

# Sensitive and specific assays for C3 nephritic factors clarify mechanisms underlying complement dysregulation

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**C3 nephritic factors are autoantibodies that prolong the half-life or prevent regulation of the alternative pathway C3 convertase, resulting in uncontrolled complement activation. They are strongly associated with renal disease but their role in pathogenesis remains controversial. Here we optimized and compared a panel of assays to identify and interrogate nephritic factor activities. Of 101 patients with histologic or clinically evident disease, 48 were positive in some or all assays. In the presence of properdin, binding of autoantibody was detected in 39 samples and convertase stabilization was detected in 36. Forty-two of 48 nephritic factors tested prevented convertase decay by factor H, and most of these by decay accelerating factor (28) and complement receptor 1 (34). Representative properdin-independent nephritic factors had no effect on C5 cleavage and terminal pathway activity, while properdin-dependent nephritic factors enhanced activity. Biacore analysis of four purified IgG samples confirmed resistance to decay and showed that properdin-independent nephritic factors increased convertase half-life over 50-fold, whereas properdin-dependent nephritic factors increased the half-life 10- to 20-fold and also increased activity of the C3 convertase up to 10-fold. Thus, our study provides a rational approach to detect and characterize nephritic factors in patients.**

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Complement is a key component of innate immunity.<sup>1</sup> The complement alternative pathway (AP) is critical for amplification; the key amplifying enzyme is the AP C3 convertase, C3bBb. C3b, surface-bound or in fluid phase, binds factor B (fB) in a Mg<sup>2+</sup>-dependent manner and presents it for cleavage by factor D (fD). The enzyme, Bb, remains attached to C3b and cleaves more C3 to C3b, driving amplification. C3bBb is naturally labile, dissociating with a half-life of 90 s at 37 °C; binding of properdin extends half-life 10-fold.<sup>2</sup> Newly formed C3b binds indiscriminately pathogens and host cells; damage to self is restricted by complement regulators in the fluid phase (factor H; fH) and on membranes (decay accelerating factor, DAF; membrane cofactor protein; CR1, complement receptor 1) that accelerate decay of C3bBb (fH, DAF, CR1) or are cofactors for inactivation of C3b by factor I (fI, membrane cofactor protein, CR1).<sup>3,4</sup>

Uncontrolled AP activation following overwhelming infection or polytrauma contributes to the systemic inflammatory response syndrome.<sup>5</sup> AP control is also compromised by complement protein mutations or by autoantibodies that stabilize the convertase.<sup>6–8</sup> Autoantibodies targeting the AP C3 convertase, C3 nephritic factors (C3NeF), are associated with renal disease, present in 50% of patients with membranoproliferative glomerulonephritis Types I and III, and 80% with dense deposit disease (DDD; previously named membranoproliferative glomerulonephritis Type II).<sup>9,10</sup> C3NeF are also associated with partial lipodystrophy<sup>11</sup> and, rarely, poststreptococcal glomerulonephritis, systemic lupus erythematosus, and meningococcal meningitis.<sup>11–13</sup> C3NeF are occasional findings in healthy individuals, provoking the suggestion that they are part of the normal immune repertoire and causing some to question whether C3NeF are causal for the associated pathologies or merely markers of disease.<sup>14–17</sup>

Numerous studies have explored mechanisms of action of C3NeF; all bind C3bBb while some also bind C3b or

Bb alone.<sup>18</sup> C3NeF stabilize the convertase, markedly increasing half-life,<sup>19</sup> and inhibit accelerated decay mediated by fH, DAF, and CR1, although reported efficiency of inhibition differed between studies.<sup>20-22</sup> Some reported a requirement for properdin for C3NeF stabilization of C3bBb, separating C3NeF into properdin-dependent and -independent groups with different effects *in vivo*.<sup>23</sup>

Despite strong disease association and effects on C3 convertase stability, the pathogenic significance of C3NeF is uncertain because C3NeF activity correlates poorly with C3 consumption and disease progression, and C3NeF are found in healthy individuals.<sup>15</sup> There is further uncertainty regarding whether C3NeF are primary causes of disease or secondary consequences and modifiers of the disease process. These uncertainties are exacerbated by lack of robust, reproducible assays. Few routine laboratories measure C3NeF, and methods are variable, technically complex, and lack standardization and appropriate quality control. These problems are exacerbated because C3NeF are heterogeneous between and even within individuals, and some assays may detect only subsets of C3NeF. Some C3NeF additionally stabilize the C5 convertase, causing C5 consumption and terminal pathway activation, sometimes without significantly activating C3,<sup>23</sup> these are easily missed using C3 cleavage assays;<sup>24</sup> these C3NeF may require properdin in the convertase.<sup>23</sup>

We set out to define a suite of assays that enabled reproducible detection of all C3NeF in patient samples. For a subset of the identified C3NeF, we performed in-depth analyses of target binding, convertase stabilization, dysregulation, and escape from decay acceleration. The assays described are optimized to permit reproduction in other laboratories. Adoption into routine use will improve the capacity to diagnose and monitor patients with C3NeF-associated diseases.

## RESULTS

### Detection of C3NeF in patient samples

Serum samples were available from 101 patients presenting with histological and/or clinical evidence of DDD (>80%), membranoproliferative glomerulonephritis I/III, or, rarely, other renal pathologies, and proven AP activation (low C3, fB), and 25 healthy controls (Table 1). Samples were screened for C3NeF using the five assays described in Methods: C3 convertase stabilization enzyme-linked immunosorbent assay (ELISA; COS), C3 convertase with added properdin ELISA (COS-P), C3NeF immunoglobulin G (IgG)-binding ELISA (COIg), hemolytic assay (HA), and fluid-phase C3 cleavage assay (FPC). For some samples, insufficient volume or other technical matters prevented analysis in HA and/or FPC assays. All assays were repeated at least twice.

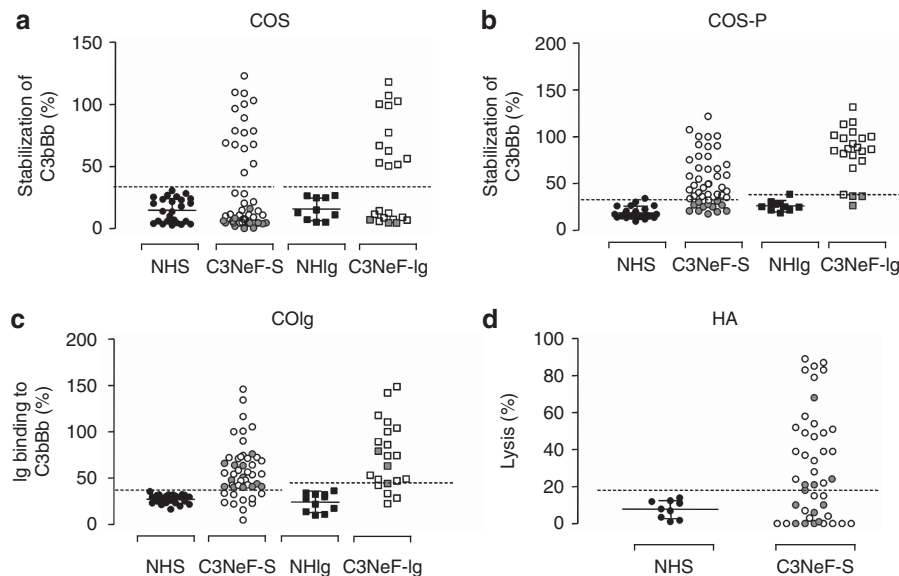
The ELISA measures the capacity of C3NeF to bind (COIg) and stabilize (COS and COS-P) the AP C3 convertase, whereas activity assays measure C3 cleavage by C3NeF-stabilized convertase either directly (FPC) or by developing hemolysis (HA; Table 1; Figure 1). Normal controls were used

**Table 1 | Identification of C3NeF-containing samples**

Sample	COS		COS-P		COIg		HA	FPC
	Serum	Ig	Serum	Ig	Serum	Ig	Serum	Serum
Control mean	14.5	15.8	18.4	26.3	26.6	24.2	13.9	(-) ve
Control mean+2 s.d.	33.5	33.7	32.1	37.4	36.7	43.9	17.5	
1	15.0	7.3	65.3	84.6	33.4	NA	49.0	(-) ve
2	<b>44.9</b>	<b>56.2</b>	<b>70.3</b>	<b>115.7</b>	<b>74.4</b>	<b>42.2</b>	NA	(+) ve
3	1.9	NA	<b>38.3</b>	NA	22.7	NA	0	(+) ve
4	0.4	5.7	<b>37.7</b>	<b>81.9</b>	23.6	<b>148.6</b>	0	(+)ve
5	<b>68.8</b>	<b>50.4</b>	<b>78.9</b>	<b>113.3</b>	<b>71.0</b>	<b>51.9</b>	0	(+) ve
6	11.4	14.3	<b>65.9</b>	<b>105.0</b>	<b>72.8</b>	<b>47.0</b>	0	(+)ve
7	<b>52.2</b>	<b>52.8</b>	<b>79.2</b>	<b>87.1</b>	<b>105.3</b>	<b>74.4</b>	0	(+) ve
8	0.0	5.4	<b>57.9</b>	26.4	<b>50.9</b>	<b>79.1</b>	<b>58.0</b>	(-) ve
9	28.0	12.4	<b>57.6</b>	<b>85.0</b>	<b>53.6</b>	<b>33.2</b>	0	(+) ve
10	4.4	4.1	<b>40.3</b>	36.4	<b>64.1</b>	<b>44.1</b>	<b>79</b>	(+)ve
11	21.4	8.9	<b>48.1</b>	36.5	<b>72.2</b>	<b>63.0</b>	<b>47.0</b>	(-) ve
12	6.1	7.0	<b>43.2</b>	NA	<b>55.5</b>	<b>48.9</b>	<b>39.6</b>	NA
13	<b>88.9</b>	NA	<b>100.3</b>	NA	<b>71.3</b>	NA	6.8	(+) ve
14	14.1	NA	<b>49.4</b>	NA	27.7	NA	51.8	(+) ve
15	10.8	11.5	25.1	<b>38.1</b>	<b>47.8</b>	<b>48.6</b>	<b>28.8</b>	(+) ve
16	10.1	8.5	<b>38.5</b>	<b>74.1</b>	<b>43.8</b>	<b>86.0</b>	15.6	(+) ve
17	20.4	NA	<b>58.9</b>	NA	<b>47.8</b>	NA	<b>21.6</b>	(+) ve
19	<b>78.6</b>	<b>107.0</b>	<b>91.6</b>	<b>101.5</b>	<b>42.7</b>	<b>22.3</b>	<b>89.0</b>	(+) ve
20	<b>108.8</b>	NA	121.7	NA	<b>116.3</b>	NA	<b>85.2</b>	(+) ve
21	<b>96.4</b>	<b>102.4</b>	<b>100.9</b>	<b>98.3</b>	<b>90.0</b>	<b>89.2</b>	<b>83.5</b>	(+)ve
22	11.0	6.8	<b>45.9</b>	<b>80.0</b>	26.2	<b>74.0</b>	<b>83.5</b>	(+) ve
23	<b>100.0</b>	<b>100.0</b>	<b>100.0</b>	<b>100.0</b>	<b>100.0</b>	<b>100.0</b>	<b>52.7</b>	(+) ve
29	<b>109.5</b>	<b>99.0</b>	<b>152.6</b>	<b>86.6</b>	<b>100.6</b>	<b>104.0</b>	<b>54.7</b>	(+) ve
32	<b>78.6</b>	<b>62.4</b>	<b>41.7</b>	<b>92.5</b>	<b>47.2</b>	<b>28.5</b>	NA	NA
34	<b>77.0</b>	<b>77.2</b>	<b>107.5</b>	<b>98.4</b>	<b>134.3</b>	<b>117.6</b>	NA	(+) ve
38	<b>122.9</b>	<b>117.8</b>	<b>66.6</b>	<b>131.7</b>	<b>146.1</b>	NA	<b>87.9</b>	(-) ve
42	<b>103.0</b>	NA	<b>75.2</b>	NA	<b>65.9</b>	NA	<b>22.3</b>	(+) ve
47	<b>67.5</b>	NA	<b>45.5</b>	NA	<b>72.3</b>	NA	<b>37.4</b>	(-) ve
54	4.2	NA	<b>33.4</b>	NA	22.0	NA	0	NA
56	<b>67.7</b>	<b>66.7</b>	<b>89.5</b>	<b>88.6</b>	<b>56.5</b>	<b>142.0</b>	<b>34.6</b>	NA
57	<b>64.3</b>	<b>51.5</b>	<b>90.2</b>	<b>66.4</b>	<b>42.8</b>	<b>110.7</b>	<b>39.4</b>	NA
59	7.6	NA	<b>45.2</b>	NA	<b>33.3</b>	NA	<b>39.6</b>	NA
62	4.3	NA	<b>35.0</b>	NA	<b>58.5</b>	NA	<b>49.0</b>	(+) ve
64	4.6	NA	20.7	NA	<b>63.9</b>	NA	10.8	NA
65	3.8	NA	27.0	NA	<b>76.1</b>	NA	0	NA
70	28.5	NA	27.1	NA	<b>40.9</b>	NA	<b>68.1</b>	NA
73	5.0	NA	20.6	NA	<b>68.6</b>	NA	<b>18.1</b>	NA
74	5.7	NA	25.1	NA	<b>40.3</b>	NA	10.8	NA
81	4.8	NA	<b>36.8</b>	NA	4.8	NA	7.9	NA
84	5.2	NA	<b>35.7</b>	NA	<b>54.4</b>	NA	NA	(+) ve
85	14.6	NA	<b>54.7</b>	NA	<b>32.3</b>	NA	4.7	NA
86	7.2	NA	17.5	NA	<b>63.5</b>	NA	<b>24.7</b>	NA
89	10.8	NA	27.6	NA	<b>40.8</b>	NA	<b>21.1</b>	NA
91	7.7	NA	19.7	NA	<b>42.1</b>	NA	15.0	NA
92	8.2	NA	31.2	NA	<b>54.1</b>	NA	3.5	NA
93	9.1	NA	30.1	NA	<b>40.8</b>	NA	0	NA
94	4.5	NA	20.6	NA	<b>40.0</b>	NA	1.8	NA
96	6.2	NA	<b>35.8</b>	NA	15.6	NA	<b>24.2</b>	NA

Abbreviations: C3NeF, C3 nephritic factor; ELISA, enzyme-linked immunosorbent assay; NA, not assayed.

Serum or immunoglobulin (Ig) from patients or healthy donors were tested using three different ELISAs, which detected (i) stabilization of the convertase without additional properdin (COS), (ii) stabilization of C3 convertase with additional properdin, (COS-P); or (iii) binding of C3NeF to the convertase (COIg). In these ELISAs, activity of C3NeF samples is expressed relative to a highly positive C3NeF sample (23), which is defined as 100%. Samples were additionally tested by hemolytic assay (HA), which indirectly detected the presence of C3NeF-stabilized C3 convertase in serum; percent lysis is given. Ability of C3NeF to promote cleavage of C3 in the fluid phase (FPC) is also shown. Control samples were run in triplicate and means and upper limits (mean+2 s.d. values) are given in the Table (apart from FPC). Samples were run in duplicate. Data are only shown from samples that were positive in one or more test. The results are a single assay set representative of at least two performed on the samples. Samples in bold are considered to be positive.



**Figure 1 | Detection of C3NeF in patient samples.** Serum or immunoglobulin (Ig) from patients or healthy donors were tested using enzyme-linked immunosorbent assay (ELISAs), which detected (a) stabilization of C3 convertase without additional properdin (COS); (b) stabilization of the convertase with additional properdin (COS-P); (c) binding of C3NeF to the convertase (COIg); or (d) hemolytic assay (HA), which indirectly detects the presence of C3NeF-stabilized C3 convertase in serum. In the ELISAs, the activity of C3NeF samples is expressed relative to a highly positive C3NeF sample (23), which is defined as 100%. Mean  $\pm$  s.d. is indicated; normal range (mean of controls + 2 s.d.) is indicated by a dashed line in each assay (a–c). To enable comparison between assays, those patient samples that are negative in the COS-P assay are colored in gray. COIg, C3NeF Ig-G-binding ELISA; COS, C3 convertase ELISA; COS-P, C3 convertase-properdin ELISA; C3NeF, C3 nephritic factor; NHLg, Ig from normal healthy donors; NHS, serum from normal healthy donors.

to set detection thresholds (mean + 2 s.d.) for each assay, except FPC where results were either positive or negative (all controls negative) (Table 1). Of 101 samples tested, 48 were positive in one or more assay. COS-P detected C3NeF in 36 of 101 samples (serum data; Figure 1b). Results, expressed relative to a chosen ‘standard’, varied up to 5-fold between samples but were internally consistent in three repeats (Figure 1; Table 1). COS detected C3NeF in only 16 samples, all strongly positive in COS-P, implying that properdin increased detection sensitivity (Figure 1a and b; Table 1). COIg detected C3NeF in 39 serum samples, 27 of which were also positive in COS-P but 12 of which were negative in convertase stabilization assays (Figure 1c; Table 1). Measured binding in COIg varied enormously between serum samples and correlated ( $r = 0.5762$ ;  $P < 0.0001$ ) with COS-P (serum).

Twenty-six of 98 patient samples tested in HA were positive; of these, five were negative in COS-P and five (different samples) were negative in COIg (Figure 1c; Table 1). Ten C3NeF ‘missed’ in the HA assay were low positive in COS-P or COIg assays. Twenty-three of 82 samples tested were positive in FPC (Figure 1d; Table 1); all of these were positive in COS-P serum assay, except sample 15, which was positive when purified IgG was used.

All C3NeF-positive samples were tested for binding convertase component proteins (C3b, fB, Bb) immobilized on ELISA plates; no sample gave a reading above background in the presence of ethylenediaminetetraacetic acid (EDTA) to block activation on the plate, indicating that all C3NeF bound neopeptides in the assembled convertase.

### C3NeF prevent C3 convertase regulation

To test whether C3NeF binding affected C3 convertase regulator binding and control of convertase, an ELISA-based functional assay was used. Nickel-stabilized C3bBb formed in the presence of C3NeF was incubated with fH, soluble DAF (sDAF), or soluble CR1 (sCR1) titrated to cause complete decay of nickel-stabilized convertase in the absence of C3NeF. Forty-two of 48 C3NeF tested completely or substantially (>20% residual convertase) prevented fH-mediated decay; 39 blocked sCR1 decay (34 of which also blocked fH decay), and 30 blocked sDAF decay (28 of which also blocked fH; Table 2).

### Real-time analysis of C3NeF stabilization of convertase

Representative C3NeF, either highly stabilizing and positive in COS and COS-P (29 and 21) or weakly stabilizing and positive only in COS-P (16 and 22), were tested for their capacity to bind C3bBb by surface plasmon resonance (SPR). These assays required purified patient IgG at high concentration and were thus possible only for samples available in sufficient volume (at least 2 ml). fB, fD, and C3NeF IgGs were flowed over immobilized C3b, and convertase formation and decay were measured (Figure 2). Binding signal was increased when fB/fD were flowed in the presence of each C3NeF, but was much greater for samples 29 and 22 (Figure 2a and d) compared with samples 21 and 16 (Figure 2b and c). Increased binding signal in the presence of C3NeF could be a consequence of either enhanced convertase formation (more Bb binding C3b) or binding of C3NeF to C3bBb; these events

**Table 2 | Decay of C3NeF-stabilized C3 convertase by fH, sDAF, and sCR1**

Sample	Percentage residual C3 convertase		
	fH	sCR1	sDAF
1	82.1	48.1	50.4
2	82.2	60.6	63.9
3	81.2	0	0
4	81.0	0	0
5	82.3	83.5	90.6
6	82.1	88.5	98.1
7	100.0	80.7	90.8
8	82.0	9.6	3.5
9	81.9	55.7	58.5
10	4.6	0	0
11	55.4	62.8	61.7
12	25.4	0	0
13	100.0	76.0	71.0
14	74.5	32.8	37.6
15	67.3	92.4	99.5
16	46.2	31.3	23.9
17	73.1	26.5	25.0
19	96.2	81.0	70.7
20	99.2	82.0	81.7
21	100.0	73.4	70.8
22	94.9	34.6	35.0
23	85.8	62.6	57.4
29	89.8	72.1	64.7
32	84.5	9.6	9.4
34	79.0	28.0	17.0
38	94.8	75.8	62.8
42	77.9	79.9	79.6
47	46.1	63.0	66.5
54	31.7	32.9	16.1
56	98.1	79.6	74.4
57	93.0	73.1	68.0
59	50.3	14.4	16.1
62	46.3	48.4	44.4
64	32.7	12.8	16.6
65	59.0	25.0	31.5
70	94.4	74.6	77.6
73	20.4	28.4	16.5
74	17.7	35.2	27.0
81	14.6	22.1	12.5
84	3.3	27.5	22.2
85	23.8	27.1	17.8
86	21.5	80.0	80.3
89	11.6	23.5	13.5
91	3.6	24.4	13.0
92	26.9	26.2	12.4
93	24.7	26.3	17.8
94	24.1	9.6	7.8
96	52.4	56.8	47.5

Abbreviations: Bb, larger cleavage fragment of factor B; COS-P, C3 convertase-properdin enzyme-linked immunosorbent assay; C3NeF, C3 nephritic factor; ELISA, enzyme-linked immunosorbent assay; fH, factor H; sCR1, soluble complement receptor 1; sDAF, soluble decay accelerating factor.

The convertase ELISA (COS-P) was adapted to determine whether C3NeF affected accelerated decay mediated via fH, sDAF, or sCR1. After enzyme formation, plate-bound convertase was subjected to 20 min of accelerated decay prior to detecting residual convertase with anti-Bb antibody. Results are presented as percent residual convertase; values above 20% indicate a decay-resistant C3NeF; values below 20% represent decay-sensitive C3NeF. The results are a single assay set representative of at least two performed on the samples.

are influenced by C3NeF concentration, affinity for C3bBb, and the capacity to prevent decay, which is impossible to analyze by SPR. Injection of sDAF to remove nonstabilized enzyme (Figure 2; gray control trace) showed that the

amount of residual stabilized enzyme was much greater for sample 29 than the other tested C3NeF, but in all cases stabilized enzyme was resistant to further sDAF decay (Figure 2). Strongly stabilizing C3NeF markedly extended C3 convertase half-life (sample 29 >2 h; sample 21 >4 h), whereas weakly stabilizing C3NeF increased half-life to a much less extent (sample 16, 40 min; sample 22, 42 min; unstabilized convertase ~5 min). SPR showed that C3NeF requiring properdin for detection in ELISA (COS negative) stabilized C3bBb even in the absence of properdin, albeit much less efficiently than C3NeF detected without added properdin (COS positive). Properdin-requiring C3NeF likely cause even greater increased half-life of properdin-stabilized convertases, which is impossible to test by SPR because properdin-stabilized convertase was not decayed by DAF and resisted dissociation by regeneration buffer.

### C3NeF binding enhances C3 convertase activity

To investigate whether C3NeF binding altered convertase C3-cleaving capacity, C3b was immobilized on chip and C3NeF-stabilized convertase generated by flowing fB, fD, and C3NeF-IgG representative of the range of characteristics observed in the samples (strong positive in COS: samples 29 and 21; positive only in COS-P: samples 16 and 22). Nonstabilized C3 convertase was decayed by sDAF and residual C3NeF-stabilized convertase was measured. C3 was flowed for 2 min to deposit nascent C3b covalently on-chip. Chip-bound Bb and C3NeF were removed by flowing 10 mmol/l sodium acetate, pH 4, 1 mol/l NaCl, and newly deposited, covalently bound C3b on-chip was measured (Figure 3). Control convertase, formed by flowing fB and fD in the presence of NHlg (Ig from normal healthy donors), caused deposition of ~2.5 C3b molecules per enzyme resonance unit (RU; Figure 3a; Table 3); convertase stabilized by C3NeF from samples 16 and 22 markedly increased convertase activity (8- to 10-fold increased C3b binding per enzyme RU); sample 21 increased C3 deposition 4-fold, whereas sample 29 had little effect on activity (Table 3).

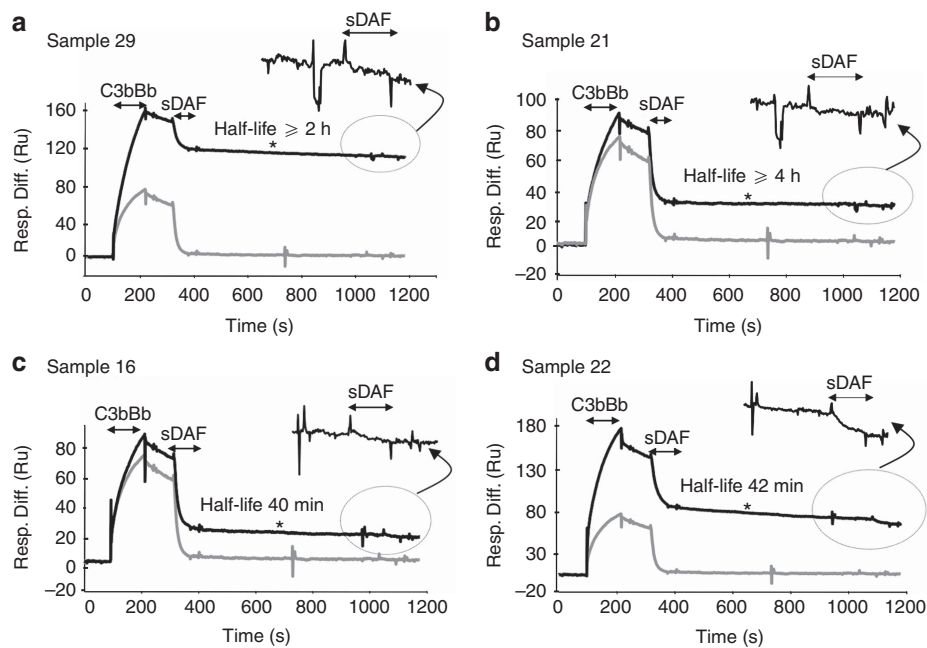
### C3NeF enhances C5 convertase activity

To assess whether C3NeF influenced C5 convertase activity, C3bBbC3b was assembled on antibody-coated sheep erythrocytes (EA) in the presence of C3NeF-Ig to stabilize convertase before developing lysis by adding R3 diluted in EDTA. The absence of active C3 in R3 prevents further C5 convertase formation; lysis therefore measures preformed C5 convertase. Those C3NeF that enhanced C3 convertase activity (samples 16 and 22; Table 3; Figure 3) also enhanced lysis in C5 convertase assay, indicating that they increased C5 convertase stability and/or activity; C3NeF samples 29 and 21 had no effect on C5 convertase (Figure 4).

## DISCUSSION

C3NeF, autoantibodies against the AP C3 convertase, strongly associate with chronic AP activation in disease. Involvement in different pathologies with variable outcomes suggests that





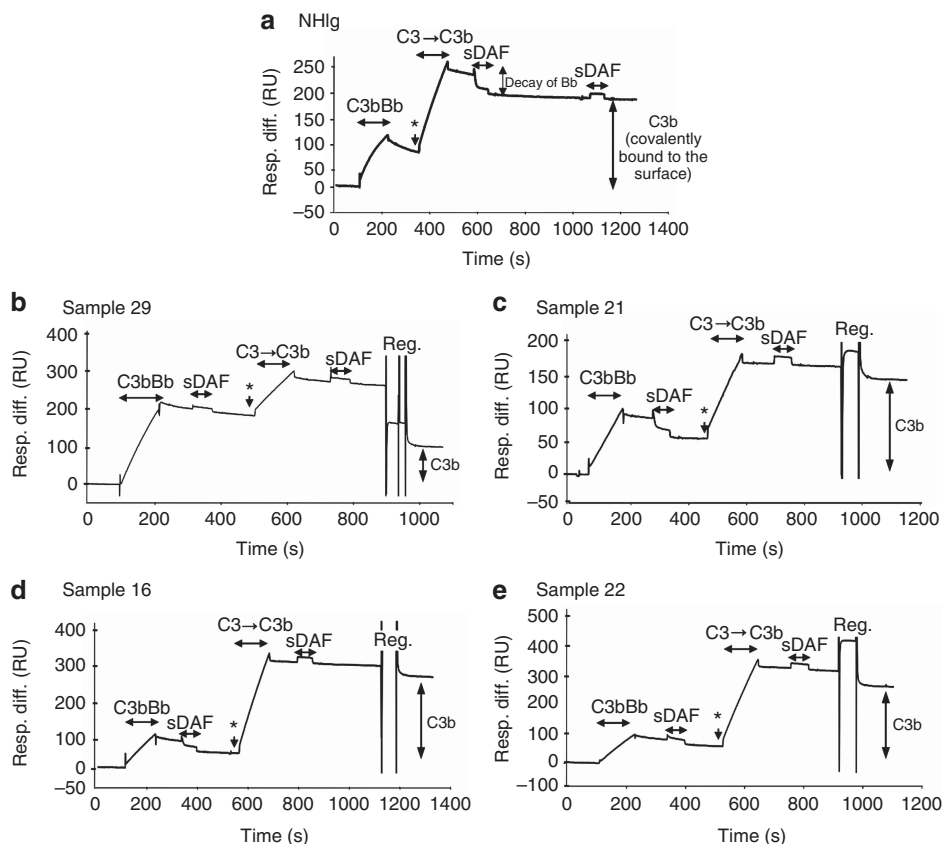
**Figure 2 | Sensorgram illustrating real-time C3 convertase formation in the presence of C3NeF-containing IgG.** C3 convertase was formed by flowing 40  $\mu$ l of a mix containing factor B (180  $\mu$ g/ml) and factor D (1  $\mu$ g/ml) in the presence of C3NeF-containing IgG (400  $\mu$ g/ml, black line) or normal human IgG (gray line) over C3b immobilized on a CM5 Biacore chip (IgG from (a) sample 29, (b) sample 21, (c) sample 16, (d) sample 22). The convertase was left to spontaneously decay for 100 s before injecting 20  $\mu$ l of sDAF (8  $\mu$ g/ml) across the surface. Notice that active larger cleavage fragment of factor B subunit is completely decayed away when C3 convertase is formed in the presence of Ig from normal healthy donors, and that in the presence of C3NeF varying amounts of C3 convertase remained bound to the surface. In all cases, the C3bBb–C3NeF remaining on the surface was very stable; decay curve indicated by asterisk. Half-life after removal of nonstabilized enzyme is calculated as  $\ln 2/k_d$ . C3NeF, C3 nephritic factor; IgG, immunoglobulin G; RU, resonance unit; sDAF, soluble decay accelerating factor.

C3NeF are heterogeneous, binding and influencing C3bBb function and regulation in diverse ways. C3NeF levels fluctuate in individuals with time and do not correlate with disease.<sup>16</sup> Confusion over their clinical relevance is, in part, because they are difficult to measure. Current assays are complicated, laborious, require expertise, and scarce reagents, and prone to artifact. Methods are inconsistent between laboratories and there is no national/international quality control.

To address these problems and identify optimal assays for routine detection of C3NeF, a panel of assays was established and optimized to identify C3NeF in patient samples. The COS-P assay, measuring the capacity of C3NeF to prolong half-life of properdin-stabilized C3bBb, was reproducibly positive in 36 of 101 samples, albeit with a wide range of values. In the absence of added properdin (COS), only 16 of 101 samples were positive, all of these were strongly positive in COS-P, indicating that these assays differed in sensitivity, and that additional stability conferred by properdin enabled detection of weaker binding C3NeF. The COIg assay, measuring binding of C3NeF IgG to nickel-stabilized convertase, was positive in 39 of 101 samples, 27 of which were positive in COS-P. The assays showed a strong correlation, but each assay alone missed some C3NeF, which was positive in the other assay. The COIg assay will miss any non-IgG C3NeF; we explored this possibility in those COS-P-

positive samples negative in COIg using commercial anti-IgM, but, without a positive control, were unable to obtain reproducible results. The HA assay was positive in 26 of 98 samples tested; all HA positives were also positive in COS-P and/or COIg, suggesting that HA was specific but insensitive, missing many C3NeF detected by other assays. The FPC was positive in 23 of 82 samples tested, all also positive in COS-P and/or COIg, demonstrating specificity but insensitivity. HA and FPC missed different C3NeF and not all missed samples were of low activity in COS-P and COIg. No C3NeF bound individual convertase components, demonstrating specific recognition of neoepitopes in intact convertase.

By SPR, purified IgGs from two samples strongly positive in all three ELISAs bound C3bBb to create highly stable convertases with half-lives in excess of 2 h (unstabilized half-life being 5 min), whereas IgGs from samples weakly positive in COS-P and COIg and negative in COS caused 8-fold increase in convertase half-life. Several reports suggest that C3NeF block accelerated decay.<sup>20–22,25</sup> Almost all C3NeF tested here blocked accelerated decay of the convertase by fH, sDAF, and sCRI; only 6 of 48 C3NeF tested failed to significantly inhibit fH decay, whereas 18 failed to inhibit decay by sDAF and 9 decay by sCRI. Inhibition of accelerated decay was confirmed by SPR. Most C3NeF therefore have dual actions, preventing both spontaneous and accelerated decay. C3NeF sensitive to regulation by fH and other



**Figure 3 | Real-time analysis of C3NeF-stabilized C3 convertase activity.** C3 convertase was formed by flowing factor B (fB) and factor D in the presence of 400  $\mu\text{g}/\text{ml}$  of C3NeF-containing IgG (from (b) sample 29, (c) sample 21, (d) sample 16, (e) sample 22) or normal human IgG (NHlg, (a)) over the amine-coupled C3b. Where C3NeF was included, all nonstabilized C3 convertase was decayed by sDAF. The enzyme substrate, C3 (200  $\mu\text{g}/\text{ml}$ ), was flowed across the surface, resulting in covalent deposition of C3b onto the chip surface. Bound Bb and C3NeF were removed with 10 mmol/l sodium acetate pH 4/1 mmol/l NaCl ('Reg'), and the amount of C3b covalently bound to the surface was measured. Asterisk indicates C3bBb-C3NeF complex remaining on surface after removal of nonstabilized convertase. Bb, larger cleavage fragment of factor B; C3NeF, C3 nephritic factor; fB, factor B; IgG, immunoglobulin G; NHlg, Ig from normal healthy donors; RU, resonance unit; sDAF, soluble decay accelerating factor.

**Table 3 | Activity of the C3NeF-stabilized C3 convertase as assessed by C3b deposition on the surface of a Biacore chip**

Sample	Amount of C3bBb-C3NeF complex (RU)	Calculated amount of Bb <sup>a</sup> (RU)	C3b deposited on the chip (RU)	Ratio of C3b/Bb bound on the chip
NHlg	64	64 (no C3NeF)	167	2.6
29	169	49	92	1.9
21	49	14	127	9.1
16	33	9	246	27
22	46	13	255	20

Abbreviations: Bb, larger cleavage fragment of factor B; C3NeF, C3 nephritic factor; IgG, immunoglobulin G; NHlg, Ig from normal healthy donors.

C3NeF-stabilized convertase was formed on the surface of a Biacore chip and substrate, C3, was flowed across for 120 s. C3NeF and bound Bb were removed with regeneration buffer and amount of nascent C3b deposited on the chip surface per resonance unit (RU) of Bb comprising the convertase was calculated.

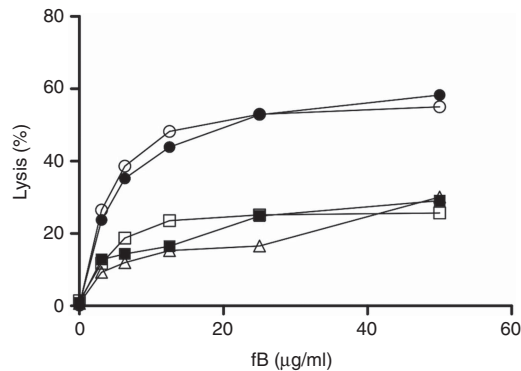
<sup>a</sup>Amount of Bb present=(total RU  $\times$  60)/(60+150). This assumes mass of Bb is 60 kDa, mass of IgG 150 kDa and a 1:1 complex of Bb and IgG is formed. The results are from a single experiment representative of at least two performed on the samples.

regulators are likely less pathogenic *in vivo*; indeed, one report showed that fH-sensitive C3NeF are not associated with hypocomplementaemia, implying that fluid-phase activation is controlled.<sup>25</sup> C3NeF that are fH-resistant but

DAF and/or CR1-sensitive likely mediate effects in the fluid phase, leading to different pathologies; these are excellent targets for therapy using recombinant sCR1 and/or DAF.<sup>26-28</sup>

To cause pathology, C3NeF-stabilized convertase must retain its capacity to cleave C3. This was demonstrated by SPR for four selected C3NeF IgGs. Each C3NeF-stabilized convertase cleaved C3 and deposited C3b on the chip; however, convertases stabilized by strongly binding C3NeF were much less efficient C3 cleavers than convertases stabilized by weak-binding C3NeF. These findings illustrate heterogeneity and suggest that pathological consequences cannot be predicted by strength of binding; strong binders may reduce efficiency of the enzyme by restricting engagement with substrate. Structural analyses of convertase-C3NeF complexes are needed to test these contentions.

A subset of C3NeF enhance C5 cleavage and terminal pathway activation.<sup>29,30</sup> The same four C3NeF IgGs used in SPR analysis were tested in HAs designed to interrogate C5 convertase stabilization and/or activity. Two caused enhanced lysis via the terminal pathway, whereas the other two did not. Those C3NeF causing enhanced lysis were 'weak' C3



**Figure 4 | Effect of C3NeF on terminal pathway.** C5 convertase was assembled on the surface of sheep E by incubating antibody-coated sheep erythrocytes-C3b with factor B (fB), factor D, and C3NeF-containing immunoglobulin (Ig; samples 16, 21, 22, or 29) or NHlg. Lysis was developed by incubating cells with serum/ ethylenediaminetetraacetic acid lacking active C3 (R3). Only C5 convertase formed in the first step was able to trigger lysis, as no further C3 could be cleaved. Lysis of cells was measured by release of hemoglobin. Samples are identified as follows: sample 16 (●); sample 21 (■); sample 29 (□), sample 22 (○), or NHlg (△). NHlg, Ig from normal healthy donors.

convertase binders, requiring extra properdin or nickel for detection, with short stabilized half-lives, and efficient C3 cleavers.

While this manuscript was in review, a report appeared describing the development of assays for C3NeF.<sup>31</sup> Patients with biopsy-proven DDD were screened for C3NeF using C3NeF assays comprising an ELISA measuring IgG binding immobilized convertase that resembled COIg, except without added properdin, and erythrocyte-based convertase-stabilization assays with or without properdin resembling COS and COS-P, respectively, except that the end point was hemolysis. These were compared with 'traditional' assays based on activation, immunoelectrophoresis, and immunofixation, which resembled FPC. The convertase-stabilization assays required serum depleted of fH, which must be prepared fresh because it rapidly autoactivates, restricting these assays to specialist complement laboratories; in contrast, our plate-based assays require no specialist reagents and are readily translatable to routine use. Of 32 DDD patients screened, 53% were positive in IgG-binding ELISA, 69% in C3 convertase stabilization without properdin, and 78% in the presence of properdin; the 'traditional' assays detected C3NeF in 69%. These data support our finding that optimal assays for C3NeF involve measurement of IgG binding to convertase or stabilization of properdin-stabilized convertase. Importantly, both studies demonstrate that, even with optimized assays, not all patients with a presumptive or confirmed diagnosis have detectable C3NeF; more work is needed to explore whether these patients harbor unusual C3NeF missed in the available assays.

We confirm previously noted heterogeneity of C3NeF, describe convertase-stabilizing activity and other activities of selected C3NeF, and provide assays for C5 convertase

stabilization. We deliberately abandon terms and subgroups used historically to categorize C3NeF, instead stressing that each C3NeF must be treated as a unique entity with properties that predicate its pathological effects. We show that simple ELISA for convertase stabilization and/or binding is most reliable for C3NeF detection. We propose a rational approach in which serum samples from patients presenting with relevant history are first screened for AP activation using standard assays (C3, C4, fB, hemolytic activity (CH50; AP50), activation markers such as terminal complement complex where available). Where AP activation is identified (low C3, fB, AH50; elevated terminal complement complex), samples are screened for C3NeF using COS-P, which identifies most C3NeF. Clinically suspicious samples negative in COS-P are screened in COIg, which detects weakly or nonstabilizing C3NeF missed in COS-P. Serum samples negative in COIg are rescreened using purified IgG to detect fH-sensitive C3NeF that can be missed in serum. Where appropriate, positive samples are further screened in specialist centers to identify those conferring resistance to fH regulation or stabilizing C5 convertase.

## MATERIALS AND METHODS

### Samples

Sera from 101 patients presenting with histological and/or clinical evidence suggesting DDD (>80%), membranoproliferative glomerulonephritis Type I, proliferative glomerulonephritis, cortical necrosis, systemic lupus erythematosus, or vasculitis were collected with local ethical approval. All sera were screened at the source for evidence of AP activation (low C3, low fB, low AP50 ± elevated terminal complement complex), and only AP activation-positive sera were included. Control sera were obtained from 25 healthy controls (Cardiff University Research Ethics Committee Ref. 10/26). To purify C3NeF-IgG, serum was applied to Protein G (GE Healthcare, Little Chalfont, UK) equilibrated with phosphate-buffered saline (PBS). Bound IgG was eluted using 0.1 mol/l glycine, pH 2.5, and dialyzed into PBS. Control IgG was purified from C3NeF-negative serum.

### Complement components and antibodies

C3 was prepared by classical chromatography, and fB and fH by affinity chromatography as described.<sup>32,33</sup> C3b and Bb were prepared from these reagents by AP activation as described.<sup>32</sup> Protein concentrations were derived from  $A_{280}$  (fH:  $1.95 \text{ cm}^{-1}(\text{mg/ml})^{-1}$ , fB:  $1.43 \text{ cm}^{-1}(\text{mg/ml})^{-1}$ ; C3:  $0.98 \text{ cm}^{-1}(\text{mg/ml})^{-1}$ ; Ig:  $1.4 \text{ cm}^{-1}(\text{mg/ml})^{-1}$ ). FD and properdin were from Complement Technology (Tyler, TX). Soluble recombinant DAF (sDAF) was from Professor S. Lea (University of Oxford); soluble recombinant CR1 (sCR1) was from T Cell Sciences (Needham, MA). Rabbit polyclonal anti-fB was from Complement Technology. Peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). R3 was generated by treating plasma with hydrazine (50 mmol/l, pH 8.5) for 2 h at 37 °C followed by dialysis against PBS, 20 mmol/l EDTA.

### C3NeF stabilization ELISA with (COS-P) and without (COS) properdin

ELISA plates (Medisorb, Nunc, VWR, Lutterworth, UK) were coated with C3b (1 µg/ml in PBS, 1 h, room temperature), blocked in PBS/1% bovine serum albumin, and washed in assay buffer (2.5 mmol/l

sodium barbitone, pH 7.4, 71 mmol/l NaCl, 0.15% Tween, 1 mmol/l  $MgCl_2$ ). Convertase was formed by adding 50  $\mu$ l test serum (1/50 in assay buffer) or purified Ig (50  $\mu$ g/ml), immediately followed by 50  $\mu$ l of a mixture comprising fB (1  $\mu$ g/ml) and fD (0.2  $\mu$ g/ml) with (COS-P) or without (COS) properdin (0.5  $\mu$ g/ml) in assay buffer. After 30 min at 37 °C, plates were washed in assay buffer and residual Bb detected using goat anti-fB (1:1000; 1 h, 37 °C), followed by peroxidase-conjugated donkey anti-sheep Ig (1:5000; 1 h). Color was developed using *o*-phenylenediamine dihydrochloride (Sigma-Aldrich, Poole, UK). Assay blank was obtained by omitting fB and serum/Ig; a sample known to be strongly positive for C3NeF was used as positive control and results expressed relative to this: percentage of stabilization =  $100 \times (A_{492} \text{ test} - A_{492} \text{ blank}) / (A_{492} \text{ C3NeF-positive control} - A_{492} \text{ blank})$ . In some experiments, convertase was formed in the presence of 1 mmol/l  $NiSO_4$  to stabilize, plates were washed and incubated with 100  $\mu$ l fH (15  $\mu$ g/ml), sDAF (5  $\mu$ g/ml), or sCR1 (10  $\mu$ g/ml) in assay buffer at room temperature for 20 min before the measurement of residual Bb. Percentage decay was calculated as  $100 \times [(A_{492} \text{ test sample in absence of the complement regulatory protein (CReg)} - A_{492} \text{ of blank}) - (A_{492} \text{ test sample in presence of the CReg} - A_{492} \text{ of blank})] / (A_{492} \text{ test sample in absence of CReg} - A_{492} \text{ blank})$ . Percent residual convertase is 100 minus percent decay.

### C3NeF-binding ELISA (COlg)

To test binding of C3NeF to preformed convertase, ELISA plates were coated with C3b and convertase formed by incubation with fB (1  $\mu$ g/ml), fD (0.2  $\mu$ g/ml), properdin (0.5  $\mu$ g/ml), and sample as described above, except that assay buffer contained 1 mmol/l  $NiSO_4$ . Plates were washed and bound Ig detected using peroxidase-labeled anti-human IgG (1:5000). A strongly positive C3NeF sample was used as positive control. Background was obtained by omitting fB, fD, and serum/Ig. Absorbance was measured at 492 nm, and bound C3NeF calculated as  $100 \times (A_{492} \text{ test sample} - A_{492} \text{ blank}) / (A_{492} \text{ C3NeF positive control} - A_{492} \text{ blank})$ .

### C3NeF fluid-phase C3 cleavage assay

Equal volumes of test serum, NHS (serum from normal healthy donors), and assay buffer (2.5 mmol/l sodium barbitone pH 7.4, 71 mmol/l NaCl, 0.15% Tween, 7 mmol/l  $MgCl_2$ , 20 mmol/l ethylene glycol bis( $\beta$ -aminoethylether)-*N,N,N',N'*-tetraacetic acid) were mixed and incubated (37 °C, 3 h). Cleavage of C3 was detected by western blot using rabbit polyclonal anti-human C3 (in-house). Controls included NHS and patient serum, incubated as described above, in the presence of 20 mmol/l EDTA. Results were expressed as positive (significant C3 cleavage) or negative (no C3 cleavage above controls).

### C3NeF HA

Sheep erythrocytes ( $1.5 \times 10^7$ /ml in AP buffer; 5 mmol/l sodium barbitone, pH 7.4, 150 mmol/l NaCl, 7 mmol/l  $MgCl_2$ , 10 mmol/l ethylene glycol bis( $\beta$ -aminoethylether)-*N,N,N',N'*-tetraacetic acid) were incubated with a mixture containing equal amounts of NHS and patient serum for 10 min at 30 °C.<sup>34</sup> Cells were washed and incubated for 1 h at 37 °C with rat serum diluted 1:10 in PBS/20 mmol/l EDTA to develop lysis. Cells were centrifuged and absorbance in supernatant measured at 410 nm. As controls, cells were incubated with AP buffer (0% lysis) or 0.01% Triton X-100 (100% lysis). Percentage lysis was calculated as follows:  $100 \times (A_{410} \text{ test sample} - A_{410} \text{ 0% control}) / (A_{410} \text{ 100% lysis} - A_{410} \text{ 0% control})$ .

### C3NeF binding to immobilized C3b, fB, or Bb

ELISA plates (Medisorb, Nunc) were coated with C3b, fB, or Bb at 1  $\mu$ g/ml, blocked in PBS/1% bovine serum albumin, and then incubated with 50  $\mu$ l of C3NeF-positive test serum diluted 1/50 in assay buffer containing 20 mmol/l EDTA. Plates were washed and C3NeF binding detected using peroxidase-conjugated anti-human IgG (1:5000). Each incubation period was for 1 h at 37 °C. Color was developed as above and absorbance measured at 492 nm.

### Stabilization and activity of convertase assessed by SPR

SPR analyses required sufficient patient IgG to flow at high concentrations, and thus was limited to samples in which adequate volume was available for IgG purification. To measure stabilization, C3 convertase was formed on-chip by flowing fB (180  $\mu$ g/ml) and fD (1  $\mu$ g/ml) over immobilized C3b (700 RU) with NHlg or C3NeF-IgG (400  $\mu$ g/ml) in Biacore buffer (10 mmol/l HEPES, pH 7.4, 150 mmol/l NaCl, 1 mmol/l  $MgCl_2$ , 0.005% surfactant P20). Convertase was left to spontaneously decay for 100 s, and 20  $\mu$ l of sDAF (8  $\mu$ g/ml) was injected to decay nonstabilized convertase. The half-life ( $t_{1/2}$ ) of C3NeF-stabilized convertase was estimated from dissociation rate ( $k_d$ ) ( $t_{1/2} = \ln 2 / k_d$ ). To measure activity, convertase was formed on-chip as above; nonstabilized enzyme was decayed by flowing sDAF. C3 (200  $\mu$ g/ml in Biacore buffer) was flowed; nascent C3b bound covalently to the surface as described.<sup>33</sup> Residual Bb and C3NeF were removed by flowing 10 mmol/l sodium acetate, pH 4, 1 mol/l NaCl, and deposited C3b was measured using the BiaEvaluation 4.1 software.

### C3NeF-stabilized terminal pathway activity

Antibody-sensitized ShE (EA) were prepared by incubating 2% ShE with amboceptor (1:4000, 30 min, 37 °C; Siemens Healthcare, Erlangen, Germany); EA were washed into complement fixation diluent (CFD; Oxoid, UK), and C3b deposited by incubating with fB- and fH-depleted serum containing the C5 inhibitor, OmCI, as described.<sup>35</sup> EA were resuspended to 2% (v/v) in AP buffer (5 mmol/l sodium barbitone, pH 7.4, 150 mmol/l NaCl, 7 mmol/l  $MgCl_2$ , 10 mmol/l ethylene glycol bis( $\beta$ -aminoethylether)-*N,N,N',N'*-tetraacetic acid), and 50- $\mu$ l aliquots were incubated with dilutions of fB and 0.5  $\mu$ g/ml fD in the presence or absence of C3NeF-IgG (100  $\mu$ g/ml; 15 min, 37 °C) to form C5 convertase. Lysis was developed by adding 50  $\mu$ l R3 diluted 1:25 in PBS, 20 mmol/l EDTA.

### Statistics

Unpaired *t*-test with Welch's correction was used for inter-group comparison. Data were analyzed using GraphPad Prism 3.0 (GraphPad, San Diego, La Jolla, CA).

### DISCLOSURE

THG acts as a scientific advisor for Alexion Pharmaceuticals. BPM acts as a scientific advisor for Baxter. None of the authors have any conflicting interests, financial or other, to disclose.

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