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QUANTIFICATION OF FLUORESCEIN-STAINED DRUSEN ASSOCIATED WITH AGE-RELATED MACULAR DEGENERATION

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Abstract

Background—Previous studies of age-related macular degeneration have not quantified the number of drusen that accumulate fluorescein. Histopathologic studies have demonstrated druse subregions with different degrees of hydrophobicity, and these subregions might potentially exhibit different degrees of fluorescein uptake.

Methods—We evaluated macular drusen from 35 age-related macular degeneration patients by measuring druse area in color digital images and fluorescein angiograms, using 2 morphometric methods.

Results—Of 828 drusen evaluated, 405 had a corresponding fluorescein angiogram signal. About half of all drusen per eye (49.57%) stained in each participant. Among fluorescein-stained drusen, druse size measured in color images did not differ significantly from the sizes measured in corresponding fluorescein images ($P = 0.8105$), across the range of druse sizes.

Conclusion—These findings indicate that our understanding of drusen subregion staining may not directly correlate to in vivo observations of macular drusen in age-related macular degeneration.

Keywords

age-related macular degeneration; drusen; fluorescein angiography; fundus photography

Age-related macular degeneration (AMD) is the leading cause of visual loss in individuals aged >50, affecting >10 million people in the United States.^{1–3} The “dry” form of AMD is associated with drusen, focal extracellular deposits between the retinal pigment epithelium and the inner collagenous layer of Bruch membrane. Indicative of long-term relative risk for advanced disease,⁴ drusen identified by retinal fundus examination are typically divided into two main morphologic phenotypes, “hard” and “soft,” distinguishable by the appearance of their edges.^{5,6} Soft drusen, conveying higher risk for progression, outnumber hard drusen in macula of donor eyes with AMD.⁷ Drusenoid material can also appear in the subretinal space,^{8,9} potentially complicating the identification of drusen in the fundus.

Recent studies^{10–16} of drusen have revealed constituents that are hydrophilic (e.g., Complement Factor H, vitronectin, tissue inhibitor of metalloproteinases-3), hydrophobic (e.g., esterified and unesterified cholesterol), and amphiphilic (e.g., Apolipoprotein B). Substructures within drusen include cores enriched in unesterified cholesterol, nonfibrillar amyloid, and peanut agglutinin-binding carbohydrates, shells enriched in esterified cholesterol, and spherical assemblies of β -amyloid that bind complement.^{15–19} These components may represent evidence of formative processes like nucleation or uplifting of lipoprotein layer in inner Bruch membrane, and it is not known whether they can be realized clinically in patients.

Fluorescein ($C_{20}H_{10}O_5Na_2$, molecular weight 376.27 Da) is a hydrophilic dye used in the angiographic evaluation of AMD.^{20–22} In circulation, 70% of injected fluorescein is bound to plasma proteins, and the remainder is unbound within the aqueous phase.²³ Both bound and unbound fluorescein preferentially diffuse into hydrophilic areas and avoid hydrophobic areas. Because many drusen routinely bind this dye and persistently hyperfluoresce in late phases of fluorescein angiography (FA), Bird and Marshall²⁴ and Pauleikhoff et al²⁵ postulated that FA could potentially reveal specific druse constituents. Ex vivo studies support this notion, as fluorescein binds to subretinal pigment epithelium lesions with high content of histochemically detected phospholipids and binds poorly to areas with neutral lipid content.¹⁰

We sought to determine if the frequency of macular druse types and the substructure of various druse components demonstrated by recent laboratory studies were detectable in AMD patients. Like Russell et al,²⁶ who analyzed patients with Early-Adult Onset Grouped Drusen, we compared the sizes of drusen in color fundus photographs to the sizes of the same drusen in FA. Different sizes in these two imaging modalities would suggest differential localization of fluorescein among hydrophilic and hydrophobic druse subregions.

Methods

Institutional Review Board approval was obtained at the University of Alabama at Birmingham under protocol number X081031004. We performed a retrospective chart review of patients with a primary diagnosis of AMD seen in a multiphysician vitreoretinal specialist practice between 2002 and 2008. These patients were treated primarily by a single provider in the practice. Billing codes were used to select patients with a primary diagnosis of AMD, and 38 patients with known wet AMD in one eye and dry AMD in the fellow eye were selected. Patients were excluded based on the presence of other underlying vitreoretinal disease that might preclude accurate analysis of retinal pathology (i.e., diabetic retinopathy, chorioretinal scarring, etc.). The dry eye was chosen for analysis of drusen without occlusion by neovascular changes on FA.

Standard imaging protocols were used to obtain fundus photographs and fluorescein images using a TopCon TRC RetCam-50Ex. Two images (color and FA) were chosen from each study that best represented the patient's retina. Either color or red-free images were obtained before injection of intravenous fluorescein dye. In accordance with previous protocols, late-phase images (>4 minutes time lapse) were obtained of both eyes during the study. Fluorescein images were considered to be "late" images if they were taken after 4 minutes from fluorescein injection. Color and FA images were then processed using IGOR Pro 6 imaging software (Version 6.04, Wavemetrics, Inc, Lake Oswego, OR) in a no-loss compression file format (.tiff) to directly overlay the 2 images. Rapid alternation of the images was used to assist accurate alignment. Images were aligned so that the major and mid-sized vessels were aligned (Figure 1). Measurements expressed in pixels were

normalized for each fundus photograph using a standard measure of 125 μm attributed to the size of a retinal vein as it crosses the disk margin. This measurement, derived from cadaveric studies, could vary across our subjects, causing a “rubber ruler” effect. Therefore, drusen from different eyes were compared using ratios rather than absolute sizes.

Drusen were identified on color images and FA. Images were then processed by identifying and measuring individual drusen using a “bounding box” to determine the area encompassed by the druse. Using hairline cursor software accurate to 0.5 pixels, a box was placed around each druse. Corresponding angiographic coordinates were then evaluated for a detectable fluorescent region. If a corresponding angiographic druse was identified by the investigator, this area was also measured using the bounding box. For the purpose of plotting druse area, areas of boxes were converted to areas of equivalent circles by multiplying by $\pi/4$.

A second observer (J.S.P.) repeated these measurements with a randomly selected subset of 6 eyes and 160 drusen from the original population to provide an interobserver comparison of the bounding box technique. A validation of measurement was conducted using digital planimetry with a second subset of six representative eyes. Here, the observer directly encircled the drusen present in each image to measure the circumscribed areas reported in pixels using an Intuos 3 Graphics Tablet (Model PTZ-630; Wacom, Seattle, WA) and the program IP Lab (Version 3.9.3 r4; Biovision, Mountain View, CA).

The subset analysis between observers using the bounding box technique showed good interobserver correlation of measurement with a Pearson correlation coefficient equal to 0.98 for drusen measured in fundus photography and 0.94 for drusen measured on FA. Using digital planimetry, the bounding box technique was validated with a Pearson correlation coefficient of 1.00 for fundus photography and 1.00 for drusen measured on FA.

A mixed model was used to compare drusen size as measured by fundus photography and fluorescein angiography to account for the clustering of drusen within individual patients. Additionally, an intraclass correlation coefficient was calculated to quantify the consistency of measurements between fundus photography and fluorescein angiography. Finally, a Bland–Altman plot was used to evaluate the uniformity of the agreement between measurements made from the two imaging modalities.

Results

The average age of the 38 study participants was 80 years (minimum: 61 years; maximum: 96 years); 25 were women. Three patients were excluded because of poor-quality images precluding drusen detection. Of the remaining 35 eyes, 31 had at least 1 druse with a corresponding angiographic image for comparison. Of the 828 drusen identified, 405 had a corresponding fluorescein angiographic image, approximately half of which (49.6%) were illuminated by angiography (Figure 2). Some patients had no stained drusen (e.g., Patient 19, Figure 2). The sizes of staining and nonstaining drusen in each eye differed significantly ($P < 0.05$). The average area of drusen that bound fluorescein was 43% larger than the average area of drusen that did not.

We then compared normalized drusen area as determined from the color fundus photograph with the normalized area of the same druse as determined from the fluorescein image. The intraclass correlation coefficient for these data is 0.89, indicating a high degree of agreement between measurements made in the 2 imaging modalities. Figure 3 presents the Bland–Altman plot showing the agreement between areas measured in FA and color photographs. Data points are represented as logarithms to allow all values to be scalable. While the majority of points fall along the line indicating no difference, the slight upward slope of the

regression line indicates that for smaller-sized drusen the angiogram tends to yield smaller values compared with photography; the converse is true for larger drusen.

Discussion

We initiated this study because druse types and substructures within drusen likely contain materials of different relative hydrophobicity and thus, potentially differential binding capacity for fluorescein. We compared the clinical appearance of the same drusen seen in fundus photography and fluorescein angiography, using digital technology to directly measure and compare drusen between the two imaging modalities. Our study is the first in the era of digital imaging to quantify the ratio of fluorescent versus nonfluorescent drusen. Approximately half of all drusen visible on clinical examination show sustained fluorescein uptake into the late phases of the study, consistent with previous clinical impressions.²⁵ It is possible that the nonstaining drusen include subretinal drusenoid debris (reticular pseudodrusen). These lesions, which are extracellular aggregations of druse-like material in the subretinal space,^{8,27} are visible in color fundus images but have a negligible angiographic signature.²⁸ We also found that stained drusen were included in all observed sizes. Of note, we did find that stained drusen were larger on average than their nonstaining counterparts. Finally, we did not find evidence consistent with druse subregions exhibiting different relative fluorescein-binding capacity, as the size of drusen visible in color fundus images that also stained on FA were only subtly different in the two imaging modalities.

We expected to find more angiographic heterogeneity on the basis of previous studies. Bird and Marshall²⁴ and Pauleikhoff et al²⁵ noted that fluorescein-stained drusen in different patterns and believed that this correlated with their chemical composition. Pauleikhoff et al¹⁰ showed a poor in vitro correlation of fluorescein dye to areas of high content of histochemically detected neutral lipid within drusen, but an intense stain correlated with areas of high phospholipids. Russell et al²⁶ specifically investigated the angiographic appearance of drusen in Early-Adult Onset Grouped Drusen and demonstrated characteristic shells of high presumed hydrophobicity. These authors speculated that their observations could extend to AMD, based on clinical observations. Our results do not confirm this expectation for AMD patients. Of note, we analyzed a large sample of clinically discernable drusen, whereas Russell et al²⁶ selected only the six largest drusen in each patient. This combination of a different disease and different sampling methods most likely explains the discrepancy between our results and those of Russell et al.²⁶

We acknowledge that several aspects of our study limited our ability to detect druse heterogeneity. First, our study did not examine multiple angiographic time points to distinguish early-staining “hard” drusen from late-staining “soft” drusen. Second is a small sample of patients ($n = 38$) whose data may not be applicable to the general AMD population. Extension of the study to a broader group of patients would help ascertain if a difference truly exists between patients. Finally, the resolution of digital imaging might not be accurate enough to either measure the difference in druse area or permit discernment of subregions between fundus photographs and FA.

A major strength of our study is its use of digital imaging techniques that enabled the study of a very large sample of >800 drusen. We attribute this success to at least two technological advances, the use of digital image formats that allowed straightforward alignment and 2 interactive methods for druse measurement (bounding box and digital planimetry). Previous studies used encircling methods and standardized circles from grading systems to estimate the size of individual drusen from either film images^{5,29} or digital images.^{30–32} Our methods showed a high level of interobserver agreement, making an argument for further use of these digital overlay techniques in analysis of retinal pathology.

The methods we used have potential utility for improving the detection and analysis of drusen in the management of nonneovascular AMD.^{33,34} Previous studies have sought to automatically detect drusen using computerized algorithms. Computer-assisted drusen detection has been difficult to achieve, in part because drusen must be distinguished from a varying background of macular pigment, retinal pigment epithelium, and choroid in the fundus.³² Thus, the ability to detect drusen and their fluorescein signals has lower sensitivity than clinical observations by retina specialists.^{35–40} When background fundus reflectance is smoothed, semiautomated druse detection for classification and longitudinal study is facilitated.⁴¹ Based on our work, a clinically complementary approach, possible in patients for whom FA is available, is using the fluorescein as a contrast agent to impart a distinguishing characteristic to half of the drusen. Our methods provide a fast and reproducible means of measuring drusen and their FA signals. This will aid in further classifying drusen load and possibly distinguishing between subtypes based on size and FA signal.

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Fig. 1.

Color and fluorescein images of typical participant fundus. Full-view color images (**A-1**) were analyzed for clinically apparent drusen (**A-2**). These images were then compared with the corresponding full-view fluorescein angiograms (**B-1**) to examine for fluorescence in corresponding areas (**B-2**). Rapid alternation of overlying images (**C-1**) allowed accurate analysis of each druse. Drusen with corresponding fluorescein signals are indicated by black arrowheads in all images. Drusen without corresponding fluorescein signals are indicated by white arrows.

Fig. 2.
Staining versus nonstaining drusen in each participant.

Fig. 3.
Bland–Altman plot of individual drusen with line of best fit.