

# Causes of Alternative Pathway Dysregulation in Dense Deposit Disease

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## Summary

**Background and objectives** This study was designed to investigate the causes of alternative pathway dysregulation in a cohort of patients with dense deposit disease (DDD).

**Design, setting, participants, & measurements** Thirty-two patients with biopsy-proven DDD underwent screening for C3 nephritic factors (C3Nefs), factor H autoantibodies (FHAs), factor B autoantibodies (FBAs), and genetic variants in *CFH*. C3Nefs were detected by: ELISA, C3 convertase surface assay (C3CSA), C3CSA with properdin (C3CSAP), two-dimensional immunoelectrophoresis (2DIEP), and immunofixation electrophoresis (IFE). FHAs and FBAs were detected by ELISA, and *CFH* variants were identified by Sanger sequencing.

**Results** Twenty-five patients (78%) were positive for C3Nefs. Three C3Nef-positive patients were also positive for FBAs and one of these patients additionally carried two novel missense variants in *CFH*. Of the seven C3Nef-negative patients, one patient was positive for FHAs and two patients carried *CFH* variants that may be causally related to their DDD phenotype. C3CSAP was the most sensitive C3Nef-detection assay. C3CSAP and IFE are complementary because C3CSAP measures the stabilizing properties of C3Nefs, whereas IFE measures their expected consequence—breakdown of C3b.

**Conclusions** A test panel that includes C3CSAP, IFE, FHAs, FBAs, and genetic testing for *CFH* variants will identify a probable cause for alternative pathway dysregulation in approximately 90% of DDD patients. Dysregulation is most frequently due to C3Nefs, although some patients test positive for FHAs, FBAs, and *CFH* mutations. Defining the pathophysiology of DDD should facilitate the development of mechanism-directed therapies.

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## Introduction

Dense deposit disease (DDD; also known as membranoproliferative GN type 2 or MPGN2) is a rare renal disease characterized by electron-dense deposits that localize to the lamina densa of the glomerular basement membrane in a segmental, discontinuous, or diffuse pattern (1–3). The deposits lack substructure and appear as dark homogeneous smudges. Laser micro-dissection with mass spectrometry of glomeruli has identified complement components of both the alternative pathway (AP) and the terminal complement cascade in diseased glomeruli consistent with fluid-phase dysregulation of the C3 and C5 convertases (4).

The genetics of DDD is complex. Mutations have been identified in several complement genes, although DDD patients more frequently carry specific variants of several different genes that define a DDD complement haplotype or “complotype” (5–9). The DDD complotype is associated with increased AP activity even in control sera, suggesting that the development of DDD is favored when basal levels of complement activity are elevated (9).

The triggers of AP dysregulation in DDD are unknown, although 55% of adult DDD patients and 80% of pediatric DDD patients reportedly circulate autoantibodies to C3 convertase called C3 nephritic factors (C3Nefs) (10,11). First identified in 1969 by Spitzer *et al.* (12), C3Nefs were described as a substance in serum that increases AP activity from basal “tick-over” levels. Tick-over is the process by which hydrolysis of a reactive thioester in C3 generates small amounts of an initial C3 convertase called C3(H<sub>2</sub>O)Bb. This convertase generates C3bBb, which is quickly inactivated by protein regulators of complement activation (RCA).

C3Nefs compromise C3 convertase regulation by blocking RCA access and control, prolonging the *t*<sub>1/2</sub> of C3 convertase from a few seconds to 4 to 60 minutes (13–15). The consequence is massive C3 consumption and exceedingly low levels of serum C3, a hallmark of DDD (13,16,17). Vast amounts of C3b generate more C3 convertase and also C5 convertase, the convertase of the terminal complex cascade. C5 convertase produces soluble C5b-7, clusterin, and vitronectin complexes, which bind C8 and C9 to form inactive but soluble membrane attack complex (sMAC), clusterin,

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and vitronectin (18). C3 degradation products and sMAC deposit in the glomerular basement membrane as the dense deposits of DDD (4).

Although the presence of C3Nefs would seem to be pathogenic because they impair C3 convertase regulation, the relationship between C3Nefs and DDD is unclear (10,11,19–21). Not only has it been difficult to correlate C3Nef assays with clinical parameters of disease, but C3Nefs are also reportedly not unique to DDD. They have been detected in healthy individuals and in patients with partial lipodystrophy, meningococcal meningitis, and poststreptococcal acute GN (22–25). This diversity suggests that there may be many C3Nefs with different functional activity, a possibility that has not been widely studied because C3Nef detection assays are laborious and of limited availability. The purpose of this study was to investigate causes for AP dysregulation in a cohort of 32 DDD patients by completing a comprehensive panel of functional and genetic tests that included the following: five C3Nef detection assays, screens for factor H and factor B autoantibodies (FHAA, FBAA, respectively), and sequence analysis of *CFH*.

## Materials and Methods

### Patients and Controls

Thirty-two patients with DDD were enrolled in this study under guidelines approved by the institutional review board of the University of Iowa. DDD was diagnosed by renal biopsy, which showed characteristic immunostaining for C3 and the presence of intramembranous electron-dense deposits by electron microscopy (2,3). Whole blood was used as a source of genomic DNA and serum. Control sera were obtained from 87 de-identified blood donors with no history of renal disease.

### Reagents

Gelatin veronal buffer (GVB)-EDTA and GVB-EGTA-Mg<sup>2+</sup> were purchased from Boston Bioproducts Inc (Worcester, MA). (AP activation requires Mg<sup>2+</sup>, whereas classic and lectin pathway activation requires Ca<sup>2+</sup> and Mg<sup>2+</sup>. EGTA chelates Ca<sup>2+</sup> allowing AP activation when Mg<sup>2+</sup> is added; EDTA chelates Ca<sup>2+</sup> and Mg<sup>2+</sup>, preventing all complement activation.) Complement proteins and factor H (FH)-depleted serum were purchased from Complement Technology Inc (Tyler, TX). Factor B (FB)-partially inactivated/FH-depleted serum was generated by incubating FH-depleted serum in a 50°C water bath for 5 minutes and used as source of C3b deposition onto sheep erythrocytes. Testing incubation for optimized FB inactivation was done at 5 and 10 minutes, which yielded 90% and 50% hemolysis, respectively, after adding FB and factor D (FD) back and immediately incubating in rat EDTA serum. Sheep erythrocytes in Alsever's buffer were purchased from Colorado Serum Company (Denver, CO) and washed twice with GVB-EGTA-Mg<sup>2+</sup> buffer. Rat serum was purchased from Pel-Freez Biologicals (Rogers, AR).

### IgG Purification

Patient IgG was purified using the Melon Gel IgG Purification Kit (Thermo Scientific, Rockford, IL) and adjusted to 1 mg/ml.

### C3Nef Assays

**ELISA.** Purified human C3b (5 µg/ml) was coated on a 96-well microtiter plate pretreated with 0.25% glutaraldehyde in PBS and allowed to sit overnight at 4°C (26). After washing three times with 1× PBS, free reactive sites were blocked with 1% BSA in 1× PBS for 1 hour at 37°C. FB (5 µg/ml) and FD (0.25 µg/ml) in GVB + 2mM NiCl<sub>2</sub> were added to the washed wells for 30 minutes at 37°C, followed by three washes with GVB-EDTA. A C3b-only well was used as a control.

Patient serum (1:100 dilution) was added to a C3 convertase well and a C3b-only well for a 25-minute 37°C incubation. Plates were washed with PBST (1× PBS with 0.5% Triton-X) and incubated for 30 minutes at room temperature (RT) with horseradish peroxidase-labeled goat anti-human IgG specific for the γ chain (Sigma-Aldrich Corp, St. Louis, MO). Enzymatic activity was measured using TMB (3,3',5,5'-tetramethylbenzidine) and OD was read at λ450. Results were expressed as the ratio of OD<sub>C3bBB</sub>/OD<sub>C3b</sub>. A positive result was defined as OD<sub>C3bBB</sub>/OD<sub>C3b</sub> >1.28, which exceeds the average OD<sub>ratio</sub> + 2 × SD<sub>ratio</sub> (average OD<sub>C3bBB</sub>/OD<sub>C3b</sub>=0.95; SD=0.16) for 87 controls.

**C3 Convertase Stabilizing Assay.** The C3 convertase stabilizing assay (C3CSA) is a novel assay that measures the ability of C3Nefs to stabilize surface-bound C3 convertase on sheep erythrocytes. Sheep erythrocytes were coated with C3b by adding 45 µl of FB-partially inactivated/FH-depleted serum to 1 ml of washed sheep erythrocytes (1×10<sup>9</sup>/ml), followed by a 30-minute incubation at 37°C. Cells were then washed three times with GVB-EGTA-Mg<sup>2+</sup> buffer and resuspended in 1 ml GVB-EGTA-Mg<sup>2+</sup> buffer. The use of FB-partially inactivated/FH-depleted serum allowed maximum deposition of C3b on sheep erythrocytes without generating membrane attack complex (MAC), because the wash steps remove unattached complement proteins preventing formation of MAC until rat EDTA serum is added later.

To form C3 convertase, FB (5 µl of 1 mg/ml) and FD (5 µl of 0.1 mg/ml) were added to 0.5 ml of C3b-coated sheep erythrocytes (1×10<sup>9</sup>/ml) and incubated at 30°C for 5 minutes. After incubation, 0.5 ml ice-cooled GVB-EDTA buffer was added; cells were used immediately.

To perform the assay, 100 µl of sheep erythrocytes (5×10<sup>8</sup>/ml) prepared as described above and 25 µl of patient-purified IgG were added to 100 µl GVB-EDTA buffer for 5 minutes on ice. The C3 convertase was allowed to decay at 30°C for 20 and 60 minutes. At each time point, 50 µl was removed and mixed with an equal amount of rat EDTA serum (1:9 diluted in GVB-EDTA buffer) in a 96-well microplate and incubated at 37°C for 1 hour. We added 150 µl of ice-cooled GVB-EDTA buffer after the incubation period and cells were centrifuged at 1000 × g for 15 minutes. The supernatant was transferred to a clean well to measure OD at λ415. C3CSA activity was reported as a function of hemolysis at 20 minutes.

**C3 Convertase Stabilizing Assay with Properdin.** The C3 convertase stabilizing assay with properdin (C3CSAP) assay, which is also novel, is similar to the C3CSA, although properdin is included in the protocol to generate a properdin-containing C3 convertase. In brief, the C3CSA protocol was repeated adding properdin (5 µl, 1 mg/ml) when forming the convertase. The remaining steps to

prepare the sheep erythrocytes were identical. To perform the assay, the convertase was allowed to decay after adding patient-purified IgG and activity was measured at 30 and 80 minutes. Results were reported as a function of hemolysis at 30 minutes.

**Two-Dimensional Immunoelectrophoresis.** Two-dimensional immunoelectrophoresis (2DIEP) was based on the protocol described by Davies and Norsworthy (27). Briefly, 15  $\mu$ l of normal human serum was mixed with 3  $\mu$ l of patient serum in PBS containing 10 mM EGTA-Mg<sup>2+</sup> (AP activation possible) or 10 mM EDTA (complement activation not possible) as a parallel control. After a 45-minute incubation at 37°C, migrations were performed in Seakem ME agarose (Lonza Group Ltd, Basel, Switzerland). Antihuman C3 antibody (MP Biomedical, Fisher Scientific Inc, Pittsburgh, PA) was added in the second gel run and the gel was then stained with Coomassie Blue G250 (Bio-Rad Laboratories, Hercules, CA). C3 convertase activity was quantified as the C3 fragment/C3 ratio (ImageQuant; GE Healthcare, Piscataway, NJ).

**Immunofixation Electrophoresis.** C3 degradation products were detected by immunofixation electrophoresis (IFE) (28). Ten microliters of normal human serum were mixed with 10  $\mu$ l of patient serum in PBS containing 10 mM EGTA-Mg<sup>2+</sup> or 10 mM EDTA (as a control) and incubated for 45 minutes at 37°C. C3 or C3 degradation products were resolved by electrophoresis on precasted agarose Titan gels (Helena Laboratories, Beaumont, TX). After electrophoresis, antihuman C3 antibody (MP Biomedical, Fisher Scientific Inc, Pittsburgh, PA) was added and gels were stained with acid blue and de-stained. Mobility changes of C3 after activation were quantitated as the C3 fragment/C3 ratio using AlphaEaseFC software (Cell Biosciences, Santa Clara, CA).

#### FHAA and FBAA

FHAA and FBAA were detected by ELISA. In brief, either FH or FB (both at 5  $\mu$ g/ml) (Complement Technology Inc) was coated on a 96-well microtiter plate and allowed to sit overnight at 4°C. After washing three times with 1 $\times$  PBST, free reactive sites were blocked with Ultra-block (AbD Serotec, Raleigh, NC) for 1 hour at RT. Patient serum (1:50 dilution) was added for 1 hour at RT. Plates were washed and incubated for 1 hour at RT with horseradish peroxidase-labeled goat anti-human IgG specific for the  $\gamma$  chain. After a final washing, enzymatic activity was measured using TMB, and OD was read at  $\lambda$ 450. A positive result was an OD >0.15 for FHAA and >0.05 for FBAA, which exceed the mean + 2 $\times$  SDs for 87 controls.

#### Additional Measurements and Screening

**sMAC.** sMAC was measured using the MicroVue sC5b-9 Plus EIA Assay (Quidel, San Diego, CA).

**Alternative Pathway Functional Activity.** Alternative pathway functional activity (APFA) was measured using the Wieslab Complement AP Assay (Wieslab AB, Lund, Sweden).

**DNA Extraction, Mutation Screening, and Analyses.** Genomic DNA was extracted from blood samples using standard protocols (PAXgene Blood DNA Kit; Qiagen, Valencia, CA). Coding regions and intron-exon boundary

junctions of *CFH* (NM\_000186) were amplified and screened using bi-directional sequencing as described (7).

#### Statistical Analyses

Renal survival time was estimated using the nonparametric Kaplan–Meier method (29).

## Results

### Patient Data

Patients ranged in age from 8 to 49 years (median, 19 years) and carried the diagnosis of DDD for 1 to 17 years (median, 3 years). Eleven patients had ESRD and were dialysis dependent. The estimated renal  $t_{1/2}$  was approximately 12 years (95% confidence interval, 6– $\infty$ ). (Table 1, Figure 1).

### C3Nef Assays

Twenty-five patients were positive for C3Nefs by at least one assay (Figure 2).

**ELISA.** C3Nefs were detected by ELISA in 17 of 32 patients (53%) and in 17 of 25 patients (68%) positive for C3Nefs by at least one C3Nef assay. IgG binding to C3 convertase was detected in 7 of 87 control sera samples (8%); however, these samples also bound C3b alone and had OD<sub>C3bBb</sub>/OD<sub>C3b</sub> ratios <1.28.

**C3CSA.** C3CSA results were positive in 22 of 32 patients (69%) and in 22 of 25 patients (88%) who were positive for at least one C3Nef assay. C3CSA activity reported as a function of hemolysis at 20 minutes was highly variable although intra- and inter-assay coefficients of variation were minimal (approximately 2% intra-assay variation; approximately 5% inter-assay variation) (Figure 3A). All patients who were positive by ELISA were positive by C3CSA (Figure 2).

**C3CSAP.** C3CSAP was the most sensitive assay for C3Nef detection and was positive in 25 of 32 patients (78%). In patients 18, 22, and 23, for example, there was background lysis by C3CSA (normal) but lysis was 62%, 79%, 73%, respectively, by C3CSAP, whereas in patient 24, lysis increased from 29% by C3CSA to 63% by C3CSAP (Figure 3B). Intra- and inter-assay variation was comparable with that for C3CSA.

**2DIEP.** 2DIEP detected C3b breakdown products in 22 of 32 DDD patients (69%) (Figure 2).

**IFE.** IFE performed identically to 2DIEP, detecting C3b breakdown products in the same 22 patients (69%) (Figure 2).

### FHAA and FBAA

FHAA and FBAA were detected in one (3%) and three (9%) patients, respectively. The FHAA-positive patient (patient 28) was negative for C3Nefs, whereas the three FBAA-positive patients (patients 7, 8, and 22) were positive for C3Nefs. No patients negative for C3Nefs were positive for FBAA.

### Additional Measurements and Screening

**sMAC.** sMAC was elevated in 3 of 32 (9%) patients.

**APFA.** APFA was normal in all control sera and decreased in 26 of 32 (81%) patients.

**FH Mutation Screening.** Twenty-six of 32 patients (81%) carried at least one copy of the FH His402 polymorphism;

Table 1. Patient data <sup>a</sup>																
Patient No.	Age (yr)	Sex	Age at Dx (yr)	Years with Dx	ELISA (Titer)	C3CSA (%)	C3CSAP (%)	2DIEP	IFE	sMAC (mg/L)	CKD Stage	Dialysis	CFH Genotype	APFA	FHAA	FBAA
1	18	M	15	3	1:100	58	48	0.33	0.50	ND <sup>b</sup>	2	No	His402	57.4	—	—
2	16	M	14	3	1:200	76	64	0.35	0.92	0.27	3	No	His402	5.4	—	—
3	20	M	19	1	1:400	72	53	2.15	1.82	0.13	5	Yes	heterozygote	0.7	—	—
4	13	M	10	3	1:100	36	36	0.28	0.43	ND	1	No	homozygote	30.8	—	—
5	27	F	11	17	1:100	107	89	1.53	1.21	0.15	5	Yes	heterozygote	7.2	—	—
6	18	M	12	7	1:400	48	44	0.32	0.51	ND	2	No	heterozygote	40.4	—	—
7	11	F	7	5	1:200	43	62	0.18	0.35	ND	5	Yes	homozygote	0.1	—	+
8	8	M	7	1	1:200	31	24	0.55	0.58	ND	2	No	homozygote	71.6	—	+
9	27	F	13	15	1:200	36	29	0.28	0.38	0.08	5	Yes	heterozygote	39.1	—	—
10	36	M	35	1	1:400	66	41	0.39	0.51	0.15	3	No	homozygote	64.1	—	—
11	13	F	8	6	1:400	83	68	0.45	1.02	0.32	5	Yes	homozygote	12.4	—	—
12	15	F	14	1	1:400	108	64	0.26	0.85	0.14	4	Yes	homozygote	13.5	—	—
13	8	F	8	1	1:800	103	93	2.14	1.19	0.2	5	Yes	heterozygote	2.9	—	—
14	19	M	18	1	1:200	99	69	0.40	0.94	0.16	3	No	heterozygote	84.4	—	—
15	9	M	8	1	1:800	63	48	0.22	0.31	0.3	3	No	heterozygote	20	—	—
16	25	M	24	1	1:400	42	25	0.18	0.45	0.22	3	Yes	heterozygote	39.5	—	—
17	20	M	8	12	1:100	66	89	0.11	0.28	0.45	5	Yes	heterozygote	0.1	—	—
18	21	M	18	3	—	17	62	0.18	0.36	0.19	5	Yes	heterozygote	45.8	—	—
19	16	M	11	7	—	83	67	0.21	0.42	ND	1	No	homozygote	0.1	—	—
20	31	M	29	2	—	28	26	0.19	0.33	ND	1	No	heterozygote	83.4	—	—

**Table 1. (Continued)**

Patient No.	Age (yr)	Sex	Age at Dx (yr)	Years with Dx	ELISA (Titer)	C3CSA (%)	C3CSAP (%)	2DIEP	IFE	sMAC (mg/L)	CKD Stage	Dialysis	CFH Genotype	APFA	FHAA	FBAA
21	28	M			—	31	26	0.08	0.29	0.07	5	Yes	His402	71.9	—	—
22	14	M	13	1	—	19	80	0.24	0.38	3	1	No	heterozygote Tyr402 homozygote c.2509G>A, p. Val837Ile; c.3435G>C, p. Glu1145Asp	0.2	—	+
23	12	F	6	6	—	17	73	0.06	0.28	ND	5	Yes	His402	4.2	—	—
24	22	M	9	13	—	29	63	0.23	0.39	0.71	5	No	homozygote Tyr402	7.4	—	—
25	21	F	14	7	—	38	28	0.20	0.37	0.03	1	No	homozygote His402	114.7	—	—
26	20	M	16	5	—	15	18	0.13	0.28	ND	5	Yes	heterozygote His402	39.3	—	—
27	21	M	20	2	—	17	19	0.14	0.26	1.08	3	No	heterozygote; c.1548T>A, p. Asn516Lys His402	4.3	—	—
28	42	F	28	14	—	18	15	0.13	0.17	0.16	5	Yes	homozygote; c.2867C>T, p. Thr956Met His402	36.0	+	—
29	14	M	12	2	—	14	19	0.09	0.16	ND	4	No	heterozygote Tyr402	0.1	—	—
30	14	M	11	3	—	14	19	0.10	0.24	ND	1	No	homozygote Tyr402	13.1	—	—
31	49	F	44	5	—	16	18	0.05	0.19	ND	1	No	homozygote His402	28.1	—	—
32	18	M	17	2	—	17	17	0.08	0.25	0.31	3	No	homozygote Tyr402	54.8	—	—

<sup>a</sup>C3CSA, C3 convertase stabilizing assay: nl <20% at 20 minutes; C3CSAP, C3CSA with properdin: nl <20% at 30 minutes; 2DIEP, 2-dimensional immunoelectrophoresis: nl <0.15; IFE, immunofixation electrophoresis: nl <0.30; sMAC, soluble membrane attack complex or C5b-9: nl <0.30 mg/L; APFA, alternative pathway functional assay: nl, 65–130.  
<sup>b</sup>ND, not done because plasma sample not available.

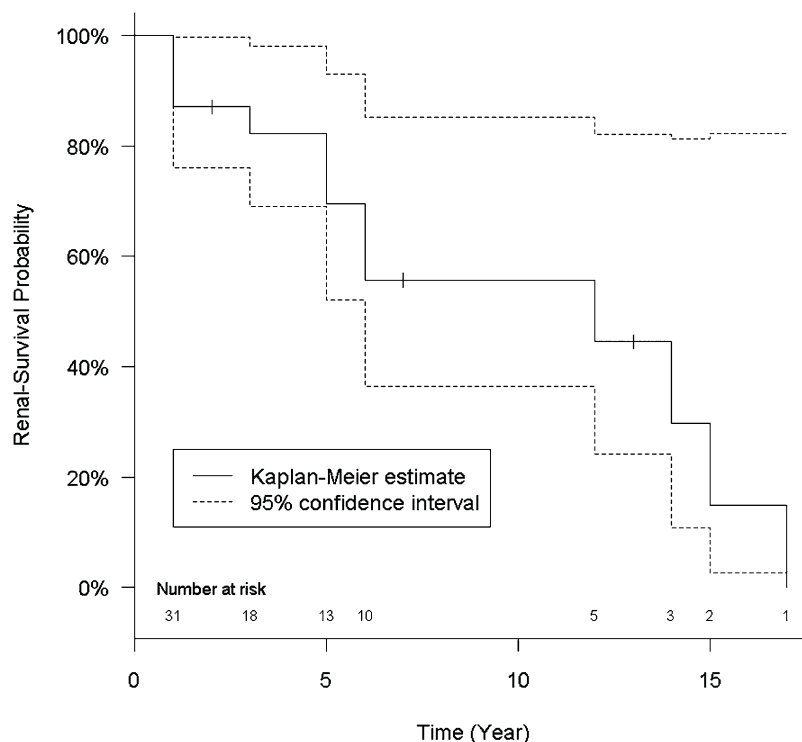


Figure 1. | The renal  $t_{1/2}$  as estimated by the nonparametric Kaplan–Meier method was approximately 12 years (95% confidence interval, 6– $\infty$ ).

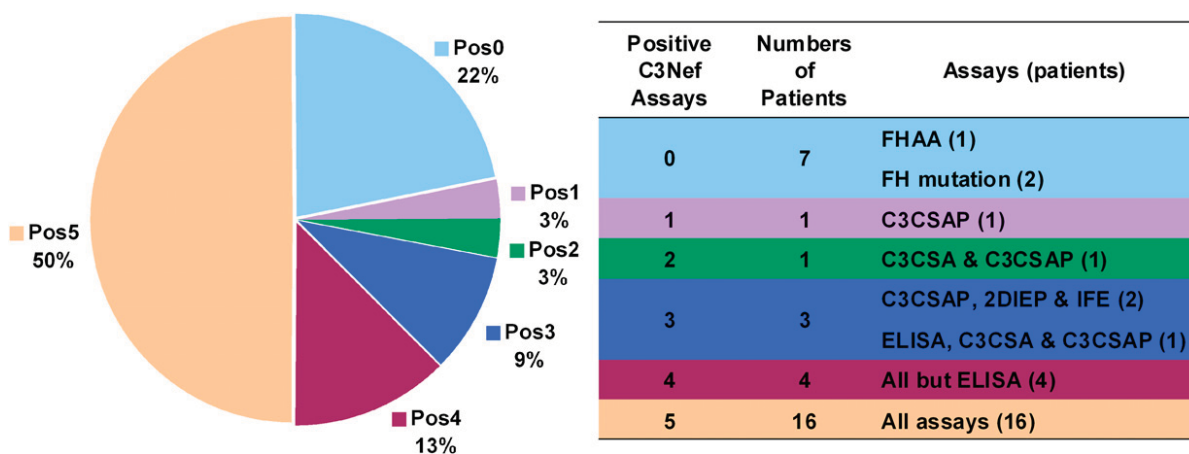
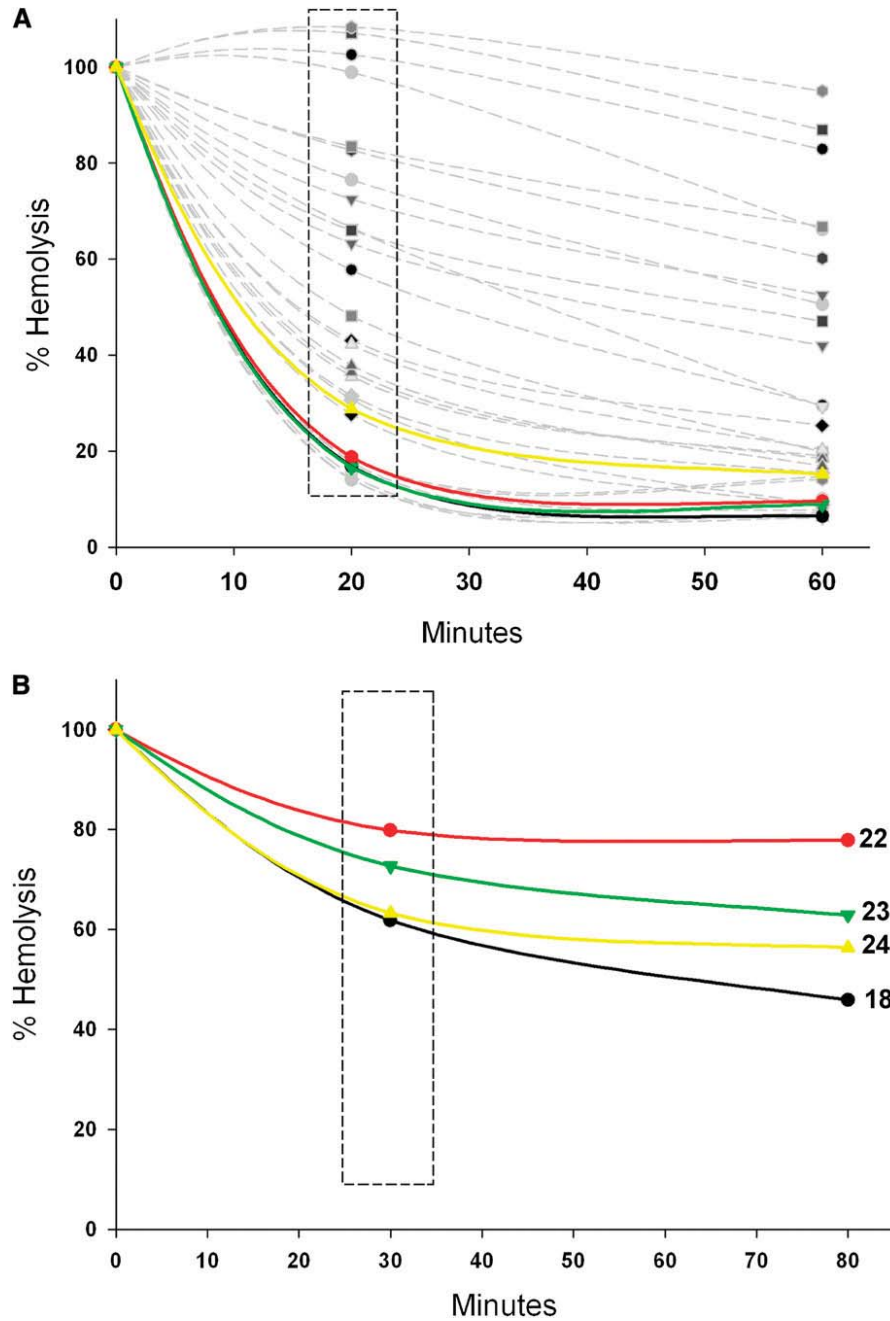


Figure 2. | **Alternative pathway dysregulation in 32 patients with dense deposit disease.** C3Nefs were detected in 25 patients and in the 7 C3Nef-negative patients, 1 had FHAA and 2 had *CFH* mutations. Four patients were negative for all investigations completed in this study (see patient details, Table 1). Of the C3Nef assays, ELISA was the least sensitive (maximum sensitivity only 68%), whereas the best performing assay was C3CSAP. C3Nef, C3 nephritic factor; FHAA, factor H autoantibody; C3CSAP, C3 convertase surface assay with properdin; C3CSA, C3 convertase stabilizing assay; FH, factor H; 2DIEP, two-dimensional immunoelectrophoresis; IFE, immunofixation electrophoresis.

three patients (9%) also carried *CFH* mutations. Patient 22 carried two novel missense variants, one in short consensus repeat (SCR) 14, c.2509G>A p.Val837Ile, and the second in SCR19, c.3435G>C p.Glu11145Asp; patient 26 carried a novel missense variant, c.1548T>A p.Asn516Lys, in SCR9; and patient 27 carried a missense variant that has been reported in patients with atypical hemolytic uremic syndrome (aHUS), c.2867C>T p.Thr956Met, in SCR16 (30,31).

## Discussion

DDD is caused by uncontrolled fluid-phase activity of the AP secondary to dysregulation of the AP C3 convertase, C3bBb (32). In this study, we found that the number of years with disease correlated with CKD stage. These findings are similar to those reported by other investigators (2,33,34). By completing a comprehensive panel of functional and genetic tests that included assays for C3Nefs, FHAA and FBAA, and mutation screening *CFH*, we were



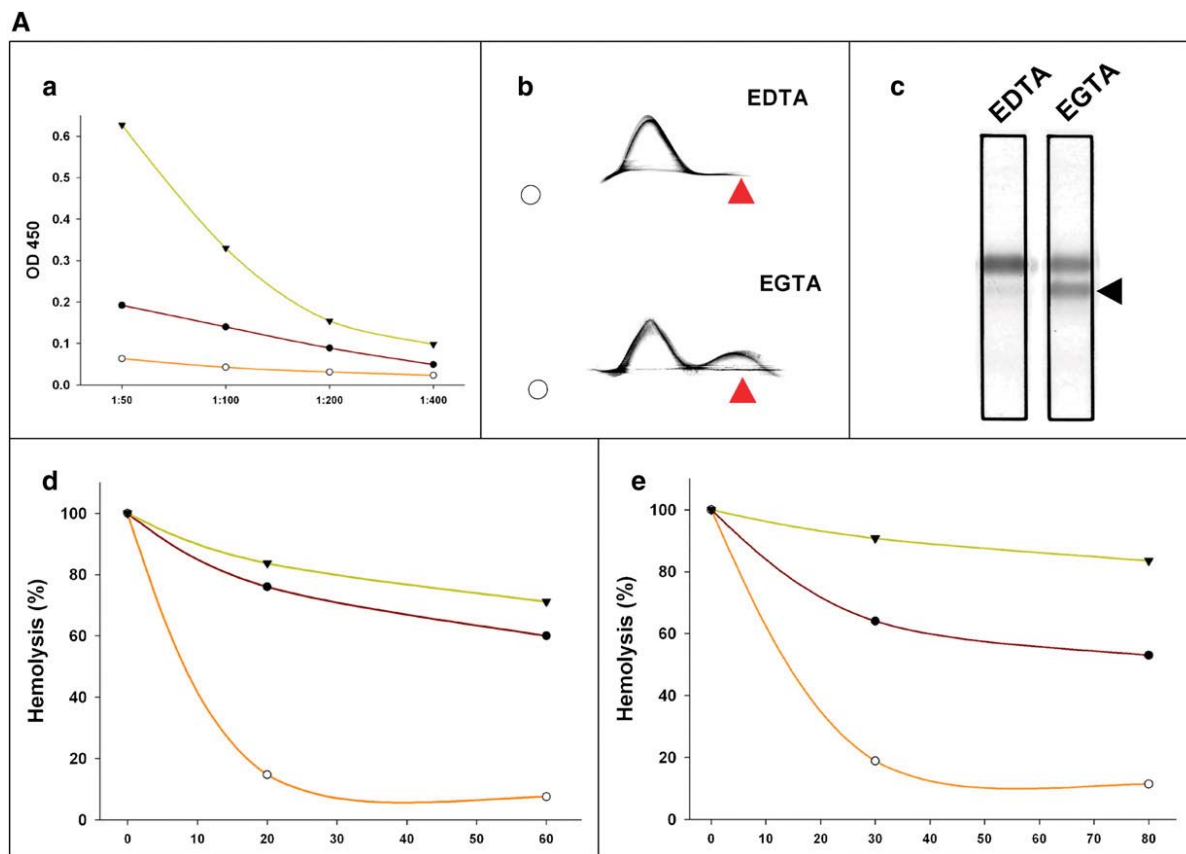
**Figure 3. | C3CSA and C3CSAP.** (A) C3CSA using purified IgGs from 32 patients with dense deposit disease showed that the stabilizing abilities of IgG C3Nefs varied considerably among patients. (B) C3CSAP was more sensitive than C3CSA in detecting C3Nefs at any time during the course of disease. In four patients with dense deposit disease, for example, C3CSAP was strongly positive, whereas C3CSA was either weakly positive (29%; patient 24, yellow) or negative (patient 18, black; patient 22, red; and patient 23, green). C3CSA, C3 convertase surface assay; C3CSAP, C3 convertase surface assay with properdin; C3Nef, C3 nephritic factor; FHAA, factor H autoantibody.

able to identify a probable etiology for C3 convertase dysregulation in 28 patients (87.5%).

Of the C3Nef assays, the simplest was the ELISA, a well described test that measures the amount of IgG in patient serum that binds to C3 convertase built *in vitro* in the presence of Ni<sup>2+</sup> (26). Ni<sup>2+</sup> is used in place of Mg<sup>2+</sup> to generate a C3 convertase with a longer *t*<sub>1/2</sub>. Our data suggest that this assay has a high false-negative rate, perhaps because the conformation of C3 convertases formed artificially on plastic

surfaces with Ni<sup>2+</sup> differs from their conformation on cell membranes. As a consequence, C3Nef binding may be weak and unable to survive repeated washings.

The second and third assays, C3CSA and C3CSAP, are novel and measure the affinity of patient-purified IgG to stabilize C3 convertase on sheep erythrocytes. The use of the Melon exclusion gel made purification of patient IgG simple and straightforward, and C3CSA and C3CSAP are simpler and easier to perform, in contrast to the assay



**Figure 4. | C3Nef test battery.** (A) Patient 2 is a 16-year-old male with stage 3 CKD in whom C3Nefs were detectable using all five assays: (a) ELISA, (b) 2DIEP, (c) IFE, (d) C3CSA, and (e) C3CSAP. Consistent with this finding, both C3 and fluid-phase AP activity were low (C3=5 mg/dl; normal range, 86–184 mg/dl; AP activity, 5.4%; normal range, 65%–130%). sMAC was normal. (B) Patient 23 is a dialysis-dependent 12-year-old female in whom only C3CSAP was positive. Her C3 was undetectable and fluid-phase AP activity was 4.2%. (b) Red arrowhead, C3 breakdown product peak; (c) black arrowhead, C3 breakdown product line; (d and e) line colors: test sample, red; negative control, orange; positive control, green. EGTA chelates  $\text{Ca}^{2+}$  allowing AP activation when  $\text{Mg}^{2+}$  is added. EDTA chelates  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , preventing all complement activation. C3Nef, C3 nephritic factor; 2DIEP, two-dimensional immunoelectrophoresis; IFE, immunofixation electrophoresis; C3CSA, C3 convertase surface assay; C3CSAP, C3 convertase surface assay with properdin; AP, alternative pathway; sMAC, soluble membrane attack complex.

described in 1990 by Ohi *et al.*, which was designed to build C3b on sheep erythrocytes through C4b2a (35).

The two final assays, 2DIEP and IFE, measure C3 breakdown products to infer the presence of C3Nefs. Although these two assays performed identically in our cohort, 2DIEP is reportedly insensitive to C3Nefs with low activity (27,36). Both assays lack specificity because there are other types of C3-splitting factors, including heat-labile C3 activating factors found in poststreptococcal GN (37–39). We prefer IFE because it is easier and less labor intensive than 2DIEP.

Of these five assays, C3CSAP was the most sensitive for C3Nef detection at any stage of disease (Figure 4). C3CSAP and IFE can be considered complementary because C3CSAP measures the stabilizing properties of C3Nefs, whereas IFE measures their expected consequence—change of motility of C3 after activation in serum. These two tests are also highly specific because we failed to identify C3Nefs using either of these assays in our control population.

We did find 8% of controls positive by ELISA, but positivity was for C3b and not for C3bBb. We therefore reviewed the three reports describing C3Nef-positivity in normal individuals (22,40,41). In the Tedesco *et al.* report

(41), the patient had no known infection and C3 positivity was inferred on the basis of a positive IFE result. However, in the reports by Gewurz *et al.* (22) and Karstop *et al.* (40), the patients had disseminated gonococcal infection and tonsillitis, respectively, which may have been the basis for C3Nef positivity. In support of this possibility, Rasmussen *et al.* (24) screened 132 patients with meningococcal disease and found one C3Nef-positive assay, and Fremeaux-Bacchi *et al.* (25) found C3Nefs transiently in three patients with poststreptococcal acute GN.

Only one patient in our study was positive for FHAAs. These antibodies have been reported in other DDD patients and in aHUS (42,43). The different phenotypes reflect antibody specificity for either the amino- or carboxy-terminal SCRs of FH, respectively. There has also been a single report describing a FBAA-positive DDD patient (44). In both that patient and the three FBAA-positive DDD patients in our study, the antibody also showed affinity for C3 convertase but not for other individual complement proteins.

Three patients carried mutations in *CFH*. Although functional studies of the mutant FH proteins were not done, the novel p.Asn516Lys variant in patient 26 lies in SCR9, a



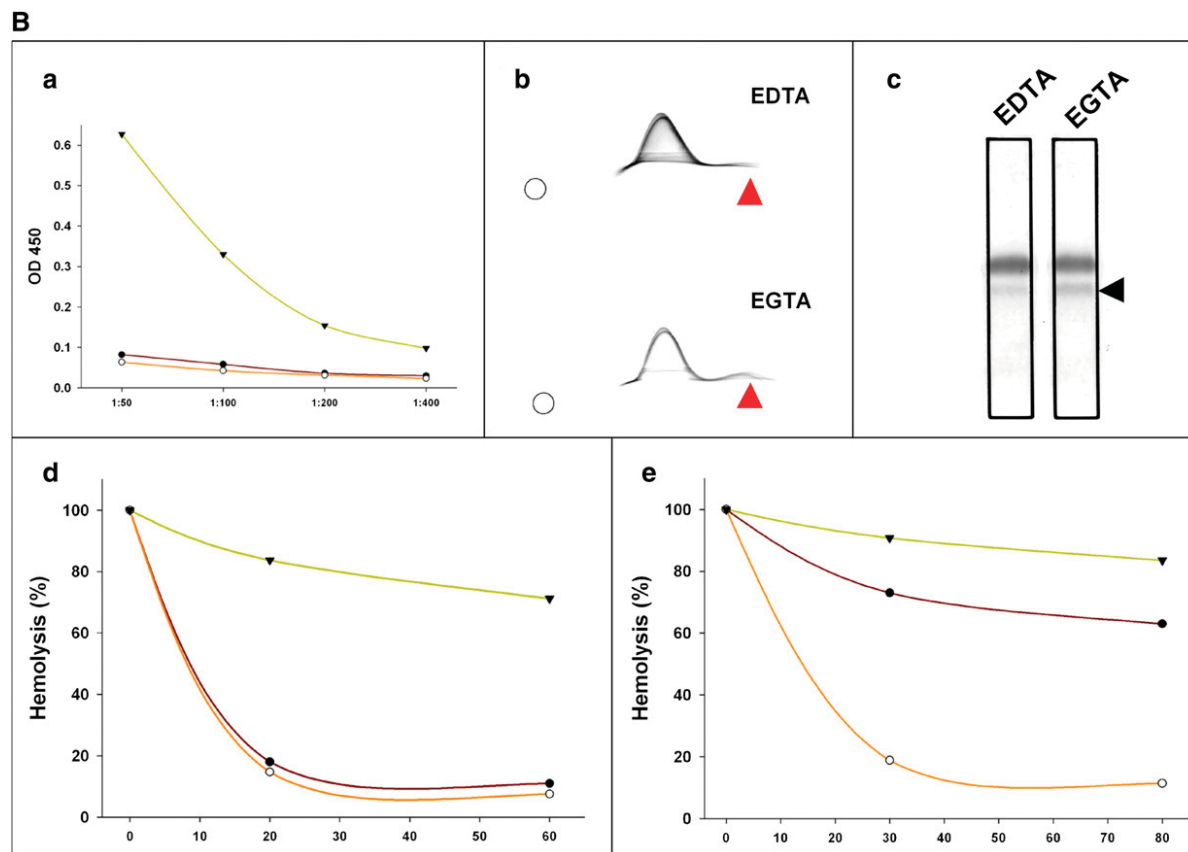


Figure 4. | Continued

known C3b binding site, suggesting that this change from a polar uncharged amino acid to a positively charged amino acid could alter FH binding affinity for C3b (5). The two novel variants in patient 22 were in SCR14 (p.Val837Ile) and SCR19 (p.Glu1145Asp), whereas the previously reported p.Thr956Met variant in patient 27 was found in a patient with aHUS (30,31). The phenotypic difference may reflect modifying genetic factors as variants in several other complement genes that have been reported in both DDD and aHUS, consistent with data showing that these diseases are genetically complex (9,31). In the four patients in whom we could not identify a cause for complement dysregulation, a more comprehensive evaluation should be completed (6,9).

Disease-specific therapies for DDD are not currently available; however, treatment options that should be considered would restore C3 convertase control, impair C3 convertase activity, or remove C3 breakdown products from the circulation (3). Eculizumab, a mAb that blocks C5 activation, has been approved for patients with paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. Although the benefit of targeting MAC in DDD patients is difficult to assess, our data show that only three patients had elevation of sMAC suggesting that the response of DDD patients to eculizumab might be marginal. Clinical trials evaluating its effect on DDD should be designed to follow sMAC as a biomarker of disease activity and eculizumab response.

We were able to identify a possible cause for AP dysregulation in approximately 90% of DDD patients in this study. This degree of analysis in this size cohort represents the

largest reported to date for DDD. On the basis of these findings, we recommend that all DDD patients undergo a multi-tiered evaluation for C3Nefs, FHAAs, FBAAAs, and genetic variants in *CFH*. The additional data from these diagnostic tests in a larger cohort would validate the clinical value of these tests and may offer important insights into treatment as anticomplement therapies are tested in these patients. To that end, standard operating procedures are available and can be requested for all of the assays we have reported.

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**Disclosures**

None.

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