Article

Identification of hydroxyapatite spherules provides new insight into sub-retinal pigment epithelial deposit formation in the aging eye

Richard B. Thompson[a], Valentina Reffatto[b], Jacob G. Bundy[c], Előd Körtvély[d], Jane M. Flinn[e], Antonio Lanzirotti[f], Emrys A. Jones[c], David S. McPhail[g], Sarah Fearn[g], Karsten Boldt[d], Marius Ueffing[d], Savanjeet Guy Singh Ratu[b], Laurenz Pauleikhoff[b], Alan C. Bird[b] and Imre Lengyel[b]

[a]Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine,Baltimore, MD 21201 USA;
[b]UCL Institute of Ophthalmology, EC1Y 8TB London, UK;
[c]Department of Surgery and Cancer, Imperial College London, South Kensington, London SW7 2AZ, UK;
[d]Universitatsklinikum Tubingen, Centre for Ophthalmology, Tubingen, Germany;
[e]Department of Psychology, George Mason University, Fairfax, VA USA;
[f]Center for Advanced Radiation Sources, The University of Chicago, Chicago, IL 60439 USA;
[g]Department of Materials, Imperial College London, South Kensington, London SW7 2AZ, UK

__Corresponding author__:
Imre Lengyel, PhD
Department of Ocular Biology and Therapeutics
UCL Institute of Ophthalmology
11-43 Bath Street, London
EC1V 9EL, UK
Tel:+44-(0)2076086894
Fax: +44(0)2076086862
email: i.lengyel@ucl.ac.uk

Abstract:

Accumulation of protein- and lipid-containing deposits external to the retinal pigment epithelium (RPE) is common in the aging eye, and has long been viewed as the hallmark of age-related macular degeneration (AMD). The cause for the accumulation and retention of molecules in the sub-RPE space, however, remains an enigma. Here we present fluorescence microscopy and x-ray diffraction evidence for the formation of small (0.5-20 micron diameter), hollow, hydroxyapatite (HAP) spherules in the Bruch's membrane in human eyes. These spherules are distinct in form, placement, and staining from the well-known calcification of the elastin layer of the aging Bruch's membrane. Secondary ion mass spectrometry (SIMS) imaging confirmed the presence of calcium phosphate in the spherules, and identified cholesterol enrichment in their core. Using HAP-selective fluorescent dyes we show that all types of sub-RPE deposits in the macula as well as in the periphery contain numerous HAP spherules. Immunohistochemical labeling for proteins characteristic of sub-RPE deposits, such as complement factor H, vitronectin, and amyloid beta, revealed that HAP spherules were coated with these proteins. HAP spherules were also found outside the sub-RPE deposits, ready to bind proteins at the RPE-choroid interface. Based on these results we propose a novel mechanism for the growth and possibly even the formation of sub-RPE deposits, namely that the deposit growth and formation begins with the deposition of insoluble HAP shells around naturally occurring cholesterol-containing extracellular lipid droplets at the RPE-choroid interface; proteins and lipids then attach to these shells, initiating or supporting the growth of sub-RPE deposits.

Keywords:

Significance Statement

Proteins and lipids accumulating in deposits external to the RPE represent a barrier to metabolic exchange between the retina and the choroidal capillaries. With time, these deposits can lead to age-related macular degeneration (AMD), the most common cause of blindness in the elderly in the developed world. It remains unclear how sub-RPE deposits are initiated and grow to clinically relevant features. Using a combination of high resolution analytical techniques we found that tiny hydroxyapatite (bone mineral) spherules with cholesterol-containing cores are present in all examined sub-RPE deposits, providing a scaffold to which proteins adhere. If the spherules are important in initiating sub-RPE deposit formation this may provide attractive new approaches for early identification and treatment of AMD.

Introduction

A major feature of the aging retina is the deposition of proteins[1] and lipids[2] external to the retinal pigment epithelium (RPE), leading to the formation of sub-RPE deposits that can be focal (drusen) or diffuse (basal linear and basal laminar deposits)[3, 4]. Sub-RPE deposits increase in number and size with age, and are believed to impair metabolic exchange between the choroidal blood circulation and the retina[5]. This blockage of nutrient and waste flow to and from the highly active photoreceptors is widely suspected of inducing degeneration of the sensory retina, particularly in the macula, eventually leading to age-related macular degeneration. Due to this correlation between AMD and sub-RPE deposit formation, substantial effort has been devoted to determining the composition and origin of sub-RPE deposits, with a view to developing better diagnosis, prevention, and treatment for AMD[4]. Although AMD does not necessarily follow the same course in all patients[6], it is acknowledged that progression of sub-RPE deposit formation is a major factor in a large proportion of cases.

There is compelling evidence for the production and secretion of cholesterol-containing lipids and lipoprotein particles, secreted at least partly by the RPE[7, 8], that are recruited and retained in the aging Bruch's membrane[9-14]. However, there is no unifying explanation of how and why proteins are recruited and retained at the RPE/choriocapillaris interface. Given the high degree of protein heterogeneity within sub-RPE deposits, it was puzzling why specific proteins, like beta amyloid or complement factor H, appear therein as hollow, spherule-like structures [15, 16]. Here, we identify numerous hollow hydroxyapatite (HAP) spherules with lipid cores in all examined sub-RPE deposits, with proteins bound to the spherules' surfaces. Individual HAP spherules are also present external to the RPE and we propose that these HAP spherules are involved in protein deposition at the RPE/choroid interface and contribute to the growth of sub-RPE deposits.

Results and Discussion

As part of our ongoing studies to determine the composition of sub-RPE deposits[17, 18], we collected micro-focused synchrotron X-ray diffraction (µXRD) patterns from isolated sub-RPE deposits from human cadaver eyes. The µXRD pattern clearly identified hydroxyapatite (HAP) in sub-RPE deposits (**Figure 1A and B**), demonstrating that this highly insoluble form of calcium phosphate ($Ca_5(PO_4)_3OH$, also called hydroxylapatite), ordinarily found in bones and teeth, is present in sub-RPE deposits. Ectopic calcification of soft tissues has been associated with the general aging process, and significant calcification of the collagenous layer of Bruch's membrane is typically present in AMD[19]. This calcification can be readily labelled with Von Kossa's stain[20] that indicates the presence of calcium phosphates. However, compared with other calcium phosphates, HAP is much less soluble, more stable, mechanically harder, and to our knowledge solubilized physiologically only with acid treatment[21]. Small internal spherules with calcium content have been noted in sub-RPE deposits before[20, 22] but HAP has not been associated with these spherules.

We imaged HAP in sub-RPE deposits in human cadaver eyes by confocal fluorescence microscopy using four stains that are known to label HAP in bones and teeth. These were tetracycline[23] and its analog Bone-Tag 680 RD[24], Alizarin Red S[25] and Xylenol Orange[26]. Three of these compounds have differing HAP-binding moieties (**Supporting Information FigureS1**), making it unlikely that the very similar fluorescent staining with each

compound was due to nonspecific binding to some other molecule(s), such as a protein. These stains are specific for HAP in contrast to dibasic calcium or zinc phosphates (**Supporting Information Figure S2**) and they all displayed very similar staining patterns in freshly dissected flat mounts of human cadaver retinas (**Supporting Information Figure S3).** Bone Tag 680 RD fluorescence (depicted as magenta in **Figure 1C-D**) and used in most of the experiments) was present in small (0.5–20 µm diameter, average size ~3 µm) spherules within all sub-RPE deposits. This fluorescence was quite distinct from the typical tissue autofluorescence (depicted in green in **Figure 1F-H**) (27) or pigment granules. The spherules typically exhibited a hollow appearance, indicating that the dyes did not stain the core of the spherules (**Figure 1D-H**). This was not simply a consequence of poor dye penetration, as sectioned specimens with HAP stained on the cut face appeared hollow as well (supplementary information **Movie M1**). The number of HAP spherules varied between sub-RPE deposits: in some there were only a few, in others there were dozens present (**Figure 1D-F).** In addition, we identified spherules that were not surrounded by the autofluorescence typical of sub-RPE deposits(27) (**Figure 1G and H**). Essentially identical spherules were observed in sub-RPE deposits in all of the 30 eyes from different donors we received in a 6 month period, with an age range of 43-96 years (Supporting Information Table T1).There was a direct relationship between the thickness of the sub-RPE deposits and the number and size of drusen on the one hand, and the number of HAP labeled spherules on the other. There were no obvious gender differences in the labeling, though there were variations in retinal location. Some eyes contained large, soft drusen-like deposits in the macula (Figure 1A, and labelled with *** in Table S1), suggesting that these donors had early AMD. Large areas covered by diffuse deposits were reminiscent of basal laminar or linear deposits (Fig 1B). With our current light microscopy approach using unfixed tissues we were not able to unambiguously identify the different subtypes of sub-RPE deposits. However, all eyes contained hard drusen-like deposits mainly located at the retinal periphery (Figure 1C-D and Table 1, labelled with *). Spherules were occasionally seen in isolation in the sub-RPE space (Figure 1E-F).

The similarity of form and hollow nature of the spherules raised the question of what may be in the center that promotes the formation of the HAP shell. Lipids are actively transported through the Bruch's membrane(2, 10) and can be coated by HAP in vivo(28). Therefore, unfixed whole mount samples were stained with Nile Red, a fluorescent dye for neutral lipids. Nile Red labeled a large number of spherical inclusions with diameters similar to HAP spherules in these samples (**Supporting Information Figure S4**). Unfortunately, double labeling with Nile Red and the HAP dyes was unsuccessful due to incompatibilities of the reagents. Therefore, we analysed the composition of flat mounted and freeze-dried (**Figure 2E**) sub-RPE deposits by mass spectrometry imaging. For this experiment sub-RPE deposits (a druse on **Figure 2**) were "milled" (without fixation or mechanical sectioning) using a focused ion beam (FIB) to reveal the inner structure of the druse (**Figure 2A and B**) while preserving the native chemical composition. The milled samples were then analysed with time-of-flight secondary ion mass spectrometry (TOF-SIMS), which is well suited to this investigation as it combines high lateral resolution (~250 nm) and high sensitivity(29), and images molecular ions and organic molecules over a wide mass range. A multivariate image analysis (non-negative matrix factorization score plot) clearly demonstrated chemically distinct spherular structures inside the drusen (**Figure 2F**). Further analysis of selected ions provided detailed and independent maps of calcium phosphate (**Figure 2C**), organic molecules such as cholesterol (**Figure 2D**), ions characteristic of phosphatidylcholine (**Figure 2G**), and overall protein signature (**Figure 2H**). Neither the readily-identified m/z 184 ion, derived from phosphocholine headgroups, nor those of protein signatures showed enrichment in spherules. The ionic signatures for other phospholipids and/or triglycerides were not detected at this spatial resolution. However, the spherules in sub-RPE

deposits clearly contain calcium phosphate (these experiments did not distinguish the different calcium phosphates) and they are also associated with cholesterol and/or cholesteryl esters in accord with previous observations on the composition of extracellular lipid droplets in the Bruch's membrane and sub-RPE deposits (30, 31).

The shells shown in **Figure 1** are strikingly similar to images previously obtained by immunohistochemical staining of proteins earlier identified as constituents of sub-RPE deposits, such as amyloid beta(15, 16), complement factor H (CFH)(32) and vitronectin(33). HAP is well known to bind proteins and for this reason is widely used as a stationary phase for chromatography(34). Consequently we tested the hypothesis that the HAP spherules promote the growth of the protein-rich sub-RPE deposits by binding proteins present in the sub-RPE space. Co-labelling for HAP with Bone-Tag 680 RD and antibodies for selected proteins revealed a very distinct staining for each of amyloid beta, vitronectin, and complement factor H on the outer surface of the HAP spherules (**Figure 2I,J,K,** respectively**)**. Triple labeling with amyloid beta (red), complement factor H (green) and HAP (magenta) shows that these proteins can bind to the same HAP spherule simultaneously, and in some surface areas their immunostaining is so near to each other that they show co-localisation (yellow) (**Figure 2L**). Not all HAP spherules are positive for the tested proteins (**Figure 2K and Supporting Information Figure S5**), possibly reflecting localised events, such as site-specific secretion or highly localised inflammatory reactions in a single cell. It is also interesting to note that while there is an enrichment of immunolabeling on the HAP surfaces for vitronectin (**Figure 2G**) this protein shows also a diffuse labeling around the spherules. Given that in these experiments we used whole mount immunolabeling and antibody penetration into all deposits equally could not be ascertained, quantification of the proportion of HAP spherules with the different antibodies was not feasible. Importantly, HAP spherules are present in some cases where no discernable protein deposit is present (as assessed by autofluorescence; **Figure 1G,H**), suggesting that HAP spherule formation may precede protein build-up and the consequent formation of this component of sub-RPE deposits. Moreover, the spherules described here differ overtly from the 100 nm matrix vesicles implicated in bone formation, which have distinct crystals of HAP enclosed within a lipid shell(35). It is also important to note that immunostaining for several sub-RPE deposit-associated proteins did not show a spherular staining pattern(36), and therefore it appears that there is a selectivity of protein binding to HAP.

To elucidate further the selective binding of proteins to HAP, ARPE-19 cells (a widely used cell model for RPE studies) were grown in a stable-isotope-labeled amino acid-containing culture medium. Secreted proteins were bound to externally added magnetic HAP-coated beads; the same beads without HAP coating were used as controls. Bound proteins were identified by quantitative mass spectrometry-based proteomic analysis. The proteins in this complex mixture exhibited selective binding to HAP (**Supporting Information Table T1**) that included previously identified sub-RPE deposit-associated proteins (labelled red in **Supporting Information Table T1**): Alpha crystalline B (gene: CRYAB), Amyloid-like protein 2 (APLP2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH); histone H2A.x (H2AFX), proteasome subunit beta type 6 (PSMB6), and vimentin (VIM). This experiment was not designed to exactly replicate the conditions in the aging retina in vivo, particularly since we did not culture cells until they were well differentiated, nor were the Bruch's membrane and choroid present. Therefore we did not expect to identify all sub-RPE-associated proteins in this mixture as many would be secreted in very small quantities from "unchallenged" RPE cells. Furthermore, many of the sub-RPE deposit-associated proteins originate from blood(7). This experiment was designed to show that

proteins have different affinities to HAP and that several proteins actually do not bind to the HAP surface under these conditions (**Supporting Information Table T2)**. Evidently, HAP can provide a surface for preferential binding and hence concentration of proteins, forming a scaffold for more extensive and less specific binding of proteins and can lead to sub-RPE deposit growth.

Based on these observations we propose a novel mechanism for the growth, and perhaps the initiation, of sub-RPE deposits with implications for the development of AMD depicted in **Figure 3**. Sub-RPE deposit growth, and maybe even formation, may be mediated, at least partly, by the formation of HAP shells on cholesterol-containing, naturally present extracellular lipid droplets at the RPE/choroid interface (**Figure 3**). This calcification process in drusen can be distinguished from that in atherosclerotic plaques(37, 38) where, instead ofextracellular lipid droplets, HAP is deposited on large cholesterol crystals. Whether the HAP deposition in the two diseases share similarities has yet to be determined, but it is interesting to note that there were suggestions that the two diseases might be related to each other (39). Furthermore, the micron sized extracellular lipid droplets in the core of the HAP spherules in sub-RPE deposits are likely to differ from the smaller (<200 nm) lipoprotein particles described by Curcio et al. (11), therefore HAP spherule formation underneath the RPE appears to be a novel phenomenon. Once the HAP-coated spherules are formed they have the capacity to bind different proteins and so provide nucleation sites for the growth of sub-RPE deposits by promoting further protein binding and lipid deposition and entrapment, leading to a self-driven oligomerisation process and the growth of sub-RPE deposits to clinically relevant size (**Figure 3**).
Mild to moderate calcification of the elastic layer of the Bruch's membrane is commonly observed in aging eyes(20) and can be a sign of accelerated aging(19). Needle-like calcifications, presumed but not proven to be HAP, were described in the Bruch's membrane in pseudoxanthoma elasticum(40); these however differ from the HAP spherules in location and morphology (Supplementary Figure S6). Classic freeze-fracture and scanning electron micrographs show calcium- and phosphorus-containing spherules similar in size to those shown in this study(41), but this has generally been interpreted as dibasic calcium phosphate and not as HAP, and was not associated with the binding of proteins and the entrapment of lipids. Therefore, HAP spherule formation on the surface of lipid droplets in the inner aspect of the Bruch's membrane is a newly recognized phenomenon that differs from previously described calcifications. Hydroxyapatite need not form as a result of a "maturing" process from previously deposited amorphous calcium phosphate: HAP can be directly formed in physiological concentrations of calcium and phosphate at physiological pH(42) and this appears to be the case for the sub-RPE space. We note that hydroxyapatite is much harder and less soluble than dibasic calcium phosphate, with the former only being re-solubilized by acid secretions in bone remodeling(43). Interestingly, calcified drusen are usually associated with end stage AMD (geographic atrophy) (44). It is known that drusen resolve as GA develops and it is possible that the calcific material represents HAP spherules that are resistant to clearing during this process. Sub-RPE deposit formation in the macula is the hallmark of AMD, but deposits are also present in the aging eye without recognized AMD, especially in the peripheral retina(18). Based on post mortem grading for AMD(45) we examined several eyes with sub-RPE deposits in the macula, and all these contained numerous HAP spherules. However a precise correlation with clinical characterization of drusen, such as hard and soft, could not be ascertained by examination of non-fixed samples. Therefore, understanding how the HAP shells form on the ubiquitous cholesterol-containing droplets (9, 10) and how particular proteins bind to these surfaces and initiate the formation of sub-RPE deposits, will be key to improving our understanding of forms of AMD in which this process is important, and may lead to novel early intervention strategies, perhaps before sight-threatening conditions develop.

Methods

HAP Fluorescent Labeling and Immunohistochemistry of Deposit Proteins

Thirty human eyes of donor ages between 43 and 96 years were obtained from the Moorfields Eye Hospital Eye Depository and were post mortem phenotyped based on the Alabama grading for post mortem tissues (45). Samples were obtained within 24 h of death. Full Local Research Ethics Committee approval and appropriate consent were obtained in each case. The protocol of the study adhered to the tenets of the Declaration of Helsinki regarding research involving human tissue. Flat mounts of Bruch's membrane/choroid were prepared, after removal of the neurosensory retina and the RPE cells as described previously (27). It is important to note that none of the samples were fixed for these experiments to avoid post-mortem chemical interference. Of these samples, six had extensive sub-RPE deposits resembling soft drusen or basal linear/laminar deposits when examined under white light (Nikon LMZ 1500 stereo microscope), a further 10 had numerous focal deposits (hard drusen) in the macula, while all 30 had manifested peripheral, mainly focal, sub-RPE deposits (in Table 1 (*) indicates the presence of hard drusen while (***) indicates extensive focal and/or large diffuse sub-RPE deposits). Basal linear and laminar deposits could not be differentiated on whole mounts and, given the nature of the experiments and the incompatibilities of methodologies for resampling, unambiguous identification of the subtypes of sub-RPE deposits using electron microscopy was not possible(27). In some experiments fixed, paraffin-embedded samples were obtained from the Pathology Archive at the UCL Institute of Ophthalmology. Based on pathology reports describing the presence of different type of sub-RPE deposits in the macula or at the peripheral retina, suitable eyes were selected and 7 um sections were stained for HAP.
Flat mounts were stained with 1 mg/ml Alizarin Red S (Sigma-Aldrich; Excitation wavelength: 532 nm, Emission wavelength: 620 nm), 20 µM Bone-Tag 680RD (Li-Cor, Lincoln, Nebraska; Ex: 620 nm, Em: 680 nm), 1 mg/ml tetracycline (Sigma-Aldrich; Ex: 405 nm, Em: 570 nm) or 1mg/ml Xylenol Orange (Sigma-Aldrich; Ex: 532 nm Em:570 nm) all in aqueous buffer for 20 minutes at room temperature. Nile Red (Invitrogen; Ex: 532 nm, Em: 620 nm) was first dissolved in acetone and then diluted in aqueous buffer to 3.5 mg/ml and tissues were incubated for 20 minutes at room temperature.
Immunohistochemistry was also performed on unfixed flat mounted Bruch's membrane/choroid tissue, obtained as above. Flat mounts were blocked with goat serum, then incubated with primary and secondary antibodies for 2 hours each at 30 °C. The primary antibodies used were anti-complement factor H (Santa Cruz; 1/100 dilution), anti-vitronectin (AbDSerotec, 1/200 dilution) and anti-amyloid β (6E10, Covance, 1/100 dilution). Alexa-Fluor 488 Goat anti-rabbit and Alexa-Fluor 568 Goat anti rat secondary antibodies were from Invitrogen and used in 1/1000 dilution. Samples were imaged using a Zeiss LSM700 confocal microscope through a 63x/1.2 NA Zeiss Neofluar objective.

Mapping of Drusen Constituents by TOF-SIMS Imaging

Flat mounts on glass slides were transported and stored at -20°C, then immediately prior to analysis were placed in a freeze dryer for four hours to ensure the samples were compatible with the ultra-high vacuum conditions of the SIMS instruments. In order to expose the internal structure of drusen, a focused ion beam (FIB) milling approach was implemented using an FEI FIB200-SIMS. Following gold sputter coating to ensure a conducting surface, the glass slide was mounted on the sample stage. Using an increased ion beam current, the upper 1µm was removed sequentially until the required region of the drusen was reached (Figure 2).

For mass spectrometry imaging at sub-micron resolution over m/z 0-880, the slide was immediately transferred to a TOF.SIMS 5 (ION-TOF, Münster, Germany) secondary ion mass spectrometer. The system comprises a bismuth primary ion beam, operating at 25 kV and tuned to use the Bi_3^+ cluster for greater secondary ion yield, and a low energy electron flood gun for charge compensation. Ionic species sputtered from the surface under the bismuth bombardment are steered into a reflectron time-of-flight mass analyser.

All data analysis and visualization was performed using in-house written MATLAB functions. Non-negative matrix factorization was performed on the data in order to reduce its dimensionality into five chemically distinct factors. Peaks identified as strongly localized to the spherules were identified from the factors and single ion images produced (Figure 2). The overall protein signature was based on summing a combination of characteristic immonium ions; the phosphatidylcholine (PC) distribution was visualized by the PC head-group peak at m/z 184.07; and cholesterol by its $[M+H-H_2O]^+$ion at m/z 369.38.

Synchrotron µX-Ray DiffractionAnalysis of Drusen

Synchrotron micro-focused X-ray diffraction analyses (µXRD) of drusen were conducted at beamline X26A, National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY USA. Tissue samples containing drusen were flat mounted on 4µm thick Ultralene® film for analysis. Monochromatic X-rays were used tuned to an incident wavelength of 0.70926 Å, corresponding to 17.481 keV energy, using a channel-cut Si(111) monochromator crystal. The incident beam was focused to a spot size of 9 (H) x 5 (V) µm on the sample using Rh-coated silicon mirrors in a Kirkpatrick-Baez geometry. The X-ray diffraction from the sample was measured using a Rayonix SX165 CCD area detector. Calibrations and corrections for detector distortions (camera–sample distance, camera tilt and rotation, and the beam center on the camera plane) were done using Fit2D™ software (46) and corrected using NIST SRM 674a corundum standard and silver behenate. The 2D area detector data was integrated in to 2□-intensity using Fit2D and HAP was then identified by comparison with reference powder-diffraction patterns (ICDD 2003) using the Match software (Crystal Impact).

Identification of HAP-Bound Proteins Secreted from Cultured RPE Cells by SILAC

Cell culture For stable isotope labeling with amino acids in cell culture (SILAC) experiments, ARPE-19 cells were grown in SILAC DMEM:F12 medium (PAA) supplemented with 10% dialyzed fetal bovine serum (PAA), 1% (v/v) penicillin-streptomycin and $^{12}C_6,^{14}N_2$ lysine plus $^{12}C_6,^{14}N_4$ arginine (light medium) or $^{13}C_6$ lysine plus $^{13}C_6,^{15}N_4$ arginine (heavy medium). 0.5 mM proline was added to all SILAC media to prevent arginine-to-proline conversion(47). All amino acids were purchased from Silantes. Sub-confluent cultures were thoroughly washed with PBS and kept in serum-free medium for 24 hours. Label-swap replication was used for enhanced reliability in affinity ratios. Equal amounts of "heavy" supernatants were incubated either with BcMag™ hydroxyapatite-coated magnetic silica beads (Bioclone, San Diego) or with unmodified silica beads (negative control). "Light" supernatants were processed in the same way. After incubating three hours on a rotator at 25°C, bound proteins were washed with serum/free medium (2X 15 min). Finally, the beads were re-suspended in 30 µl of 50 mmol/L ammonium bicarbonate (ABC) and subjected to on-bead tryptic digestion. The corresponding samples

(eluates from light-labeled HAP and heavy-labeled control beads, and vice versa) were mixed as described earlier(48) before being subjected to LC-MS/MS.

Mass spectrometry and data analysis LC-MS/MS analysis was performed on an Ultimate3000 nano HPLC system (Dionex) coupled to a LTQ OrbitrapXL mass spectrometer (Thermo Fisher Scientific, Waltham, USA) by a nano spray ion source. For SILAC experiments, all acquired spectra were processed and analyzed using MaxQuant version 1.3.0.5 (*www.maxquant.org*) and the human-specific SwissProt (http://www.maxquant.org/) in combination with Mascot (version 2.2; Matrix Science). Cysteine carbamidomethylation was selected as fixed modification; methionine oxidation and protein acetylation were allowed as variable modifications. The peptide and protein false discovery rates were set to 1%. Contaminants like keratins were removed. Proteins identified and quantified by at least 2 peptides per experiment were considered for further analysis. Each experiment consisted of a forward and a reverse labeling approach (label swapping) to exclude label-specific effects. A P value of 0.001 was selected as threshold for significant enrichment or alteration.

Study approval.

Thirty human eyes with sub-RPE deposits were obtained from the Moorfields Eye Hospital Eye Depositorywithin24 h of death. Full Local Research Ethics Committee approval and appropriate consent was obtained in each case. The protocol of the study adhered to the tenets of the Declaration of Helsinki regarding research involving human tissue.

Acknowledgments:

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Figure 1:

Hydroxyapatite (HAP) is present in sub-RPE deposits as spherular structures. X-ray diffraction pattern (A) and radial intensity profile (B) confirmed the presence of hydroxyapatite in isolated sub-RPE deposits. Red lines in B indicate the position of diffraction peaks for pure HAP powder. Deposits contain numerous HAP spherules(magenta, stained with Bone Tag 680RD) in sub-RPE deposits(C-F).Optical sectioning using confocal microscopy revealed that HAP forms spherules throughout macular large drusen (D) as well as in hard drusen (E and F), but were also seen in the absence of the typical autofluorescence (green) of sub-RPE deposits and Bruch's membrane (G and H).

Figure 2:

Identification of molecular constituents of the inner core and outer surface of HAP spherules by TOF-SIMS. Secondary electron (A) and secondary ion (B) images of the inner structures after FIB millingof a deposit (E).The non-negative matrix factorisation score plot demonstrates chemically distinct spherules (F) inside the deposit. Positive ion SIMS images confirmed the selective enrichment of calcium phosphate (C) and cholesterol (D), but not phosphatidylcholine head-group (G) or combined signals from protein fragments (H) associated with the HAP spherules. Immunohistochemical fluorescent labeling with protein-specific antibodies revealed that HAP spherules (magenta on fig I-L) were found coated with amyloid beta (I, red), vitronectin (J, yellow), complement factor H (K, green), or co-labeled by a combination of these proteins (L, red:amyloid beta; green:complement factor H). Not all the spherules are labelled by the antibodiesused (L and K) indicating diverse protein binding to HAP spherules. Scale bars are10 µm on panels A-H, and 2 µm on panels I-L.

Figure3:

A simplified diagram of the proposed mechanism for the growth of sub-RPE deposit. Deposit growth is associated with, maybe even initiated by, the precipitation of HAP (magenta) onto the naturally present, micron sized cholesterol-containing extracellular lipid droplets (black). Consequently different proteins (blue) bind to the HAP surface, which facilitates further deposition in a self-driven oligomerisation process, leading ultimately to the formation of the macroscopic sub-RPE deposits (yellow). Melanocytes are depicted as brown particles in the RPE.