Functional Candidate Genes in Age-Related Macular Degeneration: Significant Association with *VEGF*, *VLDLR*, and *LRP6*

Jonathan L. Haines,[1] *Nathalie Schnetz-Boutaud,*[1] *Silke Schmidt,*[2] *William K. Scott,*[2] *Anita Agarwal,*[3] *Eric A. Postel,*[4] *Lana Olson,*[1] *Shannon J. Kenealy,*[1] *Michael Hauser,*[2] *John R. Gilbert,*[2] *and Margaret A. Pericak-Vance*[2]

PURPOSE. Age-related macular degeneration (AMD) is a retinal degenerative disease that is the leading cause of blindness worldwide for individuals over the age of 60. Although the etiology of AMD remains largely unknown, numerous studies have suggested that both genes and environmental risk factors significantly influence the risk of developing AMD. Identification of the underlying genes has been difficult, with both genomic screen (locational) and candidate gene (functional) approaches being used. The present study tested candidate genes for association with AMD.

METHODS. Eight genes (α-2-macroglobulin [*A2M*], creatine kinase [*CKB*], angiotensin-converting enzyme [*DCP1*], interleukin-1α [*IL1A*], low-density lipoprotein receptor–related protein 6 [*LRP6*], microsomal glutathione-*S*-transferase 1 [*MGST1*], vascular entothelial growth factor [*VEGF*], and very low density lipoprotein receptor [*VLDLR*]) were tested for genetic linkage and allelic association, using two independent datasets: a family-based association dataset including 162 families and an independent case-control dataset with 399 cases and 159 fully evaluated controls.

RESULTS. Test results suggested that genetic variation in five of these genes (*IL1A*, *CKB*, *A2M*, *MGST1*, and *DCP1*) is unlikely to explain a significant fraction of the risk of developing AMD in this population. *LRP6* showed evidence both for linkage (heterogeneity lod [HLOD] = 1.14) in the family-based dataset and for association ($P = 0.004$) in the case-control dataset. *VEGF* showed evidence of linkage (HLOD = 1.32) and demonstrated significant independent allelic association in both the family-based ($P = 0.001$) and case-control ($P = 0.02$) datasets.

VLDLR showed evidence of association in both the family based ($P = 0.03$) and case-control ($P = 0.01$) datasets.

CONCLUSIONS. These data suggest that *LRP6*, *VEGF*, and *VLDLR* may play a role in the risk of developing AMD. (*Invest Ophthalmol Vis Sci.* 2006;47:329–335) DOI:10.1167/iovs.05-0116

A ge-related macular degeneration (AMD), often referred to as age-related maculopathy (ARM), is a degenerative disease of the retina that causes progressive impairment of central vision and is the leading cause of irreversible vision loss in older Americans. The prevalence of the disease increases with age, afflicting 9% of the population over age 65 and 28% over age 75.[1,2] It is estimated that 2 million people in the United States alone are blind as a result of AMD.[3]

Although the etiology of AMD is largely unknown, numerous studies indicate that risk factors include age, gender, ethnicity, smoking, hypertension, and diet. Familial aggregation,[4–7] twin,[8–10] and segregation analysis[11,12] studies also suggest a significant genetic contribution to the disease. However, it is clear from these data and from the results of multiple genomic screens[13–19] that the underlying genetic etiology of AMD is complex and thus involves multiple genes, risk factors, and interactions. Although numerous regions of interest have been identified by these genome screens, only two regions, on chromosomes 1 and 10, have been consistently identified by the majority of studies. No region has been identified in all studies with a genome-wide significance level indicative of a single-locus major effect.

Complementing a genomic screening (e.g., locational) approach is the direct testing of candidate genes proposed because their putative functions are related to the known AMD pathology. One such set of genes consists of those already known to be responsible for Mendelian macular and retinal dystrophies that share common features with AMD. However, genes *ELOVL4* (Stargardt disease),[20] bestrophin (Best disease),[21,22] *TIMP-3* (Sorsby fundus dystrophy),[23] and peripherin (retinal degeneration)[24–26] have failed to convincingly demonstrate association with AMD. *ABCA4* (formerly *ABCR;* Stargardt disease) may account for a small percentage of AMD cases,[27] but this result is not universally confirmed.[28–33] Another set of candidate genes can be identified by a putative functional relationship with AMD. This set includes the toll-like receptor 4 (*TLR4*) gene,[34] chemokine receptors (*CX3CR1*),[35] and genes involved in cellular detoxification.[36] The respective studies either have not yet been replicated or did not find any initial association.

In contrast, the apolipoprotein E (*APOE*) gene, which is involved in lipid transport and distribution, has consistently demonstrated a protective effect for the APOE-ε4 allele on disease risk in white AMD populations (Klaver CCW, et al. *IOVS* 1996;37:ARVO Abstract 1920).[37–41] A few studies have also suggested a modest increase in disease risk with the ε2 allele,[37,40,41] with one study reporting a sex-specific effect in

TABLE 1. Description of Study Datasets

Characteristic	Family Dataset*		Case-Control Dataset	
	Affected	**Unaffected**	**Cases**	**Controls**
AMD grade				
1	0	69	0	124
2	0	28	0	35
3	84	0	91	0
4	51	0	55	0
5	179	0	253	0
Total	314	97	399	159
Female (%)	67.8	66.0	64.2	58.5
Mean age at examination	73.9	66.1	76.2	66.4

* Discordant sibpair (DSP) only (57); total DSPs (111); 2 affected sibs (80); 3 affected sibs (18); 4 affected sibs (5); 5 affected sibs (2).

males.[41] Most recently, variation in the gene for complement factor H (*CFH*) has been identified as a major risk factor for AMD, and likely explains the genetic linkage signal on chromosome 1.[42–44]

Additional candidate genes can be identified using a number of different strategies related to known or proposed gene function. Interleukin-1α (*IL1A*) and creatine kinase (*CKB*) were both chosen for study because cDNA microarray experiments demonstrated that their expression was significantly altered in AMD retinal tissue compared to normal tissue.[45] Because of the confirmed role of *APOE*, we chose three genes that interact with *APOE*, very low density lipoprotein receptor (*VLDLR*), α-2-macroglobulin (*A2M*), and low-density lipoprotein receptor–related protein 6 (*LRP6*).[46–48] Microsomal glutathione-*S*-transferase 1 (*MGST1*), which is involved in oxidative stress pathways, was chosen because oxidative stress has been hypothesized to play a role in AMD.[49] Angiotensin-converting enzyme (*DCP1*), involved in the renin-angiotensin pathway and the control of blood pressure, had a previously published association.[50] *VEGF* was examined because of its role in vascular growth and because it is a target for inhibition therapy in neovascular AMD.[51] The goal of the present study was to determine whether any of these candidate genes demonstrated initial association in multiple independent datasets and would thus be worthy of more detailed examination.

MATERIALS AND METHODS

Datasets

The datasets consisted of multiplex families (≥2 affected sampled family members), discordant sibpairs, singleton cases, and controls (Table 1). All individuals participating in this study were recruited in the southeastern United States and evaluated by Duke University Medical Center and Vanderbilt University Medical Center. Stereoscopic fundus photographs were available for all participating individuals,

TABLE 2. Genes and Markers

Gene Symbol	Gene Name	Chromosome	Location (Mb)	Genomic Size (kb)	SNP	SNP Number	MAF*
IL1A	Interleukin-1α	2	106.9	11.5	hCV431488	1	0.42
					hCV7628620	2	0.49
					hCV11725573	3	0.48
					hCV1839912	4	0.28
VEGF	Vascular endothelial growth factor	6	45.3	14.4	hCV8311614	1	0.34
					hCV1647373	2	0.48
					hCV1647372	3	0.35
					hCV1647366	4	0.33
					hCV1647360	5	0.46
VLDLR	Very low density lipoprotein receptor	9	2.5	32.7	hCV16173551	1	0.46
					hCV1595792	2	0.32
					hCV1595778	3	0.26
					hCV1595773	4	0.47
					hCV16173550	5	0.41
					hCV15884692	6	0.19
					hCV7589159	7	0.23
A2M	α-2-Macroglobulin	12	10.8	48.2	hCV2682746	1	0.32
					hCV2682734	2	0.33
					hCV2682719	3	0.19
					hCV2682701	4	0.37
LRP6	Low-density lipoprotein receptor-related protein 6	12	17.4	123.4	hCV2685141	1	0.11
					hCV345771	2	0.45
					hCV9891803	3	0.47
					hCV2685192	4	0.46
MGST1	Microsomal glutathione-*S*-transferase 1	12	21.7	17.3	hCV2684682	1	0.45
					hCV2684692	2	0.45
					hCV1585290	3	0.35
					hCV2684712	4	0.47
CKB	Creatine kinase	14	84.0	3.2	hCV1292621	1	0.26
					hCV1292615	2	0.35
DCP1	Angiotensin-converting enzyme	17	55.9	20.5	hCV473036	1	0.28
					hCV589777	2	0.40
					hCV1247713	3	0.42
					hCV1247717	4	0.44
					hCV1247681	5	0.37

MAF, minor allele frequency.
* Calculated from the case-control dataset.

TABLE 3. Linkage Results

Gene	Grade 3, 4, 5	Grade 5
IL1A	0.00	0.25 (D)
VEGF	1.32 (D)	1.08 (D)
VLDLR	0.22 (D)	0.34 (D)
A2M	0.00	0.00
LRP6	1.14 (D)	0.54 (D)
MGST1	1.61 (R)	0.79 (R)
CKB	0.20 (R)	0.25 (D)
DCP1	0.00	0.00

Data are HLOD scores; all results are from two-point analyses. D, maximum score found under the dominant model; R, maximum score found under the recessive model.

including all patients, their participating relatives, and controls. All protocols were approved by the appropriate institutional review boards and conformed to the tenets of the Declaration of Helsinki. All individuals provided informed consent before participating in the study.

Disease severity was graded using a slightly modified version[28] of established classification systems.[52] Severity was assessed on a scale of 1 to 5: grade 1, no AMD features; grade 2, only small or nonextensive intermediate drusen; grade 3 ("early" AMD), extensive intermediate drusen (deposits of 63 to 125 μm totaling or exceeding the area of a

350-μm-diameter circle), large drusen, and/or drusenoid RPE detachments; grade 4 ("atrophic" AMD), geographic atrophy; and grade 5, neovascular/exudative disease (Table 1). Individuals were classified by the grade of disease in their more severely affected eye.

The mean age at examination differed substantially between affected and unaffected individuals. Because of the insidious nature of onset in AMD and the severity of the disease in most of our patients, the age of onset in the subjects with AMD was likely to be many years earlier than the age at examination and thus closer to the age at examination of the control subjects. We included age at examination as a covariate in our statistical analyses.

Molecular Analysis

Genomic DNA was extracted from blood using standard protocols and a commercial system (Puregene; Gentra Systems, Minneapolis, MN). All markers were single nucleotide polymorphisms (SNPs) and were identified using the Ensembl (www.ensembl.org), dbSNP (www.ncbi.nlm. nih.gov/projects/SNP), and Celera (www.celera.com) databases. Multiple SNPs spanning each gene were chosen using a hierarchy of nonsynonymous coding change, minor allele frequency > 0.10, availability, location within the gene, and ease of genotyping. A total of 35 SNPs were genotyped for the eight genes (Table 2).

Laboratory personnel were blinded to pedigree structure, affection status, and location of quality control samples. Duplicate quality control samples were placed both within and across 96-well plates, and

A2M

	1	2	3	4
1		1.00	1.00	0.98
2	0.97		1.00	0.94
3	0.10	0.09		1.00
4	0.30	0.29	0.11	

MGST1

	1	2	3	4
1		0.47	0.43	0.48
2	0.11		0.64	0.91
3	0.18	0.21		0.99
4	0.20	0.37	0.84	

CKB

	1	2
1		0.96
2	0.63	

VLDLR

	1	2	3	4	5	6	7
1		0.46	0.02	0.21	0.31	0.46	0.16
2	0.10		0.89	0.54	0.51	1.00	0.64
3	0.00	0.15		0.87	0.81	0.89	0.74
4	0.04	0.12	0.23		0.97	0.54	0.68
5	0.07	0.09	0.15	0.75		0.51	0.74
6	0.10	1.00	0.15	0.12	0.09		0.64
7	0.01	0.20	0.05	0.10	0.10	0.20	

DCP1

	1	2	3	4	5
1		0.04	0.26	0.22	0.05
2	0.00		0.91	0.85	0.46
3	0.03	0.46		0.99	0.42
4	0.02	0.42	0.93		0.43
5	0.00	0.10	0.15	0.15	

VEGF

	1	2	3	4	5
1		0.98	0.98	0.03	0.03
2	0.43		0.99	0.18	0.12
3	0.25	0.56		0.24	0.36
4	0.00	0.02	0.05		0.90
5	0.00	0.01	0.05	0.29	

IL1A

	1	2	3	4
1		1.00	0.99	0.97
2	0.79		0.99	0.95
3	0.79	0.98		0.94
4	0.48	0.38	0.37	

LEGEND

Shade	LD value
	0.75–1.00
	0.50–0.74
	0.25–0.49
	0.00–0.24

LRP6

	1	2	3	4
1		1.00	0.98	0.98
2	0.15		0.99	0.99
3	0.14	0.97		0.99
4	0.14	0.95	0.98	

FIGURE 1. LD measurements for each gene. D' is given in the upper right half, r^2 in the lower left half.

TABLE 4. Family-Based Association Results

Gene	SNP	Grade 3, 4, 5		Grade 5	
		PDT	HBAT*	PDT	HBAT*
IL1A	1	0.60		0.38	
	2	1.00	0.79	0.24	0.52
	3	0.56	0.87	0.16	0.48
	4	0.75	0.41	0.42	0.64
VEGF	1	0.07		0.15	
	2	**0.001**	**0.005**	0.08	0.79
	3	**0.03**	0.05	0.38	0.85
	4	0.49	**0.03**	0.88	0.41
	5	0.92	0.59	0.54	0.48
VLDLR	1	0.45		0.57	
	2	0.23	1.00	0.20	0.64
	3	0.41	0.56	0.10	0.12
	4	0.64	0.62	0.68	0.38
	5	0.44	0.76	0.92	0.35
	6	0.05	0.19	**0.03**	0.14
	7	0.21	0.24	0.52	0.36
A2M	1	0.50		0.39	
	2	0.58	0.52	0.39	0.67
	3	0.61	0.30	1.00	0.71
	4	0.83	0.37	0.64	0.87
LRP6	1	0.23		0.05	
	2	0.82	0.68	0.63	0.83
	3	0.81	0.51	0.29	0.31
	4	0.81	0.35	0.32	0.66
MGST1	1	0.59		1.00	
	2	0.34	0.16	0.88	0.39
	3	0.16	0.15	0.38	0.63
	4	0.10	0.13	0.42	0.42
CKB	1	0.57		0.37	
	2	0.13	0.50	0.12	0.06
DCP1	1	0.48		0.32	
	2	0.56	0.09	0.73	0.05
	3	0.28	0.96	0.34	0.84
	4	0.18	0.54	0.37	0.66
	5	**0.03**	0.16	0.07	0.45

Association data are *P*-values; values in boldface were considered significant.

* Included for pairwise adjacent SNP combinations.

equivalent genotypes were required for all quality control samples to ensure accurate genotyping. Hardy-Weinberg calculations were performed for each marker, and Mendelian inconsistencies (in the multiplex families) were identified using PedCheck.[53] Suspect genotypes were re-read or retested. All SNPs were required to have >95% of possible genotypes.

Statistical Analysis

Genotyping data were analyzed for two different disease models defined by the most severe status in either eye: grades 3, 4, and 5 combined, and grade 5 alone. Grade 4 was not examined separately because it represented only a small portion of the overall dataset. Two-point heterogeneity lod (HLOD)-score analyses were computed using Allegro.[54] Parametric analyses were performed using autosomal dominant and autosomal recessive models with respective disease allele frequencies of 0.01 and 0.14 to model a common susceptibility allele. Marker allele frequencies were obtained from the dataset by counting all independent chromosomes.

Allelic association studies within the family dataset were performed using the allelic pedigree disequilibrium test (PDT).[55] Multilocus haplotype analysis using two adjacent SNPs within a gene was performed using the haplotype based association test (HBAT).[56] Multilocus haplotype analysis was not done using more than two adjacent SNPs because of concerns about sensitivity to small haplotype frequencies. Linkage disequilibrium (LD) calculations were performed using the

graphical overview of linkage disequilibrium method.[57] Logistic regressions for the case-control data were calculated using SAS Version 8[58]; the SNPs were modeled assuming an additive effect and adjusted for age and sex. Nominal significance was declared if $\alpha < 0.05$.

RESULTS

Linkage Analysis

Three genes, *VEGF*, *MGST1*, and *LRP6*, generated modestly positive HLOD scores for at least one SNP (1.32, 1.14, and 1.61, respectively; Table 3). *MGST1* and *LRP6* are located within 4 Mb of each other on chromosome 12p. The *VEGF* HLOD scores were relatively insensitive to grade, remaining above 1.0 in both categories. However, scores for *MGST1* and *LRP6* fell below 1.0 when the analyses were restricted to only grade 5.

LD Analysis

The results of LD calculations are given in Figure 1. All SNPs were in Hardy-Weinberg equilibrium. The SNPs within *IL1A*, *A2M*, *LRP6*, and *CKB* were all in strong LD within each gene. The SNPs in *VEGF* appeared to fall into two distinct blocks (SNPs 1–3 and SNPs 4 and 5). In *VLDLR*, SNPs 2 to 7 were in strong LD with each other; SNP 1 was in only moderate LD with the rest. In *MGST1*, SNPs 2 to 4 were in strong LD with each other; SNP 1 was in only moderate LD with the rest. In *DCP1*, SNPs 2 to 4 were in strong LD with each other; SNP 5 was in only moderate LD with them, and SNP1 was in low LD with both groups.

Family-Based Association Analyses

Only *VEGF* and *DCP1* showed nominally significant results for any SNP (Table 4) for the grade 3, 4, and 5 analysis. Of particular interest was SNP 2 of *VEGF* ($P = 0.001$) which was almost significant in the grade 5 analysis ($P = 0.08$). The HBAT results confirmed the PDT results in *VEGF* (SNP 1 to 2, $P = 0.005$; SNP 3 to 4, $P = 0.03$) in the grade 3, 4, and 5 analysis. *VLDLR* had one nominally significant result ($P = 0.03$) in the grade 5 analysis.

Case-Control Association Analysis

IL1A, *VLDLR*, and *LRP6* all showed nominally significant results in the independent case-control dataset for the grade 3, 4, and 5 analysis (Table 5). The *IL1A* results for SNPs 1 to 3 were insensitive to grade, remaining significant in the grade 5 analyses. These SNPs were all in significant LD with each other (Fig. 1). The *VLDLR* results for SNP 6 were also insensitive to grade, and SNP 2 became nominally significant in the grade 5 analyses. These two SNPs were in strong LD with each other. Similarly, all the SNPs in *LRP6* showed significant results in both grades, with the results becoming more significant in the grade 5 analyses (Table 5). These four SNPs were all in strong LD with each other. The only other significant result that appeared was for *VEGF*, for SNP 1 ($P = 0.02$) in the grade 5 analysis.

DISCUSSION

Unraveling the genetics of AMD has been difficult. Although a few rare variants have been associated with AMD,[27] virtually all the genetic effect remains to be explained. Two complementary approaches can be used to tackle this problem. The genome screen approach has identified some common chromosomal regions,[13] and work is ongoing to identify these genes in this and other datasets.[17,59] Because of the underlying complexity, however, linkage analyses are unlikely to identify the locations of all relevant genes, and thus the examination of

TABLE 5. Case-Control Association Results

Gene	SNP	Grade 3, 4, 5	Grade 5	OR	Lower CI	Upper CI	Risk Allele
IL1A	1	**0.03**	**0.02**	1.37	1.03	1.81	C
	2	**0.05**	**0.05**	1.33	1.00	1.77	A
	3	**0.02**	**0.03**	1.40	1.05	1.86	A
	4	0.99	0.65	1.00	0.74	1.36	T
VEGF	1	0.16	**0.02**	1.24	0.92	1.69	C
	2	0.41	0.27	1.13	0.85	1.49	C
	3	0.90	0.75	1.02	0.76	1.37	C
	4	0.76	0.48	1.05	0.77	1.43	T
	5	0.59	0.85	1.08	0.81	1.43	A
VLDLR	1	0.11	0.08	1.26	0.95	1.68	T
	2	0.07	**0.04**	1.31	0.97	1.77	G
	3	0.09	0.12	1.30	0.96	1.78	C
	4	0.85	0.81	1.03	0.77	1.36	A
	5	0.85	0.98	1.03	0.77	1.37	A
	6	**0.01**	**0.01**	1.58	1.10	2.28	C
	7	0.77	0.59	1.05	0.74	1.50	C
A2M	1	0.33	0.35	1.16	0.86	1.57	C
	2	0.42	0.39	1.13	0.84	1.52	C
	3	0.49	0.46	1.15	0.77	1.70	A
	4	0.21	0.20	1.20	0.90	1.60	A
LRP6	1	**0.02**	**0.01**	1.76	1.10	2.83	G
	2	**0.02**	**0.007**	1.40	1.06	1.86	T
	3	**0.03**	**0.009**	1.37	1.03	1.82	G
	4	**0.02**	**0.004**	1.41	1.07	1.87	C
MGST1	1	0.19	0.12	1.21	0.91	1.60	A
	2	0.56	0.26	1.10	0.81	1.49	T
	3	0.78	0.84	1.04	0.78	1.38	A
	4	0.95	0.97	1.01	0.76	1.34	A
CKB	1	0.79	0.52	1.04	0.76	1.43	C
	2	0.90	0.88	1.01	0.76	1.36	A
DCP1	1	0.24	0.35	1.20	0.89	1.61	C
	2	0.77	0.78	1.04	0.78	1.39	C
	3	0.41	0.34	1.13	0.85	1.49	G
	4	0.36	0.26	1.14	0.86	1.51	C
	5	0.10	0.21	1.27	0.95	1.69	C

Association data are *P*-values; values in boldface were considered significant. OR, odds ratio; CI, confidence interval.

functional candidate genes is a useful complementary approach.

We examined 35 SNPs in the eight functional candidate genes. These SNPs were chosen initially for availability of assays, informativeness, and spacing across the gene. For this initial screen of candidates, we did not attempt to perform a comprehensive analysis of all polymorphisms in each gene. Our LD results confirm the emerging idea of haplotype blocks, with the SNPs in several genes (*IL1A, A2M, LRP6, MGST1,* and *CKB*) in strong LD across the entire gene. *VEGF, VLDLR,* and *DCP1* each had two relatively independent blocks of SNPs. These data suggest that we effectively reduced the number of independent SNP results from 35 to 11.

Of the genes chosen for study here, *A2M* did not demonstrate any nominally significant results. *IL1A* showed nominally significant results only in the case-control analysis. *DCP1* showed nominally significant results only in the family-based association analysis. Two genes, *MGST1* and *CKB*, generated modestly interesting LOD scores (>1.0), although none reached levels proposed as suggestive or significant.[60] The lack of consistently positive results for these genes strongly suggests that they do not play a significant role in the etiology of AMD. However, our dataset does not have sufficient power to detect more modest effects of these genes, and we cannot exclude the possibility that they may have a small effect.

Of more interest are the results for *LRP6, VLDLR,* and *VEGF. LRP6* generated a modestly interesting HLOD score of 1.14 in the multiplex families, and independently generated

the strongest association results in the case-control analysis (*P* = 0.004 in the more severe grade 5 clinical group). Although the linkage results for *VLDLR* were unimpressive (HLOD = 0.34), it generated a nominally significant result in the family-based association analysis (*P* = 0.03) and an independent significant result in the case-control dataset (*P* = 0.01). *VEGF* had a maximal LOD score of 1.32, the strongest family-based association result (*P* = 0.001, grade 3, 4, and 5) and a moderate case-control association result (*P* = 0.02, grade 5).

The nominally significant results must be interpreted cautiously, since we genotyped multiple SNPs and performed the analyses under two different clinical models. Given the LD results, we have effectively studied only 11 independent blocks of polymorphisms along with two clinical groups, resulting in 22 tests per dataset. The most conservative correction for such multiple comparisons (Bonferroni) would suggest an adjusted *P*-value of 0.002.

In this light, the *LRP6* case-control results are only on the border of significance. They are, however, independently supported by the linkage results, and thus this gene must remain of some interest. Functionally, *LRP6* is a low-density lipoprotein receptor involved in vasculature remodeling pathways.[48] Neither the family-based or case-control *VLDLR* results survive this correction. However, the functional role of *VLDLR* as a cell surface receptor for Reelin and the nominal results in two independent datasets suggest that this gene should be examined further. Finally, the family-based *VEGF* result remains

significant even with this conservative correction. Combined with the nominal result in the case-control dataset, the interesting genetic linkage results, and its functional role in vascular growth and regeneration,[51] this is perhaps our most strongly implicated gene in the etiology of AMD.

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