Cluster Analysis Identifies Distinct Pathogenetic Patterns in C3 Glomerulopathies/Immune Complex–Mediated Membranoproliferative GN

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ABSTRACT

Membranoproliferative GN (MPGN) was recently reclassified as alternative pathway complement–mediated C3 glomerulopathy (C3G) and immune complex–mediated membranoproliferative GN (IC-MPGN). However, genetic and acquired alternative pathway abnormalities are also observed in IC-MPGN. Here, we explored the presence of distinct disease entities characterized by specific pathophysiologic mechanisms. We performed unsupervised hierarchical clustering, a data-driven statistical approach, on histologic, genetic, and clinical data and data regarding serum/plasma complement parameters from 173 patients with C3G/IC-MPGN. This approach divided patients into four clusters, indicating the existence of four different pathogenetic patterns. Specifically, this analysis separated patients with fluid-phase complement activation (clusters 1–3) who had low serum C3 levels and a high prevalence of genetic and acquired alternative pathway abnormalities from patients with solid-phase complement activation (cluster 4) who had normal or mildly altered serum C3, late disease onset, and poor renal survival. In patients with fluid-phase complement activation, those in clusters 1 and 2 had massive activation of the alternative pathway, including activation of the terminal pathway, and the highest prevalence of subendothelial deposits, but those in cluster 2 had additional activation of the classic pathway and the highest prevalence of nephrotic syndrome at disease onset. Patients in cluster 3 had prevalent activation of C3 convertase and highly electron-dense intramembranous deposits. In addition, we provide a simple algorithm to assign patients with C3G/IC-MPGN to specific clusters. These distinct clusters may facilitate clarification of disease etiology, improve risk assessment for ESRD, and pave the way for personalized treatment.

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Membranoproliferative GN (MPGN) is a chronic nephropathy characterized by capillary wall thickening and mesangial expansion due to increased matrix deposition and hypercellularity.[1] A classification on the basis of immunofluorescence (IF) has been recently proposed that divides MPGN into C3 glomerulopathy (C3G), with dominant glomerular C3 deposition and little or no Ig deposition (C3 greater than or equal to

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two grades of order of magnitude greater than any other immune reactant), and immune complex–associated membranoproliferative GN (IC-MPGN), with significant glomerular Ig deposition.[2,3] C3G is further divided into dense deposit disease (DDD) and C3GN, the latter lacking the intramembranous highly electron-dense deposits.[3] The term C3G is also used to define other proliferative patterns or even nonspecific alterations sharing C3-dominant glomerular staining.[3]

The current classification is on the basis of the assumption that C3G arises from abnormalities in the control of the complement alternative pathway, whereas IC-MPGN derives from the deposition of immune complexes that trigger the classic complement pathway.[1] Consequently, genetic screening in complement genes and assays for C3 nephritic factor (C3NeF) is recommended in C3G, and conversely, evaluation for infections, autoimmune diseases, or monoclonal gammopathy is recommended in IC-MPGN.[1,3]

The IF-based classification represents an advance toward an etiology-based diagnostic approach of these diseases, but some issues require further investigation. First, the widely accepted "C3 dominant" criterion for the definition of C3G was established using patients with DDD as a reference group and has not been validated using patients with known dysregulation of the complement alternative pathway.[4] Second, up to 16% of patients shift from C3G to IC-MPGN and vice versa when a kidney biopsy is repeated.[4,5] Third, the distinction between DDD and C3GN is not always clear cut, and there are a few borderline cases.[3] Fourth, C3GN and DDD may not reflect different underlying causes, because both may affect different members of the same pedigree.[1,6–8] Fifth, complement alternative pathway dysregulation is common in patients with idiopathic IC-MPGN, because most carry C3NeF, an autoantibody stabilizing the alternative pathway C3 convertase; autoantibodies against the two components of the alternative pathway C3 and C5 convertases, complement factor B (CFB) and C3b; and/or complement gene mutations.[9–11] Additionally, about one half of patients with IC-MPGN have low serum C3 with normal C4 levels, indicating activation of the alternative pathway of complement without significant activation of the classic pathway.[9] Altogether, the assumptions that Ig-negative MPGN is complement mediated and that Ig-positive MPGN is immune complex mediated have yet to be validated.

Recently, unsupervised cluster analysis has been introduced as an objective data-driven method for exploring whether patients can be separated into relatively homogeneous groups characterized by specific pathophysiologic mechanisms.[12–14] Simplistically, subjects with many commonalities are placed close together, and subjects with many dissimilarities are placed farther apart. This approach was successfully used to identify disease subtypes in asthma, Parkinson disease, and chronic obstructive pulmonary disease, with an effect on clinical practice.[12,15,16]

In this study, we explored whether cluster analysis on the basis of histology findings, serum/plasma complement profile, genetic data, and clinical features could be a tool for disclosing, in patients with C3G and patients with IC-MPGN, distinct disease entities characterized by specific pathophysiologic mechanisms. Through cluster analysis, we identify four groups of patients with relatively homogeneous phenotypes. Patients with known alternative pathway abnormalities are separated from those without, the latter clustering into a unique group. Patients with alternative pathway abnormalities are further divided into three groups with distinct underlying disease pathogenetic mechanisms. These clusters are useful for better understanding the pathogenesis of the disease and predicting the risk of progression to ESRD.

Significance Statement

C3 glomerulopathies (C3G) and membranoproliferative glomerulonephritis (MPGN) are uncommon forms of glomerulonephritis characterized by high risk of progression to ESRD. This manuscript describes results, using data from a large cohort of patients with C3G/MPGN, of unsupervised cluster analysis, an objective data-driven method to explore whether patients can be separated into homogeneous groups characterized by specific pathophysiologic mechanisms. Based on histologic, biochemical, genetic and clinical features, investigators identified four groups of patients with distinct phenotypes and differing renal survival. These clusters may be useful for better understanding the multifaceted molecular mechanisms underlying C3G/MPGN, and ultimately improve prediction of risk of ESRD and treatment response.

RESULTS

Description of the Cohort

The histologic, biochemical, genetic, and clinical features of the 173 patients, classified according to the recent C3G/IC-MPGN classification,[2,3] are reported in Table 1. We have included all patients with C3G, even if their light microscopy is not MPGN. Sixty-eight patients (39%) had C3GN, 25 (14%) had DDD, and 80 (46%) had IC-MPGN. Kidney biopsy was performed after medians of 0.4, 1.1, and 0.4 years from the onset for C3GN, DDD, and IC-MPGN, respectively, without significant differences between groups. C3G diagnosis captured 25 of 28 patients with intramembranous highly electron-dense deposits (Table 2), confirming previous studies.[3,4]

Age of onset and sex distribution did not differ between groups. Complement gene likely pathogenic variants (LPVs) were identified in 25%, 16%, and 16% of patients with C3GN, DDD, and IC-MPGN, respectively (Supplemental Tables 1 and 2, Table 1). C3NeF prevalence was higher in DDD (78%) versus C3GN (38%) and IC-MPGN (40%). In all three histologic groups, the majority of the patients carried LPVs and/or C3NeF, with a higher prevalence in DDD (83%) versus C3GN (56%) and IC-MPGN (49%).

Only 59% of patients with complement gene LPVs and/or C3NeF and 56% of patients with low C3 and normal C4 had a biopsy diagnosis of C3G. Consistently, compared with patients with IC-MPGN, patients with C3G did not show a significantly higher prevalence of LPVs and/or C3NeF (64% versus 49%) or a higher prevalence of low C3 and normal C4 (78% versus

Table 1. Clinical features, complement assessment, genetic screening, and histologic features in patients classified according to the C3G/IC-MPGN classification

Variable	C3GN	DDD	IC-MPGN	Overall *P* Value
N	68	25	80	
Sex, % men	60	56	53	0.64
Data at onset				
Age, yr	18.2 (±16.1)	15.3 (±11.2)	20.8 (±15.8)	0.26
Microhematuria, %	85	92	82	0.50
Gross hematuria, %	37	44	29	0.34
Proteinuria, %	93	88	90	0.77
Nephrotic syndrome, %	29	24	43	0.12
Renal impairment, %	19	4	23	0.10
Trigger event, %	34	32	25	0.49
Familiarity for nephropathy, %	18	12	13	0.70
Serum C3, mg/dl	50.9 (±41.8)	33.8 (±36.1)	49.8 (±39)	0.16
Serum C4, mg/dl	21.8 (±8.9)	24.4 (±9.6)	21.1 (±10.6)	0.35
Plasma SC5b-9, ng/ml	1163 (±1289)	545 (±524)	1120 (±1248)	0.08
Low serum C3 and normal serum C4, %	74	84	70	0.38
LPV carriers, %	25	16	16	0.53
C3NeF positive, %	38	78[a,b]	40	0.002
LPV carriers and/or C3NeF, %	56	83[a,b]	49	0.02
Data during follow-up, %				
Nephrotic syndrome	46[b]	52	70	<0.01
High BP	34	24[b]	49	0.04
CKD	31	28	44	0.17
ESRD	10	4	9	0.76
Thrombotic microangiopathy	6	0	4	0.67
Histologic features				
Time onset to biopsy, yr, median (IQR)	0.4 (0.0–2.9)	1.1 (0.3–4.0)	0.4 (0.1–2.3)	0.29
Light microscopy				
Sclerotic glomeruli, %	8 (±17)	2 (±7)	8 (±14)	0.17
Crescents, %	3 (±11)	6 (±20)	7 (±18)	0.40
Degree of mesangial proliferation[c]	1.7 (±1)	1.8 (±0.7)	1.9 (±0.9)	0.19
Degree of endocapillary proliferation[c]	1 (±1.1)	0.9 (±1.1)	1.2 (±1)	0.29
Degree of interstitial inflammation[c]	0.5 (±0.8)	0.6 (±0.8)	0.9 (±0.8)[a]	0.03
Degree of interstitial fibrosis[c]	0.4 (±0.7)	0.2 (±0.4)	0.6 (±0.8)	0.07
Degree of arteriolar sclerosis[c]	0.3 (±0.8)	0 (±0.1)	0.3 (±0.7)	0.16
IF[c]				
C3	2.7 (±0.5)	2.8 (±0.3)	2.6 (±0.7)	0.12
IgA	0 (±0.1)	0.1 (±0.3)	0.5 (±0.8)[a,d]	<0.001
IgG	0.2 (±0.4)	0.2 (±0.4)	1.6 (±1.1)[a,d]	<0.001
IgM	0.3 (±0.4)[b]	0.7 (±0.7)[a,b]	1.4 (±0.9)	<0.001
C1q	0.1 (±0.3)	0.1 (±0.3)	1.2 (±1)[a,d]	<0.001
Fibrinogen	0.3 (±0.7)	0.3 (±0.8)	0.3 (±0.7)	0.98
Electron microscopy, %				
Mesangial deposits	72	48	58	0.08
Subepithelial deposits	57[b]	8[a,b]	37	<0.001
Subepithelial hump-like deposits	27	8	14	0.63
Subendothelial deposits	71	12[a,b]	80	<0.001
Intramembranous granular deposits	54	0[a,b]	47	<0.001
Intramembranous highly electron-dense ribbon-like deposits	0	100[a,b]	4	<0.001

Quantitative variables are expressed as mean (±SD) unless otherwise specified. Serum C3: reference 90–180 mg/dl; serum C4: reference 10–40 mg/dl; plasma SC5b-9: reference ≤400 ng/ml. IQR, interquartile range.

[a]Significantly different versus C3GN.

[b]Significantly different versus IC-MPGN.

[c]Degrees of mesangial proliferation, endocapillary proliferation, interstitial inflammation, interstitial fibrosis, and arteriolar sclerosis as well as IF findings were graded using a scale of 0–3, including 0, trace (0.5+), 1+, 2+, and 3+.

[d]Significantly different versus DDD.

Table 2. Sensitivity and specificity of the C3-dominant or -isolated criteria on IF to capture the presence of intramembranous electron-dense deposits, LPVs, and/or C3NeF and low serum C3 and normal C4 in patients with C3G/MPGN

Categories	Prevalence		*P* Value	C3G	
	C3G	IC-MPGN		Sensitivity, %	Specificity, %
All patients					
Intramembranous electron-dense deposits	0.27	0.04	<0.001	89	53
LPVs and/or C3NeF	0.64	0.49	0.07	59	56
Low serum C3 and normal C4	0.78	0.70	0.23	56	55
Only patients with C3G and isolated C3 versus patients with IC-MPGN and 3+ staining in any of Ig or C1q[a]					
Intramembranous electron-dense deposits	0.24	0.03	0.03	89	56
LPVs and/or C3NeF	0.53	0.47	0.62	52	55
Low serum C3 and normal C4	0.76	0.68	0.46	52	58

[a]Patients with C3G and 3+ C3 staining and no Ig or C1q staining on IF and patients with IC-MPGN and 3+ staining in at least one of the Igs or C1q.

70%) (Table 2). To overcome bias due to immune-reactant intensity misevaluation,[7] we repeated the analyses including only the extremes of the phenotypic continuum: patients with C3G and 3+ C3 and absent Ig or C1q staining (n=34) versus patients with IC-MPGN and 3+ staining in at least one of the Ig classes or C1q (n=34) (Supplemental Figure 1, Table 2). Even with this setting, the prevalence of LPVs and/or C3NeF and the prevalence of low C3 and normal C4 were not significantly higher in C3G versus IC-MPGN (53% versus 47%, respectively, and 76% versus 68%, respectively).

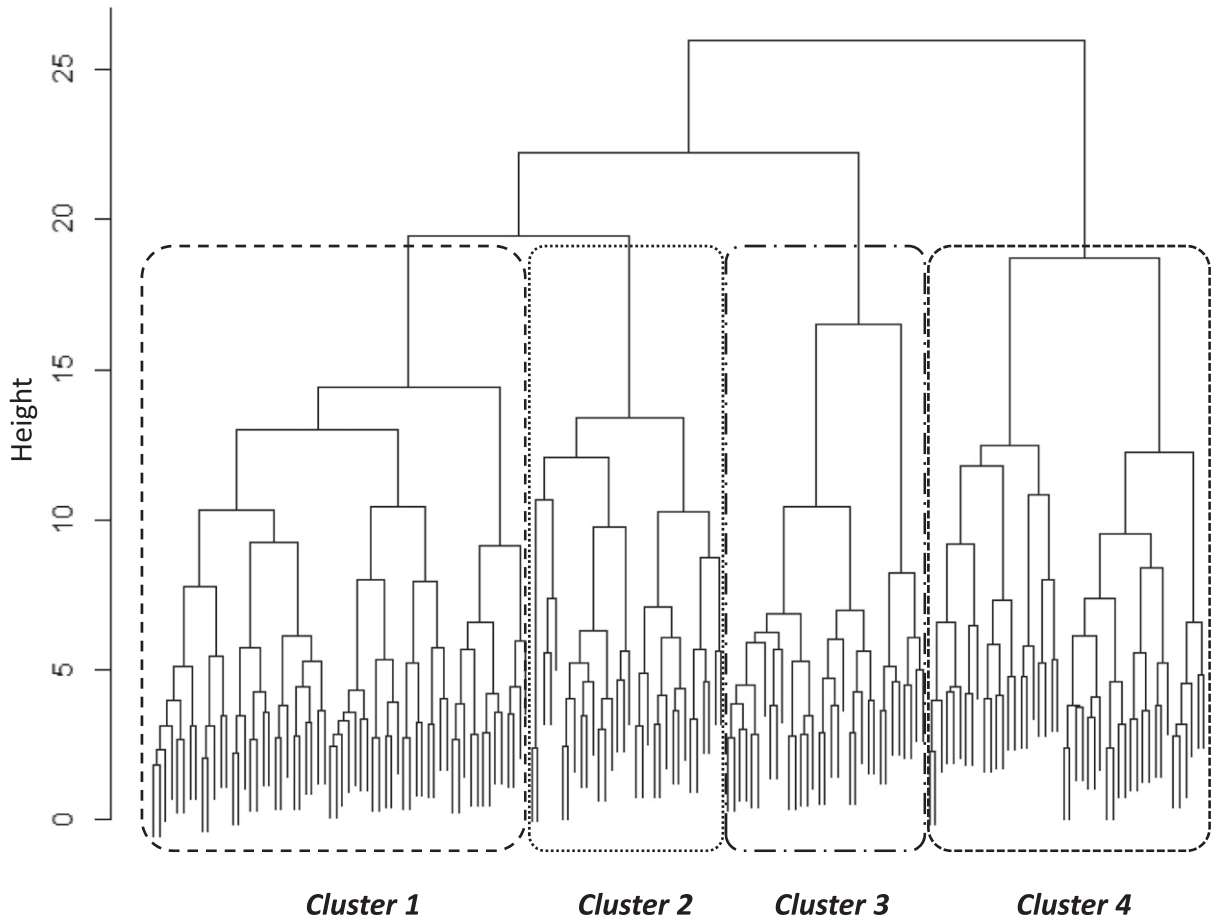

Figure 1. Dendrogram illustrating the identification of 4 clusters in 173 patients with C3G or IC-MPGN. Each vertical line at the extremity of the dendrogram (bottom) represents a patient, and the length of the vertical lines represents the degree of dissimilarity between patients or groups of patients. The boxes define the four clusters.

Table 3. Clinical features, complement assessment, genetic screening, and histologic features in patients classified according to the clusters obtained through cluster analysis

Variable	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Overall *P* Value
N	62	32	33	46	
Sex, % men	61	41	55	61	0.24
Data at onset					
Age, yr	15 (±11.6)	17.2 (±13)	14.5 (±10.4)	28.8 (±19.8)[a,b,c]	<0.001
Microhematuria, %	90	93	94	64[a,b,c]	<0.001
Gross hematuria, %	37	34	50	20[a,c]	0.04
Proteinuria, %	93	94	85	89	0.51
Nephrotic syndrome, %	29	72[a,c,d]	24	24	<0.001
Renal impairment, %	10	19	6	39[a,c]	<0.001
Trigger event, %	38	31	23	22	0.29
Familiarity for nephropathy, %	23[c]	16	3	11	0.05
Serum C3, mg/dl	34.7 (±30.2)	34.5 (±37.9)	31 (±25.6)	86.7 (±35.6)[a,b,c]	<0.001
Serum C4, mg/dl	21.1 (±10.3)	19.1 (±13)	23.5 (±7.9)	23.6 (±7.5)	0.17
Plasma SC5b-9, ng/ml	1494 (±1325)[c,d]	1558 (±1285)[c,d]	591 (±1062)	416 (±395)	<0.001
Low serum C3 and normal C4, %	81	72[c]	94	50[a,b,c]	<0.001
LPV carriers, %	32	22	15	4[a,b]	0.004
C3NeF positive, %	53	53	75	9[a,b,c]	<0.001
LPV carriers and/or C3NeF, %	75	63	79	14[a,b,c]	<0.001
Data during follow-up, %					
Nephrotic syndrome	52	88[a,c,d]	52	50	0.003
High BP	26	44	27	63[a,c]	<0.001
CKD	18[b,c,d]	47	36	54	<0.001
ESRD	2[d]	6	6	22	0.003
Thrombotic microangiopathy	2	0	0	13[a,c]	<0.01
Histologic features					
Time onset to biopsy, yr, median (IQR)	0.6 (0.2–3.6)	0.2 (0.1–1.7)	0.6 (0.2–1.8)	0.4 (0.0–3.3)	0.31
Light microscopy					
Sclerotic glomeruli, %	4 (±8)	8 (±15)[c]	1 (±3)	17 (±21)[a,b,c]	<0.001
Crescents, %	1 (±4)	3 (±9)	18 (±31)[a,b,d]	4 (±10)[a]	<0.001
Degree of mesangial proliferation[e]	2 (±0.9)	1.4 (±1.1)[a,c]	2 (±0.8)	1.7 (±0.9)	0.01
Degree of endocapillary proliferation[e]	1.2 (±1.1)	1.6 (±1)	1.3 (±1.2)	0.5 (±0.8)[a,b,c]	<0.001
Degree of interstitial inflammation[e]	0.5 (±0.6)[b,d]	0.8 (±0.6)	0.6 (±0.8)	1 (±1)[c]	<0.001
Degree of interstitial fibrosis[e]	0.3 (±0.6)	0.5 (±0.6)	0.3 (±0.6)	0.8 (±0.9)[a,c]	<0.001
Degree of arteriolar sclerosis[e]	0.2 (±0.5)	0.2 (±0.5)	0 (±0.2)	0.6 (±1)[a,b,c]	<0.001
IF[e]					
C3	2.7 (±0.5)	2.7 (±0.5)	2.8 (±0.3)	2.5 (±0.7)[a,c]	0.03
IgA	0.1 (±0.4)	0.7 (±0.8)[a,c,d]	0.1 (±0.3)	0.2 (±0.7)	<0.001
IgG	0.4 (±0.7)[d]	2 (±0.9)[a,c,d]	0.5 (±0.8)	1 (±1.2)	<0.001
IgM	0.7 (±0.8)	1.2 (±0.9)	0.8 (±0.7)	0.8 (±1)	0.12
C1q	0.3 (±0.5)[d]	1.6 (±0.9)[a,c,d]	0.3 (±0.6)	0.6 (±1)	<0.001
Fibrinogen	0.4 (±0.8)	0.6 (±0.9)	0.3 (±0.8)	0 (±0.1)[a,b,c]	0.01
Electron microscopy, %					
Mesangial deposits	83[b,c,d]	52	44	53	<0.001
Subepithelial deposits	57[c,d]	38	19	36	0.004
Subepithelial hump-like deposits	20	14	19	16	0.88
Subendothelial deposits	84	93	9[a,b,d]	66[a,b]	<0.001
Intramembranous granular deposits	59	41	16[a,b,d]	41	<0.001
Intramembranous highly electron-dense ribbon-like deposits	7	0	73[a,b,d]	0	<0.001

Quantitative variables are expressed as mean (±SD) unless otherwise specified. Serum C3: reference 90–180 mg/dl; serum C4: reference 10–40 mg/dl; plasma SC5b-9: reference ≤400 ng/ml. IQR, interquartile range.

[a]Significantly different versus cluster 1.

[b]Significantly different versus cluster 2.

[c]Significantly different versus cluster 3.

[d]Significantly different versus cluster 4.

[e]Degrees of mesangial proliferation, endocapillary proliferation, interstitial inflammation, interstitial fibrosis, and arteriolar sclerosis as well as IF findings were graded using a scale of 0–3, including 0, trace (0.5+), 1+, 2+, and 3+.

Table 4. Overlap between histologic groups and clusters

Histologic Diagnosis	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Overall *P* Value[a]
C3GN	42	2	4	20	
DDD	4	0	21	0	
IC-MPGN	16	30	8	26	<0.001

[a]*P* value was calculated with the Fisher exact test.

Clustering Analyses

To investigate the presence of relatively homogeneous groups of patients on the basis of histologic, biochemical, genetic, and clinical features at onset, we performed unsupervised hierarchical cluster analysis using 34 variables reduced to 13 principal components (Supplemental Table 3). Cluster analysis identified four clusters (Supplemental Figure 2, Figure 1, Table 3). Initially, cluster 4 was separated from the others. It included patients with a lower prevalence of LPVs and/or C3NeF, normal or mildly altered serum C3 levels, and later onset. Subsequently, cluster 3, characterized by a higher prevalence of intramembranous electron-dense deposits, was divided from clusters 1 and 2, which showed highly increased plasma levels of soluble terminal complement complex SC5b-9. Finally, compared with cluster 1, cluster 2 included patients with stronger IgG, IgA, and C1q staining.

Cluster 1 included 62 patients with a high prevalence of LPVs and/or C3NeF (75%), low serum C3 (35±30 mg/dl), and very high plasma SC5b-9 levels (1494±1325 ng/ml). The mean age of onset was 15.0 years old. These patients were characterized by few crescents (1%±4%; $P<0.001$) in the kidney biopsy compared with the other clusters. On EM, mesangial deposits (83%) were more frequently detected compared with the other clusters ($P<0.001$).

Cluster 2 included 32 patients who, like those in cluster 1, had a high prevalence of LPVs and/or C3NeF (63%), low serum C3 (35±38 mg/dl), high plasma SC5b-9 (1558±1285 ng/ml), and a high prevalence of subendothelial deposits (93%; $P<0.001$ versus clusters 3 and 4). However, this cluster differed from the others due to stronger glomerular staining of IgG (2.0±0.9; $P<0.001$), IgA (0.7±0.8; $P<0.001$), and C1q (1.6±0.9; $P<0.001$) and a higher prevalence of nephrotic syndrome at onset (72%; $P<0.001$).

Patients in cluster 3 ($n=33$) showed a high prevalence of LPVs and/or C3NeF (79%) and low serum C3 (31±26 mg/dl) similar to clusters 1 and 2, but plasma SC5b-9 levels were significantly lower (591±1062; $P<0.001$ versus clusters 1 and 2), suggesting a different underlying pattern of complement activation. Histologically, this cluster was characterized by a higher prevalence of crescents (18%±31%; $P<0.001$) and intramembranous highly electron-dense deposits (73%; $P<0.001$) and fewer subendothelial deposits (9%; $P<0.001$) versus the other clusters.

Finally, cluster 4 included 46 patients with a lower prevalence of LPVs and/or C3NeF (14%; $P<0.001$), higher serum C3 (87±36 mg/dl; $P<0.001$), later age of onset (29±20 years old; $P<0.001$), and lower prevalence of microhematuria (64%; $P<0.001$) versus the other clusters. This cluster also had more sclerotic glomeruli (17%±21%; $P<0.001$) and arteriolar sclerosis (0.6±1.0; $P<0.001$) and less endocapillary proliferation (0.5±0.8; $P<0.001$) versus the other clusters. Interestingly, six of seven patients who initially presented C3G or IC-MPGN with bright glomerular C3 staining and later developed thrombotic microangiopathy fell into cluster 4.

Clusters 1 and 4 were composed prevalently of patients with C3GN and patients with IC-MPGN (Table 4). Clusters 2 and 3 included mostly patients with IC-MPGN (94%) and patients with DDD (64%), respectively. Interestingly, 21 of 25 patients with DDD and all three patients with IC-MPGN with intramembranous highly electron-dense deposits were included in cluster 3.

Cluster analysis was crossvalidated by half-splitting the cohort and repeating clustering and by the kmeans technique (Supplemental Table 4).

Algorithm for Cluster Identification on the Basis of Features Available at Onset

To select the minimum set of features available at onset, which could be used to assign patients to the different clusters, we performed a three-step analysis using binomial and multinomial logistic regression (Supplemental Tables 5 and 6). Seventeen different features were associated with one or more clusters: number of alternative pathway abnormalities (LPVs and C3NeF); serum C3; plasma SC5b-9; glomerulosclerosis; mesangial proliferation; endocapillary proliferation; IgG, C1q and fibrinogen glomerular staining; mesangial, subepithelial, subendothelial, and intramembranous highly electron-dense deposits; hematuria at onset; proteinuria at onset; familiarity for nephropathy; and the complement factor H (*CFH*) V62I variant. To make up a more robust list of features to assign patients to clusters, we repeated the analyses, adopting a 0.001 significance threshold (Supplemental Table 7, Table 5). The number of alternative pathway abnormalities and low serum C3 levels reduced the probability of belonging to cluster 4. C1q glomerular deposits (≥1+) increased the probability of belonging to cluster 2, and intramembranous electron-dense deposits increased the probability of belonging to cluster 3 (Table 5). The concordance of the multinomial logistic regression model using only the above four variables with the original cluster analysis was 75%. We used the above four features to design a three-step algorithm (Figure 2). Table 6 shows the features of the four algorithm-based clusters. The concordance of the algorithm-based clusters with the cluster analysis was 74%.

Prognostic Significance of the Clusters

We evaluated whether the four clusters identified by the unsupervised hierarchical cluster analysis were characterized by a different renal outcome. Kaplan–Meier analyses showed that

Table 5. Results of the multivariate multinomial logistic regression showing the features available at onset that independently predict the clusters adopting a significance *P* value threshold of 0.001

Feature	Prevalence, %	Group Versus Reference[a]	β	RR (e^{β})	*P* Value
No. of alternative pathway abnormalities[b]	45:12	4	−2.3	0.10	<0.001
Serum C3[c]	23:61	4	−2.0	0.14	<0.001
Intramembranous highly electron-dense deposits	16	3	3.8	44	<0.001
Glomerular C1q deposits ≥1+	38	2	3.2	25.2	<0.001

Only associations with a $P<0.001$ are shown. RR, relative risk.
[a]The group with the greatest number of patients (cluster 1) was taken as the reference group.
[b]Abnormalities include the alleles with LPVs and C3NeFs; the prevalence rates of patients with one and two abnormalities are reported.
[c]Serum C3 subdivided as normal (≥90 mg/dl), low (between ≥50 and <90 mg/dl), and very low (<50 mg/dl); the prevalence rates of patients with low and very low C3 are reported.

patients in the fourth cluster have a higher risk of ESRD compared with those in the other three clusters (Figure 3A). The higher degree of glomerulosclerosis in cluster 4 could account for worse renal outcome, because in the whole cohort, this parameter predicted ESRD (Supplemental Table 8). Similar results were obtained with Kaplan–Meier analyses of renal outcome in the algorithm-identified clusters (Figure 3B). At variance, when patients were grouped according to the C3G/IC-MPGN classification, no differences in renal outcome were observed (Figure 3C).

We compared the treatment received by patients in the four clusters (Supplemental Table 9). We observed a higher prevalence of patients treated with intensified immunosuppression in cluster 2, probably reflecting the higher prevalence of nephrotic syndrome at onset in this cluster. However, the renal outcome of this cluster is not different from those of clusters 1 and 3.

Seventeen patients underwent kidney transplantation (three, four, three, and seven in clusters 1–4, respectively). Disease recurred in five of 15 for whom information was available (two, two, and one in clusters 2–4, respectively).

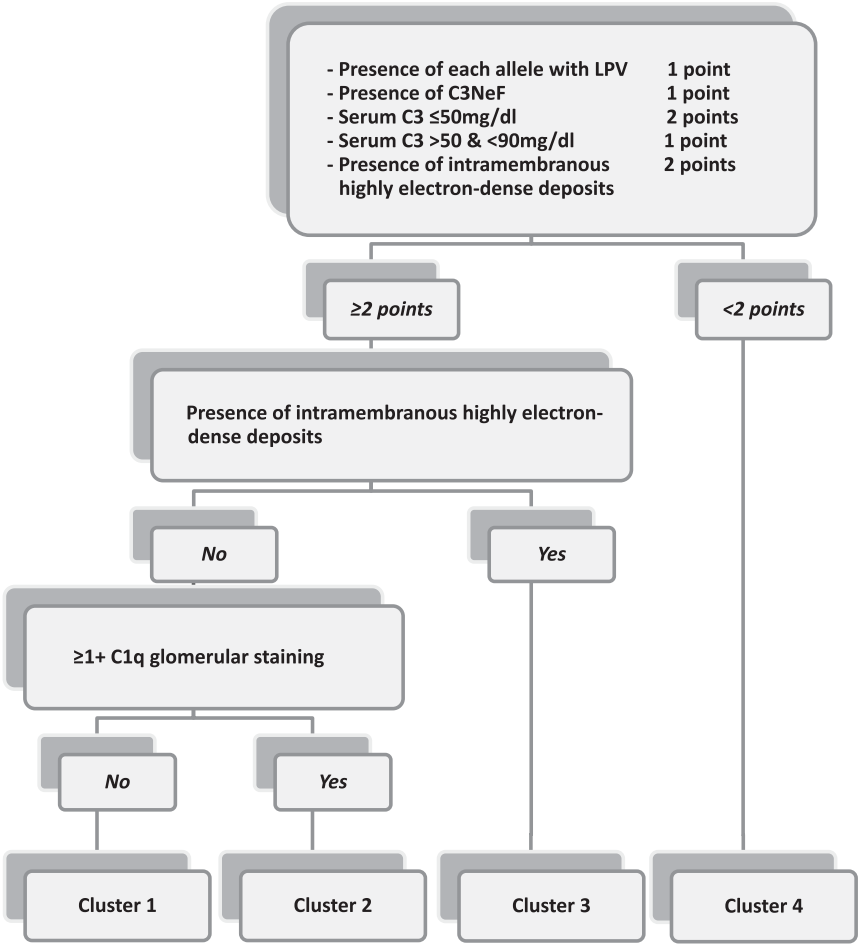

Figure 2. Algorithm to assign patients to the different clusters. A three-step algorithm was created based on features available at onset to assign patients with C3G/IC-MPGN to specific clusters.

Differences in Complement Gene LPV Distribution, C3NeF Activity, and Antifactor H Antibodies between Clusters

We observed that LPVs affecting *C3* and *CFB* were more frequent in clusters 1 (19%) and 2 (13%) versus clusters 3 (3%) and 4 (2%) (Figure 4A). Similar results were obtained using the clusters identified by the algorithm (Figure 4B). At variance, no differences in LPV distribution were observed between histologic groups or between C3G and IC-MPGN (Supplemental Figure 3).

C3NeFs of patients in cluster 1 showed a lower C3 convertase stabilizing activity (66%±35%) versus that in clusters 2 and 3, and differences were significant among clusters identified by algorithm (Figure 4, C and D). At variance, no differences in C3NeF activity were observed between histologic groups or between C3G and IC-MPGN (Supplemental Figure 3).

Antifactor H antibodies were measured in 93 patients and found positive in five (one of 34, one of 17, three of 20, and zero of 22 in clusters 1–4, respectively; clusters 1–3 versus 4: $P=0.34$) (Supplemental Table 2).

Table 6. Clinical features, complement assessment, genetic screening, and histologic features in patients classified according to the clusters obtained through the three-step algorithm for cluster definition

Variable	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Overall *P* Value
N	56	40	28	49	
Sex, % men	57	48	57	61	0.62
Data at onset					
Age, yr	15.3 (±12.4)	18 (±12.7)	15 (±10.8)	26.3 (±19.8)[a,b,c]	<0.001
Microhematuria, %	89	87	93	73	0.08
Gross hematuria, %	36	30	43	31	0.65
Proteinuria, %	93	98	86	86	0.17
Nephrotic syndrome, %	32	68[a,c,d]	21	18	<0.001
Renal impairment, %	13	23[c]	4	31[a,c]	0.01
Trigger event, %	32	26	32	29	0.92
Familiarity for nephropathy, %	16	13	11	17	0.89
Serum C3, mg/dl	31.6 (±21.2)	21 (±18.8)[a,c]	35.6 (±35.6)	96.2 (±28.8)[a,b,c]	<0.001
Serum C4, mg/dl	21.6 (±9.7)	20.6 (±12.6)	24.2 (±9.2)	21.8 (±7.5)	0.53
Plasma SC5b-9, ng/ml	1297 (±1281)[c,d]	2003 (±1389)[a,c,d]	523 (±508)[d]	308 (±154)	<0.001
Low serum C3 and normal C4, %	95	78	86	42[a,b,c]	<0.001
LPV carriers, %	34	25	14	2[a,b]	<0.001
C3NeF positive, %	52	60	79	7[a,b,c]	<0.001
LPV carriers and/or C3NeF, %	75	75	83	9[a,b,c]	<0.001
Data during follow-up, %					
Nephrotic syndrome	48	90[a,c,d]	50	47	<0.001
High BP	27	45	25	57[a,c]	0.004
CKD	27	45	25	47	0.06
ESRD	5	8	4	16	0.20
Thrombotic microangiopathy	2	0	0	12[a,b]	0.01
Histologic features					
Time onset to biopsy, yr, median (IQR)	0.4 (0.1–2.1)	0.3 (0.0–1.9)	0.4 (0.4–3.8)	0.4 (0.0–3.0)	0.23
Light microscopy					
Sclerotic glomeruli, %	4 (±8)	6 (±12)	2 (±6)	16 (±21)[a,b,c]	<0.001
Crescents, %	6 (±17)	3 (±8)	6 (±19)	6 (±17)	0.73
Degree of mesangial proliferation[e]	2 (±0.9)	1.7 (±1.1)	1.9 (±0.8)	1.6 (±1)	0.22
Degree of endocapillary proliferation[e]	1.3 (±1.2)[d]	1.6 (±1)[c,d]	1 (±1.1)	0.6 (±0.8)	<0.001
Degree of interstitial inflammation[e]	0.4 (±0.6)[b,d]	0.8 (±0.7)	0.7 (±0.9)	1 (±0.9)	0.001
Degree of interstitial fibrosis[e]	0.3 (±0.6)	0.5 (±0.8)	0.2 (±0.4)	0.8 (±0.9)[a,c]	0.003
Degree of arteriolar sclerosis[e]	0.1 (±0.5)	0.2 (±0.5)	0.1 (±0.2)	0.6 (±1)[a,b,c]	<0.001
IF[e]					
C3	2.7 (±0.5)	2.7 (±0.5)	2.8 (±0.3)	2.5 (±0.7)[a,c]	0.04
IgA	0.1 (±0.4)[b,d]	0.4 (±0.7)[c]	0.1 (±0.3)	0.4 (±0.8)	<0.01
IgG	0.3 (±0.8)	1.5 (±1)[a,c]	0.4 (±0.7)	1.2 (±1.2)[a,c]	<0.001
IgM	0.6 (±0.8)	1.3 (±0.9)[a,c,d]	0.7 (±0.6)	0.8 (±1)	0.001
C1q	0 (±0.1)[c,d]	1.7 (±0.7)[a,c,d]	0.2 (±0.5)[d]	0.7 (±0.9)	<0.001
Fibrinogen	0.4 (±0.8)	0.5 (±0.9)	0.3 (±0.7)	0.1 (±0.3)[a,b]	0.05
Electron microscopy, %					
Mesangial deposits	72	72	50	50[a,b]	0.04
Subepithelial deposits	48	49	11[a,b,d]	43	<0.01
Subepithelial hump-like deposits	22	14	11	20	0.55
Subendothelial deposits	77	87	11[a,b,d]	70	<0.001
Intramembranous granular deposits	60	54	0[a,b,d]	40	<0.001
Intramembranous highly electron-dense ribbon-like deposits	0	0	100[a,b,d]	0	<0.001

Quantitative variables are expressed as mean (±SD) unless otherwise specified. Serum C3: reference 90–180 mg/dl; serum C4: reference 10–40 mg/dl; plasma SC5b-9: reference ≤400 ng/ml. IQR, interquartile range.

[a]Significantly different versus cluster 1.

[b]Significantly different versus cluster 2.

[c]Significantly different versus cluster 3.

[d]Significantly different versus cluster 4.

[e]Degrees of mesangial proliferation, endocapillary proliferation, interstitial inflammation, interstitial fibrosis, and arteriolar sclerosis as well as IF findings were graded using a scale of 0–3, including 0, trace (0.5+), 1+, 2+, and 3+.

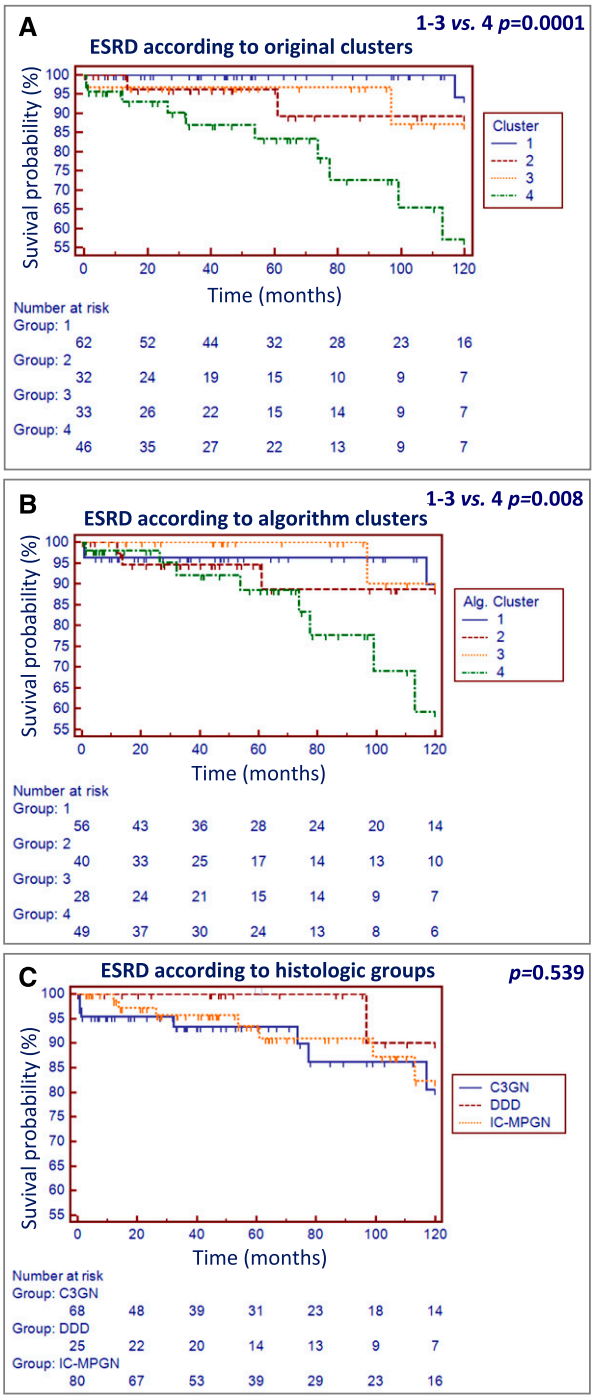

Figure 3. Patients in cluster 4 have poor renal outcomes. Kaplan–Meier renal survival analysis according to (A) the groups obtained by the cluster analysis, (B) the clusters defined by the three-step algorithm, and (C) the histologic groups.

DISCUSSION

Here, in a large cohort of 173 patients with C3G and patients with IC-MPGN, we integrated histology features with clinical, biochemical, and genetic parameters and performed unsupervised cluster analysis. Through this approach, we succeeded in distinguishing patients with massive fluid-phase complement activation from patients with solid-phase activation. Moreover, we identified three distinct pathogenetic mechanisms within patients with fluid-phase complement activation. We have also provided a simple algorithm to assign patients to the four clusters, which combines histology features with complement profile, all parameters usually available in these patients.

The four clusters were characterized by peculiar clinical phenotypes, IF and EM features, and complement abnormalities, indicating the existence of multifaceted molecular mechanisms underlying C3G/IC-MPGN. Finding that patients in clusters 1–3 have very low serum C3 levels, a high prevalence of LPVs in genes of the alternative complement pathway, and/or C3NeF strongly indicates that fluid-phase alternative pathway dysregulation plays a major role. These data represent an advancement to the "C3-dominant" staining criterion,[3] which showed low sensitivity for capturing patients with complement gene LPVs and/or C3NeF or patients with low serum C3 and normal C4.

Cluster analysis evidenced particular features that distinguish clusters 1–3 from each other. The very high SC5b-9 plasma levels in patients from clusters 1 and 2 suggest massive complement activation until the terminal pathway. This possibility is consistent with findings that, in clusters 1 and 2, there is a prevalence of LPVs in *C3* and *CFB*, which encode the two components of both the C3 convertase (C3bBb) and the C5 convertase (C3bBbC3b) of the alternative pathway. Conceivably, C3NeFs in clusters 1 and 2 may stabilize the C5 convertase efficiently, resulting in high SC5b-9 plasma levels, as confirmed by findings of C3NeFs, which alongside C3 convertase, stabilize the C5 convertase.[17,18] In these two groups, C3 and C5 complement activation products formed in the fluid phase would accumulate in the glomerulus, forming amorphous deposits along the glomerular membrane layers. However, unlike cluster 1, in cluster 2, the alternative pathway abnormalities are combined with glomerular C1q deposits, which are always associated with IgG and/or IgM deposits. This finding strongly suggests that classic complement pathway activation may be required additionally to trigger the disease in patients in cluster 2, because the classic pathway is initiated by C1q binding to Ig-antigen complexes.[19] One could argue that cluster 1 represents a more advanced phase of the same pathologic process underlying cluster 2, in which the C1q and IgG deposits have been cleared. We discard this hypothesis on the basis of the time from onset to biopsy not differing between the two clusters. In addition, the significantly higher prevalence of nephrotic syndrome at onset and during follow-up in cluster 2 supports the existence of a particular pathogenetic mechanism in this cluster.

Regarding cluster 3, the low serum C3 levels associated with normal or mildly increased plasma SC5b-9 levels indicate prevalent activation of the alternative pathway C3 convertase in fluid phase. We find that C3NeFs isolated from patients from cluster 3 stabilize C3 convertase very efficiently. Moreover, LPVs identified in cluster 3 mostly affect genes encoding com-

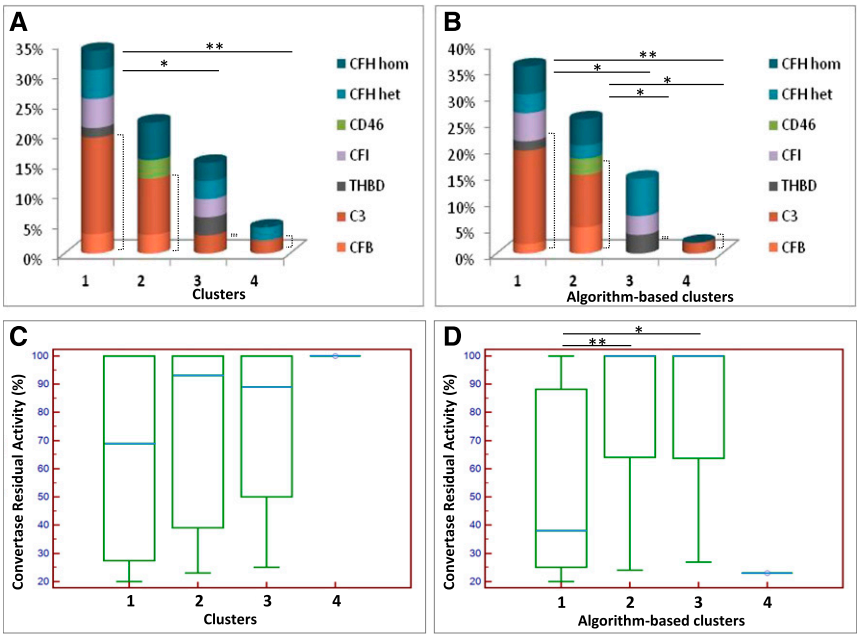

Figure 4. The four clusters show differences in distribution of LPVs and in C3NeF residual activity. (A and B) Distribution of the LPVs according to (A) the clusters and (B) the algorithm-based clusters. LPVs in *C3* and *CFB* are over-represented in clusters 1 and 2 compared with cluster 3. *$P<0.05$; **$P<0.01$. (C and D) C3NeF residual activity evaluated by hemolytic assay in C3NeF-positive patients according to (C) the clusters and (D) the algorithm-based clusters. C3NeFs of patients in cluster 1 stabilize alternative pathway C3 convertase less efficiently than those of patients in clusters 2 and 3. The central box represents the values from the 25th to 75th percentiles. The blue lines represent the medians. Lines extend from the minimum to the maximum values. *$P<0.05$; **$P<0.01$.

plement regulators, namely *CFH*, complement factor I (*CFI*), and thrombomodulin (*THBD*), further differentiating this cluster from the first two. Altogether, these peculiar abnormalities result in intramembranous highly electron-dense deposits, so that the large majority of patients with DDD fall into this cluster. These findings are consistent with previous data showing that dysregulation of the C3 convertase prevails over dysregulation of the C5 convertase in DDD, where the highly electron-dense, midlayer deposits may represent a continuous, slow buildup of C3 breakdown products.[18,20]

Remarkably, in the cluster analysis, we considered the presence of C3NeF and LPVs as variables but not the C3NeF efficiency to stabilize C3 convertase or which genes were affected by the LPVs. Finding differences in C3NeF activity and LPV localization between clusters confirms our approach's validity for identifying groups with distinct pathogenetic mechanisms.

One of the most intriguing findings here is the identification of a group of patients, cluster 4, with a unique complement phenotype. Unlike clusters 1–3, cluster 4 is characterized by a low prevalence of complement gene LPVs and/or C3NeF and likely, normal serum C3 and plasma SC5b-9. The bright C3 glomerular staining suggests that local solid-phase complement activation on glomerular cells or along the glomerular basement membrane occurs in cluster 4.[21] This hypothesis is further supported by the finding that cluster 4 includes six of seven patients who, during follow-up, developed thrombotic microangiopathy, a condition associated with cell surface complement activation on glomerular endothelial cells.[21] Alternatively, continuous low-grade fluid-phase C3 activation could contribute to glomerular C3 deposits.

We speculate that, in cluster 4, there is a low level of complement activation with continuous glomerular deposition of complement effector molecules, leading to chronic progressive subclinical injury until presenting with irreversible damage. Indeed, patients in this cluster present later onset, more glomerulosclerosis, and more advanced interstitial and arteriolar lesions. This would translate into a higher risk of ESRD,[22,23] as observed in our cohort. The latter is consistent with previous data from our group showing a higher risk of ESRD in patients with C3G/IC-MPGN without LPVs or C3NeF.[10] Indeed, LPVs in known disease-associated complement genes are rare in cluster 4, although we were able to identify two LPVs, one in *CFH* and one in *C3*. Interestingly, another *C3* mutation resulting in solid-phase restricted complement activation on podocytes and glomerular endothelial cells has recently been reported in two patients with C3GN, adult onset, progression to ESRD, and normal C3 levels,[24] all features shared with cluster 4. In addition, internal duplications and genomic rearrangements affecting CFHR genes have been reported in patients with C3G who, like patients in cluster 4, usually show intense C3 glomerular deposits but a normal serum complement profile.[25–27]

In conclusion, by using a data-driven statistical approach, we identify clusters of patients with C3G/IC-MPGN and distinct underlying mechanisms characterized by different clinical features and renal survival. The newly identified clusters may be useful for better defining the multifaceted molecular mechanisms underlying C3G/IC-MPGN and to predict the risk of ESRD and the response to anticomplement therapies. Patients from clusters 1 and 2, characterized by intense C5 convertase activation, may be more likely to respond to anti-C5 blockade, which is consistent with published data showing that a high level of plasma SC5b-9 was potentially a marker of responsiveness.[20,28] Patients from cluster 3 might benefit from new molecules under clinical development, such as factor D or CFB inhibitors that target the C3 convertase of the alternative pathway of complement.[29] Finally, emerging complement inhibitors targeting C3 activation products on cell surfaces, such as TT30,[30] might be helpful for blocking solid-phase restricted complement activation in patients in cluster 4. The latter may lack the side effects of unselective C3 inhibitors, such as infections and autoimmunity.

CONCISE METHODS

Through the Italian Registry of MPGN, we recruited 173 patients with C3G or IC-MPGN classified according to the recent C3G/IC-MPGN classification.[2,3] Briefly, patients with "dominant C3" glomerular staining (intensity greater than or equal to two magnitude orders greater than other immune reactants) were considered C3G, whereas patients with an MPGN pattern and significant Ig deposits were considered IC-MPGN. By EM, C3G was further classified as DDD or C3GN.[3] We excluded patients with MPGN secondary to autoimmune diseases, monoclonal gammopathy, infections (HBV, HCV, and HIV) or neoplasms, atypical hemolytic uremic syndrome preceding or concomitant to MPGN onset, and no available IF, EM, or DNA samples. All participants provided informed written consent. The study was approved by the Ethics Committee of the Azienda Sanitaria Locale of Bergamo.

Clinical data were recorded using standardized case report forms. Screening of *CFH*, *CD46* (encoding membrane cofactor protein), *CFI*, *CFB*, *C3*, and *THBD* gene exons was performed by amplicon-based next generation sequencing.[10] C3NeF activity was determined by assessing the ability of plasma-purified IgGs to stabilize cell-bound C3bBb convertase.[31] Serum C3 and C4 levels were assessed by kinetic nephelometry.[32] Plasma SC5b-9 levels were measured using an ELISA-based commercial kit. Detailed methods are provided in Supplemental Material.

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None of the activities listed in the Disclosures have had any influence on the results or interpretation in this article.

A complete membership list of the registry of MPGN/C3G is in Supplemental Appendix.

DISCLOSURES

M.N. has received honoraria from Alexion Pharmaceuticals for giving lectures and participating in advisory boards. G.R. has consultancy agreements with AbbVie, Alexion Pharmaceuticals, Bayer Healthcare, Reata Pharmaceuticals, Novartis Pharma, AstraZeneca, Otsuka Pharmaceutical Europe, and Concert Pharmaceuticals. G.R. did not accept personal remuneration; compensations are paid to his institution for research and educational activities. The other authors declare that no financial conflict of interest exists.

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See related editorial, "Clusters Not Classifications: Making Sense of Complement-Mediated Kidney Injury" on pages 9–12.

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