

# Complement Factor H Autoantibodies and Age-Related Macular Degeneration

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**PURPOSE.** In this case-control study, the hypothesis that factor H autoantibodies are associated with age-related macular degeneration (AMD) was examined.

**METHODS.** One hundred AMD patients (median age, 78 years), 98 age-matched control subjects (median age, 78 years) known not to have AMD, and 100 healthy blood donors (median age, 43 years) were enrolled. An enzyme-linked immunosorbent assay (ELISA) was used to screen for complement factor H autoantibodies and either quantitative polymerase chain reaction (qPCR) or multiplex ligation-dependent probe amplification (MLPA) were performed to measure the copy number of the gene encoding complement factor H-related protein 3 (*CFHR3*).

**RESULTS.** There was a significant difference in the median complement factor H autoantibody titer between the three groups (AMD patients, 196 reference units [RU]); age-match control subjects, 316 RU; and blood donor control subjects, 121 RU; Kruskal-Wallis test,  $P < 0.001$ ). Pair-wise comparison (Mann-Whitney test) showed that all three groups were significantly different from each other. Two different thresholds were used in the healthy blood donors to identify individuals with complement factor H autoantibodies. Both suggested that the prevalence

of factor H autoantibodies was decreased in AMD patients. The *CFHR3* copy number was measured as a surrogate for the deletion of the genes encoding complement factor H-related proteins 3 and 1 (*CFHR3/1*). The allele frequency of the deletion was significantly higher in the age-matched control subjects than in the AMD patients (22.2% vs. 8.2%).

**CONCLUSIONS.** The level of factor H autoantibodies is lower in AMD patients than in age-matched control subjects. (*Invest Ophthalmol Vis Sci.* 2010;51:5858–5863) DOI:10.1167/iovs.09-5124

It is well accepted that naturally occurring variability in the genes encoding both regulators and activators of the complement system are associated with susceptibility to age-related macular degeneration (AMD). The complement system can be activated by three routes: the classic, the lectin, and the alternative pathways.<sup>1</sup> All three of these pathways lead to generation of the pivotal molecule C3b. Accumulation of undesirable quantities of C3b is avoided by the synthesis of regulatory proteins by the host that inhibit C3b formation from C3, both on cell surfaces and in plasma. They include the serum protein factor H and transmembrane regulators, such as membrane cofactor protein, decay-accelerating factor, and complement receptor 1. A series of studies published in 2005 identified a sequence variant (c.1277T>C; p.Tyr402His, rs1061170) in the gene encoding complement factor H (*CFH*) as a major susceptibility factor for AMD.<sup>2–5</sup> The Tyr402His variant of factor H lies in the seventh short consensus repeat (SCR). Since then, both SNPs and haplotype blocks in other complement genes, including those encoding factor B (*CFB*), factor I (*CFI*), *C2*, and *C3* have been shown to be associated with AMD.<sup>6–10</sup> In addition, a deletion in the RCA cluster that leads to loss of the genes encoding complement factor H-related proteins 1 and 3 (*CFHR3*, *CFHR1*) is associated with protection against the development of AMD.<sup>11,12</sup> Other risk factors for AMD besides age and complement include smoking, racial background, obesity, and sequence variants in the *ARMS2/HTRA1* region on chromosome 10.<sup>13,14</sup>

It is also well established that complement dysregulation predisposes to the development of the renal disease atypical hemolytic uremic syndrome (aHUS). Mutations have been found in the genes encoding both complement regulators (*CFH*, *CFI*, and *MCP*) and complement activators (*CFB* and *C3*) in ~50% of patients.<sup>15</sup> In addition factor H autoantibodies have been described in a further ~10% of patients.<sup>16–18</sup> These antibodies have been shown to block the C-terminal recognition domain of complement factor H,<sup>17</sup> an area where it is known that *CFH* mutations associated with aHUS cluster.<sup>19</sup> Moreover, it has been shown that most patients with factor H autoantibodies have a complete deficiency of complement factor H-related proteins 1 and 3,<sup>18</sup> secondary to the aforementioned deletion that is associated with protection against

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AMD. This deletion occurs as a result of nonallelic homologous recombination within segmental duplications in the regulators of complement activation gene cluster on the long arm of chromosome 1, region 32.

The observation that a high percentage of patients with dense deposit disease (DDD, also known as type II membranoproliferative glomerulonephritis [MPGN]) have drusen led to the identification of the aforementioned sequence variant in *CFH* as a susceptibility factor for AMD.<sup>20</sup> To date, there has been one report of a complement factor H autoantibody in association with MPGN.<sup>21</sup> The Tyr420His variant of factor H lies in SCR 7 of complement factor H, an area known to have a role in binding to glycosaminoglycans (GAGs) and possibly CRP on cell surfaces. It has been shown that the affinities of the two allelic variants for GAGs are significantly different,<sup>22,23</sup> consistent with a local structural difference in GAG-binding sites.<sup>24</sup> Similar results for the binding to CRP have been reported,<sup>25</sup> but whether this is physiological is uncertain.<sup>26–28</sup> Antibodies binding specifically to this region may therefore have a similar modulator effect on ligand binding and predispose to AMD. This forms the basis of the hypothesis that we tested in the present study. To examine this hypothesis, we screened for complement factor H autoantibodies in a cohort of AMD patients and two control samples: one age-matched and the other derived from blood donors.

## METHODS

### Subjects

Plasma and DNA samples were available from 100 patients with AMD, 98 age-matched normal control subjects known not to have AMD, and 100 healthy blood donors (blood donor control subjects). The AMD patients and age-matched control subjects are a subgroup of a larger cohort that has been reported previously.<sup>8</sup> The individuals within this subgroup were chosen at random from the larger cohort, and the control subjects were age and sex matched. The AMD patients were recruited between 2004 and 2006 from ophthalmic clinics in Dundee, Inverness, and the Lothian region of Scotland. The age-matched control subjects were recruited from the same sources and included spouses, subjects who had undergone cataract surgery, and the Lothian birth cohort.<sup>29</sup> The subjects were examined by an ophthalmologist, and data were collected regarding medical history, lifestyle, and smoking history. Color, stereoscopic fundus photography of the macular region was performed in all subjects. A study investigator graded images; for validation, images from 100 case subjects and control subjects were independently graded at the Moorfields Reading Centre ( $\kappa = 0.84$ ). Written informed consent was obtained from all subjects. The research protocol was in accordance with the provisions of the Declaration of Helsinki. Approval for the study was obtained from a multicenter research ethics committee (MREC/03/0/41).

### Complement Factor H Autoantibody Assay

This assay was undertaken in all three groups. Flexible, 96-well plates were coated with 5  $\mu\text{g}/\text{mL}$  of purified complement factor H (Merck Chemicals, Ltd., Nottingham, UK) or molar equivalents of complement factor H fragments (short consensus repeats [SCRs] 1–4, 6–8, 8–15, and 19–20)<sup>30,31</sup> or molar equivalents of a complement factor H-related protein 1 fragment (SCRs 4–5)<sup>31</sup> in pH 7.6 coating buffer (AbDserotec, Kidlington, UK) and incubated overnight at 4°C. The plates were then washed three times with PBS/Tween 0.01%, followed by blocking for 45 minutes (200  $\mu\text{L}$  per well of Ultrablock; AbDserotec) at room temperature. At this point, a duplicate plate was set up with a blocking solution to act as a background binding control. After blocking, a 1:50 dilution of sera in PBS/Tween 0.01% was loaded (50  $\mu\text{L}$ ) in triplicate onto both plates and incubated for 1 to 2 hours. The plates were washed three times and then blocked again. Goat anti-human IgG horse radish peroxidase (HRP; Stratech Scientific, Newmarket, UK) at

1:4000 was added and incubated for 1 hour at room temperature. The plates were washed twice with PBS/Tween 0.01% and twice with PBS. Tetramethyl benzidine (TMB) readymade standard kinetic solution (AbDserotec) was added to each well for 7 minutes exactly, before the reaction was stopped with 10% sulfuric acid. Absorbance at an optical density of 450 nm ( $\text{OD}_{450}$ ) was established with a plate reader (SpectraMax 190; MDS Analytical Technologies, Ltd., Coventry, UK). Triplicate data were analyzed, and mean blocking agent-only readings (Ultrablock; AbDserotec) were subtracted from the mean complement factor H readings, to control for nonspecific/false-positive readings. Purified complement factor H was batch tested for reactivity with anti-human IgG antibody before use in the ELISA. For the anti-factor H assay, a standard curve was generated from a known positive (a kind gift from Marie-Agnes Dragon-Durey, Hôpital Européen Georges Pompidou, Paris, France) and the  $\text{OD}_{450}$  value for the 1:50 dilution given an arbitrary 10,000 relative units. Linear-regression curve fit analysis was preformed, and reference units (RU) were calculated for each sample accordingly (Prism, ver. 3; GraphPad, San Diego, CA).

### Measurement of *CFHR3* Copy Number

The *CFHR3* copy number was measured to determine the allele frequency of the *CFHR3/1* deletion in all three groups. In the AMD and age-matched control subjects, the *CFHR3* copy number was measured using quantitative polymerase chain reaction (qPCR). Two sets of primers and minor groove binder (MGB) probes (Applied Biosystems [ABI], Warrington, UK) were designed on computer (Primer Express ver. 2.0; ABI). Each set consisted of a pair of primers and a 3'-fluorescent-tagged probe (Table 1). One pair of primers and a fluorescein amidite (FAM) 5'-labeled probe were designed inside intron 3 of *CFHR3*. Another pair of primers and a 5'-labeled probe (VIC; ABI) was designed inside the  $\beta$ -globin (*HBB*) gene and used as an endogenous control. Each qPCR was performed in triplicate using 384-well optical-reaction plates (ABI). Five-microliter reactions contained 1  $\mu\text{L}$  of DNA (10 ng/ $\mu\text{L}$ ) used as template, 2.5  $\mu\text{L}$  of 2 $\times$  PCR master mix (TaqMan Universal; ABI), 0.2  $\mu\text{L}$  of each primer at 10  $\mu\text{M}$ , 0.2  $\mu\text{L}$  of each probe at 1  $\mu\text{M}$ , and 0.3  $\mu\text{L}$  of  $\text{dH}_2\text{O}$  (Invitrogen-Gibco; Paisley, Scotland, UK). Reactions were performed in real time, with the absolute quantitation (standard curve) setting on a real-time PCR system (HT7900; ABI). Conditions used in the qPCR were as follows: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. After completion of PCR, fluorescence was read by using the system software (SDS; ABI), and the resulting cycle thresholds (Ct) were exported to a spreadsheet and analyzed (Excel; Microsoft Redmond, WA).

In the blood donor control subjects, the *CFHR3* copy number was measured with multiplex ligation-dependent probe amplification<sup>52</sup> (MLPA; SALSA MLPA kit, P236-A1 ARMD; MRC Holland). In this kit are six probes for *CFHR3*. Details of these are given in Table 2.

### Statistics

Values for antibody titer within the three groups are expressed as the median (range). Comparisons between groups were made using the nonparametric Kruskal-Wallis and Mann-Whitney Tests. The allele fre-

**TABLE 1.** Primer and Probe Sequences for the *CFHR3* qPCR Dosage Analysis

Name	Primer Sequence (5'–3')	5' Fluorescent Dye
CFHR3-F	TGGGCATTAGTCAAGAATACAGTAAAA	—
CFHR3-R	ATTAATGCCGCTTCAATATGACTTT	—
CFHR3-Probe	AATTAGAACACAATACTTGTGGC	6-FAM
Bglobin-F	GGGAGAGCCATCTATTGCTT	—
Bglobin-R	TGGTGTCTGTTTGGAGTTGCTAGT	—
Bglobin-Probe	TTGCTTCTGACACAACCTG	6-VIC

TABLE 2. MLPA Probes Used to Determine *CFHR3* Copy Number

Gene, Exon	Ligation Site	Partial Sequence (20 Nucleotides Adjacent to Ligation Site)
<i>CFHR3</i> , Exon 1	Intron 1	AGGTAAGTTA-AAAGAGATCT
<i>CFHR3</i> , Exon 2	Intron 1	CATTTCTTGTG-TGGAATTACA
<i>CFHR3</i> , Exon 3	Intron 3	CGGACGACAG-TCTCAGACTT
<i>CFHR3</i> , Exon 4	Intron 4	GGGTATATG-AATTCTTACA
<i>CFHR3</i> , Exon 6	Intron 5	TTCCCAACA-TCACAGCAGA
<i>CFHR3</i> , Exon 6	1003-1002 reverse	TCCCTTCCCG-ACACACTGCT

quency of the *CFHR3/1* deletion in the three groups was compared by using the  $\chi^2$  test.

## RESULTS

### Subjects

The median age of the AMD patients was 78 years (range, 53-96; 38 men and 62 women); that of the age-matched normal control subjects was 78 years (range, 48-92; 38 men and 60 women); and that of the healthy blood donors was 43 years (range, 18-68; 44 men and 56 women).

Of the AMD patients, 19 had severe non-neovascular (dry), and 81 had severe neovascular (wet) changes. There was no significant difference in smoking history between the AMD patients and the age-matched control subjects.

### Complement Factor H Autoantibodies

In ELISAs, the median (range) antibody titer (in RU) in the three groups was AMD patients, 196 (0-1495); age-matched control subjects, 316 (0-1743); and blood donor control subjects, 121 (0-3104) (Fig. 1; Kruskal-Wallis Test  $P < 0.001$ ). Pairwise comparisons by Mann-Whitney test showed that all three groups were significantly different from one another (AMD patients versus blood donor control subjects,  $P < 0.05$ ; AMD patients versus age-matched control subjects,  $P < 0.01$ ; age-matched control subjects versus blood donor control subjects,  $P < 0.001$ ). In the three groups, there was no significant difference in median antibody titer between the men and women (AMD patients, male 190 vs. female 206; age-matched control subjects, male 317 vs. female 316; blood donor control subjects, male 123 vs. female 108). The relationship between autoantibody titer and age is shown as a composite scattergram (Fig. 2). Although this result suggests that the prevalence of factor H autoantibodies increases with age, there was no relationship between age and autoantibody titer in the individual groups (AMD patients,  $r^2 = 0.090$ ,  $P = 0.375$ ; age-matched control subjects,  $r^2 = -0.086$ ,  $P = 0.401$ ; blood donor control subjects,  $r^2 = 0.034$ ,  $P = 0.739$ ). The threshold for determining autoantibody positivity in the AMD patients was calculated in two ways. First, we used the mean antibody titer +2SD from the blood donor control subjects. This is the method that most groups,<sup>16,17</sup> including our own,<sup>33</sup> have used, but it does not take into account the non-normal distribution. The mean antibody titer +2SD in the blood donor control subjects was 810 RU, and accounting for individual sample variance, a value over 900 RU was taken as indicative of the presence of a complement factor H autoantibody. In both the AMD patients and the age-matched control subjects, there were eight individuals with an autoantibody titer greater than this threshold. In the blood donor control group, there was only one. Second, to take into account the non-normal distribution of antibody titer in all three groups, we used the 0.975 fractile, as recommended by the International Federation of Clinical Chemistry,<sup>34</sup> of the blood donor control subjects and

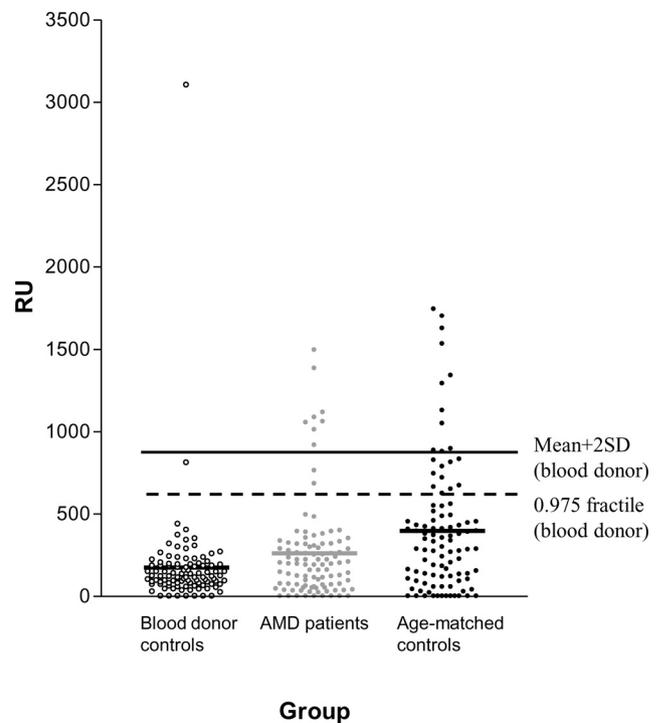


FIGURE 1. Complement factor H autoantibody titer in blood donor control subjects, AMD patients, and age-matched control subjects. *Horizontal solid line*: the threshold of 900 RU, which was derived from the mean +2 SD of the autoantibody titer in the blood donor control subjects. *Dashed line*: threshold of 624 RU which represents the 0.975 fractile of the blood donor control subjects.

derived a threshold of 624 RU. With this threshold, there would be 21 age-matched control subjects and 10 AMD patients who were autoantibody positive. This frequency is significantly different between the two groups ( $\chi^2 = 4.895$ ,  $df = 1$ ,  $P = 0.027$ ). The two thresholds are shown in Figure 1.

### Binding of Autoantibodies to CFH and CFHR1 Fragments

The binding sites of the autoantibodies were determined by using complement factor H fragment SCRs 1 to 4, 6 to 8, 8

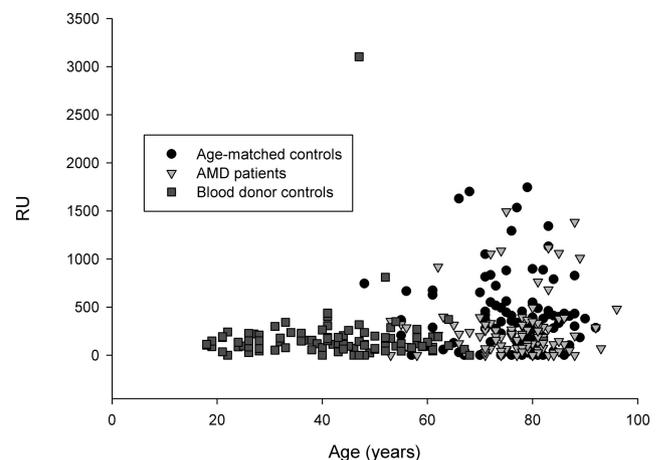
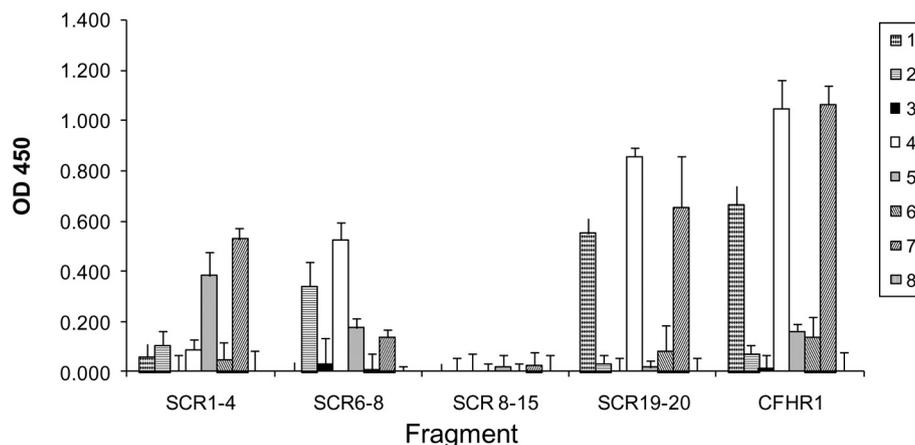
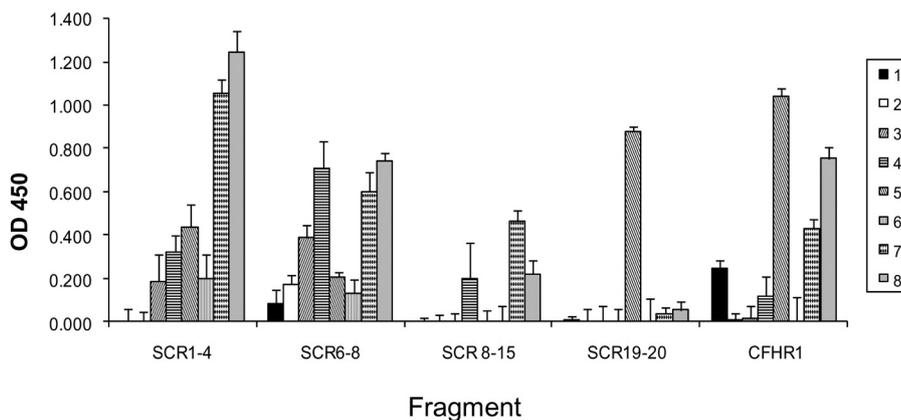


FIGURE 2. Complement factor H autoantibody titer versus age for the three groups: AMD patients  $r^2 = 0.090$ ,  $P = 0.375$ ; age-matched control subjects  $r^2 = -0.086$ ,  $P = 0.401$ ; and blood donor control subjects  $r^2 = 0.034$ ,  $P = 0.739$ .

a) Age-matched controls



b) AMD patients



**FIGURE 3.** Autoantibody reactivity with short factor H fragments. Autoantibody binding (represented as OD<sub>450</sub>) to factor H fragments (corresponding to SCRs 1-4, 6-8, 8-15, and 19/20) and a factor H-related protein 1 fragment (SCRs 4/5) was assessed with ELISA. Data are expressed as the mean ± SD of results in three experiments. (a) Results of the age-matched control subjects; (b) AMD patients. Each bar represents one subject.

to 15, and 19/20 and a complement factor H-related protein 1 fragment SCR 4/5 (Fig. 3). There was evidence of binding by autoantibodies from both the AMD patient group and the age-matched control subjects to all these fragments, with the exception of complement factor H SCRs 8 to 15, which was not recognized by the age-matched control subjects. There was a bias in the AMD patients (7/8) for stronger autoantibody reactivity against SCRs 1 to 8 of complement factor H compared with the age-matched control subjects (2/8), but given the overall spectrum of binding and the level of interaction, it is unlikely to be significant.

**CFHR3 Copy Number**

The *CFHR3* copy number was used as a marker of the *CFHR3/1* deletion. Homozygous deletion of *CFHR3/1* was significantly more frequent in the age-matched control subjects than in the AMD patients (age-matched control subjects 5.6% vs. AMD patients, 0%;  $\chi^2 = 14.3$ ,  $df = 2$ ,  $P = 8.0 \times 10^{-4}$ ). The frequency of homozygous deletion of *CFHR3/1* in the blood donor control subjects was 2%, which was not significantly different from either the AMD patients or the age-matched control subjects. Likewise, the allele frequency of the *CFHR3/1* deletion was significantly greater in age-matched control subjects than in the AMD patients (age-matched control subjects 22.2% vs. AMD patients, 8.2%;  $\chi^2 = 14.6$ ,  $df = 1$ ,  $P = 1.3 \times 10^{-4}$ ; Table 3). The allele frequency of the *CFHR3/1* deletion was also significantly

greater in the blood donor control subjects than in the AMD patients (blood donor control subjects 15.5% vs. AMD patients, 8.2%;  $\chi^2 = 5.1$ ,  $df = 1$ ,  $P = 0.024$ ). The *CFHR3/1* deletion frequency in all three groups is consistent with Hardy-Weinberg equilibrium.

**TABLE 3.** *CFHR3* Copy Number and Allele Frequency

	AMD Patients (n = 98)	Age-Matched Control (n = 90)	Blood Donor Control (n = 100)
<i>CFHR3</i> copy number			
0	0	5	2
1	16	30	27
2	82	55	71
<i>CFHR3</i> allele frequency*			
Deleted	16 (8.2%)	40 (22.2%)	31 (15.5%)
Present	180	140	169

\* AMD patients vs. age-matched controls;  $\chi^2 = 14.6$ ,  $df = 1$ ,  $P = 1.3 \times 10^{-4}$ ; AMD patients vs. blood donor controls  $\chi^2 = 5.1$ ,  $df = 1$ ,  $P = 0.024$ ; age-matched controls vs. blood donor controls  $\chi^2 = 2.818$ ,  $df = 1$ ,  $P = 0.093$ .

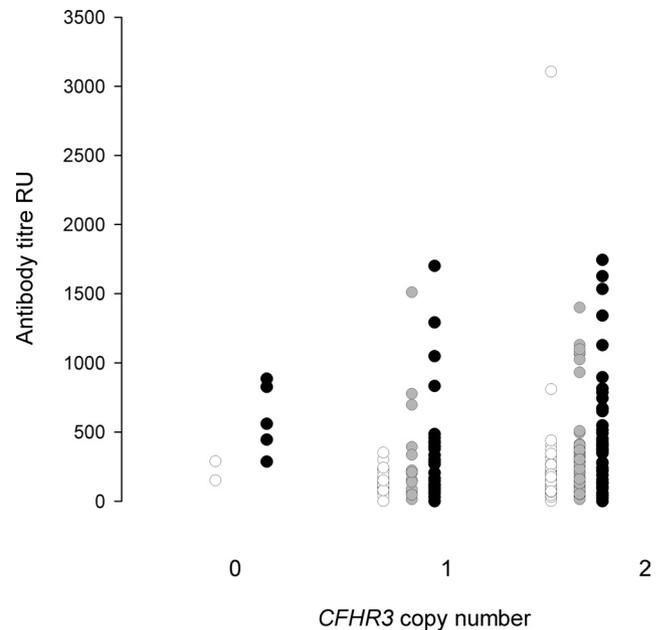
### CFHR3/1 Deletion and Complement Factor H Autoantibodies

There were no AMD patients with the homozygous *CFHR3/1* deletion. As reported, there were eight AMD patients, eight age-matched control subjects, and one blood donor control with complement factor H autoantibodies. Of these 17 individuals, 13 had two copies of *CFHR3* and 4 had one copy (3 age-matched control subjects and 1 AMD patient). Figure 4 shows the antibody titer for each of the groups, according to *CFHR3* copy number. There was no evidence of an association between *CFHR3* copy number and autoantibody titer in the three groups (Kruskal-Wallis test: AMD patients,  $P = 0.893$ ; age-matched control subjects,  $P = 0.123$ ; blood donor control subjects,  $P = 0.360$ ) (Table 4, Fig. 4).

### DISCUSSION

In this study, we examined the hypothesis that complement factor H autoantibodies are associated with AMD. We found, by using a threshold derived from the mean +2SD autoantibody of the blood donor control subjects, that complement factor H autoantibodies were present in 1 (1%) of 100 blood donor control subjects, 8 (8%) of 100 AMD patients, and 8 (8.2%) of 98 age-matched control subjects. Using a lower threshold derived from the 0.975 fractile of the blood donor control subjects, we found that 21 (21%) age-matched control subjects and 10 (10%) AMD patients were autoantibody positive. The hypothesis that we set out to test in this study was that the prevalence of factor H autoantibodies is increased in AMD patients. However, the results showed the opposite, in that the complement factor H autoantibody titer was significantly higher in the age-matched control subjects, and the prevalence of autoantibody positivity was significantly greater in the age-matched control subjects when we used the lower 0.975 fractile blood donor threshold. We believe that it would be tenuous to suggest from this that factor H autoantibodies protect against the development of AMD. However, the observation is fascinating. We have confirmed again that the deficiency of factor H-related protein 1 is associated with a decreased risk of AMD, and we speculate that this protein may have additional immunomodulatory properties.

Although determining the relation of age to the presence of factor H autoantibodies was not a goal of this study, the results suggest that the prevalence of factor H autoantibodies was greater in the older age groups. The median age of the blood donor control subjects (48 years) was substantially less than that of the AMD patients and age-matched control subjects (both 78 years), and the complement factor H autoantibody titer was significantly lower in the blood donor control subjects than in either the age-matched control subjects or the AMD patients. However, within each group there was no association between age and autoantibody titer. To test the hypothesis that the prevalence of factor H autoantibodies in-



**FIGURE 4.** Factor H autoantibody titer for each of the three groups according to *CFHR3* copy number. (○), Blood donor control subjects, (●), AMD patients, (●), age-matched control subjects.

creases with age necessitates a prospective study, but it has been known for many years that the prevalence of both organ-specific and systemic antibodies increases with age.<sup>35</sup> Factors that may predispose to this phenomenon include immune senescence involving a decline in naive T cells with a compensatory accumulation of memory T cells, thymic atrophy, chronic inflammation, and age-associated changes in epigenetic phenomenon.<sup>36,37</sup> Whether such autoantibodies are disease-predisposing or disease-causing is uncertain.

As in other studies, we have shown that deletion of *CFHR3* and *CFHR1* is associated with a lower prevalence of AMD.<sup>11,12,38</sup> Complement factor H-related protein 1 lacks the regulatory domain of factor H but has been shown to inhibit C5 convertase activity.<sup>39</sup> The C-terminal region of complement factor H-related protein 1 (SCRs 3, 4, and 5) has a high degree of homology with factor H (SCRs 18, 19, and 20) and thus can compete with complement factor H for the same binding sites on cell surfaces.<sup>40</sup> The same deletion has been shown to be associated with complement factor H autoantibodies in aHUS.<sup>16–18,33</sup> The autoantibodies in most such patients bind to SCRs 19 and 20 of complement factor H and thus block the recognition domain.<sup>17</sup> In this study, we have examined both the relationship of complement factor H autoantibodies to the *CFHR3/1* deletion and mapped their binding sites. In the 17 individuals found in this study to be positive for complement factor H autoantibodies (using the higher threshold of mean +2SD), 13 had two copies of *CFHR3* and 4 had one copy, suggesting that only 4 of the 17 carried the *CFHR3/1* deletion. These autoantibodies were at low levels compared with most of those found to be associated with aHUS. We also found evidence of binding to multiple segments of the complement factor H molecule, indicative of a polyclonal antibody response in contrast to the limited clonality seen in aHUS.

In summary, we have found that the prevalence of factor H autoantibodies is decreased in AMD patients compared with age-matched control subjects.

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**TABLE 4.** Factor H Autoantibody Titer

<i>CFHR3</i> Copy Number	AMD Patients	Age-Matched Control	Blood Donor Control
0		560	219
1	166	276	138
2	202	354	114

Data are in median reference units (RU). Kruskal-Wallis test: AMD patients  $P = 0.893$ , age-matched control  $P = 0.123$ , blood donor control  $P = 0.360$ .

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