Allelic Variants of Complement Genes Associated with Dense Deposit Disease

Maria Asuncion Abrera-Abeleda,*† Carla Nishimura,† Kathy Frees,† Michael Jones,† Tara Maga,*† Louis M. Katz,§ Yuzhou Zhang,† and Richard J.H. Smith*†‡

*Interdisciplinary PhD Program in Genetics, †Department of Otolaryngology–Head and Neck Surgery, and ‡Department of Medicine, University of Iowa, Iowa City, Iowa; and §Mississippi Valley Regional Blood Center, Davenport, Iowa

ABSTRACT

The alternative pathway of the complement cascade plays a role in the pathogenesis of dense deposit disease (DDD). Deficiency of complement factor H and mutations in CFH associate with the development of DDD, but it is unknown whether allelic variants in other complement genes also associate with this disease. We studied patients with DDD and identified previously unreported sequence alterations in several genes in addition to allelic variants and haplotypes common to patients with DDD. We found that the likelihood of developing DDD increases with the presence of two or more risk alleles in CFH and C3. To determine the functional consequence of this finding, we measured the activity of the alternative pathway in serum samples from phenotypically normal controls genotyped for variants in CFH and C3. Alternative pathway activity was higher in the presence of variants associated with DDD. Taken together, these data confirm that DDD is a complex genetic disease and may provide targets for the development of disease-specific therapies.


The complement system is an integral arm of innate immunity that facilitates lysis, opsonization, and clearance of pathogens.1 Its three initiating arms—the classical, lectin, and alternative pathways—respond to different triggers to generate an amplifying complex known as the C3 convertase (C3bBb). The classical pathway typically requires antibodies for activation, whereas the mannose-binding lectin and alternative pathways are activated by antigens and C3 hydrolysis, respectively.

C3 hydrolysis is the reaction of a thioester on C3 with water to form C3(H2O). The process occurs spontaneously, and consequently the alternative pathway (AP) is continuously active, albeit at a low rate.2 Unchecked, C3(H2O) reacts with complement factor B (fB) to generate the initial C3 convertase C3(H2O)B, which is converted to C3(H2O)Bb in the presence of complement factor D (fD). Additional cleavage of C3 to C3b leads to formation of C3 convertase, which continues to catalyze the cleavage of C3 to C3a and C3b in a potent amplification loop.3 C3b molecules that associate with C3 convertase form C5 convertase (C3bBbC3b), which cleaves C5 to initiate the terminal complement cascade (TCC). The TCC culminates in formation of membrane attack complex, a multimeric transmembrane channel comprised of C5b, C6, C7, C8, and polymeric C9 that causes osmotic lysis of target pathogens.4,5

Because the AP does not recognize target-specific activators and is constitutively active, a number of strategies have evolved to regulate AP activity and discriminate between activating (pathogenic)
and nonactivating (self) surfaces.6 The major regulator of AP in plasma is complement factor H (fH). fH accelerates the decay of C3bBb, acts as a cofactor for complement factor I (fI)-mediated proteolytic inactivation of C3b, and competes with fB for binding to C3b.7,8 When fH has high affinity for a surface or for surface-bound C3b, AP activation is stopped; if affinity is low, AP activation proceeds, and opsonization and lysis occur.

The AP has been implicated in the pathogenesis of dense deposit disease (DDD), a rare renal disease that affects two of 1,000,000 persons and progresses to end-stage renal failure in half of patients within 10 years of diagnosis.9–13 DDD is named for the pathognomonic amorphous electron-dense deposits that must be present in the glomerular basement membranes on renal biopsy to make the diagnosis. Affected patients typically present with nonspecific signs and symptoms of glomerular damage such as nephrotic syndrome, hypertension, hematuria, and proteinuria; however, evidence of AP dysregulation is also present.14 C3 serum levels are often exceedingly low, and in most patients, autoantibodies to C3 convertase known as nephritic factors (C3Nefs) are found.15,16 A few DDD patients also have autoantibodies to fH (FHAA) or fB (FBAA).17–19 Over the long term, DDD patients develop visual impairment secondary to ocular deposits known as drusen in Bruch’s membrane.20

Mutations and polymorphisms in CFH have been associated with DDD, and on that basis we hypothesized that novel alterations and known polymorphisms in other complement genes may play a role in DDD.21,22 Because the AP is continuously activated at a low rate in human plasma, we also hypothesized that AP activity among individuals differs and that controls carrying DDD “risk” alleles have intrinsically higher AP activity compared with other controls.

**RESULTS**

**DDD Cases and Controls**

Sixty-six patients with biopsy-proven DDD were ascertained in nephrology divisions and enrolled in this study. The control group was comprised of 165 age-, gender-, and ethnicity-matched individuals. The status of the control group with respect to age-related macular degeneration and/or other complement-mediated disease was unknown (Table 1). All patients and controls were American Caucasians who identified themselves as of Northern European heritage. Patients with ambiguous/unknown race were excluded from the analysis. All procedures were approved by the Institutional Review Board of the University of Iowa, Carver College of Medicine.

**Novel Sequence Alterations**

Genomic DNA extracted from blood samples of patients and controls (PAXgene Blood DNA Kit, Qiagen, Valencia, California) was used to amplify and bidirectionally sequence the coding regions and intron-exon boundary junctions of C3 (NM_000064), CFH (NM_001736), CFHR5 (NM_030787), C3aR1 (NM_004054), C5aR1 (NM_001736), CR1 (NM_000065), and ADAM19 (NM_033274). Novel missense variants in DDD patients were identified in four genes: C3 p.K1203R, C3aR1 p.L84S, CR1 p.V1222L, and ADAM19 p.G507S (Table 2). These changes were not found in any controls and are not reported in single nucleotide polymorphism (SNP) databases. Each variant was found in a single DDD patient except C3aR1 p.L84F, which was found in two patients. PolyPhen predicted this sequence change to be possibly damaging; SIFT predicted C3 p.K1203R to be possibly damaging; and Align GVGD predicted that ADAM19 p.G507S is likely to interfere with protein function. CR1 p.V1222L was classified as benign by PolyPhen, SIFT, and Align GVGD.

**Association Analyses**

A CNV in C4A, which renders a null allele, and 18 SNPs were selected for association analysis based on possible function and data from other complement-mediated diseases like age-related macular degeneration and atypical hemolytic uremic syndrome (aHUS). The SNPs included four in C3 (rs2230199, rs2230201, rs10347286, and rs2230203), five in CFH (rs3753394, rs800292, rs1061170, rs3753396, and rs1065489), three in CFHR5 (rs9427661, rs9427662, and rs800292), two in C5aR1 (rs4467185 and rs11008897), three in CR1 (rs738467, rs2274567, and rs3811381) and one in ADAM19 (rs1422795). Five SNPs were eliminated from further consideration because they were in linkage disequilibrium with nearby SNPs included in the analysis (C3 rs2230203; CFHR5 rs9427661; CR1 rs2274567 and rs3811381; and C5aR1 rs11008897).

Using a chi-squared test of independence without correction, the C4A CNV and five SNPs with a P-value <0.05 were

<table>
<thead>
<tr>
<th>Gene</th>
<th>Variant</th>
<th>Patients</th>
<th>ConSeq</th>
<th>PolyPhen</th>
<th>SIFT</th>
<th>Align GVGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3</td>
<td>c0.3607 A&gt;G p.K1203R</td>
<td>1</td>
<td>1</td>
<td>Benign</td>
<td>Affects protein function</td>
<td>Least likely to interfere with function</td>
</tr>
<tr>
<td>C3aR1</td>
<td>c0.250 C&gt;T p.L84F</td>
<td>2</td>
<td>4</td>
<td>Possibly damaging</td>
<td>Tolerated</td>
<td>Least likely to interfere with function</td>
</tr>
<tr>
<td>CR1</td>
<td>c0.3664 G&gt;T p.V1222L</td>
<td>1</td>
<td>2</td>
<td>Benign</td>
<td>Tolerated</td>
<td>Least likely to interfere with function</td>
</tr>
<tr>
<td>ADAM19</td>
<td>c0.1522 G&gt;A p.G507S</td>
<td>1</td>
<td>1</td>
<td>Benign</td>
<td>Tolerated</td>
<td>Most likely to interfere with function</td>
</tr>
</tbody>
</table>
considered nominally associated with the DDD phenotype \([CFH]\) p.Y402H (rs1061170), C3 p.R102G (rs2330199), C3 p.P314L (rs1047286), CFHR5 [−20 T>C (rs9427662), and ADAM19 p.S284G (rs1422795)]. With Bonferroni correction \((\alpha = 0.004)\), ADAM19 SNP rs1422795 \((P = 5.49E-05)\), C4A CNV \((P = 3.29E-05)\), and C3 SNP rs1047286 \((P = 0.0018)\) were associated with DDD. With correction for multiple testing using the false discovery rate method described by Benjamini and Hochberg to minimize the likelihood of rejecting false negatives, C3 SNP rs2330199 \((P = 0.0065)\), CFH SNP rs1061170 \((P = 0.019)\), and CFHR5 SNP rs9427662 \((P = 0.02)\) were also associated with DDD \((\alpha = 0.021)\).

The reference haplotype, which is the most common haplotype in both groups, has OR \(95\% \text{ CI}\) 1.00 to 3.51, where the minor allele was protective (Table 3).

### Haplotype Analyses

Haplotype analyses were performed using an expectation-maximization (EM) algorithm for all genes. In CFH, four SNPs—331 T>C \((rs3753394)\), p.V62I \((rs800292)\), p.Y402H (rs1061170), and p.Q673 \((rs3753396)\)—define a low-risk (protective) haplotype: CATA or haplotype 2 (odds ratio \([OR]\), 0.58; confidence interval \([CI]\), 0.33 to 1.00) (Table 4). A haplotype analysis by Pickering and colleagues using the same SNPs produced similar results (OR, 0.42; CI, 0.14 to 1.24).

For C3, a linkage disequilibrium analysis was done to identify haploblocks. Haplotype analysis was then performed using SNPs in the haplotype block p.R102G (rs2230199), p.R304 G>A \((rs2230201)\), p.P314L \((rs1047286)\), and p.P518 C>A \((rs1047286)\). The “at-risk” haplotype was GGTA (haplotype 2) (Table 5).

### Gene-Gene Interaction

To evaluate SNP-based gene-gene interactions, we selected SNPs based on known protein interactions \((i.e., C3 \text{ binds to } \text{fH and CFHR5})\) and applied multifactor dimensionality reduction. This process predicted a synergistic interaction between \(CFH\) p.V62I and C3 p.P314L (Figure 1). Main effect and two- and three-variant combination analyses were performed using \(CFH\) p.Y402H, \(CFH\) p.V62I, C3 p.R102G, and C3 p.P314L, and the EM algorithm to predict allele combination frequencies (Table 6). To determine significant allele combinations in DDD patients, we computed ORs, 95% CIs, and \(P\)-values. The most significant SNP combination was \(CFH\) p.Y402H × \(CFH\) p.V62I × C3 p.P314L, which had the highest OR. \(CFH\) p.Y402H × C3 p.P314L had the second

### Table 3. Risk and protective alleles for developing DDD

<table>
<thead>
<tr>
<th>Gene</th>
<th>Variant</th>
<th>Allele</th>
<th>Frequency</th>
<th>OR 95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFHR5</td>
<td>−20 T&gt;C</td>
<td>6</td>
<td>0.045</td>
<td>37</td>
<td>0.11</td>
</tr>
<tr>
<td>CFH</td>
<td>Y402H</td>
<td>64</td>
<td>0.48</td>
<td>121</td>
<td>0.37</td>
</tr>
<tr>
<td>C3</td>
<td>R102G</td>
<td>43</td>
<td>0.36</td>
<td>68</td>
<td>0.21</td>
</tr>
<tr>
<td>C3</td>
<td>P314L</td>
<td>44</td>
<td>0.33</td>
<td>65</td>
<td>0.20</td>
</tr>
<tr>
<td>ADAM19</td>
<td>S284G</td>
<td>58</td>
<td>0.44</td>
<td>82</td>
<td>0.25</td>
</tr>
<tr>
<td>C4A</td>
<td>Deletion</td>
<td>24</td>
<td>0.18</td>
<td>19</td>
<td>0.06</td>
</tr>
</tbody>
</table>

### Table 4. Haplotype analysis of CFH SNPs using SNPStats

<table>
<thead>
<tr>
<th>Allele</th>
<th>Control Frequency</th>
<th>DDD Frequency</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.3218</td>
<td>0.4048</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>A</td>
<td>0.2420</td>
<td>0.1743</td>
<td>0.58 (0.33 to 1.00)</td>
<td>0.05</td>
</tr>
<tr>
<td>G</td>
<td>0.1050</td>
<td>0.1084</td>
<td>—</td>
<td>NS</td>
</tr>
<tr>
<td>T</td>
<td>0.1193</td>
<td>0.1050</td>
<td>—</td>
<td>NS</td>
</tr>
<tr>
<td>A</td>
<td>0.0159</td>
<td>0.0180</td>
<td>—</td>
<td>NS</td>
</tr>
<tr>
<td>G</td>
<td>0.0065</td>
<td>0.0246</td>
<td>—</td>
<td>NS</td>
</tr>
</tbody>
</table>

### Table 5. Haplotype analysis for C3 SNPs using SNPStats

<table>
<thead>
<tr>
<th>Allele</th>
<th>Control Frequency</th>
<th>DDD Frequency</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.6417</td>
<td>0.5004</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>G</td>
<td>0.1354</td>
<td>0.2389</td>
<td>2.12 (1.24 to 3.60)</td>
<td>0.0061</td>
</tr>
<tr>
<td>T</td>
<td>0.1305</td>
<td>0.1095</td>
<td>1.13 (0.63 to 2.03)</td>
<td>0.54</td>
</tr>
<tr>
<td>A</td>
<td>0.0218</td>
<td>0.0565</td>
<td>—</td>
<td>NS</td>
</tr>
</tbody>
</table>
highest OR. These data mean that likelihood of developing DDD increases with the presence of two or more risk alleles in **CFH** and **C3**.

### Complement Activity

To determine whether the fH-C3 interaction had a functional consequence, we tested AP activity in genotyped controls. Using unconditional logistic regression, a significant association was noted between APH50 and **CFH** p.V62I, **C3** p.R102G, and **C3** p.P314L (**P** = 0.0193, 0.0166, 0.0054, respectively). Pearson goodness-of-fit **P**-values indicated that the predicted model was a good model for the observed data (**P** > 0.05), and the OR indicated that the presence of risk allele **CFH** p.V62, **C3** p.G102, or **C3** p.L314 doubled the risk of a low APH50 (high complement activity).

The association between APH50 and **C3** p.R102G and **C3** p.P314L was verified using a Mann-Whitney U-test by comparing the medians between controls homozygous for **C3** R102 or **C3** P314 with controls either homozygous or heterozygous for **C3** G102 or **C3** L314. Controls carrying either **C3** G102 or **C3** L314 (in heterozygosity or homozygosity) had significantly lower APH50 values and therefore higher AP activity than controls homozygous for either **C3** R102 or **C3** P314 (**P** = 0.045 and 0.0176, respectively). There were no differences in protein levels between groups (**C3** G102 or **C3** L314 **C3** levels, 1.29 ± 0.95 and 1.34 ± 0.95 mg/ml, respectively; **C3** R102 or **C3** P314 **C3** levels, 1.19 ± 0.92 and 1.15 ± 0.92 mg/ml, respectively; **P** > 0.05) (Figure 2, A and B).

In two variant combinations, controls with at least one copy of both **C3** G102 and **C3** L314 demonstrated lower APH50 values (higher AP activity) than controls homozygous for both **C3** R102 and **C3** P314 (**P** = 0.0329). Again, there were no differences in protein levels between groups (**C3** G102 and **C3** L314 **C3** levels, 1.29 ± 0.93 mg/ml; **C3** R102 and **C3** P314 (1.18 ± 0.93 mg/ml) (**P**-value = 0.5956) (Figure 2C).

Comparison of major and minor alleles of **CFH** H402Y showed that higher %AP values (lower AP activity) were present in controls homozygous for **CFH** Y402 (**P** = 0.0345) com-

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**Table 6. ORs of main effects and variant combinations between **CFH** and **C3****

<table>
<thead>
<tr>
<th>Risk Alleles</th>
<th>DDD Frequency</th>
<th>Control Frequency</th>
<th>OR</th>
<th>95% CI</th>
<th><strong>P</strong>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main effects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CFH</strong> Y402H</td>
<td>H</td>
<td>47 (0.71)</td>
<td>93 (0.56)</td>
<td>1.92</td>
<td>1.04 to 3.54</td>
</tr>
<tr>
<td><strong>CFH</strong> V62I</td>
<td>V</td>
<td>23 (0.35)</td>
<td>73 (0.44)</td>
<td>0.67</td>
<td>0.37 to 1.22</td>
</tr>
<tr>
<td><strong>C3</strong> R102G</td>
<td>G</td>
<td>37 (0.56)</td>
<td>57 (0.36)</td>
<td>2.42</td>
<td>1.35 to 4.33</td>
</tr>
<tr>
<td><strong>C3</strong> P314L</td>
<td>L</td>
<td>37 (0.56)</td>
<td>58 (0.35)</td>
<td>2.35</td>
<td>1.32 to 4.21</td>
</tr>
<tr>
<td>Two-variant combinations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CFH</strong> Y402H × <strong>CFH</strong> V62I</td>
<td>H × V</td>
<td>32 (0.49)</td>
<td>56 (0.34)</td>
<td>1.83</td>
<td>1.02 to 3.27</td>
</tr>
<tr>
<td><strong>CFH</strong> Y402H × <strong>C3</strong> R102G</td>
<td>H × G</td>
<td>10 (0.15)</td>
<td>14 (0.08)</td>
<td>2.61</td>
<td>1.30 to 5.24</td>
</tr>
<tr>
<td><strong>CFH</strong> Y402H × <strong>C3</strong> P314L</td>
<td>H × L</td>
<td>12 (0.18)</td>
<td>12 (0.07)</td>
<td>3.58</td>
<td>1.72 to 7.45</td>
</tr>
<tr>
<td><strong>CFH</strong> V62I × <strong>C3</strong> R102G</td>
<td>V × G</td>
<td>17 (0.27)</td>
<td>27 (0.16)</td>
<td>1.63</td>
<td>0.95 to 2.79</td>
</tr>
<tr>
<td><strong>CFH</strong> V62I × <strong>C3</strong> P314L</td>
<td>V × L</td>
<td>21 (0.32)</td>
<td>24 (0.15)</td>
<td>2.47</td>
<td>1.43 to 4.27</td>
</tr>
<tr>
<td><strong>C3</strong> R102G × <strong>C3</strong> P314L</td>
<td>G × L</td>
<td>18 (0.27)</td>
<td>29 (0.18)</td>
<td>1.87</td>
<td>1.14 to 3.07</td>
</tr>
<tr>
<td>Three-variant combinations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CFH</strong> Y402H × <strong>CFH</strong> V62I × <strong>C3</strong> R102G</td>
<td>H × V × G</td>
<td>10 (0.15)</td>
<td>14 (0.09)</td>
<td>2.74</td>
<td>1.26 to 5.94</td>
</tr>
<tr>
<td><strong>CFH</strong> Y402H × <strong>CFH</strong> V62I × <strong>C3</strong> P314L</td>
<td>H × V × L</td>
<td>12 (0.18)</td>
<td>12 (0.07)</td>
<td>4.51</td>
<td>2.01 to 10.13</td>
</tr>
<tr>
<td><strong>CFH</strong> Y402H × <strong>C3</strong> R102G × <strong>C3</strong> P314L</td>
<td>H × G × L</td>
<td>7 (0.11)</td>
<td>10 (0.06)</td>
<td>2.49</td>
<td>1.05 to 5.91</td>
</tr>
<tr>
<td><strong>CFH</strong> V62I × <strong>C3</strong> R102G × <strong>C3</strong> P314L</td>
<td>V × G × L</td>
<td>16 (0.24)</td>
<td>22 (0.13)</td>
<td>2.06</td>
<td>1.09 to 3.89</td>
</tr>
</tbody>
</table>

**P** < 0.05 are significant; DDD, **n** = 66, controls, **n** = 165.
pared with controls carrying at least one copy of \( CFH \) H402 (\( CFH \) H402 fH serum levels, 2.51 ± 0.91 mg/ml; \( CFH \) Y402 fH serum levels, 2.17 ± 0.84 mg/ml; \( P > 0.05 \)) (Figure 2D). Although AP activity in controls homozygous for both \( CFH \) H402 and C3 G102 could not be studied due to the rarity of the \( CFH \) H402 plus C3 G102 homozygous genotype (approximately one per 230 persons), we did compare controls homozygous for C3 G102, C3 L314, or \( CFH \) H402 (risk alleles) to controls homozygous for C3 R102, C3 P314, or \( CFH \) Y402 and noted consistently greater AP activity associated with DDD risk alleles (\( P < 0.05 \)). This observation was also true when AP activity was compared between controls homozygous for both C3 G102 and C3 L314 versus controls homozygous for both C3 R102 and C3 P314 (\( P < 0.05 \)) (Table 7). These data confirm that AP activity is greater in controls with DDD risk alleles compared with controls without these alleles, supporting a genetic basis for differences in normal AP activity.

### DISCUSSION

The AP and TCC form a complex network of pathways with amplification loops and cascades. Dysregulation of this system underlies two rare renal diseases: aHUS and DDD. In aHUS, dysregulation occurs at the cell surface, and multiple mutations in complement genes and their functional impact have been characterized in affected patients.25–32 DDD is rarer than aHUS and has not been studied as thoroughly.13,14,21,33 It is caused by fluid-phase dysregulation of the C3 and C5 convertases that leads to accumulation of complement debris—C3b breakdown products and sMAC—in renal glomeruli.34

We identified novel missense sequence variants in four genes (\( C3 \), \( C3aR1 \), \( CR1 \), and \( ADAM19 \)) in DDD patients. Although we have not determined the effect of these variants on complement activity, the rarity of these changes and their presence only in DDD patients suggests that they may have functional significance. In testing gene-gene interactions, we also found that four SNPs in \( CFH \) and \( C3 \)—namely \( CFH \) p.Y402H, \( CFH \) p.V62I, C3 p.R102G, and C3 p.P314L—are associated with DDD and that the presence of two or more of these risk alleles increases the ORs of developing DDD (Table 6). The additive effect of these SNPs on DDD risk is consistent with the known interaction of fH and C3 and defines a predisposing at-risk complement haplotype or “complotype” in DDD patients.

The functional consequence of the DDD at-risk complotype is increased AP activity. Functional studies of the first
CFH SNP, Y402H, has shown that the H402 variant decreases binding to C-reactive protein and heparin and alters affinity for ocular membranes.35–37 Our data show that it is also associated with increased AP activity. The second CFH SNP, p.V62I, is in SCR1 of fH and contributes to a C3b binding site important for fluid-phase complement control through fH-mediated cofactor activity and fH/fB competition for C3b.38 It is interesting to note that despite finding that the protective allele CFH p.62 was more frequently found in controls than DDD patients, CFH p.V62I was not associated with the DDD phenotype in this study (chi-squared test of independence and Cochran-Armitage trend test). In an earlier study, in contrast, we found that it was (chi-squared test of independence), a discrepancy that may be due to the small number of cases and controls in both studies, which can cause inflated type I or II errors using a chi-squared test of independence.22 However, it is also possible that the main effect of CFH p.V62I is not independent but synergistic with another variant, consistent with our gene-gene interaction analysis, which found that CFH p.V62I and C3 p.P314L together are associated with DDD.

The associated C3 SNPs are in the macroglobulin (MG) domains of C3. C3 p.R102G is also known as the C3 F/S allele and lies in MG1 near the thioester domain (TED).39,40 Based on the three-dimensional structure of C3 using SNPs3D, it is on the protein surface. With arginine at this position, an electronegative interface is created on MG1 that can interact with the strong electropositive region on the exposed TED surface to stabilize C3b. In the absence of stabilizing electrostatic interactions, because glycine is not electronegative, TED binding to target surfaces could be affected.41,42

The second C3 SNP, p.P314L, is also known as the HAV4–1± allele and lies in MG3, a binding site for fB.43 SNPs3D places this SNP on the protein surface, and PolyPhen and SIFT predict the change to be probably damaging, with an effect on function. The C3 L314 allele also reacts with the monoclonal antibody, HAV4–1, consistent with a structural alteration that exposes or creates an epitope recognized by the antibody. Both C3 SNPs are associated with aHUS and systemic lupus erythematosus.27,44–46

Synergism between CFH p.V62I and C3 p.P314L may occur because the fH variant decreases binding to C3b, whereas the C3 variant increases binding to fB. These interactions would promote fB association with C3b to form C3 convertase while simultaneously decreasing fH affinity for and regulation of C3b. The predicted outcome would be a more active AP, which we demonstrated in functional assays of AP activity using genotyped control serum. However, it is also possible that the major effect of these interactions is to expose novel epitopes on C3 convertase that favor the development of C3Nefs. Consistent with this second possibility, Finn and Mathieson have shown an association between C3 G102 and the presence of C3Nefs in serum.47

In summary, we have shown that DDD patients segregate a complotype that is comprised of risk alleles in CFH and C3. This complotype is associated with higher AP activity, which is consistent with the interaction of these two proteins. The DDD complotype may predispose to disease development by facilitating generation of autoantibodies like C3Nefs. These data also suggest that deep sequencing of all complement genes in DDD patients is warranted to better define the complotype of this complex disease. Determining the complex genotype associated with DDD may provide insight into the care of affected patients by identifying complotypes associated with disease progression and clinical outcome.

### CONCISE METHODS

#### Mutation Screening and Analyses

Genomic DNA was extracted from blood samples of patients and controls using commercially available kits (PAXgene Blood DNA Kit, Qiagen, Valencia, California). Coding regions and intron–exon boundary junctions of C3 (NM_000064), CFH (NM_000186), CFHR5 (NM_030787), C3aR1 (NM_004054), CSaR1 (NM_001736), CR1 (NM_000651), and ADAM19 (NM_033274) were amplified and screened for sequence variants and polymorphisms by bidirectional sequencing.22 To identify the C4A deletion, two sets of primers were used that detected a nondeleted C4A (5.4 kb) band and/or a deleted C4A (5.2 kb) band.48 The quantitative alleles, H/L, for CR1 were determined by restriction digestion with HindIII. The H allele generates a 1.8-kb restriction digestion band, whereas the L allele generates 1.3- and 0.5-kb bands.49 Possible functional effects of variants were predicted using ConSeq, PolyPhen, and SIFT.50–52 Align GVGD,53,54 and SNPs3D.55

#### Alternative Pathway Functional Studies

Functional studies were completed on 102 anonymized blood and serum samples obtained from the Mississippi Valley Regional Blood Center in Davenport, Iowa. Serum was immediately frozen and kept at −80°C until use. DNA was extracted from leukocytes using standard techniques (PAXgene Blood DNA Kit, Qiagen, Valencia, California). Each sample was genotyped and screened for CFH and C3 SNPs associated with DDD (C3 p.R102G, p.R304, p.P314L, p.P518; CFH −331 T>C, p.V62I, p.Y402H, p.Q673).

#### Alternative Pathway Hemolytic Assay

The AP hemolytic assay was based on a standard hemolytic assay protocol.56 Absorbance (Abs) was measured at 415 nm using a microplate reader. Fractional hemolysis for each reading was computed as

![Table 7. AP activity associated with homozygosity for the listed genotypes](image-url)
percentage hemolysis \( (y) = \frac{(\text{Abs sample} - \text{Abs blank})}{(\text{Abs positive control} - \text{Abs blank})} \times 100 \). Each value was plotted against serum dilution \((x\text{-axis})\) for each individual to create an S-shaped curve, which was converted to a linear function of serum dilution \((x)\) versus \(y/(1 - y)\). The volume at which \(y/(1 - y) = 1\) was defined as the APH50, i.e., that volume of serum in which 50% of red blood cells hemolyze. This definition means that the lower the APH50, the more active the AP complement activity.\(^{57}\)

### Alternative Pathway Functional Immunoassay

The AP functional immunoassay (Wieslab COMPL AP330 kit; AL-PCO Immunoassays, Salem, New Hampshire) utilizes microtiter strips coated with specific activators of the AP. We measured absorbance at 405 nm and calculated %AP as \(\frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{negative control}}}{\text{Abs}_{\text{positive control}} - \text{Abs}_{\text{negative control}}} \times 100\). Lower %APs are associated with greater AP complement activity.\(^{58}\)

### Statistical Analyses

The chi-squared test of independence was used to detect differences in allele frequencies between cases and controls; \(P\)-values < 0.05 were considered significant.\(^{59}\) The Cochran-Armitage trend test was used to identify linear trends in genotypes and to compare these trends between cases and controls.\(^{60}\) Haplotype analyses for all genes performed using an EM algorithm in Haploview and SNPStats programs.\(^{61,62}\) For each polymorphism, ORs, 95% CI, and Hardy-Weinberg equilibrium were computed using SNPStats.\(^{61}\) Correction for multiple hypothesis testing was performed using Bonferroni correction and false discovery rate at \(P < 0.05\) considered significant.

### References


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**DISCLOSURES**

None.


