Lack of association of *CFD* polymorphisms with advanced age-related macular degeneration

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Purpose: Age-related macular degeneration (AMD) is the most common cause of irreversible central vision loss worldwide. Research has linked AMD susceptibility with dysregulation of the complement cascade. Typically, complement factor H (*CFH*), complement factor B (*CFB*), complement component 2 (*C2*), and complement component 3 (*C3*) are associated with AMD. In this paper, we investigated the association between complement factor D (*CFD*), another factor of the complement system, and advanced AMD in a Caucasian population.

Methods: Six single nucleotide polymorphisms (SNPs), rs1683564, rs35186399, rs1683563, rs3826945, rs34337649, and rs1651896, across the region covering *CFD,* were chosen for this study. One hundred and seventy-eight patients with advanced AMD and 161 age-matched normal controls were genotyped. Potential positive signals were further tested in another independent 445 advanced AMD patients and 190 controls. χ^2 tests were performed to compare the allele frequencies between case and control groups.

Results: None of the six SNPs of *CFD* was found to be significantly associated with advanced AMD in our study.

Conclusions: Our findings suggest that *CFD* may not play a major role in the genetic susceptibility to AMD because no association was found between the six SNPs analyzed in the *CFD* region and advanced AMD.

Age-related macular degeneration (AMD) is the most common cause of irreversible central vision loss worldwide [1]. It can be divided into early and advanced (late) forms. Early AMD is characterized by the presence of drusen or pigmentary abnormalities in the retinal pigment epithelium (RPE), while advanced AMD includes two clinical types—geographic atrophy of the RPE (dry AMD) and choroidal neovascularization (wet AMD). Age-related macular degeneration is a complex disease caused by the combination of genetic predisposition and environmental factors [2-4]. The inheritance of AMD is polygenic and multifactorial: race, age, smoking, body mass, and body mass index have all been associated with AMD [2,5-9]. In the past five years, significant progress has been made in our understanding of AMD genetics through studies that identified specific single nucleotide polymorphisms (SNPs) significantly associated with AMD. The discovery of the strongest associations of AMD with the variants of complement factor H (*CFH*; OMIM 134370) [10-13] and *ARMS2/HTRA1* (age-related maculopathy susceptibility 2, OMIM 611313; the high

temperature requirement factor A1, OMIM: 602194) [14-17] has led to new hypotheses regarding the pathogenesis of this disease. *CFH* is primarily associated with the formation of drusen that often characterizes both types of advanced AMD in Caucasian populations, whereas *ARMS2/HTRA1* is mainly associated with wet AMD [18]. Other than these two major loci, three other members of the complement system, complement component 2 (*C2*; OMIM 217000), complement component 3 (*C3*; OMIM 120700), and complement factor B (*CFB*; OMIM 138470), were also found to be associated with AMD [10,19-21]. Most of these genes are complement pathway-associated genes, with the products participating in the alternative complement pathway and playing important roles in the complement system. Many studies have provided evidence that the complement system is involved in the pathogenesis of AMD and may have a pivotal effect on the formation and development of the disease [13,22-24]. However, the specific role of the complement system in the etiology of AMD is difficult to elucidate, and questions remain as to whether other components from this system are associated with AMD.

Complement factor D (CFD) is a protein of the trypsin family encoded by the *CFD* gene (OMIM 134350) and is involved in the alternative complement pathway of the complement system [25,26]. CFD is unique among serine

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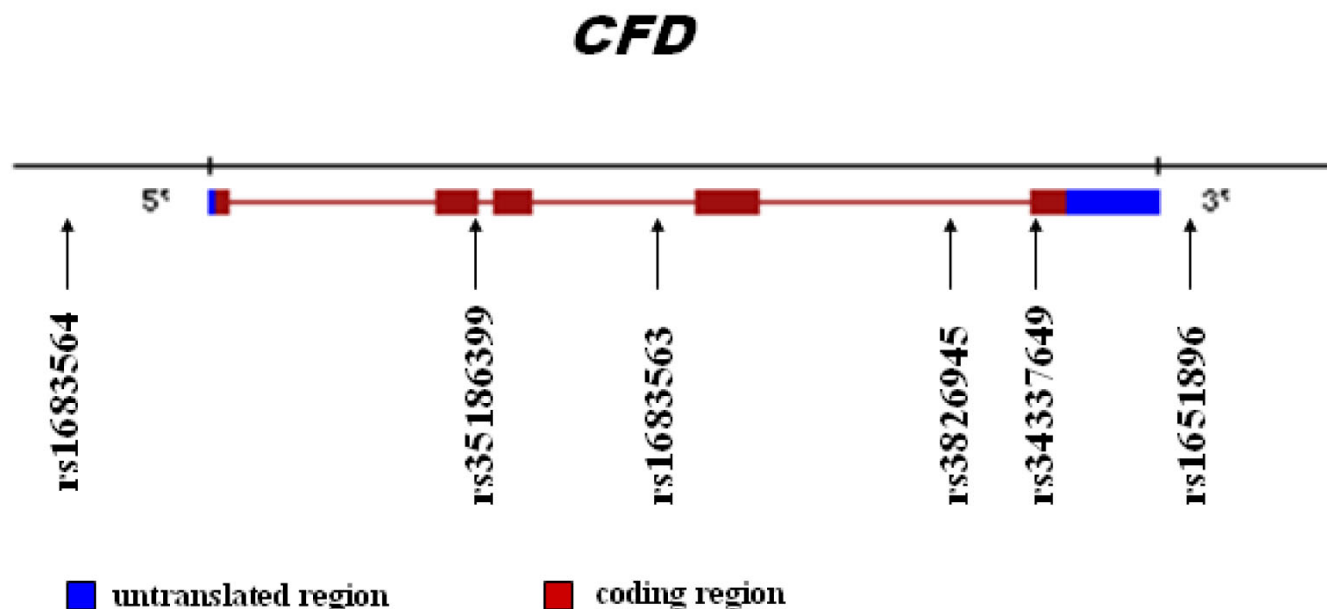
CFD

Figure 1. Six single nucleotide polymorphisms (SNPs) on complement factor D (*CFD*). Shown is the corresponding location of each SNP on *CFD* (chromosome:19; location:19p13.3) that was chosen in this study.

TABLE 1. ASSOCIATION BETWEEN 5 SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) ON COMPLEMENT FACTOR D (*CFD*) AND ADVANCED AGE RELATED MACULAR DEGENERATION (AMD)

SNP	Minor allele	Frequency in case	HWE in case	Frequency in control	HWE in control	Frequency in HapMap	p (allelic)
rs1683564	G	0.424	8.96E-01	0.409	1.57E-01	0.460 (CEPH)	0.710
rs35186399	A	0.006	9.97E-01	0.003	9.99E-01	N.A.	0.658
rs1683563	G	0.371	3.47E-01	0.414	8.21E-01	0.353	0.253
rs3826945	G	0.346	9.18E-01	0.309	9.68E-01	0.351	0.318
rs34337649	T	0	N.A.	0	N.A.	N.A.	N.A.

Shown are minor alleles for each SNP on *CFD*; frequencies for our advanced AMD, control groups and HapMap European groups; Hardy–Weinberg equilibrium (HWE) test; and P(allelic) values. (The P(allelic) value cannot be calculated when there is a count equal to zero.)

proteases in that it requires neither enzymatic cleavage for expression of proteolytic activity nor inactivation by a serpin for its control [27]. It is best known for its role in humoral suppression of infectious agents and has a high level of expression in fat, suggesting a role for adipose tissue in immune system biology (CFD). In this study, we investigated the association of *CFD* with advanced AMD in a Caucasian population.

METHODS

Subjects and clinical diagnosis: This study was approved by the Institutional Review Board of the University of California, San Diego. Informed consent was signed by all subjects before participation in the study. One hundred and seventy-eight nonfamilial advanced AMD patients and 161 age-matched normal controls (60 years or older with no drusen or RPE changes) as well as an independent replication cohort with 445 nonfamilial advanced AMD patients and 190 age-matched normal controls were recruited using the standard ophthalmic

examination protocol. Grading was performed using a standard grid classification suggested by the International Age-related Maculopathy (ARM) Epidemiological Study Group for the age-related maculopathy and age-related macular degeneration group [28]. All the participants were Caucasian.

Genotyping: Six SNPs, rs1683564, rs35186399, rs1683563, rs3826945, rs34337649, and rs1651896 at the *CFD* locus were chosen, which were either tag SNPs or those that might affect the function of *CFD* (Figure 1). A Caucasian cohort of 178 advanced AMD patients was genotyped and allele frequencies were compared with 161 age- and ethnicity-matched normal controls by laboratory personnel blinded to the case/control status. Potential positive findings were tested for replication in an independent cohort of 445 advanced AMD patients and 190 normal controls.

Genotypes of six SNPs were achieved by primer extension of multiplex PCR products followed with a

TABLE 2. ASSOCIATION BETWEEN COMPLEMENT FACTOR D (*CFD)* SINGLE NUCLEOTIDE POLYMORPHISM (SNP) rs1651896 AND ADVANCED AGE RELATED MACULAR DEGENERATION (AMD) IN THE DISCOVERY, REPLICATION AND COMBINATION COHORTS

Cohort	Phenotype	N	MAF (A)	HWE	p (allelic)
Discovery	Advanced AMD	178	0.374	4.21E-01	0.07
Cohort	Control	161	0.307	9.97E-01	
Replication	Advanced AMD	445	0.372	9.52E-01	0.882
Cohort	Control	190	0.376	8.41E-01	
Combination	Advanced AMD	623	0.372	9.05E-01	0.223
Cohort	Control	351	0.345	9.21E-01	
HapMap-CEU	Control	112	0.321		

Shown are the corresponding numbers of the *CFD* SNP rs1651896 for both advanced AMD and control groups in the discovery cohort, replication cohort, and combination cohort. Frequencies for the minor allele in our cohorts and HapMap European group, hardy Weinberg equation (HWE) test, and P(allelic) values are given.

SNaPshot using an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

Statistical analysis: Deviation from Hardy–Weinberg equilibrium was assessed, with a statistical significance level of 0.01. A chi-square test was performed to assess evidence for association. The statistical significance level was adjusted by Bonferroni correction. Linkage disequilibrium (LD) patterns and haplotype blocks were defined using Haploview 4.1.
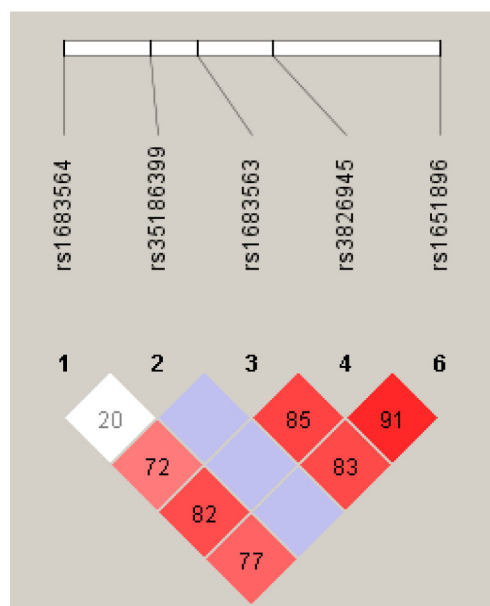

Figure 2. Linkage disequilibrium (LD) plot of six single nucleotide polymorphisms (SNPs) on complement factor D (*CFD)*. The physical position of each SNP is shown above the plot. Darker shades of red indicate higher values of the LD coefficient (D'). The numbers in pink and white squares show the % of D' between SNPs with incomplete LD. Blue squares with no number indicate a pairwise linkage disequilibrium of 1 between SNPs, supported by logarithm (base 10) of odds (lod) scores < 2.

RESULTS

We genotyped six SNPs that tag the majority of *CFD* haplotypes and investigated allelic association with AMD. Four SNPs, rs1683564, rs35186399, rs1683563, and rs3826945, located either at the promoter region, exon, or intron of *CFD*, exhibited no association with advanced AMD in our study population. The SNP rs34337649, in exon 5 of *CFD*, showed no polymorphisms in our data (Table 1).

Of the six SNPs, rs1651896, which is located at the 3′ region of *CFD*, was found to be marginally associated with advanced AMD (P(allelic)=0.07, risk allele A: 37.4% in cases versus 30.7% in controls) in a Caucasian cohort of 178 advanced AMD cases and 161 normal controls. The result was further investigated in an independent replication cohort of 445 advanced AMD patients and 190 controls. No further significant association was observed for either the replication cohort (P(allelic)=0.882, risk allele A: 37.2% in cases versus 37.6% in controls) or the combined cohort (P(allelic)=0.223, risk allele A: 37.2% in cases versus 34.5% in controls, Table 2).

Linkage disequilibrium patterns and haplotype blocks were defined by combining the six SNPs (Figure 2). No significant association was found between the haplotypes and AMD phenotypes. The SNP rs34337649 was not shown in the plot because it was not a detected polymorphism in our data set.

DISCUSSION

The alternative complement pathway is one of three distinct complement pathways and is important in the clearance and recognition of pathogens in the absence of antibodies [29] (Figure 3). It is triggered by spontaneous C3 hydrolysis to form C3a and C3b, which makes C3b capable of binding to a pathogenic membrane surface. After binding with an activator membrane, C3b is bound by CFB to form C3bB. Complement factor H acts as one of the primary regulators by inhibiting the binding of CFB to C3b and also by degrading C3b. In the presence of CFD, C3bB is cleaved into C3bBa and C3bBb
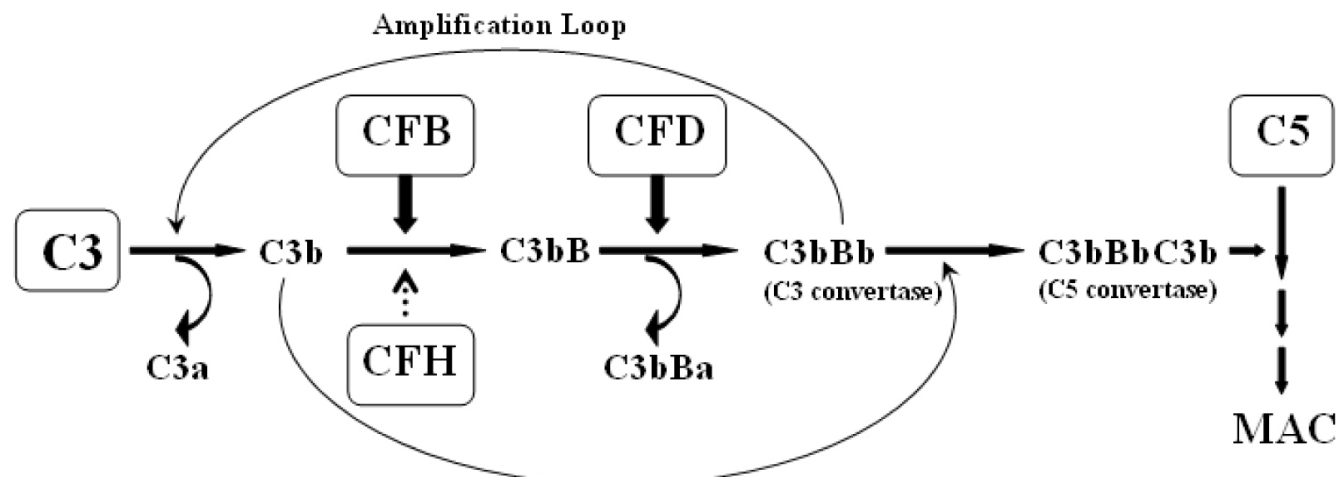
Amplification Loop

Figure 3. The alternative complement pathway. The principal components of the alternative complement pathway from C3 to the stage of membrane attack complex (MAC) formation are shown in detail. Complement factor H (CFH) with the dotted arrow acts as an inhibiting regulator while Complement factor B (CFB) and Complement factor D (CFD) with the filled arrows are activators.

(C3 convertase). After hydrolysis of C3, C3 convertase and C3b become C3bBbC3b, which cleaves C5 into C5a and C5b. A membrane attack complex (MAC) is formed through subsequent reactions, which inserts into the cell membrane and initiates cell lysis [18,30,31]. In this pathway, CFB and CFD are involved in the activation and amplification loop, whereas CFH is a fluid phase inhibitor. To keep the complement activity under control, the competition between CFH and CFB binding to C3b on the host or pathogen cells needs to be tightly regulated. How this control is achieved is not completely clear [18].

Compared with other complement factors, CFH, C3, and CFB have been associated with AMD in many independent studies. However, the exact role they play in the pathogenesis of AMD and whether their effects are mediated through the complement system or through another pathway is poorly understood. Previously, CFD was studied using a *CFD* $^{-/-}$ mouse model, with the conclusion that eliminating the alternative pathway was neuroprotective and reduced photoreceptor susceptibility to light-induced damage [25]. Contrary to this finding, no significant association between *CFD* SNPs and advanced AMD was found in this study. Due to the dearth of information about the relationship between *CFD* and AMD, further research in other populations may be warranted. Our results suggest it is unlikely that *CFD* is a major functional candidate gene conferring risk for AMD. However, due to the relatively small sample size, this study has limited power to detect minor effects. Extended cohorts will be needed to confirm the genetic role of *CFD*. Future research should also focus on a comprehensive understanding of genetic variants throughout all pathways of the complement system. By identifying high risk variants and improving our understanding of the complement system, early intervention

for patients at risk of developing AMD and novel gene-based treatments may become a reality.

REFERENCES
1. Chappelow AV, Kaiser PK. Neovascular age-related macular degeneration: potential therapies. Drugs 2008; 68:1029-36. [PMID: 18484796]
2. Montezuma SR, Sobrin L, Seddon JM. Review of genetics in age related macular degeneration. Semin Ophthalmol 2007; 22:229-40. [PMID: 18097986]
3. Patel N, Adewoyin T, Chong NV. Age-related macular degeneration: a perspective on genetic studies. Eye (Lond) 2008; 22:768-76. [PMID: 17491602]
4. Khan JC, Thurlby DA, Shahid H, Clayton DG, Yates JR, Bradley M, Moore AT, Bird AC, Genetic Factors in AMD Study. Smoking and age related macular degeneration: the number of pack years of cigarette smoking is a major determinant of risk for both geographic atrophy and choroidal neovascularisation. Br J Ophthalmol 2006; 90:75-80. [PMID: 16361672]
5. Bressler SB, Munoz B, Solomon SD, West SK. Racial differences in the prevalence of age-related macular degeneration: the Salisbury Eye Evaluation (SEE) Project. Arch Ophthalmol 2008; 126:241-5. [PMID: 18268216]
6. Clemons TE, Milton RC, Klein R, Seddon JM, Ferris FL 3rd. Risk factors for the incidence of Advanced Age-Related Macular Degeneration in the Age-Related Eye Disease Study

(AREDS) AREDS report no. 19. Ophthalmology 2005; 112:533-9. [PMID: 15808240]

7. Klein R, Klein BE, Tomany SC, Cruickshanks KJ. The association of cardiovascular disease with the long-term incidence of age-related maculopathy: the Beaver Dam eye study. Ophthalmology 2003; 110:636-43. [PMID: 12689879]

8. Seddon JM, Cote J, Davis N, Rosner B. Progression of age-related macular degeneration: association with body mass index, waist circumference, and waist-hip ratio. Arch Ophthalmol 2003; 121:785-92. [PMID: 12796248]

9. van Leeuwen R, Klaver CC, Vingerling JR, Hofman A, de Jong PT. Epidemiology of age-related maculopathy: a review. Eur J Epidemiol 2003; 18:845-54. [PMID: 14561043]

10. Katta S, Kaur I, Chakrabarti S. The molecular genetic basis of age-related macular degeneration: an overview. J Genet 2009; 88:425-49. [PMID: 20090206]

11. Hageman GS, Anderson DH, Johnson LV, Hancox LS, Taiber AJ, Hardisty LI, Hageman JL, Stockman HA, Borchardt JD, Gehrs KM, Smith RJ, Silvestri G, Russell SR, Klaver CC, Barbazetto I, Chang S, Yannuzzi LA, Barile GR, Merriam JC, Smith RT, Olsh AK, Bergeron J, Zernant J, Merriam JE, Gold B, Dean M, Allikmets R. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. Proc Natl Acad Sci USA 2005; 102:7227-32. [PMID: 15870199]

12. 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; 114:2168-73. [PMID: 18054635]

13. Anderson DH, Radeke MJ, Gallo NB, Chapin EA, Johnson PT, Curletti CR, Hancox LS, Hu J, Ebright JN, Malek G, Hauser MA, Rickman CB, Bok D, Hageman GS, Johnson LV. The pivotal role of the complement system in aging and age-related macular degeneration: Hypothesis re-visited. Prog Retin Eye Res 2010; 29:95-112. [PMID: 19961953]

14. Yang Z, Camp NJ, Sun H, Tong Z, Gibbs D, Cameron DJ, Chen H, Zhao Y, Pearson E, Li X, Chien J, Dewan A, Harmon J, Bernstein PS, Shridhar V, Zabriskie NA, Hoh J, Howes K, Zhang K. A variant of the HTRA1 gene increases susceptibility to age-related macular degeneration. Science 2006; 314:992-3. [PMID: 17053109]

15. Dewan A, Liu M, Hartman S, Zhang SS, Liu DT, Zhao C, Tam PO, Chan WM, Lam DS, Snyder M, Barnstable C, Pang CP, Hoh J. HTRA1 promoter polymorphism in wet age-related macular degeneration. Science 2006; 314:989-92. [PMID: 17053108]

16. Andreoli MT, Morrison MA, Kim BJ, Chen L, Adams SM, Miller JW, DeAngelis MM, Kim IK. Comprehensive analysis of complement factor H and LOC387715/ARMS2/HTRA1 variants with respect to phenotype in advanced age-related macular degeneration. Am J Ophthalmol 2009; 148:869-74. [PMID: 19796758]

17. Cameron DJ, Yang Z, Gibbs D, Chen H, Kaminoh Y, Jorgensen A, Zeng J, Luo L, Brinton E, Brinton G, Brand JM, Bernstein PS, Zabriskie NA, Tang S, Constantine R, Tong Z, Zhang K. HTRA1 variant confers similar risks to geographic atrophy and neovascular age-related macular degeneration. Cell Cycle 2007; 6:1122-5. [PMID: 17426452]

18. DeWan A, Bracken MB, Hoh J. Two genetic pathways for age-related macular degeneration. Curr Opin Genet Dev 2007; 17:228-33. [PMID: 17467263]

19. Seddon JM, Reynolds R, Maller J, Fagerness JA, Daly MJ, Rosner B. Prediction model for prevalence and incidence of advanced age-related macular degeneration based on genetic, demographic, and environmental variables. Invest Ophthalmol Vis Sci 2009; 50:2044-53. [PMID: 19117936]

20. Kaur I, Katta S, Reddy RK, Narayanan R, Mathai A, Majji AB, Chakrabarti S. 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. [PMID: 19696172]

21. Despriet DD, van Duijn CM, Oostra BA, Uitterlinden AG, Hofman A, Wright AF, ten Brink JB, Bakker A, de Jong PT, Vingerling JR, Bergen AA, Klaver CC. Complement component C3 and risk of age-related macular degeneration. Ophthalmology 2009; 116:474-80. [PMID: 19168221]

22. Zanke B, Hawken S, Carter R, Chow D. A genetic approach to stratification of risk for age-related macular degeneration. Can J Ophthalmol 2010; 45:22-7. [PMID: 20130705]

23. Nussenblatt RB, Liu B, Li Z. Age-related macular degeneration: an immunologically driven disease. Curr Opin Investig Drugs 2009; 10:434-42. [PMID: 19431076]

24. Gehrs KM, Jackson JR, Brown EN, Allikmets R, Hageman GS. Complement, age-related macular degeneration and a vision of the future. Arch Ophthalmol 2010; 128:349-58. [PMID: 20212207]

25. Rohrer B, Guo Y, Kunchithapautham K, Gilkeson GS. Eliminating complement factor D reduces photoreceptor susceptibility to light-induced damage. Invest Ophthalmol Vis Sci 2007; 48:5282-9. [PMID: 17962484]

26. Sprong T, Roos D, Weemaes C, Neeleman C, Geesing CL, Mollnes TE, van Deuren M. Deficient alternative complement pathway activation due to factor D deficiency by 2 novel mutations in the complement factor D gene in a family with meningococcal infections. Blood 2006; 107:4865-70. [PMID: 16527897]

27. Volanakis JE, Narayana SV. Complement factor D, a novel serine protease. Protein Sci 1996; 5:553-64. [PMID: 8845746]

28. Bird AC, Bressler NM, Bressler SB, Chisholm IH, Coscas G, Davis MD, de Jong PT, Klaver CC, Klein BE, Klein R, Mitchell P, Sarks JP, Sarks SH, Soubrane G, Taylor HR, Vingerling JR. An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. Surv Ophthalmol 1995; 39:367-74. [PMID: 7604360]

29. Stahl GL, Xu Y, Hao L, Miller M, Buras JA, Fung M, Zhao H. Role for the alternative complement pathway in ischemia/reperfusion injury. Am J Pathol 2003; 162:449-55. [PMID: 12547703]

30. Hecker LA, Edwards AO, Ryu E, Tosakulwong N, Baratz KH, Brown WL, Charbel Issa P, Scholl HP, Pollok-Kopp B, Schmid-Kubista KE, Bailey KR, Oppermann M. Genetic control of the alternative pathway of complement in humans and age-related macular degeneration. Hum Mol Genet 2010; 19:209-15. [PMID: 19825847]

31. Scholl HP, Charbel Issa P, Walier M, Janzer S, Pollok-Kopp B, Börncke F, Fritsche LG, Chong NV, Fimmers R, Wienker T,

Holz FG, Weber BH, Oppermann M. Systemic complement activation in age-related macular degeneration. PLoS ONE 2008; 3:e2593. [PMID: 18596911]