Genetic Association with Response to Intravitreal Ranibizumab in Patients with Neovascular AMD

Barbara Kloeckener-Gruissem,[*,1,2,3] *Daniel Barthelmes,*[3,4,5] *Stephan Labs,*[1] *Christian Schindler,*[6] *Malaika Kurz-Levin,*[4] *Stephan Michels,*[4,7] *Johannes Fleischhauer,*[4] *Wolfgang Berger,*[1,8] *Florian Sutter,*[*,4,8] *and Moreno Menghini*[3,4]

Purpose. Neovascular age-related macular degeneration (AMD) resulting in decreased central vision severely impairs affected individuals. Current standard treatment is an intravitreal anti-VEGF therapy (ranibizumab), but responses to treatment show large variability. Genetic factors that influence AMD and that affect the outcome of ranibizumab treatment were sought within a sample of Swiss patients.

Methods. Changes in visual acuity (VA) after initiation of anti-VEGF treatment were observed during 12 months, and percentiles of VA were calculated. Genotypes of polymorphisms in known AMD susceptibility loci (*CFH*, *CFB*, *HTRA1*, *AMRS2*, and *VEGFA*) as well as not yet reported AMD-associated genes (*KDR*, *LRP5*, and *FZD4*) were determined, and their frequencies were compared.

Results. Of the 309 eyes included in the study, 243 completed VA assessment. On average, 3.9 ±2.6 ranibizumab injections were administered. Based on the change in visual acuity, two responder groups were established: 63 eyes were assigned to the poor responders (≤25th percentile) and 63 eyes to the good responders (≥75th percentile). Individuals with genotype CC of p.Y402H in *CFH* had a decreased chance of positive treatment outcome compared with those with the CT and TT genotypes ($P = 0.005$ and $P = 0.006$). In this study, the genotype combination of AG at *CFH* with CT at *FZD4* (SNP rs10898563) promised an increased chance of positive treatment outcome ($P = 0.004$). Furthermore, the association with the known genetic susceptibility loci *CFH*, *HTRA1*, and *AMRS2* were confirmed, and a risk-conferring polymorphism in one new locus, *LRP5*, was identified.

Conclusions. Genetic predisposition may account for the variability in response to anti-VEGF treatment. (*Invest Ophthalmol Vis Sci.* 2011;52:4694–4702) DOI:10.1167/iovs.10-6080

Age-related macular degeneration (AMD) remains the leading cause of progressive, irreversible visual impairment in the elderly population in Western countries. Advanced stages of the disease involve either the development of choroidal neovascularization or atrophic changes in the central macula. The newly formed vessels tend to leak and to form a fibrovascular tissue, thereby severely affecting the neural tissue of the central retina. As a result, patients have low visual acuity (VA), eventually leading to legal blindness (vision <20/200). Although the mechanisms leading to the disease are not yet fully understood, it is well established that several factors influence this process of neovascularization and angiogenesis. One of the most important seems to be the production of vascular endothelial growth factor (VEGF).[1,2]

Intravitreal injection of the anti-vascular endothelial growth factor (anti-VEGF) compounds, ranibizumab (Lucentis; Novartis, Basel, Switzerland; and Genentech Inc., South San Francisco, CA) and bevacizumab (Avastin; Roche, Basel, Switzerland) is currently the standard treatment for neovascular AMD. In the pivotal clinical trials MARINA[3] and ANCHOR,[4] monthly injections of ranibizumab demonstrated a gain in VA of 6.6 and 10.7 ETDRS letters after 24 months. Several years of clinical application of anti-VEGF drugs have shown a broad range of responses, with a substantial fraction of patients experiencing further deterioration of VA despite intensive and regular treatment.[5] Few studies have been undertaken to investigate the relationship between genetic factors and photodynamic therapy (PDT) or intravitreal injection of anti-VEGF compounds.[6–8]

Genetic factors that influence the development of AMD have been primarily identified through association studies with DNA sequence variants.[9] Several of the identified proteins respond to systemic and local inflammation.[10] Among them are components of the complement system,[11,12] such as complement factor H (CFH)[13–15] and component factors C3, C2, and B.[16,17] Other physiological responses, such as stress responses, may also play a role in AMD. One such stress response protein is the serine protease, which accumulates within drusen,[18] a structure that forms between the retina and Bruch's membrane and is considered a typical pathologic feature of AMD. The protein is encoded by *HTRA1*,[18,19] and DNA sequence variants at this locus have been shown to associate with AMD. An SNP at the genetically linked locus *ARMS2* (*LOC387715*; age-related maculopathy susceptibility 2 gene), which encodes a mitochondrial protein, was also shown to associate with AMD.[20,21] Vascular endothelial growth factor (VEGF) is involved in the process of vascularization and as such has been a candidate

From the [1]Institute of Medical Molecular Genetics, University of Zurich, Schwerzenbach, Switzerland; the [2]Department of Biology, ETHZ (Eidgenössische Technische Hochschule Zürich), Zurich, Switzerland; the [4]Department of Ophthalmology, University Hospital of Zurich, Zurich, Switzerland; the [5]Save Sight Institute, University of Sydney, Sydney, Australia, the [6]Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland; and the [7]Augenklinik, Stadtspital Triemli, Zurich, Switzerland.

[3,8]These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Supported by an unrestricted research grant from Novartis Pharma Schweiz, AG; the Swiss National Foundation (DB); and the Walter and Gertrud Siegenthaler Foundation, Zurich, Switzerland (DB).

Submitted for publication June 18, 2010; revised October 12, November 12, and December 6, 2010; accepted December 7, 2010.

Disclosure: **B. Kloeckener-Gruissem**, Novartis (F); **D. Barthelmes**, Novartis (F); **S. Labs**, Novartis (F); **C. Schindler**, Novartis (F); **M. Kurz-Levin**, Novartis (F); **S. Michels**, Novartis (F); **J. Fleischhauer**, Novartis (F); **W. Berger**, Novartis (F); **F. Sutter**, Novartis (F, C); **M. Menghini**, Novartis (F)

*Each of the following is a corresponding author: Barbara Kloeckener-Gruissem, University of Zurich, Institute of Medical Molecular Genetics, Schwerzenbach, Switzerland; kloeckener@medgen.uzh.ch. Florian Sutter, University Hospital of Zurich, Department of Ophthalmology, Zurich, Switzerland; florian@sutter-adler.ch.

target for AMD. Especially in the context of anti-VEGF treatment it is noteworthy that DNA sequence variants in the *VEGFA* gene were found to be associated with AMD,[22,23] although these results have not been confirmed in all patient populations.[24,25]

The purpose of the study was to search for genetic factors that associate with treatment outcome. For that, retinal lesion type and the patient's age and sex were assessed. For genetic analysis, we chose several of the previously identified and published SNP sequence variants that have been shown to confer increased risk or protective properties on AMD to test the effect of these factors in our patient sample. Furthermore, we tested additional sequence variants in loci, which encode proteins involved in angiogenesis and vascularization.

METHODS

Participants

Demographic and clinical data of patients treated with ranibizumab for neovascular AMD, at the University Hospital Zurich, were recorded and used for analysis. The recruiting and analysis stretched over a period of approximately 3 years. The MARINA[3] and ANCHOR[4] trials criteria regarding age, VA, and lesion type (minimally classic, predominantly classic, and occult) were applied. Prior treatments other than ranibizumab were not an exclusion criterion. In patients in whom the second eye became eligible for treatment during the course of the study, both eyes were included and handled separately. Patients were given a full explanation of the nature and purpose of the study. Anti-VEGF treatment with ranibizumab was initiated if the diagnosis of neovascular AMD was established and the patient consented to the treatment. The study was conducted according to the Declaration of Helsinki and was approved by the local ethics committee. DNA of individuals representing the general population was used as the control for SNP analysis.[26] These individuals were not age matched and did not undergo clinical assessment for the present study.

Clinical Data Collection

Ophthalmic baseline examinations were performed before the start of the treatment. The initial clinical assessment included testing of best corrected visual acuity (BCVA) with ETDRS-like logMAR charts,[27] dilated funduscopy, optical coherence tomography (OCT), color fundus photography, and fundus fluorescein angiography. The diagnosis of neovascular AMD was established by known retina specialists (FS, MK, JF, SM). The lesion type of the choroidal neovascularization was determined by assessing fluorescein angiography (FA) and defined according to the AREDS trial classification,[28,29] the Macular Photocoagulation Study (MPS),[30] the Verteporfin in Photodynamic Therapy Study (VIP),[31] and the Treatment of Age-Related Macular Degeneration with Photodynamic Therapy Study (TAP).[32,33] At baseline, one intravitreal administration of 0.5 mg ranibizumab was given. Subsequent injections were performed only if signs of lesion activity (i.e., subretinal fluid, cystoid macular edema, sub- or intraretinal bleeding, or active lesion in FA) were present. Follow-up visits were scheduled monthly, but could vary due to patients' personal needs. VA, dilated funduscopy, and OCT were always performed during follow-up visits. FA was repeated only if deemed necessary by the treating ophthalmologist or in cases where a deterioration of VA could not be explained by funduscopy or OCT. The course of VA was monitored during a follow-up time of 12 months. In all eyes with completed 12-month visits and complete datasets, percentiles were calculated for the range of changes in VA at month 12. Two responder groups were established: poor responders (PRs; $\leq$25th percentile) and good responders (GRs; $\geq$75th percentile).

DNA Preparation

Venous blood was collected in EDTA tubes, and genomic DNA was extracted as described.[34] DNA concentration was adjusted to 10 ng/μL. SNP genotyping was performed either by DNA sequencing or by the genetic analysis technology (TaqMan; Applied Biosystems, Inc. [ABI], Rotkreuz, Switzerland) in both cases, the chemistry was supplied by ABI. Sequencing primers were designed by Primer3 (ver. 4.0; http://frodo.wi.mit.edu/primer3, provided in the public domain by Massachusetts Institute of Technology, Cambridge, MA) and synthesized by Microsynth (http://www.microsynth.ch; Balgach, Switzerland) (Table 1). PCR conditions were 50 ng genomic DNA, 5 μM primers, 0.2 mM dNTPs, and 0.6 U DNA polymerase (HotFire; Solis BioDyne, Tartu, Estonia) were mixed. Annealing temperatures varied between 54°C and 60°C, depending on the individual primer pair. The denaturing and elongation steps lasted for 1 minute each. Each temperature cycle was repeated 35 times. For the SNP assay analysis (TaqMan; ABI), either predesigned or custom-made assays were used. Associated software (Software SDS2.2; ABI) was applied for genotype calling. The allele calling of the SNP assays was verified by DNA sequence analysis from 15 DNA samples for each assay yielding 100% concordance. In addition, genotyping of 10% of all samples were repeated for each SNP.

Genetic Analysis

Previously described risk factors for AMD (*CFH* [rs1061170], *HTRA1* [rs11200638], *VEGF* [rs1413711], *ARMS2* [rs10490924], and *CFB* [rs641153]) were analyzed. Furthermore, we included SNPs in three candidate genes that play a role in angiogenesis and vascularization (*KDR* [rs7671745], *FZD4* [rs10898563], and *LRP5* [rs3736228]; Table 1). The criteria for choosing the SNPs within *KDR* and *FZD4* were based on the frequency of the minor allele (not below 40% within European populations on the basis of HapMap information, 2009; http://www.hapmap.org; developed and maintained in the public domain by a multinational consortium of scientists) as well as the availability of the TaqMan assay from ABI. The SNP in *LRP5* has been used in other association studies.[48]

Statistical Analysis

Statistical calculations were performed with either a commercially available software package (IBM SPSS Statistics 19; SPSS Inc. Chicago, Il) or open-access Internet portals from Vassar College (Poughkeepsie, NY; http://faculty.vassar.edu/lowry/odds2x2.html) and the University of Kansas (Lawrence, KS). Normally distributed data were presented as the mean $\pm$ SD. Normality was assessed with the Shapiro-Wilk test. Odds ratios from independent samples were compared as described.[49] The eight SNPs were analyzed independently. The χ^2 test of goodness of fit and independence was performed on the genotype data. The Bonferroni correction was applied for multiple testing. The specific number of tests and the appropriate corrections are given in the table legends. We used logistic regression to determine the effect of different genotypes on the likelihood of a favorable treatment outcome (software SAS, ver. 9.2; SAS, Cary, NC). Since there were only a small number of patients with two treated eyes, we refrained from using standard methods to account for correlation of outcomes within subjects and determined confidence intervals of the ORs by the bootstrap method. Resampling was performed separately in patients with one or with two treated eyes and iterated 5000 times.[50,51]

RESULTS

Characteristics of Patient Cohort and Effect of Treatment

In total, 309 eyes of 267 patients were enrolled in the study. Forty-two (13.6%) eyes had received treatment for neovascular AMD other than ranibizumab. In 215 patients, 243 eyes (154 female, 89 male) had a complete dataset at month 12 and were included in the final analysis. In 28 patients, both eyes were included in the study. The mean age of the patients at study entry was 78.9 $\pm$ 7.0 years (78.3 $\pm$ 7.4 in the women, 79.8 $\pm$

Table 1. Characteristics of the Genetic Loci, the SNPs, and the Method of Investigation

Gene	SNP ID	Nucleotides	Frequency (%)	Location	Method Sequencing (Primer)/Assay	Reference
ARMS2 (age-related maculopathy susceptibility 2)	rs10490924	G/T	80/20	A69S	For_ACCCAGGACCGATGGTAACT Rec_AAGCACCTGAAGGCTGGTTA	Rivera et al.[21]; Weger et al.[35]; Kanda et al.[36]; Leveziel et al.[37]; Kaur et al.[38]; Xu et al.[39]; Francis et al.[40]; Jakobsdottir et al.[41]
HTRA1 (HtrAserine peptidase 1)	rs11200638	A/G	N/A	−625G>A	For_ATGCCACCCACAACAACTTT Rev_GGGGAAAGTTCCTGCAAATC	Rivera et al.[21]; Weger et al.[35]; Kanda et al.[36]; Leveziel et al.[37]; Kaur et al.[38]; Xu et al.[39]; Francis et al.[40]
CFH (complement factor H)	rs1061170	T/C	72/28	Y402H	Taqman assay (ABI)	Klein et al.[42]; Haines et al.[14]; Edwards et al.[13]; Maller et al.[43]; Thakkinstian et al.[12]; Narayanan et al.[44]; Jakobsdottir et al.[41]
CFB (complement factor B)	rs641153	G/A	94/6	R32Q	For_CAGGTACGTGTCTGCACAGG Rev_TCTGGAGGTAAGCGAGGGTA	Gold et al.[16]; Spencer et al.[45]; Jakobsdottir et al.[41];
VEGFA (Vascular endothelial growth factor)	rs1413711	A/G	N/A	+674C>T (intron 1)	Taqman assay (ABI)	Churchill et al.[22]; Haines et al.[23]; Richardson et al.[25]; Lin et al.[47]; Boekhoorn et al.[24]
FZD4 (frizzled homolog 4 [*Drosophila*])	rs10896563	A/G	54/46	+581 3′UTR	Taqman assay (ABI)	This study
LRP5 (low-density lipoprotein receptor-related protein 5)	rs3736228	C/T	86/14	A1330V (exon 18)	Taqman assay (ABI)	This study
KDR (kinase insert domain receptor; vascular endothelial growth factor receptor 2 precursor)	rs7671745	A/G	57/43	Intron 22	For_CCCTTCCGTATGAAGCTGAA Rev_TTCTTGTGCTCCCCAAGACT	This study

Allele frequencies were taken from HapMap (http://www.hapmap.org) unless no information was available (N/A). The DNA sequences for primers are given as 5′ to 3′. The first five loci have been shown to affect AMD (Reference).

6.4 in the men, $P = 0.19$). Baseline VA was 54.0 ± 14.5 ETDRS letters (53.7 ± 15.0 in the women, 54.4 ± 13.8 in the men; $P = 0.81$) and was comparable among lesion types (Table 2). The baseline VA in the responder groups was significantly higher in PRs than in GRs ($P = 0.005$). The mean changes of VA in all eyes after a period of 12 months are displayed in Figure 1A.

In all eyes, there was an average increase of 1.9 ± 11.0 ETDRS letters (change from 54.0 ± 14.5 to 55.9 ± 17.1) after 12 months. Eyes in the PR group (≤25th percentile, which corresponds to a loss of 5 or more letters) lost on average 12.7 ± 5.6 letters (change from 55.4 ± 13.9 to 42.9 ± 16.2 letters), whereas eyes in the GR group (≥75th percentile, which corresponds to a gain of 11 or more letters) gained on average 14.8 ± 4.2 letters (change from 48.2 ± 14.6 to 62.6 ± 14.9 letters; Fig. 1). The difference between the average loss in the PRs and average gain in the GRs is approximately 20 ETDRS letters. Based on these changes, 63 eyes of 57 patients were allocated to the PR group and 63 eyes of 59 patients to the GR group. Two patients had one eye allocated to the PR group and the other eye to the GR group.

All three lesion types were found in both groups at a comparable frequency (Table 2). An average of 4.2 ± 2.3 ranibizumab injections were administered to the group that turned out to show a poor response (PR), whereas eyes demonstrating a good response (GR) had received 4.1 ± 2.5 injections ($P = 0.78$). Although the follow-up scheme was not fixed and the individual patient's needs were taken into account when scheduling the visits, across all groups and classifications (PR, GR, sex, and lesion type) the number of visits was close to 10 per 12 months (9.7 ± 2.1 in the PR group, 9.6 ± 2.1 in the GR group; $P > 0.05$; Table 2; i.e., the variation was rather low).

Genetic Analysis

SNP genotype and allele frequencies of the previously reported genetic factors *CFH*, *HTRA1*, and *ARMS2* were found to be significantly different in patients compared with the control group, whereas those in *CFB* and *VEGFA* did not (Table 3A). We also examined a possible effect of VEGF receptor 2, encoded by the gene *KDR*, as well as that of two factors playing an important role in the process of vascularization: the low density lipoprotein receptor–related protein 5 (*LRP5*) and the frizzled homolog 4 (*FZD4*).[52] The minor allele (T) and its genotype of SNP rs3736228 in *LRP5* were found at a significantly higher frequency in patients compared with controls ($P < 0.0006$). The size of the effect (odds ratio [OR]) was calculated for the minor allele (as defined in a normal population), as well as the corresponding homozygous genotype. Odds ratios calculated for those SNPs that showed significant differences in frequency (*CFH*, *HTRA1*, *ARMS2,* and *LRP5*) ranged from the lowest value of 1.70 (*LRP5*: T allele) to the highest value of 5.24 (*LRP5*: TT genotype), all showing statistical significance; confidence intervals and *P* values are given in Table 3B. We also analyzed potential interactions between loci and their three possible genotypes. The combinations of genotypes at *HTRA1* with *FZD4*, *LRP5* and *KDR*; at *ARMS2* with *KDR* and *LRP5*; and at *CFH* with *LRP5* yielded statistical significance ($P < 0.0001$; with $\alpha \leq 0.0055$; detailed data not shown).

The differences in response to treatment could not be explained by demographic and therapeutic characteristics (Table 2) but rather by genetic components as outlined below. Comparison of the genotype frequencies at SNP rs1061170 in *CFH* between the two responder groups indicated statistically significant differences (Table 4). Thirty-eight percent of the PRs carried the CC genotype rs1061170 in *CFH* compared with 15% of the GRs. With the CC genotype as the reference category, the OR for the CT genotype was 3.94 (95% CI, 1.64–11.43; $P = 0.005$), whereas the OR for the TT genotype alone was not statistically significant (OR, 2.67; 95% CI, 0.89–9.14; $P = 0.08$). In a combined analysis, individuals carrying the CT or TT genotype fared better than those with the CC genotype (OR, 3.42; 95% CI, 1.4–9.42; $P = 0.006$). Analysis of the SNP rs10898563 in *FZD4* did not reveal a statistically significant change in treatment outcome (Table 4B).

Because of the expected genotypic complexity of the disease, we performed a pair-wise analysis among the studied SNPs in *CFH* and *FZD4*. Patients who were simultaneously heterozygous at both SNPs had a better chance of improving VA (Table 4C). This genotype combination was found in 36% of the GRs compared with only 13% of the PRs (OR, 3.66; 95% CI, 1.58–11.11; $P = 0.004$). The chance for positive treatment outcome is significantly elevated in individuals carrying the genotype combination AG;CT, compared with those with the other possible genotypes.

Discussion

The sequence variant p.Y402H of the complement factor H (CFH) is the most consistently found genetic susceptibility locus for both AMD forms and most ethnic groups. With the exception of several Asian populations, individuals who carry the variant C leading to the amino acid histidine at p.402 are between 2.4 and 4.6 times more likely to be affected by AMD (reviewed in Ref. 53). Our results concur. Of particular interest

Table 2. Clinical Characteristics

	n	Female, *n* (%)	Male, *n* (%)	Age	Baseline VA	Change in VA	Visits	Injections
All AMD	243	154 (63)	89 (37)	78.9 ± 7.0	54.0 ± 14.5	1.9 ± 11.0	9.5 ± 2.4	3.9 ± 2.6
Occult	156	100 (64)	56 (36)	78.8 ± 7.2	54.8 ± 14.9	2.4 ± 10.5	9.5 ± 2.5	4.1 ± 2.6
Min-classic	34	24 (70)	10 (30)	78.9 ± 6.5	52.8 ± 12.2	3.4 ± 11.4	9.9 ± 1.7	3.4 ± 2.5
Pred-classic	53	30 (57)	23 (43)	79.2 ± 7.3	52.1 ± 15.1	−0.7 ± 11.9	9.2 ± 2.5	3.6 ± 2.3
25th Percentile	63	39 (62)	24 (38)	79.1 ± 7.0	55.3 ± 13.7*	−12.6 ± 7.2	9.7 ± 2.1	4.2 ± 2.3
Occult	33	20 (60)	13 (40)	80.7 ± 6.0	56.9 ± 12.2	−13.1 ± 6.8	9.8 ± 2.1	4.2 ± 2.2
Min-classic	9	6 (67)	3 (33)	77.3 ± 7.8	54.1 ± 9.0	−12.7 ± 5.6	10.4 ± 2.0	4.4 ± 2.5
Pred-classic	21	13 (62)	8 (38)	78.9 ± 8.3	53.2 ± 18.0	−11.7 ± 8.6	9.2 ± 2.0	4.0 ± 2.5
75th Percentile	63	39 (62)	24 (38)	77.7 ± 6.8	48.2 ± 14.6*	14.3 ± 3.7	9.6 ± 2.1	4.1 ± 2.5
Occult	41	26 (63)	15 (37)	77.3 ± 7.2	47.0 ± 16.2	14.2 ± 3.6	9.7 ± 2.3	4.4 ± 2.6
Min-classic	10	7 (70)	3 (30)	79.5 ± 7.6	48.6 ± 13.3	14.8 ± 4.2	9.6 ± 1.4	4.0 ± 2.6
Pred-classic	12	6 (50)	6 (50)	77.8 ± 4.9	51.8 ± 10.1	14.3 ± 3.7	9.3 ± 2.0	3.1 ± 2.1

Demographic and clinical data of all eyes. The 25th percentile represents the PR group and the 75th percentile, the GR group. Demographic data are comparable among all eyes. min-classic, minimally classic lesions; pred-classic, predominantly classic lesions.

* The difference in baseline VA in the PR and GR groups is statistically significant ($P = 0.005$); the average number of visits and injections are not.

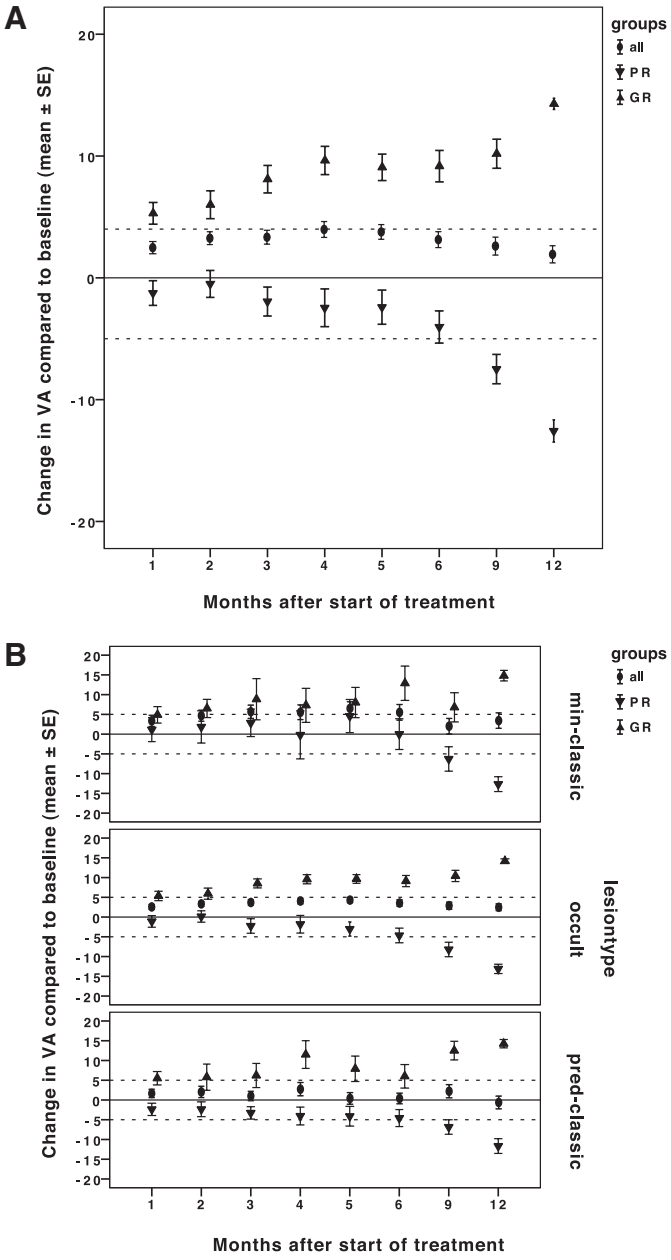

FIGURE 1. **(A)** The change in ETDRS letters compared with baseline in PRs, GRs, and all patients over the study period of 12 months. **(B)** Similarly, the change in ETDRS letters compared with baseline in PRs, GRs, and all patients assigned to the three different lesion types.

is that finding that *CFH* influences the response to intravitreal ranibizumab therapy. Individuals who carry the CT genotype at SNP rs1061170 in *CFH* are more likely to experience a positive outcome (i.e., an increase in VA) after ranibizumab treatment. Of interest, treatment with the other anti-VEGF compound, bevacizumab, showed a similar association with improved VA.[7] Strong support for the involvement of FZD4 comes from our observation that individuals who were simultaneously heterozygous for the SNPs in both loci, *CFH* and *FZD4*, seem to fare better after ranibizumab treatment than did those who did not have this genotype combination ($P = 0.004$). *FZD4* encodes a seven-transmembrane domain protein that functions as receptor of Wnt proteins and Norrin protein and has been implicated in development and/or maintenance of retinal vasculature.[52] Our results suggest that, in this context, the FZD4 protein may influence ranibizumab therapy results, although probably at a rather complex level, involving more than one factor.

Complement factor H plays an important role in the innate immune system, specifically in the regulation of induction of the alternative pathway.[11] The C-variant (p.Y402H) of *CFH* is postulated to result in aberrant activation of the complement pathway leading to reduced removal of cellular debris along the macular retinal pigment epithelial cells, most likely due to reduced binding capabilities to C-reactive protein (CRP).[54] This modified activation of the complement cascade may lead to an enhanced inflammatory response,[55] which ultimately may lead to increased levels of VEGF.[56] Until the exact process, mechanism, and consequences of the treatment are understood, this argument remains speculative. Nevertheless, we propose that anti-VEGF treated patients who carry the *CFH* CC genotype experience a reduced response due to a possible inferior capacity to reduce the level of VEGF-A in the retina.

VEGF-A was suggested to be a risk factor for neovascular AMD on the basis of the association of SNPs in the promoter and intron of the gene.[22] Hence, we find it possible that the treatment outcome could also be influenced by *VEGFA*. As we did not find a *VEGFA* association in our data set, we suggest that other components of the VEGF system, in particular VEGF-A receptors, may influence AMD and the response to treatment.

For that purpose, we investigated sequence variants of the VEGF-A receptor 2 (VEGFR2). It is encoded by the gene kinase insert domain receptor (*KDR*).[57] The intronic sequence variant that we tested in *KDR* lies within one of the haplotype blocks that were recently reported to lack association with AMD in a Caucasian population.[58] We did not find an association for *KDR* alone or in combination with other SNPs.

In addition to the VEGF system, the product of the frizzled homolog 4 gene also influences angiogenesis. Supportively, in our analysis the heterozygous AG genotype in *FZD4* of SNP rs10898563 in combination with the CT genotype in *CFH* of SNP rs1061170 demonstrated an effect on the response to treatment.

Our data emphasize the heterogeneity of genetic factors that can influence an individual's response to ranibizumab treatment. Furthermore, we were able to confirm previously reported associations with AMD (*CFH*, *HTRA1*, and *ARMS2*). In addition, one of the candidate genes for angiogenesis, *LRP5*, showed an increased risk for neovascular AMD in our patient cohort. The low-density lipoprotein receptor–related protein 5 is an essential co-receptor of the Wnt-signaling pathway. $Lrp5^{-/-}$ knockout mice show a lack of development of a complete vasculature in the deeper plexus of the retina and Müller cell–specific glutamine transporter *Slc38a5* expression is significantly reduced.[59] These observations make *LRP5* a promising candidate to affect AMD. It could directly interfere with retinal vasculature, but an indirect effect is equally possible, for example, via the glutamine transporter SLC38A5. A similar situation has recently been reported, where association of *LIPC* and HDL with AMD is thought likely to be due to another factor, acting within a common pathway of lipid metabolism.[60]

The general validity of our results has to be confirmed in additional patient cohorts. The combination of various genetic factors could modify the effect on AMD exerted by single loci as first shown by Maller et al.[43] and later repeated by others. Our data, however, did not confirm these observations, possibly due to patient heterogeneity.

Our results may have been further affected by the heterogeneity in the treatment regimen. Of interest is the finding that PR showed a statistically significant higher baseline VA compared with GR. It could be argued that a ceiling or flooring effect may

TABLE 3. Allele and Genotype Characteristics in Patients and Controls

A. Frequencies*

Locus/SNP	Group	AF (%)	*P* ($\alpha \leq 0.00625$)	Genotype *n* (%)	*n* (%)	*n* (%)	*P* ($\alpha \leq 0.00625$)
CFH rs1061170		C		CC	CT	TT	
	Controls	36		46 (16)	112 (40)	124 (44)	
	Patients	51	<0.0001	71 (27)	122 (47)	66 (25)	<0.00001
HTRA1 rs11200638		A		AA	AG	GG	
	Controls	23		16 (6)	83 (33)	152 (61)	
	Patients	42	<0.0001	49 (19)	115 (45)	92 (36)	<0.00001
ARMS2 rs10490924		T		GG	GT	TT	
	Controls	20		141 (66)	60 (28)	12 (6)	
	Patients	42	<0.00001	92 (36)	117 (45)	50 (19)	<0.00001
LRP5 rs3736228		T		CC	CT	TT	
	Controls	15		202 (71)	78 (27)	5 (2)	
	Patients	23	0.0006	157 (61)	78 (30)	22 (9)	0.0006
CFB rs641153		C		CC	CT	TT	
	Controls	91		222 (83)	43 (16)	3 (1)	
	Patients	94	NS (0.07)	230 (88)	30 (11)	1 (0)	NS (0.19)
VEGF rs1413711		G		AA	AG	GG	
	Controls	47		60 (21)	149 (52)	78 (27)	
	Patients	46	NS (0.81)	57 (22)	125 (48)	77 (30)	NS (0.69)
FZD4 rs10898563		G		AA	AG	GG	
	Controls	62		75 (40)	81 (44)	30 (16)	
	Patients	61	NS (0.71)	90 (36)	123 (49)	36 (14)	NS (0.48)
KDR rs7671745		G		AA	AG	GG	
	Controls	31		21 (11)	72 (39)	92 (50)	
	Patients	38	NS (0.035)	39 (15)	117 (45)	103 (40)	NS (0.10)

B. Odds Ratios†

Locus SNP	Allele/Genotype	OR	95% CI	χ^2	*P* ($\alpha \leq 0.0125$)
CFH rs1061170	C	1.83	1.44–2.34	24.08	<0.0001
	CC	1.94	1.28–2.94	9.82	0.002
HTRA1 rs11200638	A	2.40	1.83–3.15	40.47	<0.0001
	AA	3.48	1.92–6.30	18.48	<0.0001
ARMS2 rs10490924	T	2.94	2.18–3.95	52.92	<0.0001
	TT	4.01	2.07–7.75	19.15	<0.0001
LRP5 rs3736228	T	1.70	1.26–2.31	11.91	0.001
	TT	5.24	1.96–14.06	13.22	0.0003
CFB rs641153	C	1.54	0.97–2.45	3.39	0.07
	CC	1.54	0.94–2.51	2.97	0.08
VEGF rs1413711	A	1.03	0.81–1.31	0.06	0.81
	AA	1.07	0.71–1.61	0.1	0.75
FZD4 rs10898563	G	0.95	0.72–1.25	0.14	0.71
	GG	0.88	0.52–1.49	0.23	0.63
KDR rs7671745	G	0.74	0.56–0.98	4.44	0.035
	GG	0.67	0.46–0.98	4.35	0.037

* Allele and genotype frequencies are compared between patients and controls. Number (*n*), fractions (%), allele frequency (AF) and *P* values are given. *P* values were adjusted by application of the Bonferroni correction for multiple testing (eight SNPs); thus significance is reached with $\alpha \leq 0.00625$. SNPs with significant differences are separated by a line space from those that are not (NS).

† Statistical analysis of sequence variants and AMD phenotypes. ORs with 95% CI. The χ^2 values are based on Pearson calculation with 1 *df*. *P* values were adjusted with the Bonferroni correction for multiple testing (four tests); thus, significance is reached with $\alpha \leq 0.0125$. SNPs with significant ORs are separated by a line space from those that are not (NS).

have influenced the results in our study (i.e., the PRs "can only lose" because they have a rather high starting VA, whereas the GRs "can only gain" since they are starting from a lower VA). To check whether such a ceiling/flooring effect may have been present, all patients were grouped, according to the baseline VA: 25 to 35 letters, 36 to 45 letters, 46 to 55 letters, and so forth. An analysis of these groups created with respect to the baseline VA did not reveal a significant imbalance for the distribution of eyes to GR and PR (i.e., eyes with a rather low or rather high VA at baseline were well distributed to GR and PR within each group; data not shown). Therefore, a floor or ceiling effect in our study seems unlikely. Furthermore, it is conceivable that the amount of the injected compound and/or variations in the injection frequency could have affected the outcome. A significantly higher average number of intravitreal injections of ranibizumab have recently been reported for patients carrying the p.*CFH* Y402H variant.[8] In our study, however, the injection frequency was similar for GRs and PRs.

The heterogeneity of the treatment regimen seems unlikely to have influenced the observed response to treatment. Rather genetic factors appear to contribute to patients' response to anti-VEGF treatment. Undoubtedly, further support must be

TABLE 4. Ranibizumab Treatment Results

A. Genotype Frequencies from the PR and GR Groups

Locus/SNP	Allele/Genotype	Frequency *n* (%)* PR	GR
CFH rs1061170	CC	24 (38)	9 (15)
	CT	23 (37)	34 (58)
	TT	16 (25)	16 (27)
FZD4 rs10896563	AA	25 (41)	14 (24)
	AG	30 (49)	36 (61)
	GG	6 (10)	9 (15)

B. Odds Ratios†

Locus/SNP	Reference Category	Genotype	OR	95% CI	*P* (0.025)
CFH rs1061170	CC	CT	3.94	1.64–11.43	0.005
	CC	TT	2.67	0.89–9.14	0.083
	CC	CT and TT	3.42	1.4–9.42	0.006
FZD4 rs10896563	AA	AG	2.14	0.98–5.33	0.058
	AA	GG	2.68	0.70–18.65	0.148
	AA	AG and GG	2.23	0.97–5.6	0.055

C. Frequencies of Genotype Combinations CT (*CFH*) and AG (*FZD4*) in the PR and GR Groups†

Locus/SNP/Genotypes	Frequency (%) PR (%)	GR (%)	OR	95% CI	*P* (0.025)
CFH rs1061170 CT; *FZD4* rs10898563 AG	13	36	3.66	1.58–11.11	0.004

Association of SNPs rs1061170 (*CFH*) and rs10898563 (*FZD4*) with treatment outcome.

* Total number, *n*; fractions, %.

† The Bonferroni correction for multiple testing was applied (two tests); thus significance is reached with $\alpha \leq 0.025$. Lower and upper limit of bootstrap at 95% for confidence interval (CI).

obtained to implement the predictive consequences of individually tailored medical treatments.

Acknowledgments

The authors thank Jaya Balakrishnan, Esther Glaus, and Philippe Reuge for help with extraction of genomic DNA; Walther Hänseler and Brenna Neghaiwi for help with genotyping; Gabor Matyas for maintenance of the sequencing facility; Tamara Meierhofer, Flavia Schatzmann, and Lena Steuri for help with clinical data entry; and last but not least, all the patients for participating in the study. We recognize the vast amount of effort in this field by many researchers and apologize for not being able to cite everyone.

References

1. Grisanti S, Tatar O. The role of vascular endothelial growth factor and other endogenous interplayers in age-related macular degeneration. *Prog Retin Eye Res.* 2008;27:372–390.
2. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling: in control of vascular function. *Nat Rev Mol Cell Biol.* 2006;7:359–371.
3. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med.* 2006;355:1419–1431.
4. Brown DM, Kaiser PK, Michels M, et al. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *N Engl J Med.* 2006;355:1432–1444.
5. Menghini M, Kurz-Levin MM, Amstutz C, et al. Response to ranibizumab therapy in neovascular AMD: an evaluation of good and bad responders. *Klin Monbl Augenheilkd.* 2010;227:244–248.
6. Feng X, Xiao J, Longville B, et al. Complement factor H Y402H and C-reactive protein polymorphism and photodynamic therapy response in age-related macular degeneration. *Ophthalmology.* 2009;116:1908–1912 e1901.
7. Brantley MA Jr, Fang AM, King JM, Tewari A, Kymes SM, Shiels A. Association of complement factor H and LOC387715 genotypes with response of exudative age-related macular degeneration to intravitreal bevacizumab. *Ophthalmology.* 2007;114:2168–2173.
8. Lee AY, Raya AK, Kymes SM, Shiels A, Brantley MA Jr. Pharmacogenetics of complement factor H (Y402H) and treatment of exudative age-related macular degeneration with ranibizumab. *Br J Ophthalmol.* 2009;93:610–613.
9. Swaroop A, Chew EY, Rickman CB, Abecasis GR. Unraveling a multifactorial late-onset disease: from genetic susceptibility to disease mechanisms for age-related macular degeneration. *Annu Rev Genomics Hum Genet.* 2009;10:19–43.
10. Tuo J, Bojanowski CM, Chan CC. Genetic factors of age-related macular degeneration. *Prog Retin Eye Res.* 2004;23:229–249.
11. Gehrs KM, Jackson JR, Brown EN, Allikmets R, Hageman GS. Complement, age-related macular degeneration and a vision of the future. *Arch Ophthalmol.* 2010;128:349–358.
12. Thakkinstian A, Han P, McEvoy M, et al. Systematic review and meta-analysis of the association between complement factor H Y402H polymorphisms and age-related macular degeneration. *Hum Mol Genet.* 2006;15:2784–2790.

13. Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. *Science.* 2005;308:421–424.
14. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. *Science.* 2005;308:419–421.
15. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. *Proc Natl Acad Sci U S A.* 2005;102:7227–7232.
16. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. *Nat Genet.* 2006;38:458–462.
17. Kaur I, Katta S, Reddy RK, et al. The involvement of complement factor B and complement component C2 in an Indian cohort with age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2010;51:59–63.
18. Yang Z, Camp NJ, Sun H, et al. A variant of the HTRA1 gene increases susceptibility to age-related macular degeneration. *Science.* 2006;314:992–993.
19. Dewan A, Liu M, Hartman S, et al. HTRA1 promoter polymorphism in wet age-related macular degeneration. *Science.* 2006;314:989–992.
20. Fritsche LG, Loenhardt T, Janssen A, et al. Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. *Nat Genet.* 2008;40:892–896.
21. Rivera A, Fisher SA, Fritsche LG, et al. 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.* 2005;14:3227–3236.
22. Churchill AJ, Carter JG, Lovell HC, et al. VEGF polymorphisms are associated with neovascular age-related macular degeneration. *Hum Mol Genet.* 2006;15:2955–2961.
23. Haines JL, Schnetz-Boutaud N, Schmidt S, et al. Functional candidate genes in age-related macular degeneration: significant association with VEGF, VLDLR, and LRP6. *Invest Ophthalmol Vis Sci.* 2006;47:329–335.
24. Boekhoorn SS, Isaacs A, Uitterlinden AG, et al. Polymorphisms in the vascular endothelial growth factor gene and risk of age-related macular degeneration: the Rotterdam Study. *Ophthalmology.* 2008;115:1899–1903.
25. Richardson AJ, Islam FM, Guymer RH, Cain M, Baird PN. A tag-single nucleotide polymorphisms approach to the vascular endothelial growth factor-A gene in age-related macular degeneration. *Mol Vis.* 2007;13:2148–2152.
26. Moskvina V, Holmans P, Schmidt KM, Craddock N. Design of case-controls studies with unscreened controls. *Ann Hum Genet.* 2005;69:566–576.
27. Ferris FL 3rd, Kassoff A, Bresnick GH, Bailey I. New visual acuity charts for clinical research. *Am J Ophthalmol.* 1982;94:91–96.
28. Risk factors associated with age-related macular degeneration: a case-control study in the age-related eye disease study: Age-Related Eye Disease Study Report Number 3. *Ophthalmology*. 2000;107:2224–2232.
29. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. *Arch Ophthalmol*. 2001;119:1417–1436.
30. Moisseiev J, Alhalel A, Masuri R, Treister G. The impact of the macular photocoagulation study results on the treatment of exudative age-related macular degeneration. *Arch Ophthalmol.* 1995;113:185–189.
31. Verteporfin therapy of subfoveal choroidal neovascularization in age-related macular degeneration: two-year results of a randomized clinical trial including lesions with occult with no classic choroidal neovascularization: Verteporfin in Photodynamic Therapy Report 2. *Am J Ophthalmol*. 2001;131:541–560.
32. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: one-year results of 2 randomized clinical trials–TAP report: Treatment of Age-Related Macular Degeneration with Photodynamic Therapy (TAP) Study Group. *Arch Ophthalmol*. 1999;117:1329–1345.
33. Bressler NM. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: two-year results of 2 randomized clinical trials-tap report 2. *Arch Ophthalmol.* 2001;119:198–207.
34. Zuercher J, Neidhardt J, Magyar I, et al. Alterations of the 5′untranslated region of *SLC16A12* lead to age-related cataract. *Invest Ophthalmol Vis Sci.* 2010;51:3354–3361.
35. Weger M, Renner W, Steinbrugger I, et al. Association of the HTRA1 -625G>A promoter gene polymorphism with exudative age-related macular degeneration in a Central European population. *Mol Vis.* 2007;13:1274–1279.
36. Kanda A, Chen W, Othman M, et al. A variant of mitochondrial protein LOC387715/ARMS2, not HTRA1, is strongly associated with age-related macular degeneration. *Proc Natl Acad Sci U S A.* 2007;104:16227–16232.
37. Leveziel N, Zerbib J, Richard F, et al. Genotype-phenotype correlations for exudative age-related macular degeneration associated with homozygous HTRA1 and CFH genotypes. *Invest Ophthalmol Vis Sci.* 2008;49:3090–3094.
38. Kaur I, Katta S, Hussain A, et al. Variants in the 10q26 gene cluster (LOC387715 and HTRA1) exhibit enhanced risk of age-related macular degeneration along with CFH in Indian patients. *Invest Ophthalmol Vis Sci.* 2008;49:1771–1776.
39. Xu Y, Guan N, Xu J, et al. Association of CFH, LOC387715, and HTRA1 polymorphisms with exudative age-related macular degeneration in a northern Chinese population. *Mol Vis.* 2008;14:1373–1381.
40. Francis PJ, Zhang H, Dewan A, Hoh J, Klein ML. Joint effects of polymorphisms in the HTRA1, LOC387715/ARMS2, and CFH genes on AMD in a Caucasian population. *Mol Vis.* 2008;14:1395–1400.
41. Jakobsdottir J, Conley YP, Weeks DE, Ferrell RE, Gorin MB. C2 and CFB genes in age-related maculopathy and joint action with CFH and LOC387715 genes. *PLoS One.* 2008;3:e2199.
42. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science.* 2005;308:385–389.
43. Maller J, George S, Purcell S, et al. Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. *Nat Genet.* 2006;38:1055–1059.
44. Narayanan R, Butani V, Boyer DS, et al. Complement factor H polymorphism in age-related macular degeneration. *Ophthalmology.* 2007;114:1327–1331.
45. Spencer KL, Hauser MA, Olson LM, et al. Protective effect of complement factor B and complement component 2 variants in age-related macular degeneration. *Hum Mol Genet.* 2007;16:1986–1992.
46. Richardson AJ, Islam FM, Guymer RH, Cain M, Baird PN. A tag-single nucleotide polymorphisms approach to the vascular endothelial growth factor-A gene in age-related macular degeneration. *Mol Vis.* 2007;13:2148–2152.
47. Lin JM, Wan L, Tsai YY, et al. Vascular endothelial growth factor gene polymorphisms in age-related macular degeneration. *Am J Ophthalmol.* 2008;145:1045–1051.
48. Richards JB, Rivadeneira F, Inouye M, et al. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. *Lancet.* 2008;371:1505–1512.
49. Altman DG, Bland JM. Interaction revisited: the difference between two estimates. *BMJ.* 2003;326:219.
50. Davison AC, Hinkley DV. *Bootstrap Methods and their Application*. Cambridge, UK: Cambridge University Press; 1997.
51. Zeger SL, Liang KY, Albert PS. Models for longitudinal data: a generalized estimating equation approach. *Biometrics.* 1988;44:1049–1060.
52. Ye X, Wang Y, Cahill H, et al. Norrin, frizzled-4, and Lrp5 signaling in endothelial cells controls a genetic program for retinal vascularization. *Cell.* 2009;139:285–298.
53. Katta S, Kaur I, Chakrabarti S. The molecular genetic basis of age-related macular degeneration: an overview. *J Genet.* 2009;88:425–449.
54. Ormsby RJ, Ranganathan S, Tong JC, et al. Functional and structural implications of the complement factor H Y402H polymor-

phism associated with age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2008;49:1763–1770.

55. Laine M, Jarva H, Seitsonen S, et al. Y402H polymorphism of complement factor H affects binding affinity to C-reactive protein. *J Immunol.* 2007;178:3831–3836.
56. Bressler SB. Introduction: understanding the role of angiogenesis and antiangiogenic agents in age-related macular degeneration. *Ophthalmology.* 2009;116:S1–S7.
57. Shibuya M, Claesson-Welsh L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Exp Cell Res.* 2006;312:549–560.
58. Fang AM, Lee AY, Kulkarni M, Osborn MP, Brantley MA Jr. Polymorphisms in the VEGFA and VEGFR-2 genes and neovascular age-related macular degeneration. *Mol Vis.* 2009;15:2710–2719.
59. Xia CH, Liu H, Cheung D, et al. A model for familial exudative vitreoretinopathy caused by LPR5 mutations. *Hum Mol Genet.* 2008;17:1605–1612.
60. Neale BM, Fagerness J, Reynolds R, et al. Genome-wide association study of advanced age-related macular degeneration identifies a role of the hepatic lipase gene (LIPC). *Proc Natl Acad Sci U S A.* 2010;107:7395–7400.