Genome-Wide Analyses Demonstrate Novel Loci That Predispose to Drusen Formation

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Purpose. To test whether genes for drusen formation are independent of age-related macular degeneration (AMD) pathogenesis.

Methods. A genome-wide model-free linkage analysis was performed, using two semiquantitative drusen traits, size and type, on two sets of data: (1) 325 individuals (225 sib pairs) from the Beaver Dam Eye Study (BDES), and (2) 297 individuals (346 sib pairs) from the Family Age Related Maculopathy Study (FARMS). Apolipoprotein E (APOE) genotypes were used as a covariate in a multipoint sibpair analysis.

Results. The authors found evidence of linkage on 19q13.31 (*D19S245*), with size of drusen in both the BDES ($P = 0.0287$) and the FARMS ($P = 0.0013$; $P = 0.0005$, combined). In the BDES, type showed linkage evidence on 3p24.3 (*D3S1768*; $P = 0.0189$) and 3q25.1 (*D3S2404*; $P = 0.0141$); the linkage on 3p24.3 was also found with size (*D3S1768*; $P = 0.0264$). In the FARMS, size showed evidence of linkage at 5q33.3 (*D5S820*; $P = 0.0021$), 14q32.33 (*D14S1007*; $P = 0.0013$), and 16p13.13 (*D16S2616*; $P = 0.0015$) and type at 21q21.2 (*D21S2052*; $P = 0.0070$). For size in the FARMS, there was a small increase in *P*-value at marker *D19S245* from 0.0044 to 0.0111, and from 0.0044 to 0.0064, when the $\varepsilon 4$-carrier and the $\varepsilon 3$-carrier genotype were the covariates, respectively.

Conclusions. The results show that APOE effects may be mediated early in the progression of ARM to AMD and thus may not be detected by standard genome scans for more severe disease. (*Invest Ophthalmol Vis Sci.* 2005;46:3081–3088) DOI: 10.1167/iovs.04-1360

Age-related maculopathy (ARM) is a complex degenerative eye disorder that primarily affects the macular region of the eye. Progression from ARM to age-related macular degeneration (AMD) is the main cause of blindness in the elderly population in the United States. Approximately 1.7 million Americans 40 years of age or older have the advanced stages and 7.3 million show the earlier stages of AMD.[1–3] Despite extensive research on AMD, the effect of drusen formation on AMD pathogenesis remains unknown. One assumption in this process is that drusen represent an early stage in the natural history of disease and that other genes are necessary for the progression to exudative or neovascular stages of the disease. Hamdi and Kenney[4] proposed that drusen formation is independently controlled by genes affecting many retinal dystrophies, citing as examples the presence of drusen in other retinal dystrophies such as RDS-associated pattern dystrophy, Stargardt disease, dominant drusen dystrophy, glomerulonephritis type II, and North Carolina macular dystrophy. They also proposed that progression to advanced neovascular stages of AMD invokes separate genetic pathways.

So far, six genes have been associated with AMD. They include the ATP-binding cassette rim protein (*ABCA4*),[5,6] apolipoprotein E (*APOE*),[7,8] angiotensin converting enzyme (*ACE*),[9] complement factor H (*CFH*),[10–12] hemicentin-1,[13] and fibulin 5 (*FBLN5*).[14] Of these genes, *APOE* is an attractive candidate for AMD pathogenesis. A recent study showed that the APOE protein is present in the human retina and the retinal pigment epithelium (RPE),[15] and age-related alteration of lipoprotein biosynthesis at the level of the RPE and/or Bruch's membrane may be a significant contributing factor in drusen formation and AMD pathogenesis.[16] Genetic association between AMD and the *APOE* gene has been observed in mouse models,[17,18] as well as in human case–control studies,[7,8,19–21] although this association has not been observed in all studies.[22] In those studies in which this association was observed, the APOE epsilon 4($\varepsilon 4$) variant had a protective effect and the epsilon 2($\varepsilon 2$) variant displayed an increased risk for AMD.

To test whether genes that promote drusen formation are independent of AMD pathogenesis, we conducted genome scans on two separate phenotypes: maximum drusen size and severe drusen type. We used data from two different cohorts, the Beaver Dam Eye Study (BDES) and the Family Age Related Maculopathy Study (FARMS), to test this hypothesis. The BDES has families with all grades of ARM severity, from early to late, and has previously been analyzed for the genetics of ARM.[23] The FARMS data set includes families ascertained through probands with severe AMD, and a genome scan for AMD has also been conducted.[24] We compared the results obtained from our previous scans of maculopathy with those of drusen components to identify novel and shared candidate regions.

Methods

Subjects

The study population consisted of two cohorts: the BDES and FARMS. The BDES includes a community sample of 4926 subjects between 43 and 86 (average, 65.13) years of age at baseline, and a detailed description of this cohort is given elsewhere.[23] In brief, 2783 subjects in 602 pedigrees were collected from initial screen-

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Supported in part by Grant GM28356 from the National Institute of General Medical Sciences; U10-EY06594, EY10605, EY13438, and EY015810 from the National Eye Institute; and Grant RR03655 from the National Center for Research Resources.

Submitted for publication November 22, 2004; revised April 5, 2005; accepted April 12, 2005.

Disclosure: **G. Jun**, None; **B.E.K. Klein**, None; **R. Klein**, None; **K. Fox**, None; **C. Millard**, None; **J. Capriotti**, None; **K. Russo**, None; **K.E. Lee**, None; **R.C. Elston**, None; **S.K. Iyengar**, None

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ing. From these, 105 sibships were selected for genotyping and linkage analysis.

The FARMS data were ascertained through index cases with advanced AMD with 306 individuals in 34 families between 20 and 90 (average, 62.81) years of age recruited from the Retinal Clinic at the University of Wisconsin.[24] The Internal Review Boards at the University of Wisconsin and Case Western Reserve University approved these studies, and informed consent was obtained from all participants. The research was performed in accordance with the principles of the Declaration of Helsinki.

Phenotypic Evaluation

The size and type of drusen were judged according to the Wisconsin Age-Related Maculopathy Grading Scheme, the detailed description of which is found elsewhere.[25] For each eye a severity score was assigned to each drusen size category phenotype as follows: none = 0, questionable = 1, <63 μm in diameter = 2, ≥63 μm to <125 μm in diameter = 3, ≥125 to <250 μm in diameter = 4, ≥250 μm in diameter = 5, and reticular drusen = 6. Similarly, a severity score was assigned for each drusen type category phenotype as follows: none or hard indistinct = 0, hard distinct = 1, soft distinct = 2, and soft indistinct or reticular = 3.[26]

For each phenotype, measurements for the right and left eyes were averaged at each time point. However, if a score was missing for either eye, then the score for the available eye was substituted for the missing score. Overall, substitution occurred 6.45% of the time for size (n = 2308) and 13.03% for type (n = 2288) in the BDES and 5.63% for size (n = 284) and 18.98% for type (n = 274) in the FARMS. The BDES cohort had data that were collected at 5-year intervals for up to three time points. To include the same or a similar amount of information on each individual, the mean scores of the right and left eyes were averaged separately for size and type over two time points whenever possible. When scores at all three time points were available, the first and last mean scores were averaged; otherwise, the mean scores at the two available time points were averaged. At least two time points of measurements were available for size and type in 75.23% and 74.48% of participants, respectively.

We used multiple regression models to examine the effects of gender, age (at baseline for the BDES), history of heavy drinking, and history of smoking, along with the first-order interactions of these effects, assuming independence of all observations. In the BDES, we found age, age^2, history of heavy drinking, interaction between age and history of heavy drinking, and interaction between history of heavy drinking and history of smoking, to be nominally significant at the 5% level in predicting drusen size. Drusen type was predicted by age, age^2, and interaction between age and history of smoking. In contrast, for the FARMS, both size and type were predicted solely by age and age^2.

To minimize the variance among individuals due to age differences, we used the regression model from the previous section. First, we calculated the residuals for each observation from the final regression model. Next, we obtained the predicted value for an individual at age 80 from the final model. Finally, we added the residuals to this predicted value to obtain our final phenotypes.

Genotyping and Error Checking

After extracting DNA from the blood samples, we used a fluorescence-based genotyping method for the genome scan.[23,24] We genotyped 351 markers on 22 chromosomes using the Weber panel 8 marker set in the BDES, which has an average marker spacing of 11 cM. In addition, we included markers in fine-mapped regions of chromosomes 3, 5, 12, and 16 from our previous genome scan.[23] Our analysis included extended coverage of additional fine-mapped regions, which consisted of 25, 8, 10, and 4 markers on chromosomes 3, 5, 12, and 16, respectively. We also genotyped individuals from 34 extended families in the FARMS with 381 markers on 22 autosomes, by using the Weber panel 10 of microsatellite markers. This covered the genome at an average marker spacing of 8.85 cM. Two additional markers, *D1S406* and *D1S236*, in close proximity to the *ABCA4* gene, were also genotyped. We also included 4, 25, and 14 markers on chromosomes 1, 12, and 15, respectively, from our previous study.[24] As a result, the average inter-marker distance of fine-mapped regions on chromosomes 1, 12, and 15 decreased to 3.13, 3.40, and 2.86 cM, respectively. Approximately 77% of the markers were shared between the two screening sets.

On the basis of the initial findings from the genome scans, *APOE* was selected to be genotyped. *APOE* genotyping was performed by using the previously described restriction endonuclease (*Hba*I) method followed by agarose electrophoresis.[27] We included all the individuals who were originally genotyped (n = 325 in the BDES; n = 297 in the FARMS). Seven individuals from FARMS were set to "missing" in subsequent covariate analysis, because their genotypes were not available.

Inconsistencies of the genotypes within families were examined using MARKERINFO in S.A.G.E. (ver. 4.5; available at http://darwin.cwru.edu/sage/, provided by the Case Western University, Cleveland, OH). In total, 0.69% and 0.24% of the data were treated as missing in the BDES and the FARMS, respectively. In addition, we checked misclassification of relationships in each pedigree using RELTEST in S.A.G.E. (ver. 4.5). In the BDES, we reclassified 6 individuals in 3 full sibships as unrelated and 21 individuals in 12 full sibships as half-sibs.[23] In the FARMS, we cleaned genotyping errors, as well as reclassified four individuals in four full sibships as half sibs, and deleted three individuals in three full sibships because they were unrelated.[24]

Linkage Analyses

To perform a Box-Cox power transformation of the data to maximize power, we performed segregation analysis with SEGREG in S.A.G.E. (ver. 4.5) on these values from the entire BDES sample (size: N = 2308; type: N = 2288) to obtain appropriate population-based transformation parameters. Before transforming the phenotypes in both the BDES and the FARMS, we examined the correlations between size and type. Size and type correlate highly in both the BDES (0.875) and FARMS (0.862). Transformation of the data with the power parameter λ_1 = 1.45 for both size and type obtained from the population-based study (BDES) led to retention of the original correlation structure of 0.874 in the BDES and 0.857 in the FARMS. Since the population-based parameter altered the correlation little between size and type after transformation, subsequent analyses were conducted by using this transformation. We also checked the correlations between drusen phenotypes and 15-step AMD severity scale (AMD score).[23]

Sibling correlations for the size and type were estimated using the FCOR program in S.A.G.E. (ver. 4.5). We performed linkage analyses on the power transformed size and type phenotypes, separately in the BDES and the FARMS, using the GENIBD and SIBPAL programs in S.A.G.E. (ver. 4.5). A weighted combination of squared trait difference and squared mean-corrected trait sum (W4 option) was used as the dependent variable in a Haseman-Elston regression model.[28,29]

In regions where both the BDES and the FARMS showed linkage, we combined the P-values of the two independent tests, using the Fisher method: letting P_1 and P_2 be the two P-values, $-2\sum_{i=1}^{p} \mathrm{Ln}(P_i)$ is compared with the χ^2 distribution with four degrees of freedom.[30]

Candidate Gene Covariate Analysis

We coded the *APOE* alleles in two different ways: First, for the additive model, we counted the number of a specific allele, and coded an individual as having 0, 1, or 2 alleles of that type. Second, for the dominant model, we ascertained the presence of a certain allele (e.g., $\varepsilon 2$) and coded for its absence or presence as 0 or 1, without regard for homozygosity or heterozygosity for that allele. For example, the three different genotypes $\varepsilon 2/\varepsilon 3$, $\varepsilon 3/\varepsilon 3$, and $\varepsilon 3/\varepsilon 4$ share the same value (code = 1) in the $\epsilon 3$ dominant model (E3Dom), but in the $\varepsilon 3$ additive model (E3Add) they were coded as 1, 2, and 1, respectively. We performed model-free linkage analysis with the newly derived covariate included in the model to determine whether inclusion of the

TABLE 1. Phenotypic Description of Genotyped Individuals in the BDES and the FARMS

Average Severity Scale*	BDES (n = 325)		FARMS (n = 290)	
	Total (%)	AMD (SD)†	Total (%)	AMD (SD)†
Drusen size				
Missing‡	1 (0.31)	—	10† (3.45)	—
0	1 (0.31)	1 (0.00)	10 (3.45)	3.64 (5.59)
>0 to ≤1	16 (4.92)	1.22 (0.31)	34 (11.72)	2.54 (3.74)
>1 to ≤2	115 (35.38)	2.43 (0.51)	94 (32.41)	2.89 (2.51)
>2 to ≤3	114 (35.08)	4.21 (1.41)	63 (21.72)	4.73 (2.78)
>3 to ≤4	60 (18.46)	6.91 (2.18)	35 (12.07)	9.16 (3.87)
>4 to ≤5	17 (5.23)	10.31 (1.86)	39 (13.45)	11.71 (2.14)
>5	1 (0.31)	10 (0.00)	5 (1.72)	11.6 (1.98)
Drusen type				
Missing‡	2 (0.62)	—	20 (6.90)	—
0	8 (2.46)	1 (0.00)	12 (4.14)	3.17 (5.06)
>0 to ≤1	154 (47.38)	2.60 (0.71)	148 (51.03)	3.04 (2.50)
>1 to ≤2	114 (35.08)	4.91 (1.89)	52 (17.93)	6.71 (3.98)
>2	47 (14.46)	8.72 (2.24)	58 (20.00)	11.30 (2.50)

* Scales were calculated by averaging left and right eye.
† The 15-scale AMD severity score was averaged over individuals in the same category.
‡ Missing = inability to grade; see Methods section for additional details of phenotype.

covariate (*APOE* genotype) eliminates the observed linkage in this region on chromosome 19. We compared each covariate model with the baseline model (no covariate).

RESULTS

Description of Phenotypes

The BDES and FARMS data sets included 325 and 290 genotyped individuals with available phenotypes, respectively. We found larger sized drusen (≥250 μm in diameter or reticular) in the FARMS (15.17%) than in the BDES (5.54%). For type, the number of individuals with soft drusen (distinct or indistinct/reticular) was higher in the BDES (49.54%) than in the FARMS (37.93%). We observed that both drusen size and type correlated highly with the AMD score. The correlations between drusen phenotypes (size and type) and the AMD score in the BDES are 0.8192 and 0.8215 for the size and type, respectively; in the FARMS, 0.7310 and 0.7458 for the size and type, respectively. Table 1 summarizes the phenotypic description in the BDES and FARMS.

Genome Scan

The BDES data set consisted of 225 sib pairs with size and 224 sib pairs with type, available in 105 families; the FARMS data set comprised 346 sib pairs with both size and type measures available. The correlations between siblings for size and type were 0.1760 and 0.2174 in the BDES, respectively; 0.1074 and 0.0858 in the FARMS, respectively. In addition, the sibling correlations between size and type in the BDES and the FARMS were 0.1961 and 0.1043, respectively. The result of the genome scans for the BDES and the FARMS are presented in Figures 1 and 2, respectively. In addition, Table 2 displays markers that had a nominal multipoint significance level of $P \leq 0.01$ in either study. In the FARMS, we found linkage for size at five markers on chromosomes 5, 14, 16, and 19, and for type at one marker on chromosome 21 (Table 2). In the BDES, although we did not detect regions with a nominal significance level of $P \leq 0.01$, we observed the shared regions on chromosomes 3 and 19, for both size and type, with a nominal significance level of $P \leq 0.05$ (Table 3).

The region with the highest linkage peak was found on chromosome 19 (19q13.31), for drusen size, in the FARMS (Fig. 2), and there was a similar but weaker peak in the BDES (Fig. 1). The location of the linkage peak was near *D19S245* in the FARMS (46 cM; $P = 0.0013$) and in the BDES (50 cM; $P = 0.0243$), and the combined *P*-value for the two data sets is 0.0005.

We observed additional linkage signals in the FARMS (Fig. 2; Table 2) on three chromosomes (5q33.3, 14q32.33, and 16p13.13) with size. We detected two closely located peaks on chromosome 5 near *D5S816* (138 cM; $P = 0.0204$) and at *D5S820* (160 cM; $P = 0.0021$). The linkage evidence on chromosome 14 was seen near the long arm at *D14S1007* (126 cM; $P = 0.0013$). On chromosome 16, the signal was seen at *D16S2616* (11 cM; $P = 0.0015$). With type, we observed a linkage signal on chromosome 21 at *D21S2052* (22 cM; $P = 0.0070$).

In addition, we found interesting regions on chromosome 3 (3p24.3 and 3q25.1) in the BDES that showed similar linkage signals with size and type (Fig. 1; Table 3). At *D3S1768* on chromosome 3, we found a linkage signal with both size (55.98 cM; $P = 0.0264$) and type (60 cM; $P = 0.0189$). The second signal on chromosome 3 near *D3S2404* (166 cM) illustrated evidence of linkage with type ($P = 0.0141$).

Covariate Analysis with *APOE* Alleles

We chose to follow up the region on chromosome 19 because this chromosome gave the most significant results and the *APOE* gene lies within the 1-lod decrease in significance interval. Moreover, previous association studies suggested *APOE* alleles are associated with AMD.[7,8,19–21] Overall the *APOE* genotype and allele frequency distributions in the BDES and FARMS are shown in Table 4. The most frequent genotype was $\varepsilon3/\varepsilon3$, both in the BDES (59.38%) and the FARMS (54.48%).

A summary of the findings in the BDES and FARMS with *APOE* genotype as a covariate is shown in Table 5. Inclusion of *APOE* as a covariate in linkage models led to a decrease in linkage signals compared with baseline (i.e., without a covariate). The *P*-value at marker *D19S245* in the FARMS was increased by approximately one order of magnitude from

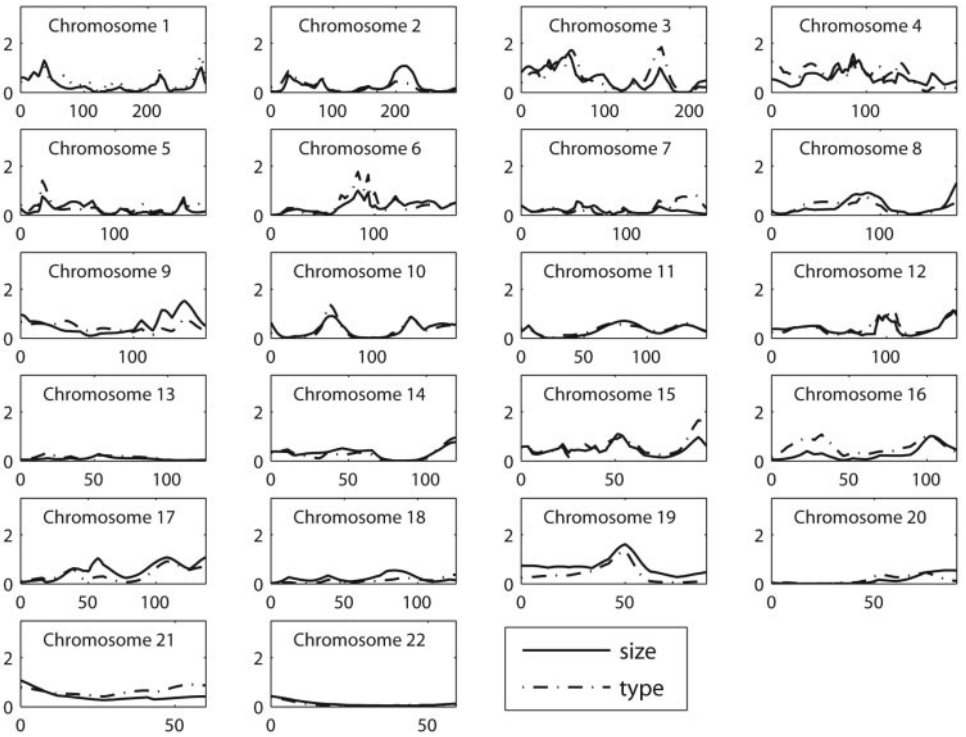

FIGURE 1. Multipoint results of the genome-wide linkage analysis for size and type in the BDES. For each chromosome, genetic distance (in centimorgans) is plotted on the *x*-axis against $-\log_{10}(P)$ on the *y*-axis.

0.0044 to 0.0111 with the presence of ε4 (E4Dom), but did not change much (from 0.0044 to 0.0064) with the presence of ε3 (E3Dom). However, the linkage signal did not entirely disappear, even after the APOE alleles were accounted for. This implies that the linkage peak on chromosome 19 is not fully accounted by the *APOE* gene, and a novel locus, close to *APOE*, may be additionally associated with large soft drusen. We did not find the same covariate effect in the BDES (Table 5). We detected similar covariate effects using additive models. We have listed these covariate models and their effects for the FARMS data set in Table 6. The additive models E2E3Add and E3E4Add showed results similar to those of the E3Add model.

DISCUSSION

The most significant finding was detected on chromosome 19 (at q13.21) with size in both the BDES and the FARMS. Because this region was concordant in both studies, we

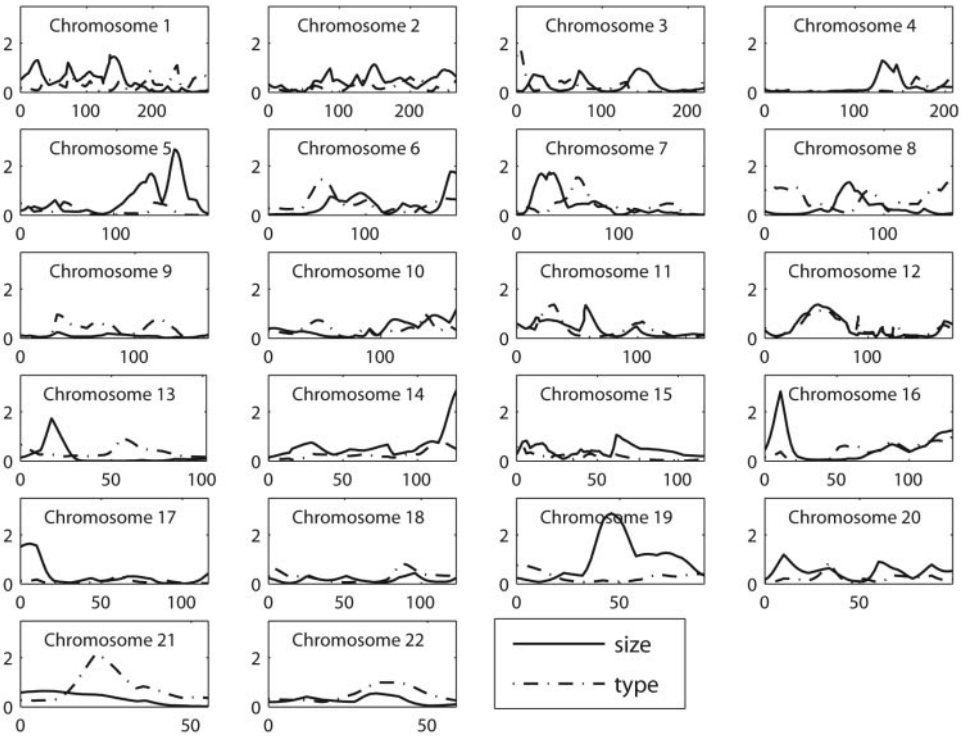

FIGURE 2. Multipoint results of the genome-wide linkage analysis for size and type in the FARMS. For each chromosome, genetic distance (in centimorgans) is plotted on the *x*-axis against $-\log_{10}(P)$ on the *y*-axis.

TABLE 2. Multipoint *P*-Values in the BDES and the FARMS on Chromosomes 5, 14, 16, 19, and 21 for Drusen Size and Type, Where the Nominal Significance Level in Either Study Is $P < 0.01$

FARMS		BDES	
Phenotype and Location (cM)	***P*-Value**	**Phenotype and Location (cM)**	***P*-Value**
Size		Size	
Chromosome 5:		Chromosome 5:	
158.0	0.0043	*D5S820* (159.68)	0.8895
D5S820 (160.0)	0.0021	160.0	0.8826
162.0	0.0023	162.0	0.8208
164.0	0.0032	164.0	0.7142
166.0	0.0060	166.0	0.5629
168.0	0.0146	168.0	0.4024
170.0	0.0383	170.0	0.2753
Chromosome 14:		Chromosome 14:*	
122.0	0.0060		
124.0	0.0025		
D14S1007 (126.0)	0.0013		
Chromosome 16:		Chromosome 16:*	
8.0	0.0083		
10.0	0.0025		
D16S2616 (11.0)	0.0015		
12.0	0.0029		
Chromosome 19:		Chromosome 19:	
38.0	0.0290	*D19S433* (34.0)	0.2288
40.0	0.0076	36.0	0.1838
D19S433 (42.0)	0.0025	38.0	0.1455
44.0	0.0016	40.0	0.1177
46.0	0.0013	*D19S245* (42.0)	0.0995
48.0	0.0016	44.0	0.0616
D19S245 (49.0)	0.0019	46.0	0.0393
50.0	0.0023	48.0	0.0287
52.0	0.0045	*D19S178* (50.0)	0.0243
54.0	0.0117	52.0	0.0326
56.0	0.0323	54.0	0.0497
Type		Type	
Chromosome 21:		Chromosome 21:	
D21S2052 (22.0)	0.0070	22.0	0.3441
24.0	0.0096	24.0	0.3603

* Markers are not in this screening set.

propose that this can be considered as a replication. After combining the probabilities, our result on chromosome 19 was more significant in aggregate ($P = 0.0005$) than when individually considered ($P = 0.0013$ in FARMS and $P = 0.0243$ in BDES).

The *APOE* gene, mapped on 19q13.2, is an intriguing candidate gene residing close to the chromosome 19 linkage signal. Numerous association studies of this gene have confirmed the association between *APOE* alleles and AMD.[7,8,19–21] We have revealed evidence of linkage at that location using a genome scan, whereas others have not done so.[23,24,31–36] This may be because most previous genome-wide linkage studies, including ours,[23,24] have focused on the late stages of disease with AMD affection status, as opposed to focusing on the early stages of the disease. We were able to find some *APOE* gene involvement on large drusen presence, although our results suggest that a novel locus for drusen formation might reside close to the *APOE* locus. Thus, it may be necessary to examine this chromosomal region more carefully for other genes in linkage disequilibrium with *APOE*.

At the linkage signal on 14q32.33, we did not find known genes that might be involved in AMD or drusen. However, the Usher syndrome type 1A gene (*USH1A*; 14q32) maps near this region.[37,38] For the region on 21q21.2, amyloid beta A4 precursor protein (*APP*; 21q21) is considered as an interesting candidate gene. In a recent study,[39] a macromolecular assembly was observed that contains amyloid β as well as activated complement components in drusen, suggesting that some of the pathogenic pathways that give rise to drusen and AMD may be shared with other neurodegenerative diseases.

Our results on 3p24.3, 5q33.3, and 16p13.13 are consistent with those reported in previous genome scans with AMD phenotypes. Using size and type of drusen, we were able to confirm the linkage signals on both arms of chromosomes 3 found in our previous BDES genome scan,[23] where the 15-scale AMD severity score used included drusen phenotypes as a semiquantitative trait. TIMP4, the tissue inhibitor of metalloproteinase mapped on 3p25 could be a potential candidate gene, because TIMP4 immunoreactivity was detected in situ from human RPE choroids.[40] A chemokine receptor (CCR2) on 3p21 could also be considered as a candidate gene, because *ccl2* or *ccr2* aged knockout mice exhibit cardinal features of AMD, including accumulation of lipofuscin and drusen beneath the RPE, photoreceptor atrophy, and choroidal neovascularization.[41]

Our finding of linkage on chromosome 5 agrees with the result of our previous genome scan,[24] but the shape of the signal is refined with drusen size as the phenotype (Fig. 2). This region was also identified by a genome scan,[34] using ARM

TABLE 3. Multipoint *P*-Value for Drusen Size and Type in the BDES on Chromosomes 3 and 19, Where the Nominal Significance Level Is $P < 0.05$

Size		Type	
Location (cM)	***P*-Value**	**Location (cM)**	***P*-Value**
Chromosome 3:		Chromosome 3:	
46.0	0.0485	46.0	0.0555
48.0	0.0408	48.0	0.0617
50.0	0.0377	50.0	0.0714
52.0	0.0372	52.0	0.0826
D3S2432 (52.38)	0.0373	*D3S2432* (52.38)	0.0847
54.0	0.0289	54.0	0.0458
D3S1768 (55.98)	0.0264	*D3S1768* (55.98)	0.0240
56.0	0.0265	56.0	0.0240
58.0	0.0327	58.0	0.0200
60.0	0.0482	60.0	0.0189
62.0	0.0816	62.0	0.0221
64.0	0.1442	64.0	0.0316
D3S2409 (65.07)	0.1882	*D3S2409* (65.07)	0.0396
66.0	0.2016	66.0	0.0483
158.0	0.3080	158.0	0.0332
160.0	0.2282	160.0	0.0221
D3S1299 (161.39)	0.1970	*D3S1299* (161.39)	0.0209
162.0	0.1658	162.0	0.0181
164.0	0.1008	164.0	0.0175
D3S1746 (164.06)	0.0997	*D3S1746* (164.06)	0.0177
166.0	0.1777	166.0	0.0141
D3S2404 (166.73)	0.1353	*D3S2404* (166.73)	0.0176
168.0	0.1626	168.0	0.0238
170.0	0.2228	170.0	0.0453
Chromosome 19:		Chromosome 19:	
D19S245 (42.0)	0.0995	*D19S245* (42.0)	0.1538
44.0	0.0616	44.0	0.1020
46.0	0.0393	46.0	0.0684
48.0	0.0287	48.0	0.0508
D19S178 (50.0)	0.0243	*D19S178* (50.0)	0.0429
52.0	0.0326	52.0	0.0765

affection status, and also showed a bifurcation in the shape of the linkage signal in this region. The authors suggested glutathione peroxidase 3 (*GPX3*; 5q33.1) as a potential candidate gene.

A recent genome scan showed linkage on 16p using AMD affection status and ordered subset analysis.[42] This region harbors MMP25 on 16p13.3, a type of matrix metalloproteinase protein similar in function to MMP3 on 11q23. This is likely to be a good candidate gene because the pattern of MMP immunoreactivity was detected only on the surface of drusen, but was absent in the central part of drusen, implying that drusen are regions lacking proteolysis activity.[40]

TABLE 4. *APOE* Genotypes and Alleles in the BDES and FARMS

APOE Characteristic	BDES ($n = 325$)	FARMS ($n = 290$)
Genotype		
ε2/ε2	0 (0.00%)	0 (0.00%)
ε2/ε3	46 (14.15%)	44 (15.17%)
ε2/ε4	1 (0.31%)	6 (2.07%)
ε3/ε3	193 (59.38%)	158 (54.48%)
ε3/ε4	71 (21.85%)	65 (22.41%)
ε4/ε4	9 (2.77%)	6 (2.07%)
Missing	5 (1.54%)	11 (3.79%)
APOE allele		
ε2	47 (7.23%)	50 (8.62%)
ε3	503 (77.38%)	425 (73.28%)
ε4	90 (13.85%)	83 (14.31%)

Our approach differs from other previous genome-wide linkage studies for AMD or ARM in two respects. First, we have isolated drusen phenotypes from the AMD trait, to investigate the relationship between AMD and drusen phenotypes. Furthermore, we categorized size and type, adapting the scale of severity to cover a wide range of clinical phenotypes, and used them as quantitative traits to provide greater power to detect linkage. Second, we conducted linkage analyses on two independent data sets. As a result, the finding of one analysis not only acts as a replicate to the other, but also as a reference to investigate the similarity and difference of pattern and location of a linkage signal.

Although there are various criteria to characterize drusen phenotypes, we defined the severity of the disease phenotype by maximum involvement of type and size based on the natural progression of drusen in ARM. Only some progress from small, hard (HD) drusen to soft distinct (SD) drusen and even less progress to soft indistinct (SI) drusen. We used this approach deliberately, because in these analyses we are looking for the most severe phenotype, not the presence of a specific type of drusen, knowing that approximately 90% or more of subjects have at least one HD in one eye. Therefore, if we use a criterion that includes HD, we would end up with HD only, HD and SD, and HD and SI, as well as HD, SD, and SI. We believe this would give less chance to

TABLE 5. Multipoint Model-Free Linkage Analysis of Drusen Size in the BDES and FARMS Including *APOE* Alleles as a Covariate in the Regression

FARMS				BDES			
Location	**—***	**E3Dom†**	**E4Dom**	**Location**	**—***	**E3Dom†**	**E4Dom**
38.0	0.0356	0.0229	0.0385	*D19S433*	0.2478	0.2423	0.2362
40.0	0.0169	0.0062	0.0111	36.0	0.1993	0.1944	0.1854
D19S433	0.0098	0.0022	0.0041	38.0	0.1573	0.1533	0.1421
44.0	0.0057	0.0020	0.0039	40.0	0.1265	0.1232	0.1109
46.0	0.0041	0.0026	0.0049	*D19S245*	0.1059	0.1034	0.0907
48.0	0.0040	0.0045	0.0082	44.0	0.0655	0.0644	0.0560
D19S245	0.0044	0.0064	0.0111	46.0	0.0415	0.0412	0.0361
50.0	0.0053	0.0069	0.0125	48.0	0.0299	0.0300	0.0267
52.0	0.0101	0.0099	0.0192	*D19S178*	0.0250	0.0254	0.0230
54.0	0.0248	0.0180	0.0351	52.0	0.0336	0.0342	0.0299
56.0	0.0617	0.0353	0.0664	54.0	0.0513	0.0525	0.0442

* The baseline model with no covariate is shown.

† In the dominant model the presence of the specific allele in a genotype is coded as 1; its absence is coded as 0.

identify disease susceptibility genes for large soft drusen, which are known to be associated with progression to more advanced AMD. Analyzing drusen area within the central macula is an alternative approach, since the extent of the macular area covered by drusen correlates highly with size and type. In our previous analyses,[23,24] we used drusen area as the least-severe steps of our AMD score. Comparing our current results with the previous results should further refine the relationship between AMD and the drusen phenotypes as defined by our group.

In view of our findings from covariate analysis for the *APOE* gene, we cannot definitively conclude that this gene is the source of the linkage signal on chromosome 19. Equally, we cannot rule out the possibility that linkage methods to identify variants with low to moderate effect sizes (e.g., *APOE* alleles) may not have sufficient power in family-based studies. We also observed that the two phenotypes, size and type, correlated highly in both the BDES and the FARMS (see the Methods section). However, for type, we failed to provide evidence of linkage on chromosome 19 in the FARMS. In contrast, we observed a weak linkage peak in the BDES. This may be because the number of individuals with soft drusen was smaller in the FARMS than in the BDES (Table 1), and consequently the effect size may not be big enough. Alternatively, the genes present in individuals with end-stage disease, as applicable to the FARMS, are unique. These two hypotheses are equally supported by the fact that the correlation between drusen phenotypes and the AMD score (see the result section) was higher in the BDES than in the FARMS.

When we tested the AMD score as a covariate to understand the relationship between the drusen phenotypes and AMD phenotype for the observed linkage on chromosome 19, inclusion of the AMD score did not modify the linkage results at the marker *D19S245* (baseline: $P = 0.0016$, with covariate: $P = 0.0013$) in the FARMS, or at the marker *D19S178* (baseline: $P = 0.0377$, with covariate: $P = 0.0293$) in the BDES. This supports our hypothesis that among linkage regions for the drusen phenotypes a few may be independently acting on the AMD pathogenesis, and the region on chromosome 19 may be one of them.

In summary, our evidence of linkage on chromosomes 14 and 19 suggest that specific loci for drusen formation, in particular drusen size, are present. These data imply that *APOE* and other novel loci are associated with large drusen. Similarly, the region on chromosome 21 may be linked to the type of drusen. These regions may be exclusively involved in drusen formation, because no genome scans have identified these regions for ARM or AMD. In contrast, the loci on chromosomes 3, 5, and 16 confirmed published genome-wide linkage reports for ARM or AMD. Thus, our results, together with other previous findings, have provided better insights into the intertwined nature of multiple genetic influences on AMD, implying that different genes act at different stages of the disease, which contribute separately or together to the final AMD disease outcome. In the

TABLE 6. Extended Covariate Analysis for Drusen Size in the FARMS, Using Model-Free Multipoint Linkage Analysis of the Candidate Region on Chromosome 19

Location	**—**	**E3Dom**	**E4Dom**	**E2Dom***	**E3Add†**	**E4Add**
38.0	0.0356	0.0229	0.0385	0.0277	0.0275	0.0329
40.0	0.0169	0.0062	0.0111	0.0078	0.0076	0.0095
D19S433	0.0098	0.0022	0.0041	0.0028	0.0075	0.0035
44.0	0.0057	0.0020	0.0039	0.0027	0.0058	0.0034
46.0	0.0041	0.0026	0.0049	0.0035	0.0032	0.0045
48.0	0.0040	0.0045	0.0082	0.0059	0.0057	0.0077
D19S245	0.0044	0.0064	0.0111	0.0082	0.0079	0.0106
50.0	0.0053	0.0069	0.0125	0.0091	0.0087	0.0118
52.0	0.0101	0.0099	0.0192	0.0133	0.0128	0.0174
54.0	0.0248	0.0180	0.0351	0.0242	0.0232	0.0314
56.0	0.0617	0.0353	0.0664	0.0471	0.0447	0.0590

* In the dataset, there are no $\varepsilon 2/\varepsilon 2$ homozygous genotypes.

† In the additive model the covariate is the number of a specific allele in a genotype.

future, we hope to investigate several different aspects of AMD and its progression, providing new perspectives for the underlying genetic mechanism of AMD.

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