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Sub-retinal drusenoid deposits in human retina: Organization and composition

Martin Rudolf¹, Goldis Malek², Jeffrey D. Messinger¹, Mark E. Clark¹, Lan Wang¹, and Christine A. Curcio¹

¹Department of Ophthalmology, University of Alabama at Birmingham

²Department of Ophthalmology and Pathology, Duke University

Abstract

We demonstrate histologically sub-retinal drusenoid debris in three aged human eyes, two of them affected by age-related maculopathy. By postmortem fundus examination, the lesions appeared drusen-like, i.e., they were pale spots apparently at the level of the retinal pigment epithelium (RPE). Light and electron microscopy revealed aggregations of membranous debris, the principal constituent of soft drusen, in the sub-retinal space. Immunohistochemistry and confocal microscopy confirmed the presence of molecules typically associated with drusen (positive for unesterified cholesterol, apoE, complement factor H, and vitronectin) without evidence for molecules associated with photoreceptors (lectins and opsins), Müller cells (glial fibrillary acid protein and cellular retinal binding protein, CRALPB), or RPE (CRALPB). The fact that a drusenoid material, sharing some markers with conventional drusen, can occur on opposite faces of the RPE, suggests deranged polarity of normally highly vectorial processes for basolateral secretion from RPE, and that overproduction of secreted materials and direction of secretion are independently specified processes. In the future, drusenoid sub-retinal debris might be more frequently revealed by emerging high-resolution imaging techniques.

Keywords

Drusen; age-related maculopathy; membranous debris; sub-retinal space; imaging; complement factor H; apolipoprotein E; cholesterol

Introduction

Drusen are extracellular deposits that accumulate between the retinal pigment epithelium (RPE) basal lamina and the inner collagenous layer of Bruch's membrane in aging human eyes (Green and Enger, 1993). Drusen type, size, number, total area and/or degree of confluence are risk factors for the development of age related maculopathy (ARM), the leading cause of irreversible blindness in people over the age of 65 years (van Leeuwen et al., 2003). Although

Correspondence to: Christine A. Curcio.

Corresponding Address: Christine A. Curcio, PhD Department of Ophthalmology 700 South 18th Street Room H020 Callahan Eye Foundation Hospital University of Alabama School of Medicine Birmingham AL 35294-0009, USA (205) 325-8632; FAX (205) 325-8634 Email: curcio@uab.edu.

Current address of M. Rudolf is Department of Ophthalmology, University of Schleswig-Holstein, Lübeck, Germany

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the pathobiology of drusen is not fully understood, recent studies have identified many druse constituents such as cholesterol, apolipoproteins B and E, acute phase proteins such as vitronectin, and complement components such as factor H (Anderson et al., 2001; Crabb et al., 2002; Li et al., 2007; Li et al., 2006; Malek et al., 2003; Mullins et al., 2000).

Basal linear deposit, a diffusely distributed lesion also located external to the RPE basal lamina, contains membranous debris, defined as variably sized coils of multilamellar, uncoated membranes (Sarks et al., 2007; Sarks et al., 1980). Although often described as vesicles (i.e., closed membranes with aqueous interiors), membranous debris may represent in part the cholesterol-enriched surface of sub-optimally preserved solid lipoprotein particles (Curcio et al., 2005b), and we use the term here as a convenient descriptor. In a classic 1988 paper on geographic atrophy (Sarks et al., 1988) and in subsequent studies (Sarks et al., 2007), John and Shirley Sarks showed that membranous debris constituted the principal component of lesions in four extracellular locations (soft drusen, basal linear deposit, basal mounds, and aggregations within the sub-retinal space) and in one intracellular location (vacuoles within the RPE). Membranous-debris-containing soft drusen and basal linear deposit are recognized as the specific lesions of ARM (Curcio and Millican, 1999; Green and Enger, 1993; Sarks et al., 2007), but the aggregations of membranous material between photoreceptor outer segments and apical RPE are more enigmatic, as they have been described only rarely. As originally illustrated by low magnification transmission electron microscopy (TEM) (Sarks et al., 1988), they are well organized, spherical, and homogeneous aggregations of membranous debris said to be present only where photoreceptors are also present. Their coherent morphology suggests a specific formative process rather than the consequences of post-mortem retinal detachment. More recently (Curcio et al., 2005a), we showed that deposits in the sub-retinal space of eyes with ARM (and importantly, also with attached retinas) were highly enriched in unesterified cholesterol labeled by filipin. These aggregations resembled drusen in the same eyes with regard to staining intensity and morphology of the stained material. Unlike drusen, however, sub-retinal aggregations contained little esterified cholesterol.

More information about sub-retinal debris (SRD) would be useful, for two reasons. First, it might elucidate some aspects of biogenesis of conventionally placed drusen. Second, it may assist the interpretation of sub-retinal findings revealed by clinical retinal imaging techniques with near-histological resolution, such as ultrahigh resolution or spectral domain optical coherence tomography (Michels et al., 2008; Pieroni et al., 2006; van Velthoven et al., 2007). Herein we describe SRD within the maculae of three donor eyes, two with funduscopically-identifiable ARM and another with drusenoid spots in the fundus that were exclusively SRD confirmed by histology.

Methods

The Institutional Review Board at the University of Alabama at Birmingham approved use of human tissues in this study. Eyes were preserved within 6 hr of donor death by immersion in 4% paraformaldehyde in 0.1M phosphate buffer for 24 hrs following corneal removal and stored in 1% paraformaldehyde at 4°C until used. Stereo color images of macula and periphery of each eye (Curcio et al., 1998) were taken with a dissecting scope (SMZ-U, Nikon Instruments Inc., Melville NY) after complete removal of the anterior segment and vitreous using 35 mm film (EPJ320T, Kodak, Rochester NY). The number of macular drusen was determined for each eye from images of the post-mortem fundus taken with the retina in place.

Two eyes (Case 1 and Case 2, Table 1) were prepared as described previously (Rudolf et al., 2008). Under stereomicroscopic guidance, macular RPE-capped drusen were mobilized from Bruch's membrane as part of RPE-retina sheets, placed into BEEM capsules (Electron Microscopy Sciences, Hatfield PA), and covered with a 0.75%-agarose/5%-sucrose solution.

Blocks were trimmed, post-fixed in 1% osmium in 0.1 M sodium cacodylate buffer, 1% tannic acid (gallotannin, C₁₄H₁₀O₉), and 1% paraphenylenediamine (OTAP method) (Curcio et al., 2005b; Guyton and Klemp, 1988) and embedded in epoxy resin (PolyBed 812; Polysciences, Warrington PA). One- μ m-thick sections were cut with a Leica Ultramicrotome (Ultracut UCT, Leica Mikrosysteme AG, Vienna, Austria) and stained with 1% toluidine-O-blue. Sections were examined and photographed with a 40x planapochromat objective on an Eclipse 80i microscope (Nikon Instruments Inc., Melville NY). Lengths of SRD along the RPE were measured with an ocular reticule. Images were captured with a Retiga 4000R Fast digital camera and Qcapture v2.8.1 software (Qimaging, Burnaby BC, Canada).

One eye (Case 3, Table 1) was prepared for cryosections as described (Malek et al., 2003). The retina/RPE/choroid from a horizontal belt containing the fovea and optic nerve head and extending from the nasal to temporal ora serrata were removed from the sclera. A sample for histochemistry and immunohistochemistry that was 10 mm (nasal to temporal) by 6 mm (superior to inferior), including the fovea and the temporal half of the optic nerve head, was cut from the macula with a razor blade. The sample was cryo-protected by infiltration with successive solutions of 10%, 20%, and 30% sucrose in phosphate buffer, 4:1 30% sucrose/OCT (Histoprep, Fisher Scientific, Pittsburgh PA) solution and 2:1 30% sucrose/OCT solution for 30 minutes each and then frozen in liquid nitrogen. Specimens were sectioned at 10 μ m (CM3000 cryostat, Leica Microsystems Inc., Bannockburn IL). Consecutive sections were collected on gelatin-subbed slides, dried at 40-60°C for at least 2 hours, and stored at -20°C until used.

Cryostat sections were stained with Gill's formulation #3 hematoxylin (Fisher Scientific) for histopathologic evaluation. Filipin (Sigma-Aldrich, St Louis MO) was used to visualize unesterified and esterified cholesterol, the latter after extraction and hydrolysis, as described (Malek et al., 2003). For lectin labeling, cryostat sections were incubated with rhodamine-conjugated PNA (*Arachaea hypogaea* agglutinin) or WGA (*Triticum vulgaris* agglutinin), purchased from Vector Laboratories (Burlingame CA) and EY Laboratories (San Mateo CA) respectively (Table 2). Unlabeled adjacent sections were used to distinguish between lectin binding and autofluorescence. For immunofluorescence, primary antibodies were obtained from the sources indicated in Table 2. Rhodamine-conjugated secondary antibodies (donkey anti-rabbit, 1:100, and goat anti-mouse, 1:200) were obtained from Jackson ImmunoResearch (West Grove PA). Alexa 594 and 488 conjugated secondary antibodies (1:200-500) were obtained from Invitrogen (Carlsbad CA). Negative control sections were routinely processed with each experiment and included samples incubated with an irrelevant antibody or without the primary antibody.

Sections were viewed on one of two systems for wide field epifluorescence: 1) a Zeiss Axiophot with 10X and 40X plan apochromat objectives, 3 filter cubes (excitation, barrier, and fluorophor: 360/40-460 nm, DAPI; 480-535 nm, FITC; and 560-630 nm, Cy3), AxioCam MRm digital camera, and AxioVision v4.6 image processing software; or 2) a Nikon Optiphot2 with a 20X plan apochromat objective, 3 filter cubes (420-520 nm, filipin; 546/10-590 nm, rhodamine; and 450/490-520 nm, autofluorescence), SensiCam camera (Cooke, Auburn Hills MI), and IPLab imaging software (BD Biosciences, Exton PA). Sections were also examined by confocal microscopy using a Nikon Eclipse 90i equipped with 3 lasers and Metamorph v7.5 software (Molecular Devices, Sunnyvale CA). All images of experimental and control sections were exposed at matched times on the same microscope.

Cases 1 and 3 were examined by TEM (Case 2 could not due to a soft block). For Case 3, 2 $\times$ 2 mm size blocks at the macular boundary but still containing grossly visible lesions were postfixed in 2% osmium and embedded in epoxy resin (PolyBed 812; Polysciences, Warrington PA). From Cases 1 and 3, gold sections were stained (3% uranyl acetate for 8 min, 0.2% lead

citrate for 6 min) and examined with a 1200 EXII electron microscope (JEOL USA, Peabody MA). TEM sections were photographed with an AMTXR-40 camera (Advanced Microscopy Techniques, Danvers MA). All images were assembled into composites using Photoshop CS2 (Adobe Systems, USA) with adjustments for exposure and contrast only.

Genotypes for two genes suspected of conferring risk for ARM (Montezuma et al., 2007), complement factor H (CFH) and HtrA serine peptidase 1 (HTRA1), were determined using genomic DNA isolated from aldehyde-fixed neurosensory retina with the Recover All Total Nucleic Acid kit (Ambion Inc., Austin TX). Before extraction, tissue was treated with ascending concentrations of ethanol (30% to 100%). Retina was digested using protease at 50° C for 48 hr according to the manufacturer's instructions. DNA concentration was determined by spectrophotometry (NanoDrop™ 1000, Thermo Fisher Scientific, Wilmington DE). Genotyping for CFH (rs1061170) and HTRA1 (rs11200638) was carried out using Taqman SNP Genotyping Assays with primers custom-designed by the manufacturer (Applied Biosystems, Foster City CA) and run on a PTC 200 Thermocycler PCR system (MJ Research, Reno NV).

Results

Gross appearance and light microscopic histopathology

Table 1 summarizes characteristics of Cases 1-3, all from non-diabetic donors without ophthalmic history and at least one risk allele for CFH and HTRA1. Figure 1 shows the appearance of the post-mortem macula and 1- μ m sections.

Case 1 had 5-7 irregularly spaced, variably sized large drusen near the fovea (Figure 1A-1), consistent with early ARM. Most drusen were soft and lost their contents upon isolation (Figure 1A-2), as was the case for about 1/3 of macular drusen isolated in this manner (Rudolf et al., 2008). Therefore, in the histological sections the vacant space external to the RPE was scalloped, because it previously contained drusenoid material (Figure 1A-2, arrows) in large drusen and in extensive basal linear deposits. The RPE was attached to the retina, and between RPE and photoreceptor outer segments were aggregations of an organized material (Figure 1A-2, arrowheads), sometimes with full-length outer segments inserted between adjacent aggregations (not shown). Individual sections had 1-7 aggregations (length, 14.8-177.8 μ m) and 1-5 drusen. Within sections, drusen and aggregation numbers were not related.

At post-mortem fundus examination Case 2 (not shown) had a thickened operculum around the fovea and 30-34 large white drusen that were irregularly spaced, glistening, and calcified, also consistent with early ARM (Rudolf et al., 2008). Case 2 had a widespread, thick layer of basal laminar deposit (including early and late stages) and drusen that were mostly lost when isolated (Figure 1C, 'd'). Overlying the RPE encasing drusen, however, were well-formed pillars of debris in the sub-retinal space (Figure 1C, arrowheads). Aggregation prevalence, relative to drusen, was similar to that for Case 1, but Case 2 had fewer very large aggregations (length, 12.3-98.8 μ m). Interestingly, Case 2's fellow eye (Rudolf et al., 2008) exhibited 45-50 grossly visible drusen but no histologically detectable SRD.

Case 3 had 50 small pale spots of relatively uniform size and spacing, particularly in the temporal macula (Figure 1B-1). Unlike drusen in Cases 1 and 2, which were visible under both epi- and trans-illumination, these spots were seen best with epi-illumination, and upon histological analysis, proved to be 24.7-190.1 μ m-long aggregations located in the sub-retinal space only. Case 3 had no drusen conventionally positioned in the sub-RPE compartment. For this reason, we do not consider Case 3 ARM. Nor did our limited tissue sample reveal phenomena such as leukocyte infiltration that would lead us to suspect diseases with an

inflammatory etiology. SRD aggregations tightly apposed by outer segments (Figure 1B-2) will be shown in more detail below.

Ultrastructure of SRD

Figure 2 shows the ultrastructure of SRD in Cases 1 and 3. Case 1 had membrane-bounded profiles with medium density, homogeneous interiors that were dispersed throughout a flocculent, surrounding material (Figure 2A). The membranes in the photoreceptor outer segments surrounding these aggregations appeared normal within the limits of post-mortem delay to preservation. Case 3 appeared different, perhaps due to use of a different post-fixation method. The sub-retinal aggregations had numerous electron-dense closed membranous profiles with empty interiors (Figure 2B). These membranous profiles were identical in morphology to those enclosed within basal laminar deposit in the sub-RPE compartment, and they differed from the nearby outer segment disk membranes by being more electron-dense. Note that the outer segments did not appear normal in Case 3, as they were bloated, but they did connect with the inner segments (not shown), and the electron densities of the sub-retinal and outer segment membranes were always distinct from each other. We also observed that bundles of filaments surrounded the sub-retinal aggregations on their lateral aspects, and these filaments were confirmed as apical microvilli connected to the RPE perikaryon (Figure 2C).

Case 3: morphology and composition of SRD

Figures 3 and 4 and Table 2 show more detail about the morphology and composition of the aggregations present in the temporal macula of Case 3, for which many cryo-sections were available. In hematoxylin-stained sections, the aggregations are clearly visible as organized material between, and in contact with, the RPE and photoreceptor outer segments (Figure 3A). They were clearly distinguishable from very small drusen that were rarely found in the same eye (Figure 3C). There was a loss of photoreceptor nuclei over larger aggregations (Figure 3A-D), and in some cases, the debris breached the overlying outer nuclear layer (Figures 3C, D). Not all aggregations were large enough to impact photoreceptors in this manner, as some were low in height and diffuse in shape (e.g., Figure 1B-2).

Like drusen (Li et al., 2007; Li et al., 2006; Malek et al., 2003) and SRD previously described in ARM (Curcio et al., 2005a), SRD in Case 3 contained abundant unesterified cholesterol in amorphous and reticulated staining patterns (Figure 4A). Also like drusen (Anderson et al., 2001), SRD contained apoE immunoreactivity (Figure 4B, negative control-4C). With growing evidence of a significant role for the complement system in ARM pathogenesis, we evaluated the distribution of CFH (Figure 4D), a regulatory protein previously demonstrated in drusen (Hageman et al., 2005). We also checked for another abundant druse protein, the acute phase responder, vitronectin (Hageman et al., 1999) (Figure 4E). Both CFH and vitronectin localized to sub-retinal aggregations of all sizes and to the vasculature of the choriocapillaris and choroid. To exclude the possibility that membranous material in the aggregations was derived directly from photoreceptors, we examined sections probed with antibodies to opsins (not shown) and with lectins that bind specifically to cone or rod-associated interphotoreceptor matrix (PNA Figure 4F, 4G, and 4H-red and WGA not shown). SRD was universally negative, despite strong staining within the photoreceptor layer. Similarly, to exclude the participation of retinal glial cells (astrocytes and Müller cells), which can become activated during retinal detachment and injury (Lewis et al., 2005) and can invade sub-RPE deposits (Kuntz et al., 1996), we examined sections probed with antibodies to glial fibrillary acidic protein (GFAP) and cellular retinaldehyde-binding protein (CRALBP). SRD was unreactive for both proteins (Figure 4F, 4G, and 4H-green). CRALBP has also been described within RPE cells and their apical processes (Bonilha et al., 2004; Bunt-Milam and Saari, 1983). Accordingly, CRALBP immunoreactivity in RPE apical processes of our samples was elaborate, appearing to extend

around rather than within the sub-retinal aggregations (Figure 4G, 4H, and 4I-brightfield), consistent with ultrastructural observations (Figure 2C).

Discussion

In this report we expanded the description of drusenoid SRD originally formulated by the Sarks (Sarks et al., 1988). The presence of attached outer segments and the lack of an associated basal lamina (see Figure 2B) argue against SRD being artifactual displacement of conventional drusen into the sub-retinal space. In three eyes, two with ARM, we show organized material with membranous contents distinct from outer segments. In the third eye, the material was sufficiently reflective to permit its visualization in the fundus, but due to the lack of conventional drusen and the abnormal outer segments, we cannot call this eye ARM. Its identity remains uncertain. Other diseases with lesions in the same location and a presumably similar appearance would include disorders like multiple evanescent white dot syndrome (MEWDS) which in contrast to our cases typically appear much earlier in life (Nguyen et al., 2007; Quillen et al., 2004).

SRD is negative for photoreceptor and glial cell markers and positive for the universally agreed-upon druse components complement factor H, vitronectin, apoE, and unesterified cholesterol. The apical microvilli of the RPE cells, which are specialized for interaction with photoreceptor outer segments, contain the retinoid processing protein CRALBP and appear to extend around the SRD. Published TEM images have shown membranous material identical to the contents of soft drusen enclosed intracellular vacuoles within the RPE (Curcio and Millican, 1999; Sarks et al., 1988; Sarks et al., 2007). It has been speculated that these vacuoles represent an episodic release of debris that will form drusen at the basolateral RPE surface. The fact that a similar material, sharing some markers, can occur on opposite faces of the RPE (see our Figure 2B), is consistent with the idea (Curcio et al., 2005a) that deranged polarity of normally highly vectorial processes for basolateral secretion is involved. SRD differed from drusen, however, as other druse constituents (apoB, apoA-I, and esterified cholesterol) thought to represent components of an RPE-produced lipoprotein (Li et al., 2006) were only minimally present (not shown), and SRD was considerably less autofluorescent than typical hard drusen. We caution that we cannot exclude the possibility that SRD contains components of plasma origin that arrive in the sub-retinal space via extravasation from the choroidal capillaries and focal breakdown of the outer blood-retinal barrier. Nevertheless, our data raise the possibility that to the extent that drusenoid debris is produced by the RPE, overproduction of secreted materials and the direction in which it is secreted appear to be independently specified processes.

Our results have implications for clinical studies, adding to the ways in which yellow-white lesions at the level of the RPE at fundus examination are famously not necessarily drusen in histological sections (Anderson et al., 2006; Bressler et al., 1994). We do not know the extent to which soft macular drusen in the fundus may include sub-retinal material over their apices or around their borders, as implied by our Figures A-2 and C. Nor do we know the prevalence of this phenomenon among ARM patients in general. It may be a small minority but so far we have observed SRD in 15-22% in 2 cohorts of differently processed eyes (2 of 9 eyes of 7 donors; 4 of 26 eyes of 20 donors (Curcio et al., 2005a; Rudolf et al., 2008)). Our findings suggest that drusen in color fundus photographs may not correspond precisely to drusen revealed by late staining in fluorescein angiograms. We would expect that even if SRD is made of material identical to that found in drusen, the intact outer blood-retinal barrier would prevent absorption of fluorescein and therefore make visualization of apically located lesions difficult. It should prove informative to superimpose color fundus and angiographic images for ARM patients, as was done for patients with the inherited disorder early-onset adult grouped drusen (Russell et al., 2004). We can also speculate about the appearance of SRD by new imaging techniques like ultra-high resolution, spectral domain, or polarization-sensitive OCT. Soft

drusen, which like the sub-retinal lesions also contain principally membranous debris, usually impress as spaces of low reflectance under a hyper-reflective RPE layer in a characteristic druse shape (Michels et al., 2008; Pieroni et al., 2006; van Velthoven et al., 2007). Both light microscopic and OCT studies indicate that soft drusen contain little internal structure (Michels et al., 2008; Pieroni et al., 2006; Rudolf et al., 2008). By analogy, SRD could be represented by uniform, low reflective, or empty spaces between the photoreceptor layer and the RPE. Since SRD can vary in shape between thin plaques to prominences reaching deep into the neurosensory retina, various presentations in OCT imaging should be possible. In ARM eyes (Cases 1 and 2) the photoreceptor layer was thinned above all lesions. If SRD encroaches on the neurosensory retina, the reflective bands representing the penetrated retinal layers may become discontinuous. Finally, eyes that have drusenoid material in the sub-retinal as well as the sub-RPE space may have different risk levels for choroidal neovascularization, as this material would not provide an initial cleavage plane easily accessible to newly invading choriocapillaries.

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Figure 1. Maculas of eyes with sub-retinal drusenoid debris

A-1, B-1: post-mortem fundus appearance, produced using a stereomicroscope and epi- and trans-illumination of eyecups after removal of the anterior segments. White bars, 1 mm. **A-2, B-2, C:** One μm -thick sections stained with toluidine-O-blue and photographed using a 40X plan fluor objective. ONL, outer nuclear layer; RPE, retinal pigment epithelium; Ch, choroid. RPE appears thicker in A-2 and C than B-2, because it was sectioned at a slightly oblique angle. Black bars, 50 μm . **A-1:** Case 1, left eye. Optic nerve head is at left edge of the panel. Arrow indicates several large drusen. **A-2:** Case 1. Sheet of retina with attached RPE was removed from Bruch's membrane. Arrowheads point to two domes of SRD. Little of the druse contents remain at the base of the RPE in this eye (arrows). **B-1:** Case 3, left eye. Arrows indicate some regularly scattered small drusenoid spots. **B-2:** Case 3. Arrowheads indicate two areas of SRD. More striking examples from Case 3 are shown in Figure 3. **C:** Case 2. Arrowheads indicate two domes of SRD. Some dislodged druse contents are visible (d).

Figure 2. Ultrastructure of sub-retinal drusenoid debris

RPE, retinal pigment epithelium. OS, outer segments. Bar in each panel, 10 μ m. **A.** Case 1. Aggregation of SRD contains flocculent material with interspersed globules of homogeneous material bounded with electron-dense lines (arrowheads). Retina is at the top and choroid at the bottom of the panel. **B.** Case 3. Aggregation of SRD, delimited by dashed lines, contains abundant membranous loops (arrowheads) resembling similar material within basal laminar deposit (arrowhead) external to the RPE. Photoreceptor OS also appear full of expanded membranous profiles, of a much lesser electron density. The RPE cell layer, which spans the panel, is not uniform in either morphology or pigmentation. A true basal lamina is located external to the RPE (paired arrows), but not in relation to the SRD. **C.** Case 3. Within SRD (between the dotted line and the RPE), bundles of apical microvilli are apparent (pairs of wavy arrows).

Figure 3. Morphology of sub-retinal drusenoid debris in Case 3

A-D: Cryo-sections, 10 μm -thick, stained with Gill's hematoxylin #3. Bar in A, 50 μm , applies to all panels. OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segments; RPE, retinal pigment epithelium; C, choroid. **A.** Lesions (arrowhead) were typically located in the sub-retinal space sandwiched between the photoreceptor OS and the RPE. **B-D.** Occasionally the lesions penetrated the OS layer, IS layer, and ONL. In C, arrowheads delineate the outer border of the lesion, and the arrow indicates a tiny druse. C and D are serial sections demonstrating the progression of the debris through the photoreceptors.

Figure 4. Composition of sub-retinal drusenoid debris in Case 3

Cryo-sections, 10 μm -thick. OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segments; OS, outer segments; RPE, retinal pigment epithelium; Ch, choroid. **A-C:** Wide-field epi-fluorescence. Bar in C, 50 μm , applies to panels A-C. **A.** Filipin-stained unesterified cholesterol is abundant, in amorphous and reticulated staining patterns. SRD is delineated by yellow arrowheads. The autofluorescent RPE is out of focus. **B.** SRD is apolipoprotein E-immunoreactive. **C.** Non-immune serum control section shows no fluorescence in the SRD. **D-F:** Confocal fluorescence. Bar in D, 100 μm , applies to panels D-F. **D.** SRD is CFH-immunoreactive. Section is closer to the fovea than the sections shown in the other panels, and the Henle fiber layer of the OPL is very thick. **E.** SRD is vitronectin-immunoreactive. **F.** SRD

shows no reactivity for PNA (red), which stains cone matrix sheaths, or GFAP (green), which localizes exclusively to Müller cell end feet. **G-I:** Epifluorescence. Bars in G and I (applies to H as well), 100 μm . **G.** CRALBP (green) staining is present throughout Müller cells and in the apical processes of the RPE cells, but is absent from the SRD. SRD shows no reactivity for PNA (red). **H.** Higher magnification of the SRD in panel G, demonstrates CRALBP immunoreactivity within apical processes of RPE cells. **I.** Brightfield image of SRD depicted in H. SRD is delineated by yellow arrowheads. Nuclei in panels D-H were stained with 1 $\mu\text{g}/\text{ml}$ Hoechst (blue).

Table 1

Donors and Eyes

Case # ^a	Age	Gender	Histopathologic description: diagnosis	CFH ^b	HTRA1 ^c
1	76	F	Large soft drusen, thick BlamD: ARM	TC	<u>AG</u>
2	91	F	Large soft drusen, thick BlamD: ARM	TC	<u>AA</u>
3	86	M	Multiple aggregations of sub-retinal debris: unknown	TC	<u>AG</u>

^a All donors were Caucasian.

^b Complement factor H, SNP rs1061170 (Y402H). Risk allele C is underlined.

^c HtrA serine peptidase 1, SNP, rs11200638 (–625G/A). Risk allele A is underlined.

Table 2
Labeling of sub-retinal debris

Label	Source	Dilution	SRD
Antibodies			
GFAP	Dako	1:100	—
CRALBP	Gift from John C Saari	1:2500	—
Rhodopsin (R4D2)	Gift from Robert Molday	1:50	—
Red-green opsin	Gift from Jeremy Nathans	1:50	—
ApoE	Calbiochem	1:100	+
ApoB	Polysciences, Inc.	1:100	+/-
ApoA-I	Polysciences, Inc.	1:100	+/-
Complement Factor H	Quidel	1:200	+
Vitronectin	Santa Cruz	1:200	+
Lectins			
PNA	EY Laboratories	1:1000	—
PNA	Vector Laboratories	1:500	—
WGA	EY Laboratories	1:200	—
Lipid Stains			
Filipin	Sigma	500 µg/ml	+ UC +/- EC

SRD: sub-retinal debris, UC: unesterified cholesterol, EC: esterified cholesterol, present (+), minimal present (+/-), absent (-).