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Candidate gene analysis suggests a role for fatty acid biosynthesis and regulation of the complement system in the etiology of age-related maculopathy

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Age-related maculopathy (ARM) is a leading cause of visual impairment in elderly Americans and is a complex genetic disorder. Hypothesized pathways for the etiology of ARM include cholesterol and lipoprotein metabolism and transport, extracellular matrix integrity, oxidative stress and inflammatory/immunologic processes. This study investigates 21 polymorphisms within 15 candidate genes whose products function within these pathways by performing family and case–control genetic association studies using clearly affected familial cases ($n = 338$ families, 796 individuals), clearly affected, unrelated sporadic cases ($n = 196$) and clearly unaffected, unrelated controls ($n = 120$). Two genes demonstrated significant association with ARM status. A Met299Val variant in the elongation of very long chain fatty acids-like 4 (*ELOVL4*) gene was significantly associated with ARM in the case–control allele ($P = 0.001$), case–control genotype ($P = 0.001$) and case–control family ($P < 0.0001$) tests. A Tyr402His variant in exon 9 in the complement factor H (*CFH*) gene was also significantly associated with ARM in the case–control allele ($P < 0.0001$), case–control genotype ($P < 0.0001$) and case–control family ($P < 0.0001$) tests. All of these results remain significant after adjusting for false discovery rates to control for the impact of multiple testing. In addition, the *CFH* variant appears to play a role in exudative and atrophic disease, whereas the *ELOVL4* variant may play a greater role in exudative disease in our population. These results support a potential role for multiple pathways in the etiology of ARM, including pathways involved with fatty acid biosynthesis and the complement system.

INTRODUCTION

Age-related maculopathy (ARM) is one of the leading causes of blindness of the elderly in the United States (1). Genetic susceptibility plays a role in ARM etiology with heritability estimates ~45% (2). Several studies have used a genome-wide linkage approach to detect regions of the genome, which potentially contain genes involved in the etiology of ARM. Several regions of the genome have been implicated in multiple studies and strengthen support for these regions. Linkage to the 1q31 region has been the most consistent with seven separate analyses, implicating this region in the etiology of ARM (3–9). The region 10q26 has also received considerable support (3,4,6–8,10,11). Several other regions of the genome have been implicated in different studies with less replication.

In addition, many studies have taken a candidate gene approach to the study of the genetic etiology of ARM, implicating the genes for hemicentin (*FIBL-6*), elongation of very long chain fatty acids-like 4 (*ELOVL4*), angiotensin 1 converting enzyme (*ACE*), apolipoprotein E (*APOE*), paraoxonase (*PON1*), manganese superoxide dismutase (*SOD2*), cystatin C (*CST3*), ATP-binding cassette transporter (*ABCA4*) and the complement factor H (*CFH*) in ARM

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etiology. The gene for hemicentin, also known as fibulin 6 (*FIBL-6*), located at 1q25–q31 has been implicated in one large ARM family (12). In addition, *FIBL-6* has homology to the EGF-containing fibulin-like extracellular matrix protein 1 gene known to be mutated in diseases characterized by early-onset drusen including Doyne's honeycomb and malattia leventinese macular degeneration (13). Mutations in *ELOVL4*, located at 6q14, which result in the production of a truncated protein have been associated with autosomal dominant macular dystrophy (14–16). *ACE*, located at 17q23, contains a well-characterized variant known to influence expression of ACE and this variant has been implicated in diabetic retinopathy and was shown to have a protective effect for ARM in one study (17). *APOE*, located at 19q13, has been associated with ARM in several studies, with the E4 allele potentially having a protective role (18–20). *PON1*, located at 7q21 and *SOD2*, located at 6q25, have been implicated in the exudative form of ARM each in one study (21,22). *CST3*, located at 20p11, has been associated with disease in German ARM subjects in one study (23). *ABCA4*, located at 1p21, has been implicated in ARM (24); however, our group has not been able to replicate this finding in our familial samples (11). A Tyr402His polymorphism within exon 9 of the *CFH* gene, located at 1q31, has been implicated in ARM in four previous studies to date (25–28).

Several hypotheses exist concerning the etiology of ARM including the involvement of cholesterol and lipoprotein metabolism and transport, extracellular matrix integrity, oxidative stress and inflammatory/immunologic processes. Support for the involvement of cholesterol and lipoprotein metabolism and transport include the commonalities between atherosclerotic deposits and drusen deposits on the retina of some ARM patients (29) and the significant contribution of cholesterol to the composition of drusen and the potential link between serum cholesterol levels and ARM etiology (30). Additional support for the role of cholesterol and lipoprotein metabolism and transport in ARM stems from the overlap of retinal degeneration in diseases such as abetalipoproteinemia, angioid streaks and from the fact that the retina is capable of *de novo* synthesis, assembly and secretion of lipids (31). Support for the involvement of extracellular matrix integrity involves the age-related changes, which have been noted in the retinal pigment epithelium and Bruch's membrane of the macula in ARM patients with the earliest signs of ARM found at Bruch's membrane (32). The accumulation of abnormal extracellular matrix resulting in thickening of Bruch's membrane is a hallmark of advanced ARM (33), and it has been suggested that this thickening might be due to impaired degradation of the extracellular matrix at this site (34). Additional support for the involvement of the integrity of the extracellular matrix in ARM relates to the evidence implicating genes involved with the extracellular matrix in early onset forms of macular degeneration. Examples include the involvement of the EGF-containing fibulin-like extracellular matrix protein 1 *(EFEMP1)* in malattia leventinese and Doyne's honeycomb retinal dystrophy (13), the involvement of the tissue inhibitor of metalloproteinases-3 (*TIMP3*) gene in Sorsby's fundus dystrophy (35) and the cadherin-3 gene in juvenile macular degeneration (36,37). Oxidative damage has also been hypothesized to play a role in the etiology of ARM, and a role of oxidative damage has been supported by cellular studies and clinical trials. Clinical trials have supported the intake of antioxidants to slow the progression of ARM (38); therefore, supporting a possible role for oxidative damage in ARM. In addition, the composition of the retina makes it a prime target for oxidative damage given its high consumption of oxygen and exposure to light (39) as well as studies demonstrating that lipid peroxidation occurs in the retina with the greatest extent of lipid peroxidation occurring in the macula (40). It has been suggested that inflammatory and immune responses may play a role in ARM etiology. This has been supported by the observation that chronic inflammation of the retina is related to the neovascularization and exudation from these new vessels often observed in the early stages of ARM (41). Animal studies investigating retinal wound healing also support the influence of the immune response and the inflammation on the development of retinal degeneration (42).

Although there are additional models of pathogenesis of ARM, our group has initially taken the route of investigating candidate genes found predominantly within our linked regions of 1q31 and 17q25 (3,4,11) with functions that fit within the hypotheses described earlier. A brief description of selected candidates can be found in Table 1.

RESULTS

Identification of previously unknown variants for *FIBL-6*, *RGS16* and *PRELP*

RGS16: An A → G transition was identified in intron 3 of the *RGS16* gene at nucleotide 2164 based on GenBank accession no. AF009356.
FIBL-6: A GATA tetranucleotide repeat polymorphism was identified within intron 58. The range of PCR generated fragment sizes was 154–218 bp.
PRELP: A C → A transversion was identified within the untranslated region of exon 3 of the *PRELP* gene at nucleotide 1561 based on GenBank accession no. NM002725.

Allele frequency estimation

Estimates of the allele frequencies (Table 2) identify four polymorphisms with relatively large frequency differences between cases and controls. The *ELOVL4* polymorphism: Met299Val, the intronic *CFH* polymorphism: rs10922093, the exon 9 CFH polymorphism: Tyr402His and allele 97 of the *VLDLR* repeat polymorphism. Also, three of the ApoH polymorphisms (ApoH160, ApoH107 and ApoH335) are not very polymorphic at all, and so are not considered further.

Hardy–Weinberg equilibrium testing

In the controls, only one locus, *ACE*, is significantly out of Hardy–Weinberg equilibrium (HWE). In the cases, both *ACE* and *FIBL-6* are significantly out of HWE with $P < 0.0001$. There is also some evidence that *LAMC1* and

Table 1. Description of candidate genes investigated

Gene	Locus	Gene function	References
Hemicentin (*FIBL-6*)	1q25–q31	Extracellular matrix protein, potentially involved with cell migration	(61)
Laminin C1 (*LAMC1*) Laminin C2 (*LAMC2*)	1q25	Extracellular matrix protein, component of the extracellular basal lamina of the retinal pigmented epithelium	(62)
Regulator of G-protein signaling 16 (*RGS16*)	1q25	Desensitizes G-protein mediated signaling through GTP hydrolysis and recycling of transducin which is involved with signal transduction in photoreceptor cells	(63,64)
Oculomedin (*OCLM*)	1q31	Function is not fully understood although its shared homology with neuromedin K suggests a potential vasoactive neuropeptide role. OCLM is expressed in the retina and trabecular cells after mechanical stretching	(65)
Glutaredoxin 2 (*GLRX2*)	1q31	Redox regulating enzyme in the mitochondria that is involved in defense against oxidative damage and is expressed in the retina	(66,67)
Proline arginine-rich end leucine-rich repeat protein (*PRELP*)	1q32	Connective tissue glycoprotein in the extracellular matrix and binds several ligands including heparin and perlecan	(68)
Complement factor H (*CFH*)	1q31	Member of the regulator of complement activation gene cluster on 1q32. Glycoprotein involved in the regulation of immune defense mechanisms, protection of tissues from damage by complement activation and binds C-reactive protein, heparin and sialic acid and interacts with a variety of microorganisms	(69–71)
Transforming growth factor beta 2 (*TGFB2*)	1q41	Growth factor involved in the wound healing process of proliferative vitreoretinopathy	(72)
Elongation of very long chain fatty acids-like 4 (*ELOVL4*)	6q14	Biosynthesis of fatty acids, expressed in photoreceptor cells of adult retina	(14)
Very low density lipoprotein receptor (*VLDLR*)	9p24	Fatty acid and triglyceride metabolism and able to bind apolipoprotein E rich VLDL	(73)
Angiotensin 1 converting enzyme (*ACE*)	17q23	Enzyme involved with systemic blood pressure regulation	(74)
Apolipoprotein H (*APOH*)	17q24	Glycoprotein that functions in lipid and triglyceride metabolism and is highly expressed in the retina	(75)
Integrin beta 4 (*ITGB4*)	17q25	Transmembrane receptor involved with maintenance of basal cell-matrix adhesion and binding of laminins	(76)
Apolipoprotein E (*APOE*)	19q13	Involved in lipid metabolism and transport within the central nervous system	(77,78)

APOE are out of HWE in the cases (*P*-values 0.028 and 0.056, respectively).

Association testing

When the allele test is applied to the case–control sample, four loci (*FIBL-6*, *CFH*, *ELOVL4* and *VLDLR*) generate significant results (Table 3). Allele-based logistic regression allowing for sex and age effects for the two-allele markers generates similar conclusions (data not shown). Under the genotype test, only two of these, *CFH* and *ELOVL4*, are significant. When the CLUMP test is applied to the three multiallelic markers, *FIBL-6* generates only moderately significant results (*P*-value close to 0.01). When we analyze the family and the case–control data simultaneously, using the case–control association analysis of pedigree data, CCREL (43), *CFH* and *ELOVL4* are significant. Figure 1 shows the genotype distributions for the Met299Val polymorphism in *ELOVL4* and the Tyr402His polymorphism in *CFH* for the case–control sample. Haplotype-based association testing for the two polymorphisms in the *CFH* gene (rs10922093 and Tyr402His) was significant ($P < 0.001$), whereas that for the APOHin6, APOH154 and APOH266 variants was not significant ($P = 0.906$; minimum haplotype-specific $P = 0.267$).

Results for the clinical subphenotypes, exudative and atrophic forms of the disease, are summarized in Table 4. We find that the Tyr402His variant in the *CFH* gene is

Table 2. Allele frequencies for all variants. The 'case–control' column gives the difference between the allele frequency estimates in the cases and in the controls

Locus	Allele	Mendel				Allele counting						Case–
		Family data		Type A affecteds		Control			Case			Control
		p	SE	p	SE	p	SE	Number of alleles	p	SE	Number of alleles	
ACE	1	0.328	0.015	0.313	0.016	0.342	0.031	234	0.358	0.025	374	0.016
ACE	2	0.672	0.015	0.687	0.016	0.658	0.031	234	0.642	0.025	374	−0.016
APOHin6	1	0.750	0.014	0.749	0.015	0.711	0.030	232	0.746	0.023	354	0.035
APOHin6	2	0.251	0.014	0.251	0.015	0.289	0.030	232	0.254	0.023	354	−0.035
ApoH107	1	0.969	0.006	0.968	0.007	0.983	0.008	232	0.967	0.009	362	−0.016
ApoH107	2	0.031	0.006	0.032	0.007	0.017	0.008	232	0.033	0.009	362	0.016
ApoH154	1	0.093	0.009	0.096	0.010	0.086	0.018	232	0.070	0.013	384	−0.016
ApoH154	2	0.907	0.009	0.904	0.010	0.914	0.018	232	0.930	0.013	384	0.016
ApoH160	1	0.001	0.001	0.001	0.001	0.009	0.006	228	0.021	0.007	380	0.012
ApoH160	2	0.999	0.001	0.999	0.001	0.991	0.006	228	0.979	0.007	380	−0.012
ApoH266	1	0.257	0.014	0.249	0.015	0.221	0.028	222	0.228	0.022	372	0.007
ApoH266	2	0.744	0.014	0.752	0.015	0.779	0.028	222	0.772	0.022	372	−0.007
ApoH335	1	1.000	0.000	1.000	0.000	1.000	0.000	236	0.987	0.006	380	−0.013
ApoH335	2	0.000	0.000	0.000	0.000	0.000	0.000	236	0.013	0.006	380	0.013
ELOVL4	1	0.897	0.010	0.900	0.010	0.786	0.027	234	0.890	0.016	382	0.104
ELOVL4	2	0.103	0.010	0.100	0.010	0.214	0.027	234	0.110	0.016	382	−0.104
GRLX2	1	0.726	0.016	0.725	0.017	0.708	0.030	236	0.743	0.023	366	0.035
GRLX2	2	0.274	0.016	0.275	0.017	0.292	0.030	236	0.257	0.023	366	−0.035
CFH rs10922093	1	0.208	0.013	0.213	0.014	0.290	0.029	238	0.200	0.021	380	−0.090
CFH rs10922093	2	0.790	0.013	0.787	0.014	0.710	0.029	238	0.800	0.021	380	0.090
CFH Tyr402His	1	0.391	0.018	0.388	0.019	0.685	0.032	216	0.372	0.026	336	−0.313
CFH Tyr402His	2	0.609	0.018	0.612	0.019	0.315	0.032	216	0.628	0.026	336	0.313
ITGB4	1	0.752	0.014	0.752	0.015	0.757	0.028	230	0.789	0.021	370	0.032
ITGB4	2	0.249	0.014	0.248	0.015	0.243	0.028	230	0.211	0.021	370	−0.032
LAMC1	1	0.539	0.016	0.529	0.017	0.529	0.032	238	0.550	0.026	380	0.021
LAMC1	2	0.461	0.016	0.471	0.017	0.471	0.032	238	0.450	0.026	380	−0.021
LAMC2	1	0.735	0.014	0.737	0.015	0.716	0.031	218	0.700	0.024	370	−0.016
LAMC2	2	0.265	0.014	0.263	0.015	0.284	0.031	218	0.300	0.024	370	0.016
OCLM	1	0.908	0.009	0.911	0.010	0.898	0.020	236	0.918	0.014	366	0.020
OCLM	2	0.092	0.009	0.089	0.010	0.102	0.020	236	0.082	0.014	366	−0.020
PRELP	1	0.824	0.012	0.830	0.013	0.868	0.022	228	0.849	0.018	378	−0.019
PRELP	2	0.176	0.012	0.170	0.013	0.132	0.022	228	0.151	0.018	378	0.019
RGS16	1	0.582	0.016	0.584	0.017	0.575	0.033	226	0.590	0.025	374	0.015
RGS16	2	0.418	0.016	0.416	0.017	0.425	0.033	226	0.410	0.025	374	−0.015
TGFB2	1	0.395	0.016	0.390	0.017	0.393	0.032	234	0.420	0.026	352	0.027
TGFB2	2	0.606	0.016	0.610	0.017	0.607	0.032	234	0.580	0.026	352	−0.027
APOE	2	0.101	0.011	0.102	0.012	0.108	0.021	222	0.126	0.017	374	0.018
APOE	3	0.812	0.014	0.809	0.016	0.784	0.028	222	0.799	0.021	374	0.015
APOE	4	0.088	0.010	0.089	0.011	0.108	0.021	222	0.075	0.014	374	−0.033
FIBL-6	154	0.004	0.002	0.003	0.002	0.004	0.004	230	0.008	0.005	364	0.004
FIBL-6	158	0.177	0.012	0.181	0.013	0.122	0.022	230	0.102	0.016	364	−0.020
FIBL-6	162	0.124	0.010	0.123	0.011	0.130	0.022	230	0.085	0.015	364	−0.045
FIBL-6	164	0.007	0.003	0.005	0.002							
FIBL-6	166	0.038	0.006	0.038	0.006	0.057	0.015	230	0.030	0.009	364	−0.027
FIBL-6	170	0.109	0.010	0.114	0.011	0.091	0.019	230	0.118	0.017	364	0.027
FIBL-6	174	0.045	0.006	0.040	0.007	0.074	0.017	230	0.044	0.011	364	−0.030
FIBL-6	178	0.005	0.002	0.003	0.002	0.004	0.004	230	0.030	0.009	364	0.026
FIBL-6	182	0.001	0.001	0.001	0.001	0.009	0.006	230	0.022	0.008	364	0.013
FIBL-6	186	0.001	0.001	0.001	0.001	0.004	0.004	230	0.003	0.003	364	−0.001
FIBL-6	190	0.032	0.005	0.031	0.006	0.009	0.006	230	0.049	0.011	364	0.040
FIBL-6	194	0.133	0.011	0.144	0.012	0.170	0.025	230	0.165	0.019	364	−0.005
FIBL-6	198	0.200	0.013	0.195	0.013	0.213	0.027	230	0.236	0.022	364	0.023
FIBL-6	202	0.109	0.010	0.103	0.010	0.104	0.020	230	0.071	0.013	364	−0.033
FIBL-6	206	0.015	0.004	0.018	0.004	0.004	0.004	230	0.008	0.005	364	0.004
FIBL-6	214	0.001	0.001	0.000	0.000	0.004	0.004	230	0.016	0.007	364	0.012
FIBL-6	218	0.001	0.001	0.001	0.001	0.000	0.000	230	0.011	0.005	364	0.011
VLDLR	97	0.318	0.017	0.322	0.018	0.266	0.030	214	0.347	0.025	354	0.081
VLDLR	100	0.010	0.003	0.009	0.004							
VLDLR	103	0.024	0.005	0.020	0.005	0.019	0.009	214	0.011	0.006	354	−0.008
VLDLR	106	0.365	0.017	0.345	0.019	0.332	0.032	214	0.322	0.025	354	−0.010
VLDLR	109	0.274	0.016	0.296	0.018	0.355	0.033	214	0.319	0.025	354	−0.036
VLDLR	112	0.004	0.002	0.004	0.003							
VLDLR	115	0.005	0.002	0.004	0.003	0.028	0.011	214	0.000	0.000	354	−0.028

The allele frequency differences that contribute to our significant findings are underlined.

Table 3. Results of association analyses

Locus	HWE tests Number of alleles	Association tests Case–control sample Case	Control	OR	95% CI for OR		Allele test	Genotype text	CLUMP	Family and CC CCREL
FIBL-6	17	<0.0001	0.356				0.011	0.034	0.011	
LAMC1	2	0.028	0.271	0.92	0.67	1.27	0.611	0.059		0.871
LAMC2	2	0.387	0.243	1.08	0.75	1.56	0.711	0.283		0.738
RGS16	2	0.449	0.179	0.94	0.67	1.31	0.680	0.810		0.800
OCLM	2	0.339	0.333	0.79	0.45	1.39	0.467	0.703		0.489
GRLX2	2	0.701	0.661	0.84	0.58	1.21	0.293	0.643		0.475
CFH rs10922093	2	0.262	0.658	0.61	0.42	0.89	0.011	0.042		0.006[a]
CFH Tyr402His	2	0.743	0.179	3.67	2.56	5.28	<0.0001[a]	<0.0001[a]		<0.0001[a]
PRELP	2	0.149	0.688	1.17	0.73	1.89	0.477	0.346		0.240
TGFB2	2	0.439	1.000	1.12	0.80	1.57	0.548	0.680		0.858
ELOVL4	2	0.706	0.782	0.45	0.29	0.71	0.001[a]	0.001[a]		<0.0001[a]
VLDLR	7	0.300	0.828				0.007[a]	0.145	0.217	
ACE	2	<0.0001	<0.0001	1.07	0.76	1.51	0.720	0.477		0.654
APOHin6	2	1.000	0.267	0.84	0.58	1.22	0.392	0.453		0.267
APOH154	2	1.000	0.592	0.80	0.44	1.46	0.527	0.820		0.883
APOH266	2	0.404	0.782	0.96	0.64	1.43	0.839	0.959		0.504
ITGB4	2	0.664	0.612	0.83	0.56	1.23	0.371	0.615		0.812
APOE	3	0.056	0.609				0.345	0.494	0.369	

All test results are given as *P*-values, which are underlined if $P \leq 0.01$. CC, case–control.
[a]Significant after adjusted for FDR level of 5%.

involved in all forms of the disease ($P < 0.0001$ in all tests). The Met299Val variant in the *ELOVL4* gene is clearly involved in the exudative form of the disease ($P < 0.01$ in all tests); however, two out of the three tests do not show significance for the atrophic form of the disease, but this could be due to low power from the low number of atrophic cases (exudative = 130 and atrophic = 57). Combined analysis of the family and case–control data does show that the Met299Val variant in *ELOVL4* may be involved in the atrophic form of the disease ($P < 0.0001$).

Multiple testing

When we use a false discovery rate (FDR) (44,45) of 0.05 for the 54 association *P*-values (allele, genotype, CLUMP and CCREL tests) in Table 3, we obtain a *P*-value cutoff of 0.007. Using this cutoff, we obtain one or more significant association tests results for the rs10922093 and Tyr402His variants within the *CFH* gene, the Met299Val variant in the *ELOVL4* gene and the repeat variant in the *VLDLR* gene. These are noted in Table 3.

DISCUSSION

The complex genetics of ARM is apparent given the number of regions of the genome that have been implicated through linkage analyses, as well as the many candidate gene association studies that have been published. The fact that most of the genes known to be involved with early onset, single gene maculopathies are not strongly involved in ARM adds to the complexity of ARM genetics. Replication of linkage findings on chromosomes 1q31 and 10q26, however, offers encouragement that determining the genetic basis of susceptibility to ARM is feasible.

Most of the results presented in this paper are non-significant findings. With our sample size, the case–control allele test has power >80% at alpha = 0.01 to detect an odds ratio greater than 1.90 when the allele frequency is 0.20 in the controls and an odds ratio greater than 2.84 when the control allele frequency is 0.05. In addition, the association tests utilized for this study rapidly lose power as allelic heterogeneity increases (46). Therefore, these non-significant findings should not be interpreted as necessarily excluding these genes from playing a role in ARM. Indeed, a much larger pooled study (20), of which our data was a part of, found that the E4 allele of *APOE* was protective against ARM; although we see the same trend here in terms of the E4 allele being more frequent in controls than that in cases, our smaller study does not have enough power to reach significance on its own for *APOE*.

ELOVL4 appears to be significantly associated with ARM status in the case–control simulated χ^2 test ($P = 0.001$ for allele test; $P = 0.001$ for genotype test) as well as in the family and case–control tests ($P < 0.0001$). Finding association across several types of analyses strengthens the potential relationship between *ELOVL4* and ARM status. An OR of 0.45 (95% CI: 0.29–0.71) for *ELOVL4* could potentially indicate that having a valine at residue 299 is protective. Exon 6 of the *ELOVL4* gene was evaluated in the cases using dHPLC and the only variant that could be identified was the Met299Val variant. None of the other reported mutations in exon 6, including the 5 bp deletion reported by Zhang *et al.* (14) in several families with Stargardt-like and autosomal dominant maculopathies, was found in any of our cases. The

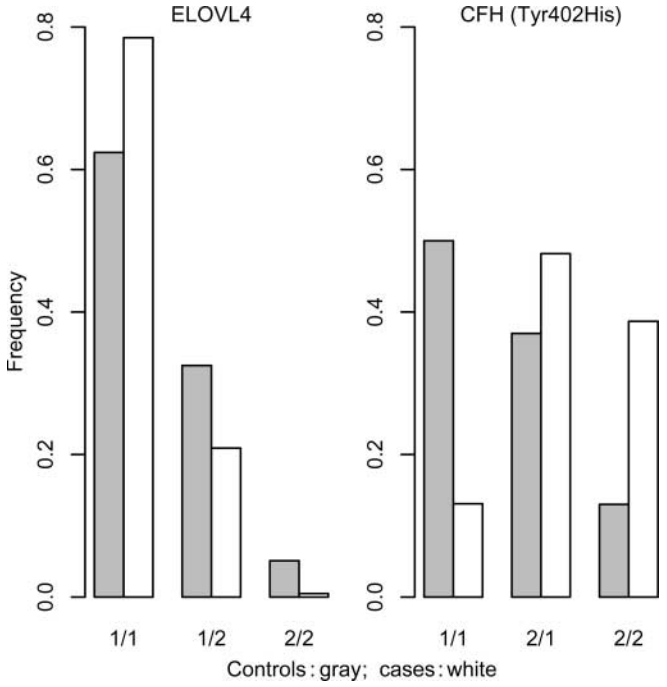

Figure 1. The genotype distribution for ELOVL4 and CFH (Try402His). Gray bars are controls and white bars are cases.

Table 4. Results of association analyses for sub-clinical types

		Case–control sample				Family and CC	
	OR	95% CI for OR		Allele test	Genotype test	CCREL	CLUMP
CFH rs10922093							
Exudative	0.63	0.41	0.95	0.038	0.096	0.002	
Atrophy	0.38	0.21	0.70	0.002	0.005	0.012	
CFH Tyr402His							
Exudative	3.46	2.33	5.13	<0.0001	<0.0001	<0.0001	
Atrophy	4.76	2.87	7.91	<0.0001	<0.0001	<0.0001	
ELOV4							
Exudative	0.41	0.24	0.68	<0.0001	0.002	<0.0001	
Atrophy	0.53	0.28	1.00	0.051	0.135	<0.0001	
VLDLR							
Exudative				0.114	0.396		0.232
Atrophy				0.017	0.166		0.106
FIBL-6							
Exudative				<0.0001	0.013		0.003
Atrophy				<0.0001	0.001		0.047

All test results are given as *P*-values, which are underlined if $P \leq 0.01$. CC, = case–control.

only variant that was genotyped in both the cases and the controls was the Met299Val variant due to the reported low frequency of other non-synonymous variants in *ELOVL4*. This Met299Val variant represents an amino acid change from one that is hydrophilic to one that is hydrophobic, indicating that it could have functional consequences; however, it should be noted that the amino acid at position 299 in the ELOVL4 protein is not conserved across species. It is interesting to note that to date all of the mutations in *ELOVL4* that have been implicated in earlier-onset maculopathy have been truncation mutations (14–16) and it is possible that this missense variant is responsible for the more common, ARM, which is represented by our subject population. In addition, one previous study did not find an association of the Met299Val variant in *ELOVL4* with ARM using a case–control analyses (47). This discrepancy may be explained by our use of familial and sporadic samples and their use of only sporadic samples, it may be due to the over-representation of exudative cases in our study versus atrophic samples in their study or it may be due to differences in population substructure or number of subjects investigated. The issue of different proportions of end-stage disease between the two populations may be a valid concern given the results of the clinical subphenotype analyses (Table 4), which indicate that the *ELOVL4* variant may play a greater role in exudative disease versus atrophic disease.

FIBL-6 and *VLDLR* appear to be significantly associated with ARM status in the case–control simulated χ^2 analysis (allele test $P = 0.011$ and $P = 0.007$, respectively). *FIBL-6* is within our linked region on 1q31. The *FIBL-6* gene is ~7 cM from the *CFH* gene on chromosome 1 and for this reason we would not expect linkage disequilibrium to be present between these two loci. Although *FIBL-6* is also significant for both the genotype test and the CLUMP test, none of these associations remained significant after adjusting for the FDR. *VLDLR* is only significant for the allele test, however, this association remained after adjusting for the FDR and may indicate that further investigations are warranted. Furthermore, in the absence of a strong linkage signal at 9p24 where the *VLDLR* gene resides, the collective evidence that *VLDLR* is involved in ARM is not compelling at this time.

Complement factor H *(CFH)*, located within our linked region of 1q31, is significantly associated with ARM status in all of our tests of association for the Tyr402His variant (Table 3); this strong signal is consistent with the findings of others (25–28). In addition, our clinical subphenotype analyses indicate that the variants in the *CFH* gene most likely play a role in exudative and atrophic disease (Table 4). This finding supports a role for immune defense mechanisms in the etiology of ARM. Data provided by studying individuals with abnormalities in the complement system suggest that endothelial cells are not adequately protected during an inflammatory insult (48) and that this may lead to predisposition to thrombotic microangiopathy (49), which could help to explain the initial stages of ARM. The joint distribution of the Tyr402His variant for *CFH* and the Met299Val variant for *ELOVL4* (Fig. 1) is independently distributed in cases ($P = 0.840$) and in controls ($P = 0.832$).

These data support a potential role for genes involved in multiple pathways for ARM susceptibility particularly those involving fatty acid biosynthesis and the complement system with some evidence for extracellular matrix integrity and lipid metabolism, contributing to the complex nature of ARM etiology.

MATERIALS AND METHODS

Subjects and DNA extraction

To ensure homogeneity and to avoid population substructure, we restricted analyses to Caucasian subjects; this results in relatively little loss of information since the vast majority of our subjects are Caucasians. We also restricted our attention to Type A affecteds, which fall into our most stringent model for clinical classification. Individuals in this category were classified as 'affected' only if they were clearly affected with ARM based on the extensive/coalescent drusen, the pigmentary changes (including pigment epithelial detachments) and/or the presence of end-stage disease (geographic atrophy and/or choroidal neovascular membranes).

Subjects fall into three categories: familial ($n = 338$ families, 796 individuals), sporadic, unrelated, Type A affecteds ($n = 196$) and unaffected, unrelated controls ($n = 120$). Sporadic affected subjects were 61% females with a median age of onset of 72 years and unaffected controls were 54% females with a median age of assessment of 75 years. Control individuals were required to have no macular pathology other than a small number (less than 15) small hard drusen in the macula and no evidence of significant extramacular drusen. Recruitment and clinical characterization of the familial samples have been previously described (3,4,11). Sporadic affecteds and control subjects were recruited from two sources. One source was the initial contacts made during our recruitment of ARM families and these included individuals who were not eligible for our initial family-based studies (because of lack of an affected family member who was willing to participate) and spouses of affected individuals. A second source of sporadic cases and controls have been from the vitreo-retinal practice of the University of Pittsburgh Medical Center Eye Center, family members and community members who became informed of our studies from public information lectures and acquaintances. Clinical information was obtained on sporadic cases and controls and affection status assigned in the same manner as those who participated in our familial cohorts. The informed consent process for all subjects was approved by the institutional review board of the University of Pittsburgh. Genomic DNA was extracted from leukocytes obtained from whole blood collected into EDTA vacutainers using a simple salting-out procedure (50).

Clinical subphenotypes of advanced ARM

The affected sporadic cases were subphenotyped with respect to the presence of choroidal neovascular membranes (exudative disease) ($n = 130$) and RPE atrophy (geographic atrophy) ($n = 57$). The presence of a choroidal neovascular membrane in either or both eyes was considered sufficient for the designation of 'exudative' disease, whether or not geographic atrophy was also identified. The definition of geographic atrophy includes individuals with and without choroidal neovascular membranes, but with the additional requirement that geographic atrophy had to be present in at least one eye that had no history or no evidence of a choroidal neovascular membrane. This stipulation is necessary because clinicians often refer to a patient as having RPE atrophy or there is photographic evidence of RPE atrophy after treatment or spontaneous resolution of a choroidal neovascular membrane. Clinical studies have shown a high concordance of geographic atrophy between the two eyes of affected individuals, thus it is reasonable to use the presence of geographic atrophy in one eye (with no evidence of a choroidal neovascular membrane) to meet the clinical definition for subphenotyping.

Criteria for selection of polymorphisms within the candidate genes

Polymorphisms were selected based on several criteria for prioritization. First, polymorphisms with a high heterozygosity score were chosen to increase the likelihood of obtaining informative data to detect an association with ARM even if the allele was not disease-causing, but in linkage disequilibrium with a true causative gene. Secondly, polymorphisms that result in a non-synonymous amino acid change were chosen and thirdly, polymorphisms that were likely to be informative and had allele frequency differences between Caucasians and African Americans were chosen.

New variant identification

Previously unknown informative variants for *FIBL-6*, *RGS16* and *PRELP* were identified for this project. Primer sequences, PCR annealing temperatures as well as temperature(s) for dHPLC evaluation for appropriate fragments are listed in Table 5. The *FIBL*-6 gene was too large to evaluate the entire gene for variants; therefore, a tandem repeats finder program (51) was used to identify potential repeat polymorphisms and a potential tetranucleotide polymorphism was detected in intron 58. The search for variants in the *RGS16* gene was conducted by SSCP of PCR amplified fragments covering all five exons of the gene. Each fragment was heat denatured and single stranded fragments resolved on a precast 6% TBE NuPAGE Novex polyacrylamide gel (Invitrogen). Unique conformers were selected for sequencing. The search for variants in the *PRELP* gene was conducted by PCR amplification of each of the three exons of the gene then evaluated by dHPLC using the WAVE DNA fragment analysis system (transgenomic). The reference sequences for the PCR generated fragments were obtained from the UCSC genome bioinformatics website (genome.ucsc.edu) using the human genome browser gateway and imported into the WAVE Maker software version 4.0 (transgenomic) for the generation of an appropriate melting temperature. The injected sample was 10 μl of the PCR product and a modified buffer gradient was used, which went from 35 to 59% buffer B (0.1 M TEAA/25% acetonitrile), while simultaneously going from 65 to 41% buffer A (0.1 M TEAA) with a constant flow rate of 0.9 ml/min. The volume of the injection loop of the autosampler was 100 μl, the waste volume was 50 μl and the run time was 14.0 min/sample using the fast clean setting for the accelerator. Initially, five pools of ten unrelated DNA samples were utilized for dHPLC, allowing for the detection of any variant with an allele frequency of at least 1%. When a variant chromatogram was detected from the pooled samples, all of the individual samples in the pool were evaluated and the variant samples selected for sequen-

Table 5. Primer, annealing and dHPLC temperatures for evaluation of genes for informative variants

Gene/exon	Primer sequences	Annealing temperature (°C)	dHPLC evaluation temp (°C)
PRELP Exon 1	CTGGGAGATCAGATCTTCTAGCT (F) CTGCACACACCTGCCTCTCC (R)	57	68
PRELP Exon 2 Fragment 1	CTGCATCACCTGGATCATGA (F) TGATGAAGTTGTTCTGGAGATAG (R)	55	63
PRELP Exon 2 Fragment 2	GATTTCCCATCTGCCCTCTAC (F) GTTGTGGGCCAGGTTGAGC (R)	57	63
PRELP Exon 2 Fragment 3	GCTCCTGGATCTCCAGCACA (F) CTCGATGCTATTGTTGTTGAGG (R)	55	63
PRELP Exon 3 Fragment 1	CATCTTGGCCGCCAGCCTCC (F) CCAGCCTTCAAGTGCGGTCA (R)	55	63
PRELP Exon 3 Fragment 2	CATGGCCCTACTCCGCCACC (F) TTTAGACACATCTGTGTTTCTGGAGC (R)	59	62
RGS16 Exon 1	AGCCTGCCACCATCCTGCCT (F) CCCCAAGCAGTTGGACAACC (R)	58	NA
RGS16 Exon 2	CATAAACCTCTCCACACTGG (F) TCTCTAGGGAGGTGTTGCCA (R)	55	NA
RGS16 Exon 3	GCTAGAATTTGTACTATGCT (F) GCAGATGAGTACACATGTGC (R)	48	NA
RGS16 Exon 4	CAGCTGACCTATTTCCTCTT (F) GGGCATATGGGGGCTCTAAC (R)	52	NA
RGS16 Exon 5	CAACTCACCCTGTGTGTGTC (F) CCTCTCTTCCCGGCTGGCTT (R)	58	NA
FIBL6	GTACTTTCATTGGATACATC (F) CCCACAATGCCGAGGTGTCTAATC (R)	61	NA

cing. Variants identified by dHPLC and SSCP were sequenced using dRhodamine dye terminator cycle sequencing reactions on the ABI377 (Applied Biosystems).

Genotyping methods

Five different genotyping methods were used over the course of this project. These include RFLP, fluorescent PCR for STR sizing, dHPLC, TaqMan and insertion/deletion evaluation. Two unrelated CEPH samples were genotyped for each variant and included on each gel, in each dHPLC run and in each TaqMan tray to assure internal consistency in genotype calls. In addition, double-masked genotyping assignments were made for each variant, compared and each discrepancy addressed using raw data or regenotyping. Table 6 lists the techniques and reaction conditions for each variant that used RFLP or fragment size analysis for genotype assignment.

Size-based variant evaluation. STR variants were genotyped by sizing fragments using the ABI377 and genotyper 2.5 software (Applied Biosystems).

dHPLC evaluation. The Met299Val SNP within exon 6 of *ELOVL4* was genotyped for sporadic cases and controls using RFLP and dHPLC was utilized to genotype familial subjects. Using dHPLC, heterozygotes were initially identified and genotyped by the presence of heteroduplexes. Samples demonstrating homoduplexes were spiked with a known met/met homozygote and samples resulting in heteroduplex formation were genotyped as val/val homozygotes and those that remained homoduplex were genotyped as met/met homozygotes.

TaqMan evaluation. Genotype data was collected using 5′ exonuclease assay-on-Demand TaqMan assays (Applied Biosystems) for variants selected for the *ITGB4* (rs820168), *GRLX2* (rs6665069), *OCLM* (hCV1462335), *TGFB2* (rs2009112), one of the *APOH* (rs1544556) SNPs and *CFH* (rs10922093). Amplification and genotype assignments were conducted using the ABI7000 and SDS 2.0 software (Applied Biosystems).

Statistical analyses: error checking

For the family data, genotypes at each locus were checked for Mendelian inconsistencies with the use of PedCheck (52), which uses genotype elimination to identify subtle inconsistencies.

Allele frequency estimation

Allele frequencies were estimated separately using all family members and only Type A affected family members using Mendel version 5 (53) . This approach properly takes all known familial relationships into account, while estimating the allele frequencies. For the unrelated cases and controls, allele frequencies were estimated by allele counting.

Tests for HWE

For the two-allele SNPs, we tested for HWE using an exact test (54) as implemented in the HWE exact function of the R genetics package (55,56); empirical *P*-values were estimated by simulation with 10 000 replicates. For the three multi-allelic markers, we tested for HWE using the Markov Chain Monte Carlo approach of Guo and Thompson (57), which is implemented in Mega2 (58). This approach can more properly handle the sparse tables encountered when analyzing multiallelic markers.

Table 6. Method and reaction conditions for genotyping using RFLP or fragment size analysis.

Polymorphism	Method of genotyping (restriction enzyme if RFLP)	Primer sequences	Annealing temperature (°C)
LAMC1 7122 G → A	RFLP (MspI)	GCCTTAATCTACAGCCTTGCTCTCC (F) ATTCAGATTTTATTATAAATAAAATACTG (R)	51
LAMC2 112A → C	RFLP (MslI)	ACAGCGGAGCGCAGAGTGAG (F) TTCCCTCCTGGAGGTGGCCC (R)	63
PRELP[a] exon 3	RFLP (DraIII)	CATGGCCCTACTCCGCCACC (F) TTTAGACACATCTGTGTTTCTGGAGC (R)	59
ELOVL4 Met299Val	RFLP (BspHI) dHPLC	AGATGCCGATGTTGTTAAAAG (F) CATCTGGGTATGGTATTAAC (R)	50
RGS16[a] intron 3	RFLP (AciI)	CAGCTGACCTATTTCCTCTT (F) GGGCATATGGGGGCTCTAAC (R)	52
APOE Cys112Arg Arg158Cys	RFLP (HhaI)	Methodology previously described by Kontula *et al.* (79)	
APOH Ser107Asn	RFLP (Tsp509I)	GAAATTTACCTGTTTATGTTT (F) TGTGCTCAGTGTGTTAACTG (R)	51
APOH Arg154His	RFLP (DraIII)	CCATCATCTGCCCTCCACCA (F) CCCTGCATTCTGGTAATTTAGTCCAA (R)	59
APOH Ala160Asp	RFLP (PvuII)		59
APOH Val266Leu	RFLP (RsaI)	GACCAATTTGTGTAGGTGTACTCATCTACT (F) CTCTCCTTGGTACACCACAGTGG (R)	60
APOH Trp335Ser	RFLP (BstBI)	GAAATGATTGTTTCTCTTAGAATGT (F) TGGATGAACAAGAAACAAGTG (R)	51
FIBL-6[a] intron 58	STR	FAM - GTACTTTCATTGGATACATC (F) CCCACAATGCCGAGGTGTCTAATC (R)	61
VLDLR 5′ UTR	STR	Methodology was described previously by Okuizumi *et al.* (80) with the forward primer 5′ fluorescently labeled with FAM	
ACE intron 16	INS/DEL	Methodology was previously described by Tiret *et al.* (74)	
CFH Tyr402His	RFLP (NlaIII)	TCTTTTTGTGCAAACCTTTGTTAG (F) CCATTGGTAAAACAAGGTGACA (R)	52

[a]New variants characterized during this project and described in the results section.

Analysis of case–control data

Using the unrelated cases and the unrelated controls, we tested for association between the variants and the ARM disease status. χ^2 tests were done using the χ^2 function of the R package and simulated *P*-values using 10 000 replicates were obtained. For the three multi-allelic markers, the T4 test of CLUMP package (59) was used. The T4 test seeks the grouping of alleles that maximizes the χ^2 statistic and adjusts for the multiple testing by using simulation to generate empirical *P*-values.

Case–control and family data

Association testing for SNPs using the combined family and case–control data was done using the case–control association analysis of pedigree data (CCREL) (43). While testing for association, this approach permits one to use related individuals in the family data by calculating the effective number of cases. Independent cases and controls can also be used in this analysis. CCREL allows us to perform haplotype-based association for a window width of a maximum of three markers. For the APOHin6, APOH154 and APOH266 variants within the *APOH* gene and rs10922093 and Tyr402His variants within the *CFH* gene haplotype-based association testing was carried out and the *P*-values reported. R package for the analysis (CCREL) were provided by the authors (43), but, as currently implemented, this is only applicable to two-allele systems. TDT or PDT type analyses are not feasible for our data, since such testing requires that parents be genotyped, which we essentially have none due to the late onset of AMD.

Multiple testing issues

To provide a measure of the impact of multiple testing, we employ the FDR approach of Benjamini and Hochberg (44) and Storey (45), as implemented in the fdr.control program of the R library Gene TS (60). The FDR approach controls the expected proportion of false positives among the significant tests.

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