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Autofluorescence Characteristics of Early, Atrophic, and High-Risk Fellow Eyes in Age-Related Macular Degeneration

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Abstract

Purpose—To assess the relationships of drusen, pigment, and focally increased autofluorescence (FIAF) and the reticular pattern of hypoautofluorescence, to distinguish the combined photographic and AF characteristics of early, atrophic, and high-risk fellow eyes in AMD.

Methods—In a retrospective interinstitutional clinical study, AF and color photograph pairs of 221 eyes were examined: 166 eyes of 83 patients with bilateral large, soft drusen, with and without geographic atrophy (GA), and 55 fellow eyes of 55 patients with unilateral choroidal neovascularization (CNV). Forty-two eyes (one eye from each of 42 patients with early or atrophic AMD) were divided into four groups: 14 with drusen only, 9 with drusen and pigment abnormalities, 11 *fellow* eyes of patients with unilateral GA, and 8 eyes of patients with bilateral GA (acronyms for the groups: D-D, D-Pig, D-GA and GA-GA, respectively). The 55 fellow eyes of patients with CNV were divided into three groups: 19 eyes with no FIAF (CNV-0), 16 with FIAF without reticular AF (CNV-1), and 20 eyes with reticular AF and/or pseudodrusen (CNV-R). Image pairs of eyes with FIAF were registered, and drusen, pigment, and FIAF were segmented using automated background leveling and thresholding. All 221 eyes were surveyed for reticular AF and reticular pseudodrusen. The main outcome measures were (1) the fraction and relative probability of FIAF colocalizing with drusen and pigment and (2) the presence or absence of reticular AF and reticular pseudodrusen.

Results—The mean fractions of FIAF that colocalized with large drusen were: D-D group, 0.46 ± 0.21; D-Pig group, 0.42 ± 0.29; D-GA group, 0.13 ± 0.09; and GA-GA group, 0.11 ± 0.12. Comparisons between groups showed significant differences when comparing either the D-D group or the D-Pig group with either the D-GA group or the GA-GA group (*P* between 0.0001 and 0.015), whereas other comparisons were nonsignificant (Mann-Whitney rank sum test). The mean probabilities of FIAF colocalizing with large drusen relative to chance (1.0) were: D-D group, 4.7 ± 2.5; D-Pig group, 4.3 ± 2.3; D-GA group, 1.4 ± 0.8; and GA-GA group, 1.8 ± 1.3, with similar significant differences as for the colocalization fractions. The mean probability of FIAF colocalizing with small to intermediate drusen in the D-D group was 1.5 ± 1.3, which was not significantly different from chance. In the D-Pig group, the median probability of FIAF colocalizing with pigment abnormalities was 10.0 (range, 1.1–51.0). The AF patterns in 15 of 19 eyes in the CNV-0 group were normal; the remainder had nonreticular hypoautofluorescence only. In the CNV-1 group, the relations

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of FIAF with drusen and pigment were similar to those in the early AMD groups. CNV-R comprised 20 of 55 eyes in the CNV group, but reticular autofluorescence and/or pseudodrusen were found in only 14 of 166 eyes of the early and atrophic groups. Of the 34 total eyes with reticular AF or pseudodrusen, 28 had both, 4 had reticular AF only, and 2 had reticular pseudodrusen only.

Conclusions—There are clear relationships between AF patterns and clinical AMD status. In early AMD, FIAF's colocalization with large, soft drusen and hyperpigmentation is several times greater than chance, suggesting linked disease processes. In advanced atrophic AMD, FIAF is found mostly *adjacent* to drusen and GA, suggesting that dispersal of drusen-associated lipofuscin is a marker of atrophic disease progression. In the neovascular case, a large group of fellow eyes have no FIAF abnormalities, suggesting that lipofuscin is not a major determinant of CNV. However, reticular hypoautofluorescence, consistent with widespread inflammatory damage to the RPE, appears to be a highly sensitive imaging marker for the disease that determines reticular pseudodrusen and is strongly associated with CNV.

Drusen, observed in color fundus photographs, are the hallmark of age-related macular degeneration (AMD),[1–9] which is the leading cause of blindness in the developed world.[10] The current standard for grading drusen is manual assessment of stereo pairs at the light box. We have developed an automated digital technique to measure the drusen area[11–13] that is comparable to the standard technique in precision and reproducibility.

Autofluorescence (AF) images obtained with the scanning laser ophthalmoscope (SLO) are derived largely from lipofuscin accumulated in the retinal pigment epithelium (RPE).[14,15] It is believed that metabolic activity of the RPE correlates with the content of lipofuscin in RPE cells.[16] In humans, lipofuscin accumulates with age within the lysosomal compartment.[17] Excessive lipofuscin accumulation in the RPE, as imaged by focally increased autofluorescence (FIAF), has been proposed to be a marker of RPE disease and photoreceptor cell degeneration.[18,19] Many previous studies have been made on correlations of change in AF distribution with pathologic features.[14,20–24] Markedly decreased autofluorescence, especially over larger areas, is equivalent to GA.[25] Reticular AF was identified as a specific subtype of AF by Lois et al.[26] and is characterized as a grouping of ill-defined hypofluorescent lesions against a background of mildly elevated AF. Its role in the AMD disease process has not yet been defined.

Our goal was to provide some insight into the disease processes of AMD by clarifying some of the complex relationships between AF abnormalities and AMD lesions. For example, we will present evidence that the photographic counterpart of reticular AF is reticular pseudodrusen, lesions whose high-risk association with CNV is well known.[27] The term reticular pseudodrusen, according to the nomenclature of Arnold et al.,[27] refers to a yellowish interlacing network that lies below the RPE and above the largest choroidal vessels, most commonly near the superior arcades. Although FIAF appears to be associated with GA progression,[28] it has also been suggested that drusen and FIAF are independent markers for AMD.[21,26]

However, prior conclusions about a lack of association between drusen and FIAF have been based on examination of the entire spectrum of AMD lesions, from small drusen to choroidal neovascularization (CNV), without regard for lesion subtype. Furthermore, comparison of image pairs to determine colocalization is often difficult if images are acquired by different cameras (i.e., the fundus camera and the SLO). We propose herein to focus somewhat more precisely on AF patterns in those higher risk eyes with large, soft drusen (diameter >125 μm), with or without pigment abnormalities or geographic atrophy (GA), and fellow eyes of CNV. We also offer an improvement in precision by exact registration of the AF and fundus images. This method enables rigorous pixel-to-pixel comparison, removing the need to judge drusen and FIAF location in a side-by-side, subjective manner.[21,26] Moreover, we can use our

automated techniques for identification of drusen, extend them to FIAF lesions, and thus measure FIAF and drusen colocalization objectively, as a normalized colocalization coefficient (NCC).

Methods

Patient Selection

AF and color photograph pairs of 166 eyes of 83 patients with bilateral large, soft drusen (>125 μm), with or without pigment abnormalities and GA, but without evidence of CNV, and 55 fellow eyes of 55 patients with unilateral CNV, were selected retrospectively, either from an AMD study database at the Institute of Ophthalmology in the United Kingdom or from patients imaged at the Columbia University Eye Institute. The study adhered to the tenets of the Declaration of Helsinki and received approval by the institutional review board of New York Presbyterian Hospital (New York, NY) and ethics approval at King's College Hospital and the Institute of Ophthalmology.

Photographic Phenotypes

For a detailed study of FIAF in the early and GA phenotypes, we chose a representative sample (42 patients) divided into patients with bilateral large, soft drusen only (one eye chosen at random); patients with large, soft drusen and pigment abnormalities (one eye chosen at random); patients with unilateral GA (fellow eye of GA chosen); and patients with bilateral drusen and GA (one eye chosen at random). We used the acronyms D-D (n = 14), D-Pig (n = 9), D-GA (n = 11), and GA-GA (n = 8), respectively, to label these four groups.

AF Phenotypes

We then inspected the AF scans of the 42 early and GA phenotypes. Because our prior work suggested that the presence of GA was associated with marked changes in the distribution of FIAF with respect to drusen (Busuioc M, et al. *IOVS* 2004;45:ARVO E-Abstract 2961) and because focally decreased AF (FDAF) is the AF hallmark of GA,[28] we redivided the images into two groups: If FDAF was present in either the study eye *or* the fellow eye, the eye was assigned to FDAF1. The remaining eyes, without FDAF in either the study eye or fellow eye, were assigned to FDAF0. Thus, group FDAF1 necessarily included all eyes in the photographic groups D-GA and GA-GA, but also included 2 eyes from the D-Pig group and 2 from the D-D group (23 eyes total). Group FDAF0 consisted of the remaining 19 eyes (Table 1).

The fellow eyes of patients with CNV were subdivided into three groups based on their AF characteristics: CNV-0 (no focal FIAF abnormalities, 19 eyes), CNV-1 (FIAF present without reticular AF, 16 eyes), and CNV-R (reticular AF or pseudodrusen present, 20 eyes).

Our survey for reticular AF and reticular pseudodrusen included all 166 eyes from the early and atrophic group and the 55 fellow eyes from the CNV group. Because the presentation of reticular pseudodrusen is often quite subtle, we studied the color photographs, both in their original state and as highly contrast-enhanced versions, to facilitate identification. If either AF or photographic reticular disease were present, we noted whether it was generalized or confined to a quadrant. If both were present, we noted in which quadrants they coincided (See Fig. 1 for an example in which the pathology coincided in both eyes). If they coincided in a quadrant, we attempted to determine which individual photographic and AF lesions corresponded. We distinguished reticular AF from those atrophic drusen patterns that were more central, darker, and more scattered than the lower-contrast reticular AF that filled a whole region homogeneously. Such atrophic drusen patterns were not included as reticular AF.

The photographic and AF image pairs of the 42 eyes in the drusen and atrophic subgroups and of the 16 eyes in CNV-1 were precisely registered (MatLab ver. 7.0; The MathWorks, Natick, MA) for lesion segmentation and colocalization analysis. Corresponding points from AF and color images were selected based on vessel landmarks such as bifurcation points. The points were used to determine the image transformation necessary for alignment and resizing. Superimposition and flicker comparison were used to determine the accuracy of the registration, which was redone if necessary.

Image Analysis

Color Photograph Segmentation—Drusen pixel identification (segmentation) and area measurements in the color photographs were performed in the 3000-μm diameter region by the automated mathematical model– based digital technique previously described.[12] When the 6000-μm diameter region was used, two additional annuli of outer radii, 4500 and 6000 μm, were used for extension of the model to this larger region. Drusen segmentation in the extended region was then performed exactly as before. We also applied a morphologic filter to separate large drusen (>125 μm diameter) from small and intermediate drusen. Analysis was performed on the subset of large drusen in each image group. Separate analysis of the small to intermediate drusen subset was performed on the D-D group. Areas of hyper- or hypopigmentation were manually segmented in the D-Pig group. One image in the D-Pig group had only intermediate drusen after the filter was applied, but demonstrated striking colocalization of FIAF and hypopigmentation. Hence, it was included in the analysis of pigmentary abnormalities only.

AF Segmentation—To make quantitative assessments of abnormal AF relative to the image background and to perform this thresholding efficiently and uniformly in the setting of significant background variability, the AF background was leveled with a six-zone mathematical model in a manner completely analogous to that used for drusen segmentation. Briefly, we used a 600-μm central disc, three annular zones (600–1000, 1000–2000, and 2000–3000 μm diameter), and two outer annular zones (3000–4500 and 4500–6000 μm). Areas of clinical GA were first manually segmented and masked from the image to be leveled by the model. The model has been tested for accuracy on normal AF scans (Smith RT, et al. *IOVS* 2005;46:ARVO E-Abstract 4300),[29] as has been described. The details of implementation for GA and FIAF have also been described.[30] After leveling the background, the mean and SD σ of the resultant image in each of two regions were used to define the thresholds. These regions were the 3000-μm region and the 3000- to 6000-μm annulus. The thresholds were set at 2.0 σ above and below the mean, to determine the FIAF and FDAF, respectively.

We also tested the thresholds 2.5 and 1.5 σ above the mean for FIAF in the D-D group to see how results were affected.

Colocalization Measurements

All areas of total drusen and FIAF were expressed as decimal fractions of the 6000 μm region (i.e., numbers between 0 and 1). If this region was not available in both images, the 3000 μm region was used. Thus,

$$p\,(\mathrm{D}) = \text{total Drusen},$$

and

$$p\,(\mathrm{FIAF}) = \text{total} \quad \text{FIAF},$$

The standard spatial colocalization of FIAF with respect to drusen, C, was defined as the area of those FIAF pixels that coincided with drusen, $p(\mathrm{D} \cap \mathrm{FIAF})$, divided by the total area of FIAF, also a ratio between 0 and 1:

$$\mathrm{C}=\frac{p(\mathrm{D} \cap \mathrm{FIAF})}{p(\mathrm{FIAF})}. \tag{1}$$

These area relationships have a simple interpretation in the terminology of probability. The fractional area of drusen in the region may be thought of as the probability $p(\mathrm{D})$ that any pixel is a drusen pixel. Similarly, the fractional area of FIAF is the probability $p(\mathrm{FIAF})$ that any pixel has FIAF. If the colocalization of drusen and FIAF is random—that is, if the occurrence of drusen and FIAF are independent events, then a basic law states that the probability that any pixel is both a drusen pixel and an FIAF pixel is given by the product of the probabilities:

$$p(\mathrm{D} \cap \mathrm{FIAF}) = p(\mathrm{D}) \cdot p(\mathrm{FIAF}). \tag{2}$$

Rearranging equation 2, we see that the statement that drusen and FIAF occur independently is equivalent to the equation

$$\mathrm{NCC}=\frac{p(\mathrm{D} \cap \mathrm{FIAF})}{p(\mathrm{D}) \cdot p(\mathrm{FIAF})}=1, \tag{3}$$

where NCC (the *normalized* colocalization coefficient) is defined for any image pair as the ratio calculated. By similar reasoning, consider a case in which the occurrence of drusen and FIAF were linked or were *not* independent events, in which for example, the probability that drusen and FIAF would occur together was twice that which would be predicted by chance alone. In the language of probability, the corresponding equation would be:

$$p(\mathrm{D} \cap \mathrm{FIAF}) = 2p(\mathrm{D})\, p(\mathrm{FIAF}). \tag{4}$$

In this case, the NCC defined in equation 2 would be equal to 2. And conversely, an NCC of 2 is equivalent to a probability that drusen and FIAF will occur together that is two times greater than that expected if they were independent. In general, therefore, the NCC is the probability that drusen and FIAF will colocalize relative to that expected if they were independent.

The relationship between the *normalized* colocalization coefficient, NCC and C, the standard colocalization of FIAF with drusen, follows from equations 1 and 3:

$$\mathrm{NCC}=\frac{C}{p(\mathrm{D})}. \tag{5}$$

Thus, the NCC is simply C normalized by the fraction of drusen in the image. Both quantities have intuitive interpretations: if, for example, C is 0.5, that means that half of the FIAF overlays the drusen and half is related to “something else.” If in this same image, the fractional area of drusen, $p(\mathrm{D})$, is only 0.1, NCC = 0.5/0.1 = 5, indicating that the colocalization of drusen and FIAF was five times greater than expected by chance. Intuitively, a large fraction of the FIAF has landed on a relatively small amount of drusen, suggesting a strong relationship. In contrast, if in this image $p(\mathrm{D})$ was 0.5, then the NCC = 0.5/0.5 = 1, indicating a colocalization no greater

than that expected by chance. Thus, even though half the FIAF still landed on drusen (C = 0.5), half the macula was drusen anyway, and so this result would be expected by chance alone.

For a sample mathematical modeling of an AF image, segmentations of the corresponding AF/drusen image pair, FIAF, and drusen colocalization measurement and NCC calculation, see Figures 2A–C for AF image modeling and segmentation, Figures 2D and 2E for drusen image segmentation, and Figure 2F for FIAF and drusen colocalization and NCC calculation). The templates shown in this and all figures are the standard grading fields of 1000-, 3000- and 6000-μm diameter.

In the group D-Pig we also measured the NCC of FIAF with pigment abnormalities (Fig. 3 hyperpigmentation, Fig. 4 hypopigmentation). The example in Figure 4 has no large drusen, so was not included in drusen colocalizations for this group. In the GA-GA group, we also measured the fraction of FIAF in the 250-μm border zone surrounding the GA in the AF image and classified the pattern of FIAF according to the published scheme of Bindewald et al.[31]

Colocalizations of drusen and pigment abnormalities were performed on CNV-1 (images with FIAF), as described for the D-D and D-Pig groups. Regional colocalizations (2000 μm^2) were performed on selected cases from the CNV-R group between reticular pseudodrusen and the FDAF lesions of reticular AF.

Statistics

All comparisons between groups were performed with the Mann-Whitney rank-sum test, a nonparametric statistic appropriate for complex measurements that may not be normally distributed.

Results

The mean colocalization of FIAF with drusen in the D-D group was 0.46 ± 0.21 with a mean NCC of 4.7 ± 2.5 (Fig. 2). If thresholds for FIAF were defined by +2.5 or +1.5 σ above the image mean instead of 2.0 σ, the mean colocalization of drusen with FIAF and mean NCC were 0.58 ± 0.22, NCC 5.5 ± 3.3 and 0.42 ± 0.20, NCC 3.3 ± 1.3, respectively (Fig. 3, effect of varying the threshold for FIAF in an image from the D-Pig group).

In the D-Pig group, the mean FIAF colocalization and NCC (for FIAF 2.0 σ above the image mean) were 0.42 ± 0.29 and 4.3 ± 2.3, respectively (Fig. 3, hyperpigmentation example; Fig. 4, hypopigmentation example), in the D-GA group 0.13 ± 0.09 and 1.4 ± 0.8 (Fig. 5), and in the GA-GA group 0.11 ± 0.12 and 1.8 ± 1.3 (Fig. 6).

Comparisons of colocalization measurements between groups showed significant differences when comparing either the D-D group or the D-Pig group to either the D-GA group or the GA-GA group (P between 0.0001 and 0.015). FIAF and drusen colocalizations in the D-D group and the D-Pig groups were not significantly different, nor were they significantly different in the D-GA and GA-GA groups. These statements also held true for the normalized colocalization coefficients (P for significant tests, between 0.0001 and 0.038). The mean probability of FIAF's colocalizing with small/intermediate drusen in the D-D group was 1.5 ± 1.3, a nonsignificant difference from chance.

In the D-Pig group, there were seven images with hyperpigmentation, one with hypopigmentation, and one with both. For the eight total images with hyperpigmentation, the fractional area occupied by the pigment abnormalities was relatively small (median 0.007), causing the measured colocalization of FIAF with hyperpigmentation to vary widely (median 0.06; range, 0.03– 0.38). However, the median NCC of FIAF with hyperpigmentation was 10.0

(Fig. 3), with a minimum of 1.1, and the remaining values ranging from 3.3 to 51.0. The image with the NCC of 3.3 also had hypopigmentation, with an NCC of FIAF with hypopigmentation equal to 1.3. For the image with only hypopigmentation, the colocalization of FIAF with hypopigmentation was 0.48 and the NCC was 20.9 (Fig. 4). (There were no large drusen in this last image, and so colocalizations of FIAF with drusen from this image were not included in the results for the D-Pig group).

In the GA-GA group, the mean fraction of FIAF in the border zone relative to the FIAF in the entire image was 0.51 ± 0.19. The patterns of FIAF as defined by Bindewald et al.[31] were evenly divided, with four each of the diffuse and focal subtypes. The mean NCCs (1.85 and 1.7, respectively) were not significantly different for these subtypes. In both the D-GA and G-G groups, FIAF was often also found adjacent to drusen but not coincident with drusen (Figs. 5, 6). The drusen with FIAF around them were often themselves regions of FDAF (Fig. 6).

For the AF groups FDAF0 and FDAF1, the mean colocalizations with drusen and the NCCs were 0.44 ± 0.22, NCC = 5.2 ± 2.1 and 0.18 ± 0.20 ($P < 0.0005$), NCC = 1.6 ± 1.0 ($P < 0.0001$), respectively. In particular, C and NCC decreased significantly in the *fellow eyes* of eyes with GA (D-GA group) even when GA was not clinically obvious in the study eye, relative to eyes without FDAF in either eye (Fig. 5). A similar finding held if the FDAF1 eyes were removed separately from the D-D and D-Pig groups. That is, on removal of the FDAF1 eyes from the D-D group (respectively, from the D-Pig group), the NCC increased from 4.7 ± 2.5 to 5.1 ± 2.4 (respectively, from 4.3 ± 2.3 to 5.4 ± 1.5).

There were 19 eyes in CNV-0 without FIAF, of which 15 had normal AF and another 4 had variable FDAF lesions. In the 16 images in the CNV-1 group with FIAF, the mean NCC of FIAF with drusen was 7.3 ± 4.8 and for the seven of these images with hyperpigmentation, the median NCC with pigment was 9.2 (range, 4.2–35.0).

There were 20 eyes in the CNV-R group with the reticular pattern of hypoautofluorescence and/or reticular pseudodrusen on color photography. Of these, 15 had both, 4 had reticular AF only, and one had reticular pseudodrusen only. The reticular AF pattern (19 eyes) was confined to the region of the superior arcades in 9 eyes and was generalized in 10 eyes. When reticular pseudodrusen were also present (15 eyes), they were present in the same (11 eyes) or fewer (4 eyes) quadrants as the reticular AF pattern. Reticular pseudodrusen were present in only one eye in which reticular AF could not be identified, perhaps due to marginal AF scan quality. Where reticular pseudodrusen were not present, the AF reticular lesions had no particular association with other evident fundus abnormalities. Reticular AF and/or pseudodrusen were present in 14 of 166 eyes of the early and atrophic groups. All 14 had evidence of reticular pseudodrusen on color photography. One patient had bilateral reticular pseudodrusen and an AF image in the left eye that could not be graded for reticular AF. In these 14 eyes, the quadrantic distribution of the two types of lesions essentially coincided.

In summary, there were 34 eyes from all the groups studied that showed definite reticular AF and/or pseudodrusen, and of these, 28 had both, 4 had reticular AF only, and 2 had reticular pseudodrusen only. Thus, there were perhaps only two eyes among the entire group of 221 surveyed that had reticular pseudodrusen *without* evidence of reticular AF, demonstrating that AF imaging was over 90% sensitive for the presence of reticular pseudodrusen. Colocalization of individual lesions when reticular AF and reticular pseudodrusen coincided was successful, however, in only a minority[4] of these 28 cases, because of low lesion contrast. In each of these 4 cases, image registration and colocalization were performed over a 2000-μm^2 subregion where lesion density and contrast were greatest. In these cases, the reticular drusen colocalized mostly with the hypoautofluorescent lesions and avoided the areas of FIAF (Fig. 7).

Discussion

The findings in this study suggest that the relationships of AF lesions to photographic fundus abnormalities in AMD, while complex, fall into clear patterns that depend on the disease state: early (large soft drusen, pigmentary abnormalities), atrophic, or neovascular.

The overall picture for early and atrophic AMD that emerges is this: When large, soft drusen are present with or without pigmentary abnormalities, but without atrophy as seen by focally decreased AF, then FIAF colocalizes with the drusen to an extent several times greater than chance alone. In the presence of pigment abnormalities, a substantial portion of the FIAF may also colocalize with these (in which case the colocalization with the drusen themselves would necessarily be somewhat less). It should be noted that the relationship of FIAF and drusen, as measured by the normalized colocalization coefficient (NCC), applies symmetrically. This correlation suggests that large, soft drusen and FIAF are manifestations of initially linked disease processes affecting the RPE-Bruch's membrane-choriocapillaris complex, leading to abnormal accumulations of lipofuscin in the affected RPE overlaying the drusen. An alternate explanation could simply be that the measurement of FIAF is affected systematically by the presence of drusen. Thus, if there were significant RPE hypopigmentation overlaying the drusen, then even if the lipofuscin content were normal, the measured AF could be elevated by unmasking.[32] FIAF correlates even more highly with pigment abnormalities when they are present, and appears to come from melanolipofuscin in pigment clumps[21] and unmasked lipofuscin in depigmented RPE. Indeed, when RPE is hypopigmented but viable and not atrophic, FIAF would be expected without any increase in lipofuscin content due to unmasking. Thus, this is the second mechanism of those proposed for FIAF over drusen. Regarding hyperpigmentation, some pigment clumps show no FIAF, suggesting that these have melanin only, and some are the brightest AF lesions in the fundus (Figs. 3D, 3E). Thus, the aggregation of melanolipofuscin in these eyes seems to be a highly nonuniform process.

With progression to geographic atrophy, FIAF is no longer found over drusen, but is found mostly *adjacent* to drusen and GA, as is reflected in significant decreases in colocalization. Remarkably, the presence of FDAF in either eye seems to be associated with a marked shift to this distribution, regardless of the original photographic classification. Thus, an eye with soft drusen only and without clinical geographic atrophy, will tend to show FIAF dispersed from the drusen if FDAF is present (Fig. 5). Dispersal of drusen-associated lipofuscin may therefore be a marker for disease progression to the atrophic form of AMD.

These observations appear to be consistent with the work of others. In early AMD, Lois et al. [26] pointed out that parafoveal large, soft drusen were often associated with FIAF, in agreement with our findings. However, they also felt FIAF and drusen were otherwise independent. This conclusion can now be explained in light of the significant and heretofore unrecognized change in the pattern when there is concomitant RPE atrophy, as seen in the images with FDAF. If these disease states (with and without FDAF) were grouped together in previous qualitative studies,[21,26] then the corresponding relationships would be obscured. We consistently found high colocalization of FIAF with large, soft drusen throughout the macula in eyes without FDAF, even in the fovea where our methods of background leveling in the AF scan can reveal FIAF that is partially masked by luteal pigment, and peripherally (see Fig. 8, for examples of each). We also noted the specificity of the correlation with FIAF for the large, soft drusen types only. For small and intermediate drusen, the relationship to FIAF was indeed variable and weak. It is not clear whether this represents a fundamental change for increasing drusen size, or simply reflects difficulty imaging and analyzing the smaller lesions.

The relation of AF to drusen was investigated quantitatively by Delori et el.[18] In these four eyes, the pattern was characterized by FDAF in the center of the drusen 60 to 175 μm in size,

surrounded in some cases by FIAF in an annular pattern. For larger and confluent drusen, there were multifocal areas of FDAF, or a heterogeneous pattern. The fact that there was notable FDAF in these eyes would place them in our AF category of FDAF1. Hence, their observations are consistent with our finding that FIAF in this category is not particularly colocalized with drusen (mean NCC = 1.6 ± 1.0) but is often adjacent to drusen (Figs. 5, 6).

This relationship of FIAF to the progression from early to atrophic AMD and our previous work on FIAF and the progression of geographic atrophy[30] can also be unified. Thus, the current findings suggest that with disease progression there is lipofuscin dispersal and/or new lipofuscin around GA and atrophic drusen, which is then seen, for example, in the junctional zone of GA as FIAF. In this scenario, then, lipofuscin adjacent to GA is *secondary* to the GA/AMD process— different from the theory that lipofuscin already in the junctional zone is *primary* because it actually drives the development of new GA, perhaps due to toxicity.[25] If the latter were the case, then FIAF in the junctional zone should have some predictive power about the location of subsequent new GA, but our previous paper suggests that it does not.[30] On the other hand, if lipofuscin accumulations are *secondary* to the disease process, there is no reason for their presence to predict the location of future atrophy. For example, the mechanisms of this dispersal could be similar to those proposed by Hageman et al.[33] for accumulation of debris between Bruch's membrane and the RPE. Hence, results in both papers are consistent with a disease model in which shifting lipofuscin accumulations as measured by FIAF are secondary rather than primary to the atrophic process.

In the case of neovascular AMD, there were two striking differences with the early/atrophic groups. First, within the group CNV-0 of 19 eyes without FIAF, there was simply the size of the subgroup, 15 of the 55 eyes examined, with normal AF. Because a fellow eye of an eye with CNV is at high risk also for CNV, one might have expected a high incidence of AF abnormalities in these eyes consistent with a more advanced disease state. These findings suggest that abnormal lipofuscin accumulations may not play a significant role in many cases of neovascular AMD.

The second difference was that 20 (36%) of 55 eyes in the CNV group had reticular AF and/or pseudodrusen, whereas this pattern occurred in only 14 (8.4%) of 166 eyes with soft drusen and/or GA. To our knowledge, it has not been reported that the reticular form of AF is strongly associated with the CNV disease phenotype. It has been noted, however, that reticular pseudodrusen are associated with a high incidence of CNV (Arnold et al.[27]), and, like reticular pseudodrusen, reticular AF has a predilection for the superior arcades. In their study, 66% of patients with reticular pseudodrusen had or developed CNV. Herein, 28 of 32 of the eyes with reticular AF in this study also had reticular pseudodrusen, which corresponded in whole or in part with the AF lesions. In contrast, there were only two examples of eyes with reticular pseudodrusen that did not have some corresponding reticular AF. It hence appears that reticular AF defines essentially the same pathology as reticular pseudodrusen, and is also slightly more sensitive. Of interest, the pathologic correlate for reticular pseudodrusen found by Arnold et al.[27] in one eye was not drusenlike material at all (hence, their nomenclature pseudodrusen), but rather a decrease in choroidal vascularity. Particularly, they noted that while reticular pseudodrusen size did not fit intercapillary pillar spacing, it was consistent with a reticular pattern of fibrosis involving the larger choroidal veins in front of the deepest layer of arteries. Their ICG study of this entity was negative. However, they later found in another study[34] an altered ICG appearance with the Heidelberg SLO (Heidelberg Engineering, Heidelberg, Germany): In the early phase, there was a loss of sharpness of the large choroidal vessel outlines. In the mid and late phases, there was a pattern of hypofluorescent dots corresponding to the extent of the pseudodrusen, suggesting a similarity to the hypoautofluorescent dots of the reticular AF pattern in this study. The current findings are consistent with the viewpoint that reticular pseudodrusen are simply the fundus appearance of a disease process that affects

subretinal structures and that, when it damages the RPE, is imaged as reticular AF. The etiology of these lesions is unclear, but the appearance of these multifocal, regularly distributed lesions is consistent with inflammation. This could be significant given that inflammatory pathways (the complement pathways) are now known to be the main substrate for the genetics of AMD.[35,36]

The technical aspects of the study also merit comment. Quantification of FIAF was performed with care throughout, but it should be recognized that there is no universally accepted standard. That suggested by von Ruckmann et al.[21] involved graded elevations of gray level above the local background determined manually. The current technique, also used in our work on GA progression,[30] may be considered a refinement of that suggestion: level the background globally by the mathematical model and apply any desired definition of FIAF automatically. For example, we used the definition of FIAF as 2 SD above the local mean; however, extraction of FIAF defined by elevations of 1.5 or 2.5 SD was also easy to do. This in turn allowed an excellent test of the robustness of key conclusions: Did they remain valid over a range of definitions of FIAF? For example, with an increasingly strict definition of FIAF (1.5, 2.0, and 2.5 SD above local mean), the colocalizations with drusen were all significant and increased monotonically (see the Results section). Thus, the conclusion was robust to varying the definition of FIAF. It also showed that the higher the AF signal, the more likely it was to localize with large, soft drusen in the D-D group, which was an interesting finding in itself. With hyperautofluorescent pigment figures as in the D-Pig group, the colocalization of the bright AF signals might instead shift away from drusen to the pigment figures. An example in which these varying definitions of FIAF are applied is shown in Figure 3.

A strength of the study, therefore, was the precise demonstration of complex relationships between several important components of AMD that naturally suggest new disease models to be evaluated. A second strength was the development of a set of robust image analysis tools for implementing such analyses.

This study has several limitations. First, in the case of early to atrophic AMD, even though drusen–lipofuscin colocalization results were significantly different between the atrophic and nonatrophic groups, the four photographic subgroups were relatively small, However, the most important distinction was perhaps between the larger classes of eyes with and without AF evidence of atrophy (FDAF1 and FDAF0), with 23 and 19 eyes from as many patients, respectively, and with highly significant results that summarize the key finding of the paper for the early and atrophic groups: FIAF definitely colocalizes with large, soft drusen when atrophy is not present and definitely does not when atrophy is present. A second limitation of the study is that it was cross-sectional. Hence, even though significant differences in lipofuscin–drusen relationships were demonstrated between the early and atrophic groups, inferences about mechanisms for the changed patterns (i.e., lipofuscin dispersal), would be better supported by a prospective study of patients who advanced from early to atrophic AMD. Likewise, our observations demonstrate an association between three diseases (reticular pseudodrusen, reticular AF, and CNV) rather than a predictive value of any one for another. For pseudodrusen as a risk factor for CNV, we cite prior literature.[27,37] Divergent longitudinal findings based on the Age Related Eye Disease Study (AREDS) data have also recently been reported (Armstrong JR, et al. *IOVS* 2005;46: ARVO E-Abstract 220), suggesting that pseudodrusen do not increase risk for CNV but may in fact increase risk for GA over 5 years. Possible implications are that the latency of CNV from pseudodrusen is longer than 5 years, or that there was an ascertainment bias in one study or another. Recognition of pseudodrusen from color photographs alone can be difficult, and a detailed review of the entire AREDS database was not undertaken. The incidence of pseudodrusen in that study in eyes with large drusen was 7%, reassuringly consistent with the 8.4% we found in the early and atrophic groups, but further prospective data are clearly needed.

Finally, it should be remembered that our AF measurements are relative, and subject to degradation by the lens, rather than absolute values obtained spectroscopically,[18] and that the exact nature of the fluorophores imaged by AF is also not fully known. The best-studied source is lipofuscin, itself a mixture, with A2E predominant.[38] However, other sources of fluorescence such as sub-RPE deposits and Bruch's membrane have been identified in AMD eyes (Marmorstein et al.[39]). These authors found in in vitro cross sections that the 488-nm excitation wavelength stimulates substantial blue-green emissions from these structures. The quantitative contribution of these emissions to in vivo AF is unknown. Soft drusen may also contain intrinsic fluorescent material, as in Best's disease.

In summary, combined autofluorescence and photographic image analysis revealed dramatically divergent paths leading to atrophy and CNV. Progression from early to atrophic AMD appeared to be accompanied by shifting of lipofuscin accumulations from RPE overlaying soft drusen to adjacent areas around drusen and GA. Lipofuscin itself, however, may not be a primary determinant of *future* atrophy.[30] In the contrasting neovascular case, despite the fact that there was a small cohort of high-risk fellow eyes with significant FIAF and high colocalization with fundus abnormalities, even more fellow eyes of eyes with CNV had normal to minimal AF findings, suggesting that abnormal lipofuscin accumulations are not involved in many cases of neovascular AMD. Finally, reticular AF, which is a pattern of *hypo*autofluorescent lesions, and photographic reticular pseudodrusen appear to be imaging markers for the same unknown underlying pathologic process. This process is strongly associated with CNV, and may well be a multifocal inflammatory insult that tends to incite neovascularization, particularly in genetically susceptible individuals.

Acknowledgments

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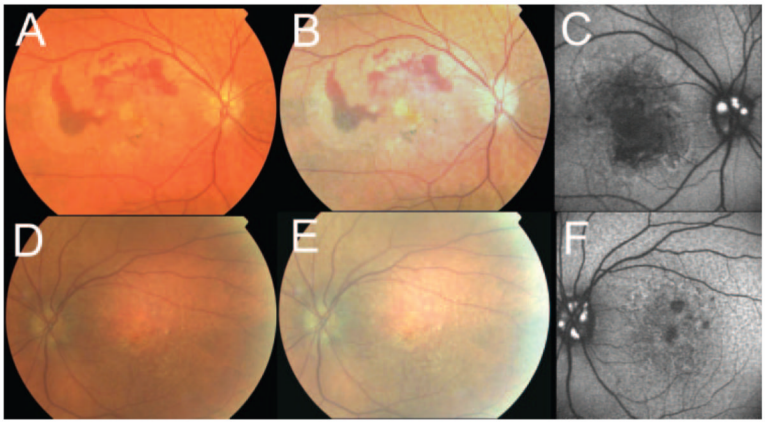

Figure 1.

Reticular autofluorescence and reticular pseudodrusen. (**A**, **D**) Original color photographs of right and left eyes of a patient with unilateral CNV in the CNV-R group. The reticular pseudodrusen are difficult to appreciate. Optic disc drusen were also incidentally present. (**B**, **E**) The photographs were contrast enhanced for better visualization of the reticular pseudodrusen. They were present in all quadrants (**E**) and were residually present above the optic nerve (**B**). (**C**, **F**) Corresponding AF images. The optic disc drusen showed characteristic hyperautofluorescence. Reticular AF was residually present above the optic nerve (**C**) and was also present in all quadrants (**F**). Hence, in both eyes the damage demonstrated by reticular AF corresponded very closely to that suggested by the reticular pseudodrusen in the phorographs.

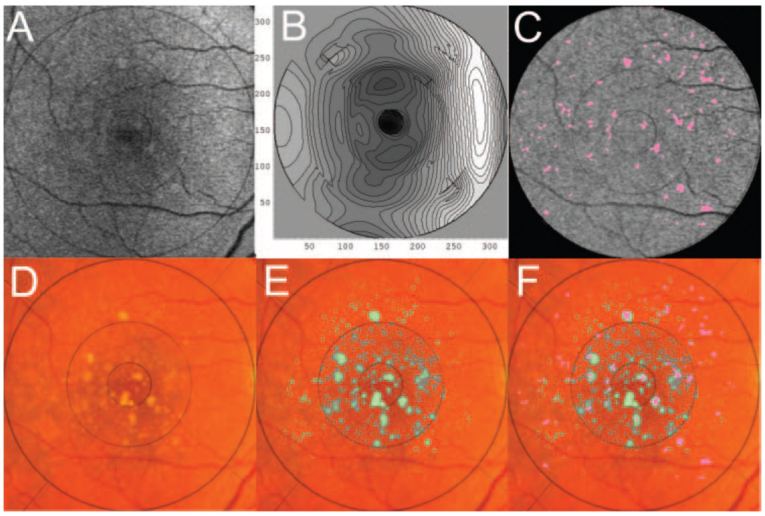

Figure 2.

Autofluorescence modeling and image leveling for colocalization of FIAF with drusen. An eye from the D-D group (large, soft drusen without pigment abnormalities in both eyes). The original AF image (**A**) and original fundus image (**D**) were registered in the 6000-μm region. Note the exact correspondence of retinal vessels, indicating good image registration. (**B**) The computed geometric model of the AF image background (presented as a contour graph) used in leveling the AF image. (**C**) The leveled AF image obtained by subtracting (**B**) from (**A**). Note the very uniform background compared to (**A**), with FIAF (2 SD above background) superimposed in *pink*. The fractional area of FIAF in this region, *p*(FIAF), was 0.028. (**D**) Original fundus image. (**E**) The segmented drusen (*green*) superimposed on the fundus image. The fractional area of drusen, *p*(D), was 0.083. (**F**) The FIAF superimposed on the drusen overlaying the fundus image. Of this total FIAF, the proportion colocalizing with drusen, C, was 0.36. Hence, in this image pair, the normalized colocalization coefficient of FIAF with drusen, NCC, was C/*p*(D) = 0.36/0.083 = 4.3, indicating that the probability of colocalization was 4.3 times greater than chance alone.

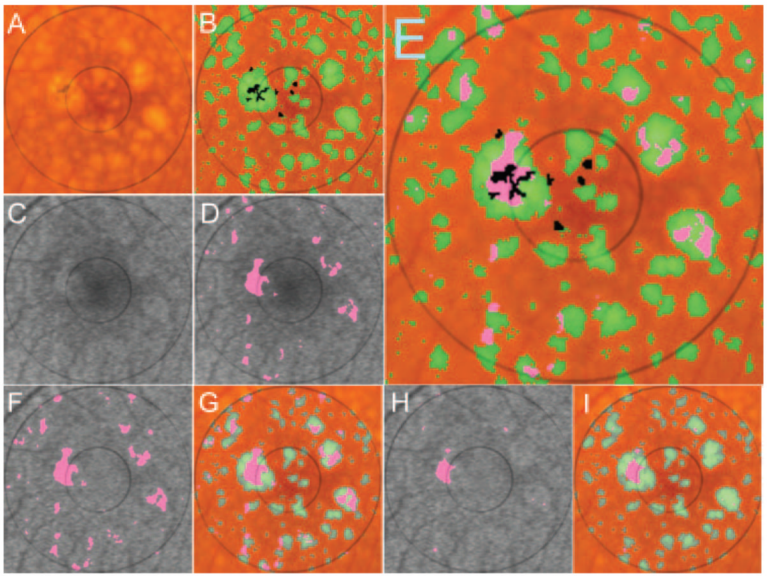

Figure 3.

Autofluorescence, drusen, and hyperpigmentation. The effect of varying the FIAF threshold. (**A**) Color photograph of an eye in the D-Pig group with large, soft drusen and hyperpigmentation, cropped to the 3000-μm region for illustration. (**B**) Hyperpigmentation labeled in *black* (0.012 of the 3000-μm region) and drusen segmentation labeled in *green* (0.26 of the region). (**C**) AF image; (**D**) FIAF (labeled in *pink*) defined as 2.0 SD above the mean of the leveled AF image (leveled image shown in **F** and **H**) and comprising 0.032 of the region. (**E**) Hyperpigmentation superimposed on the FIAF and both superimposed on the drusen. Note the intense FIAF signal from the area of the pigment clumps at the nasal edge of the fovea. The pigment clumps centrally, however, have no FIAF. The colocalization of FIAF with drusen was 0.90 and with hyperpigmentation was 0.12. The normalized colocalization coefficients for FIAF were 0.90/0.26 = 3.5 with drusen and 0.12/0.012 = 10.0 with hyperpigmentation. *Bottom row*: Effect of varying the FIAF threshold. (**F**, **H**) The FIAF (*pink*) was redefined as 1.5 and 2.5 SD, respectively, above the mean of the leveled AF image and then comprised 0.055 and 0.011 of the region, respectively. As expected, the regions defined as FIAF enlarged at the lower threshold and contracted and contained only brighter pixels at the higher threshold. (**G**, **I**) The FIAF from (**F**, **H**) superimposed on the drusen yielded colocalizations (normalized colocalization coefficients) of C = 0.86 (NCC = 3.3) and C = 0.88 (NCC = 3.4), respectively. Thus, despite varying FIAF threshold, the colocalization ratios changed little.

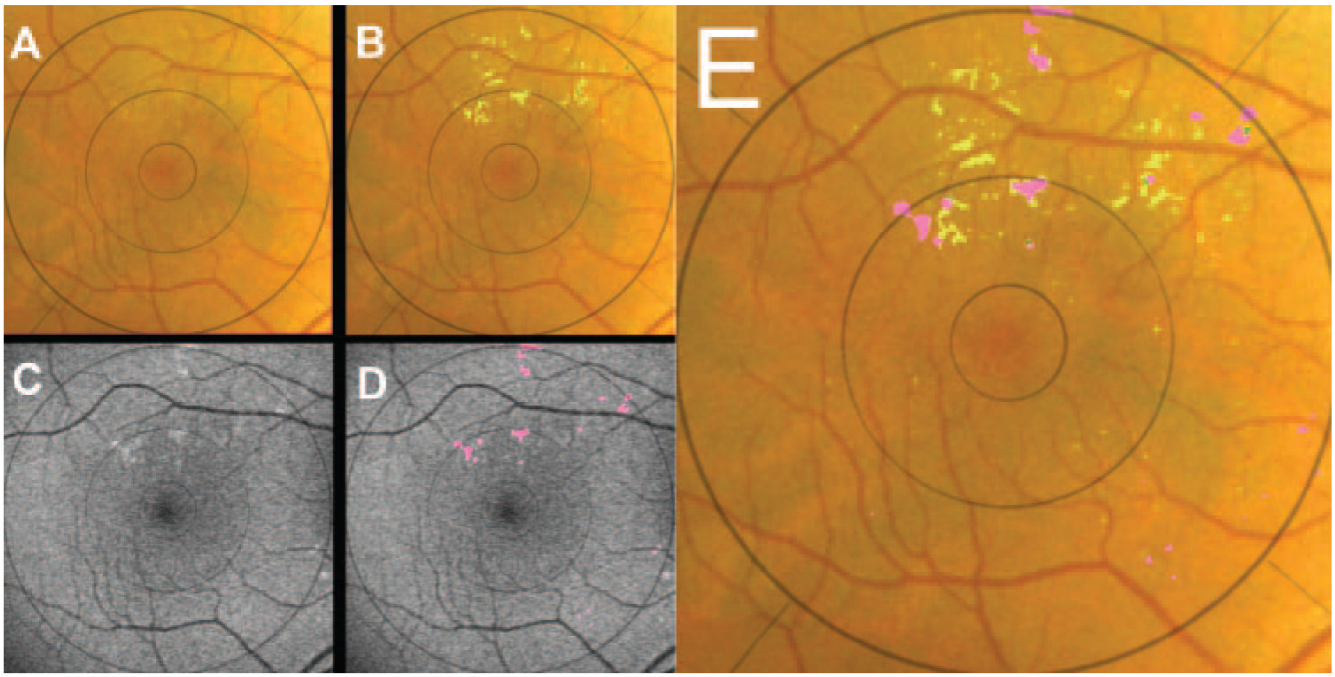

Figure 4.

Autofluorescence and hypopigmentation. (**A**) Color photograph of an eye in the D-Pig group with hypopigmentation (6000 μm region). (**B**) Hypopigmentation (*yellow*; 0.023 of the region) and a few intermediate drusen (*green*). (**C**)AF image. (**D**) FIAF image (*pink*; 0.0075 of the region). (**E**) FIAF superimposed on the hypopigmentation and drusen in (**B**). The colocalization of FIAF with hypopigmentation was 0.48, and the normalized colocalization coefficient (NCC) was 0.48/ 0.023 = 20.9.

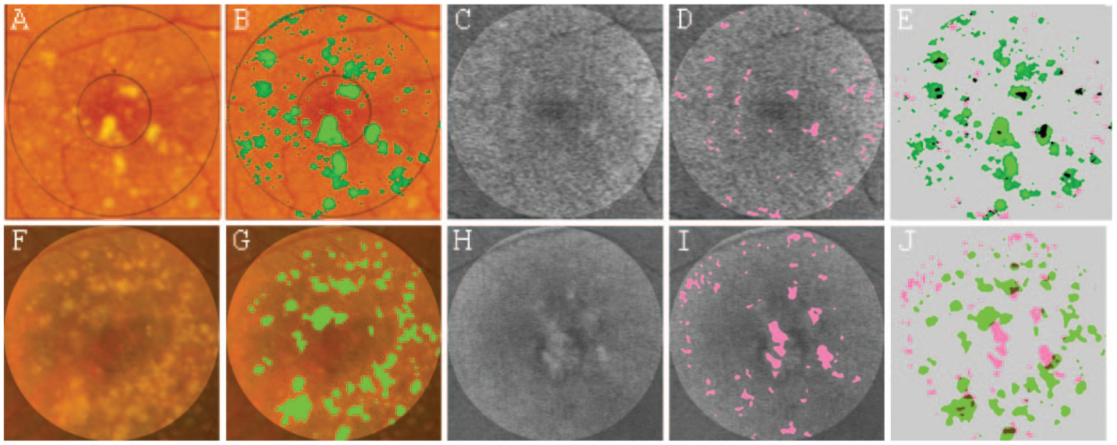

Figure 5.

Comparison of soft drusen only and drusen-GA groups. (**A**–**E**) An eye from the D-D group (soft drusen only). (**F**–**J**) An eye from the D-GA group (fellow eye has GA). (**A**, **F**) The fundus photographs have been cropped to the 3000-μm region for illustration. (**B**, **G**) The segmentation of drusen (*green*) using the automated leveling and threshold software. (**C**, **H**) The corresponding AF images, registered with their respective fundus photographs. (**D**, **I**) The segmentation (*pink*) of the FIAF. (**E**, **J**) The drusen (*green*) overlaid on the FIAF image (*pink*). *Black*: areas of colocalization of the two markers. There was a higher proportion of FIAF that colocalized with drusen in the D-D image (C = 0.45), compared with the D-GA image (C = 0.14). In fact, in the D-GA image, there were large clusters of FIAF *adjacent to* but not colocalizing with the drusen. The fundus image (**F**) of the D-GA eye showed early transitional signs of GA, and the AF image (**H**) also appeared as FDAF, which is the AF marker for GA. In this eye, the FIAF was redistributed from the drusen and some centrally was found colinear with the choroidal vessels that underlay the early GA lesions.

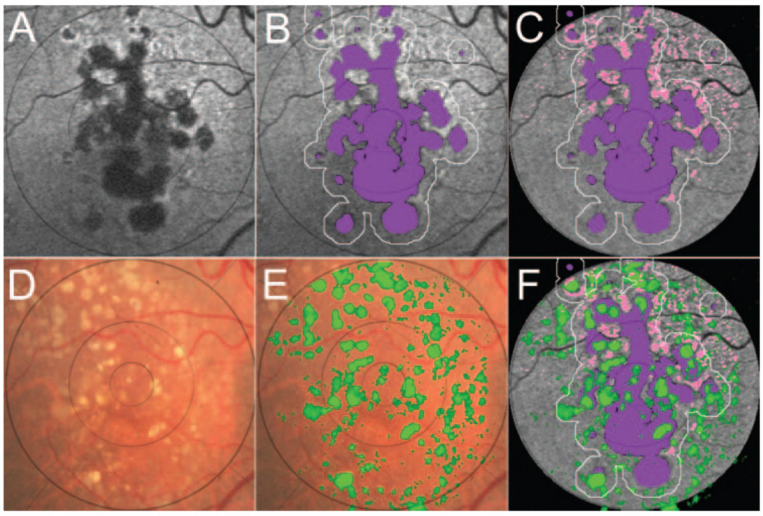

Figure 6.

Drusen and FIAF in bilateral geographic atrophy. (**A**) is the original AF image and (**D**) is the color photograph, 6000-μm region. The AF image is segmented in (**B**) to identify the GA lesions (*purple*), consisting of a central lesion and several satellite lesions of markedly decreased AF (fractional proportion of GA = 0.29). *White outline*: the border zone, defined as the 250-μm annulus surrounding all GA lesions. (**C**) The AF image background was leveled by the math model. FIAF (*pink*, 0.024) was then defined globally as all lesions 2 SD above background. Of the total FIAF, nearly all was clearly within the GA border zone (fractional proportion, 0.78). The color photo (**D**) yielded the drusen segmentation in (**E**) (*green*, p(D) = 0.27). In (**F**) the AF segmentation in turn was overlaid on the drusen to show that a relatively low level of FIAF colocalized with drusen (C = 0.24). In this image, the normalized colocalization of FIAF with drusen was also low (NCC = 0.24/0.27 = 0.89), indicating a colocalization slightly *less* than random chance. Inspection of the image reveals the reason: many clusters of FIAF were actually *adjacent to* drusen but did *not overlay* the drusen (compare with Fig. 4J).

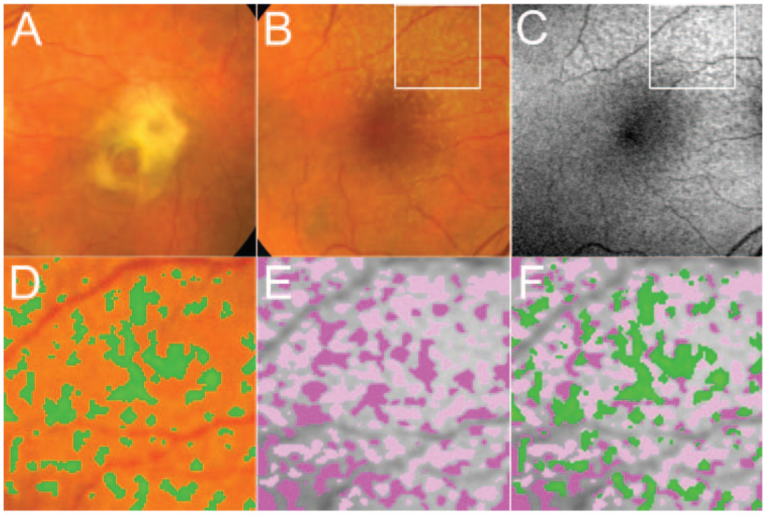

Figure 7.

Reticular pseudodrusen and reticular hypoautofluorescence in the fellow eye of a patient with unilateral CNV. (**A**) The left eye has end-stage CNV. (**B**) The fundus photograph of the fellow right eye shows reticular pseudodrusen that are most abundant superonasally. (**C**) The AF image of the right eye has been registered to the fundus photograph and shows a reticular pattern throughout the macula that is most prominent superonasally. (**D**, area outlined in **B**) Reticular pseudodrusen segmented in green and comprising 19% of the region (*p*Dr = 0.19). (**E** area outlined in **C**) Reticular autofluorescent lesions (excluding vessels; *purple*) and the areas of mildly elevated FIAF around them (*pink*). The area of reticular FDAF is pAF = 0.20. (**F**) Pseudodrusen from (**D**) superimposed on the AF image (**E**), yielding fractional and normalized colocalizations of reticular AF with reticular pseudodrusen C = 0.42 and NCC= 2.3. By contrast, the NCC of the FIAF in this section with the reticular pseudodrusen was 0.46 — illustrated clearly in (**F**), in which the pseudodrusen are seen to fall almost entirely on areas of background or decreased AF and largely avoid areas of FIAF.

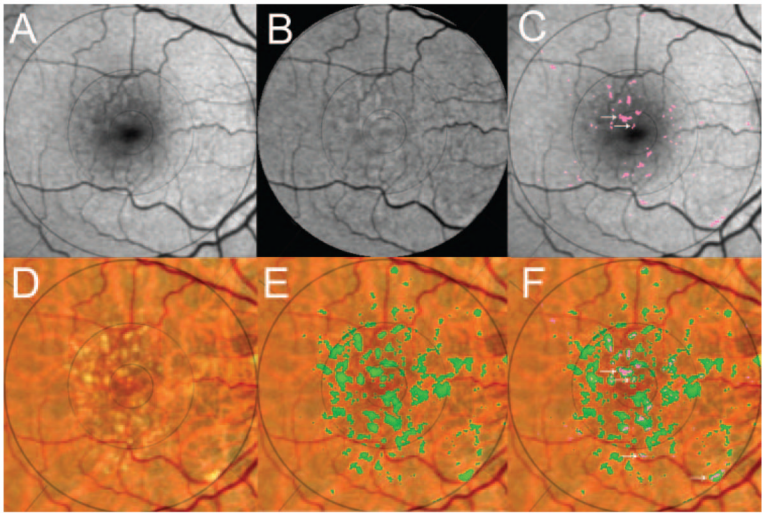

Figure 8.

Central and peripheral colocalization of drusen and FIAF. An eye from the D-D group (large, soft drusen only). The original AF image (**A**) and original fundus image (**D**), 6000-μm region. (**B**) AF image, leveled by the background model. FIAF, which was originally dim centrally, became distinct after leveling. (**C**) The FIAF segmentation superimposed on (**B**), p(FIAF) = 0.0082. *Arrows*: the FIAF, more visible in the central zone, and some FIAF peripherally. (**E**) The drusen segmentation superimposed on (**D**) p(D) = 0.11. (**F**) The colocalization of FIAF with the drusen obtained by superimposing (**C**) and (**E**) is almost complete: C = 0.82, NCC = C/p(D) = 7.7. *Arrows*: FIAF in the fovea overlaying drusen in the central zone and also FIAF at the inferonasal border of the 6000-μm region overlaying a druse.

Table 1

Categorization of Images Based on Phenotype

Categories	Photo Phenotype	AF Phenotype	
		FDAF1	FDAF0
D-D	14	2	12
D-Pig	9	2	7
D-GA	11	11	0
GA-GA	8	8	0
Total	42	23	19