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Genome-wide association study of age-related macular degeneration identifies associated variants in the *TNXB–FKBPL–NOTCH4* region of chromosome 6p21.3

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Age-related macular degeneration (AMD) is a leading cause of visual loss in Western populations. Susceptibility is influenced by age, environmental and genetic factors. Known genetic risk loci do not account for all the heritability. We therefore carried out a genome-wide association study of AMD in the UK population with 893 cases of advanced AMD and 2199 controls. This showed an association with the well-established AMD risk loci *ARMS2* (age-related maculopathy susceptibility 2)–*HTRA1* (HtrA serine peptidase 1) ($P = 2.7 \times 10^{-72}$), *CFH* (complement factor H) ($P = 2.3 \times 10^{-47}$), *C2* (complement component 2)–*CFB* (complement factor B) ($P = 5.2 \times 10^{-9}$), *C3* (complement component 3) ($P = 2.2 \times 10^{-3}$) and *CFI* ($P = 3.6 \times 10^{-3}$) and with more recently reported risk loci at *VEGFA* ($P = 1.2 \times 10^{-3}$) and *LIPC* (hepatic lipase) ($P = 0.04$). Using a replication sample of 1411 advanced AMD cases and 1431 examined controls, we confirmed a novel association between AMD and single-nucleotide polymorphisms on chromosome 6p21.3 at *TNXB* (tenascin XB)–*FKBPL* (FK506 binding protein like) [rs12153855/rs9391734; discovery $P = 4.3 \times 10^{-7}$, replication $P = 3.0 \times 10^{-4}$, combined $P = 1.3 \times 10^{-9}$, odds ratio (OR) = 1.4, 95% confidence interval (CI) = 1.3–1.6] and the neighbouring gene *NOTCH4* (Notch 4) (rs2071277; discovery $P = 3.2 \times 10^{-8}$, replication $P = 3.8 \times 10^{-5}$, combined $P = 2.0 \times 10^{-11}$, OR = 1.3, 95% CI = 1.2–1.4). These associations remained significant in conditional analyses which included the adjacent *C2–CFB* locus. *TNXB*, *FKBPL* and *NOTCH4* are all plausible AMD susceptibility genes, but further research will be needed to identify the causal variants and determine whether any of these genes are involved in the pathogenesis of AMD.

INTRODUCTION

Age-related macular degeneration (AMD) is a leading cause of visual loss in Western populations (1,2), reducing the quality of life of tens of millions of older people worldwide. It affects the macular region of the retina, which has a high density of photoreceptors for detailed central vision. Early in the disease, deposits called drusen form along Bruch's membrane, which separates the retinal pigment epithelium (RPE) from the underlying choroid (3). The later stages of the disease are characterized by focal atrophy of the RPE and overlying photoreceptors (geographic atrophy, GA) and/or growth of new blood vessels from the choroid through Bruch's membrane into the RPE (choroidal neovascularization, CNV) (3). Both of these processes can result in the loss of central vision.

Susceptibility to AMD is influenced by age, environmental and genetic factors (3). Smoking is the most important environmental risk factor (4). Striking progress has been made in understanding the genetics of AMD (5,6). Common sequence variants in the complement pathway genes *CFH* (7–10), *C2* (complement component 2)–*CFB* (complement factor B) (11,12) and *C3* (complement component 3) (13,14) are established risk factors and there is another risk locus in the vicinity of *CFI* (complement factor I) (15,16). This and other evidence points to the activation of the alternative complement pathway as an important component of the pathogenesis of AMD (17,18). Variants at the *ARMS2* (age-related maculopathy susceptibility 2)–*HTRA1* (HtrA serine peptidase 1) locus are strongly associated with AMD (19,20), but the mechanism is uncertain (21). More recently reported risk loci at *LIPC* (hepatic lipase) (22–24), *CETP* (cholesteryl ester transfer protein) (22–24), *TIMP3* (TIMP metallopeptidase inhibitor 3)–*SYN3* (synapsin III) (22–24) and *VEGFA* (vascular endothelial growth factor A) (24) implicate lipid metabolism, matrix homeostasis and control of angiogenesis as additional factors in the pathogenesis of AMD. The known genetic risk variants do not account for all the heritability of AMD. We have therefore carried out a genome-wide association study (GWAS) in the UK population to identify additional susceptibility loci.

RESULTS

We conducted a GWAS in the UK population obtaining genotypes for 893 cases and 2199 controls that passed quality control metrics (Materials and Methods, Table 1, Supplementary Material, Table S1). Subjects were typed with either the Illumina 300k array (150 cases) or the Illumina 550k array (743 cases and 2199 controls). Quality control criteria (Materials and Methods) were not met for 24 125 single-nucleotide polymorphisms (SNPs) in the 300k array and 68 531 SNPs in the 550k array. A small number of additional SNPs were excluded after visual inspection of their cluster plots so that subsequent analyses were based on 286 135 and 488 867 genotyped SNPs from the 300k and 550k arrays, respectively. We imputed variants using the CEU HapMap II reference panel and combined results from the two arrays following imputation for a total of 2 272 849 SNPs (Materials and Methods). Unless otherwise specified, the results discussed here did not show evidence of heterogeneity between the two platforms. The genomic inflation factor (λ) was 1.014 using all the genome-wide results and 1.007 after excluding the test statistics observed at the previously reported *CFH*, *C2–CFB* and

Table 1. Gender, age, phenotype and geographic origin of subjects in the discovery samples

	Cases ($n = 893$)	Controls ($n = 2199$)
Female, n (%)	494 (55.3)	1117 (50.8)
Mean age ± SD, years	78.6 ± 7.5	44–45[a]
Phenotype		
Unexamined		2199
CNV	593	
GA	182	
CNV and GA	118	
Geographic origin		
L	80	156
NMESES	548	1373
NW	163	272
S	102	398

CNV, choroidal neovascularization; GA, geographic atrophy; L, London; NMESES, North Midlands, East, Southeast and South of England; NW, Northwest of England; S, Scotland.
[a]Controls were selected from the 58BC (46,47).

ARMS2–HTRA1 loci; the quantile–quantile plots showed no widespread departure from the expected distribution of *P*-values (Supplementary Material, Figs S1 and S2). Therefore, no further correction for inflation was applied to the test statistics. Replication studies were conducted on a sample of up to 1411 advanced AMD cases and 1431 controls (Supplementary Material, Table S2).

Previously reported definite and probable AMD-associated loci

An association at genome-wide significance level ($P < 5 \times 10^{-8}$) was observed at the well-established AMD susceptibility loci *ARMS2–HTRA1* (rs10490924/rs2284665, $P = 2.7 \times 10^{-72}$), *CFH* (rs10801555, an almost perfect proxy for rs1061170, $P = 2.3 \times 10^{-47}$) and *C2–CFB* (rs541862, a perfect proxy for rs641153, $P = 5.2 \times 10^{-9}$). At the *CFH* locus, an analysis conditional on rs10801555 confirmed a second independent association with rs1329428 ($P = 1.1 \times 10^{-10}$) (12,22,25). For the reported independent signal at SNP rs9332739 in *C2* (12), we observed weak statistical support with $P = 0.02$ and no association when conditioning on rs541862 ($P = 0.78$). These results are presented in Table 2 together with association signals at other previously reported definite or probable AMD risk loci.

At the *LIPC* locus, the reported SNP rs493258 (22,23) showed weak evidence of association ($P = 0.04$) but in the same direction as previously reported [odds ratio (OR) = 0.89 and 95% confidence interval (CI) = 0.79–0.99 for allele T]. There was no evidence of an association with the reported functional variant rs10468017 in this gene ($R^2 = 0.42$; $P = 0.11$, OR = 0.91 and 95% CI = 0.80–1.03 for allele T) (24). We found an association with SNP rs943080 at the *VEGFA* locus ($P = 1.6 \times 10^{-3}$, a perfect proxy for the reported SNP rs4711751, OR = 1.20 and 95% CI = 1.07–1.35 for allele T) (24), but no association with the other reported SNP rs833069 ($P = 0.18$, OR = 0.92 and 95% CI = 0.82–1.04) (26). At the CFI locus, evidence of association was found with the previously reported SNP rs7690921 in *CCDC109B* (coiled-coil domain containing 109B) ($P = 3.6 \times 10^{-3}$, OR = 1.19 and 95% CI = 1.06–1.34 for allele T) (16,23), but not for rs10033900 ($P = 0.22$) (16) or rs2285714 ($P = 0.92$) (16,22). We did not find support for the previously reported association with variants at *CETP* (rs3764261, $P = 0.26$) (22–24) or *SYN3-TIMP3* (rs9621532, $P = 0.48$) (22–24).

At the *APOE* (apolipoprotein E) locus, the two SNPs that determine the E2/E3/E4 *APOE* alleles (rs429358 and rs7412) were not typed and could not be imputed. However, there was evidence of an association with rs2075650 ($P = 3.1 \times 10^{-6}$) in the same region, although with some degree of heterogeneity between the 300k and 550k arrays ($P = 0.06$). SNP rs2075650 lies within the *TOMM40* (translocase of outer mitochondrial membrane 40 homolog) gene and is in modest linkage disequilibrium (LD) with rs429358 ($R^2 = 0.2$). We genotyped rs2075650 and *APOE* SNP rs429358 in our replication samples (Supplementary Material, Table S2) and confirmed the known protective effect of the C allele of rs429358 ($P = 2.8 \times 10^{-6}$; heterogeneity $P = 0.04$; OR = 0.68; 95% CI = 0.58–0.80) and the association with rs2075650 ($P = 0.0037$; OR = 0.78; 95% CI = 0.67–0.92 for allele G), although some heterogeneity across the follow-up samples was observed ($P = 0.03$) (Table 3). However, the association with rs2075650 became non-significant after conditioning on rs429358 ($P = 0.64$).

Novel loci

Using a threshold of $P < 10^{-6}$ and excluding previously known associations, we identified four novel associated SNPs. For one of these, rs12231166 ($P = 6.2 \times 10^{-7}$) at 12q23.1, we genotyped the proxy rs476497 ($R^2 = 0.92$) in our replication samples (Supplementary Material, Table S2), but found no evidence of association (replication $P = 0.97$) (Table 3).

The other novel SNPs were rs2071277 in *NOTCH4* (Notch 4) ($P = 3.2 \times 10^{-8}$), rs12153855 in *TNXB* (tenascin XB) ($P = 4.3 \times 10^{-7}$) and the imputed SNP rs9391734 (a perfect proxy for rs12153855) in *FKBPL* (FK506 binding protein like) ($P = 4.3 \times 10^{-7}$), all located at 6p21.3 and ~250 kb from *C2–CFB* (Fig. 1). These SNPs are in low LD with rs541862 ($R^2 = 0.02$ and $R^2 = 0.01$ for rs2071277 and rs12153855/rs9391734, respectively), the SNP in *CFB* which has a well-established association with AMD (11,12).

Although our discovery data showed no overall evidence of residual population structure (Supplementary Material, Fig. S2), the stratification by geographic region used in our association analysis might be unsatisfactory in genomic regions such as 6p21.3 which contain long-range haplotypes that can be sensitive to population structure. Therefore, we also performed an association analysis by including the first two principal components (Supplementary Material, Fig. S3A) as covariates in the logistic regression model and repeated analogous analysis after excluding ethnic outliers (Supplementary Material, Fig. S3B). We observed negligible effect of these adjustments on the *P*-values and the effect size estimates (Supplementary Material, Table S3).

Conditioning for the effect of rs541862 ($P = 5.2 \times 10^{-9}$) did not eliminate evidence of association for several SNPs spanning the *TNXB–NOTCH4* locus (Supplementary Material, Table S4, Fig. 1B). Together with rs541862 in *CFB*, we

Table 2. Association analysis in the discovery samples for previously reported definite or probable AMD susceptibility loci

Locus	Reported SNP/proxy SNP (R^2)	Reference	Chr	Position (bp)	Effect allele (EA)/other allele	EA frequency Cases	Controls	P	OR	95% CI
ARMS2–HTRA1	rs10490924	(19,20)	10	124 204 440	T/G	0.46	0.21	2.7×10^{-72}	3.00	2.64–3.40
CFH	rs1061170/rs10801555 (0.97)	(10)	1	194 926 880	G/A	0.40	0.60	2.3×10^{-47}	0.43	0.38–0.48
	rs1410996/rs1329428 (1.0)	(12)	1	194 969 430	T/C	0.21	0.39	3.1×10^{-43}	0.40	0.35–0.45
C2–CFB	rs641153/rs541862 (1.0)	(11,12)	6	32 024 930	T/C	0.95	0.90	5.2×10^{-9}	1.97	1.56–2.49
	rs9332739	(12)	6	32 011 783	G/C	0.97	0.96	0.02	1.47	1.06–2.04
VEGFA	rs4711751/rs943080 (1.0)	(24)	6	43 934 605	T/C	0.54	0.49	1.6×10^{-3}	1.20	1.07–1.35
	rs833069	(26)	6	43 850 557	T/C	0.66	0.68	0.18	0.92	0.82–1.04
C3	rs2230199	(13,14)	19	6 669 387	G/C	0.76	0.79	2.2×10^{-3}	0.80	0.69–0.92
CCDC109B–PLA2G12A–CFI	rs7690921	(23)	4	110 787 610	T/C	0.36	0.32	3.6×10^{-3}	1.19	1.06–1.34
	rs10033900	(16)	4	110 878 520	T/C	0.51	0.49	0.22	1.07	0.96–1.20
	rs2285714	(22)	4	110 858 259	T/C	0.45	0.46	0.92	0.99	0.89–1.11
LIPC	rs493258	(22,23)	15	56 475 172	T/C	0.46	0.48	0.04	0.89	0.79–0.99
	rs10468017	(24)	15	56 465 804	T/C	0.26	0.27	0.11	0.91	0.80–1.03
CETP	rs3764261	(22-24)	16	55 550 825	C/A	0.66	0.67	0.26	0.93	0.83–1.05
SYN3–TIMP3	rs9621532	(22-24)	22	31 414 511	C/A	0.05	0.05	0.48	0.91	0.69–1.20

Chr, chromosome; bp, base pair; Effect allele, allele for which the ORs and 95% CIs were calculated; *P*, *P*-value from the stratified analysis using the Mantel's extension of the 1 df Cochrane–Armitage trend test. Heterogeneity *P*-value for SNP rs10468017 = 0.03.

selected rs12153855 in *TNXB* for follow-up, which showed a conditioned $P = 9.0 \times 10^{-6}$, and rs2071277 and rs3132946 in *NOTCH4* for which we observed some residual evidence of association after controlling for both rs541862 and rs12153855 ($P = 3.8 \times 10^{-3}$ and $P = 3.5 \times 10^{-3}$, respectively). Corresponding replication results are summarized in Table 3. As expected, the known protective association at the C allele of rs541862 was confirmed ($P = 8.8 \times 10^{-17}$; OR = 0.53; 95% CI = 0.46–0.62 when combining all samples). Association at both rs2071277 and rs12153855 was consistently corroborated across the replication samples ($P = 3.8 \times 10^{-5}$ and 3.0×10^{-4}, respectively) and reached genome-wide significance when combining all samples ($P = 2.0 \times 10^{-11}$; OR = 1.30; 95% CI = 1.20–1.41 for allele C of rs2071277; $P = 1.3 \times 10^{-9}$; OR = 1.44; 95% CI = 1.28–1.63 for allele C of rs12153855). Repeating the conditional analysis, we found that both rs12153855 in *TNXB* and rs2071277 in *NOTCH4* conferred independent risk beyond the known signal at the *C2–CFB* locus as shown in Table 4. Either conditioning on rs541862 and rs12153855 or on rs541862 and rs2071277 did not eliminate the effect at the third SNP with a conditioned *P*-value of 9.0×10^{-4} for rs2071277 and 2.5×10^{-4} for rs12153855.

To further characterize the association in this region, we carried out a haplotype analysis of rs541862, rs12153855 and rs2071277. Four haplotypes at frequency >1% accounted for 98.9% of the total haplotypes observed (Table 5). The most frequent haplotype TTT was used as reference to calculate ORs. Distinct levels of AMD risk with non-overlapping CIs were associated with these four haplotypes ($P = 9.1 \times 10^{-23}$). The known protective haplotype (CTT) tagged by the C allele of rs541862 was present in 9.4% of controls and 5.1% of cases after combining all samples. The other two haplotypes TTC and TCC that do not harbour the protective C allele of rs541862 significantly differed from the baseline haplotype TTT with OR = 1.14 (95% CI = 1.05–1.25) and OR = 1.47 (95% CI = 1.29–1.67), respectively.

Previously reported putative AMD-associated loci

A literature search for studies reporting evidence of an association between a common SNP and AMD identified a total of 47 loci (Materials and Methods) in addition to the established and probable loci discussed earlier (Table 2). We reviewed our GWAS discovery data for the most associated SNP reported at each of these putative loci (or a proxy for that SNP). Association results for 45 of these SNPs were available as summarized in Supplementary Material, Table S5. Using a Bonferroni-corrected *P*-value threshold of 0.001 to account for multiple testing, only rs429608 in *SKIV2L* (superkiller viralicidic activity 2-like) was clearly significant ($P = 1.0 \times 10^{-6}$). This SNP lies 13 kb from the established *C2–CFB* locus ($R^2 = 0.38$) and had been reported to confer an independent protective effect (27). In our conditional analysis of the extended *C2–CFB* region, the association signal at rs429608 disappeared after conditioning on rs550605 (*C2*), rs541862 (*CFB*) or rs438999 (*SKIV2L*) (Supplementary Material, Table S6). SNP rs13095226 in *COL8A1* (collagen, type VIII, alpha 1) approached the Bonferroni-corrected level of significance ($P = 4.1 \times 10^{-3}$), and rs17778253 in the same gene ($R^2 = 0.74$) showed stronger evidence of association ($P = 4.3 \times 10^{-4}$). However, replication studies for rs13095226 and rs17778253 showed no statistical support for association ($P = 0.37$ and 0.27, respectively) (Table 3).

Secondary analyses

Genome-wide association analyses were repeated for the subgroups of subjects affected by CNV only and GA only. Only SNPs at the *CFH* and *ARMS2–HTRA1* loci showed association at a genome-wide level of significance in both subgroups. Evidence of association was observed at the newly identified SNPs rs12153855/rs9391734 in *TNXB–FKBPL* in both subgroups ($P = 5.0 \times 10^{-4}$, OR = 0.70, 95% CI = 0.58–0.86 and $P = 5.0 \times 10^{-6}$, OR = 0.51, 95% CI =

Table 3. Association analysis for SNPs in *TNXB* and *NOTCH4* and other SNPs typed in the replication studies

SNP	Locus	Chr	Position (bp)	Effect allele (EA)/other allele	Discovery samples (893 cases, 2199 controls)		English replication samples (1267 cases, 1102 controls)		Scottish replication samples (144 cases, 329 controls)		Replication samples combined (1411 cases, 1431 controls)		All samples combined (2304 cases, 3630 controls)		
					EA frequency (cases/controls)	*P*	EA frequency (cases/controls)	*P*	EA frequency (cases/controls)	*P*	*P*	Het *P*	*P*	OR (95% CI)	Het *P*
Newly identified SNPs															
rs12153855	*TNXB*	6	32 182 782	C/T	0.023/0.011	4.3×10^{-7}	0.016/0.008	6.8×10^{-5}	0.022/0.019	0.9266	3.0×10^{-4}	0.0959	1.3×10^{-9}	1.44 (1.28–1.63)	0.3892
rs2071277	*NOTCH4*	6	32 279 661	C/T	0.277/0.219	3.2×10^{-8}	0.271/0.222	2.6×10^{-5}	0.222/0.212	0.5481	3.8×10^{-5}	0.3004	2.0×10^{-11}	1.30 (1.20–1.41)	0.1869
Other SNPs typed in the replication studies															
rs13095226	*COL8A1*	3	100 878 962	C/T	0.124/0.100	4.0×10^{-3}	0.105/0.099	0.4807	0.128/0.113	0.5205	0.3690	0.7481	0.0059	1.19 (1.05–1.35)	0.5970
rs17778253	*COL8A1*	3	100 917 792	C/T	0.113/0.084	4.3×10^{-4}	0.095/0.087	0.3432	0.112/0.098	0.5501	0.2703	0.8408	0.0011	1.24 (1.09–1.42)	0.5490
rs541862	*CFB*	6	32 024 930	C/T	0.003/0.010	5.2×10^{-9}	0.005/0.012	9.3×10^{-9}	0.003/0	0.0777	2.2×10^{-9}	0.5955	8.8×10^{-17}	0.53 (0.46–0.62)	0.9112
rs3132946	*NOTCH4*	6	32 298 006	A/G	0.009/0.019	2.7×10^{-3}	0.024/0.019	0.7380	0.028/0.022	0.9694	0.7691	0.8690	2.9×10^{-2}	0.88 (0.79–0.99)	0.4089
rs12231166	*GOLGA2P5–ACTR6*	12	20 332 348	C/A	0.164/0.116	6.2×10^{-7}	0.134/0.135	0.9312	0.135/0.127	0.7379	0.9687	0.7313	5.3×10^{-4}	1.22 (1.09–1.37)	0.0026
rs2075650	*TOMM40*	19	50 087 459	G/A	0.105/0.149	3.1×10^{-6}	0.007/0.022	5.6×10^{-4}	0.016/0.006	0.2288	0.0037	0.0261	8.4×10^{-8}	0.73 (0.65–0.82)	0.0148
rs429358	*APOE*	19	50 103 781	C/T	N/A	N/A	0.006/0.024	3.4×10^{-7}	0.008/0.003	0.7469	2.8×10^{-6}	0.0414	2.8×10^{-6}	0.68 (0.58–0.80)	0.0414

Chr, chromosome; bp, base pair; *P*, *P*-value from the stratified analysis using the Mantel's extension of the 1 df Cochrane–Armitage trend test; Het *P*, heterogeneity *P*-value. For the GOLGA2P5–ACTR6 locus, proxy SNP rs476497 (R^2=0.92) was followed up in the replication samples.

0.38–0.68 in the CNV only and GA only subgroup analyses, respectively). The evidence of association at SNP rs2071277 in *NOTCH4* was stronger in the CNV-only subgroup than in the GA-only subgroup ($P = 3.5 \times 10^{-6}$, OR = 0.73, 95% CI = 0.64–0.84 and $P = 0.07$, OR = 0.82, 95% CI = 0.66–1.01, respectively), but there was no statistically significant difference between the two subgroup analyses ($P = 0.81$).

DISCUSSION

We have carried out the first GWAS of AMD in the UK population. As expected, we found association with the well-established susceptibility loci *ARMS2–HTRA1* (19,20), *CFH* (7–10), *CFB–C2* (11,12), *C3* (13,14), *CFI* (15,16) and *APOE* (28). There was also evidence of association for more recently reported risk loci at *VEGFA* (24) and *LIPC* (22–24), but not *CETP* or *TIMP3–SYN3* (22–24). Our discovery experiment had limited power to detect these weaker associations because of the sample size and the use of younger unexamined controls (29).

At the *CFI* locus, the strongest association was with an SNP in the neighbouring *CCDC109B* gene as reported by others (23) rather than with the originally reported SNP rs10033900 closer to *CFI* (16). This is unsurprising as we have previously reported lack of association with rs10033900 in two independent UK case–control samples (15) and it was a subset of cases from these studies that was used in our GWAS. The other reported association at this locus with rs2285714 in *PLA2G12A* (phospholipase A2, group XIIA) (22) showed no evidence of association in our discovery analysis. Further studies are needed to clarify the origin of the association signal in this region.

The association at the *CFH* locus on chromosome 1q32 was extensive, as found in other GWAS (22–24). It spanned a megabase of DNA encompassing many genes. Conditional analysis of the region identified two independent association signals, one characterized by SNP rs10801555 (an almost perfect proxy for rs10611760, Y402H) and the other by rs1329428 (a proxy for rs1410996). These two independent association signals have been reported previously (12,22,25).

On chromosome 10q26, there was a strong association spanning the *ARMS2* and *HTRA1* genes as found by others (22–24), but high LD prevented further localization of the association signal.

On chromosome 6p21.3, there was a strong association with rs541862, a perfect proxy for a nonsynonymous change R32Q (rs641153) in *CFB* which has a well-established association with AMD, the minor allele being protective (11,12). This was one of several strongly associated SNPs spanning *C2*, *CFB*, *RDBP* (RD RNA-binding protein) and *SKIV2L* in a region of high LD (Fig. 1A) which makes the dissection of the statistical association difficult. Support for rs641153 as the causal variant comes from functional studies showing that the 32Q variant of *CFB* has reduced activity compared with 32R because of weaker binding to *C3b* (30). Another associated SNP from this region, rs438999 (R151Q) in the *SKIV2L*, has also been suggested as a causal variant (31), and a protective effect of rs429608 in the same gene has been reported (27). Our data showed evidence of association

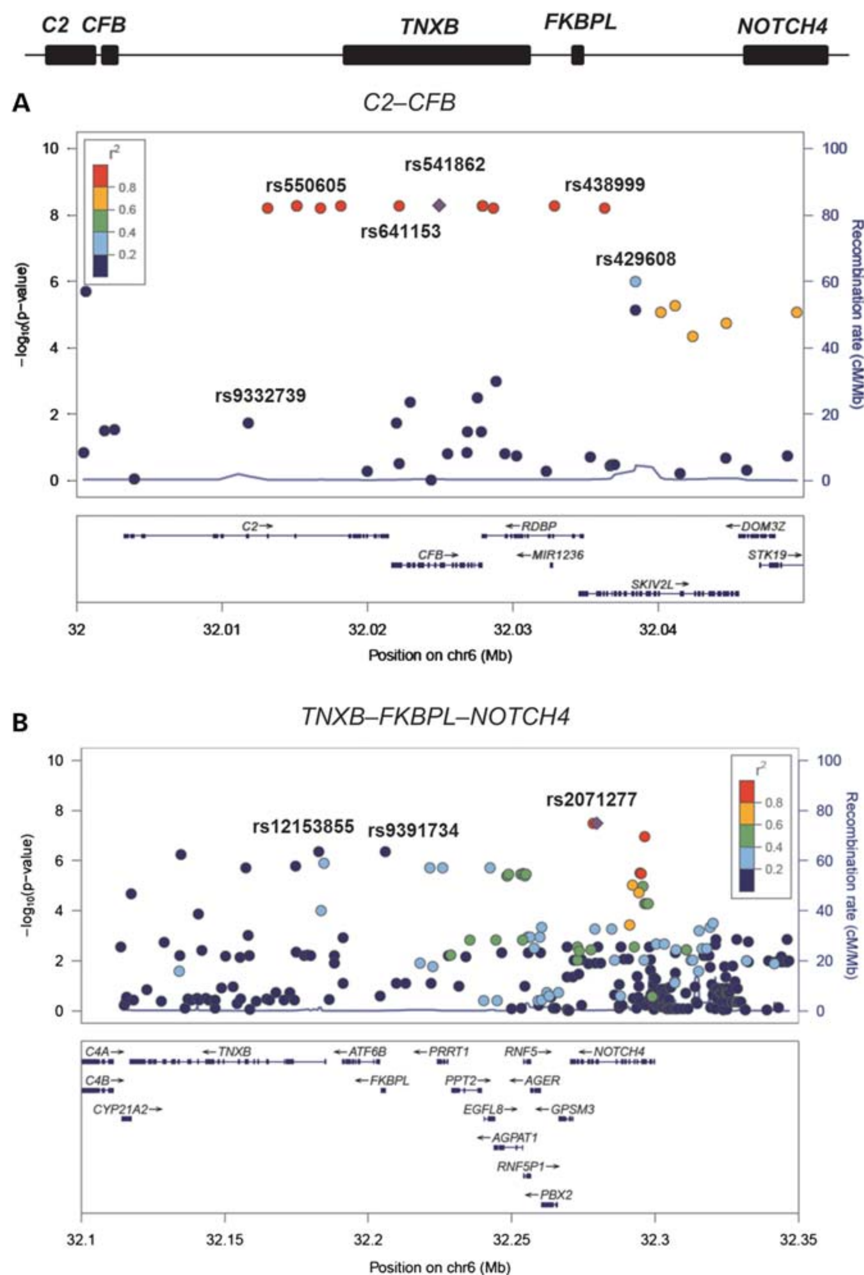

Figure 1. Regional plots of association on chromosome 6p21.3 in the discovery samples (**A**) for the established association signal at the *C2–CFB* locus (**B**) for the newly identified association in the *TNXB–FKBPL–NOTCH4* region. The most associated SNP in each region is denoted by a purple diamond and other SNPs by circles coloured to reflect their degree of LD with the most associated SNP (based on HapMap II).

with both rs438999 and rs429608, but conditional analysis did not provide support for an association at *SKIV2L* independent of the signal from rs541862 in *CFB* (Supplementary Material, Table S6).

On chromosome 6p21.3, we also found a strong association reaching genome-wide significance for SNP rs2071277 in the *NOTCH4* gene some 250 kb from *CFB*. Conditional analysis confirmed that this was independent of the *C2–CFB* signal represented by rs541862 and also provided support for a second independent association with rs12153855 in the intervening *TNXB* gene. Only four haplotypes defined by these three SNPs were observed (Table 5), including the protective haplotype CTT characterized by a C allele at rs541862 which is the well-established protective association reported for *C2–CFB*. The other three haplotypes had a T allele at rs541862. The commonest of these was TTT, which we designated as the reference haplotype. The next most common haplotype TTC was associated with an increased risk for AMD, giving an OR of 1.14 (95% CI 1.05–1.25); the fourth haplotype TCC was associated with higher risk, the OR being 1.47 (95% CI 1.29–1.67).

Both rs12153855 in *TNXB* and rs2071277 in *NOTCH4* are intronic and unlikely to be causal variants. SNP rs12153855 has a perfect proxy rs9391734 in the 5′ untranslated region

Table 4. Conditional association analysis in the extended C2/CFB region

Test SNP	Gene	Chr	Position (bp)	Conditioning SNP(s)	Discovery samples (893 cases, 2199 controls)		Replication samples (1411 cases, 1431 controls)		All samples combined (2304 cases, 3630 controls)	
					Unconditioned *P*	Conditioned *P*	Unconditioned *P*	Conditioned *P*	Unconditioned *P*	Conditioned *P*
rs541862	*CFB*	6	32 024 930	rs541862	4.9×10^{-9}	N/A	2.2×10^{-9}	N/A	8.8×10^{-17}	N/A
rs12153855	*TNXB*	6	32 182 782		4.2×10^{-7}	9.0×10^{-6}	2.9×10^{-4}	0.0024	1.3×10^{-9}	1.6×10^{-7}
rs2071277	*NOTCH4*	6	32 279 661		3.1×10^{-8}	2.3×10^{-5}	3.8×10^{-5}	0.0048	2.0×10^{-11}	1.1×10^{-6}
rs3132946	*NOTCH4*	6	32 298 006		2.7×10^{-3}	4.9×10^{-4}	0.77	0.32	0.03	2.5×10^{-3}
rs541862	*CFB*	6	32 024 930	rs541862, rs12153855	4.9×10^{-9}	N/A	2.2×10^{-9}	N/A	8.8×10^{-17}	N/A
rs12153855	*TNXB*	6	32 182 782		4.2×10^{-7}	N/A	2.9×10^{-4}	N/A	1.3×10^{-9}	N/A
rs2071277	*NOTCH4*	6	32 279 661		3.1×10^{-8}	3.8×10^{-3}	3.8×10^{-5}	0.05	2.0×10^{-11}	9.1×10^{-4}
rs3132946	*NOTCH4*	6	32 298 006		2.7×10^{-3}	3.5×10^{-3}	0.77	0.58	0.03	0.02
rs541862	*CFB*	6	32 024 930	rs541862, rs2071277	4.9×10^{-9}	N/A	2.2×10^{-9}	N/A	8.8×10^{-17}	N/A
rs12153855	*TNXB*	6	32 182 782		4.2×10^{-7}	2.0×10^{-3}	2.9×10^{-4}	0.04	1.3×10^{-9}	2.5×10^{-4}
rs2071277	*NOTCH4*	6	32 279 661		3.1×10^{-8}	N/A	3.8×10^{-5}	N/A	2.0×10^{-11}	N/A
rs3132946	*NOTCH4*	6	32 298 006		2.7×10^{-3}	0.04	0.77	0.95	0.03	0.21
rs541862	*CFB*	6	32 024 930	rs541862, rs3132946	4.9×10^{-9}	N/A	2.2×10^{-9}	N/A	8.8×10^{-17}	N/A
rs12153855	*TNXB*	6	32 182 782		4.2×10^{-7}	7.7×10^{-5}	2.9×10^{-4}	0.003	1.3×10^{-9}	1.2×10^{-6}
rs2071277	*NOTCH4*	6	32 279 661		3.1×10^{-8}	2.2×10^{-3}	3.8×10^{-5}	0.01	2.0×10^{-11}	1.4×10^{-4}
rs3132946	*NOTCH4*	6	32 298 006		2.7×10^{-3}	N/A	0.77	N/A	0.03	N/A
rs541862	*CFB*	6	32 024 930	rs541862, rs12153855, rs3132946	4.9×10^{-9}	N/A	2.2×10^{-9}	N/A	8.8×10^{-17}	N/A
rs12153855	*TNXB*	6	32 182 782		4.2×10^{-7}	N/A	2.9×10^{-4}	N/A	1.3×10^{-9}	N/A
rs2071277	*NOTCH4*	6	32 279 661		3.1×10^{-8}	0.06	3.8×10^{-5}	0.09	2.0×10^{-11}	0.01
rs3132946	*NOTCH4*	6	32 298 006		2.7×10^{-3}	N/A	0.77	N/A	0.03	N/A
rs541862	*CFB*	6	32 024 930	rs541862, rs12153855, rs2071277	4.9×10^{-9}	N/A	2.2×10^{-9}	N/A	8.8×10^{-17}	N/A
rs12153855	*TNXB*	6	32 182 782		4.2×10^{-7}	N/A	2.9×10^{-4}	N/A	1.3×10^{-9}	N/A
rs2071277	*NOTCH4*	6	32 279 661		3.1×10^{-8}	N/A	3.8×10^{-5}	N/A	2.0×10^{-11}	N/A
rs3132946	*NOTCH4*	6	32 298 006		2.7×10^{-3}	0.04	0.77	0.92	0.03	0.22

Chr, chromosome; bp, base pair; *P*, *P*-value from the stepwise logistic regression using the Wald test on the beta coefficients. LD based on HapMap II: R^2(rs541862, rs12153855) = 0.01; R^2(rs541862, rs2071277) = 0.02; R^2(rs541862, rs3132946) = 0.02; R^2(rs12153855, rs2071277) = 0.14; R^2(rs12153855, rs3132946) = 0.02; R^2(rs2071277, rs3132946) = 0.17.

Table 5. Association analysis for the common haplotypes spanning *CFB–TNXB–NOTCH4*

Haplotype			Discovery samples (893 cases, 2199 controls)		Replication samples (1411 cases, 1431 controls)		All samples combined (2304 cases, 3630 controls)			
rs541862 (CFB)	rs12153855 (TNXB)	rs2071277 (NOTCH4)	OR	95% CI	OR	95% CI	Haplotype frequency Cases	Controls	OR	95% CI
T	T	T	1		1		0.423	0.449	1	
T	T	C	1.18	1.04–1.34	1.13	1.00–1.27	0.394	0.359	1.14	1.05–1.25
T	C	C	1.62	1.35–1.94	1.35	1.12–1.62	0.132	0.098	1.47	1.29–1.67
C	T	T	0.54	0.42–0.71	0.58	0.46–0.73	0.051	0.094	0.56	0.48–0.67

TTT, TTC, TCC and CTT account for 98.9% of the total haplotypes observed.

of the neighbouring gene *FKBPL*, which might have functional significance. So we have identified novel associated variants but do not have evidence to pinpoint the causal variants responsible for these associations, as is the case with other susceptibility loci for AMD and many other complex diseases. All three genes could play a role in the pathogenesis of AMD as we review in what follows. However, further research will be needed to determine whether or not this is the case. Other disease associations have been reported for this region of chromosome 6 and notably an association with dementia and variants near *NOTCH4* and *AGER* (advanced glycosylation end product-specific receptor) (32).

TNXB encodes the extracellular matrix protein tenascin-X, which is present in the macular Bruch's membrane/choroid complex (33). Various functions have been attributed to tenascin-X, which could have relevance to AMD. It has been shown to modulate collagen deposition (34). There is also evidence that it modulates the mechanical properties of collagen networks (35) and is involved in the maturation and/or maintenance of collagen and elastin networks (36); both collagen and elastin are present in Bruch's membrane. In addition, forms of tenascin-X have been shown to bind to *VEGFA* and *VEGFB* (vascular endothelial growth factor B) and enhance their pro-angiogenic activity (37).

FKBPL encodes the *FKBP*-like protein which belongs to the family of *FK506*-binding proteins and has a role in intracellular signalling (38). Of particular interest in the context of AMD, *FKBPL* also appears to have a separate role in controlling angiogenesis as a secreted protein that inhibits endothelial cell migration, tubule formation and angiogenesis through a *CD44*-mediated pathway (39).

NOTCH4 encodes one of the four cell surface receptors of the Notch signalling pathway which has a key role in cell differentiation decisions across many cell types and at different stages of cell lineage progression (40). Notch signalling is important in embryonic development, the regulation of tissue homeostasis and the maintenance of stem cells. Notch signalling is particularly important in vascular development, not only in embryogenesis but also in pathological angiogenesis, prompting research on Notch pathway inhibitors as potential agents to disrupt tumour-related angiogenesis for cancer therapy (41).

Notch signalling plays an important role in the developing retinal vasculature by regulating the specification of endothelial cells into stalk and tip cells (42). In studies of mice, *NOTCH4* is strongly expressed in retinal arterial endothelial cells (43). Moreover, Notch signalling has been shown to influence pathological angiogenesis in a study of laser-induced CNV in rats (44). Following laser photocoagulation, inhibition of Notch signalling by intravitreal or subcutaneous administration of the gamma secretase inhibitor *DAPT* resulted in a substantial increase in the CNV volume compared with controls; conversely, stimulation of Notch signalling by intravitreal administration of Jagged1 peptide, a Notch ligand, substantially reduced the CNV volume (44). In the same report, evidence was presented that Notch signalling contributes to angiogenic homeostasis by providing a counterbalance to proangiogenic pathways such as that mediated by *VEGF* (44). This led the authors to propose the Notch pathway as a potential therapeutic target for the treatment of CNV, which is the severest form of AMD (44). This might complement the use of anti-*VEGF* antibodies, which is the mainstay of current treatment. Our finding that SNPs at the *NOTCH4* locus are associated with AMD provides support for the involvement of Notch signalling in AMD and points specifically to a role for the *NOTCH4* receptor. However, if this were mediated through an alteration in angiogenic homeostasis, we might expect the association to be specifically with CNV. Most of the association signal does indeed come from CNV because this is the condition affecting most of the cases in our study, but the subgroup analysis suggests that there is also an association in the minority of cases with GA. This raises the possibility that other functions of the Notch pathway may be more important, such as its role in the differentiation of immune cells, including macrophages, which, as recent evidence suggests, may play a key part in the pathogenesis of AMD (45).

In summary, our UK GWAS has shown the expected association between AMD and known risk loci at *ARMS2–HTRA1*, *CFH*, *C2–CFB*, *C3*, *CFI–CCDC109B* and *APOE* and with more recently reported risk loci at *LIPC* and near *VEGFA*. In addition, we have found novel associations with variants in *TNXB–FKBPL* and the neighbouring gene *NOTCH4* on chromosome 6p21.3 which are independent of the associated SNPs at the nearby *C2–CFB* locus. *TNXB*, *FKBPL* and *NOTCH4* are all plausible AMD susceptibility genes, but further research will be needed to identify the causal variants and determine whether any of these genes are involved in the pathogenesis of AMD.

MATERIALS AND METHODS

Cases and controls

Cases for the discovery experiment were selected from existing Cambridge and Edinburgh AMD case–control sample collections (14). All cases selected for the GWAS had at least one eye affected by CNV and/or GA. For cases genotyped using the 300k platform, preference was given to cases with early onset. Control genotyping data were obtained from the British 1958 Birth Cohort (58BC) (46,47). For cases genotyped using the 300k platform, controls matched by geographic area were selected from the 58BC subjects used in the Wellcome Trust Case Control Consortium study (48). For cases genotyped using the 550k platform, controls matched by geographic area were selected from the 58BC subjects genotyped as controls for the Type 1 Diabetes Genetics Consortium (T1DGC) study (46). The replication studies used cases and controls from the Cambridge and Edinburgh sample collections and from case–control collections from London and Southampton in the UK. All the studies used in this research followed the tenets of the Declaration of Helsinki and had appropriate ethical approval. All participants gave informed written consent.

Cambridge AMD study subjects

Cases and controls were recruited from ophthalmic clinics in London, the South East of England and the North West of England between 2002 and 2006 (14). Almost all the controls were the spouses or partners of index cases and the remainder were friends of cases. All subjects described themselves as ‘white’ rather than ‘other’ on a recruitment questionnaire. Subjects were examined by an ophthalmologist, and health, lifestyle and smoking data were collected. All subjects had colour, stereoscopic fundus photography of the macular region, and the images were graded at the Reading Centre, Moorfields Eye Hospital, London, using the International Classification of Age-related Maculopathy and Macular Degeneration (49).

Edinburgh AMD study subjects

The majority of cases and controls were recruited from ophthalmic clinics in Edinburgh, Dundee and Inverness between 2004 and 2006 (14). Controls were predominantly recruited from cataract clinics in the same centres but some spouses were also included. Cases and controls were all examined by an ophthalmologist, and visual acuities, health, lifestyle and smoking data were collected. Members of the 1921 Lothian Birth Cohort (50) were examined by an ophthalmologist and those found to have AMD and a similar number of unaffected controls from the Cohort were also included in the study. All cases and controls had colour, stereoscopic fundus photography of the macular region and images were graded by an ophthalmologist working on the study, using the International Classification of Age-related Maculopathy and Macular Degeneration (49); for validation, 100 cases and controls were independently graded at the Moorfields Reading Centre (kappa statistic = 0.84).

London AMD study subjects

Cases were recruited from Moorfields Eye Hospital, London. Controls were recruited from spouses, partners or friends of cases, or were from local residential homes for the elderly within 8 km of the hospital. All subjects were of Caucasian descent. Cases were examined by an ophthalmologist and had colour, stereoscopic fundus photography of the macular region with grading of the photographs at the Reading Centre, Moorfields Eye Hospital, according to the International Classification of Age-related Maculopathy and Macular Degeneration (49). Cases were excluded if they had retino-choroidal inflammatory disease, diabetic retinopathy, branch retinal vein or artery occlusion or any other cause of visual loss other than amblyopia. The ophthalmic examination included Snellen acuity, slit-lamp examination and biomicroscopic fundoscopy. Auto-fluorescence images were taken of the macula, and fluorescein angiography was performed when CNV was suspected. For patients presenting with visual dysfunction in the second eye, retrospective data were gathered from hospital records concerning previous acuities. Moreover, any colour images or fluorescein images relating to previous visual loss were located from the hospital archive. All images were digitized. Controls had the same evaluation as cases and were excluded if they had drusen >63 μm or more extensive evidence of AMD. Each participant was interviewed specifically for the study, and a family history, smoking history and other medical history were taken.

Southampton subjects

Cases were ascertained through the Southampton Eye Unit or research clinics in Guernsey, UK. Controls were spouses/partners of AMD cases, patients attending eye clinics with unrelated eye disease or their spouses/partners. All participants were white and >55 years of age. All cases and controls were evaluated by an experienced retinal specialist and had dilated eye examination. Phenotyping was based on the Age-Related Eye Disease Study (AREDS) classification system (51), in cases taking into account the results of fundus photography and fluorescein angiography and in controls based on clinical findings alone.

Genotyping

Genotyping of cases for the discovery experiment used genomic DNA extracted from peripheral blood leucocytes. Cases were typed either at the British Heart Foundation Glasgow Cardiovascular Research Centre, using the Illumina Infinium HumanHap300 BeadChip, or at the Wellcome Trust Clinical Research Facility, Edinburgh, using the Illumina Infinium HumanHap550v3 BeadChip, according to the manufacturer’s protocols. Controls from the 58BC had been previously genotyped using the Illumina Infinium HumanHap550v1 BeadChip (58BC controls) (48) and the Illumina Infinium HumanHap550v3 BeadChip (T1DGC controls) (46).

The replication studies used genomic DNA extracted from the peripheral blood. Genotyping was carried out by the Medical Research Council Human Genetics Unit (MRC HGU) in Edinburgh, the Centre National de Génotypage (CNG) in France or the commercial company KBioscience.

At MRC HGU and CNG, genotyping was carried out using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. KBioscience (http://www.kbioscience.co.uk) used their own proprietary KASP SNP genotyping system, a competitive allele-specific PCR incorporating a fluorescent resonance energy transfer quencher cassette (see http://www.kbioscience.co.uk/reagents/KASP.html).

Statistical analysis

Genotype and sample quality control
Genotypes for the Illumina arrays were called using the genotyping module made available in the Illumina GenomeStudio 2009.2 software that incorporates the GenTrain 2.0 clustering algorithm. Given the different platforms used in the 300k analysis, only the overlap set of genotyped SNPs was used in the analysis. SNPs were excluded if they had a minor allele frequency of <0.05, or a departure from Hardy–Weinberg equilibrium at $P < 10^{-5}$ in controls, or a call rate <0.975 either in cases or in controls in the 300k and 550k data sets separately. Genotype cluster plots were visually inspected for each putative association and results were discarded if the clusters were not clearly separated. Samples were removed if they presented inconsistencies between reported sex and sex determined by homozygosity on the X chromosome, or an excessive genetic relatedness based on IBS metrics calculated for each pair of individuals, or a call rate <0.975. To control for possible population stratification, cases were stratified according to their reported geographic origin in four strata (London; North Midlands, East, Southeast and South of England; Northwest of England; Scotland), and only controls from matching geographic regions were used.

Imputation
Autosomal imputation was performed using the R package snpStats from the BioConductor Project (http://www.bioconductor.org) and CEU HapMap II samples as the reference data set. The imputation method has been described previously (52). Briefly, for the 300k and the 550k arrays separately, HapMap SNPs were divided into those that passed quality control steps in the study (X) and those that were not typed or failed quality control (Y). Linear regression models were used to predict each SNP in Y from nearby SNPs in X. R^2 was used as a measure of accuracy of the imputation.

Discovery and replication association analyses
Single SNP association analyses were carried out using the R package snpStats (http://www.bioconductor.org). For both discovery and replication analyses, single SNP association was evaluated using the Mantel extension of the 1 degree-of-freedom (df) trend test (53), which is a stratified version of the Cochrane–Armitage test, to take into account the geographic stratification of the samples. Results from the 300k and 550k arrays were combined by pooling the respective single SNP 1 df score statistics, and corresponding combined P-values were obtained from a 1 df χ^2 distribution. Presence of heterogeneity between the 300k and 550k association results was evaluated through a 1 df χ^2 test. As an alternative procedure to control for potential population stratification, we performed principal component analysis to categorize samples in terms of their large-scale genetic ancestry, using the GCTA software (54), and HapMap III samples as reference, and repeated the association analysis including the first two principal components as covariates in the logistic regression model, using PLINK v1.07 (55). The presence of residual population structure was assessed by evaluating the genomic inflation factors (λ) (56) and examining the quantile–quantile plots of the genome-wide P-values before and after exclusion of the established AMD loci. SNPs showing association at $P < 10^{-6}$ in regions not previously reported were selected for follow-up in the replication samples. Regional association results were plotted using the LocusZoom software (http://csg.sph.umich.edu/locuszoom).

Conditional and haplotype-based association analyses
To detect secondary independent signals within associated regions, we used stepwise logistic regression models stratified by array and geographic origin after controlling for the strongest associated genotyped variants at each region added to the models in a forward fashion (R package snpStats, http://www.bioconductor.org).

Haplotype-based analysis was carried out in PLINK v1.07 (55). Array information and geographic origin were added as covariates in the analysis. Only haplotypes with overall frequency $>1\%$ were considered. The ORs and the corresponding CIs were estimated using the commonest haplotype as reference.

Signal at previously reported SNPs
A literature search was conducted to identify studies reporting an association between a common SNP and AMD. Reports of associations with established and probable AMD loci were excluded (Table 2), as were associations with deletions, rare variants, mitochondrial variants or SNP haplotypes where no single SNP showed a significant association. This led to the identification of 47 previously reported putative AMD susceptibility loci (Supplementary Material, Table S5). For the SNP reported to show the strongest evidence of association at each of these loci, we checked the corresponding signal in our discovery samples. SNPs that passed the Bonferroni correction were chosen for follow-up in our replication samples.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. J.R.W.Y. declares a financial interest in a patent that Cambridge Enterprise, on behalf of Cambridge University, has filed in North America arising from the discovery that a genetic variant in the complement *C3* gene influences the risk of developing AMD. V.C., A.T.M. and J.R.W.Y. declare a financial interest in a patent application that UCL Business has made on behalf of University College London arising from the discoveries reported here.

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REFERENCES

1. Bunce, C., Xing, W. and Wormald, R. (2010) Causes of blind and partial sight certifications in England and Wales: April 2007-March 2008. *Eye (Lond.)*, **24**, 1692–1699.
2. Klein, R., Chou, C.F., Klein, B.E.K., Zhang, X., Meuer, S.M. and Saaddine, J.B. (2011) Prevalence of age-related macular degeneration in the US population. *Arch. Ophthalmol.*, **129**, 75–80.
3. Jager, R.D., Mieler, W.F. and Miller, J.W. (2008) Age-related macular degeneration. *N. Engl. J. Med.*, **358**, 2606–2617.
4. Chakravarthy, U., Wong, T.Y., Fletcher, A., Piault, E., Evans, C., Zlateva, G., Buggage, R., Pleil, A. and Mitchell, P. (2010) Clinical risk factors for age-related macular degeneration: a systematic review and meta-analysis. *BMC Ophthalmol.*, **10**, 31.
5. Deangelis, M.M., Silveira, A.C., Carr, E.A. and Kim, I.K. (2011) Genetics of age-related macular degeneration: current concepts, future directions. *Semin. Ophthalmol.*, **26**, 77–93.
6. Swaroop, A., Chew, E.Y., Rickman, C.B. and Abecasis, G.R. (2009) Unraveling a multifactorial late-onset disease: from genetic susceptibility to disease mechanisms for age-related macular degeneration. *Annu. Rev. Genomics Hum. Genet.*, **10**, 19–43.
7. Edwards, A.O., Ritter, R., III, Abel, K.J., Manning, A., Panhuysen, C. and Farrer, L.A. (2005) Complement factor H polymorphism and age-related macular degeneration. *Science*, **308**, 421–424.
8. Hageman, G.S., Anderson, D.H., Johnson, L.V., Hancox, L.S., Taiber, A.J., Hardisty, L.I., Hageman, J.L., Stockman, H.A., Borchardt, J.D., Gehrs, K.M. *et al.* (2005) A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc. Natl Acad. Sci. USA*, **102**, 7227–7232.
9. Haines, J.L., Hauser, M.A., Schmidt, S., Scott, W.K., Olson, L.M., Gallins, P., Spencer, K.L., Kwan, S.Y., Noureddine, M., Gilbert, J.R. *et al.* (2005) Complement factor H variant increases the risk of age-related macular degeneration. *Science*, **308**, 419–421.
10. Klein, R.J., Zeiss, C., Chew, E.Y., Tsai, J.Y., Sackler, R.S., Haynes, C., Henning, A.K., SanGiovanni, J.P., Mane, S.M., Mayne, S.T. *et al.* (2005) Complement factor H polymorphism in age-related macular degeneration. *Science*, **308**, 385–389.
11. Gold, B., Merriam, J.E., Zernant, J., Hancox, L.S., Taiber, A.J., Gehrs, K., Cramer, K., Neel, J., Bergeron, J., Barile, G.R. *et al.* (2006) Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat. Genet.*, **38**, 458–462.
12. Maller, J., George, S., Purcell, S., Fagerness, J., Altshuler, D., Daly, M.J. and Seddon, J.M. (2006) Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. *Nat. Genet.*, **38**, 1055–1059.
13. Maller, J.B., Fagerness, J.A., Reynolds, R.C., Neale, B.M., Daly, M.J. and Seddon, J.M. (2007) Variation in complement factor 3 is associated with risk of age-related macular degeneration. *Nat. Genet.*, **39**, 1200–1201.
14. Yates, J.R., Sepp, T., Matharu, B.K., Khan, J.C., Thurlby, D.A., Shahid, H., Clayton, D.G., Hayward, C., Morgan, J., Wright, A.F. *et al.* (2007) Complement C3 variant and the risk of age-related macular degeneration. *N. Engl. J. Med.*, **357**, 553–561.
15. Cipriani, V., Matharu, B.K., Khan, J.C., Shahid, H., Hayward, C., Wright, A.F., Armbrecht, A.M., Dhillon, B., Harding, S.P., Bishop, P.N. *et al.* (2012) No evidence of association between complement factor I genetic variant rs10033900 and age-related macular degeneration. *Eur. J. Hum. Genet.*, **20**, 1–2.

16. Fagerness, J.A., Maller, J.B., Neale, B.M., Reynolds, R.C., Daly, M.J. and Seddon, J.M. (2009) Variation near complement factor I is associated with risk of advanced AMD. *Eur. J. Hum. Genet.*, **17**, 100–104.
17. Bradley, D.T., Zipfel, P.F. and Hughes, A.E. (2011) Complement in age-related macular degeneration: a focus on function. *Eye (Lond.)*, **25**, 683–693.
18. Khandhadia, S., Cipriani, V., Yates, J.R.W. and Lotery, A.J. (2011) Age-related macular degeneration and the complement system. *Immunobiology*, **217**, 127–146.
19. Jakobsdottir, J., Conley, Y.P., Weeks, D.E., Mah, T.S., Ferrell, R.E. and Gorin, M.B. (2005) Susceptibility genes for age-related maculopathy on chromosome 10q26. *Am. J. Hum. Genet.*, **77**, 389–407.
20. Rivera, A., Fisher, S.A., Fritsche, L.G., Keilhauer, C.N., Lichtner, P., Meitinger, T. and Weber, B.H. (2005) Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. *Hum. Mol. Genet.*, **14**, 3227–3236.
21. Friedrich, U., Myers, C.A., Fritsche, L.G., Milenkovich, A., Wolf, A., Corbo, J.C. and Weber, B.H. (2011) Risk- and non-risk-associated variants at the 10q26 AMD locus influence ARMS2 mRNA expression but exclude pathogenic effects due to protein deficiency. *Hum. Mol. Genet.*, **20**, 1387–1399.
22. Chen, W., Stambolian, D., Edwards, A.O., Branham, K.E., Othman, M., Jakobsdottir, J., Tosakulwong, N., Pericak-Vance, M.A., Campochiaro, P.A., Klein, M.L. *et al.* (2010) Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. *Proc. Natl Acad. Sci. USA*, **107**, 7401–7406.
23. Neale, B.M., Fagerness, J., Reynolds, R., Sobrin, L., Parker, M., Raychaudhuri, S., Tan, P.L., Oh, E.C., Merriam, J.E., Souied, E. *et al.* (2010) Genome-wide association study of advanced age-related macular degeneration identifies a role of the hepatic lipase gene (LIPC). *Proc. Natl Acad. Sci. USA*, **107**, 7395–7400.
24. Yu, Y., Bhangale, T.R., Fagerness, J., Ripke, S., Thorleifsson, G., Tan, P.L., Souied, E.H., Richardson, A.J., Merriam, J.E., Buitendijk, G.H. *et al.* (2011) Common variants near FRK/COL10A1 and VEGFA are associated with advanced age-related macular degeneration. *Hum. Mol. Genet.*, **20**, 3699–3709.
25. Sivakumaran, T.A., Igo, R.P. Jr., Kidd, J.M., Itsara, A., Kopplin, L.J., Chen, W., Hagstrom, S.A., Peachey, N.S., Francis, P.J., Klein, M.L. *et al.* (2011) A 32 kb critical region excluding Y402H in CFH mediates risk for age-related macular degeneration. *PLoS One*, **6**, e25598.
26. Galan, A., Ferlin, A., Caretti, L., Buson, G., Sato, G., Frigo, A.C. and Foresta, C. (2010) Association of age-related macular degeneration with polymorphisms in vascular endothelial growth factor and its receptor. *Ophthalmology*, **117**, 1769–1774.
27. Kopplin, L.J., Igo, R.P. Jr., Wang, Y., Sivakumaran, T.A., Hagstrom, S.A., Peachey, N.S., Francis, P.J., Klein, M.L., SanGiovanni, J.P., Chew, E.Y. *et al.* (2010) Genome-wide association identifies SKIV2L and MYRIP as protective factors for age-related macular degeneration. *Genes Immun.*, **11**, 609–621.
28. McKay, G.J., Patterson, C.C., Chakravarthy, U., Dasari, S., Klaver, C.C., Vingerling, J.R., Ho, L., de Jong, P.T., Fletcher, A.E., Young, I.S. *et al.* (2011) Evidence of association of APOE with age-related macular degeneration - a pooled analysis of 15 studies. *Hum. Mutat.*, **32**, 1407–1416.
29. McCarthy, M.I., Abecasis, G.R., Cardon, L.R., Goldstein, D.B., Little, J., Ioannidis, J.P. and Hirschhorn, J.N. (2008) Genome-wide association studies for complex traits: consensus, uncertainty and challenges. *Nat. Rev. Genet.*, **9**, 356–369.
30. Montes, T., Tortajada, A., Morgan, B.P., Rodriguez de Cordoba, S. and Harris, C.L. (2009) Functional basis of protection against age-related macular degeneration conferred by a common polymorphism in complement factor B. *Proc. Natl Acad. Sci. USA*, **106**, 4366–4371.
31. McKay, G.J., Silvestri, G., Patterson, C.C., Hogg, R.E., Chakravarthy, U. and Hughes, A.E. (2009) Further assessment of the complement component 2 and factor B region associated with age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.*, **50**, 533–539.
32. Bennet, A.M., Reynolds, C.A., Eriksson, U.K., Hong, M.G., Blennow, K., Gatz, M., Alexeyenko, A., Pedersen, N.L. and Prince, J.A. (2011) Genetic association of sequence variants near AGER/NOTCH4 and dementia. *J. Alzheimers Dis.*, **24**, 475–484.
33. Yuan, X., Gu, X., Crabb, J.S., Yue, X., Shadrach, K., Hollyfield, J.G. and Crabb, J.W. (2010) Quantitative proteomics: comparison of the macular Bruch membrane/choroid complex from age-related macular degeneration and normal eyes. *Mol. Cell. Proteomics*, **9**, 1031–1046.
34. Mao, J.R., Taylor, G., Dean, W.B., Wagner, D.R., Afzal, V., Lotz, J.C., Rubin, E.M. and Bristow, J. (2002) Tenascin-X deficiency mimics Ehlers-Danlos syndrome in mice through alteration of collagen deposition. *Nat. Genet.*, **30**, 421–425.
35. Margaron, Y., Bostan, L., Exposito, J.Y., Malbouyres, M., Trunfio-Sfarghiu, A.M., Berthier, Y. and Lethias, C. (2010) Tenascin-X increases the stiffness of collagen gels without affecting fibrillogenesis. *Biophys. Chem.*, **147**, 87–91.
36. Egging, D.F., van Vlijmen, I., Starcher, B., Gijsen, Y., Zweers, M.C., Blankevoort, L., Bristow, J. and Schalkwijk, J. (2006) Dermal connective tissue development in mice: an essential role for tenascin-X. *Cell Tissue Res.*, **323**, 465–474.
37. Ikuta, T., Ariga, H. and Matsumoto, K.I. (2001) Effect of tenascin-X together with vascular endothelial growth factor A on cell proliferation in cultured embryonic hearts. *Biol. Pharm. Bull.*, **24**, 1320–1323.
38. McKeen, H.D., Brennan, D.J., Hegarty, S., Lanigan, F., Jirstrom, K., Byrne, C., Yakkundi, A., McCarthy, H.O., Gallagher, W.M. and Robson, T. (2011) The emerging role of FK506-binding proteins as cancer biomarkers: a focus on FKBPL. *Biochem. Soc. Trans.*, **39**, 663–668.
39. Valentine, A., O'Rourke, M., Yakkundi, A., Worthington, J., Hookham, M., Bicknell, R., McCarthy, H.O., McClelland, K., McCallum, L., Dyer, H. *et al.* (2011) FKBPL and peptide derivatives: novel biological agents that inhibit angiogenesis by a CD44-dependent mechanism. *Clin. Cancer Res.*, **17**, 1044–1056.
40. Andersson, E.R., Sandberg, R. and Lendahl, U. (2011) Notch signaling: simplicity in design, versatility in function. *Development*, **138**, 3593–3612.
41. Kerbel, R.S. (2008) Tumor angiogenesis. *N. Engl J. Med.*, **358**, 2039–2049.
42. Hellstrom, M., Phng, L.K., Hofmann, J.J., Wallgard, E., Coultas, L., Lindblom, P., Alva, J., Nilsson, A.K., Karlsson, L., Gaiano, N. *et al.* (2007) Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature*, **445**, 776–780.
43. Claxton, S. and Fruttiger, M. (2004) Periodic Delta-like 4 expression in developing retinal arteries. *Gene Expr. Patterns*, **5**, 123–127.
44. Ahmad, I., Balasubramanian, S., Del Debbio, C.B., Parameswaran, S., Katz, A.R., Toris, C. and Fariss, R.N. (2011) Regulation of ocular angiogenesis by Notch signaling: implications in neovascular age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.*, **52**, 2868–2878.
45. Cherepanoff, S., McMenamin, P., Gillies, M.C., Kettle, E. and Sarks, S.H. (2010) Bruch's membrane and choroidal macrophages in early and advanced age-related macular degeneration. *Br. J. Ophthalmol.*, **94**, 918–925.
46. Barrett, J.C., Clayton, D.G., Concannon, P., Akolkar, B., Cooper, J.D., Erlich, H.A., Julier, C., Morahan, G., Nerup, J., Nierras, C. *et al.* (2009) Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. *Nat. Genet.*, **41**, 703–707.
47. Power, C. and Elliott, J. (2006) Cohort profile: 1958 British Birth Cohort (National Child Development Study). *Int. J. Epidemiol.*, **35**, 34–41.
48. Wellcome Trust Case Control Consortium (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*, **447**, 661–678.
49. Bird, A.C., Bressler, N.M., Bressler, S.B., Chisholm, I.H., Coscas, G., Davis, M.D., de Jong, P.T., Klaver, C.C.W., Klein, B.E.K., Klein, R. *et al.* (1995) An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. *Surv. Ophthalmol.*, **39**, 367–374.
50. Deary, I.J., Whiteman, M.C., Starr, J.M., Whalley, L.J. and Fox, H.C. (2004) The impact of childhood intelligence on later life: following up the Scottish mental surveys of 1932 and 1947. *J. Pers. Soc. Psychol.*, **86**, 130–147.
51. The Age-Related Eye Disease Study Research Group (2001) The age-related eye disease study system for classifying age-related macular degeneration from stereoscopic color fundus photographs: the Age-Related Eye Disease Study report number 6. *Am. J. Ophthalmol.*, **132**, 668–681.
52. Wallace, C., Smyth, D.J., Maisuria-Armer, M., Walker, N.M., Todd, J.A. and Clayton, D.G. (2010) The imprinted DLK1-MEG3 gene region on

chromosome 14q32.2 alters susceptibility to type 1 diabetes. *Nat. Genet.*, **42**, 68–71.
53. Mantel, N. (1963) Chi-square tests with one degree of freedom; extensions of the Mantel-Haenszel procedure. *J. Am. Stat. Assoc.*, **58**, 690–700.
54. Yang, J., Lee, S.H., Goddard, M.E. and Visscher, P.M. (2011) GCTA: a tool for genome-wide complex trait analysis. *Am. J. Hum. Genet.*, **88**, 76–82.
55. Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A., Bender, D., Maller, J., Sklar, P., de Bakker, P.I., Daly, M.J. and Sham, P.C. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.*, **81**, 559–575.
56. Devlin, B. and Roeder, K. (1999) Genomic control for association studies. *Biometrics*, **55**, 997–1004.