in cross-sectional histology of nvAMD eyes is illustrated in Figures 4 and 5, which are high-magnification and panoramic views, respectively, of the same four nvAMD eyes. Figures 4A,B,C and 5A,B,C show three nvAMD eyes with sub-RPE fibrovascular scar meeting criteria for inclusion in this analysis. A typical eye (Figures 4A, 5A) has ‘sloughed’ RPE and shed cone inner segments⁴⁰ outside the atrophy. An atypical eye (Figures 4B, 5B) has cells with spherical melanosomes (‘melanotic’)²⁹ in the continuous RPE layer and in sub-RPE scar. Figures 4C, 5C show a continuous RPE layer with some ‘sloughed’ RPE, with degeneration of the overlying photoreceptors. Figures 4D, 5D show an eye that did not meet criteria due to a sub-retinal fibrovascular scar and fibrin located within $500\ \mu\text{m}$ of the ELM descent. Of the illustrated criterion-meeting nvAMD eyes, those in Figure 4A,B and 5A,B have oblique ELM descents, and the eye in Figures 4C,5C has a curved ELM descent. At 51 transitions to atrophy, 47.4% of ELM descents were curved, 7.8% were reflected, 59.0% were oblique, and 5.9% were indeterminate. In two eyes (Figures 4A,C, 5A,C), the outer nuclear layer ended coterminously with the ELM descent. In two eyes (Figures 4B,D, 5B,D) the outer nuclear layer extended beyond the ELM descent into the atrophic area. In one eye (Figure 4C, 5C) an island of photoreceptors survived with a few RPE cells over a fibrovascular scar.

Figure 6 shows the distribution of RPE phenotypes relative to the ELM descent in 27 nvAMD eyes. At both assessment points on the non-atrophic side of the ELM descent, ‘very non-uniform’ cells predominate. ‘Sloughed’ and ‘bilaminar’ cells increase at $-100\ \mu\text{m}$. ‘Shedding’, ‘vacuolated’ and ‘intraretinal’ cells are absent. On the atrophic side, ‘dissociated’ RPE is common. ‘Subducted’ cells are found on both sides of the ELM descent, with many fewer at $-500\ \mu\text{m}$ than at $-100\ \mu\text{m}$. Only occasional cells are detected within the sub-RPE fibrovascular scar. ‘Melanotic’ cells are infrequent.

Tables 1 and 2 show thicknesses of RPE layer and BLamD. RPE thickness across all morphologies did not differ significantly between -500 and $-100\ \mu\text{m}$ ($11.8 \pm 6.0\ \mu\text{m}$ vs $12.8 \pm 6.2\ \mu\text{m}$, $p = 0.28$). Similarly, overall BLamD thickness did not differ significantly between -500 and $-100\ \mu\text{m}$ ($3.7 \pm 3.7\ \mu\text{m}$ vs $2.9 \pm 3.2\ \mu\text{m}$, $p = 0.08$), with the exception of BLamD underlying ‘very non-uniform’ RPE being thinner at $-100\ \mu\text{m}$ ($4.0\ \mu\text{m} \pm 3.9\ \mu\text{m}$ vs $2.9 \pm 3.4\ \mu\text{m}$, $p = 0.04$). To facilitate SDOCT metrics that are both histologically informed and feasible, combined thicknesses for the RPE + BLamD are shown in Table 3. Combining across all RPE morphologies, mean RPE + BLamD thickness at $-500\ \mu\text{m}$ and $-100\ \mu\text{m}$ was almost identical ($15.7 \pm 7.0\ \mu\text{m}$ and 15.4 ± 7.7 , respectively; $p=0.91$).

We compared our data in these 27 nvAMD eyes to 13 separately reported GA eyes analyzed by the same methods³⁰ (Tables 4, 5). nvAMD eyes had proportionately fewer transitions than the GA eyes, which included multilobular GA.

Regarding distribution of RPE phenotypes, nvAMD eyes had mostly ‘very non-uniform’ cells at –500 and –100 µm with few “non-uniform” (age-normal) cells (Table 4, **row 1**), thus lacking noticeable worsening towards the ELM descent. In GA, the proportion of cells considered abnormal was significantly lower at –500 µm than at –100 µm, where many ‘shedding’, ‘sloughed’, and ‘intraretinal’ cells were found. Table 4, **row 2** shows that the atrophic areas of nvAMD and GA eyes both exhibit ‘dissociated’ and ‘subducted’ cells²⁹. However, fibrovascular scars in nvAMD are solely associated with RPE-derived phenotypes ‘entombed’ and ‘melanotic’. In this study ‘subducted’ are distributed on either side of the ELM descent in nvAMD eyes and are proportionately fewer at –500 µm than at –100 µm in nvAMD than GA.

Regarding layer thicknesses, in GA, RPE thickens significantly (20.6%) approaching the ELM descent but not in nvAMD (Table 5, **row 1**). BLamD thickness is 2-fold greater in GA relative to nvAMD both inside and outside the atrophic areas but does not vary significantly across the transition in either GA or nvAMD (Table 4, **rows 3–4**). Due to RPE thickening, the combination of RPE + BLamD together thickens by 19.6% near the ELM descent in GA but not in nvAMD (Table 5, **row 3**).

Regarding ELM shape, the percentage of descents that were oblique vs (curved + reflected) is significantly higher in nvAMD (47.9%) than in GA eyes (31.9%) (Table 4, **row 5**).

Five eyes accessioned after 2006 could have conceivably received intravitreal anti-VEGF therapy, and we found that the effect of excluding these eyes from the analysis was minimal. Thicknesses of RPE, BLamD, and RPE+BLamD in the remaining 22 eyes were almost identical to those found for the full dataset, and differences between nvAMD and GA eyes reached similar levels of statistical significance (**notes to** Tables 1–3). The proportion of oblique ELM shapes increased from 47.9% to 50.0%, which although a slightly larger effect, reduced the significance to borderline ($p = 0.086$).

Discussion

Our series is the largest to date providing high-resolution histology of intact nvAMD eyes. We used epoxy sections for a comprehensive and polychromatic visualization across the entire macula and focused on cardinal features of RPE ultrastructure and outer retinal neurodegeneration. We discretized continuous variation in RPE morphology with a system of cellular phenotypes and used unbiased systematic sampling⁴¹ to develop measures amenable to biometric analysis, which allowed us to compare eyes with nvAMD to eyes with GA. Previously, Sarks et al¹⁹ examined 18 eyes with disciform (fibrovascular) scars secondary to nvAMD and noted that the margin delimiting the atrophy surrounding the scar was not hyperpigmented, in contrast to eyes with GA, in which a hyperpigmented margin is common. Due to our excluding borders with sub-retinal fibrosis from analysis, our sample is not strictly comparable to this prior study¹⁹. Yet our data support the observation of absent hyperpigmentation by showing that the predominant RPE phenotype in the transition to atrophy is ‘very non-uniform’ and not ‘sloughed’ or ‘intraretinal’. Further, we found evidence for neither worsening of RPE morphology nor thickening of the combined RPE +BLamD layer, as the ELM descent is approached from the non-atrophic side. Our data

extend the findings of Sarks et al¹⁹ by showing that ELM descents tend to be oblique rather than curved or reflected, as in GA. Histologically-guided metrics can potentially distinguish between GA and macular atrophy in SDOCT, with added value for interpreting FAF imaging.

Non-progressive dysmorphia in the transition to atrophy

We found little evidence for progressive RPE dysmorphia in the transition to atrophy that is well established for GA eyes^{27, 30, 31}. We did note that ‘bilaminar’ increased near the ELM descent, perhaps because these are precursors of ‘entombed’ cells, which are specific to fibrovascular scars.²⁹ In this and our recent GA study³⁰ we considered fully pigmented, nucleated cells in the subretinal space and within the neurosensory to be RPE, because they contained numerous RPE-characteristic granules at a concentration similar to cells in the RPE layer.⁴² In our material, it is also possible to discern non-RPE cells such as lipid-filled monocytes.^{33, 34} In GA eyes we confirmed a dramatic and progressive deterioration in RPE health within 500 µm of the ELM descent^{27, 31}. In the GA transition we found evidence for two main pathways of RPE fate, anterior migration and apoptosis.^{31, 32} The difference in RPE phenotype distribution between GA and nvAMD may be explained by the occurrence of acute, chronic or episodic exudation in nvAMD that is under different regulatory influences than those governing primary pigmentary degeneration, including the possibility of choriocapillary dysfunction early in the process.^{43, 44}

The ELM Descent is a Marker for Atrophy

For the first time for nvAMD, we used as the border of atrophy the descent of the ELM towards Bruch’s membrane. Guided by an early description by Sarks³⁹ we used this anatomical landmark in analyzing RPE phenotypes in GA³⁰. The ELM comprises junctional complexes between the Müller cells and photoreceptor inner segments^{45–47} and together with junctional complexes among RPE, bounds the subretinal space⁴⁸. The shape of the descent in atrophy is dictated by the outward and lateral extension of Müller cells^{53, 54}. Published illustrations of immunoreactivity for glial fibrillary acidic protein show how Müller cells are reactive and the scaffolding of their apices perfectly outlines curved (Figure 2C of⁴⁹; Figure 2H of⁵⁰) and reflected (Figure 4C of⁴⁹) ELM descents. Although we did not find similar published examples of oblique ELM descents, we believe that these fall along the same continuum. ELM descents of indeterminate shape were few in number and could be attributed to distortions due to fixation quality or tissue processing. We propose that curved and reflected ELM descents are related to the scrolling of Müller cells and cone photoreceptors seen in outer retinal tubulation.^{51–53} In this macula-specific gliosis, Müller cells protect surviving cones from the failing RPE-Bruch’s membrane complex by scrolling them into interconnecting tubes. We propose further that oblique ELM descents additionally reflect severe photoreceptor loss inherent in nvAMD (see below).

Interpretation of autofluorescence imaging

New information about RPE morphology in nvAMD can inform the interpretation of FAF imaging. Hyperautofluorescence can be explained by cell-autonomous and non-cell-autonomous mechanisms, some of which are assessable in the cross-sectional view of histology and SD-OCT. These include increased concentration of efficiently detected

fluorophores, increased concentration of lipofuscin granules in individual cells, loss or re-positioning of melanosomes that block 488 nm autofluorescence, RPE hypertrophy resulting in taller individual cells, and superimposition of cells resulting from RPE migration^{31, 54}. The latter two create a longer summation path length for exciting light through biologic fluorophores.^{30, 31} Reduced screening of RPE fluorophores due to reduced photoreceptor pigment can also lead to hyperautofluorescence^{55, 56}. In contrast, hypoautofluorescence can be explained by loss of lipofuscin and melanolipofuscin granules singly or in aggregates, resulting in increasingly variable and overall reduction of FAF intensity.³² Hypoautofluorescence is also due to blocking by non-fluorescent material located in more anterior retinal layers.

Several FAF patterns have been described in nvAMD. First, a contiguous lawn of FAF indicates that RPE/photoreceptor complex is physically intact and metabolically active as driven by photoreceptor outer segment renewal and retinoid cycling.²⁰ Second, a contiguous and well-demarcated area of greatly reduced FAF signal signifies photoreceptor death, with or without RPE loss^{1, 20, 21, 57} and in any case, predicts poor visual acuity at baseline and follow-up. These minimally AF areas may form a multilobular pattern typical of primary GA⁵⁸ (Figure 7A), as the underlying degeneration progresses. Third, areas of absent FAF are preceded by and surrounded by areas of an abnormally granular FAF^{21, 57}, seen as pigmentary disturbances in color imaging that are also associated with poor vision.⁴ Fourth, several published FAF images^{3, 9, 19, 22, 23, 57, 59} include a band of relatively uniform hyperautofluorescence with smoothly contoured edges surrounding atrophic areas (Figure 7B). This pattern recalls the inferior-angling gravitational tracks that typify central serous retinopathy⁶⁰ and gives the impression of fluid having washed over the RPE, like a floodplain of exudation.

Of relevance is our recent observation of a severe form of photoreceptor degeneration that occurs in 44% of nvAMD eyes and not in GA.⁴⁰ Of our 27 study eyes, many with attached retinas, 7 exhibited cone inner segments containing mitochondria shed into the subretinal space where they interspersed with 'sloughed' RPE.⁴⁰ Inner segment shedding is consistent with other neurodegenerations also involving release of cellular material⁶¹ and may be evoked by direct contact of photoreceptors with extravasated fluid. By markedly reducing the photopigment available for screening, this distinctive degeneration is a plausible contributor to a hyperautofluorescent exudative floodplain (Figure 7B, D). In contrast, hyperautofluorescence at the margin of primary GA (Figure 7A,C) is attributable to a progressive RPE dysmorphia (rounding and stacking) towards the ELM descent that increases path length for light. In nvAMD, shorter and/or fewer photoreceptors over an extended area outside the atrophic area will result in a straight, obliquely oriented ELM descent (Figure 7D), compared to curved ELM descents in primary GA (Figure 7C). In most nvAMD eyes, these processes probably occur simultaneous with an underlying primary GA. If this is true, then our observed differences in atrophy borders between GA and nvAMD eyes might have been larger, had we been able to separate primary GA from atrophy secondary to exudation. Only in eyes with simultaneous longitudinal OCT and FAF imaging^{1, 22} can these questions be definitively resolved.

Why RPE atrophy continues and possibly accelerates in the course of chronic VEGF suppression is not fully understood but several hypotheses exist. First, neovascularization may help sustain the diseased macula, in part by recapitulating choriocapillaris subjacent to RPE, and thus pharmaceutically suppressing neovascularization is deleterious.⁴ Further, the growth rate of atrophy is dependent on the type of neovascularization, with type 1 probably most resistant^{8, 10, 13}. A re-direction of blood flow from the choriocapillaris serving RPE surrounding fibrovascular tissue to the scar itself has been suggested.¹⁹ Second, VEGF has neuroprotective functions that are blunted by therapy, possibly exacerbating vision loss.⁴ Third, atrophy is proposed to arise non-specifically in response to the exudative process itself, with episodic leakage over time, mechanical damage secondary to vascular outgrowth and contraction (dramatically demonstrated via OCT-angiography⁶²), and concomitant inflammation and ischemia.³ Newly recognized forms of severe photoreceptor degeneration,⁴⁰ the non-progressive nature of RPE change at the ELM descent herein described, and FAF signatures consistent with a floodplain^{3, 9, 19, 23, 57, 59} (Figure 7) support this third model, as do recent analysis of fellow eyes to those treated with anti-VEGF agents.²⁴

Strengths, limitations, conclusions

Strengths of this study include a large number of nvAMD eyes analyzed at high-resolution, a cellular phenotyping system to discretize RPE degeneration, and unbiased sampling methods to include both the atrophic areas and surrounding less-affected tissue. Limitations were the small number of RPE flat mounts for en face viewing, lack of flat-mounts and histology from the same donor tissues, absence of Bruch's membrane thicknesses to include with the RPE+BLamD thicknesses, and lack of clinical information about treatment history and subretinal and sub-RPE fibrovascular scar components.

Nevertheless, our study represents the first quantitative description of RPE morphology in intact nvAMD eyes. We formulated new hypotheses testable in longitudinal imaging of patients receiving anti-VEGF therapy and in animal models with genetic predisposition to neovascularization.^{63, 64} Importantly our data support three anatomical markers of potential utility in assessing via SDOCT and FAF whether RPE death in nvAMD is due to an underlying degeneration or secondary to exudation, with or without VEGF suppression. Relative to eyes with GA, eyes with nvAMD lack the progressive dysmorphia and thickening of the RPE layer toward the ELM descent, have thinner combined RPE+BLamD, and tend to have obliquely oriented, straight ELM descents. Analysis of large clinical imaging datasets will help determine whether these histological features are found with sufficient reliability to merit use as biomarkers. Histology of clinically imaged eyes will be essential for determining RPE changes relate to specific neovascularization patterns, and for testing hypotheses of how exudation impacts outer retinal cells.

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Figure 1. Ex vivo imaging of a donor eye with neovascular AMD

96-year-old white woman. **A.** Color photograph shows a large atrophic area with scalloped edges, especially on the temporal side. Nasal to the fovea the retina is thickened with two areas of black intraretinal pigment corresponding to ‘melanotic’ cells of RPE origin.²⁹ Green line indicates the approximate position of the B-scan in panel D. Ruby bead⁶⁵ is 1 mm in diameter. **B.** Red-free photograph shows choroidal vessels through thinned retina and dark areas representing the black pigment in panel A. **C.** 488 nm autofluorescence shows atrophy that is multilobular on superior, nasal, and inferior aspects, and small hypoautofluorescent spots superior to the optic nerve head. In this thinned retina, autofluorescence is associated with walls of large retinal vessels. Black pigment is hypoautofluorescent. **D, E.** B-scan and corresponding submicrometer histologic section through the fovea. Red arrowheads delimit an area with sub-retinal fibrovascular membrane, as determined by inspection of the histology at higher magnification. No sub-RPE fibrovascular membranes were detected. Yellow arrowheads 1 and 2 delimit an area of continuous RPE. At the right of arrowhead 3 is also continuous RPE. ELM descents near arrowheads 1 and 3 met criteria for inclusion in the border analysis. An ELM descent near arrowhead 2 was not included, because it was 500 µm from subretinal fibrovascular scar. Teal arrowheads show groups of ‘dissociated’ RPE atop thick basal laminar deposit (stained

blue).³⁷ This deposit extends across the atrophic area and is particularly reflective in the peripapillary area (to the left of arrowhead 1) where it also contains neovessels⁶⁶.

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Figure 2. Definitions of layers and compartments within atrophic area of neovascular AMD
 80-year-old woman. Submicrometer epoxy section, toluidine blue stain. GCL, ganglion cell layer; IPL, inner plexiform layer; INL= Inner nuclear layer; OPL, outer plexiform layer; HFL, Henle fiber layer; Ch, choroid; Sc, sclera; FV scar, fibrovascular scar; ORT, outer retinal tubulation. Persistent basal laminar deposits (BLamD) (also called outer retinal corrugations in SDOCT³⁷), yellow arrowheads. ‘Subducted’ cells of hypothesized RPE origin, external to scar and resting on Bruch’s membrane²⁹, orange arrowheads. Photoreceptors have degenerated, and HFL contacts BLamD. In the analysis of atrophic areas lacking a continuous RPE layer, BLamD divided retinal and (obliterated) subretinal compartments from the sub-RPE compartment³⁸. The sub-RPE compartment in these eyes may be empty or may contain scar, neovascularization, blood, fluid, cells, and/or basal linear deposit, an extracellular lipid-rich lesion equivalent to soft drusen.

Figure 3. Variation in RPE autofluorescence and cytoskeleton in neovascular AMD

Images are from a 93-year-old female donor. **Column 1** demonstrates that RPE cells at the border of atrophy show either intact or disrupted cytoskeleton. In the atrophic zone, cells lose f-actin cytoskeleton, and condensed f-actin tangles form from remaining f-actin fragments. **Column 2** shows brightly AF lipofuscin/ melanolipofuscin granules, either within isolated cells or in extracellular granule aggregates shed into basal laminar deposit³². **Column 3** superimposes images from **columns 1 and 2** to show the relationship of AF material to the termination of organized cytoskeleton. **Column 4** shows bright field images to confirm that cells and cellular fragments also have melanosomes. **Row A** shows coterminous AF and organized cytoskeleton, with a clear boundary between intact RPE and atrophy. **Row B** shows AF stopping short of the end of organized cytoskeleton as well as extending into the atrophic area. Scale bars: 50 μ m.

Figure 4. Descent of the external limiting membrane in 4 eyes with neovascular AMD
 Panoramic views of these eyes are shown in Figure 4. ELM, external limiting membrane (green arrowheads); GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; Ch, choroid. Black arrowheads, Bruch's membrane. Bar in D applies to all panels. **A.** Oblique ELM descent in an 87-year-old man. **B.** Oblique ELM descent in an 83-year-old woman. **C.** Curved ELM descent in an 80-year-old woman. **D.** Oblique ELM descent and subretinal fibrocellular scar in a 90-year-old-man.

Figure 5. RPE morphologies near the ELM descent in neovascular AMD eyes

ELM descent (green arrowheads) and its projection on to Bruch's membrane (red arrow) are shown. The ELM descent in all four eyes is shown at higher magnification in Figure 3. RPE and BLamD morphology and thickness were analyzed at -500 and -100 μm outside atrophy (yellow ticks to the left of the red arrows) and $+500$ and $+100$ μm inside atrophy (yellow ticks to the right of the red arrows). Submicrometer epoxy sections, toluidine blue stain. GCL = Ganglion cell layer, INL= Inner nuclear layer, ONL= Outer nuclear layer. **A.** A typical eye meeting criterion for inclusion. Outside atrophy: Very non-uniform and 'sloughed' RPE cells over a thin BLamD layer. Shed cone inner segments are found between the ONL and RPE⁴⁰ (aqua arrowhead). Inside atrophy: a thicker layer of BLamD. The ONL stops at the ELM descent, which is oblique. 83-year-old woman. **B.** An atypical eye due to presence of melanotic RPE but still meeting criterion for inclusion. Outside atrophy: a continuous layer of cells containing dark polydispersed spherical granules and sub-RPE scar with melanotic cells (orange arrowhead). Inside atrophy: melanotic cells within fibrovascular scar. The ONL continues over the scar. The ELM descent is oblique. 86-year-old woman. **C.** Eye meeting criterion for inclusion. RPE layer is continuous, and photoreceptors are degenerate. The ELM descent is curved. An island of photoreceptors and several RPE cells survive over scar in the atrophic zone, at the right edge of the panel. 80-year-old woman. **D.** An eye not meeting criterion for inclusion due to sub-retinal

fibrovascular scar with Entombed RPE and fibrin²⁸ (pink arrowhead). Scar continues from the left edge to the ELM descent. Outer and inner segments of photoreceptors are degenerate. ONL continues over the scar. The ELM descent is oblique. 93-year-old woman. **A–D.** Complete histological sections are available at the Project MACULA website. **A.** <http://projectmacula.cis.uab.edu/?p=1888>. **B.** <http://projectmacula.cis.uab.edu/?p=2999>. **C.** <http://projectmacula.cis.uab.edu/?p=1696>. **D.** <http://projectmacula.cis.uab.edu/?p=1704>.

Figure 6. Distribution of RPE morphologies with respect to the ELM descent in neovascular AMD

The ELM descent (red arrow) was the point of reference. The number of assessment locations is expressed at the right of each bar. Epithelial, non-epithelial, atrophic, and subducted morphologies²⁸ are indicated by blue, green, orange, and brown bars respectively, with less affected at the top and more affected at the bottom. Percentages are referenced to the total number of RPE. RPE phenotypes do not obviously worsen in the junctional zone as the ELM descent is approached. Melanotic cells with spherical melanosomes (pink and yellow bars) are present in nvAMD eyes and not in GA eyes²⁹.

Figure 7. Proposed mechanisms for hyperautofluorescence in primary geographic atrophy and macular atrophy secondary to neovascularization in age-related macular degeneration

A. Multilobular GA with variegated hyperautofluorescence in the margins (arrows). **B.** Neovascular AMD with multilobular atrophy and also hyperautofluorescence in a floodplain configuration (arrows), attributed to the history of exudation in this eye (image courtesy of N. Phasukkijwatana, MD, and D. Sarraf, MD). **C,D.** Schematics show outer retinal cells at the descent of the ELM (orange dashed line) towards Bruch's membrane in GA (**C**) and nvAMD (**D**). The ELM is comprised of junctional complexes of Müller cells (orange) and photoreceptors (yellow, cones; green, rods). For simplicity, Bruch's membrane and neovascular membranes are not shown. **C.** The RPE layer exhibits a progressive dysmorphia towards the ELM descent.^{27, 30, 31, 54} Hyperautofluorescence is attributable to stacked and rounded RPE cells that increase path length of exciting light through biologic fluorophores.³¹ **D.** In nvAMD, the RPE is non-uniform in morphology, without worsening toward the border. Photoreceptor loss is severe due to inner segment shedding⁴⁰ believed secondary to episodic exudation. Reduced screening by photopigment in these shortened

cells also contributes to hyperautofluorescence. In most cases, as in panel B, RPE dysmorphia and reduced photopigment screening may occur simultaneously.

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Table 1

RPE thickness relative to the ELM descent in neovascular AMD eyes

RPE Morphology	–500 μm from ELM descent	–100 μm from ELM descent		
	Thickness, mean $\pm$ SD, μm ; N		<i>p</i> value, –500 vs –100	Thickness, mean $\pm$ SD, μm ; N
Overall	11.8 $\pm$ 6.0; 23	12.8 $\pm$ 6.2; 26	0.2847	12.3 $\pm$ 6.1; 27 *
Non-uniform	10.6 (n/a); 1	---	---	10.6 (n/a); 1
Very non-uniform	10.8 $\pm$ 3.0; 20	11.0 $\pm$ 3.9; 20	0.8360	10.9 $\pm$ 3.5; 25
Sloughed	23.9 $\pm$ 8.9; 4	17.4 $\pm$ 4.5; 6	0.0600	19.8 $\pm$ 6.8; 8
Shedding	---	---	---	
Bilaminar	17.7 $\pm$ 8.2; 2	22.3 $\pm$ 4.5; 6	0.3104	21.2 $\pm$ 5.4; 7
Vacuolated	---	---	---	
Intraretinal	---	---	---	

Notes: assessments were made in 27nv-AMD eyes lacking subretinal scar within $\pm$ 500 μm of the ELM descent and including eyes with sub-RPE scar

N indicates the number of donors in which measurements were made.

Thicknesses were not recorded for ‘dissociated’ RPE within the atrophic area.

P-value obtained via general estimating equations

SD, standard deviation

n/a, not applicable

* For 22 eyes accessioned before 2006, overall mean RPE thickness was 12.3 $\pm$ 6.2 μm

Table 2

Basal laminar deposit thickness relative to the ELM descent in neovascular AMD eyes

RPE Morphology	-500 μ m from ELM descent	Thickness, mean $\pm$ SD, μ m; N		-500 μ m vs -100 μ m	Thickness, mean $\pm$ SD, μ m; N		+500 μ m vs +100 μ m	Thickness, mean $\pm$ SD, μ m; N		Overall +100 μ m and +500 μ m
				<i>p</i> value			<i>p</i> value			
Overall	3.7 $\pm$ 3.7; 23		2.9 $\pm$ 3.2; 26	0.0844		3.3 $\pm$ 4.7; 27	0.0246		3.2 $\pm$ 3.5; 27*	3.7 $\pm$ 4.8; 27
Non-uniform	2.2 (n/a); 1		---	---		---	---		2.2 (n/a); 1	---
Very non-uniform	4.0 $\pm$ 3.9; 20		2.9 $\pm$ 3.4; 20	0.0404		---	---		3.5 $\pm$ 3.7; 25	---
Sloughed	2.6 $\pm$ 2.6; 4		4.5 $\pm$ 3.7; 6	0.2204		---	---		3.8 $\pm$ 3.3; 8	---
Shedding	---		---	---		---	---		---	---
Bilaminar	1.7 $\pm$ 2.4; 2		1.3 $\pm$ 1.7; 6	0.7621		---	---		1.4 $\pm$ 1.7; 7	---
Vacuolated	---		---	---		---	---		---	---
Intraretinal	---		---	---		---	---		---	---
Dissociated	---		---	---		7.5 $\pm$ 6.5; 10	0.6461		---	7.2 $\pm$ 6.6; 12
Atrophy with BLamD	---		---	---		3.4 $\pm$ 2.0; 11	0.0407		---	4.6 $\pm$ 3.2; 18
Atrophy without BLamD	---		---	---		0; 10	0.2407		---	0.3 $\pm$ 1.6; 15

Notes: assessments were made in 27nv-AMD eyes lacking subretinal scar within $\pm$ 500 μ m of the ELM descent and including eyes with sub-RPE scar

N indicates the number of donors in which measurements were made.

P-value obtained via general estimating equations

SD, standard deviation

n/a, not applicable

* For 22 eyes accessioned before 2006, overall mean BLamD thickness was $2.9 \pm 3.3 \mu$ m

Table 3

RPE + Basal laminar deposit thickness relative to the ELM descent in nvAMD

RPE Morphology	–500 µm from ELM descent	–100 µm from ELM descent	–500 µm vs –100 µm	Overall
	Thickness in µm, mean ± SD; N		<i>p</i> -value	Thickness in µm, mean ± SD; N
Overall	15.7 ± 7.0; 23	15.4 ± 7.7; 26	0.9109	15.4 ± 7.3; 27 [*]
Non-uniform	12.8 (n/a); 1	---	---	12.8 (n/a); 1
Very non-uniform	14.8 ± 5.3; 20	13.9 ± 5.8; 20	0.3788	14.3 ± 5.5; 25
Sloughed	26.6 ± 8.4; 4	21.8 ± 7.9; 6	0.1408	23.5 ± 8.0; 8
Shedding	---	---	---	---
Bilaminar	19.4 ± 10.6; 2	23.6 ± 3.6; 6	0.4423	22.6 ± 5.4; 7
Vacuolated	---	---	---	---
Intraretinal	---	---	---	---

Notes: Mean RPE + BLamD thickness by RPE grades in 27 nvAMD eyes from 27 donors

N indicates the number of donors in which measurements were made.

P-value obtained via general estimating equations

SD, standard deviation

n/a, not applicable

*

For 22 eyes accessioned before 2006, overall mean RPE+BLamD thickness was 15.0 ± 7.4 µm

Table 4

Comparison of microstructure in neovascular AMD and geographic atrophy eyes

#	Metric	Neovascular AMD (N=27)	Geographic atrophy* (N=13)	P
1	% of RPE phenotypes	'very non-uniform' in non-atrophic area		
	−500 μm	97.7%	72.2%	0.0027
	−100 μm	100.0%	96.8%	n.s.
2	RPE phenotypes in atrophic area	'dissociated', 'subducted', 'melanotic'**, 'entombed'**, 'entubulated'†	'dissociated', 'subducted', 'entubulated'†	--
3	BLamD thickness, non-atrophic area	3.25 ± 3.46 μm	7.99 ± 7.49 μm	0.017
4	BLamD thickness, atrophic area	3.70 ± 4.81 μm	6.63 ± 6.17 μm	0.047
5	Shape of ELM descent			
	Oblique	47.9%	31.9%	0.046
	Curved + reflected	52.1%	68.1%	

Notes:

*30;

**29;

†53

±500, ±100, distances from ELM descent in μm, negative is non-atrophic, positive is atrophic;

Statistical evaluation: n.s., not significant at $p < 0.05$ level.

Line 3–4 thicknesses in given locations were pooled across all RPE phenotypes.

Table 5

Comparison of RPE and BLamD thicknesses by location in neovascular AMD and geographic atrophy eyes

#	Metric	−500 μm	−100 μm	<i>P</i>
1	RPE thickness			
	Neovascular AMD	11.8 $\pm$ 6.0	12.8 $\pm$ 6.2	n.s.
	Geographic atrophy	12.1 $\pm$ 5.2	14.6 $\pm$ 6.9	0.039
2	BLamD thickness			
	Neovascular AMD	3.7 $\pm$ 3.7	2.9 $\pm$ 3.2	n.s.
	Geographic atrophy	7.2 $\pm$ 6.1	8.4 $\pm$ 8.2	n.s.
3	RPE + BLamD thickness			
	Neovascular AMD	15.7 $\pm$ 7.0	15.4 $\pm$ 7.7	n.s.
	Geographic atrophy	19.3 $\pm$ 8.2	23.1 $\pm$ 10.7	0.046

Notes: ± 500 , ± 100 , distances from ELM descent in μm , negative is non-atrophic, positive is atrophic;

Statistical evaluation: n.s., not significant at $p < 0.05$ level