Lipofuscin Redistribution and Loss Accompanied by Cytoskeletal Stress in Retinal Pigment Epithelium of Eyes With Age-Related Macular Degeneration

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PURPOSE. Lipofuscin (LF) and melanolipofuscin (MLF) of the retinal pigment epithelium (RPE) are the principal sources of autofluorescence (AF) signals in clinical fundus-AF imaging. Few details about the subcellular distribution of AF organelles in AMD are available. We describe the impact of aging and AMD on RPE morphology revealed by the distribution of AF LF/MLF granules and actin cytoskeleton in human tissues.

METHODS. Thirty-five RPE-Bruch’s membrane flatmounts from 35 donors were prepared (postmortem: ≤4 hours). Ex vivo fundus examination at the time of accession revealed either absence of chorioretinal pathologies (10 tissues; mean age: 83.0 ± 2.6 years) or stages of AMD (25 tissues; 85.0 ± 5.8 years): early AMD, geographic atrophy, and late exudative AMD. Retinal pigment epithelium cytoskeleton was labeled with AlexaFluo464-Phalloidin. Tissues were imaged on a spinning-disk fluorescence microscope and a high-resolution structured illumination microscope.

RESULTS. Age-related macular degeneration impacts individual RPE cells by (1) lipofuscin redistribution by (i) degranulation (granule-by-granule loss) and/or (ii) aggregation and apparent shedding into the extracellular space; (2) enlarged RPE cell area and conversion from convex to irregular and sometimes concave polygons; and (3) cytoskeleton derangement including separations and breaks around subretinal deposits, thickening, and stress fibers.

CONCLUSIONS. We report an extensive and systematic en face analysis of LF/MLF-AF in AMD eyes. Redistribution and loss of AF granules are among the earliest AMD changes and could reduce fundus AF signal attributable to RPE at these locations. Data can enhance the interpretation of clinical fundus-AF and provide a basis for future quantitative studies.

Keywords: AMD, lipofuscin, melanolipofuscin, autofluorescence, granule

Clinic fundus-autofluorescence (AF) has become an indispensable tool in the diagnosis and management of many chorioretinal diseases, especially AMD.1–3 It is a good indicator of retinal pigment epithelium (RPE) health because hypointense and/or hyperautofluorescent areas in fundus-AF are often associated with AMD-related extracellular deposits (sub-RPE drusen; subretinal drusenoid deposits)4,5 or lesions directly affecting the RPE (atrophy).6,7 While drusen and subretinal drusenoid deposits become clinically visible at 30 μm,8,9 changes in RPE cells are smaller than that and may not be revealed by current ophthalmoscopic imaging techniques.

Of note, compared with the voluminous studies on clinical fundus-AF, there are only few studies known to us that specifically focused on the cellular and subcellular basis of AF in AMD.10,11 Of these, only one provided high-resolution tissue photodocumentation10 to serve as visualization targets for clinical AF imaging in the way that photoreceptor histology influenced the development of adaptive optics scanning laser ophthalmoscopy.12–15

Closing this knowledge gap would help clinicians and scientists further understand clinical fundus-AF from its origin (i.e., the accumulation and distribution of AF granules in healthy and diseased RPE cells). This topic is of renewed interest due to the arrival of quantitative AF imaging technique that will standardize AF signal across clinic populations. Further, our recent work in the context of prior literature establishes that RPE cell numbers are stable in aging, implying that decreased fundus AF after 70 years cannot be attributed to RPE cell loss.10 This finding in turn implicates loss of individual granules, a shift in fluorophore composition relative to the detector sensitivity, and change in cell shape impacting path length of exciting light through fluorophores as potential cell-autonomous mechanisms underlying diminished fundus AF.

Lipofuscin (LF) and melanolipofuscin (MLF) granules of the RPE are the principal subcellular sources of AF signals in clinical fundus AF imaging.20 In this hypothesis-generating histologic survey, we report intracellular distribution of LF/MLF...
granules in RPE cells from an en face (fundus) view in healthy and AMD human RPE-BrM flatmounts. Simultaneously, the filamentous-actin (F-actin) cytoskeleton of the RPE cells was imaged to report changes in shape and size in eyes affected by AMD. Our goal was to provide a resource for the interpretation of clinical confocal-AF by exploring the cellular and subcellular basis of variation in AF. We find evidence for several pathways of LF/MLF redistribution and morphologic readouts of cellular stress.

**METHODS**

Institutional review at the University of Alabama at Birmingham (Birmingham, AL, USA) approved this study, and all procedures adhered to the Tenets of the Declaration of Helsinki.

Thirty-five human chorioretinal tissues from 35 Caucasian donors were preserved in 0.1 M phosphate-buffered paraformaldehyde (≤4.2 hours after death), cryoprotected in glycerol-buffer, and frozen at −80°C until used. Maculopathy status was determined at the time of accession, as follows: The anterior segment and vitreous was removed, and globes were inspected internally under a dissecting microscope with epi-illumination to accentuate drusen and transillumination to accentuate pigmentedary change. Stereo color photographs were graded as normal, questionable, or AMD, using the criteria of the Alabama Age-related Maculopathy Grading System (≥1 druse larger than 125 μm in diameter or an area of pigment change 500 μm in diameter). Eyes with smaller drusen or smaller areas of pigment change (questionable) were analyzed in the current study so as to include incipient AMD pathology. These eyes were considered early AMD for the purpose of analysis, because they exhibited changes consistent with those in definite AMD eyes. Of all tissues, 28 were also examined after thawing using spectral-domain optical coherence tomography (SD-OCT, Spectralis; Heidelberg Engineering, Heidelberg, Germany) to confirm absence (for healthy) or presence (for AMD) of drusen, abnormal hypo/hyperautofluorescence, or atrophic areas. During photomicroscopy and analysis of RPE-BrM flatmounts (below), we checked whether RPE morphologies visible en face were consistent with those seen in cross-sectional histology (digital sections, glossary, and bibliography at (in the public domain) http://projectmacula) and detailed separately.21,23

Retinal pigment epithelial-BrM flatmounts of the macula and near periphery (20 × 20 mm) were prepared and imaged as described.10 Briefly, retina and choroid were removed under a dissection microscope resulting in 20- to 25-μm thick RPE-BrM flatmounts that were then labeled with 647 Alexa Phalloidin (Life Technologies, Grand Island, NY, USA) to bind the F-actin cytoskeleton. Phalloidin labeling permitted semiautomatic counting (to be reported separately) and assisted in defining cell boundaries as the disease progressed. Bright field and fluorescence imaging was performed using a confocal microscope (BX51; Olympus, Tokyo, Japan) with 460- to 490-nm excitation/emission greater than 505 nm for LF/MLF-AF and 635-nm excitation/emission greater than 650 nm for phalloidin. Z-stacks in 0.4-μm steps were taken from apical to basal RPE. For LF/MLF z-stacks included the first granules in focus on the apical aspect through last granules out of focus on the basal aspect. Exposure times varied between tissues (7.0-15.5 ms/slice). For cytoskeleton, z-stacks included the apical RPE. Images were systematically acquired at predefined locations, as described.10 In AMD RPE-BrM flatmounts, additional images were captured in areas affected by drusen and/or atrophy. Bright-field images were taken to illustrate intracellular melanosome distribution. For all aforementioned imaging modes, final all-in-focus images were created using the microscope’s internal software (extended focal imaging tool, cellSens software version 7; Olympus). To further highlight F-actin bands, the background subtraction and edge detection filter (Sobel; Olympus) tools in the same software were applied. Images were also captured from 10-μm thick vertically-oriented cyrossections of additional AMD eyes retained from previous studies.24,25 In describing locations we use “cone-dominated” fovea (within 500 μm of the foveal center) and the terminology of Polyak26 for perifovea (1.25-2.75 mm from the foveal center) and periphery (10 mm from the foveal center, at the edge of the tissue).

Age-related macular degeneration affected RPE cells were also imaged using high-resolution structured illumination microscopy (HR-SIM), a multicolor microscopy technique that surpasses twice Abbe’s resolution limit.27 In this mode of microscopy a grid pattern is superimposed on the specimen, while images are simultaneously captured. This periodic light pattern allows down-modulation of high-frequency sample information that is normally not transferred by a standard wide-field microscope by converting this information to a Moiré pattern that makes it accessible for detection by HR-SIM. Postimaging processing requires specific algorithms to extract high-resolution information and to reconstruct images, resulting in significantly better resolution (∼110 nm lateral) compared with wide-field or confocal microscopy. We recently showed that HR-SIM is a suitable tool for examination of individual LF granules.28 High-resolution SIM imaging was performed on an ELYRA-S.1 system (Zeiss, Jena, Germany). Excitation was at wavelengths of 488, 561, and 642 nm using grating periods of 28 (for 488 nm) and 34 (for 561 and 642 nm) and a ×63, numerical aperture 1.40 plan apochromat oil immersion objective. Depending on excitation wavelength, approximately 100 serial sections of 90- to 110-nm axial spacing each with 15 raw images (5 grating positions × 3 rotations) were acquired. Images were exposed for 100 to 150 ms using an EMCCD camera iXon 885 (Andor Technology Ltd., Belfast, UK) cooled to −65°C. Reconstructions of raw image sequences were performed using the instrument software (ZEN 2010; Zeiss). Locations for HR-SIM imaging (in normal and AMD flatmounts) do not necessarily correspond to locations for confocal AF imaging. For HR-SIM AMD flatmount imaging, RPE cells at different stages of degranulation were chosen randomly.

**RESULTS**

Study results are based on a survey of RPE cells at 1729 locations in 25 AMD eyes (85.0 ± 5.8 years, mean ± SD) and 782 locations in 10 age-matched controls (83.0 ± 2.6 years).

Normal RPE cells in older adults are filled with AF-LF and -MLF granules, revealed by HR-SIM. Figure 1A shows homogeneously AF granules (LF) as well as granules with elongated hypo-AF cores surrounded by AF shells (MLF). At 460- to 490-nm excitation, melanosomes are minimally AF and are thus visible in dark relief against the cushion of other organelles. Space-filling nuclei also reduce AF signal within cells, a phenomenon that is especially prominent in small foveal RPE cells (Fig. 1A). High-resolution SIM images reveal highly ordered packing of granules, which are similarly sized and evenly distributed throughout the cytoplasm.

Fovea and perifovea show fewer and larger LF granules, and many more MLF granules and light blocking melanosomes, compared with periphery (compare Figs. 1A–C).

Each RPE cell is delimited by a F-actin containing and evenly labeled cytoskeleton of uniform thickness that runs in a straight line along each side, meeting at sharp vertices (arrows in Fig. 1A).
Cytoskeletons of adjacent cells form parallel lines like railroad tracks.

We observed striking intracellular granule redistribution and loss in aging and in AMD, with different patterns in the two conditions. Cells tended to exhibit reorganization of their granules in one of two ways: degranulation (granule-by-granule loss) and/or aggregation.

Degranulation is observable in both aged normal and AMD eyes (Fig. 2). Cells redistribute their granules (Fig. 2A), leading to granule-free zones in the cytoplasm (Figs. 2B, 2C). With ongoing redistribution, very few AF granules are visible (Fig. 2D). Yet the F-actin cytoskeleton is intact, indicating that these cells are still present. An additional explanation for reduced RPE-AF in healthy and AMD eyes is that some cells densely pack with melanosomes (Figs. 2E, 2F) with a normal cytoskeleton (not shown). Although it seems unlikely that these cells degranulated of LF/MLF then filled with melanosomes, it is possible that LF granules are still present, and AF is completely blocked by melanosomes.

Aggregation is the more dramatic form of LF/MLF redistribution, observable almost exclusively in AMD eyes (Figs. 3A–F). Retinal pigment epithelial cells aggregate granules into packets several micrometers in diameter (median: 5.1 μm, range: 2.5–20.9 μm; analysis of 24 aggregates in 9 RPE cells; Fig. 3A), sometimes filling the whole cell and giving it the appearance of crumbling (Fig. 3E). In most cells, however, the cytoplasm surrounding the aggregates empties of granules. Although AF associated with aggregates can be locally intense, the presence of granule-free areas in the same cells reduces total cellular AF. In late degenerative stages, as aggregates dwindle in number, AF signal diminishes even further (Figs. 3C, 3D, 3F). Degranulation can occur contemporaneously with aggregation (Figs. 3E, 3F). Remarkably, the cytoskeleton of cells undergoing LF/MLF loss as well as their neighbors seems intact, suggesting the persistence of epithelial properties.

The normally precise packing geometry of RPE becomes highly variable in AMD, and within that context, some cells enlarge excessively (Fig. 3G). Cells can grow to 4 to 5 times the typical 15-μm size (in unaffected perifovea, Supplementary Table 5 in Ach et al.10). Enlarged cells can contain hundreds of regularly packed LF granules, while the cytoskeleton also remains intact (not shown). Thus, despite the remarkable appearance, it would be difficult to say that these giant cells are not healthy.

Some RPE cells are crowded out of the monolayer, a phenomenon observed in healthy and AMD-affected eyes (Fig. 4). These mushroom-like cells have stems in the RPE mosaic, delimited by cytoskeletons of neighboring cells, and caps protruding into the subretinal space, delimited by their own encircling cytoskeletons (Fig. 4A). As mushroom cells were found in eyes with attached retinas (Fig. 4), the subretinally extruded material cannot be considered secondary to post-mortem retinal detachment. These subretinal portions also contain LF/MLF yet no melanosomes are visible. The additional volume in the cap reduces intracellular LF/MLF granule concentration but because en face AF imaging uses projection images, total AF signal from this cell would resemble those around it (Figs. 4B, 4C). Throughout, there were no obvious holes in the mosaic.

Age-related macular degeneration–specific pathology also affects RPE cytoskeleton. F-actin bundles bend slightly outward, and interior angles of vertices increase, as affected cells round (Figs. 3A, 3D). It also includes separation of the F-actin cytoskeleton of adjacent cells, interruption of individual cell cytoskeletons, and formation of stress fibers (Figs. 5, 6). Separation started with a partial (<50%) dilatation of two adjacent cytoskeletons (Fig. 5A) and continued, as if unzipping, until completely disconnected (Fig. 5C). These changes culminate in cytoskeleton interruptions, with free ends, curls, and loops (Figs. 5D, 5G). The enlargement of RPE cells also leads to the formation of stress fibers crossing each cell in arbitrary directions (Fig. 6). These fibers appear to exert additional forces on the surrounding cytoskeleton, leading to thickening at points of insertion and an overall ragged appearance. Stress-traction exerted appears to create concavities in the previously straight sides of individual cells (Figs. 6B, 6C).

**DISCUSSION**

This is the largest en face survey of the cellular and subcellular basis of RPE AF from human AMD tissues. We undertook this
The Intracellular Distribution of Autofluorescent Granules Is Spatially Organized, Implying a Regulated Process

High-resolution SIM images reveal an orderly distribution of LF/MLF granules within RPE cells in normal aged eyes. Granules similar in size and shape, and methodically arranged, give the impression that LF/MLF accumulation is a normal and regulated process and not a stochastic event brought on by disease. Intracellular distribution also depends on retinal location, which provides different LF/MLF/melanosome ratios and age. Early single-section TEM studies reported differences among macula, equatorial, and peripheral regions in intracellular granule distribution in normal RPE cells but included no or minimally characterized AMD eyes. Further, only one study of healthy eyes distinguished between RPE serving fovea (cones-only) and perifovea (cones and rods). Regular cellular functions require organized structures, of special importance for polarized and geometrically precise cells such as RPE, which are tasked with maintaining accuracy of the sampling array of photoreceptors.

In Both Aging and AMD, RPE Cells Redistribute AF Organelles by Degranulation

The term degranulation was suggested by the finding of still-intact cells containing greatly reduced numbers of AF granules. The difference between aging and disease was that degranulation was the principle means of AF reduction in aging and aggregation was more prominent in AMD. Our methods do not allow us to distinguish whether this phenomenon indicates insufficient granule formation, excessive granule deletion, or other processes suggested by the dynamic model posed above.
For example, degranulation might be part of an active control mechanism to avoid overload in cells of constant volume via active extrusion, described for melanosomes, or via autophagic renewal. Degranulation via exosome pathways is a possibility, because crystallins and other proteins are released by RPE in this manner. However, exosomes (30–100 nm) are too small to accommodate native LF/MLF granules (~1 μm) and are not visible using common light-microscopy techniques. Autofluorescence structures less than 1 μm were reported outside RPE using HR-SIM, which are still too large for exosomes.

Redistribution of AF granules by aggregation is a process specific to the examined AMD eyes and may represent classic apoptotic bodies. Aggregations varied in size, up to 20-μm diameter in our study, are large relative to previously reported autophagic bodies (diameter, 300–900 nm). Aggregations are often multiple within one cell. They have been described as intracellular “LF congregations” within RPE cells overlying drusen, and they have been illustrated in GA eyes (Figs. 5H, 5I of Rudolf et al.). We propose that the intracellular granule aggregations seen currently are the forerunners of the material released into basal laminar deposit (BLamD). Based on previous and ongoing histologic studies, nonnucleated granule-containing aggregates are shed basally into the BLamD, which always accompanies cells at this stage of degeneration (a stage previously called 2B). In a separately reported histologic survey of RPE morphology in late AMD, we renamed this cellular phenotype “shedding.” Shedding of aggregates is distinct from the apical migration of nucleated RPE cells into the neurosensory retina. Further, the shed granule aggregates are visible in clinical SD-OCT as hyperreflective spots within a split RPE-BrM band signifying BLamD.

Our observations can be compared and contrasted with TEM observations of Feeney-Burns and colleagues showing shedding of RPE cytoplasm lacking LF into small drusen of eyes without BLamD. These authors speculated that shedding of aliquots of cytoplasm by RPE cells may serve to dispose of “old or damaged membranes and organelles.” Alternatively, seminal ultrastructural studies of cells fated for regulated cell death described membrane-bounded inclusions of local cytoplasm contents as apoptotic bodies. Given the huge number of LF and MLF granules in aged human RPE, and the association with a cellular phenotype apparently on a death pathway leads to the intriguing hypothesis that AF granule aggregates are apoptotic bodies. The triggers for apoptosis remain to be determined and are suggested to involve the
inflammasome and vascular insufficiency/micronutrient deficiency.

Cell volume is modulated in response to specific stimuli and conditions. Early on, it was believed that intracellular accumulation of RPE LF granules depresses cellular function in part from occupying cellular volume but also by disrupting membranes via a detergent-like action of major bisretinoids. It is instructive to place RPE LF/MLF in the context of age-related pigments in other postmitotic cells, particularly neurons, because of the RPE's neuroectodermal origin and the extensive pathology literature on aging and neurodegeneration. Several excellent reviews collectively put age-related brain LF in a neutral role, since neurons can accumulate large quantities of granules without exhibiting vulnerability to dysfunction and death. Also, a comprehensive review by Katz and Robison states that each cell type's LF is unique to that cell type and distinguishable from truly pathologic ceroids found in rare inherited diseases. Thus, it is telling that LF-packed RPE cells from normal aged eyes look healthy, with intact cytoskeleton, precise geometry, and absence of swelling, as we previously showed using confocal microscopy.

Even Exceedingly Enlarged Cells (Fig. 3G) Can Have Unremarkable Cytoskeleton and Polygonal Shape

As a consequence of this enlargement, the RPE cell density appears qualitatively reduced; quantitative assessments are the focus of our ongoing analysis. Retinal pigment epithelial cells resembling mushrooms were rare, yet captured by two microscopy modalities in healthy and AMD eyes with attached retinas. By HR-SIM LF/MLF was visible within these cells indicating RPE origin without also revealing melanosomes. Our study cannot clarify whether mushrooming stops at the extrusion of caps or continues until whole cells are extruded into the subretinal space in a process of transdifferentiation. As known from published and separately reported histology, apically sloughed cells are a prominent phenotype in AMD eyes. Subretinally displaced pigment-bearing cells and exploded granules suggestive of vacuolated or lysed cells have been reported for mechanically diverse mouse models. The relationships among these histologic variants in humans are also described by Zanzottera et al. while their causative pathways and distribution in early and late AMD remain to be explored through further research. Another point exemplified by the mushrooms is that cells must be viewed both en face and from cross-sectional views for definitive identifications and mechanistic insight. From en face only, the AF of the mushrooms looked like their neighbors. Retinal pigment epithelial cytoskeleton changes significantly with AMD, exhibiting separations and breaks, becoming thickened and connected to stress fibers, with concomitant conversion of cell bodies from convex to concave polygons. These changes were not observable in normal aging, where we reported a mosaic dominated by five to seven neighbored RPE and a small percentage of convex-polygonal cells with greater than or equal to eight neighbors in younger and older adults.
An intact cytoskeleton is important for the maintenance of RPE cell’s shape and function, and for RPE monolayer polarity. In cell culture and mouse models, stress disequilibrates RPE cells and leads to stereotypic cell responses. Actin seems to play a key role in cell aging and apoptosis. The appearance of stress fibers was reported for various cells exposed to mechanical forces, chemicals, and wounds and support the concept of a stereotypic repertoire of RPE stress responses, to multiple stressors.

In the context of stress fibers also thickened actin bundles were observed. The cytoskeleton in the AMD affected cells in our study also showed thickened F-actin bundles, especially at areas where stress fibers insert. Focal adhesion complexes function as the structural basis for geometry sensing and transmit traction forces of the cytoskeleton to the extracellular matrix. Separation of the cytoskeleton between two adjacent RPE cells in AMD eyes might result from sub-RPE deposits including drusen and thick BLamD, which together provide an upward force from the basolateral aspect. Cell–cell contacts might suffer and get weak or lost under this permanent pressure. The impetus for cytoskeleton changes was not obvious from the images shown here, but our forthcoming work will examine relationships with underlying Bruch’s membrane pathology. Separation, breaks of cytoskeleton, and enlargement of RPE cells have also been described for mouse models with mechanistically diverse retinopathies. Notably, wild-type mice subjected to Alu RNA-induced RPE degeneration also exhibit splits, loose ends, nonpolygonal, and concave polygonal cells with ZO-1 staining very similar to our images of cytoskeleton. These findings indicate that the cytoskeleton correlates well with the distribution of zonula occludens junctions and that derangement does not require the presence of sub-RPE deposits.

**FIGURE 5.** The cytoskeleton of adjacent RPE cells separates and becomes interrupted. In early stages (A), parallel adjacent cytoskeleton bands are separated partially (<50%, between *white arrowheads*). In advanced stages (B, C) greater than 50% cytoskeleton bands are separated. Cytoskeleton interruptions are shown (D–I) with free ends, sometimes furled or pointing in different directions. Interrupted cytoskeleton is often associated with AF sub-RPE deposits ([E, H] 488-nm excitation). Donors: (A, B) 94 years, female, incipient AMD; (C) 81 years, male, geographic atrophy; (D–I) 86 years, female, late exudative AMD. F-actin labeled with AlexaFluor647-Phalloidin. Scale bars: 20 μm.
Lipofuscin Redistribution in AMD

Study Results Impact Interpretation of Clinical Fundus Autofluorescence

The early RPE cytoskeleton and LF/MLF granule distribution changes in AMD that we herein describe are currently inaccessible to clinical fundus imaging yet have considerable relevance to how these images are interpreted. Even though the pixel sampling in fundus-AF devices can reach values down to 10 μm/pixel, the optical resolution is far less. Assuming correct Nyquist sampling, the optical resolution has to be at least 20 μm, and might even be worse due to the restricted pupil and optical aberrations. This resolution is not sufficient to discern single cells without the help of adaptive optics. Our thorough histologic description, however, will serve as source for interpretation, independent from the imaging method used. For example, Delori et al. found that fundus-AF in healthy humans increases with age but decreases after age 70. The authors explained this phenomenon by preretinal screening by aged lens in addition to possible patient selection bias and methodical challenges. Loss of RPE cells can be excluded because our recent study with previous literature shows stable RPE density with age. Our data of LF/MLF redistribution suggest that this signature age-effect can now also be attributed to loss of LF/MLF granules in individual RPE cells. Furthermore, definitive evaluation of en face fundus-AF will employ synergistic information available from cross-sectional SD-OCT, as proposed. Independently, we will confirm early reports with direct clinicopathologic correlation that granule aggregates within BLamD are visible by SD-OCT. So even if the aggregates themselves are below the resolution of current fundus-AF and SD-OCT, an associated sign is visible on SD-OCT.

Our study should be viewed in light of its strengths and limitations. Technical advances included the use of the largest sample of eyes yet investigated for histologic AF in AMD, short postmortem tissues that obviated cell variability in staining and size reported by others, z-stack projections to simulate the projection image of fundus AF, and HR-SIM for 110-nm resolution of granules over large tissue areas. Limitations include absence of clinical information regarding eye donors, lack of specific marker studies to investigate mechanisms of subcellular reorganization and cell death, lack of TEM to investigate the nature of proposed intracellular organization, and inability to examine RPE and RPE-derived cells out of the RPE layer, such as intraretinal RPE of high prognostic value for progression. Furthermore, the requisite single-snapshot perspective of our en face histology does not allow simultaneous cross-section observation. Whether aggregations are specifically related to AMD has to be further elucidated.

Despite these limitations, our data enable us to meet the next challenges, which include quantifying en face AF from these flat-mounted tissues, further integrating the en face view and cross-sectional morphology, testing hypotheses about the impact of sub-RPE lipid deposits on RPE health as monitored by granule population and packing geometry, and leveraging the longitudinal view of cellular health available through clinical imaging. Upcoming systematic analysis will also include quantitative information on the frequencies of RPE degranulation and aggregation in eyes at defined stages of aging and AMD.

In summary, this is the first description of possible mechanisms for decreasing AF both in aging and AMD: degranulation of RPE cells. We have shown that redistribution and loss of AF granules are among the earliest subcellular changes in AMD. This is important for deepening the knowledge base of clinical-AF, benchmarking model systems, and for identifying molecular mechanisms for potential points of therapeutic entry.

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