The Lipid Composition of Drusen, Bruch's Membrane, and Sclera by Hot Stage Polarizing Light Microscopy

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Purpose. To detect and identify, in situ, the lipid composition of drusen, diffuse Bruch's membrane deposits, and sclera in aging human eyes using hot-stage polarizing microscopy (HSPM), a method that allows qualitative determination of lipid subtypes within histologic sections based on morphology and melting temperatures of liquid crystals as monitored by birefringence during heating and cooling.

Methods. Full-thickness buttons of the central macula and the periphery of human eyes from 17 patients were fixed in 5% calcium-buffered formalin. Frozen sections were stained with oil red O or Sudan black or were analyzed by HSPM.

Results. Birefringent anisotropic droplets ("maltese crosses") with melting characteristics of cholesterol esters were identified within diffuse Bruch's membrane deposits, drusen, and sclera. Deposits that melted from crystal to oil without any maltese cross formation when cooled were present in the sclera and are consistent with triglyceride-rich deposits. Deposits with optical properties consistent with phospholipids were identified in a single aged eye. Eyes from young donors did not show these changes.

Conclusions. HSPM is a valuable technique for evaluating the nature of lipid deposits in aging eyes. Further studies are warranted to determine whether similar changes are also present in eyes with age-related macular degeneration. (*Invest Ophthalmol Vis Sci.* 2001;42:1592–1599)

Drusen and Bruch's membrane alterations occur in aging human eyes. Histochemical and biochemical studies of age-related changes in drusen and Bruch's membrane have demonstrated the accumulation of lipids,[1–6] proteins,[7,8] and sugar-containing compounds that could represent glycolipids, glycoproteins, or proteoglycans.[9–11] Despite the many different types of compounds identified within drusen (see recent reviews),[12,13] the composition of these deposits in normal aging eyes remains incompletely understood. There are relatively few studies that investigate the lipid composition of drusen and Bruch's membrane changes, and the results of these studies vary. Early studies showed that in frozen sections, drusen demonstrate birefringence under polarized light and react with histochemical stains for all classes of lipids and neutral lipids in particular.[9] Progressive age-related accumulation of lipids in Bruch's membrane has been demonstrated using histochemical lipid stains with some eyes staining for neutral lipids, other eyes staining for phospholipids, and yet others staining for both.[1] The ratio of phospholipids to neutral lipids by thin-layer and gas chromatography correlates well with these differences, because eyes that stain for neutral lipids but not phospholipids by histochemical studies also have a lower ratio of phospholipids to neutral lipids by chromatography.[4] Other studies using chromatography have demonstrated a ratio of neutral lipids to phospholipids in Bruch's membrane and choroid almost three times higher than in the retina[14] or have reported a composition consisting largely of phospholipids, triglycerides, fatty acids, and free cholesterol but little cholesterol ester.[3] In these chromatographic studies,[3,4,14] Bruch's membrane and choroid were treated as a single tissue and, at least in one study, the RPE could not be cleanly separated from the underlying Bruch's membrane with the microdissection techniques used.[14] This makes it difficult to assign the precise localization of the extracted lipids.

Because of these variable findings, we wanted to further investigate the lipids that accumulate within drusen, Bruch's membrane, and sclera in aging human eyes and add information regarding the relative distribution of lipids throughout Bruch's membrane and drusen. This study overcomes some of the limitations of previous studies in evaluating lipid composition by using a modified polarizing light microscope in which the frozen sections are progressively heated and the thermal behavior and crystal morphology are used to identify the nature of the lipids.[15] In this way, choroidal contamination, a byproduct of microdissection,[3,4,6,14] is avoided. Using this technique, lipids can be identified by their crystal and liquid crystal morphology, melting temperature, reformation temperature, and their ability to remain in a liquid state below the temperature at which they crystallize (undercooling). Table 1[15–20] summarizes how lipids can be identified using hot-stage polarizing microscopy (HSPM) and how the melting temperatures of lipids can be correlated with fatty acid classes (saturated and unsaturated).

Cholesterol esters are a major type of nonpolar lipid that is deposited normally in such tissues as the adrenal gland and gonadal tissues and as abnormal deposits in atherosclerosis and xanthomatosis.[15] The deposits are generally intracellular cytoplasmic droplets but in disease states may form extracellular deposits.[15,20] Cholesterol ester crystals form when lipid-rich tissues are chilled. However, in native tissue at body temperature, the cholesterol esters are not present as crystals.[15,20–22] Crystals form as an artifact during the cold storage of the tissues. To return the cholesterol esters to the original state present in tissues at 37°C, a frozen sample is first heated above the crystalline melting point and then cooled back to 37°C, and observations are made under the polarizing microscope. When the tissue is cooled, at a certain temperature a liquid crystalline structure called the smectic liquid crystalline state is formed, which is identified by maltese crosses between crossed polarizers. These liquid crystals in biological systems are generally stable and do not crystallize back to the crystalline form, unless cooled to low temperatures[15,20] of approximately 10°C or less or left for long periods at room temperature.[21] The melting temperature of the maltese cross to clear isotropic fluid transition depends on the fatty acids esterified to the cholesterol

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TABLE 1. Identification of Tissue Lipids by HSPM

Lipid	Initial State at Room Temperature*	Lipid State (Crystal, Liquid Crystal, Liquid) after First Heating (Melt I) and Cooling (Cool II) to 8°C	Thermal Behavior after Second Heating
Cholesterol esters[16]	Polyunsaturated, such as cholesteryl linoleate (18:2) or linolenate (18:3); needle-shaped crystals	Liquid crystals with maltese cross pattern 	Melt to liquid (20–35°C)
	Monounsaturated, such as cholesteryl oleate (18:1) or nervonate (24:1); needle-shaped crystals	Maltese crosses	Melt to liquid (42–52°C)
	Saturated such as cholesteryl stearate (18:0) or palmitate (16:0); needle-shaped crystals	Maltese crosses, but rapidly crystallize to needles	Melt >70°C
Phospholipids and birefringent complex membrane lipids[17]	Lamellar liquid crystals; myelin figures (appears as birefringent trolley tracks)	Same 	Melt >85°C
Triglycerides[15,18]	Short needle-shaped crystals	Short needles (recrystallization occurs 20–30°C below melting point) 	Melt over broad range, depending on fatty acid composition. The more unsaturated the fatty acids, the lower the melting point.
Cholesterol monohydrate[19,20]	Parallelogram plates with edge angle of 79°C	Same 	Melts >85°C

* These tissues were stored at −70°C and kept below −10°C until sections were warmed to room temperature. Under these conditions crystals are likely to be present.

(Table 1). In polyunsaturated cholesterol esters such as cholesteryl linoleate (18:2) or linolenate (18:3), which contain two or three double bonds, respectively, the liquid crystals melt at temperatures generally below body temperatures (20–35°C). When the cholesterol esters are monounsaturated such as cholesteryl oleate (18:1) or cholesteryl nervonate (24:1), the liquid crystal maltese cross melting temperatures are higher, ranging between 42°C and 52°C. Saturated cholesterol esters such as cholesteryl stearate (18:0) or palmitate (16:0) melt at very high temperatures (>70°C).[16] However, most all biological tissues contain mixtures of these different esters, and each droplet may have a mixture of several cholesterol esters. Nevertheless, the melting point of the droplet tends to reflect the major type of ester within the droplet—that is, polyunsaturated or monounsaturated. No biological tissues have been found yet that have highly saturated cholesterol esters existing by themselves, and therefore, esters with high melting points have not been found in tissues.[15,20]

Phospholipids and complex membrane lipids are often present in pathologic deposits as multilamellar bodies or myelin figures that, when viewed by polarizing light microscopy, resemble birefringent trolley tracks.[17,20] They have very high melting temperatures, generally higher than 85°C. Triglycerides, a group of common storage fats, form short needles when frozen. These needles melt into an isotropic oil at the melting point, but when they are cooled, unlike cholesterol esters, they do not form a liquid crystalline phase (maltese crosses), but rather undercool 20°C to 30°C before recrystallizing into needles. The presence of significant amounts of triglycerides in the same droplet with cholesterol esters—that is, more than 25% triglyceride, prevents the maltese crosses from forming. Therefore, droplets that do not form maltese crosses when heated and subsequently cooled are either pure triglyceride or cholesterol ester droplets that are highly contaminated with triglycerides. We call these triglyceride-rich droplets. Free cholesterol can be present in pathologic tissue as cholesterol monohydrate crystals.[20] These are highly prevalent in atherosclerotic plaques, in gallstones, and in xanthomata. These crystals are present in uncooled tissue, and freezing does not affect them. They are characteristic plates with a 79° edge angle and melt at approximately 85°C.[19] This is the first study to analyze the nature of the lipid deposits in drusen, Bruch's membrane, and sclera using the HSPM technique with a light microscope.

METHODS

Seventeen pairs of eyes, obtained immediately after the death of the patients, were studied (National Disease Research Interchange, Philadelphia, PA). The ages of the patients ranged from 32 weeks to 92 years and are presented in Table 2. The patients had no known history of ocular disease, and gross examination of the posterior segments were normal. Pairs of whole globes or posterior poles were fixed in 5% calcium-buffered formalin for 24 to 72 hours and then rinsed twice in phosphate-buffered saline (PBS). A 6-mm corneal punch was used to obtain a full-thickness button of the macular area (central section). A second full-thickness button was obtained inferior to the vascular arcade (peripheral section). The buttons were infiltrated with 10% glycerol solution and cryopreserved in the liquid phase of partially solidified monochlorodifluoromethane gas. The buttons were stored at −70°C until sectioning. This sequence was repeated for the fellow eye of each case. Ten-micrometer cryostat sections were placed on precleaned slides (Superfrost plus; VWR Scientific, West Chester, PA).

For laminin immunohistochemistry (performed to verify the relationship of the birefringent deposits to Bruch's membrane[23]) the 10-μm sections were air dried, washed in PBS, and incubated in sodium borohydrate (0.1%) for 30 minutes. After a rinse in PBS and incubation at room temperature in 4% nonfat milk for 20 minutes, the sections were incubated for 1 hour at 37°C with anti-laminin antibody 1:25 (Sigma, St. Louis, MO). After a PBS wash, the sections were incubated with a 1:100 dilution of biotinylated horse anti-mouse IgG (heavy- and light-chain; Vector Laboratories, Burlingame, CA). After three PBS washes, the sections were incubated at room temperature for 1 hour in avidin-biotin, washed in PBS, developed in 3-amino-9-ethylcarbazole (AEC), and counterstained in hematoxylin.

Microscopy was performed on a Leitz-Dialux microscope fitted with a polarizer, analyzer, and heating–cooling stage (Leitz/Wild, Bur-

Table 2. Birefringence and Staining of Lipids in Bruch's Membrane, Drusen, and Sclera in Human Eyes

		Birefringence*†			Lipid Staining*‡					
					Bruch's Membrane		Drusen		Sclera	
Patient	Age/Sex§	Bruch's Membrane	Drusen	Sclera	Sudan Black	Oil Red O	Sudan Black	Oil Red O	Sudan Black	Oil Red O
1	32 wk/M	−	ND	−	0	0	ND	ND	0	0
2	40 wk/M	−	ND	−	0	0	ND	ND	0	0
3	5/F	−	ND	−	0	0	ND	ND	0	0
4	17/F	−	ND	NS	0	0	ND	ND	NS	NS
5	19/M	−	ND	NS	0	0	ND	ND	NS	NS
6	55/M	+	+	+	0–3+	0–3+	ND	2–3+	0–1+	0–3+
7	60/M	+	ND	−	0	0	ND	ND	0	0
8	62/M	+	ND	−	0–2+	1–4+	ND	ND	0–1+	1–2+
9	65/M	+	+	+	1–2+	0–1+	ND	ND	0–2+	0–2+
10	66/M	+	+	+	0–2+	0	3+	ND	0–1+	0–3+
11	70/M	+	ND	+	0–3+	0–4+	2–4+	0–1+	0–2+	0–2+
12	72/F	+	ND	+	0–3+	1–4+	2–3+	3–4+	1–4+	1–4+
13	75/M (1)	+	+	−	0–1+	0–3+	2+	3+	0	0–2+
14	75/M (2)	+	+	+	0–2+	2–3+	2+	ND	0–2+	0–3+
15	84/M	+	ND	−	0–3+	0–3+	ND	3+	0–1+	0–2+
16	84/F	+	+	+	0–3+	2–4+	2–4+	3–4+	1–3+	2–4+
17	92/M	+	+	+	1–3+	0–2+	3–4+	0–3+	0–3+	0–3+

* Data from central and peripheral disks of right and left eyes of each patient were combined. ND indicates that no drusen were detected in available sections. NS indicates sclera was not present in available sections.

† Birefringence in unstained sections under polarized light was recorded as present (+) or absent (−).

‡ Stain intensities were rated: none (0), low (1+), medium (2+), high (3+), and very high (4+). Two observers rated sections independently.

§ Age in years, unless otherwise noted.

lington, MA). Cross-sectional measurements (height and diameter) of drusen were made using a reticule with indexed squares mounted in an ocular insert. Photomicrographs were taken with a camera system (Microflex UFX; Nikon, Inc., Garden City, NY) using 35-mm film (Ektachrome, ASA 200; Eastman Kodak, Rochester, NY). Exposure times were standardized to aid the comparison of birefringence intensities between images. Sections used for HSPM were mounted unstained with a drop of glycerol and a glass coverslip and heated or cooled on the stage at the rate of 1°C to 2°C per minute while the tissue was constantly observed under polarized light for changes in birefringence of crystalline or liquid crystalline lipids.[15,24] Monitoring the appearance and disappearance of birefringent crystals throughout a 10-μm thick cryosection was aided by fine focal adjustments of the microscope.

Photomicrographs were taken before and after each cooling and heating run and during each run when significant changes in birefringence occurred. The temperature at which any changes occurred was also recorded. Some of the birefringent crystals in photomicrographs, necessarily taken at a fixed focal plane, were out of focus. After the birefringent patterns in the sections were observed initially and photographed at room temperature, the sections were heated to 60°C or until all birefringence had disappeared (melt I). Then, as the slide was cooled to 8°C (cool I), the temperature at which maltese crosses (liquid crystals) or other birefringent patterns began to appear (indicative of lipid recrystallization) was recorded. The slide was again heated to 60°C (melt II), and the temperature at which all maltese crosses had melted and all other birefringence disappeared was noted. The mean value of the temperatures at which the maltese crosses began to appear during cooling (cool I) and completely disappeared during heating (melt II) provided a determination of the highest melting temperature of the entire liquid crystalline droplet population. In most eyes, the birefringence of the liquid crystalline droplets disappeared over a range of temperatures. The melting temperatures of the droplets from each eye were recorded separately but were combined for clarity, because no significant differences were identified between the eyes.

Lipid staining with Sudan black B (for all lipids) and oil red O (for neutral lipids) was performed on sections closely adjacent to unstained sections studied by HSPM.[1] Some of the stained sections contained different cross-sections of the same drusen that were seen in unstained sections. Staining intensities were rated as 0, 1+, 2+, 3+, and 4+ to indicate no staining or low, medium, high, and very high staining intensities, respectively. Frequently, a wide range of staining intensities along the Bruch's membrane or across the scleral layer was noted within the same section. For example, an intensity range of 0 to 3+ indicated that some areas had no staining, whereas others had low, medium, or high staining. Two observers independently rated the stained sections, and in the few instances in which the ratings were dissimilar, the results were combined. Statistical analyses of the melting temperatures of birefringent deposits in Bruch's membrane, drusen, and sclera of all the eyes that had birefringent deposits were performed using a pooled variance *t*-test by computer (RSI software, ver. 4.3.1; BBM Software Products, Cambridge, MA). Populations were unpaired with normal distributions and equal variances. This study was approved by the human studies committee of the Boston University School of Medicine.

Results

Oil Red O and Sudan Black Staining of Bruch's Membrane, Drusen, and Sclera

The lipid staining results are presented in Table 2. These stains documented the presence of extracellular lipid deposits in nearly all eyes studied, with the exception of those 19 years of age or younger (cases 1–5). The RPE generally stained darkly with Sudan black and was lightly stained or unstained with oil red O. Bruch's membrane in aged eyes was almost always thickened compared with that in young eyes. Thickened Bruch's membrane frequently stained with Sudan black and consistently stained with oil red O. There was good correlation between the areas of positive neutral lipid staining as characterized by oil red O and birefringence of Bruch's membrane by cross-polarized light (Figs. 1A, 1B). In some eyes, sections stained positively for lipids, whereas no drusen were detected in nearly adjacent unstained sections (cases 11, 12, and 15).

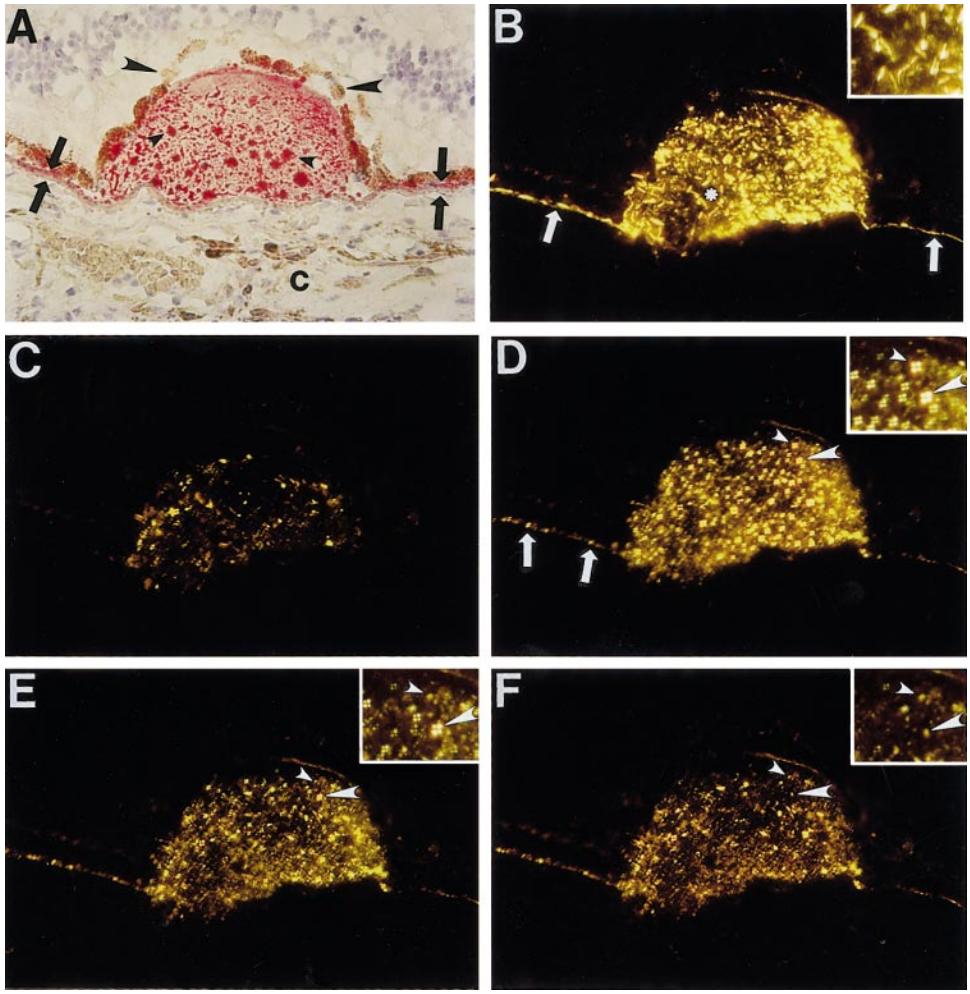

FIGURE 1. (**A**) A large druse shows positive oil red O staining. On each side of the druse is slightly thickened Bruch's membrane with oil red O staining on the inner portion (*opposing arrows*). There are intensely stained inclusions within the druse (*small arrowheads*). The RPE is disrupted above the druse (*large arrowheads*). c, choroid. (**B**) An adjacent unstained section viewed by polarized light at 21°C before heating shows a birefringent linear band in Bruch's membrane that corresponds to the oil red O stained band in (**A**). The band is intermittent at the *left* of the druse (*arrow*) and more continuous on the *right* (*arrow*). Birefringent needles are seen throughout the druse. The region with an *asterisk* has been enlarged (*inset*). (**C**) There was a marked reduction in birefringence in the druse and Bruch's membrane as the slide was progressively heated (melt I). (**D**) With progressive cooling (cool I), there was a return of focal regions of birefringence within Bruch's membrane (*arrows*), some of which were very small maltese crosses. Many maltese crosses are visible in the druse at 10°C (*arrowheads*, *inset*). The maltese crosses are characteristic of liquid crystals of cholesterol ester.[15] (**E**) As the section was progressively heated (melt II), the maltese crosses gradually disappeared. At 22°C some maltese crosses had disappeared (*small arrowhead*) and others remained (*large arrowhead*). (**F**) By 50°C (melt II), most maltese crosses had disappeared (*arrowheads*). There was a marked loss of birefringence compared with (**D**). Magnification, ×230; insets ×460.

Drusen were stained gray by Sudan black and pink by oil red O and sometimes contained intensely stained inclusions (Fig. 1A). When these stained inclusions were viewed under polarized light, some appeared slightly birefringent, suggesting that portions might be crystalline. Alternatively, another unstained birefringent crystal and the stained inclusion might be superimposed within the 10-μm-thick section. The intensities of Sudan black and oil red O staining of both the linear Bruch's membrane band and drusen were variable even within individual sections (Table 2). Scleral staining with oil red O and Sudan black (see Fig. 3A) was also variable, with some eyes showing marked staining and others minimal or no staining. This variability was also seen from one area to another within a given eye. In a few eyes, sections of sclera stained positively for lipids, whereas no birefringence was observed in nearly adjacent sections (cases 8, 13, and 15).

HSPM of Bruch's Membrane

A linear band of birefringence was identified by polarizing light microscopy from the region of Bruch's membrane in 23 of 24 eyes of the 12 patients older than 20 years (Table 2). In three (cases 7, 8, and 15; Table 2), Bruch's membrane birefringence without either drusen or scleral birefringence was observed. Linear band birefringence was seen more frequently in central sections than in peripheral sections. Indirect immunoperoxidase staining with antibodies to laminin confirmed that the birefringent band corresponded to Bruch's membrane (data

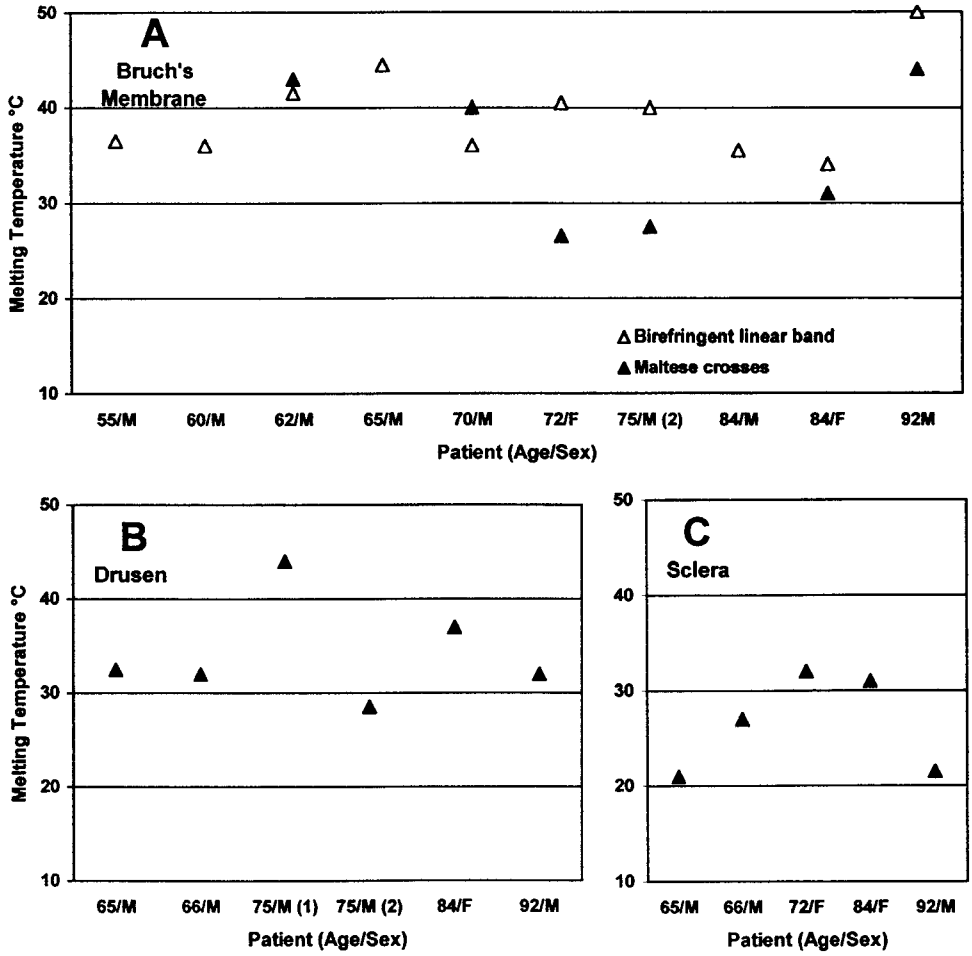

FIGURE 2. Melting temperatures of cholesterol esters in Bruch's membrane (**A**), drusen (**B**), and sclera (**C**) of aged eyes. Each plotted temperature indicates the highest temperature at which all linear band or maltese cross birefringence disappeared and represents the average of the sections studied in each pair of eyes. (**A**) (△) Birefringent thin linear band seen in Bruch's membrane; (▲) maltese crosses of Bruch's membrane found in the region of the linear band. Mean melting temperatures of the linear band and of maltese crosses within the linear band were 39.5 ± 4.9°C and 35.3 ± 7.9°C, respectively. (**B**) Melting temperatures of maltese crosses in drusen. The mean melting temperature of all these eyes was 34.3 ± 5.5°C. A small druse from the patient in case 6 showed low intensity birefringence (Table 2), but HSPM was not performed. (**C**) Melting temperatures of maltese crosses in sclera. The mean melting temperature of all these eyes was 26.5 ± 5.1°C .

not presented). Bruch's membrane birefringence occurred as a discontinuous thin band at the inner aspect of Bruch's membrane or at the RPE–Bruch's membrane interface and frequently as a continuous band (Fig. 1B). The birefringent band in Bruch's membrane disappeared after heating (Fig. 1C) and partially reappeared after cooling, partly as maltese crosses and partly as a linear band (Fig. 1D). There was a gradual disappearance of maltese cross figures, and the linear band within Bruch's membrane as the slide was again progressively heated (Figs. 1E, 1F). We therefore assumed that these linear deposits were liquid crystals similar to the adjacent maltese crosses. Melting temperatures of the linear deposit and maltese crosses within Bruch's membrane are presented in Figure 2A. The melting temperatures of the linear deposit ranged from 34.0°C to 50.0°C (mean, 39.5 ± 4.9°C). The melting temperatures of the maltese crosses within Bruch's membrane ranged from 26.5°C to 44.0°C (mean, 35.3 ± 7.9°C). There was no significant difference between the melting temperatures of the linear deposits and the maltese crosses in Bruch's membrane. These melting temperatures indicate that the main cholesterol esters present in Bruch's membrane were poly or monounsaturated.

HSPM of Drusen

Drusen were identified in 10 eyes and were often multiple. In several eyes, birefringent drusen were observed, but no drusen were detected in some or all nearly adjacent sections (cases 6, 9, 10, and 14). A total of 17 individual drusen were studied among these eyes. The cross-sectional sizes of these drusen ranged from 5 × 10 μm to 35 × 125 μm (size variability was determined in part by the position of the section cut). The largest cross-sections observed in central and peripheral regions were 35 × 125 μm (case 16) and 25 × 125 μm (case 13), respectively (Table 2). One other eye had drusen that were greater than 63 μm, a size threshold for grade 1 histopathologic ARMD,[25] in at least one dimension (20 × 75 μm; case 6). Fourteen of 17 drusen studied by HSPM showed maltese cross formation. Under polarized light, these drusen before heating contained birefringent needles that became maltese crosses after a cycle of melting and cooling (Figs. 1B–D). The summary of melting temperatures of maltese crosses within drusen are presented in Figure 2B. The temperatures ranged from 28.5°C to 44.0°C (mean, 34.3 ± 5.5°C) and were indicative of polyunsaturated and monosaturated cholesterol esters. When central and peripheral drusen in the same eye were compared, no significant differences were identified in the melting temperatures. In addition to maltese crosses, we identified a region at the margin of a drusen in one eye (case 16) with a myelin figure pattern of birefringence (see Table 1) and a higher melting point, suggesting phospholipids or other lipids such as cerebrosides that form multilamellar bilayers. The mean melting temperature of maltese crosses in drusen was similar to that of the maltese crosses in Bruch's membrane but was significantly lower ($P < 0.05$) than the mean melting temperature of the linear deposit in Bruch's membrane.

HSPM of Sclera

Scleral birefringence was identified in 8 of the 17 cases (Table 2) and appeared in central and peripheral sections in similar frequency. HSPM was performed on eyes from six of the eight donors. In one of the six cases, there was no maltese cross

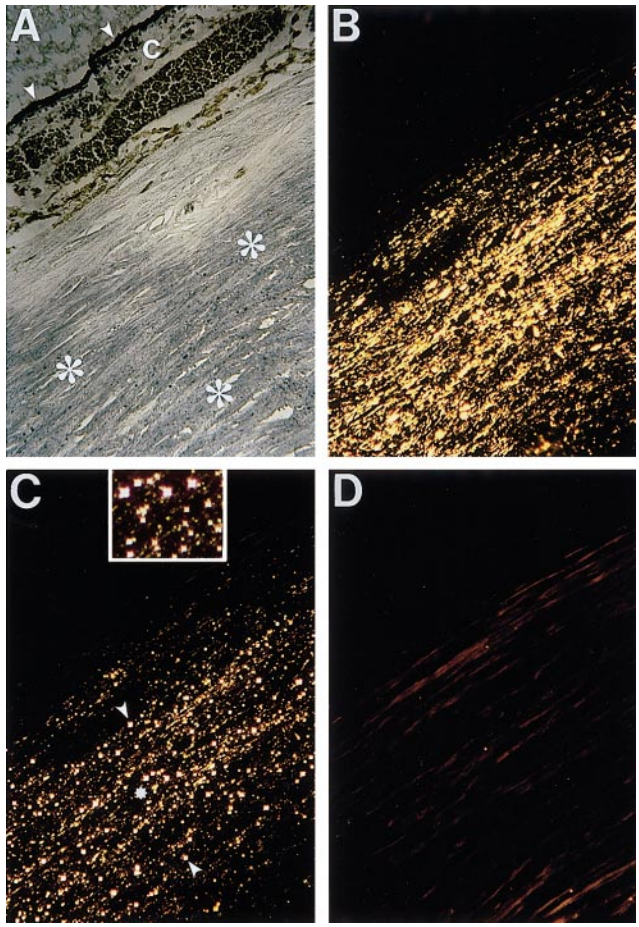

FIGURE 3. (**A**) Section demonstrating Sudan black staining of the RPE (*arrowheads*), choroid (c) and outer sclera (✻). (**B**) Adjacent unstained section under cross-polarized light showing marked crystalline birefringence before the initial melt at 22°C, which corresponds to the pattern of Sudan black staining in the outer sclera. (**C**) The section in (**B**) was first heated to 50°C, at which temperature all birefringence disappeared. The section was then slowly cooled. By 16°C many birefringent cholesterol ester droplets in the form of maltese crosses had appeared (*arrowheads*). The region with an *asterisk* (✻) is shown in the *inset*. (**D**) When the section was progressively heated to 46°C, the maltese crosses disappeared. Magnification, (**A–D**) ×92; (**C**, *inset*) ×184.

formation on cooling. No scleral birefringence was present in eyes age 19 or younger. Unstained frozen sections of sclera, such as the one illustrated in Figures 3B—D, which was nearly adjacent to a section stained with Sudan black (Fig. 3A), demonstrated birefringent needle-shaped crystals before heating (Fig. 3B). As the section was heated to 50°C, all birefringence disappeared. The section was slowly cooled, leading to a return of birefringence, mostly in the form of maltese crosses (Fig. 3C). When the section was again progressively heated, the maltese crosses disappeared (Fig. 3D). Figure 2C summarizes the melting temperatures of the maltese crosses within the sclera. The melting temperatures were somewhat variable, ranging from 21.0°C to 32.0°C (mean, 26.5 ± 5.1°C). These temperatures indicate the presence of mainly polyunsaturated cholesterol esters. In addition, small amounts of triglyceride were identified in eyes from five patients (cases 9, 10, 12, 14, and 17) and were observed in equal frequency in central and peripheral sections. In these patients, some birefringent needles melted to an isotropic oil, did not form maltese crosses when cooled, and recrystallized into short needles when undercooled 20°C to 30°C. The mean melting temperature of maltese crosses in the sclera was significantly lower than both the mean melting temperatures of the linear band in Bruch's membrane ($P < 0.01$) and the maltese crosses in drusen ($P < 0.05$), but was not different from the maltese crosses in Bruch's membrane.

In summary, the data suggest that both polyunsaturated and monounsaturated cholesterol esters were found in Bruch's membrane of all but the youngest eyes. The cholesterol esters found in drusen were predominately polyunsaturated. A few more drusen were observed in peripheral regions than in central regions, but no correlation with age could be made. In sclera, triglycerides and only low-melting-point, presumably polyunsaturated cholesterol esters were present. No deposits of cholesterol esters highly enriched in saturated fatty acids were found in any of the tissues, nor were any cholesterol monohydrate crystals seen.

DISCUSSION

Polarizing light microscopy is a useful technique that allows identification of classes of lipids and estimation of the degree of saturation of these lipids in biological tissues in situ. Triglycerides, cholesterol monohydrate crystals, phospholipid-rich liquid crystals, and cholesterol ester liquid crystals can be differentiated by their characteristic birefringence and melting behavior (see Table 1).[15,20] Because of the essentially qualitative nature of polarizing microscopy, we were not able to precisely quantify the lipid types present. However, a large area of birefringence reflects a greater amount of lipid than a small area. A principal finding of this study is the identification of lipids with birefringent morphology and melting temperatures characteristic of poly- and monounsaturated cholesterol esters both generally distributed throughout Bruch's membrane and localized to drusen in human eyes. The variability of the melting points of these cholesterol esters implies the presence of a variety of fatty acid side chains with a variable number of double bonds. Esterified and unesterified cholesterol was recently identified in aging Bruch's membrane, by using filipin staining.[26] We also identified a variably present very thin linear birefringence within Bruch's membrane with melting temperatures similar to those of the characteristic cholesterol ester liquid crystalline maltese crosses described herein. Maltese crosses in Bruch's membrane were infrequently resolved, probably because of their small size. The location of these deposits in relation to the RPE basement membrane (basal laminar deposits, basal linear deposits, diffuse Bruch's membrane thickening, or a combination of these) could not be determined. In a recent study, oil red O staining correlated with membranous debris internal to the RPE basal lamina.[27] In our study, the extent of birefringence within both the linear Bruch's membrane deposit and individual drusen corresponded well with the results of oil red O staining. This is in accord with the study of Pauleikoff et al.,[4] who found that eyes with more intense oil red O staining seemed to have higher levels of neutral lipids in Bruch's membrane-choroid extracts by gas and thin-layer chromatography.

We found evidence of phospholipids or complex membrane lipids by HSPM in the periphery of a single druse. We cannot exclude the possibility that small quantities of phospholipids existed that were below the resolution of light microscopy. These findings differ from those of Holz et al.,[3] who reported that the lipids extracted from Bruch's membrane and choroid by thin-layer chromatography consist primarily of phospholipids, triglycerides, fatty acids, and free cholesterol, but little cholesterol ester. Our study used histochemical and physicochemical techniques that showed the deposits were primarily extracellular. Holz et al. measured lipids within tissue

extracts of Bruch's membrane that could not be separated from the choroid. Little extracellular choroidal lipid was shown to be present by histochemical staining, and the investigators therefore assumed that that the lipid they detected came from Bruch's membrane. Although no study of the lipid composition of choroidal vessels exists, the highly vascular choroid may be a major source of cell membrane phospholipids, because they are clearly present in isolated cerebral microvessels in animals.[28,29] Spaide et al.[6] identified peroxidized polyunsaturated fatty acids in Bruch's membrane and choroid extracts and suggested that these lipids may cross-link with proteins and become resistant to extraction. The differences in results between our study and that by Holtz et al. could be related to choroidal contamination or changes in the extractability of lipids in their study or may simply represent variation in the composition of extracellular lipids between the two groups of patients.

The considerable variability in the extent of lipid deposits from patient to patient and unavailability of eyes in the third and fourth decades did not allow us to detect a linear relationship between age and severity of deposits. The absence of deposits in juvenile eyes strongly implies that the changes are age-related. The eyes included in this study were not known to have ARMD by clinical history or gross fundus appearance. No differences in birefringence or lipid staining were detected between small drusen (<63 μm) and larger drusen (>63 or 125 μm) that might be classified as representing early ARMD by photographic[30] or histopathologic criteria.[26] Although some investigators suggest that the composition of drusen is influenced by size,[31] others have not found size-related differences.[32] A cohort of eyes with ARMD would have to be evaluated to determine whether the changes described are unique to aging and whether they share similarities to ARMD deposits.

We have detected the presence of cholesterol ester profiles within the sclera of some aged eyes. A previous report showed an age-related increase in scleral lipids.[33] Increased concentrations of cholesterol esters[34,35] and sphingomyelin in sclera from older eyes have been reported using quantitative thin-layer chromatography techniques.[36] We have attributed the significant differences in mean melting temperatures between sclera (26.5°C) and drusen (34.3°C) or Bruch's membrane (39.5°C) as primarily due to more or less polyunsaturated cholesterol esters. However, these differences may be partially due to a higher triglyceride content associated with scleral cholesterol esters, because small amounts of triglyceride were found to be present in the sclera of some eyes. A small amount of triglycerides (maximum 3%) is soluble in cholesterol ester birefringent liquid crystals, and this causes the melting temperature of birefringent liquid crystalline deposits to decline approximately 5°C.[36] Differences in melting temperature of greater than approximately 5°C, such as those found in our study, cannot be solely attributed to triglyceride content.

In addition to drusen, Bruch's membrane, and sclera, increased cholesterol ester concentrations have also been observed within bulk connective tissues (dura mater, biceps, and psoas tendons),[37] the normal aging aortic intima,[38,39] and atherosclerotic lesions.[21,40,41] In atherosclerosis, there is progressive accumulation of lipid within intimal elastin that is primarily in the form of cholesterol esters.[42] Lipids may be transferred from lipoproteins to elastin in vitro[43] and aggregation of lipoproteins and their interaction with elastin and collagen are postulated mechanisms of lipid accumulation in vivo.[44] By electron microscopy, the abundant extracellular lipid of the atherosclerotic plaque lipid-rich core is associated with extracellular matrix constituents such as collagen and elastin. A possible relationship between extracellular cholesterol ester deposition in the eye and the mechanisms of lipid deposition described in atherosclerosis requires further study.

There is little information available regarding lipid transport mechanisms through Bruch's membrane. Serum lipoproteins participate in delivering unsaturated fatty acids from the liver to the RPE,[45] and LDL receptors are present on RPE cells, at least in vitro.[46] Apolipoprotein B, a component of lipoproteins that transport cholesterol esters, has been localized to basal laminar deposits in eyes with ARMD, but there is little information available for normal aging eyes.[47] The finding that extracellular lipid deposits in both aging human eyes and in atherosclerosis contain unsaturated or monosaturated cholesterol esters does not necessarily imply that the diseases share a common origin, because the source of the cholesterol esters within drusen cannot be determined from this study. On the other hand, the common nature of the lipids and lipoproteins implicated in both processes should stimulate further research into whether they represent parallel or homologous responses to tissue injury.

This study demonstrates the usefulness of HSPM in identifying the position and type of lipid deposits in drusen and age-related thickening of Bruch's membrane. Further studies of the lipid composition of these deposits in a group of eyes with known ARMD are warranted to determine whether their composition differs from that of deposits in elderly eyes without known macular degeneration and to further our understanding of this disorder.

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References

1. Pauleikoff D, Harper CA, Marshall J, Bird AC. Aging changes in Bruch's membrane. *Ophthalmology.* 1990;97:171–178.
2. Sheraidah G, Steinmetz R, Maguire J, et al. Correlation between lipids extracted from Bruch's membrane and age. *Ophthalmology.* 1993;100:47–51.
3. Holz FG, Sheraidah GP, Pauleikoff D, et al. Analysis of lipid deposits extracted from human macular and peripheral Bruch's membrane. *Arch Ophthalmol.* 1994;112:402–406.
4. Pauleikoff P, Zuels S, Sheraidah GS, et al. Correlation between biochemical composition and fluorescein binding of deposits in Bruch's membrane. *Ophthalmology.* 1992;99:1548–1553.
5. Curcio CA, Saunders PL, Younger PW, Malek G. Peripapillary chorioretinal atrophy. *Ophthalmology.* 2000;107:334–343.
6. Spaide, RF, Ho-Spaide WC, Browne RW, et al. Characterization of peroxidized lipids in Bruch's membrane. *Retina.* 1999;19:141–147.
7. Kliffen M, de Jong PTVM, Luider TM. Protein analysis of human maculae in relation to age-related maculopathy. *Lab Invest.* 1995; 73:267–272.
8. Hageman GS, Mullins RF, Russell SR, et al. Vitronectin is a constituent of ocular drusen and the vitronectin gene is expressed in human retinal pigment epithelial cells. *FASEB J.* 1999;13:477–484.
9. Farkas TG, Sylvester V, Archer D, et al. The histochemistry of drusen. *Am J Ophthalmol.* 1971;71:1206–1215.
10. Mullins RF, Johnson LV, Anderson DH, et al. Characterization of drusen-associated glycoconjugates. *Ophthalmology.* 1997;104: 288–294.
11. Mullins RF, Hageman GS. Human ocular drusen possess novel core domains with a distinct carbohydrate composition. *J Histochem Cytochem.* 1999;47:1533–1539.
12. Abdelsalam A, Del Priore L, Zarbin MA. Drusen in age-related macular degeneration: pathogenesis, natural course, and laser photocoagulation-induced regression. *Surv Ophthalmol.* 1999;44:1–29.
13. Guymer R, Luthert P, Bird A. Changes in Bruch's membrane with age. *Prog Retinal Eye Res.* 1998;18:59–90.

14. Gulcan HG, Alvarez RA, Maude MB, et al. Lipids of human retina, retinal pigment epithelium, and Bruch's membrane/choroid: comparison of macular and peripheral regions. *Invest Ophthalmol Vis Sci.* 1993;34:3187–3193.
15. Waugh DA, Small DM. Identification and detection of in situ cellular and regional differences of lipid composition and class in lipid-rich tissue using hot stage polarizing light microscopy. *Lab Invest.* 1984;51:702–714.
16. Small DM. *The Physical Chemistry of Lipids*. New York: Plenum Press; 1986:395–473.
17. Small DM. *The Physical Chemistry of Lipids.* New York: Plenum Press; 1986:475–522.
18. Miller KW, Small DM. Structure of triglyceride-rich lipoproteins: An analysis of core and surface phases In: AM Gotto Jr, ed. *New Comprehensive Biochemistry.* Vol 14. *Plasma Lipoproteins*. Amsterdam: Elsevier; 1987:1–75.
19. Loomis CR, Shipley DM, Small DM. The phase behavior of hydrated cholesterol. *J Lipid Res.* 1979;20:525–535.
20. Small DM. Progression and regression of atherosclerotic lesions. *Arteriosclerosis.* 1988;8:103–129.
21. Katz SS, Shipley GG, Small DM. Physical chemistry of the lipids of human atherosclerotic lesions: demonstration of a lesion intermediate between fatty streaks and advanced plaques. *J Clin Invest.* 1976;58:200–211.
22. Katz SS, Small DM, Brook JG, Lees RS. The storage lipids in Tangier disease: a physical-chemical study. *J Clin Invest.* 1977;59:1045–1054.
23. Van der Schaft TL, Mooy CM, de Bruijn WC, et al. Immunohistochemical light and electron microscopy of basal laminar deposit. *Graefes Arch Clin Exp Ophthalmol.* 1994;232:40–46.
24. Small DM. The physical state of lipids of biologic importance: cholesterol esters, cholesterol, triglyceride. In: Blank E, ed. *Surface Chemistry of Biologic Systems*. New York: Plenum Press; 1970:55.
25. Curcio CA, Medeiros NE, Millican CL. The Alabama age-related macular degeneration grading system for donor eyes. *Invest Ophthalmol Vis Sci.* 1998;39:1085–1096.
26. Curcio CA, Millican CL, Kruth HS. Cholesterol accumulates with age in human Bruch's membrane [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 2000;41(4):**S**115. Abstract nr 591.
27. Curcio CA, Millican CL, Bailey T. Lipids in human Bruch's membrane: histochemistry and ultrastructure [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 1999;40(4):**S**921. Abstract nr 4855.
28. Williams WM, Rapoport SI. Altered Composition of cerebral microvessel membrane phosphoglycerides from senescent mouse. *J Neurochem.* 1993;61:1843–1849.
29. Mooradian AD, Smith TL. The effect of age on lipid composition and order of rat cerebral microvessels. *Neurochem Res.* 1992;17:233–237.
30. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. *Surv Ophthalmol.* 1995;39:367–374.
31. Garner A, Sarks S, Sarks JP. Degenerative and related disorders of the retina and choroid. In: Garner A, Klintworth GK, eds. *Pathobiology of Ocular Disease*. New York: Marcel Dekker; 1994:642.
32. Russell S, Mullins RF, Schneider BL, Hageman GS. Location substructure and composition of basal laminar drusen compared with drusen associated with aging and age-related macular degeneration. *Am J Ophthalmol.* 2000;129:205–214.
33. Broekhuyse RM, Daemen FJM. The eye. In: Snyder F, ed. *Lipid Metabolism in Mammals.* New York: Plenum Press; 1977:145–188.
34. Feldman GL. Human ocular lipids: their analysis and distribution. *Surv Ophthalmol.* 1967;12:207–243.
35. Broekhuyse RM, Kuhlmann ED. Lipids in tissues of the eye. VI: sphingomyelins and cholesterol esters in human sclera. *Exp Eye Res.* 1972;14:111–113.
36. Croll DH, Small DM, Hamilton JA. Molecular motions and thermotropic phase behavior of cholesteryl esters with triolein. *Biochemistry.* 1985;24:7971–7980.
37. Crouse JR. Grundy SM, Ahrens EH Jr. Cholesterol distribution in the bulk tissues of man: variation with age. *J Clin Invest.* 1972;51:1292–1296.
38. Smith EB, Evans PH, Downham MD. Lipid in the aortic intima: the correlation of morphologic and chemical characteristics. *J Atheroscler Res.* 1967;7:171–186.
39. Katz SS. The lipids of grossly normal human aortic intima from birth to old age. *J Biol Chem.* 1981;256:12275–12280.
40. Smith EB. The influence of age and atherosclerosis on the chemistry of the aortic intima. *J Atheroscler Res.* 1965;5:224–240.
41. Katz SS, Shipley GG, Small DM. Physical chemistry of the lipids of human atherosclerotic lesions: demonstration of a lesion intermediate between fatty streaks and advanced plaques. *J Clin Invest.* 1976;58:200–211.
42. Kramsch DM, Franzblau C, Hollander W. The protein and lipid composition of arterial elastin and its relationship to lipid accumulation in the atherosclerotic plaque. *J Clin Invest.* 1971;50:1666–1677.
43. Kramsch DM, Hollander W, Franzblau C. The role of arterial elastin in the lipid accumulation in human atherosclerotic arteries. In: Jones RJ, ed. *Atherosclerosis*: *Proceedings of the Second International Symposium*. New York, NY: Springer–Verlag; 1970:115.
44. Guyton JR, Klemp KF. Development of the atherosclerotic core region. Chemical and ultrastructural analysis of microdissected atherosclerotic lesions from human aorta. *Arteriosclerosis Thromb.* 1994;14:1305–1314.
45. Wang N, Anderson RE. Synthesis of docosahexanoic acid by retina and retinal pigment epithelium. *Biochemistry.* 1993;32:13703–13709.
46. Noske UT, Schmidt-Erfurth U, Meyer Carsten, Diddens H. Lipidmetabolismus im retinalen Pigmetepithel (RPE). *Ophthamologe.* 1998;95:814–819.
47. Malek G, Guidry C, Bailey TM, et al. Apolipoproteins and lipid localization in eyes with age-related macular degeneration (AMD) [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 1999;40(4):**S**921. Abstract nr 4856.