# AUTOANTIBODIES IN KIDNEY DISEASES

EDITED BY: Kevin James Marchbank, Bradley Patton Dixon,
Marie-Agnes Dragon-Durey and Ashley Frazer-Abel
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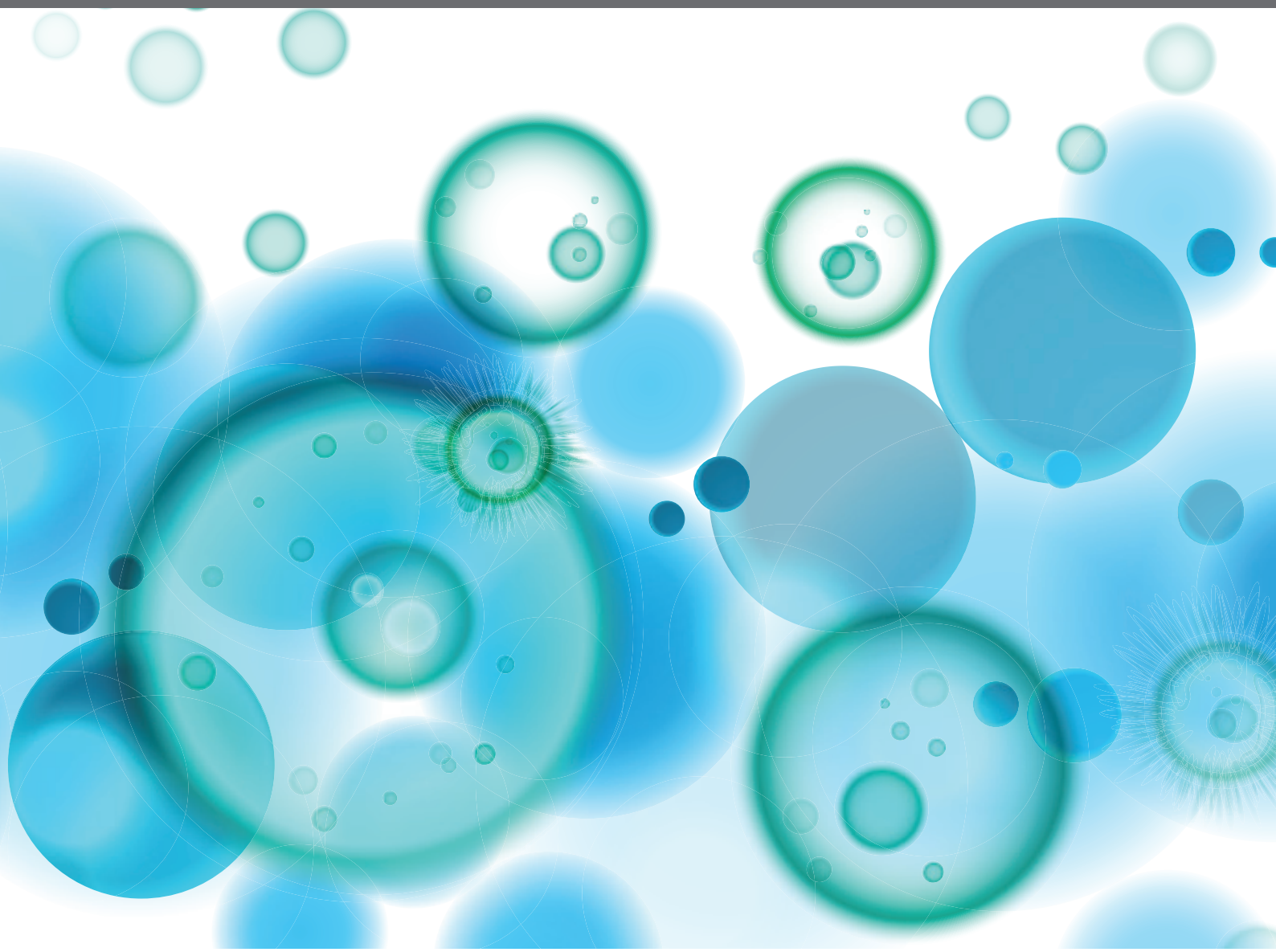

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# AUTOANTIBODIES IN KIDNEY DISEASES

Topic Editors:
**Kevin James Marchbank,** Newcastle University, United Kingdom
**Bradley Patton Dixon,** Children's Hospital Colorado, United States
**Marie-Agnes Dragon-Durey,** Université Paris Descartes, France
**Ashley Frazer-Abel,** Exsera BioLabs, United States

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EDITORIAL
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# Editorial: Autoantibodies in Kidney Diseases

*Kevin J. Marchbank*[1,2]*, *Ashley Frazer-Abel*[3], *Marie-Agnes Dragon-Durey*[4] and *Bradley P. Dixon*[5,6]

[1] Translational and Clinical Research Institute, Newcastle University, Newcastle upon Tyne, United Kingdom, [2] National Renal Complement Therapeutics Centre, Newcastle upon Tyne, United Kingdom, [3] Medicine-Rheumatology, University of Colorado Denver, Denver, CO, United States, [4] Laboratory of Immunology, Georges Pompidou European Hospital, APHP, INSERM UMRS1138, Cordeliers Research Center, Université de Paris, Paris, France, [5] Department of Pediatric Nephrology, University of Colorado Denver, Denver, CO, United States, [6] Department of Pediatric Nephrology, Children's Hospital Colorado, Aurora, CO, United States

**Keywords: autoantibody (aAb), complement, aHUS, C3G, lupus, immune complex, IgAN pathogenesis, nephrology**

## Editorial on the Research Topic

## Autoantibodies in Kidney Diseases

The purpose of this Frontier in Immunology Research Topic. Autoantibodies (aAb) in Kidney Diseases was to bring diverse knowledge, experience and view-points together into one collection highlighting the role of aAb to complement proteins in the genesis and severity of kidney disease, and providing evidence for common pathways that lead to autoantibody-induced pathology and the emergence of the aAb themselves. It was postulated that deepened insight of the crosstalk between complement and autoantibody-based pathologies would provide unique opportunities to understand and treat these diseases. Reading this collection as a body of work should stimulate vivid conversations between you and your colleagues around new avenues of investigation.

At time of writing, greater than 30 thousand views of the articles in the collection has occurred, suggesting that one key aim has already been met; the dissemination of collated thoughts and key original research on this topic. The association of anti-Complement aAb with disease has been long appreciated, yet our understanding of their roles in development of disease still remains open to interpretation. Herein, and since the days they were called immunoconglutinins (see Vasilev et al.), understanding the linkage between nephritic factors (antibodies that bind the C3/C5 convertases) or the individual components of these complexes and their modification of disease processes is as important now (see the review by Hauer et al. and Corvillo et al.) as it ever was. Our collection provides considered perspective on the mechanisms of action of nephritic factors at various stages of the complement pathway (see Hauer et al. Corvillo et al. and Zhao et al.), with different consequences/outcomes depending on titer, subclass of Ig and context. Importantly, the precision of current testing to characterize nephritic factors is also key from a clinical perspective, beautifully appraised by Corvillo et al.. Zhao et al. also reminds us that the *CFHR* gene deletions that predominately associate with anti-FH formation in aHUS do not co-associate with C3 nephritic factors in C3G and speaks to multiple routes to the generation of aAb in kidney diseases. Thus, the articles herein also provide insight into the evolution of anti-complement protein/convertase aAb by explaining the potential roles of the cryptic epitope, altered self and immune "danger signal" that undoubtedly interact with patient genetic factors to deliver the perfect storm of events that generate aAb; to generate the immune complexes (IC) or non-lethal membrane attack complex (MAC) that wreak havoc in the kidney.

Collectively, we have learned that much still remains to be discovered from anti-FH aAb associated with aHUS. Puraswani et al. noted in their large longitudinal cohort study that high anti-FH titers predict relapse, but do not correlate well with disease activity. The establishment



***Edited and reviewed by:***
*Betty Diamond,*
*Feinstein Institute for Medical Research, United States*

***Correspondence:**
*Kevin J. Marchbank*
*kevin.marchbank@newcastle.ac.uk*

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of an international reference titer has allowed a degree of correlation between high titers (over 1,000 AU/RU) and low levels of C3, confirming activation of the alternative pathway. However, correlation between low free-FH levels, predicting early relapses in patients, with high anti-FH titers as described by Puraswani et al. is less common in other studies. It likely underlines that immune complexed FH is not well-tolerated, similar to anti-dsDNA (Wang and Xia). "Stable" autoantibody titer over time appears both historically and contemporaneously a common feature of anti-complement protein aAb in many patients across the reviews and primary research in this collection.

Another major commonality is the importance of prompt detection of aAb followed by rapid delivery of clinical intervention to achieve optimal outcomes, eculizumab being particularly effective in many studies despite not altering aAb titer. Interestingly, Zhao et al. also outlined the potential for eculizumab to halt complement dysregulation at the level of C5 in the presence of C5 nephritic factors, again suggesting rationale for its use where high tire anti-complement aAb are identified early in the disease course. Through the use of highly defined control populations, Valoti et al. re-affirmed the genetic and environmental aspects of the development of anti-FH aAb clarifying the complexity of both the genesis and patho-mechanistic outcomes associated with anti-FH aAb. Wang and Xia provide a stellar review of anti-dsDNA aAbs allowing a side by side comparison of the commonality and key differences noted to anti-complement aAbs. For instance, we are reminded that antigen presentation pathways and loss of tolerance are underappreciated in the genesis of anti-complement protein aAb, and that similar mechanisms must lie behind breaks in tolerance across these diseases.

AAb targeting to cell surfaces in the kidney generates multifaceted immune activation through direct stimulation of cells as is highlighted in the review of autoimmune membranous nephritis by Liu et al. Activation of cells through IC deposition, both directly and indirectly via multiple signaling pathways, exemplifies the challenges that are faced in deciphering the mechanisms that drive kidney disease. Non-lethal effects of complement activation are a significant factor in endothelial, epithelial, podocyte, and tubulointerstitial injury in the kidney. This is further confirmed through the study by Goutaudier et al. focused on C5b-9 as a biomarker of poor prognosis in the context of antibody mediated rejection, suggesting that assembly of MAC on renal tissues, understandably, is a marker for the more severe cases of Ab mediated rejection, ineffective complement regulation and potentially a driver of GBM structural changes. Rizk et al. present a clear and compelling case for control of complement hyperactivity in IgA Nephropathy (IgAN). Historically it is well-appreciated that C3 deposition is extremely common in the glomeruli of patients with IgAN and our knowledge of the autoimmune process in IgAN is arguably the most developed. Altered self, driving auto-immunity–epitope drift, failure to correctly solubilize or clear the resultant IC systemically, and a failure to clear deposited IC in the kidney due to continued load drives AP activation and C3 deposition in the glomerular mesangium in a vicious cycle similar to other IC-driven disease discussed here. All in all, compelling evidence is given that restoration of the finely balanced control of the AP and LP in IgAN may have benefit for many patients, and together with most diseases discussed herein, with the number of complement-directed drugs coming to the clinic, targeted options for the treatment of these diseases may soon be at hand.

## AUTHOR CONTRIBUTIONS

KM wrote the initial draft of the editorial, this was reviewed principally by BD with comments from AF-A and M-AD-D. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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# Autoantibodies Against C3b—Functional Consequences and Disease Relevance

*Vasil V. Vasilev*[1†], *Maria Radanova*[2†], *Valentin J. Lazarov*[1], *Marie-Agnes Dragon-Durey*[3,4,5,6], *Veronique Fremeaux-Bacchi*[3,4,5,6] *and Lubka T. Roumenina*[4,5,6*]

[1] Nephrology Clinic, University Hospital "Tsaritsa Yoanna-ISUL," Medical University–Sofia, Sofia, Bulgaria, [2] Department of Biochemistry, Molecular Medicine and Nutrigenomics, Medical University Varna, Varna, Bulgaria, [3] Assistance Publique–Hôpitaux de Paris, Service d'Immunologie Biologique, Hôpital Européen Georges Pompidou, Paris, France, [4] INSERM, UMR_S 1138, Centre de Recherche des Cordeliers, Paris, France, [5] Sorbonne Universités, UPMC Univ Paris 06, Paris, France, [6] Université Paris Descartes, Sorbonne Paris Cité, Paris, France

The complement component C3 is at the heart of the complement cascade. It is a complex protein, which generates different functional activated fragments (C3a, C3b, iC3b, C3c, C3d). C3b is a constituent of the alternative pathway C3 convertase (C3bBb), binds multiple regulators, and receptors, affecting thus the functioning of the immune system. The activated forms of C3 are a target for autoantibodies. This review focuses on the discovery, disease relevance, and functional consequences of the anti-C3b autoantibodies. They were discovered about 70 years ago and named immunoconglutinins. They were found after infections and considered convalescent factors. At the end of the twentieth century IgG against C3b were found in systemic lupus erythematosus and recently in lupus nephritis, correlating with the disease severity and flare. Cases of C3 glomerulopathy and immune complex glomerulonephritis were also reported. These antibodies recognize epitopes, shared between C3(H2O)/C3b/iC3b/C3c and have overt functional activity. They correlate with low plasmatic C3 levels in patients. *In vitro*, they increase the activity of the alternative pathway C3 convertase, without being C3 nephritic factors. They perturb the binding of the negative regulators Complement Receptor 1 and Factor H. The clear functional consequences and association with disease severity warrant further studies to establish the link between the anti-C3b autoantibodies and tissue injury. Comparative studies with such antibodies, found in patients with infections, may help to uncover their origin and epitopes specificity. Patients with complement overactivation due to presence of anti-C3b antibodies may benefit from therapeutic targeting of C3.

**Keywords: anti-C3 autoantibodies, anti-C3b autoantibodies, complement, immunoconglutinins, autoimmunity, systemic lupus erythematosus, lupus nephritis, C3 glomerulopathy**

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***Edited by:***
*Alexandre Belot, Claude Bernard University Lyon 1, France*

***Reviewed by:***
*Josh Thurman, University of Colorado, United States*
*Marie Nathalie Sarda, Université de Lyon, France*

***Correspondence:**
*Lubka T. Roumenina*
*lubka.roumenina@crc.jussieu.fr*

*[†]These authors have contributed equally to this work*

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## INTRODUCTION

The complement system is apart of the innate immune defense (1, 2). Autoantibodies against complement components and regulators have proven pathogenic effect. These antibodies (Ab) may cause acquired functional deficiencies of the complement cascade or induce amplification of an already activated complement (3–6). This review is focused on the auto-antibodies,

recognizing the activation products of the central complement component C3. The history of their discovery, their prevalence in different diseases as well as their functional and clinical relevance are discussed. Multiple unanswered questions open avenues for future studies.

## THE COMPLEMENT COMPONENT C3—STRUCTURE AND FUNCTION

C3 is the central component of the complement system (7). It is the convergent point of the three pathways of the cascade (1, 2). C3 is a 185-kDa glycoprotein, which belongs to the α2-macroglobulin family, part of the thioester-containing protein superfamily. C3 consists of two polypeptide chains—α-chain (110 kDa) and β-chain (75 kDa), linked by one disulfide bond and by non-covalent forces (8). It contains a globular thioester domain (TED) with an intrachain thioester bond, capable to attach covalently to surfaces; eight macroglobulin (MG) domains, and CUB domain, which frames and holds the TED domain (**Figure 1A**).

C3 undergoes low-rate spontaneous conformational change, leading to hydrolysis of its thioester bond (9). This generates an activated form of the protein, called C3(H2O). It does not bind to surfaces (as it no longer possesses a thioester group), but it resembles C3b in many of its functional and structural features, despite its retention of the ANA domain. C3(H2O) is the initiator of the alternative pathway, since it binds Factor B (FB) and Factor D (FD) and generates a fluid phase initiating convertase C3(H2O)Bb, capable to cleave C3. C3 is cleaved also by the classical or alternative pathway surface C3 convertases to generate C3a (9 kDa), a small potent pro-inflammatory molecule, and C3b (177 kDa) (**Figure 1B**). Generated C3b changes conformation, uncovering the binding sites for its ligands, including FB, Factor H (FH), complement receptor 1 (CR1, CD35), properdin, etc. (10). C3b interacts with Factor B to form the C3 convertase of the alternative pathway C3bBb (11, 12). Properdin is the only positive regulator of complement, stabilizing the convertase (13). The activity of C3b is tightly regulated to avoid accidental host tissue damage by negative complement regulators, like membrane-bound CR1, membrane cofactor protein (MCP; CD46), decay acceleration factor (DAF; CD55) or circulating regulators like FH, Factor-H-like protein 1 (FHL-1), and Factor I (FI) (14, 15). FI cleaves C3b in presence of cofactors (like the regulators MCP, FH, CR1) to generate iC3b (inactivated C3b, unable to form convertases). In presence of CR1 the cleavage by FI proceeds to C3dg and finally C3d fragments, remaining attached to the surface. When iC3b is cleaved, C3c is released (**Figure 1B**).

C3 activation fragments not only participate in cleaning of pathogens, apoptotic cells, and cellular debris but also in a number of homeostatic processes. This includes tissue regeneration, synapse pruning, controlling tumor cell progression, etc. (7). Therefore, alteration of the function of the activation fragments of C3 by genetic abnormalities results in pathological conditions (7). Indeed, mutations in the *C3* gene result in an abnormal protein, promoting complement overactivation and predisposing to renal injury (atypical hemolytic uremic syndrome) due to loss of regulation or direct overactivation of the C3 convertase (16–18). On the other hand, complete C3 deficiency shows increased susceptibility to bacterial infections in early childhood (19). Critical role of intracellular C3 activation for T cells function was recently described (20). This intracellular C3 activation, as well as the C3-based recycling pathway and C3 being a driver and programmer of cell metabolism suggest that the complement system utilizes C3 to guard not only extracellular but also the intracellular environment (21).

The activation products of C3 are also a target for autoantibodies. The following parts if this review describe the functional consequences and clinical relevance of the autoantibodies targeting C3b.

## FROM IMMUNOCONGLUTININS TO ANTI-C3B AB

The notion of Ab, recognizing activated forms of C3, dates from the mid-twentieth century, when they were named immunoconglutinins (22). Like rheumatoid factors are Ab binding to IgG, immunoconglutinins are Ab, binding components of complement. By definition, the immunoconglutinins are a group of Ab, formed in response to antigenic stimulation by components of an animal's own fixed complement components C3, but sometimes C4. They react against newly-formed epitopes, created after activation of C3 and C4, when the proteins change their conformations. Immunoconglutinins appear after viral or bacterial infections, the titers peak about 2 weeks following infection and usually drop rapidly afterwards (23–26). The Ab are frequently from IgM class. In chronic infections in animal models, high titres immunoconglutinins persisted over a prolonged period of time (27). At this period, it was concluded that the immunoconglutinins are convalescence factor, helping the healing process (25). Indeed, pretreatment of mice with immunoconglutinins prior to challenge with virulent strains of bacteria resulted in prolonged survival and decreased mortality (28). It was hypothesized that immunoconglutinins could enhance the clearance of bacteria by phagocytes.

This view was challenged, when immunoconglutinins/anti-C3 activated forms Ab were established in patients with autoimmune diseases. Such Ab were detected in systemic lupus erythematosus (SLE) (29–32), lupus nephritis (LN) (33, 34), in Crohn disease (35), in some nephrotic kidney diseases (36–38), in dense deposit disease (DDD) (39), in C3 glomerulopathy (C3G), and Immune Complex glomerulonephritis (IC-GN) (40) as well as in autoimmune-prone mice (32). However, these Ab has not been detected in primary biliary cirrhosis or rheumatoid arthritis (30, 32). A single patient with atypical hemolytic uremic syndrome, positive for anti-C3b Ab was also reported (41). These Ab were IgG and were measured as anti-C3 or anti-C3b Ab by ELISA (29, 31–34, 39–41) or as immunoconglutinins (36–38). In SLE they are predominantly belonging to IgG1 and IgG3 subclasses (30).

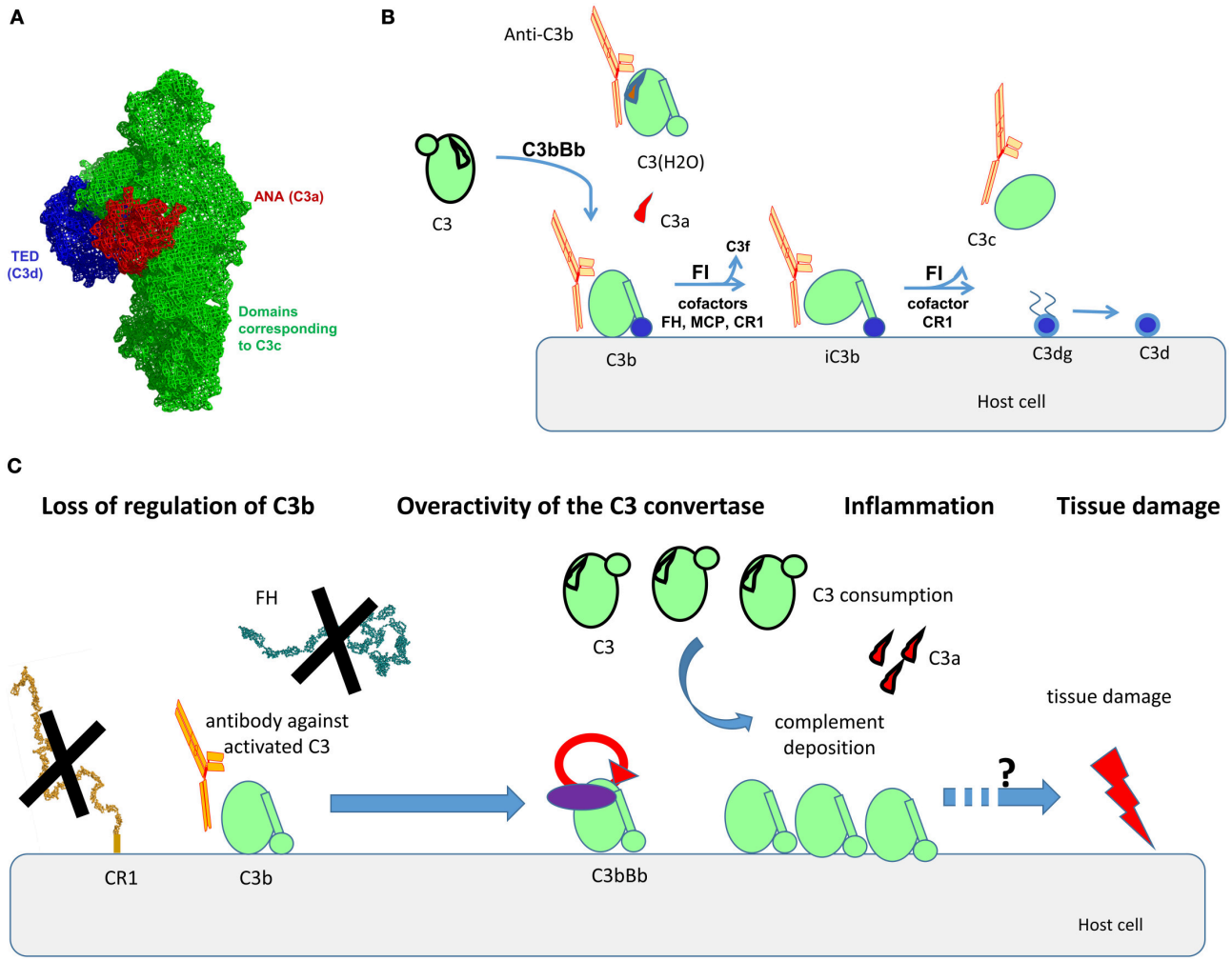


**FIGURE 1 |** Structural organization of C3, its activation forms, localization of the binding epitopes, and proposed mode of action of the anti-C3b auto-antibodies. **(A)** Structural organization of C3**,** based on the crystal structure published in Janssen et al. (8). The different domains, corresponding to the different fragments of the molecule are depicted with different color. Of note are the ANA domain, corresponding to C3a (red), the domains corresponding to C3c (green), and the TED domain, corresponding to C3d (blue). **(B)** Steps of cleavage of C3 by the C3 convertase of the alternative pathway C3bBb and generation of its activation forms. The cofactors needed for the cleavage of C3b by Factor I (FI) are indicated. The activation forms of C3, recognized by the autoantibodies (C3(H2O), C3b, iC3b, C3c) are indicated as complexed with an antibody in orange. **(C)** Proposed mode of action of the anti-C3b autoantibodies. The Ab will bind to C3b, preventing its interaction with CR1, and in the context of lupus nephritis, with Factor H (FH). This loss of regulation, together with the direct stabilization/enhancement of formation of the alternative pathway C3 convertase C3bBb will result in overactivation of complement, generating inflammation (via C3a), massive deposits of C3 activation fragments and finally, tissue damage. The exact mechanism linking complement overactivation to tissue injury and its contribution to organ injury need further studies.

## CLINICAL RELEVANCE OF THE ANTI-C3B AB

### SLE and LN

Systemic lupus erythematosus is a heterogeneous, multisystem autoimmune disease (42). Kidney involvement in SLE, also known as LN, is a common and serious organ complication that determines the quality of life and prognosis in patients with SLE and is characterized by specific clinical (nephritic or nephrotic syndrome), laboratory (proteinuria, hematuria), immune, and morphological (proliferative or non-proliferative glomerulopathy with mesangial, subendothelial, and subepithelial deposition of immune complexes, tubulointerstitial, and vascular lesions) manifestations. Different disorders of the regulation of the immune response with production of a wide range of Ab directed to various self-antigens (DNA, nuclear proteins, ribosomal proteins, and complement component C1q), are among the main characteristics of SLE and LN. The complement system plays a critical role in inflammatory and immune responses, in clearance of immune complexes and apoptotic cells, and autoreactivity to complement may have considerable pathological consequences (1, 2). The classical pathway has a predominant role in the initiation of the complement activation in SLE and LN, but the complement-mediated damage is often caused by the alternative pathway amplification loop (43).

Most of the reports in the literature related to anti-C3b concern cohorts of SLE or LN. The levels of anti-C3b Ab (measured as immunoconglutinins) were higher in active SLE compared to patients with inactive disease (29). We reported for the first time that more than 30% of LN patients (12/39) were positive for anti-C3 Ab (measured against C3 immobilized on an ELISA plate and confirmed against C3b) (33). Here again the levels of anti-C3b Ab were higher in the LN patients with severe disease, compared to a milder one. In the anti-C3b Ab-positive LN patients, the plasma levels of C4 and C3 were also lower compared to the negative ones, showing thus association of these Ab with complement activation. An additional case of LN revealed positivity against C3b, but also against FI, FB, C3, and properdin (44). The patient carried also a heterozygous mutation in the gene of C3.

In the same time Birmingham et al. showed in a bigger cohort an association of Ab against C3b with LN activity and their diagnostic and prognostic value (34). In this study, among 74 LN patients, 36% were positive for anti-C3b Ab, while only 1 out of 41 cases (2%) with non-renal SLE showed such positivity. Here again, the presence of anti-C3b Ab correlated with plasmatic C3 consumption. In the cross-sectional assessment, compared with anti-C1q IgG, anti-C3b IgG was less sensitive but more specific for lupus nephritis. In a longitudinal analysis, the rise of the levels of anti-C1q Ab were prognostic markers for LN flare only in anti-C3b Ab positive patients, thus defining anti-C3b Ab as a useful marker for to identify at risk patients for disease flare (34).

The presence of ant-C3b Ab correlated with the presence of anti-dsDNA (33). It was hypothesized that the dual presence of anti-dsDNA and anti-C1q Ab may coincide with, and possibly drive, a level of complement activation that leads to the onset of anti-C3b Ab (45).

## C3G and IC-GN

C3G is a renal disorder characterized by the presence of glomerular C3 staining in the absence of significant immunoglobulins staining (46). Subsets of C3G include DDD and C3 glomerulonephritis (C3GN). C3G is marked by the presence of glomerular deposits. They are electron dense (by electron microscopy) and localized in the lamina densa of the glomerular basement membrane in case of DDD or are subepithelial and subendothelial in patients with C3GN. The deposits in C3G contain complement components of the alternative pathway—C3b, iC3b, C3dg, C3c (47). The dysregulation of the alternative complement pathway, often caused by the presence of C3NeF, maintains a permanent activation of the complement (48).

In a cohort of 141 patients with C3G and IC-GN, only eight patients were positive for anti-C3b Ab, among which 5 were also positive for anti-FB Ab (40). These eight patients showed increased Bb fragment in plasma. Interestingly, the patients positive for anti-C3b Ab and anti-FB Ab in this cohort had higher rates of infections. Another study described two DDD patients with combined anti-C3b and anti-FB Ab (39). There is still not sufficient data that could determine the diagnostic value of anti-C3b Ab in patient with C3G and IC-GN.

# METHODS OF DETECTION AND BINDING EPITOPES

Nowadays the presence of anti-C3 activation fragments Ab can be detected routinely by ELISA (31–34, 40). More in depth characterization of the binding is done by surface plasmon resonance (SPR), allowing evaluation of the antigen-antibody interaction in real time as well as running functional tests for the formation, and regulation of the C3 convertase (33, 40, 49).

From the early studies it was known that the immunoconglutinins do not recognize native C3, but rather interact with its activated forms (22). Later studies revealed binding to immobilized C3 (hence C3(H2O)-like); C3b, iC3b, C3c with variable intensity but rarely to C3d and C3a (30, 33, 40). Therefore, the screening for diagnostic purpose should be done using coating with C3b, since it contains all recognized epitopes. C3 was also used as an antigen for the ELISA of detection (33), since it changes its conformation upon immobilization to the plastic, adopting most likely C3b(H2O)-like appearance, revealing the binding epitopes (8, 10). In some patients, positive for anti-C3b Ab, reactivity against immobilized C4 was detected as well (31, 33), but it is difficult to conclude whether these are separate Ab or a cross-reactivity of the anti-C3b ones. Nevertheless, all LN patients, positive for anti-C3b were negative for anti-C4b Ab in one of the published cohorts (34), suggesting that anti-C4/C4b Ab are most likely a separate entity occurring in some patients.

The anti-C3b Ab are different than the C3 Nephritic factor (C3Nef), since by definition C3Nef binds to the convertase C3bBb but not to its isolated components C3b and Bb (50). For one patient with very high titers of anti-C3b Ab, the C3Nef functional test remained negative (33). Moreover, anti-C3b Ab, together with Ab against FB, were detected in two unrelated patients with DDD without C3NeF activity (39).

The complexity of the C3 structure and the dramatic conformational change that the protein undergoes during its cleavage steps expose neoepitopes, which may drive pathological immune response. The ensemble of the results for the epitope mapping suggests that the immunodominant epitope is located within the domains, corresponding to the portion of the protein, which will become C3c after cleavage. This is supported by the fact that in the majority of the cases the reactivity is revealed toward C3b, immobilized C3, and C3c but rarely C3a and C3d (30, 33, 39–41). The domains corresponding to the C3c portion within C3b represent the central part of the protein, where are located the binding sites for FB (12) and numerous negative regulators, as FH, CR1, MCP, and DAF (14), **Figure 1B**. These data are relevant only to the anti-C3b Ab, found in autoimmune disease. Unfortunately, there is no epitope data about these Ab, occurring after infection. It is still unclear how the anti-C3b Ab originate and what is the immunization mechanism, but it could be explained by immune response to C3b neoepitopes. It was found that C3 could be phosphorylated at various sites, which influence its functional activity (51). It could be a reason for immunogenic properties of molecule, because increased levels of phosphorylated C3 are detected in SLE patients (52).

The activation products of C3 have diverse binding partners and functions, therefore it may not be surprising that acting on one epitope, the antibody will facilitate the pathogen clearance and on another—the complement overactivation, loss of regulation, and tissue damage. Another hypothesis could be that the binding epitopes are similar in both cases. In physiology, complement-overactivating anti-C3b IgM may help to clear the infection and disappear afterwards. Nevertheless, if they persist over time, switch to IgG, undergo epitope spreading, and reach high titers, the anti-C3b Ab could be of harm. This will be particularly relevant in autoimmune-prone background. Alternatively, C3 activation within the kidney may result in a C3b deposited in a way that presents neoeptiopes that drive anti-C3b IgG production.

## FUNCTIONAL CONSEQUENCES

It is critical to determine whether the anti-C3b Ab are protective, a disease-relevant factor or a simple epiphenomenon, one

**TABLE 1 |** Prevalence and functional consequences of the anti-C3b Ab in autoimmune diseases (cohort studies).

| | SLE (total) | SLE (non-renal) | LN | C3G/IC-GN | References |
|---|---|---|---|---|---|
| **PREVALENCE OF THE ANTI-C3b** | | | | | |
| Cohort 1, association with LN activity, frequently associated with anti-C1q Ab | 27/114 (24%) | 1/41 (2%) | 26/73 (36%) | | (34) |
| Cohort 2, association with LN activity | | | 12/39 (31%) | | (33) |
| Cohort 3 | 13/53 (25%) | | | | (32) |
| Cohort 4, association with activity | 17/20 (85%) | | | | (31) |
| Cohort 3, association with activity | 11/34 (32%) | | | | (29) |
| Cohort 4, frequently associated with infections and presence of anti-FB Ab | | | | 8/141 (6%) | (40) |
| recognized C3 fragments | | | | | (33) for LN (31) for total SLE (40) for C3G/IC-GN |
| C3(H2O) | 17/20 | | 4/4 | 8/8 | |
| C3b | 10/20 | | 4/4 | 8/8 | |
| C3c | 18/20 | | 4/4 | 4/8 | |
| iC3b | 15/20 | | 4/4 | n/a | |
| C3dg | 7/20 | | n/a | n/a | |
| C3d | 0/20 | | 0/4 | 1/8 | |
| C3a | n/a | | 0/4 | 1/8 | |
| C4/C4b | n/a | | 3/4 | 3/8 | |
| **FUNCTIONAL CONSEQUENCES** | | | | | |
| Inhibition of CR1 binding by SPR | n/a | | 7/7 | 4/4 | (33) for LN (30) for total SLE (40) for C3G/IC-GN |
| Inhibition of immune complexes release from CR1 | 2/3 | | n/a | n/a | |
| Inhibition of FH binding by SPR | n/a | | 4/4 | 0/6 | |
| Inhibition of FH cofactor activity | 3/3 | | 1/1 | n/a | |
| Inhibition of the C3 convertase dissociation by FH, western blot | n/a | | 1/1 | n/a | |
| C3 fragments deposition on resting endothelial cells, FACS | n/a | | 6/9 | 2/2 | |
| Ba generation after addition to serum, ELISA | n/a | | 5/5 | n/a | |
| Enhance C3/C5 convertase hemolytic test | n/a | | n/a | 2/7 | |
| Enhance C3 convertase by SPR | n/a | | 2/2 | 2/2 | |
| Inhibit C5 convertase hemolytic test | 2/3 | | n/a | n/a | |

among many, arising from the dysregulated immune response in the autoimmune diseases (53, 54). Although limited, the experimental studies clearly describe functional consequences for these Ab (**Table 1**). It was found that anti-C3b positive IgG, purified from plasma of patients with LN, SLE or C3G, and IC-GN, trigger overactivation of the complement cascade by the alternative pathway (30, 33, 39, 40). The mechanism of the complement activation, however, vary depending on the particular disease. In LN, C3G, and IC-GN, IgG from the patients enhanced C3 cleavage and the formation of new convertases. Moreover, they induced C3 activation fragments deposition on the surface of endothelial cells (33, 40), which could contribute to the disease process (55). These Ab, as well as the ones from SLE patients, inhibited also the interaction of C3b with its negative regulator CR1 and the Factor I-mediated cleavage (30, 33, 40). For two SLE samples, the anti-C3b Ab also perturbed the factor I-mediated release of immune complexes from CR1 (30). Nevertheless, only the tested Ab from LN perturbed the interaction of C3b with factor H (33, 40). Interestingly, these Ab differed also in terms of the stability of the formed complexes with C3b. The ones from C3G/IC-GN showed a rapid association but fast dissociation, while the C3-Ab from LN had a slow association rate but formed more stable complexes (49). This may explain in part the apparent functional differences. Based on the available functional data, the anti-C3b Ab perturb the binding of the negative regulators FH and CR1 to C3b, causing mostly loss of regulation and to a milder degree—direct overactivation of the C3 convertase.

Although the LN is clearly disease of overactivation of the classical pathway, it is important to note that the alternative pathway amplification loop is the main source of the terminal pathway complex C5b-9 (43). It is the one that generates a large number of C3b molecules that make both classical and alternative pathway C5 convertases. The capacity of anti-C3b Ab to perturb the regulation at the level of C3b would, therefore, result in an acceleration in the deposition of the C3 activation fragments, and might also result in enhanced C5b-9 deposits. The putative mechanism of action of the anti-C3b Ab is presented at **Figure 1C**.

Limited data is available for the action of anti-C3b Ab at the level of the C5 convertase. This process is not studied in LN. Nevertheless, anti-C3b/C3c Ab from two SLE patients inhibited the alternative pathway C3 convertase in an *in vitro* model, deprived of action of the complement regulators (30). On the contrary, in a relatively similar model, 2 out of 3 tested anti-C3b Ab from C3G/IC-GN patients enhanced C3/C5 convertase formation (40). Such data is not available for LN. Further studies are needed to elucidate the action of the anti-C3b Ab on the C5 convertase in different disease. It is tempting to speculate that in different pathological contexts the anti-C3b Ab will affect differently the convertases. In C3G, C3Nef, and C5Nef are described, acting differentially on the two convertases and having distinct disease associations (56).

Another aspect, which remains unexplored is the capacity of the anti-C3b Ab to bind to the C3b deposits in the kidney and to amplify the complement activation. This is possible in LN and IC-GN, where renal immunoglobulin deposits are clearly present and may, in part, be related to immune complexes, containing C3b as an antigen. In C3G, by definition, the patients do not have or have very limited, IgG deposited in the kidney. It is interesting to note that in C3G the presence of anti-C3b Ab is in fact, extremely rare, contrary to the high frequency in LN.

Apart from the action of the anti-C3b Ab at the level of the complement cascade, additional functions were described for these Ab. Suppression of apoptotic cell disposal by Ab against deposited C3 may contribute to increasing severity and/or exacerbations in different autoimmune diseases (32). Indeed, anti-C3b Ab blocked the recognition of C3b-opsinized cells by macrophages in a mouse model.

Further functional studies are needed in larger cohorts to determine to what extend the anti-C3b Ab have a pathogenic potential and whether they can transfer the disease, for example in a mouse model. Comparative studies are lacking for the anti-C3b Ab from patients/mice recovering from infections and the ones with autoimmune diseases. It is tempting to speculate that the functional consequences in the two contexts will be clearly different.

## CONCLUSION

Growing body of experimental data revealed that the anti-C3b Ab have overt functional consequences (30, 32, 33, 40) and correlate with disease severity at least in LN (33, 34). Additional clinical and experimental studies are needed to confirm their role in the disease pathogenesis and their relevance as a biomarker for the clinical practice. Nevertheless, the current state of the art shows arguments that identify the anti-C3b Ab as a potential diagnostic and prognostic marker in LN patients. The presence of anti-C3b Ab indicates a level of complement activation sufficient to initiate and accelerate the kidney damage. Therefore, these Ab should be taken into consideration in the management of LN. We hypothesize that patients with complement overactivation due to presence of anti-C3b Ab may benefit from therapeutic targeting of C3.

## AUTHOR CONTRIBUTIONS

LR, VV, and MR reviewed the literature and wrote the first draft. VF-B, M-AD-D, and VL revised the manuscript. All authors validated the submission.

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# C5b9 Deposition in Glomerular Capillaries Is Associated With Poor Kidney Allograft Survival in Antibody-Mediated Rejection

*Valentin Goutaudier[1], Hélène Perrochia[2], Simon Mucha[3], Marie Bonnet[1], Sylvie Delmas[1], Florian Garo[1], Valérie Garrigue[1], Sébastien Lepreux[4], Vincent Pernin[1,5], Jean-Emmanuel Serre[1], Ilan Szwarc[1], Pierre Merville[3,6], Annie Ramounau-Pigot[7], Céline René[7], Jonathan Visentin[6,8], Bryan Paul Morgan[9], Véronique Frémeaux-Bacchi[10], Georges Mourad[1], Lionel Couzi[3,6] and Moglie Le Quintrec[1,5]**

[1] University of Montpellier, Department of Nephrology, Dialysis and Transplantation, Lapeyronie Hospital, Montpellier University Hospital, Montpellier, France, [2] Department of Pathology, Gui de Chauliac Hospital, Montpellier University Hospital, Montpellier, France, [3] Department of Nephrology, Transplantation, Dialysis and Apheresis, Pellegrin Hospital, Bordeaux University Hospital, Bordeaux, France, [4] Department of Pathology, Pellegrin Hospital, Bordeaux University Hospital, Bordeaux, France, [5] INSERM U1183, Institute for Regenerative Medicine and Biotherapy, Saint-Eloi Hospital, Montpellier University Hospital, Montpellier, France, [6] UMR CNRS 5164, ImmunoConcEpT, Bordeaux University, Bordeaux, France, [7] Department of Immunology, Saint Eloi Hospital, Montpellier University Hospital, Montpellier, France, [8] Department of Immunology and Immunogenetics, Pellegrin Hospital, Bordeaux University Hospital, Bordeaux, France, [9] School of Medicine, Systems Immunity Research Institute, Cardiff University, Cardiff, United Kingdom, [10] Department of Immunology, Hospital European Georges Pompidou, Paris, France

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**Edited by:**
Bradley Patton Dixon,
Children's Hospital Colorado,
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**Reviewed by:**
Gaurav Gupta,
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University of Cincinnati, United States

***Correspondence:**
Moglie Le Quintrec
m-lequintrec-donnette@
chu-montpellier.fr

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C4d deposition in peritubular capillaries (PTC) reflects complement activation in antibody-mediated rejection (ABMR) of kidney allograft. However, its association with allograft survival is controversial. We hypothesized that capillary deposition of C5b9—indicative of complement-mediated injury—is a severity marker of ABMR. This pilot study aimed to determine the frequency, location and prognostic impact of these deposits in ABMR. We retrospectively selected patients diagnosed with ABMR in two French transplantation centers from January 2005 to December 2014 and performed C4d and C5b9 staining by immunohistochemistry. Fifty-four patients were included. Median follow-up was 52.5 (34.25–73.5) months. Thirteen patients (24%) had C5b9 deposits along glomerular capillaries (GC). Among these, seven (54%) had a global and diffuse staining pattern. Twelve of the C5b9+ patients also had deposition of C4d in GC and PTC. C4d deposits along GC and PTC were not associated with death-censored allograft survival ($p = 0.42$ and 0.69, respectively). However, death-censored allograft survival was significantly lower in patients with global and diffuse deposition of C5b9 in GC than those with a segmental pattern or no deposition (median survival after ABMR diagnosis, 6 months, 40.5 months and 44 months, respectively; $p = 0.015$). Double contour of glomerular basement membrane was diagnosed earlier after transplantation in C5b9+ ABMR than

in C5b9– ABMR (median time after transplantation, 28 vs. 85 months; $p = 0.058$). In conclusion, we identified a new pattern of C5b9+ ABMR, associated with early onset of glomerular basement membrane duplication and poor allograft survival. Complement inhibitors might be a therapeutic option for this subgroup of patients.

**Keywords: antibody-mediated rejection, kidney transplantation, complement, C4d, C5b9**

## INTRODUCTION

Antibody-mediated rejection (ABMR) is the most common cause of allograft failure after kidney transplantation (1). In active ABMR, donor-specific antibodies (DSA) bind to graft endothelium, and activate complement-dependent and -independent mechanisms that recruit natural killer (NK) cells, neutrophils and macrophages which contribute to inflammatory lesions [peritubular capillaritis (PTC), glomerulitis], cellular necrosis and thrombotic microangiopathy. In chronic active ABMR, a repetitive pattern of thrombotic events and inflammatory changes result in endothelial cell injury and allograft matrix remodeling, such as transplant glomerulopathy (2). The complement system plays a key role in the pathophysiology of ABMR. C4d accumulation along PTC—which reflects the ability of the DSA bound to the surface of endothelial cells to activate the classical complement pathway—is recognized as a tissue footprint marker in ABMR (3, 4). Several *in vitro* assays have recently been developed to test the ability of DSA to bind complement products. Loupy et al. (5) demonstrated that positive C1q-binding DSA in the first year after transplantation was associated with poor graft survival. Sicard et al. (6) observed that positive C3d-binding DSA at the time of ABMR diagnosis was an independent risk factor for graft loss. Moreover, Lefaucheur et al. (7) showed that ABMR in patients with predominant DSA IgG3 subclass—which is the most able to activate the complement cascade—was associated with the poorest graft survival.

However, the *in vitro* complement-fixing ability of DSA does not reflect complement activation on the endothelial cell surface and the association between positive C4d staining with allograft survival remains controversial (8–11). They both do not indicate ongoing complement-mediated endothelial injury. Complement regulatory proteins can stop at any step the complement activation cascade on endothelial cell surface.

In contrast, the deposition of the C5b9 membrane attack complex indicates complete complement cascade activation. The terminal pathway directly activates endothelial cells through sublytic concentrations of C5b9 and/or recruitment of inflammatory cells by the anaphylatoxins C3a and C5a, and can also be responsible for endothelial cell lysis (1). However, in spite of the major role the C5b9 membrane attack complex plays in this damage, it has never been evaluated *in vivo* in kidney allografts.

This study aimed to determine the frequency and location of C5b9 deposits in a well-phenotyped cohort of patients experiencing ABMR, and to evaluate their impact on allograft survival.

## METHODS

### Patients and Samples

We retrospectively selected transplant recipients with ABMR from the databases of the Departments of Pathology of two French University Hospitals (Montpellier and Bordeaux). To be included, patients had to be over 18 years and have undergone a renal biopsy that fulfilled criteria for a first histological diagnosis of (acute or chronic active) ABMR according to Banff 2015 classification from January 2008 to December 2013 at Montpellier Hospital and from January 2005 to December 2014 at Bordeaux Hospital, with positive DSA at time of biopsy. All biopsies were performed for cause: elevation of serum creatinine (>20% compared to baseline value) and/or a urine protein-to-creatinine ratio >50 mg/mmol. Complete immunofluorescence with anti-IgA, -IgG, -IgM, -C3, -C1q, -Kappa and -Lambda on frozen sections was performed in all patients. Exclusion criteria were the following: no serological evidence of anti-HLA DSA, insufficient renal tissue sample for further immunohistochemistry (i.e., <2 non-sclerosed glomeruli in each recut section), ABO-incompatible transplantation, combined transplantation, thrombotic microangiopathy and concomitant recurrent or *de novo* glomerulonephritis. The Institutional Review Board of Montpellier University Hospital approved this study (approval number: DC-2015-2473). All patients provided written informed consent to participate.

### Immunohistochemical Staining for C4d and C5b9

Staining for C4d and C5b9 was performed for all biopsies by immunohistochemistry. Briefly, paraffin-embedded sections were retrieved and cut at a thickness of 3-μm, deparaffinized and subjected to antigen retrieval. After blocking endogenous peroxidases, the sections were incubated with the relevant primary antibody. Binding of the primary antibody was visualized using the appropriate horseradish peroxidase-labeled secondary antibody and diaminobenzidine as the chromogen. Finally, the sections were counterstained with hematoxylin. The primary antibodies included rabbit monoclonal anti-human C4d (DB107, clone A24-T, dilution 1/100; DB Biotech) and mouse monoclonal anti-human C9 neoepitope (clone B7, dilution 1/50000; gift from Paul Morgan, Cardiff, United Kingdom), which is very specific to C5b9 fixation in membrane. C9

**Abbreviations:** ABMR, antibody-mediated rejection; C4d, complement split product C4d; C5b9, membrane attack complex; cg, double contour of glomerular basement membrane; DSA, donor-specific antibody; GC, glomerular capillaries; HLA, human leukocyte antigen; PTC, peritubular capillaries; TCMR, T cell-mediated rejection.

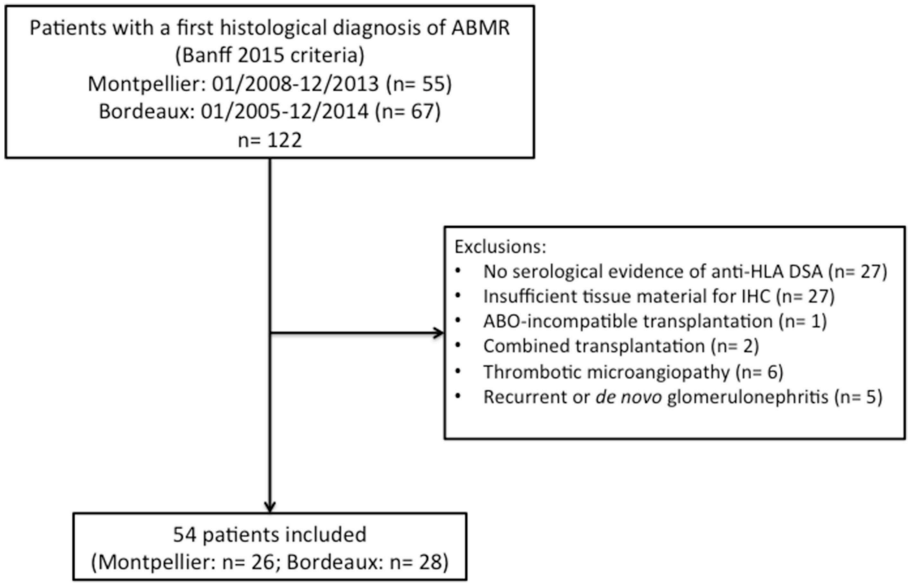


**FIGURE 1 |** Flow chart. ABMR, antibody-mediated rejection; HLA, human leukocyte antigen; DSA, donor-specific antibody; IHC, immunohistochemistry.

neoepitope detection was enhanced by the EnVision FLEX kit with linker (Dako).

The optimum antibody dilution and incubation were determined empirically for each primary antibody by performing a titration experiment and serial dilutions on positive (class 4 lupus nephritis) and negative controls (peritumoral kidney tissue).

The stained sections were evaluated by two renal pathologists who were blinded to the clinical and serological data. The sections were scored according to the presence of complement deposits along the PTC and glomerular capillaries (GC) of non-sclerosed glomeruli. Complement deposits in the glomerular mesangium and at the vascular pole were not taken into account. C4d and C5b9 deposition in the PTC were scored as minimal ($>0$ but $<10\%$ of PTC), focal (10–50% of PTC) or diffuse ($>50\%$ of PTC). C4d and C5b9 deposition in the GC were scored as segmental ($<50\%$ of capillary loops of all affected glomeruli) or global ($\geq 50\%$ of the capillary loops of at least 1 glomerulus) and focal (at least 1 glomerulus but $<50\%$ of glomeruli) or diffuse ($\geq 50\%$ of glomeruli). The staining intensity was evaluated using a semi-quantitative scoring system (negative $= 0$, weak $= +$, moderate $= ++$ and strong $= +++$).

## Detection of Donor-Specific Antibodies

All patients were tested for circulating donor-specific anti-HLA -A, -B, -Cw, -DR, -DQA1, -DQB1, and -DP antibodies in serum samples obtained at the time of biopsy using single antigen flow bead assays (One Lambda) on a Luminex platform. All beads showing a normalized mean fluorescence intensity $>500$ were considered positive.

## Statistical Analysis

Quantitative variables were expressed as a mean with standard deviation or median with first and third quartiles. Qualitative variables were expressed by counts and percentages. We compared means, medians and percentages using the Student's *t*-test (or Wilcoxon test when appropriate) and chi-square test with Yates's correction (or Fisher's exact test when appropriate). Kidney allograft survival was assessed by the Kaplan-Meier method and compared among the groups with the log-rank test. Kidney allograft survival was calculated from the date of the ABMR-defining biopsy to the date of allograft loss (return to dialysis or new transplantation). If a patient died with a functioning graft, graft survival was censored at the time of death. Cox proportional-hazards models were used to estimate the hazard ratios (HR) and 95% confidence intervals (CI) for death-censored allograft loss. The association of clinical, functional, immunological and histological parameters with allograft loss was assessed with univariate Cox regression analyses. A *P*-value $< 0.05$ was used to select variables that were then entered into a single multivariate Cox model with stepwise backward elimination. R software (version 3.2.0) was used to perform all analyses. All tests were two-sided and *P*-values $< 0.05$ were considered statistically significant.

# RESULTS

## Patient Characteristics

During the study period, 122 patients (55 at Montpellier Hospital and 67 at Bordeaux Hospital) had a first histological diagnosis of ABMR of the current graft according to Banff 2015 criteria (4). Among them, 68 were excluded for the following reasons: no serological evidence of anti-HLA DSA ($n = 27$), insufficient tissue material for C4d and C5b9 detection by immunohistochemistry ($n = 27$), ABO-incompatible transplantation ($n = 1$), combined transplantation ($n = 2$), thrombotic microangiopathy ($n = 6$) and concomitant recurrent or *de novo* glomerulonephritis ($n = 5$). Finally, 54 patients were included (**Figure 1**), with a median

**TABLE 1 |** Patient characteristics.

| Variable | *n* | Value |
|---|---|---|
| **Recipient characteristics** | | |
| Age (years), median (Q1–Q3) | 54 | 49.5 (35–59) |
| Male sex, *n* (%) | 54 | 34 (63) |
| Nephropathy, *n* (%) | 53 | |
| Glomerulonephritis | | 21 (40) |
| Autosomal dominant polykystosis | | 6 (11) |
| Interstitial nephritis | | 3 (6) |
| Malformative uropathy | | 8 (15) |
| Others | | 5 (9) |
| Unknown | | 10 (19) |
| Blood group: A/B/AB/O, *n* | 53 | 29/4/0/20 |
| Time since dialysis (months), median (Q1–Q3) | 50 | 30.5 (12–44.75) |
| **Transplant baseline characteristics** | | |
| Preemptive, *n* (%) | 52 | 4 (8) |
| Transplant number >1, *n* (%) | 54 | 19 (35) |
| Deceased donor, *n* (%) | 54 | 49 (91) |
| Expanded criteria donor, *n* (%) | 54 | 16 (30) |
| Cold ischemia time (minutes), mean ± SD | 50 | 1074.62 ± 540.78 |
| Delayed graft function, *n* (%) | 47 | 12 (26) |
| HLA A/B/DR mismatches, median (Q1–Q3) | 52 | 4 (3–5) |
| **Immunosuppressive protocol** | | |
| Induction therapy, *n* (%) | 53 | |
| Anti-thymocyte globulin | | 31 (58) |
| IL-2 receptor antagonist | | 21 (40) |
| Plasmapheresis | | 3 (6) |
| Intravenous immunoglobulin | | 6 (11) |
| Rituximab | | 3 (6) |
| Regimen at the time of biopsy | 54 | |
| None/ciclosporine/tacrolimus/mTORi, *n* | | 1/19/22/12 |
| None/mycophenolic acid/azathioprine, *n* | | 5/46/3 |
| Steroid withdrawal, *n* | | 12 (22) |
| Biology at the time of biopsy | 54 | |
| Creatininemia (μmol/l), median (Q1–Q3) | 54 | 178 (140–223) |
| eGFR, MDRD formula (ml/min/1 m732), mean +/− SD | 54 | 34.31 ± 17.71 |
| Urine protein-to-creatinine ratio (mg/mmol), median (Q1–Q3) | 45 | 34 (19–121) |
| **Characteristics of anti-HLA DSA at the time of biopsy** | | |
| Class I, *n* (%) | 54 | 31 (57) |
| Class II, *n* (%) | 53 | 47 (89) |
| Class I + II, *n* (%) | 53 | 24 (45) |
| **Immunodominant DSA** | | |
| Class I, *n* (%) | 53 | 12 (23) |
| Class II, *n* (%) | 53 | 41 (77) |
| MFI, median (Q1–Q3) | 52 | 9000 (4365.5–14125) |
| **Rejection treatment** | | |
| Steroid pulses, *n* (%) | 52 | 47 (90) |
| Switch to tacrolimus, *n* (%) | 54 | 15 (28) |
| Plasmapheresis, *n* (%) | 54 | 46 (85) |
| Intravenous immunoglobulin, *n* (%) | 54 | 43 (80) |
| Rituximab, *n* (%) | 52 | 30 (58) |
| Graft loss, *n* (%) | 54 | 33 (61) |
| Death, *n* (%) | 54 | 6 (11) |
| Follow-up (months), median (Q1–Q3) | 54 | 52.5 (34.25–73.5) |

*Q1, first quartile 1; Q3, third quartile; HLA, human leukocyte antigen; DSA, donor-specific antibody; MFI, mean fluorescence intensity; eGFR, estimated glomerular filtration rate; MDRD, modification of diet in renal disease; mTORi, mammalian target of rapamycine inhibitor.*

*Definitions: delayed graft function, dialysis within the first week after transplantation; expanded criteria donor, donor age > 60 years or 50 to 59 years with 2 of the following criteria: high blood pressure, cerebrovascular death cause, creatininemia > 132.6 μmol/l.*

follow-up of 52.5 months (range, 34.25–73.5 months). Their characteristics are summarized in **Table 1**. The median age of the patients was 49.5 years (35–59). Fifty patients (91%) had received their transplant from a deceased donor. Thirty-one patients (58%) had undergone an induction therapy with rabbit anti-thymocyte globulins. At the time of ABMR diagnosis, the mean estimated glomerular filtration rate was 34.31 ± 17.71 ml/min/1 $m73^2$, and the median proteinuria level was 34 mg/mmol (19–121). Thirty-one (57%) patients had class I anti-HLA DSA; 47 (89%) had class II anti-HLA DSA; and 24 (45%) had class I and II anti-HLA DSAs. The immunodominant anti-HLA DSAs were class I in 12 (23%) patients and class II in 41 (77%) patients with a mean fluorescent intensity of 9000 (4365.5–14125).

## Histological Parameters of ABMR-Defining Biopsies

The median time of ABMR diagnosis after transplantation was 37.5 months (range, 6.25–76 months). Twenty-eight patients (52%) had acute ABMR and 26 (48%) had chronic active ABMR, with double contour of glomerular basement membrane. Nineteen patients (35%) had a concomitant T-cell mediated rejection. Positive C4d along PTC was found in 23/28 patients (82%) with acute ABMR and 21/26 (81%) with chronic active ABMR. Forty-five patients (85%) had interstitial fibrosis and tubular atrophy score ≥1. All histological parameters are listed in **Table 2**.

## Positivity and Location of C4d and C5b9 Deposits on ABMR Biopsies

The findings are summarized in **Table 3**. C4d was positive in PTC of 44/54 biopsies (81%) and in GC of 48/54 (89%). The deposition of C4d in PTC was minimal in 3/54 biopsies (6%), focal in 11/54 (20%), and diffuse in 30/54 (56%). The staining was weak in 3/44 patients (7%), moderate in 11/44 (25%) and strong in 30/44 (68%). Glomerular C4d deposition was pseudolinear and located in the subendothelial space and/or in the glomerular basement membrane (**Figure 2**). Deposition was segmental and focal in 4/54 biopsies (7%), segmental and diffuse in 3/54 (6%)

**TABLE 2 |** Histological parameters of ABMR-defining biopsies.

| Variable | *n* | Value |
|---|---|---|
| Time after transplantation (months), median (Q1–Q3) | 54 | 37.5 (6.25–76) |
| Number of glomeruli, median (Q1–Q3) | 54 | 10 (6–14) |
| Concomitant T-cell mediated rejection, *n* (%) | 54 | 19 (35) |
| **Banff scores** | | |
| Glomerulitis (g) score | 54 | |
| ≥1, *n* (%) | | 34 (63) |
| Median (Q1–Q3) | | 1 (0–2) |
| Mesangial matrix expansion (mm) score | 54 | |
| ≥1, *n* (%) | | 31 (57) |
| Median (Q1–Q3) | | 1 (0–1) |
| Double contour (cg) score | 54 | |
| ≥1, *n* (%) | | 26 (48) |
| Median (Q1–Q3) | | 0 (0–2) |
| Interstitial fibrosis and tubular atrophy (IFTA) score | 54 | |
| ≥1, *n* (%) | | 46 (85) |
| Median (Q1–Q3) | | 1 (1–2) |
| Interstitial inflammation (i) score | 54 | |
| ≥1, *n* (%) | | 43 (80) |
| Median (Q1–Q3) | | 1 (1–2) |
| Tubulitis (t) score | 54 | |
| ≥1, *n* (%) | | 19 (35) |
| Median (Q1–Q3) | | 0 (0–1) |
| Peritubular capillaritis (ptc) score | 54 | |
| ≥1, *n* (%) | | 50 (93) |
| Median (Q1–Q3) | | 2 (1–2) |
| Arteriolar hyalinosis (ah) score | 54 | |
| ≥1, *n* (%) | | 36 (67) |
| Median (Q1–Q3) | | 1 (0–1) |
| Vascular fibrous intimal thickening (cv) score | 48 | |
| ≥1, *n* (%) | | 28 (58) |
| Median (Q1–Q3) | | 1 (0–1.25) |
| Vasculitis (v) score | 48 | |
| ≥1, *n* (%) | | 6 (12) |
| Median (Q1–Q3) | | 0 (0–0) |
| Microvascular inflammation (g + ptc) score | 54 | |
| ≥2, *n* (%) | | 41 (76) |
| Median (Q1–Q3) | | 2 (2–3) |
| C4d deposition in peritubular capillaries ≥2 (IF) | 52 | 35 (67) |

*ABMR, antibody-mediated rejection; Q1, first quartile; Q3, third quartile; IF, immunofluorescence.*

**TABLE 3 |** Location and intensity of C4d and C5b9 deposits by immunohistochemistry on ABMR biopsies ($n = 54$).

| | C4d | C5b9 |
|---|---|---|
| **Peritubular capillaries, *n* (%)** | | |
| Positive staining | 44 (81) | 1 (2) |
| **Distribution** | | |
| Negative | 10 (19) | 53 (98) |
| Minimal | 3 (6) | 0 (0) |
| Focal | 11 (20) | 1 (2) |
| Diffuse | 30 (56) | 0 (0) |
| **Intensity of positive Staining** | | |
| Weak (+) | 3 (7) | 0 (0) |
| Moderate (++) | 11 (25) | 1 (100) |
| Strong (+ + +) | 30 (68) | 0 (0) |
| **Glomerular capillaries, *n* (%)** | | |
| Positive staining | 48 (89) | 13 (24) |
| **Distribution** | | |
| Negative | 6 (11) | 41 (76) |
| Segmental and focal | 4 (7) | 4 (7) |
| Segmental and diffuse | 3 (6) | 2 (4) |
| Global and diffuse | 41 (76) | 7 (13) |
| **Intensity of positive staining** | | |
| Weak (+) | 12 (25) | 12 (92) |
| Moderate (++) | 12 (25) | 1 (8) |
| Strong (+ + +) | 24 (50) | 0 (0) |

and global and diffuse in 41/54 (76%). The staining was weak in 12/48 patients (25%), moderate in 12/48 (25%) and strong in 24/48 (50%).

C5b9 was positive in PTC of one patient (1.8%) and in GC of 13/54 biopsies (24%). Glomerular C5b9 deposition was granular and subendothelial (**Figure 2**). Deposition was segmental and focal in 4/54 biopsies (7%), segmental and diffuse in 2/54 (4%) and global and diffuse in 7/54 (13%). Glomerular C5b9 staining was weak in 12/13 patients (92%) and moderate in one (8%). All but one of the biopsies with positive glomerular C5b9 deposition had C4d deposition in PTC (1 focal and 11 diffuse) and in GC (3 segmental and diffuse and 10 global and diffuse). Only one biopsy had C5b9 deposition in PTC associated with diffuse deposition of C4d in PTC and global and diffuse deposition of C4d and C5b9 in GC. Among the patients with global and diffuse deposition of C5b9 in GC, all except one had strong (+++) C4d staining along GC and PTC (**Figure S1**).

## Death-Censored Allograft Survival After ABMR Diagnosis According to the Deposition of C4d and C5b9

During a median follow-up of 52.5 months (range, 34.25–73.5 months), 33 patients (61%) lost their graft. Overall median death-censored allograft survival after ABMR diagnosis was 43 months (range, 29–59 months). Graft survival rates were respectively 83.3, 75.9, 70.1, and 27.2% at 3 months, 1 year, 2 years, and 5 years.

C4d deposits in PTC and GC were not associated with allograft survival ($p = 0.42$ and 0.69 by log-rank-test, respectively). However, death-censored allograft survival was significantly lower in patients with global and diffuse deposition of C5b9 in GC than in those with a segmental pattern or no deposition (median survival after ABMR diagnosis: 6 months, 40.5 months, and 44 months respectively; $p = 0.015$ by log-rank-test; **Figure 3**). Graft survival rates in patients with global and diffuse deposition

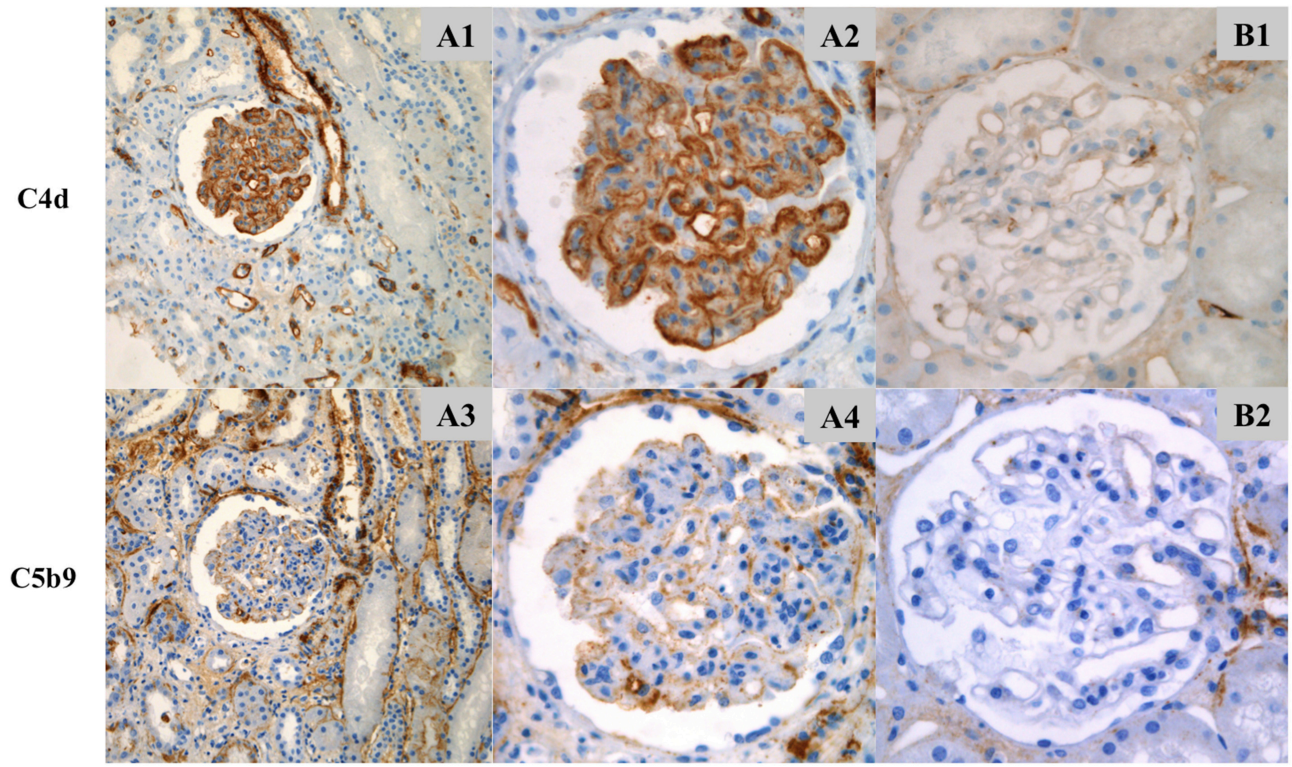


**FIGURE 2 |** Location of C4d and C5b9 deposits by immunohistochemistry. **(A1–A4)** ABMR biopsy and **(B1,B2)** normal biopsy. **(A1)** C4d staining (x20), **(A2,B1)** C4d staining (x40), **(A3)** C5b9 staining (x20) and **(A4,B2)** C5b9 staining (x40). **(A1,A2)** In ABMR biopsy, C4d deposits were located in peritubular capillaries and along the glomerular capillaries (strong intensity), in the subendothelial space and/or intramembranous (pseudolinear pattern). **(A3,A4)** C5b9 staining was negative in peritubular capillaries and positive in glomerular capillaries (weak intensity), in the subendothelial space (granular pattern). There was no mesangial deposit. Positive staining in tubular basement membranes and Bowman's capsule are of non-specific significance. **(B1,B2)** C4d and C5b9 staining was negative in the normal biopsy. Arteriolar deposits are of non-specific significance.

of C5b9 in GC were respectively 57.1, 42.9, and 28.6% at 3 months, 1 year and 2 years, compared to 85.4, 78.1, and 75.6% in patients without C5b9 deposit.

In a univariate Cox regression model (**Table S1**), the HR for death-censored allograft loss was 3.35 (95% CI, 1.46 to 9.60, $p = 0.006$) in patients with global and diffuse deposition of C5b9 in GC. In a multivariate analysis, this association was not found and the only independent risk factor for graft loss was the presence of double contour of glomerular basement membrane (adjusted HR 2.28, 95% CI 1.08 to 4.81, $p = 0.03$; **Table S2**).

## Phenotype of ABMR With Global and Diffuse Deposition of C5b9 in Glomerular Capillaries

Comparison of C5b9+ and C5b9– ABMR phenotypes is detailed in **Table 4**. The time to occurrence of ABMR after transplantation was not different between C5b9+ and C5b9– ABMR [28 (16–75) months vs. 39 (5.5–73.5) months, $p = 0.75$]. C5b9+ ABMR was associated with a worse graft function at diagnosis (median estimated glomerular filtration rate, 26 vs. 34 ml/min/1 $m73^2$, $p = 0.04$) and tended to be associated with more anti-HLA DSA [3 (2–3.5) vs. 2 (1–3), $p = 0.09$), without any other difference in DSA characteristics. The occurrence of *de novo* DSA was similar in C5b9+ ABMR to C5b9– ABMR [4/5 (80%) vs. 22/30 (73%), $p = 1.00$], in the population ($n = 35$) who had been screened for anti-HLA antibodies by solid-phase assay before transplantation. All ABMR with global and diffuse deposition of C5b9 in GC had diffuse deposition of C4d in PTC and global and diffuse deposition of C4d in GC. Moreover, all of them displayed double contour of glomerular basement membrane, with a higher mean Banff score at diagnosis than that found in C5b9– ABMR ($1.71 \pm 0.95$ vs. $0.77 \pm 1.05$, $p = 0.01$; **Figure 4**). These double contours were diagnosed earlier after transplantation in C5b9+ ABMR than in C5b9– ABMR (median time after transplantation, 28 vs. 85 months; $p = 0.058$; **Figure 5**). Among the patients with double contours, those with global deposits of C5b9 along GC were associated with a trend toward a worse death-censored allograft survival than the others (median survival after ABMR diagnosis, 6 vs. 29 months, $p = 0.078$ by log-rank test; **Figure S2**).

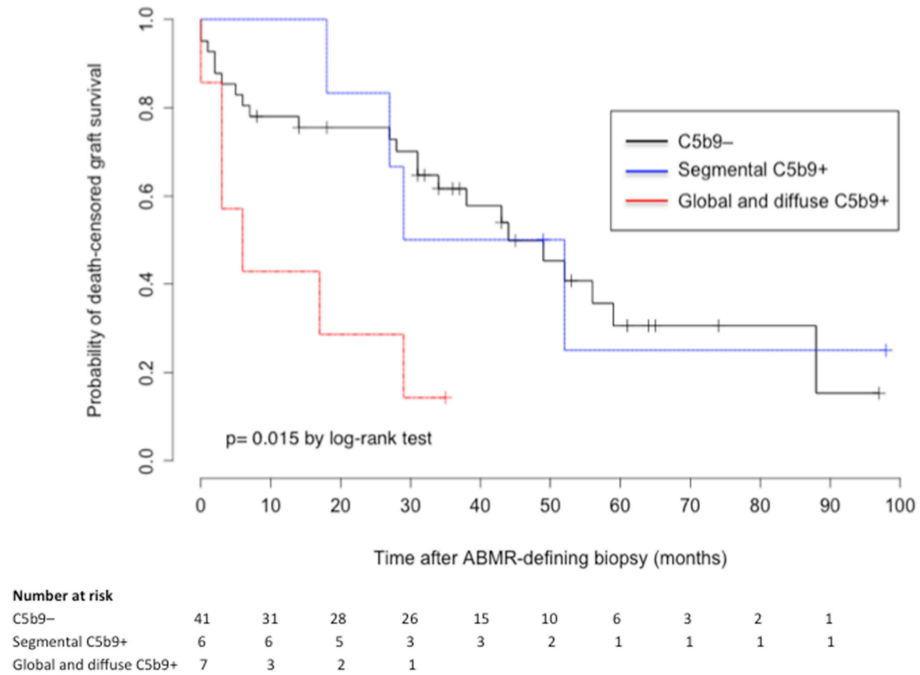


**FIGURE 3 |** Death-censored graft survival after ABMR diagnosis according to the deposition of C5b9 in glomerular capillaries. ABMR, antibody-mediated rejection; segmental C5b9+, segmental (focal or diffuse) deposition of C5b9 in glomerular capillaries; global and diffuse C5b9+, global and diffuse deposition of C5b9 in glomerular capillaries.

## DISCUSSION

This is the first study to evaluate C5b9 deposits in human kidney allografts with ABMR. In a cohort of 54 well-phenotyped kidney transplant recipients with ABMR from two centers, we observed that these deposits were present in a subgroup of patients, mainly located in the glomeruli and always associated with C4d deposits. ABMR with global and diffuse deposition of C5b9 in GC constituted a severe phenotype, with worse graft function at diagnosis and poor allograft survival. C5b9+ ABMR seems to be associated with early onset of glomerular basement membrane duplication and accelerated development of chronic glomerular lesions.

C4d deposition in PTC—a marker of active ABMR—is a footprint of the activation of the classical complement pathway after the binding of DSA to mismatched endothelial antigens (3, 4). In this study, about 80% of the patients had positive C4d in PTC and nearly 90% a pseudolinear deposition of C4d in GC. Interestingly, all the patients with C4d deposits along PTC had C4d in GC. The role of glomerular C4d in ABMR is not clearly established. The location of C4d deposits along glomerular endothelial cells, the negativity of C4d staining in T-cell mediated rejection and protocol graft biopsies (data not shown), and the strong association with positive C4d staining along PTC in our population supports the hypothesis that glomerular C4d plays a role in the pathogenesis of ABMR. Valente et al. (12) previously found similar results. In their study, glomerular C4d deposits along endothelial cells were present in cases of acute ABMR and correlated with C4d positivity along PTC. It is important to note here that in our study, patients were excluded if they had chronic/acute thrombotic microangiopathy associated with ABMR (13), immune complex-mediated glomerulonephritis and complement disease recurrence (14), because they are also associated with glomerular C4d positivity.

The endothelial C4d staining in ABMR reflects the first step of complement activation by the classical complement pathway. Although C4d staining in PTC is a helpful tool for diagnosing ABMR, its association with graft survival is controversial (10). In our study, C4d deposits along PTC and GC were not associated with graft survival. C4d is an inactive molecule, which has never been associated with endothelial injury *in vitro* and can also indicate a limited complement activation (15). The activation of the complement cascade is in fact tightly regulated by several fluid phase and membrane-bound proteins (16). Complete complement cascade activation up to the terminal pathway—expressed by positive C5b9 staining—can reflect overactivation of the complement system, defective complement regulatory proteins or an imbalance between activation and regulation. In our study, C5b9 deposits were positive in 24% of the ABMR biopsies, mainly along GC. All C5b9+ ABMR had positive C4d staining along PTC and GC indicating initiation by the classical complement pathway and full complement cascade activation. The fact that C5b9 was mainly positive in glomeruli and along with C4d deposits, supports the role of glomerular complement activation in the pathogenesis of ABMR. Of note, C5b9 is also often positive in glomeruli, but not in PTC, in thrombotic microangiopathy (13).

**TABLE 4 |** Comparison of functional, immunological and histological variables in the two patterns of antibody-mediated rejection.

| Variables | *n* | C5b9– ABMR (*n* = 47) | C5b9+ ABMR* (*n* = 7) | *P*-value |
|---|---|---|---|---|
| Time after transplantation (months), median (Q1–Q3) | 54 | 39 (5.5–73.5) | 28 (16–75) | 0.75 |
| **Functional (Biology at the Time Of Biopsy)** | | | | |
| Creatininemia (μmol/l), median (Q1–Q3) | 51 | 169 (139–212) | 258 (197–304) | 0.07 |
| eGFR, MDRD formula (ml/min/1 m732), median (Q1–Q3) | 54 | 34 (27–45.5) | 26 (13.5–31) | 0.04 |
| Urine protein-to-creatinine ratio (mg/mmol), median (Q1–Q3) | 45 | 34 (19–121) | 58 (25.5–210) | 0.78 |
| **Immunology** | | | | |
| DSA | 52 | 2 (1–3) | 3 (2–3.5) | 0.09 |
| Number, median (Q1–Q3) | | | | |
| Class I, *n* (%) | 53 | 25 (53) | 6 (86) | 0.22 |
| Class II, (*n*%) | 53 | 41 (89) | 6 (86) | 1.00 |
| Class I + II, *n* (%) | 53 | 19 (41) | 5 (71) | 0.28 |
| Immunodominant DSA | | | | |
| Class I, *n* (%) | 53 | 10 (22) | 2 (29) | 0.65 |
| Class II, *n* (%) | 53 | 36 (78) | 5 (71) | 0.65 |
| MFI, median (Q1–Q3) | 52 | 9000 (3500–14000) | 10335 (7142–13446.5) | 0.52 |
| **Histology** | | | | |
| Concomitant TCMR, n (%) | 54 | 17 (36) | 2 (29) | 1.00 |
| Glomerulitis (g) score | 54 | | | |
| ≥1, *n* (%) | | 30 (64) | 4 (57) | 1.00 |
| Median score (Q1–Q3) | | 1 (0–2) | 1 (0–1.5) | 0.92 |
| Mesangial matrix expansion (mm) score | 54 | | | |
| ≥1, *n* (%) | | 25 (53) | 6 (86) | 0.22 |
| Median score (Q1–Q3) | | 1 (0–1) | 1 (1–2) | 0.13 |
| Double contour (cg) score | 54 | | | |
| ≥1, *n* (%) | | 19 (40) | 7 (100) | 0.01 |
| Median score (Q1–Q3) | | 0 (0–2) | 1 (1–2.5) | 0.01 |
| Score, *n* (%) | | | | 0.002 |
| 0 | | 28 (60) | 0 (0) | |
| 1 | | 6 (13) | 4 (57) | |
| 2 | | 9 (19) | 1 (14) | |
| 3 | | 4 (9) | 2 (29) | |
| Interstitial fibrosis and tubular atrophy (IFTA) score | 54 | | | |
| ≥1, *n* (%) | | 40 (85) | 6 (86) | 1.00 |
| Median score (Q1–Q3) | | 1 (1–2) | 1 (1–1) | 0.49 |
| Interstitial inflammation (i) score | 54 | | | |
| ≥1, *n* (%) | | 38 (81) | 5 (71) | 0.62 |
| Median score (Q1–Q3) | | 1 (1–2) | 1 (0.5–2) | 0.91 |
| Tubulitis (t) score | 54 | | | |
| ≥1, *n* (%) | | 17 (36) | 2 (29) | 0.62 |
| Median score (Q1–Q3) | | 0 (0–1) | 0 (0−0.5) | 0.63 |
| Peritubular capillaritis (ptc) score | 54 | | | |
| ≥1, *n* (%) | | 91% (43) | 7 (100) | 1.00 |
| Median score (Q1–Q3) | | 2 (1–2) | 2 (1–2) | 0.99 |
| Arteriolar hyalinosis (ah) score | 54 | | | |
| ≥1, *n* (%) | | 30 (64) | 6 (86) | 0.40 |
| Median score (Q1–Q3) | | 1 (0–1) | 1 (1–1) | 0.65 |
| Vascular intimal fibrous thickening (cv) score | 48 | | | |
| ≥1, *n* (%) | | 23 (55) | 5 (83) | 0.38 |

*(Continued)*

**TABLE 4 |** Continued

| Variables | *n* | C5b9– ABMR (*n* = 47) | C5b9+ ABMR* (*n* = 7) | *P*-value |
|---|---|---|---|---|
| Median score (Q1–Q3) | | 1 (0–1) | 1.5 (1–2) | 0.14 |
| Vasculitis (v) score | 48 | | | |
| ≥1, *n* (%) | | 6 (14) | 0 (0) | 1.00 |
| Median score (Q1–Q3) | | 0 (0–0) | 0 (0–0) | 0.39 |
| Deposition of C4d in capillaries (IHC), *n* (%) | 54 | | | |
| Glomerular capillaries | | | | 0.85 |
| No deposition | | 6 (13) | 0 (0) | |
| Segmental and focal | | 4 (9) | 0 (0) | |
| Segmental and diffuse | | 3 (6) | 0 (0) | |
| Global and diffuse | | 34 (72) | 7 (100) | |
| Peritubular capillaries | | | | |
| None/minimal/focal/diffuse | | 10/3/11/23 | 0/0/0/7 | 0.15 |
| C5b9 deposition in peritubular capillaries (IHC) | | 0 (0) | 1 (14) | 0.13 |

**C5b9+ ABMR, antibody-mediated rejection with global and diffuse deposition of C5b9 in glomerular capillaries.*

*Q1, first quartile; Q3, third quartile; eGFR, estimated glomerular filtration rate; MDRD, modification of diet in renal disease; DSA, donor-specific antibody; MFI, mean fluorescence intensity; TCMR, T-cell mediated rejection; IHC, immunohistochemistry.*

Global and diffuse glomerular deposition of C5b9—which reflects unregulated complement activation or a massive production of complement products due to an excessive complement activation—occurred in 13% of ABMR in our study, and was associated with massive C4d deposits along PTC and GC. This characterizes a severe phenotype, with poor graft function at diagnosis and poor allograft survival. A trend for a higher number of DSA was the only factor we found to explain this uncontrolled injury. However, endothelial susceptibility to injury cannot be excluded, maybe secondary to a lack of complement regulatory proteins. Indeed, patients with segmental deposition of C5b9 in GC—which reflects partially regulated complement activation—had a similar graft survival than patients without C5b9 deposits—which reflects regulated complement activation. Moreover, the absence of C5b9 deposits along PTC could suggest a better complement regulation or the inability to form a membrane complex attack in these small vessels. Finally, it is important to note that although glomerular C5b9 staining was often weak, it was very specific because we used a mouse monoclonal anti-human C9 neoepitope—which is specific to C5b9 fixation in membrane—and it was strictly negative in negative controls. Moreover, in opposition with the intensity of C4d staining, the weakness of C5b9 staining is probably the reflection of the regulation of complement activation by endothelial cells after the cleavage of C4.

Multivariate analysis did not confirm that global and diffuse deposition of C5b9 in glomerular capillaries was associated with poor graft survival. The only independent risk factor for graft loss was the presence of double contours of glomerular basement membrane, which was associated with more than a 2-fold risk of graft loss. This result could be explained by a lack of power (there was a trend toward a lower graft survival in cg+ C5b9+ ABMR than cg+ C5b9-) but also by the fact that all patients with global and diffuse C5b9 deposits had double contours. This lesion is widely accepted to be a major prognostic factor in ABMR, possibly the most important one (17, 18). The pathogenesis of transplant glomerulopathy—currently viewed as a cardinal histological lesion of chronic ABMR—remains unclear (19). Recently, Gasim et al. (20) found that pseudolinear GC C4d correlated with the severity of glomerular basement membrane duplication by light and electron microscopy. The authors hypothesized that complement activation could be involved in this multi-layering process. However, C5b9 staining was not performed in this study. In our cohort, we observed that 25/26 (96%) patients with double contours had C4d deposition in GC indicating complement activation. Strikingly, patients who also had global and diffuse C5b9 deposits had fast onset of double contours, suggesting devastating endothelial injury. C5b9 can activate endothelial cells at sublytic concentrations and induce a pro-inflammatory state, mainly through non-canonical nuclear factor-κB signaling (21, 22). Owing to the observational nature of this study, we cannot confirm a causal link between C5b9 and double contours. Nethertheless we hypothesize that C5b9 can activate glomerular endothelial cells and accelerate the duplication of glomerular basement membrane, because we diagnosed double contours earlier in C5b9+ ABMR than in C5b9- ABMR and there was a trend toward a worse death-censored allograft survival in C5b9+ ABMR among the patients with double contours This could be a result of repeated episodes of endothelial activation, injury, and repair (19). However, the duplication of glomerular basement membrane is not specific of chronic ABMR. It has been largely described in complement-mediated diseases such as chronic thrombotic microangiopathy and membranoproliferative glomerulonephritis (13, 14), which were excluded in our study.

It is important to note that C5b9 was positive in a subgroup of patients, indicating that complement activation is often efficiently regulated. Endothelial injury can also be secondary to another complement-mediated mechanism—the recruitment of

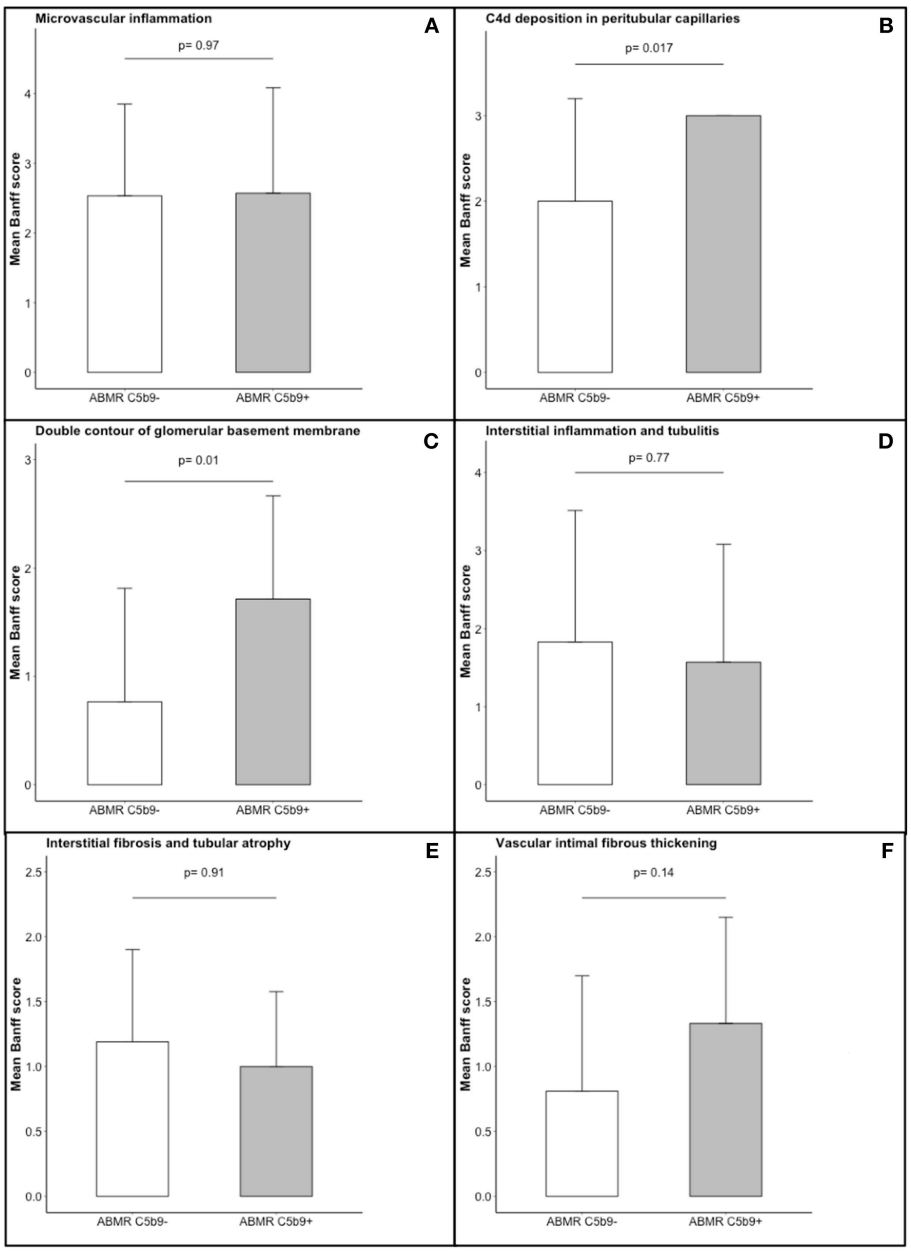


**FIGURE 4 |** Comparison of histogical features in the two patterns of antibody-mediated rejection. The following Banff scores were compared: **(A)** microvascular inflammation [sum of the Banff scores for glomerulitis and peritubular capillaritis], **(B)** C4d deposition in peritubular capillaritis, **(C)** double contour of glomerular basement membrane, **(D)** interstitial inflammation and tubulitis, **(E)** interstitial fibrosis and tubular atrophy, and **(F)** vascular intimal fibrous thickening. Each of these scores ranges from 0 to 3, with higher score indicating more severe abnormality. The T bars indicate standard errors. ABMR C5b9−, antibody-mediated rejection without global and diffuse deposition of C5b9 in glomerular capillaries; ABMR C5b9+, antibody-mediated rejection with global and diffuse deposition of C5b9 in glomerular capillaries.

inflammatory cells by the anaphylatoxins C3a and C5a—and/or to complement-independent mechanisms, such as antibody-dependent cell cytotoxicity and direct endothelial cell activation by the antigen-binding fragment of DSA (23). This relatively uncommon positivity of C5b9 could explain the conflicting results of anti-C5 therapies in the prevention and treatment of ABMR in unselected cohorts (24–26). To reassess the value of this therapy in ABMR, it could be helpful to select patients on the basis of demonstrated complement-mediated endothelial injury.

This study has limitations. Firstly, it was retrospective, and a significant number of patients were excluded for missing data (mostly insufficient tissue on recut biopsy sections). Secondly, we probably underestimated C4d-negative ABMR.

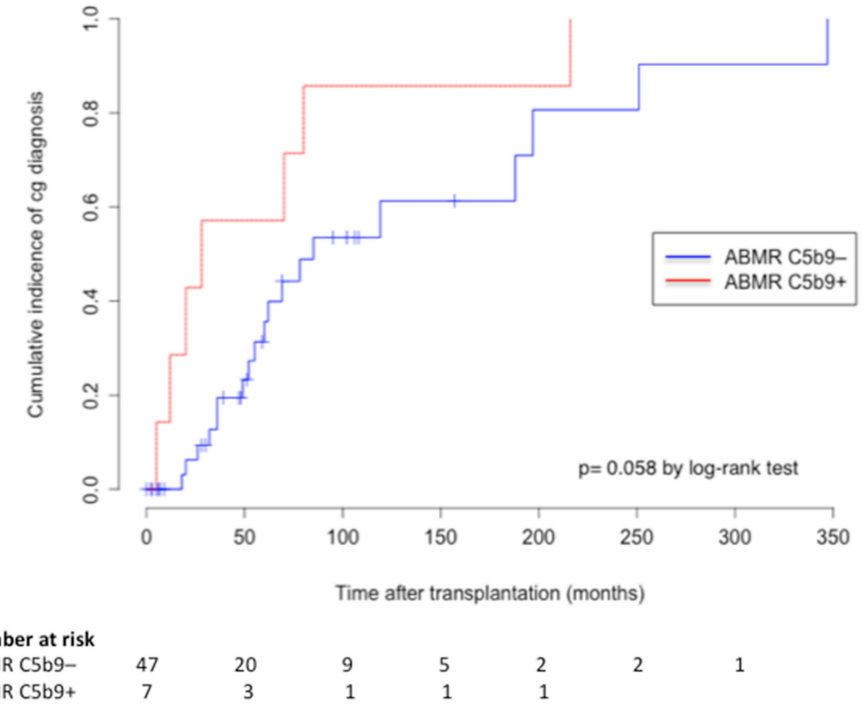


**FIGURE 5 |** Cumulative incidence of cg diagnosis after transplantation according to the deposition of C5b9 in glomerular capillaries. cg, double contour of glomerular basement membrane; ABMR C5b9–, antibody-mediated rejection without global and diffuse deposition of C5b9 in glomerular capillaries; ABMR C5b9+, antibody-mediated rejection with global and diffuse deposition of C5b9 in glomerular capillaries.

This is because, prior to the Banff 2013 classification, C4d was mandatory for a diagnosis of ABMR (27). As our study inclusion period started in 2008, some cases of C4d-negative ABMR might not have been classified as ABMR and therefore not included. Thirdly, all biopsies were performed for cause, so we did not study subclinical ABMR, which is also closely associated with graft survival (28). Lastly, unfortunately we were not able to study ultrastructural changes of glomerular basement membrane by electronic microscopy, complement abnormalities nor complement-binding DSA abilities.

In conclusion, we have defined a new pattern of ABMR of renal allografts with complete complement cascade activation up to the terminal pathway in a subgroup of patients. This constitutes a severe phenotype, with diffuse deposits of C4d along PTC, C4d/C5b9 along GC, early onset of glomerular basement membrane duplication and poor allograft survival. It could be interesting to analyze C4d and C5b9 deposits in cases of subclinical ABMR to evaluate if their positivity precedes the occurrence of double contours. Moreover, the value of anti-C5 therapies in this specific subgroup of patients should be assessed.

## AUTHOR CONTRIBUTIONS

The study was conceived and designed by VGo and ML. VGo and ML conducted analysis. VGo and ML were involved in the writing of the manuscript. VGo, ML, and all other authors contributed to the conduct of the study, recruited patients, and were involved in the review of results and final approval of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00235/full#supplementary-material

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REVIEW
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# The Emerging Role of Complement Proteins as a Target for Therapy of IgA Nephropathy

*Dana V. Rizk[1]*, Nicolas Maillard[2], Bruce A. Julian[1], Barbora Knoppova[3,4], Todd J. Green[3], Jan Novak[3] and Robert J. Wyatt[5]**

[1] Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, United States, [2] Department of Nephrology, Dialysis, Transplantation, CHU de Saint-Etienne, GIMAP, EA3064, Université Jean Monnet, COMUE Université de Lyon, Rhône-Alpes, France, [3] Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL, United States, [4] Department of Immunology, Faculty of Medicine and Dentistry, Palacky University and University Hospital, Olomouc, Czechia, [5] Department of Pediatrics, University of Tennessee Health Sciences Center, Memphis, TN, United States

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***Correspondence:**
*Dana V. Rizk*
*drizk@uabmc.edu*
*Robert J. Wyatt*
*rwyatt@uthsc.edu*

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IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis worldwide and a common cause of end-stage renal disease. Evaluation of a kidney biopsy is necessary for diagnosis, with routine immunofluorescence microscopy revealing dominant or co-dominant IgA immunodeposits usually with complement C3 and sometimes IgG and/or IgM. IgA nephropathy reduces life expectancy by more than 10 years and leads to kidney failure in 20–40% of patients within 20 years of diagnosis. There is accumulating clinical, genetic, and biochemical evidence that complement plays an important role in the pathogenesis of IgA nephropathy. The presence of C3 differentiates the diagnosis of IgA nephropathy from the subclinical deposition of glomerular IgA. Markers for the activation of the alternative and mannan-binding lectin (MBL) pathways in renal-biopsy specimens are associated with disease activity and portend a worse renal outcome. Complement proteins in the circulation have also been evaluated in IgA nephropathy and found to be of prognostic value. Recently, genetic studies have identified IgA nephropathy-associated loci. Within these loci are genes encoding products involved in complement regulation and interaction with immune complexes. Put together, these data identify the complement cascade as a rational treatment target for this chronic kidney disease. Recent case reports on the successful use of humanized anti-C5 monoclonal antibody eculizumab are consistent with this hypothesis, but a better understanding of the role of complement in IgA nephropathy is needed to guide future therapeutic interventions.

**Keywords: complement, IgA nephropathy, alternative complement pathway, mannan binding lectin complement pathway, IgAN pathogenesis, IgAN, IgAN treatment**

## INTRODUCTION

IgA nephropathy (IgAN), initially described by Berger and Hinglais in 1968 (1), is the most common primary glomerulopathy in many countries. IgAN causes end-stage renal disease in 20–40% of the patients within 20 years after diagnosis (2), and reduces life expectancy by 10 years (3). The diagnosis is based on immunofluorescence- or immunohistochemical-microscopic examination showing IgA as the dominant or co-dominant immunoglobulin in the glomerular

immunodeposits (4). These deposits may also contain IgG, IgM, or both (4). The IgA is exclusively of the IgA1 subclass (5). Complement component C3 is present in the same distribution as IgA in up to 90% of biopsies (4).

Recent studies have confirmed an autoimmune nature of IgAN. The most widely accepted mechanism for the pathophysiology of the disease entails four "hits" (**Figure 1**) (6). The first hit refers to increased levels of circulatory polymeric IgA1 with aberrant *O*-glycosylation of its hinge region. These molecules lack galactose in some *O*-glycans in the hinge region (galactose-deficient IgA1, Gd-IgA1), thus exposing *N*-acetylgalactosamine (GalNAc) as terminal glycan. The second hit is the formation of glycan-specific IgG or IgA1 autoantibodies targeting terminal GalNAc-containing hinge region of Gd-IgA1 (7). The third hit is formation of circulating immune complexes comprising Gd-IgA1 and IgG autoantibody. Other proteins can bind Gd-IgA1, such as the soluble Fcα receptor (sCD89), to form complexes, although it is not clear whether such complexes would activate complement (8). Some of the circulating immune complexes pass through fenestrae in the glomerular capillaries to enter the mesangium where they may incite cellular proliferation of mesangial cells and overproduction of extracellular matrix, cytokines, and chemokines (hit four) that potentially lead to chronic kidney damage. This proposed multi-step process is consistent with the finding that glomerular IgA immunodeposits of patients with IgAN are enriched for Gd-IgA1 (9, 10) and IgG co-deposits are of IgG1 and IgG3 subclasses, as are the circulatory IgG autoantibodies specific for Gd-IgA1 (7, 11).

Key observations in kidney transplantation support the notion that kidneys in IgAN are damaged as innocent bystanders: IgAN frequently recurs in allografts, whereas IgA deposits clear from kidneys of donors with subclinical IgAN within a few weeks after implantation into non-IgAN recipients (12).

IgAN is broadly categorized as primary or secondary, i.e., associated with a systemic disease, be it an infectious, inflammatory, or autoimmune process (13). Within primary IgAN the spectrum of disease varies substantially. The clinical presentation differs between children and adults, and the disease severity as well as gender distribution across ethnic and racial backgrounds differ widely. IgAN can manifest without extra-renal involvement, or as part of a systemic vasculitis phenotype currently referred to as IgA vasculitis with nephritis (previously Henoch Schönlein pupura nephritis) (14). About 5–8% of patients have a first- or second-degree relative with biopsy-proven IgAN or urinary abnormalities suggesting a familial occurrence or genetic predisposition for the disease (15). All these observations raise the possibility that the renal pathology phenotype we call IgAN results from different pathophysiologic processes.

In recent years, mounting pathologic, biochemical, experimental, and genetic findings have supported a pivotal role of complement activation in disease onset and progression of IgAN. In particular, the alternative and mannan-binding lectin (MBL) pathways seem to be involved. These observations, in turn, have generated tremendous interest in targeting complement pathways as an approach to treatment.

## BRIEF OVERVIEW OF THE COMPLEMENT-ACTIVATION PATHWAYS

Early knowledge of complement proteins stemmed from the 19th-century discovery of a heat-labile component of normal plasma that augmented the opsonization of bacteria by antibodies and enabled antibodies to kill some bacteria (16, 17). The name "complement" was derived from the description of the activity that "complemented" the antibacterial activity of antibodies. The complement system is an important link between innate and adaptive immunity as it participates in immunosurveillance and tissue homeostasis. The system consists of the activation cascade of ∼50 proteins located in plasma, tissues, and cells (18–20). Many of these complement-associated proteins have been described as proteases that are activated by proteolytic cleavage. This cascade of proteolytic events must be well-controlled for the system to work properly. A malfunction may result in immunodeficiency or autoimmune manifestations (21–23).

Classical, alternative, and lectin pathways are the three known ways of complement system activation (**Figure 2**). Each pathway has a different triggering mechanism; however, after creating the C3-activating enzymes (C3 convertases), the pathways share the same sequence of events that culminates with assembly of the membrane-attack complex (MAC) (26). The first activated component of the classical pathway is C1q protein that recognizes an antigen-antibody (IgG or IgM) complex and subsequently binds its partners C1r and C1s to create a protein complex named C1 ($C1q:C1r_2:C1s_2$) (27, 28). C1 has the ability to cleave complement components C2 and C4, into C2a and C4b, respectively, that interact to form C3 convertase (C4b2a). Subsequently, the cleavage of component C3 by participation of C3 convertase produces two proteins. The smaller submit C3a is an anaphylatoxin that mediates inflammation. The larger subunit C3b is an opsonin that binds covalently through a reactive thioester bond to adjacent pathogen molecule and thereby targets it for destruction by phagocytes equipped with receptors for C3b. As the next step of the cascade, C5 convertase is formed by association of C3b with C4b2a or with C3bBb (the product of cleavage from the alternative pathway). C5 convertase then releases the C5a subunit from C5 protein and the remaining C5b fragment initiates formation of the MAC. After the addition of components C6, C7, and C8, the complex C5b-8 is incorporated into the cell membrane, followed by addition of 10–16 units of C9 component that are arranged in the shape of ring, creating a pore in the membrane and leading to cell lysis and death. C3a and C5a cleavage products have inflammatory and chemo-attractant activities exerted through the corresponding C3a and C5a receptors. Moreover, complement functions include facilitation of uptake and destruction of pathogens by phagocytic cells through the specific recognition by complement receptors on phagocytes. There are six types of complement receptors: CR1-4, and C3a and C5a receptors (29, 30).

The alternative pathway is continuously activated at a low level, and is amplified on activating surfaces or in the fluid phase by bacteria, dying cells, and immune complexes. The spontaneous hydrolysis of a thioester bond in C3 component

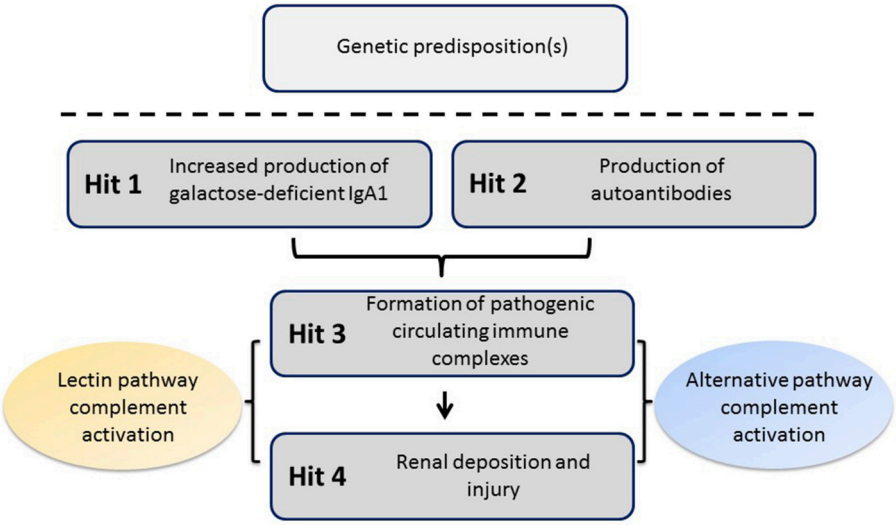


**FIGURE 1 |** Proposed four-hit pathogenesis of IgAN. Circulatory galactose-deficient IgA1 (Gd-IgA1) (Hit 1) is recognized by specific autoantibodies (Hit 2) to form circulating immune complexes (Hit 3). Some of these immune complexes deposit in the kidneys, thereby leading to mesangial activation, enhanced proliferation of mesangial cells, and ultimately kidney injury (Hit 4). Certain genetic loci have been associated with increased risk for developing IgAN. The activation of the alternative and, at least in some patients, mannan-binding lectin (MBL) pathways by immune complexes is involved in disease pathogenesis.

produces $C3b(H_2O)$ that is tightly controlled by plasma regulatory proteins (factors H [FH] and I [FI]; membrane cofactor protein [MCP, CD46]; complement receptor 1 [CR1, CD35]; decay accelerating factor [DAF, CD55]; and complement factor H-related proteins [CFHRs]) (31). Together, these regulators prevent the formation or enhance the dissociation of the alternative pathway C3 convertase (FH, CR1, and DAF) or serve as cofactors for FI-mediated inactivation of C3b to iC3b (FH, MCP, and CR1). CFHRs compete with FH. Properdin is a positive regulator of the alternative pathway, with its main role of stabilizing the alternative pathway C3 convertase (and C5 convertase). Active convertase C3bBb acts similarly to C4b2a, as it activates C5 convertase that leads to formation of MAC (29, 30).

The lectin pathway is stimulated upon binding of MBL with MBL-associated serine proteases (MASP) or ficolins to specific carbohydrate patterns. MASP-2 can also directly cleave C3 while bypassing the usual sequences to activate C4 and/or C2 (32).

Recent data have shown that renin, an aspartate protease produced by juxtaglomerular apparatus in the kidneys, can also function as a C3 convertase to activate the terminal portion of the complement cascade (33).

## ROLE OF COMPLEMENT PROTEINS IN IgAN

Complement proteins are activated in IgAN. Immunohistochemical findings of C3, properdin, C4d, MBL and C5b-9 deposits in mesangium of IgAN biopsy samples, coupled with the general absence of C1q, confirm activation of alternative and lectin pathways rather than classical pathway (24, 34, 35).

The complement cascade is regulated at several levels to prevent unwanted (uncontrolled) activation. As noted above, one of the controlling steps of the alternative pathway relies on complement FH that is present in plasma and on tissue surfaces. It has two functions, stabilizing complexes with C3b and accelerating dissociation of C3bBb. CFHRs are sequentially similar to FH and can compete with FH for C3b binding. These proteins have been studied as possible risk factors of IgAN. In a study with 1,126 IgAN patients, higher circulating levels of CFHR-5 were associated with IgAN development and progression (36, 37). Another study showed that plasma levels of FH antagonists FHR-1 and the FHR-1/FH ratio were elevated in patients with IgAN and associated with disease progression, whereas the plasma level of FHR-5 and the FHR-5/FH ratio were not. However, elevated levels of FHR-5 correlated with poor response to immunosuppressive therapy (38, 39). Moreover, gene deletions of *CFHR1,3* (protective alleles) and some rare variants of CFHR5 are associated with IgAN susceptibility.

### Complement Activation by IgA, IgG, IgM, and Immune Complexes

Human IgG antibodies can have pro- and anti-inflammatory activities, depending on the engagement of Fcγ receptors and the activation of the complement system, which, in turn, depends on the IgG subclass, hexamerization, glycosylation, and antigen density (40–49). Activation of the classical pathway by IgG (and IgM) isotypes (mostly driven by IgM and IgG1 and IgG3 subclasses, and hexameric IgG) results in production of pro-inflammatory C3a and C5a (50). This process then triggers recruitment of effector cells wherein the deposition of C3b on target cells enables recognition by C3b receptors on phagocytic and antigen-presenting cells. Moreover, the capacity of IgG to activate complement further depends on glycosylation of its Fc segment. For example, IgG can also activate the lectin pathway

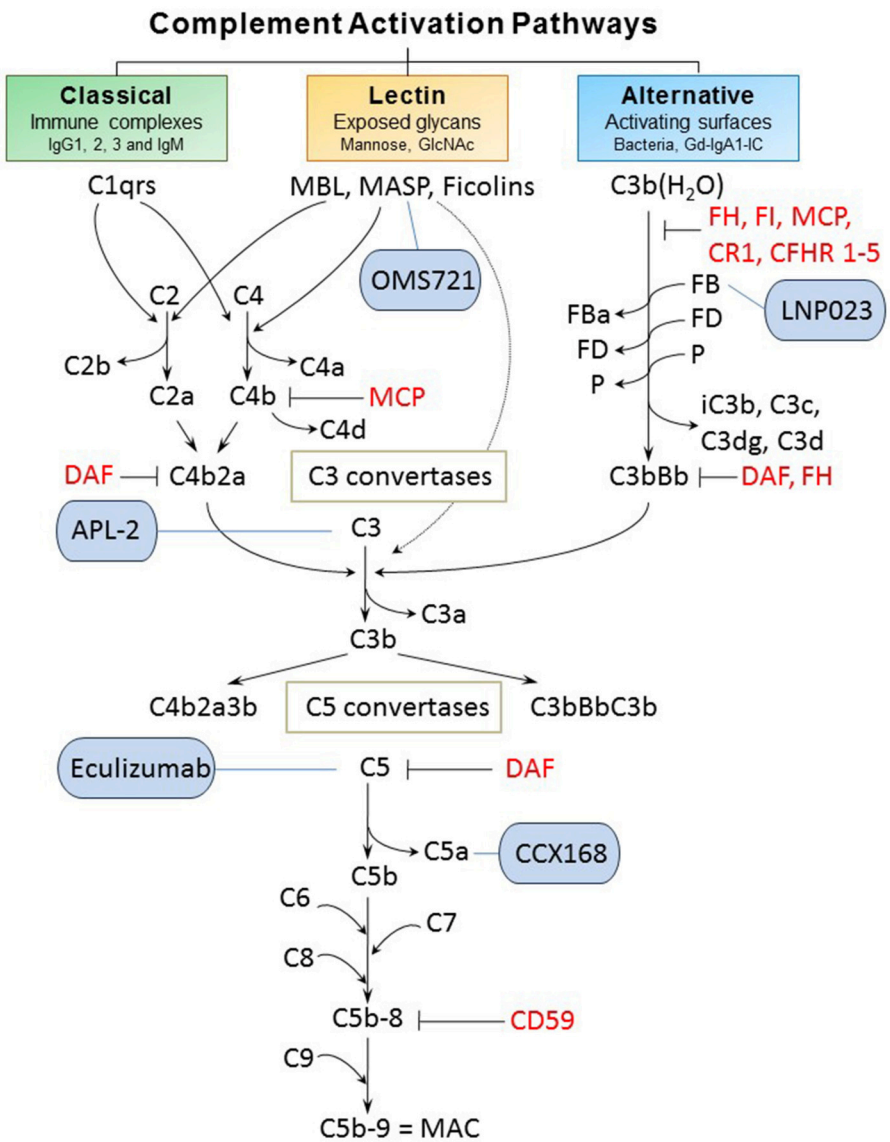


**FIGURE 2 |** Complement activation pathways and examples of complement-targeting therapeutics for IgAN. The three pathways of complement activation, classical, lectin, and alternative, are initiated by interactions of complement proteins with distinct structures. Complexes of antigen and antibody can activate the classical pathway. Mannan-binding lectin recognizes carbohydrate structures and, upon association with serine proteases (MASP, mannose-associated serine proteases), can activate the lectin pathway. Complement C3 that is covalently bound to microorganism surfaces as C3b initiates the cascade of the alternative pathway. Each pathway can ultimately generate an active C3 convertase, resulting in cleavage of C3 component into C3a and C3b fragments. C3b can interact with C4b2b or C3bBb to produce C5 convertase that cleaves C5 into C5a and C5b fragments. C5b binds to the cell membrane and serves as a platform for assembly of the membrane attack complex (MAC) (24, 25). The formation of MAC can be inhibited by membrane-bound CD59 that binds to C8 and/or C9. Several other regulatory proteins of the complement-activation pathways are shown in red. Five complement-targeting therapeutics are shown in blue rounded rectangles. These reagents include two monoclonal antibodies (eculizumab that blocks cleavage of C5; OMS721 targeting MASP-2 that inhibits its protease activity), a C5a receptor antagonist (CCX168), a low-molecular-weight inhibitor of factor B (LNP023), and an inhibitor of C3 activation (APL-2). CR1, complement receptor 1; CFHR 1-5, complement factor H-related proteins 1-5; DAF, decay-accelerating factor; FB, factor B; FD, factor D; FI, factor I; Gd-IgA1, galactose-deficient IgA1; Gd-IgA1-IC, galactose-deficient IgA1-containing immune complexes; MCP, membrane cofactor protein; P, properdin.

if the Fc glycans consist of complex *N*-glycans with terminal *N*-acetylglucosamine (i.e., galactose-deficient *N*-glycans) (51).

In contrast to IgG and IgM, human IgA does not activate complement in the fluid phase and is considered anti-inflammatory. However, differential IgA glycosylation of monomeric and polymeric IgA bound in immune complexes may positively or negatively impact complement activation (52). Mouse models have shown that autoantibodies can activate the alternative pathway and induce cell lysis and tissue damage or target autologous complement components. Such autoantibodies

may play a role in several diseases, especially vascular diseases (53). A passive mouse model of IgAN that uses pre-formed immune complexes comprised of human Gd-IgA1 and human IgG autoantibody specific for Gd-IgA1 induces hematuria and proteinuria; moreover, the glomeruli exhibit mesangial hypercellularity and deposits of IgG, IgA1, and C3 (14).

Activation of the complement system by immune complexes is less well-understood. The relative representation of immunoglobulin isotypes in an immune complex may co-determine which complement pathway or pathways are activated (54). This finding may arm researchers with tools and approaches for use in complement modulation with therapeutics (55). Selected examples relevant for IgAN are shown in **Figure 2**.

# COMPLEMENT PROTEINS AND COMPLEMENT FRAGMENTS IN PATHOLOGY

## Presence of Complement Elements in Glomeruli

A characteristic immunofluorescence-microscopy feature of IgAN renal biopsies is the almost universal glomerular deposition of some complement proteins with IgA. C3 is the most abundant, found in up to 90% of cases (56–60). C3 co-deposits could also be considered a biomarker of actual IgAN in comparison to isolated IgA deposition without renal injury. Interestingly, a European necropsy study from 753 deaths due to suicide or violent deaths (excluding persons with secondary forms of IgAN) found asymptomatic IgA deposition in the kidneys of 6.9% of individuals (61). Of those, only 4 were C3-positive (0.5%). A study from Japan evaluated the prevalence of IgA deposition among 510 kidney donors whose renal allografts were biopsied at the time of implantation. The frequency of subclinical IgA deposition was 16.1%, with concomitant C3 deposition reported in 16 individuals (3%) (62). These studies show that complement activation may distinguish isolated from nephritogenic IgA deposition. Notably, the intensity of C3 deposition by immunofluorescence studies can be influenced by genetic variations in the complement-encoding genes. Patients with at least one allele for a large deletion in the genes encoding CFHR proteins (Δ*CFHR3,1*) have less glomerular immunofluorescence staining for C3 compared to individuals with two wild-type alleles (63).

Apart from C3, other complement elements can be co-deposited with IgA. The presence of FH or properdin, suggesting activation of the alternative pathway, has been frequently found in mesangial areas of IgAN patients (59, 64, 65). More recently, mass-spectrometric analysis of micro-dissected glomeruli from IgAN kidney-biopsy specimens showed significant amounts of C3 and C5 as well as all of the complement elements located downstream from the activation cascade (C6 to C9) when compared to biopsies of normal kidneys (66). This result confirms the presence of C5b-9 in IgAN glomeruli, as shown in early immunostaining-based studies (64, 67, 68). The most important point from this study is the accumulation of alternative-pathway regulation proteins, such as FH, and also CFHR 1,2,3, and 5. Moreover, using targeted proteomic profiling, a reduced abundance of complement receptor 1 (CR1) was detected in biopsy specimens from IgAN patients with progressive vs. non-progressive disease (66, 69).

The lectin pathway is activated in some patients with IgAN (70). This subset of patients exhibits mesangial deposition of C4d, MBL, MBL-associated serine proteases (MASPs) 1 and 2 and L-ficolin (70). In a large multi-center Spanish cohort of 283 IgAN patients, C4d deposition was found in 38% of cases (71).

Hallmarks of classical pathway activation, such as C1q, are usually not detected in the glomeruli of patients with IgAN (57), although it may be found in biopsies with advanced glomerulosclerosis (72). Therefore, the presence of glomerular C4d in IgAN suggests activation of the lectin pathway rather than the classical pathway.

## Association of Complement Protein Deposition With Disease Severity and Prognosis

The intensity of mesangial C3 deposition has been negatively correlated with renal survival in a retrospective study of 343 Korean IgAN patients (58). A trend toward a similar finding was reported in a large study of French patients, although the finding did not reach statistical significance (63). The prognostic implications of detection of early (C3b, C3c, iC3b) vs. late (C3d) proteolytic products in mesangial deposits remain matters of debate. One study reported more active disease associated with C3c deposition compared to C3d (73). Another recent study found C3d and C3c/C3b/iC3b were independently associated with progressive disease in a small number of patients (38). C5b-9 deposits have been associated with disease activity in some immunostaining-based studies (38, 74). A recent study using a proteomic approach also confirmed that the presence of glomerular complement protein deposition was associated with progressive IgAN (66).

The deposition of regulatory proteins of the alternative pathway has also been related to the activity of IgAN. Patients with progressive disease had more FH and CFHRs 2 and 5 and less CR1 as assessed by mass spectrometry (66). An immunostaining approach very recently confirmed deposition of CFHR1 and CFHR5, with frequency of the finding dependent on disease severity (38). Notably, glomerular deposition of CFHR5 was significantly more frequent in biopsies from 19 patients with progressive IgAN compared to 18 stable counterparts (odds ratio [OR] 13.4 [2.2–66.9]). On the contrary, FH was less frequently deposited (OR 0.1 [0.08–0.87]) with progressive disease. These findings suggest an imbalance between CFHR5 and FH that may accentuate disease severity. Indeed, CFHRs are sequentially similar to FH and can compete with FH for C3b binding, but lack some regulatory functions. For example, CFHR1, compared to FH, lacks FI cofactor activity and the capacity to accelerate decay of C3 convertase. CFHR5 can act as FI cofactor but only at supra-physiologic concentrations, thus being less efficient than FH. Nevertheless, the promising results of these early studies need to be confirmed in larger cohorts.

The deposition of lectin pathway elements has been associated with poorer outcomes in IgAN in several studies. MBL deposits, found in about 25–35% of patients, have been associated with higher proteinuria, lower eGFR, and more severe histopathological lesions (70, 75). Several retrospective studies have confirmed the deleterious prognostic impact of mesangial C4d deposition on renal survival (71, 76, 77). In those series, the prevalence of C4d positivity ranged from 21% in the pediatric cohort up to 38% in the adult Spanish cohort (71, 76, 77).

Mesangial co-deposition of complement elements highlights the pathophysiological role of activation of the alternative and lectin pathways in IgAN and can be considered to be a biomarker of the disease itself as well as its severity. The complexity of the combinations of those deposited proteins offers a potential approach to personalize complement-targeting therapies for patients in the future.

## COMPLEMENT FRAGMENTS IN THE CIRCULATION OF IgAN PATIENTS

Despite the presence of normal or elevated C3 levels in the circulation of most Caucasian patients with IgAN, C3 activation fragments are present in about 50% of patients (78). Subsequently, larger studies showed that 45% of the patients with IgAN had a significantly elevated C3dg level (79) and 70% of pediatric IgAN patients had significantly elevated C3d/C3 ratio in the circulation (80).

Two groups examined plasma levels of activated C3 (actC3) using somewhat similar monoclonal antibodies that detected neoantigens expressed after activation of C3 (81, 82). These monoclonal antibodies were produced in the laboratories of highly accomplished complement investigators, Drs. Eberhard (Scripps) and Götze (Göttingen). The neoantigen recognized by the Scripps antibody is on iC3b, C3dg, and C3d (83), while the Göttingen antibody recognized C3b, iC3b, C3dg, and C3d (81). Data generated from use of the Scripps antibody are available only for subjects with systemic lupus erythematosus (SLE) (84). Neither antibody appears to be available today.

ActC3 in plasma was detected on one occasion for 73% of 55 adult and 57% of 28 pediatric German patients with IgAN when compared to healthy controls (82). When compared to patients with non-immune renal diseases, an elevated plasma actC3 level was found in 30% of patients with IgAN (82). There was an association with progressive loss of renal function with a single elevated actC3 level, with 75% sensitivity and 89% specificity for predicting progression. Weak, but significant, correlations were shown for degree of proteinuria and microscopic hematuria. In a subsequent study of an expanded cohort, mean plasma C3a level was higher for patients with IgAN compared to healthy controls, but mean levels for patients with stable renal function or progressive disease were similar (85). Plasma actC3 levels were near normal for the US patients with normal or minimal mesangial changes, likely corresponding to an Oxford score of M0, E0, S0, T0, and C0 (81). Patients with mesangial proliferation, crescents, or segmental glomerulosclerosis had elevated levels compared to healthy adult controls (81). Plasma C3a levels were significantly elevated for 35% of 46 adult patients with IgAN or IgA vasculitis with nephritis (86). In this study, the plasma C3a level was significantly associated with serum creatinine concentration but not 24-h urinary protein excretion. In the expanded German cohort cited above, the mean plasma C3a level was higher in adult patients with IgAN as compared to healthy controls (85). In this study, plasma C3a level did not correlate with the plasma actC3 level and the mean level did not differ between patients with stable renal function or progressive dysfunction. These findings suggest that the plasma C3a level does not supplant the plasma actC3 level for predicting decline in renal function.

Prior to the delineation of the MBL pathway (87, 88), fragments generated by activation of C4 were considered evidence of activation of the classical pathway. As noted above, we now understand that, for patients with IgAN, they are more likely generated through the MBL pathway. Significant elevation of plasma C4d/C4 ratio was found on at least one occasion for 28% of adult and 11% of pediatric patients with IgAN (80). C4-C3 complexes, assumed to indicate activation of the classical pathway, were elevated in only 8% of patients with IgAN (82).

In two early studies, soluble C5b-9 levels were normal for pediatric and adult patients with IgAN (81, 82). However, another study reported significantly elevated plasma C5b-9 levels for 17% of adult patients with IgAN (79).

Serum complement levels have also been investigated as diagnostic tools. In a Japanese study including 418 healthy individuals, and 195 IgAN and 111 non-IgAN glomerular disease patients, the pre-biopsy ratio of serum IgA to C3 (IgA/C3) was highest among IgAN patients. Additionally, it was a good diagnostic marker to distinguish IgAN from other glomerular diseases. The higher serum IgA/C3 ratio in IgAN patients compared to that in non-IgAN glomerular disease patients was driven by not only a significantly lower C3 level but also a significantly higher IgA level (89). Several studies from East Asia also suggest that the IgA/C3 ratio can be used as a prognostic marker, with higher values being associated with more severe disease histology (90) and worse clinical outcomes including urinary protein excretion, hematuria, and higher creatinine level (91). Among Japanese patients treated with corticosteroids and tonsillectomy, a higher IgA/C3 ratio was associated with a higher incidence of disease recurrence (92).

### Complement Proteins and IgA-Containing Immune Complexes in IgAN

In IgAN, the presence of IgA-containing circulating immune complexes (93–97) and association of complement-containing immune complexes with disease activity have been observed in early studies, (98–101) with many of these observations clarified in follow-up studies, as detailed in the section above. It is now thought that the pathogenic IgA1-containing immune complexes, that can activate primary human mesangial cells in culture to proliferate and produce cytokines and extracellular matrix, play a key role in the pathogenesis of IgAN (19, 33, 96, 102–109). Moreover, studies of various animal models

of IgAN also indicate complement involvement in disease development (14, 110).

Analysis of a model of immune complexes, heat-aggregated mixture of human IgG and IgA1, indicated that these mixed-immunoglobulin aggregates, but not IgA alone, activated C3 (111). Moreover, a study of IgA immune complexes formed *in vitro* from Gd-IgA1 and anti-glycan IgG antibodies in cord-blood serum indicated that the capacity of these complexes to activate proliferation of mesangial cells was dependent on a heat-sensitive serum factor, presumably complement (112). This model of formation of immune complexes *in vitro* was later enhanced by using recombinant Gd-IgA1-specific IgG derived from an IgAN patient (7, 112). Notably, these immune complexes, when formed in the presence of serum, also activate cultured primary human mesangial cells (102, 104, 105, 113).

C3 is present in IgA1-containing circulating immune complexes of patients with IgAN (114). A pilot study of IgA1-containing circulating immune complexes from IgAN patients as well as those formed *in vitro* indicated the presence of C3 products (115). Specifically, C3 α and β chains were detected in the active, large-molecular-mass immune complexes consisting of galactose-deficient IgA1 and recombinant IgG autoantibody. Targeted mass spectrometric analysis identified iC3b, C3c, and C3dg fragments in these complexes. Together, these findings are suggestive of direct binding of C3 and activation of the alternative pathway in this *in vitro* model of IgAN immune complexes (14, 35).

## GENETIC STUDIES ON THE ROLE OF COMPLEMENT PROTEINS IN IgAN

Genetic influences in the development of IgAN were first implicated by a 1985 study of a familial form of this disease (15). Although more studies followed [e.g., (116, 117)], a better appreciation of the impact had to wait until technical advances in genomics enabled genome-wide association studies (GWAS). GWAS of IgAN then provided the initial insight into the genetic architecture of IgAN by identifying specific susceptibility loci across cohorts from Europe, North America and East Asia (118–125). Common genetic variants (including those affecting the alternative complement pathway) may in part explain the geographical differences in disease prevalence worldwide (126). Serum levels of the autoantigen, Gd-IgA1, represent a heritable trait (127, 128) and two loci encoding a specific glycosylation enzyme and its chaperone are linked to this phenotype based on two recent GWAS publications (129, 130). A reader interested in more details on GWAS studies and genetics of IgAN is referred to more specialized reviews [e.g., (126, 131–135)]. Here, we will briefly present genetic and genomic data related to the role of complement in the pathogenesis of IgAN.

Among the loci associated with IgAN that are related to complement are single-nucleotide polymorphisms (SNPs) on chromosome 1q32 (centered on reference SNP ID number [rs] rs6677604) and 16p11 (rs11574637 and rs7190997). The locus on chromosome 1q32 includes a cluster of genes (*CFHR 1-5*) that encode factor H-related proteins and rs6677604 is a surrogate marker of *CFHR1,3* gene deletion (*CFHR3,1*Δ). This allele is associated with a reduced risk of developing IgAN. As CFHR peptides are involved in the regulation of the alternative pathway, the absence of these CFHR peptides may lead to a more potent inhibition of complement system by FH. This postulate is supported by a recent study; it showed that *CFHR3,1*Δ (heterozygous or homozygous) was associated with a reduced level of glomerular immune deposits (IgA, IgG, and C3) (63). These findings correspond well with the early observations about the involvement of the complement system in IgAN (35, 99) and the current understating of complement's role in IgAN (34, 136). Moreover, several studies confirmed that genetic variants in CFH, CFHR3,1, and possibly CFHR5, can differentially affect complement activation and, thus, impact predisposition to IgAN (34, 37, 137). For example, serum CFH levels are negatively associated with mesangial C3 deposition (37). For CFHR5, 28 rare and 4 common variants in amino-acid sequence were identified in a Chinese cohort and the distribution of rare variants in patients with IgAN differed significantly from that in controls (137). Moreover, some of the rare variants were functional, as shown by the reduced or increased C3b binding by recombinant CFHR5 variant proteins compared to the wild-type protein.

The second IgAN GWAS locus related to complement is on chromosome 16p11 that contains *ITGAM* and *ITGAX* genes that encode integrins αM and αX, respectively. These integrins have roles in the formation of leukocyte-specific complement receptors 3 and 4 by combining with the integrin β2 chain. ITGAM gene product, also known as CD11b, is the α-chain of the αMβ2 integrin. This leukocyte-specific integrin regulates cell activation and adhesion of neutrophils and monocytes, enabling endothelium stimulation and phagocytosis of complement-coated particles. This locus is associated with several other autoimmune diseases, including SLE (138, 139). The SLE-associated variant is related to a reduced clearance of immune complexes (140). α-X chain protein associates with β2-chain to form another leukocyte-specific integrin with functions thought to be similar to those of ITGAM.

Together, recent GWAS have currently identified 22 IgAN-associated loci. Within these loci are genes encoding products involved in complement regulation and interaction with immune complexes that may account for these associations with IgAN. These findings have provided novel insights about possible mechanisms of disease. Follow-up genetic and biochemical studies are needed to delineate the precise roles of these complement-associated genes and their alleles.

## COMPLEMENT SYSTEM AS TARGET FOR FUTURE THERAPIES

Recent scientific advances have improved our understanding of the role of complement in the pathogenesis of IgAN. This information has led to identification of new potential therapeutic targets to halt or slow the disease course. So far,

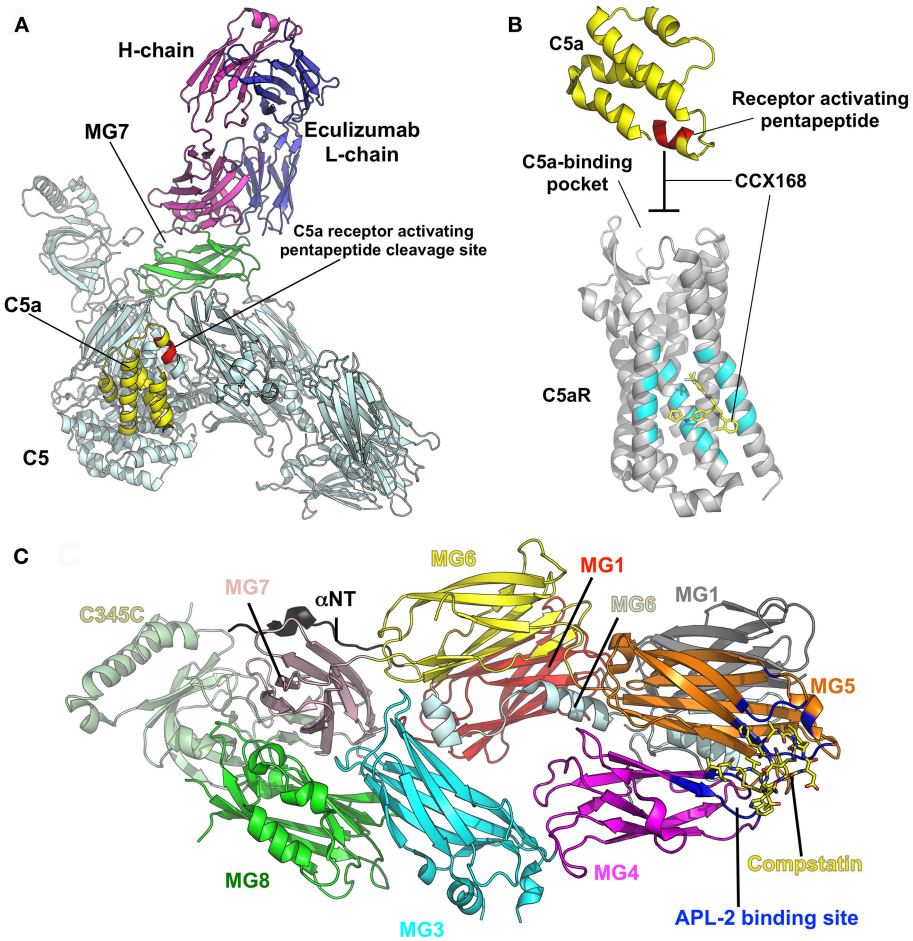


**FIGURE 3 |** Examples of therapeutic control of complement-activation pathways. **(A)** The complex of C5 and the Fab derived from eculizumab antibody (PDB ID: 5I5K) (145) are shown in cartoon model with the heavy (H) and light (L) chains of the Fab colored in magenta and blue, respectively. C5 is shaded in pale cyan, while macroglobulin domain 7 (MG7), the site of eculizumab binding on C5, is highlighted in green. C5a is yellow and the C5a-receptor-activating pentapeptide cleavage site is red. **(B)** C5a (yellow) binds to the membrane-associated C5a receptor (C5aR, gray background) *via* the C-terminal C5a pentapeptide (red). CCX168 (Avacopan, yellow stick model) binds to the surface of C5aR (pocket shaded in cyan), thereby blocking C5a binding through allosteric effects on the C5a-binding pocket. The C5a/C5aR model is based on PDB ID: 6C1R (146). **(C)** Compstatin, and APL-2 are inhibitors of activation of C3. The crystal structure of C3c (PDB ID: 2QKI) (147), a major proteolytic fragment of C3, is shown in complex with compstatin (yellow stick model). Both inhibitors bind to a site (shaded in blue) formed by the macroglobulin domains 4 and 5 (MG4, MG5). All illustrations were prepared with PyMOL (148).

treatment with complement inhibitors is limited to a few published cases reporting the use of eculizumab, a humanized monoclonal antibody that inhibits cleavage of C5 by C5 convertase, as rescue therapy. The first report came from Sweden; a young 16-year-old white male with biopsy-proven crescentic IgAN had failed to respond to corticosteroids and mycophenolate but stabilized when treated with eculizumab, although the therapeutic effects were not sustained (141). Similarly, another 16-year-old male with crescentic IgAN who had failed treatment with corticosteroids, cyclophosphamide, and plasma exchange subsequently had transient improvement in renal function with eculizumab (142). Knowing the role of complement in IgAN pathophysiology, and encouraged by these anecdotal therapeutic results, Herzog et al. used eculizumab as a rescue therapy in a 28-year-old male with post-transplant recurrent crescentic IgAN. The attempt to salvage the allograft failed but therapy was instituted after the initiation of dialysis and hence may have been too late (143). In a series of elegant experiments, Zhang et al. showed that antagonists of the receptors for C3 and C5 prevented proliferation of cultured human mesangial cells stimulated by IgA and reduced up-regulation of IL-6 and monocyte chemoattractant protein 1 (MCP-1) (144). In an experimental model of IgAN, mice deficient for C3 and C5 receptors had less proteinuria, mesangial IgA deposition, mesangial matrix expansion and hypercellularity than normal mice, but serum creatinine and blood urea nitrogen levels were similar. These experiments suggest that perhaps inhibition of receptors for C3 and C5 may be promising therapeutic interventions in the future (144).

Elucidating the role of activation of the MBL and alternative pathways in the pathophysiology of IgAN has identified new potential treatment targets. While some therapies (such as eculizumab and CCX168) may be non-specific inhibitors of the distal common pathway, others target a specific pathway more proximally (**Figure 2**). Inhibition of complement activation can be achieved with monoclonal antibodies, small molecules, and short peptides that block protein-complex formation and/or enzymatic activity. Eculizumab, a monoclonal humanized antibody, binds to complement protein C5 at the level of macroglobulin domain 7 (MG7), thus blocking cleavage of C5 by C5 convertase into pro-inflammatory components C5a and C5b (**Figure 3A**). C5a binds to the membrane-associated C5a receptor (C5aR) *via* the C-terminal C5a pentapeptide. CCX168 (Avacopan), a small molecule antagonist of the inflammatory response, binds to the surface of C5aR, thereby blocking C5a binding through allosteric effects on the C5a-binding pocket (**Figure 3B**). Compstatin, a cyclic tridecapeptide, and APL-2, a pegylated derivative of compstatin, inhibit the activation of C3 (**Figure 3C**). MASP-2 complement control protein (CCP) domain binds to C4. Upon association with MASP-2, C4 undergoes a conformational change whereby the scissile bond-containing R-loop is inserted into the catalytic site of the serine-protease domain. Cleavage yields fragments C4a and C4b. Monoclonal antibody OMS721 binds to a CCP domain of MASP-2, inhibiting the lectin pathway by blocking complex formation (**Figure 4A**). In the alternative pathway, FB binds to C3b displacing the N-terminal CCP domains. This in turn leads to rearrangement of the central helices and release of the scissile bond for proteolytic activation (**Figure 4B**). LNP023, an orally available small molecule, interferes with the alternative complement cascade by inhibition of the proteolytic activity of FB.

To date, clinical trials in the treatment of IgAN using surrogate end-points, such as doubling of serum creatinine, have been limited due to the variable course and often slowly progressing nature of the disease. The size and cost of such trials has been relatively prohibitive so far. More recently, and with guidance from the Unites States Food and Drug Administration, there has been renewed interest in testing novel therapies using "reasonably likely" surrogate end-points (such as quantitative proteinuria) that could lead to accelerated conditional drug approval (151). This change in policy has sparked the initiation of multiple clinical trials evaluating the benefits of various inhibitors of the complement cascade in IgAN (**Table 1**). Besides evaluating their efficacy, we need to assess the risks associated with the use of these drugs, infections being the primary concern. Very limited data regarding the safety of these inhibitors are available in the literature. In part, our understanding about the risks of drugs that interfere with the complement system comes from syndromes of congenital complement deficiencies. Observations that the infection rates in these children decreases as they age suggest that the role of the innate immunity becomes less prominent in the setting of maturing adaptive immunity (152). The risk of infection also depends on the level of pathway inhibition. While C5 inhibitors increase primarily the risk of neisserial infections, C3 inhibitors are likely to confer a broader infectious susceptibility warranting vaccination against several encapsulated organisms. However, even inhibitors like compstatin do not completely abrogate complement-mediated immunity against pathogens as even modest residual complement activity seems to be protective (55). Other theoretical safety concerns come from the observation that some classical complement deficiencies increase the risk of developing SLE, hence raising the concern for developing autoimmunity with complement inhibition (55). Perhaps the most information about drug safety comes from the use of eculizumab treatment that substantially increases the risk of infections with encapsulated organisms. In particular, the rate of meningococcal infection increases by 1,000-fold compared to that in the general population. It is recommended that patients contemplating treatment with eculizumab receive meningococcal vaccine at least 2 weeks prior to therapy initiation. Vaccination has reduced the risk of meningitis by 10-fold. If therapy is initiated prior to 2

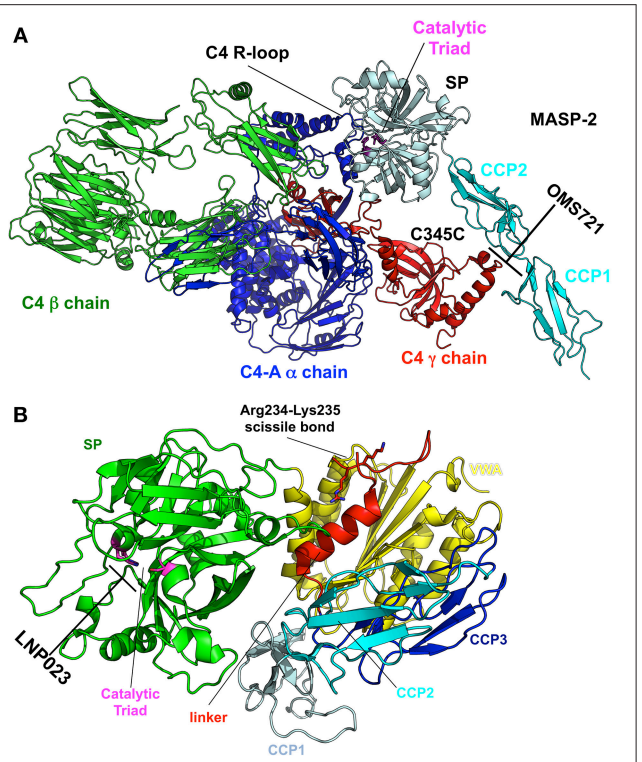


**FIGURE 4 |** Targets and therapeutic agents of the lectin and alternative complement-activation pathways. **(A)** The crystal structure of the C4/MASP-2 complex is shown (PDB ID: 4FXG) (149). MASP-2 complement control protein (CCP) domains bind to C4. Upon association with MASP-2, C4 undergoes a conformational change whereby the scissile bond containing R-loop is inserted into the catalytic site of the serine-protease (SP) domain (pale cyan with catalytic triad in magenta). Cleavage yields fragments C4a and C4b. Monoclonal antibody OMS721 binds to a CCP domain of MASP-2, inhibiting the lectin pathway by inhibiting complex formation. **(B)** The structure of factor B is shown (PDB ID: 2OK5) (150). Upon C3b binding, the N-terminal CCP domains are displaced, leading to rearrangement of the central helices and release of the scissile bond for proteolytic activation. LNP023 inhibits the proteolytic activity of factor B.

**TABLE 1 |** Registered clinical trials of complement inhibitors being tested for the treatment of patients with IgAN.

| Drug | Type | Target | Trial | ID | Phase | Sponsor |
|---|---|---|---|---|---|---|
| APL-2 | Inhibitor | C3 | Phase 2 study assessing safety and efficacy of APL-2 in glomerulopathies | NCT03453619 | 2 | Apellis Pharmaceuticals LLC. |
| CCX168 | Receptor antagonist | C5a receptor | Open-label study to evaluate safety and efficacy of CCX168 in subjects with IgAN on stable RAAS blockade | NCT02384317 | 2 | ChemoCentryx |
| LNP023 | Inhibitor | Factor B | Study of the safety and efficacy of LNP023 in patients with kidney disease caused by inflammation | NCT03373461 | 2 | Novartis Pharmaceuticals |
| OMS721 | mAb | MASP-2 | Safety study of IgAN, lupus nephritis, membranous nephropathy and C3 glomerulopathy including dense deposit disease treated with OMS721 | NCT02682407 | 2 | Omeros Corporation |
| | | | Study of the safety and efficacy of OMS721 in patients with IgAN | NCT03608033 | 3 | |

*Data from https://ClinicalTrials.gov, accessed on December 2, 2018. IgAN, IgA nephropathy; RAAS, renin angiotensin aldosterone system; ID-ClinicalTrials.gov identifier.*

weeks from the time of vaccination, antibacterial prophylaxis is recommended (153). Ultimately, the duration and extent of complement inhibition will also play a role in the safety of treatment. Several of these therapeutic agents are also being evaluated in a variety of other disorders ranging from atypical hemolytic uremic syndrome to age-related macular degeneration to various glomerulonephritides such as lupus nephritis and membranous nephropathy. The cumulative experience from all these trials will inform our future use of complement inhibitors in IgAN.

## CONCLUSION

In the past several decades, much progress has been made in understanding the role of the complement system in IgAN pathogenesis and prognosis. Data from studies of the pathology features, biochemistry of IgA1, and genetic influences on the disease and animal models confirm the involvement of the alternative and lectin pathways. Markers of complement activation are not only diagnostic but are also emerging as prognostic tools to risk-stratify disease severity. Complement components likely play significant roles in amplifying the inflammatory response for formation of immune complexes and their deposition in the glomerular mesangium. These findings have sparked marked interest in targeting the complement cascade at multiple levels in an effort to halt or slow the disease progression. Much remains to be learned about the optimal timing and intensity of use of complement inhibitors and their efficacy and safety in the treatment of patients with IgAN.

## AUTHOR CONTRIBUTIONS

DR, JN, BJ, and RW conceived the general outline. BK and TG generated the figures, with feedback from JN, DR, and BJ. All authors (DR, NM, BJ, BK, TG, JN, RW) contributed intellectually by writing assigned sections, editing and revising the drafts, and proofreading the manuscript.

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REVIEW
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# Factor B and C4b2a Autoantibodies in C3 Glomerulopathy

*Jill J. Hauer, Dingwu Shao, Yuzhou Zhang, Carla M. Nester and Richard J. H. Smith**

*Molecular Otolaryngology and Renal Research Laboratories, University of Iowa, Iowa City, IA, United States*

C3 Glomerulopathy (C3G) is a renal disease mediated primarily by dysregulation of the alternative pathway of complement. Complement is the cornerstone of innate immunity. It targets infectious microbes for destruction, clears immune complexes, and apoptotic cells from the circulation, and augments the humoral response. In C3G, this process becomes dysregulated, which leads to the deposition of complement proteins—including complement component C3—in the glomerular basement membrane of the kidney. Events that trigger complement are typically environmental insults like infections. Once triggered, in patients who develop C3G, complement activity is sustained by a variety of factors, including rare or novel genetic variants in complement genes and autoantibodies that alter normal complement protein function and/or regulation. Herein, we review two such autoantibodies, one to Factor B and the other to C4b2a, the C3 convertase of the classical, and lectin pathways. These two types of autoantibodies are identified in a small fraction of C3G patients and contribute marginally to the C3G phenotype.

**Keywords: complement dysregulation, autoantibodies, C3 glomerulopathies, factor B, C3 convertase, C5 convertase**

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****Correspondence:***
*Richard J. H. Smith*
*richard-smith@uiowa.edu*

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## INTRODUCTION

C3 Glomerulopathy (C3G) is an ultra-rare renal disease characterized by dysregulation of the alternative pathway (AP) of the complement cascade, which leads to the accumulation of complement cleavage products in the glomerular basement membrane (GBM) of the kidney. The complement cascade is comprised of over 30 proteins and can harness both the innate and adaptive immune systems to initiate and amplify an immune response in the presence of an antigen or unwanted cell debris. Activation occurs via three separate pathways: the classical, lectin, and alternative pathways. Classical pathway (CP) activation is mediated by the recognition of an antigen on a cell surface, the lectin pathway (LP) is initiated through the recognition of carbohydrates and other antigenic patterns, and the AP is spontaneously activated via the "tick-over" mechanism whereby an internal thioester bond of the C3 protein is hydrolyzed to form a functionally active form of the C3 protein ($C3_{H20}$) (1, 2).

Once initiated, all pathways converge on the formation of the C3 convertase—an enzyme complex responsible for the amplification of the complement response—which rapidly cleaves and activates additional C3 protein. If complement activation is triggered through the AP, the AP C3 convertase is formed by the interaction of cleaved C3 and Factor B proteins (C3bBb). In contrast, if CP or LP activation occurs, the classical C3 convertase is formed (C4b2a). Cleavage of C3 by C4b2a leads to formation of the AP C3 convertase, C3bBb, and over 90% of continued complement activity continues through this convertase (3). Robust C3 convertase activity generates copious C3b and facilitates the formation of the C5 convertase, which marks the beginning of the terminal complement cascade. C5 activation by the C5 convertase leads to the formation of the membrane attack complex (MAC), which is ultimately responsible for inducing lysis of the identified pathogen (4, 5).

Rigorous regulation of complement is required to prevent unwanted complement activity. Cases of complement *dysregulation* lead to a diverse set of diseases underpinned by the common characteristic of damage to host tissue. C3 Glomerulopathy (C3G) is one such example (6–8). C3G is characterized by dysregulation of the AP, which leads to C3 deposition in the glomerulus. The diagnosis requires a renal biopsy, which by consensus must show by immunofluorescence the deposition of C3 in renal glomeruli that is at least 2-fold higher than any other immune reactant. Further categorization of C3G into subtypes is made by electron microscopy. If dense, intra-membranous, sausage-like deposits are seen, the diagnosis is dense deposit disease (DDD); when the deposits are lighter, cloud-like, and sub-epithelial, or sub-endothelial, the diagnosis is C3 Glomerulonephritis (C3GN) (9, 10). There is currently no disease-specific treatment for C3G and about 50% of patients progress to end stage renal disease (ESRD) within 10 years of diagnosis (11).

The drivers of complement dysregulation in C3G can be identified in the majority of cases and include genetic variants in complement proteins and regulators, and/or autoantibodies specific to proteins or complexes of the complement cascade. The earliest description of autoantibodies that target complement proteins dates back to 1967 when C3- and C4-targeting immunoglobulins were described in the serum of several species of mammals upon stimulation with the animal's autologous, fixed complement components (12). Subsequently, autoantibodies have been described in virtually every branch of the complement system: autoantibodies targeting CP proteins (13, 14), LP proteins (15), AP proteins (16), protein complexes including the C3 (17), and C5 (18) convertases, and complement regulatory (19, 20) proteins. Autoantibodies to complement can be detected in a diverse spectrum of diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), atypical hemolytic uremic syndrome (aHUS), and C3G (21). This review is tightly focused on two types of autoantibodies that are specific for Factor B and C4b2a (also known as C4 nephritic factors or C4Nefs). We discuss the current knowledge relevant to these two antibodies, methods for their robust detection, and their more recently appreciated role in C3G (**Figure 1**).

## FACTOR B AUTOANTIBODIES

Factor B autoantibodies (FBAAs) have been identified in a small number of individuals and exclusively in patients with C3G. The first report of FBAAs was in 2010—a DDD patient was positive for an IgG that bound the cleaved Bb fragment of Factor B and recognized an epitope on the C3 convertase (C3bBb). This antibody stabilized the C3 convertase and prevented its decay; it also prevented formation of the C5 convertase thereby inhibiting terminal pathway activity. Unlike the more common C3 nephritic factor, this FBAA did not bind a neoepitope on the C3 convertase and because it prevented terminal pathway activity, it tested negative in the hemolytic assay, which is traditionally used to detect the stabilizing effect of C3 nephritic factors (C3Nefs) on C3 convertase (16). In 2011, two more patients were reported to have FBAAs, which stabilized C3 convertase activity (22). All three patients were negative for C3Nefs (16, 22).

In a larger study on a cohort of 141 patients with C3G or Ig-associated membranoproliferative glomerulonephritis (immune complex glomerulonephritis, ICGN), seven patients were positive for FBAAs, three were positive for anti-C3b IgG, and five were positive for both FBAA and anti-C3b. Ten of these 15 patients were diagnosed with ICGN. Consistent with previous reports, the patients with FBAAs alone demonstrated specific enhancement of C3bBb activity only; there was no enhancement of C5 convertase activity. Patients who were positive for both FBAAs and anti-C3b antibodies showed enhancement of both C3 and C5 convertase activity. FBAA binding was mapped to the Bb fragment of Factor B in this study (23).

Taken together, these data suggest that FBAAs are present in only a small percentage of the C3G population and drive specific over-activity of C3 convertase. Factor B autoantibodies have not yet been associated with any other complement-mediated diseases.

## C4 NEPHRITIC FACTORS

In 1979, two patients with partial lipodystrophy were reported to have a nephritic factor the activity of which was dependent on the presence of C2, thus implicating the involvement of the classical C3 convertase, C4b2a (24). Shortly thereafter, C4Nef was fully characterized in a patient with post-infectious glomerulonephritis (PIGN) and proposed to be a separate entity from C3Nef. In this study, C4Nef was demonstrated to be an IgG capable of stabilizing both cell-bound and fluid-phase classical C3 convertase (25). Further investigation has shown that C4Nefs protect the classical C3 convertase from decay mediated by CR1 and C4 binding protein (C4BP) but not from decay mediated by decay accelerating factor (DAF) (26–29).

In 1989, a study of 2 patients diagnosed with MPGN type I detected the presence of both C3Nefs and C4Nefs. Both patients had low circulating levels of C3 and C5 proteins, consistent with complement consumption due to the presences of these Nefs (30). A case study in 1993 of 100 hypocomplementemic patients with MPGN found that almost 20% were positive for C4Nefs. Half of these patients were also positive for C3Nefs. Interestingly, terminal complement pathway activity was only elevated in patients positive for both C3Nefs and C4Nefs (31). Another case report described an 18-year-old male with C3 deficiency who developed severe meningococcal meningitis. C3 consumption was driven by C4Nefs, which stabilized both C4b2a and C4b2aC3b, the C5 convertase of the classical pathway (32).

In aggregate, C4Nefs represent a possible cause of complement dysregulation in a small proportion of patients with complement-mediated diseases. Further studies are needed to elucidate their ability to stabilize both the C3 and C5 convertases of the classical pathway.

## DETECTION OF FBAA AND C4NEFS

Autoantibody detection in C3G is generally performed using ELISA and hemolytic-based assays. Both FBAA and C4Nefs have been identified using ELISA assays (23, 33, 34). For FBAAs, plates are coated with either the full length protein, the Bb fragment

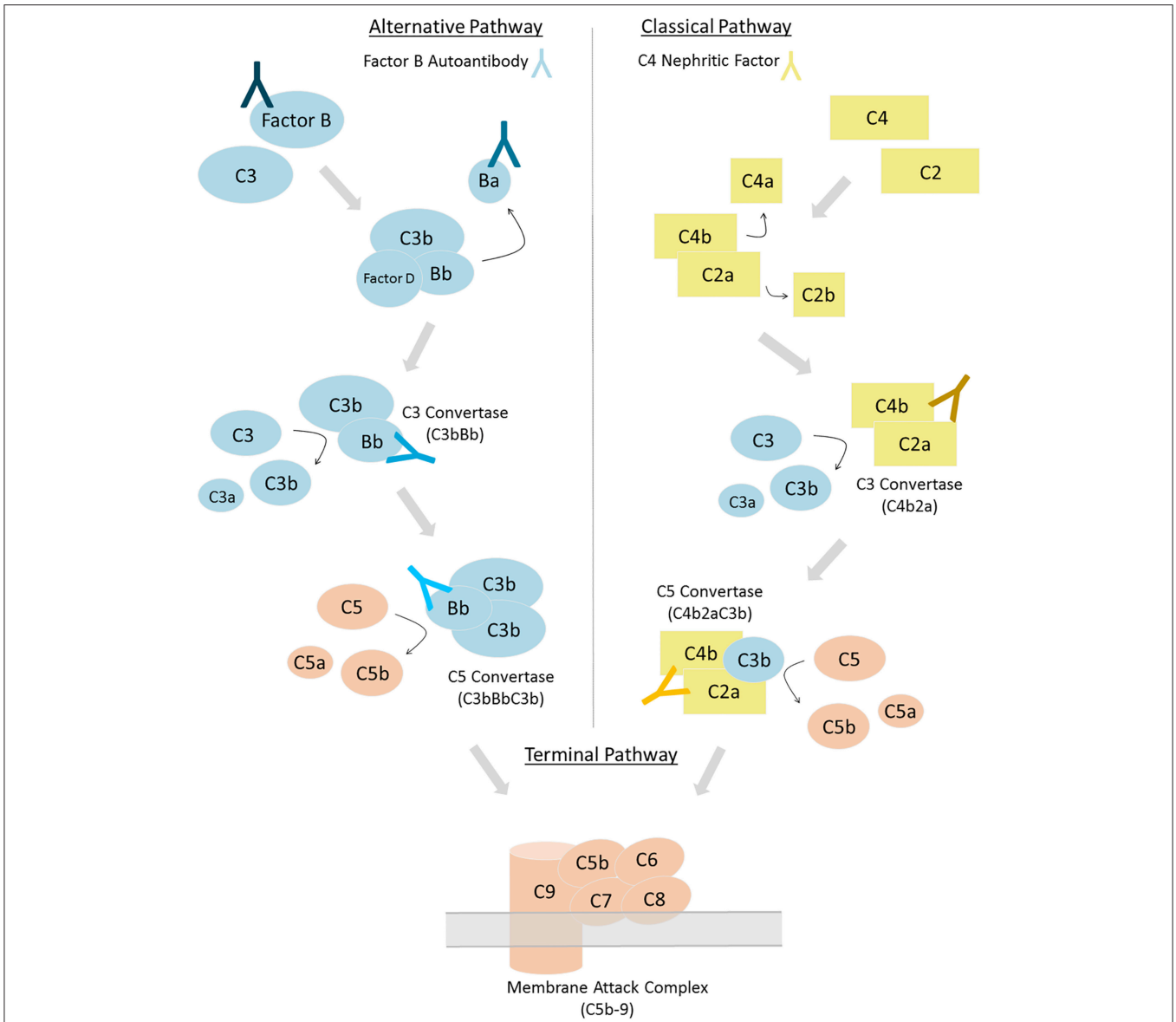


**FIGURE 1 |** Complement can be activated via three distinct pathways. Factor B autoantibodies bind to Factor B and/or its protein fragments generated via alternative pathway activation (left). C4 nephritic factors bind to the classical and lectin pathway's C3 and C5 convertases (right). Differing shades of antibodies indicate possible epitopes; however, the epitope or epitopes of factor B autoantibodies and C4 nephritic factors may vary among patients.

or the Ba fragment. After incubation with patient-purified IgG, binding can be detected using anti-human IgG (**Figure 2A**) (23). For C4Nef detection, a sandwich ELISA is used. This method indirectly detects C4Nefs by detecting the increased stabilization of the C4b2a complex. To form the CP C3 convertase (C4b2a), patient IgG is added to normal human serum (NHS). After ample time is allowed for intrinsic decay of C4b2a, the NHS-IgG mix is added to a plate coated with anti-C2 antibodies. Convertases that have not decayed (i.e., that have been stabilized by C4Nefs) are detected using an anti-C4 antibody (33). Both methods test binding of the autoantibody to its target complement protein, however neither measures functional activity (**Figure 2B**).

The hemolytic assay uses sheep erythrocytes, which are non-complement activators, to measure hemolytic activity (35). To detect C4Nefs, C4b2a is first built on sheep erythrocytes that have been antibody primed, and then the C4b2a-coated erythrocytes are incubated with patient IgG. After a fixed time during which convertase decay occurs in the absence of C4Nefs or is prevented in their presence, terminal complement components are added and lysis is quantitated. The amount of free heme reflects the degree of convertase stabilization and activity (33, 36). This assay provides an environment closer to *in vivo* conditions (**Figure 2C**).

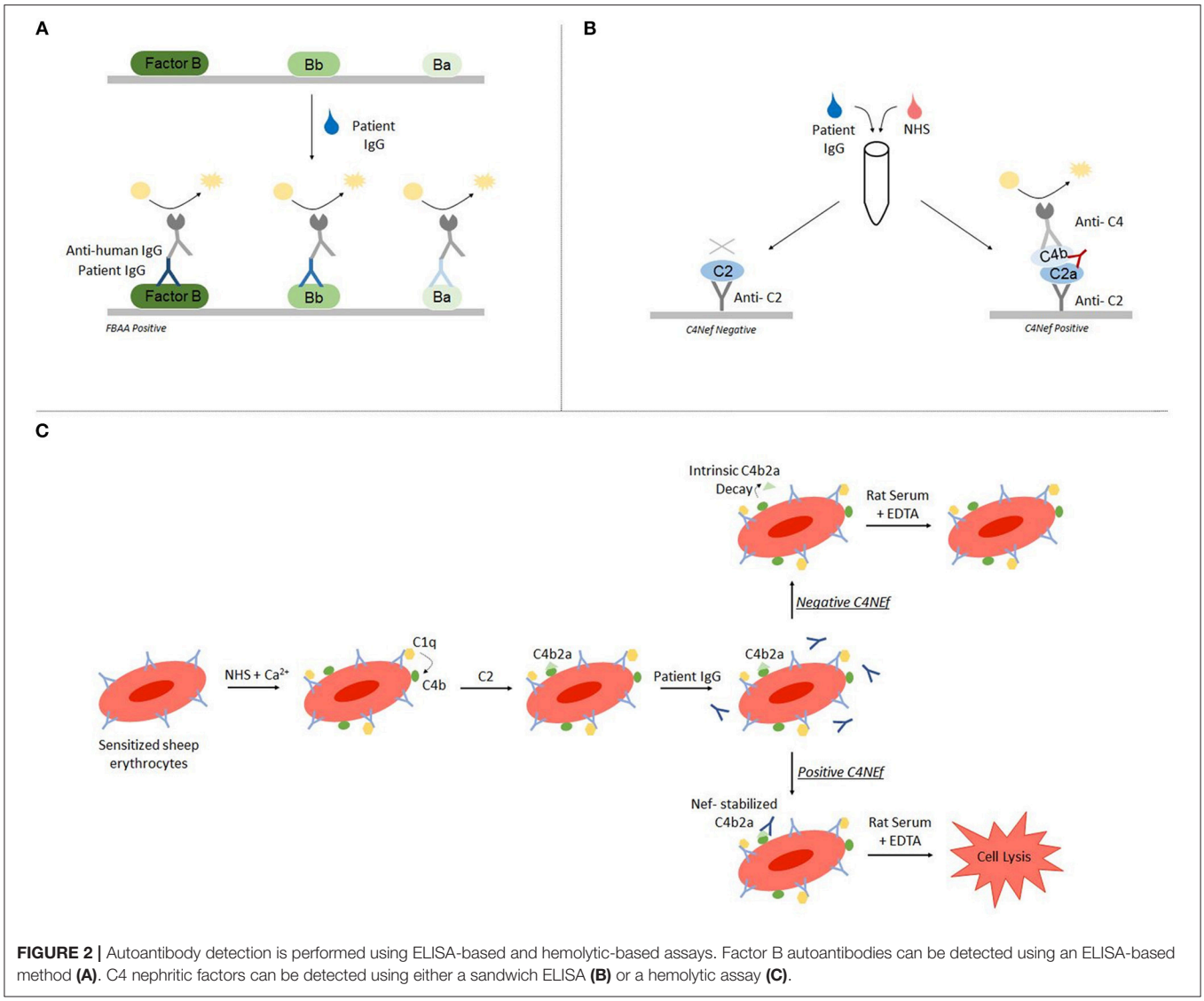


**FIGURE 2 |** Autoantibody detection is performed using ELISA-based and hemolytic-based assays. Factor B autoantibodies can be detected using an ELISA-based method **(A)**. C4 nephritic factors can be detected using either a sandwich ELISA **(B)** or a hemolytic assay **(C)**.

Most recently, Blom et al. developed a modified hemolytic-based method that is sensitive not only to Nefs but also to genetic abnormalities (37). The method uses full patient serum with the addition of a C5 inhibitor to build the convertase on sheep or rabbit erythrocytes. This method is able to determine if Nefs are resistant to the extrinsic decay of complement regulatory proteins in full patient serum (36, 38).

Each of the above assays has limitations that should be recognized and are summarized in **Table 1**.

## THE CONSEQUENCE OF FBAAS AND C4NEFS IN C3G

Many types of autoantibodies can be detected in patients with C3G. In aggregate, autoantibodies that stabilize and increase the half-life of the C3 and C5 convertases (C3Nefs and C5nefs, respectively) are identified in ∼60% of patients (36, 39). Several studies have associated these autoantibodies with complement dysregulation, which supports the hypothesis that these autoantibodies are drivers of the C3G phenotype, although this relationship is not yet well understood.

Biomarker testing is often performed on sera from C3G patients to quantitate the degree of complement dysregulation. By measuring levels of circulating C3 and its cleavage products, for example, it is possible to identify when C3 convertase overactivity occurs. When low C3 levels (indicating C3 consumption, thus complement activation) are accompanied by low C5 and high soluble C5b-9 levels, dysregulation of both the C3 and C5 convertase is highly likely.

Data on the biomarker profile of C3G patients with FBAAs are anecdotal. Marinozzi et al. identified three patients diagnosed with C3G who were positive for FBAAs. The biomarker profile of these three patients, and nine additional patients with ICGN showed only an increase in the Bb fragment of cleaved Factor B (data summarized in **Table 2**) (23).

**TABLE 1 |** Methods for detection of Factor B autoantibodies and C4 nephritic factors.

| Method | Autoantibody | Strengths | Limitations |
|---|---|---|---|
| ELISA (**Figure 2A**) | Factor B | 1. Ability to map domain of IgG binding epitope by coating ELISA plate with either FB, Bb, or Ba<br>2. Test is inexpensive and fast | 1. Anti-human IgG is used to detect FBAA binding—this tests for the presence of FBAA, but does not test the effect of FBAA on complement function<br>2. Assay is performed using patient purified IgG and is therefore unable to test the effect of complement regulators on FBAA function<br>3. Assay is performed on a protein-coated 96-well plate which does not represent *in vivo* conditions |
| ELISA (**Figure 2B**) | C4Nef | 1. Assay is conducted with patient purified IgG and normal human serum—this means nef function is tested in the presence of complement regulatory proteins<br>2. Test is inexpensive and fast | 1. Anti-C2 and anti-C4 are used to capture the classical convertase—this means the presence of C4Nefs is indirectly tested, and does not discern function of the classical convertase<br>2. Assay is performed on a protein-coated 96-well plate which does not represent *in vivo* conditions |
| Hemolytic (**Figure 2C**) | C4Nef | 1. Assay is performed using sheep erythrocytes—a more realistic representation of *in vivo* conditions<br>2. Convertase function is directly quantified by measuring hemolysis | 1. Assay is technically difficult to perform<br>2. Assay is performed using patient purified IgG and is therefore unable to test the effect of complement regulators on FBAA function<br>3. Assay does not discern where on the convertase the C4Nef is binding |

**TABLE 2 |** Biomarker profiles associated with Factor B autoantibodies and C4 nephritic factors.

| Biomarker | FBAA (23) | C4Nef (40) |
|---|---|---|
| C3 | Normal | Low |
| C3c | Not Tested | High |
| FB | Not Tested | Normal |
| Ba | Not Tested | Normal |
| Bb | High | Normal |
| C2 | Not Tested | Normal |
| C4 | Not Tested | Normal |
| C4a | Not Tested | Normal |
| C5 | Not Tested | Low |
| sC5b-9 | Normal | High |
| Properdin | Not Tested | Low |

In the most recent case series of 168 patients with C3G, five were positive for C4Nefs using the hemolytic-based assay. Of note, two of the five patients positive for C4Nefs were also positive for other autoantibodies—one patient was positive for C3Nefs and the other for Factor H autoantibodies. All five patients showed a biomarker profile consistent with dysregulation of the C3 and C5 convertase (findings summarized in **Table 2**) (40). These data are consistent with a study by Blom et al. who reported an individual with C3G positive for C4Nefs (41).

## CONCLUSIONS

C3G is a complex disease, making a composite view of complement biomarkers and complement function, coupled with a genetic analysis and a thorough screen for autoantibodies essential in every patient. FBAAs and C4Nefs drive disease in a small fraction of patients. The small numbers of patients positive for these autoantibodies makes it difficult to discern the magnitude of their role as a disease driver, but as testing for these autoantibodies becomes routine, their impact will become better defined. More precise assays, which measure not only epitopes and function but also provide data on concentration and convertase avidity, are needed to better understand the nuanced impact of these autoantibodies on disease course and outcome.

## AUTHOR CONTRIBUTIONS

JH, DS, YZ, CN, and RS contributed to the conception and design of the study. DS and YZ provided protocols for detection methods. JH performed the literature review. JH and RS wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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# Nephritic Factors: An Overview of Classification, Diagnostic Tools and Clinical Associations

*Fernando Corvillo*[1,2], *Marcin Okrój*[3], *Pilar Nozal*[1,2,4], *Marta Melgosa*[1,5], *Pilar Sánchez-Corral*[1,2] *and Margarita López-Trascasa*[1,6]*

[1] Complement Research Group, Hospital La Paz Institute for Health Research (IdiPAZ), La Paz University Hospital, Madrid, Spain, [2] Center for Biomedical Network Research on Rare Diseases (CIBERER U754), Madrid, Spain, [3] Department of Medical Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Gdansk, Poland, [4] Immunology Unit, La Paz University Hospital, Madrid, Spain, [5] Pediatric Nephrology Unit, La Paz University Hospital, Madrid, Spain, [6] Departamento de Medicina, Universidad Autónoma de Madrid, Madrid, Spain

Nephritic factors comprise a heterogeneous group of autoantibodies against neoepitopes generated in the C3 and C5 convertases of the complement system, causing its dysregulation. Classification of these autoantibodies can be clustered according to their stabilization of different convertases either from the classical or alternative pathway. The first nephritic factor described with the capacity to stabilize C3 convertase of the alternative pathway was C3 nephritic factor (C3NeF). Another nephritic factor has been characterized by the ability to stabilize C5 convertase of the alternative pathway (C5NeF). In addition, there are autoantibodies against assembled C3/C5 convertase of the classical and lectin pathways (C4NeF). These autoantibodies have been mainly associated with kidney diseases, like C3 glomerulopathy and immune complex-associated-membranoproliferative glomerulonephritis. Other clinical situations where these autoantibodies have been observed include infections and autoimmune disorders such as systemic lupus erythematosus and acquired partial lipodystrophy. C3 hypocomplementemia is a common finding in all patients with nephritic factors. The methods to measure nephritic factors are not standardized, technically complex, and lack of an appropriate quality control. This review will be focused in the description of the mechanism of action of the three known nephritic factors (C3NeF, C4NeF, and C5NeF), and their association with human diseases. Moreover, we present an overview regarding the diagnostic tools for its detection, and the main therapeutic approach for the patients with nephritic factors.

**Keywords: complement system, nephritic factor, C3 glomerulopathy, lipodystrophy, eculizumab**

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*Marie-Agnes Dragon-Durey, Université Paris Descartes, France*

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*Betty P. Tsao, Medical University of South Carolina, United States*

***Correspondence:**
*Margarita López-Trascasa*
*margarita.lopez@uam.es*

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## INTRODUCTION

The complement system is a complex molecular system with fundamental roles in apoptotic cell clearance, immune complex elimination, defense against infections, and modulation of adaptive immunity (1). Moreover, complement is capable of distinguishing between self-components and foreign agents. Through a molecular tagging system, complement labels these foreign agents to

be eliminated by opsonophagocytosis or by direct cell lysis (2, 3). The complement cascade is activated by three distinct mechanisms: the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP) (**Figure 1**). These three pathways differ mainly in the initial activation steps, but all of them converge in the activation of the C3 molecule through the generation of unstable protease complex, called C3 convertases (1–4).

The activation of CP and LP pathways is triggered by the binding of antigen–antibody complexes, and through the recognition of mannose groups on bacterial surfaces, respectively. After its activation, the resulting multi-molecular complex C3 convertase (C4b2a), is responsible for cleaved C3 molecule into C3a and C3b (**Figure 1**) (1). The next step concludes with the incorporation of C3b molecules to the C4b2a complex, and the generation of the CP/LP C5 convertase (C4b2aC3b). Finally, C5 convertase splits C5 into C5a, a pro-inflammatory anaphylatoxin, and C5b, which incorporates to the formation of the of the membrane attack complex (MAC or C5b9), along with C6, C7, C8, and C9, on the cell surface. Two soluble proteins, called vitronectin and clusterin, regulate this process, preventing its incorporation into cell membranes (1–4) (**Figure 1**).

In contrast to the CP and LP, the AP is initiated by spontaneous activation of C3 in plasma, which occurs through the "tick-over" (1, 2). This spontaneous activation results in the production of a few C3a and C3b molecules. The resulting C3b molecules can then combine with factor B (FB) to form the inactive AP pro-C3 convertase (C3bB). Then FB is cleavage by factor D (FD), generating Ba, which is released from the complex, and Bb, which remains bound. The assembled C3bBb complex is considered the functional C3 convertase of the AP, and is capable of amplifying complement activation by a feedback mechanisms that generates a large number of C3b molecules within a short time span (1–4) (**Figure 1**). There is a complement positive regulator, called properdin (P), which can bind to the C3bBb complex inducing an increase of his half-life (1, 2). Such as happened in CP and LP, generation of the AP C5 convertase (C3bBbC3b) cleaves C5 and ends in MAC formation (1, 2).

The convertase complexes dissociate spontaneously in a few minutes, a process that is critical to prevent autologous tissue injury. To prevent this damage, there is a group of soluble complement regulatory proteins (Factor H (FH), Factor I (FI) and C4BP) and membrane proteins (MCP/CD46, DAF/CD55, CR1/CD35, and CD59) with crucial roles in accelerate C3/C5 convertase dissociation, and/or inactivate C3b by proteolytic cleavage (4, 5) (**Figure 1**). A strict control between activation and regulation is necessary; as otherwise, the situation can result in the appearance of several diseases. Protein deficiencies or protein abnormal function due to genetic variations, or the presence of autoantibodies against complement components, such as nephritic factors (NFs), are associated with several diseases, including C3 glomerulopathy (C3G), membranoproliferative glomerulonephritis (MPGN), acquired partial lipodystrophy (APL) or Systemic Lupus Erythematosus (SLE) (6–8).

**Abbreviations:** AP, alternative pathway; CP, classical pathway; LP, lectin pathway; FB, Factor B; P, Properdin; FH, factor H; FI, Factor I; NF, nephritic factor; C3NeF, C3 nephritic factor; C4NeF, C4 nephritic factor; C5NeF, C5 nephritic factor; C3G, C3 glomerulopathy; DDD, dense deposit disease; IC-MPGN, immune complex-associated membranoproliferative glomerulonephritis; APL, acquired partial lipodystrophy; SLE, systemic lupus erythematosus; C3GN, C3 glomerulonephritis.

# NEPHRITIC FACTORS

## C3 and C5 Nephritic Factors

NFs were first described in 1969, based on the observation that the serum from a patient with MPGN and hypocomplementemia broke down C3 when it was mixed with normal human serum (9, 10). The activity of NFs was later attributed to the stabilization of either cell-bound or fluid phase AP complement convertase by its incorporation into C3 convertase (C3bBb); therefore, it was called C3 nephritic factor (C3NeF) (11, 12). After that, C3NeF was characterized as IgG and IgM autoantibodies with the capacity to recognize neoepitopes of the assembled AP C3 convertase (13–18) (**Figure 2A**). Studies focused to characterize C3NeF showed that IgG with stabilizing capacity mostly belonged to IgG1 and IgG3 subclasses (14, 15). Moreover, the Fab portion of the immunoglobulin retained the majority of the stabilizing function, although other studies show that the Fc portion also contributes to its functional activity (18, 19).

Several studies have reported that function of C3NeF is dependent on the presence of P. These P-dependent C3NeFs are responsible for activation of the C5 convertase, as reflected by the reduced levels of terminal complement components, and higher sC5b-9 complexes (20–23). In contrast, patients with P-independent C3NeF are characterized by low C3 levels and normal levels of component of the terminal pathway (22, 23). In a recent study, Marinozzi et al. have designed a novel functional assay that allows the identification of patients with autoantibodies with the ability to stabilize the C5 convertase of the AP, which are called C5 nephritic factors (C5NeF) (**Figure 2B**) (24). A review of previous reports establishes that this autoantibody could be identical to the P-dependent C3NeF described by Tanuma et al. (22), Paixão-Cavalcante et al. (25) and in several previous papers (20, 21). C5NeF has been detected as a unique nephritic factor (10% of cases) or as coexisting with C3NeF (39% of cases) (24). This new terminology is useful for distinguishing NFs in relation to their ability to stabilize C3 or C5 convertases.

All C3NeFs increase the half-life of C3 convertase, because they interfere with the accelerated decay by FH, DAF, and CR1 (25–28). An interesting study used a combination of ELISA and western blot assays to show the heterogeneous control of C3NeF on C3 convertase stabilization against spontaneous and accelerated decay (29). The authors demonstrated that some C3NeFs strongly stabilized the C3 convertase by preventing both spontaneous and FH-mediated decay in the absence of P. Other C3NeFs, however, need P to prevent C3 convertase dissociation (29). Regarding their epitope specificity, most C3NeFs recognize a neoepitope on the assembled C3bBb complex. However, autoantibodies against C3b and FB have recently been reported in patients with C3G, and these have the same capacity to increase the C3 convertase half-life as C3NeF (30–32). The main features for C3NeF and C5NeF are summarized in **Table 1**.

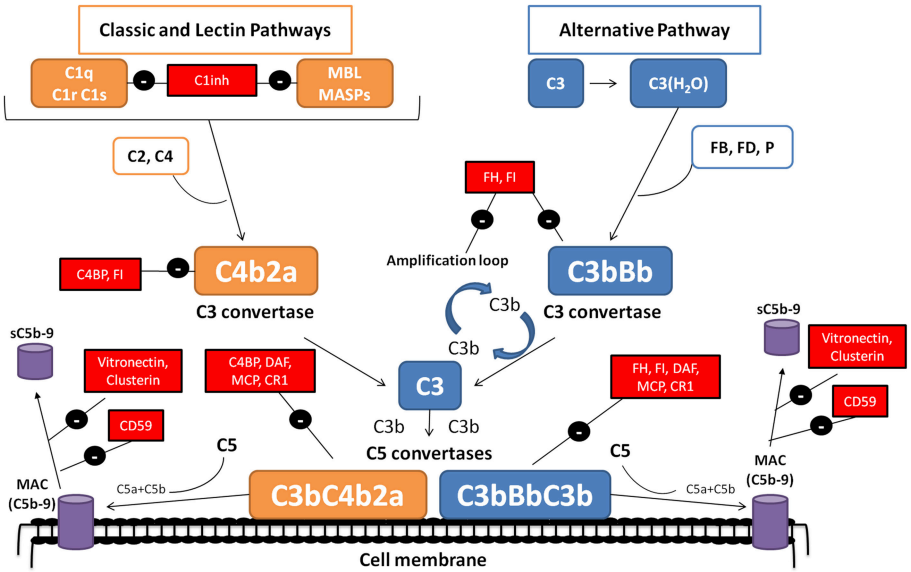


**FIGURE 1 |** The complement system. The complement system can be activated by three pathways. The classical (CP) and lectin (LP) pathways involve recognition of target-bound antibody or pathogen-specific carbohydrates by C1q or MBL, respectively. In the alternative pathway (AP), continuous, low-level activation of C3 by spontaneous hydrolysis of the internal C3 thioester, or C3 cleavage by plasma proteases, generates C3($H_2O$) or C3b. Activation by any of the three pathways leads to the generation of C3 convertase complexes (C4b2a in the CP/LP and C3bBb in the AP) that cleave C3 into C3a and C3b. Additionally, the AP C3 convertase can bind properdin (P), a positive regulator that stabilizes the enzyme, extending its half-life more than 10-fold. The C3b generated can, in turn, forms more AP C3 convertase, allowing amplification of complement activation. The binding of a new C3b molecule to the C3 convertases creates the C5 convertases (C4b2aC3b or C3bBbC3b), which cleave C5 into C5a and C5b. C5b then initiates the terminal complement pathway, which eventually leads to the formation of the membrane attack complex (C5b-9) and lysis of the target cells. Complement activation is controlled at various levels by different soluble and membrane regulatory proteins (indicated within boxes).

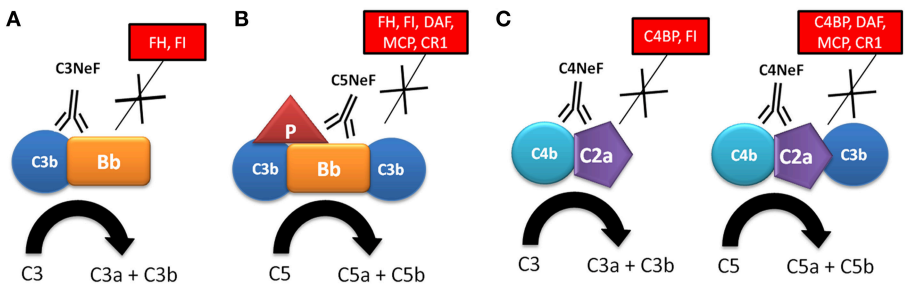


**FIGURE 2 |** Mechanism of action of nephritic factors. **(A)** C3NeF binds to a neoepitope on the C3 convertase of the alternative pathway (C3bBb), preventing its spontaneous and FH-mediated decay, and increasing its half-life and C3 consumption. **(B)** C5NeF binds to a neoepitope on the C5 convertase of the alternative pathway in the presence of properdin (P) (C3bBbC3bP), preventing its spontaneous and regulator-mediated decay, and increasing its half-life and C5 consumption. **(C)** C4NeF binds to a neoepitope on the C3/C5 convertases of the classical/lectin pathway (C4b2a/C4b2aC3b), preventing its spontaneous and regulator-mediated decay, and increasing its half-life and C3 consumption. Soluble and membrane complement regulators are indicated within boxes.

## C4 Nephritic Factor (C4NeF)

C4NeFs are autoantibodies that recognize and stabilize the CP/LP C3 convertase (C4b2a) (**Figure 2C**), increasing its half-life in the fluid and solid phase from a few minutes to several hours (41–44). They were first described in 1980 in one patient with post-infectious glomerulonephritis who had low C3 and C5 levels (41), and in 2 lupus nephritis patients (42). C4NeFs were detected in a patient with chronic glomerulonephritis (44), and in two MPGN patients with very low C3 and C5 levels who also presented C3NeF autoantibodies (45). Coexistence of both C3NeF and C4NeF in the same patient was corroborated upon screening of 100 MPGN patients, 10 of whom presented C3NeF and C4NeF, and reduced C3 and C5 levels (38). The reduced C5 levels observed in these patients is explained by the additional capacity of some C4NeFs to stabilize the C5 convertase and prevent its decay (46).

C4NeFs frequency could be underestimated. As assays for detecting C4NeFs are similar to the assays for detecting C3NeFs (see section Clinical Associations), the generation of C3 activation fragments could be erroneously attributed to the

**TABLE 1 |** Specificity and clinical associations of nephritic factors.

| | Epitope | Clinical association (frequency) |
|---|---|---|
| C3NeF | Neoepitope on assembled C3 convertase of the AP (C3bBb) | C3GN (40–50%) and DDD (70–80%) (7, 24, 25, 33, 34)<br>IC-MPGN (40–50%) (34, 35)<br>APL (70-80%) (36, 37)<br>SLE* |
| C4NeF | Neoepitope on assembled C3/C5 convertase of the CP/LP (C4b2a or C4b2aC3b) | C3G and IC-MPGN (3–9%) (38–40)<br>SLE* |
| C5NeF | Neoepitope on assembled C5 convertase of the AP (C3bBbC3bP) | C3GN (67%) and DDD (33%) (24) |

*AP, alternative pathway; CP, classical pathway; LP, lectin pathway; C3G, C3 glomerulopathy; DDD, dense deposit disease; IC-MPGN, immune complex-associated membranoproliferative glomerulonephritis; APL, acquired partial lipodystrophy; SLE, systemic lupus erythematosus; C3GN, C3 glomerulonephritis*

**The frequency of C3NeF and/or C4NeF in SLE has not been thoroughly investigated.*

presence of C3NeF instead of C4NeF; to avoid this, data on the levels of C3, C5 and terminal components in the patient's plasma should be known. Thus, C3 levels are lower in C3NeF positive patients than in C4NeF positive patients, while in C4NeF positive patients reduced levels of C5 and most terminal complement components are observed (38).

C4NeFs are most likely of the IgG3 subclass (41). *In vitro* studies with patients' purified IgG have been used to determine the molecular mechanisms of convertase stabilization by C4NeFs. Protection against C4BP-mediated decay was observed (47), and later on confirmed in another study that also showed increased resistance to spontaneous decay and to the proteolytic inactivation of C4b within the C4b2a complex (43). Resistance of C4NeF-C3 convertase to the dissociation induced by CR1 has also been shown (48). C3 and C5 convertases stabilized by C4NeF are strongly resistant to DAF-mediated decay; however, neither C3NeF nor C4NeF allow assembly of the C3 convertase in the presence of DAF (27). A recent study with C4NeFs purified from C3G patients (39) confirms the increased protection against C4BP- and CR1-mediated decay, as well as stabilization of the C5 convertase. Therefore, C4NeF seems to be a highly effective shield against the spontaneous and regulator-induced dissociation of the CP C3/C5 convertases (**Figure 2C**). The main features for C4NeF are summarized in **Table 1**.

## DIAGNOSTIC TOOLS TO DETECT NEPHRITIC FACTORS

Several methods for the evaluation of NFs have been reported in the literature. Although the old and very simple methods based on mixing normal and hypocomplementemic serum from suspected individuals and the subsequent identification of complement activation markers are still in use, they appear to be of low sensitivity; therefore, a number of more sophisticated protocols have been developed (25). Modern methods are based on measuring the binding of NFs to the pre-formed C3 convertase (see section Binding Assays), or measuring C3/C5 convertase activity in the presence of an NF-suspected sample (see section Functional Assays) (**Figure 3**). However, such methods represent a substantial challenge due to the labile nature of the C3/C5 convertases, and to a number of situations that mimic NF activity; like as presence of gain-of-function mutations in C3 and FB (49, 50). Apparently, detection of NFs remains problematic, because the 2015 European quality assessment revealed that only half of the participating laboratories properly identified C3NeF reference samples (51). Of note, there is no ideal test capable of covering all difficulties, and both binding assays and functional assays have advantages and drawbacks. Therefore, the combination of convertase assays helps not only improving the specificity of detection but also shedding light on the nature of NFs.

### Binding Assays

Binding assays use convertases reconstituted from purified components on artificial surfaces such as microplates or biosensor chips. Identification of the ligand bound to the convertase complex can give rise to a diagnosis of NF. However, ligand binding may not have any influence on enzyme function, and therefore the positive results of binding assays might not be physiologically relevant. This possibility is reflected by the fact that a certain portion of C3NeF-positive samples detected by binding assays were negative in other functional assays, possibly representing false-positive results (25). However, binding assays performed on purified convertase components allow study the kinetics of convertase dissociation, especially when performed with surface plasmon resonance techniques (52). There are several procedures described for the detection of C3NeF (53). Typically, C3b is immobilized on the microplate surface and then reacted with FB and FD. High yields of microplate-attached alternative convertase complexes are achieved by the addition of $NiCl_2$ (53) or $NiSO_4$ (25), which outperforms magnesium ions normally supporting convertase formation. The most popular binding assays are enzyme-linked immunosorbent assays (ELISAs) to detect immunoglobulins bound to preformed convertase complex (CO-Ig) (**Figure 3**). However, such assays might not identify the non-Ig C3 activating factors (54). Another version of the ELISA-based binding assay, the so-called C3NeF stabilization (COS) assay, detects plate-bound Bb fragments with a polyclonal antibody (**Figure 3**) (25). The COS assay, like assays detecting convertase-bound Igs, begins with C3b deposition followed by the addition of test serum or purified Ig fraction together with FB, FD and optionally, P (COS-P assay) (**Figure 3**). Detection of Bb fragments on the plate surface coated with C3b indicates the presence of complete AP convertase, which normally decays within minutes when no stabilizing agent is present. Importantly, experimental data by Paixão-Cavalcante et al. showed that 36 out of 101 samples collected from patients with complement-related renal diseases were found to be positive for C3NeF in a COS-P assay but only 16 out of these 36 were positive in a COS assay (25). This difference indicates that C3NeF

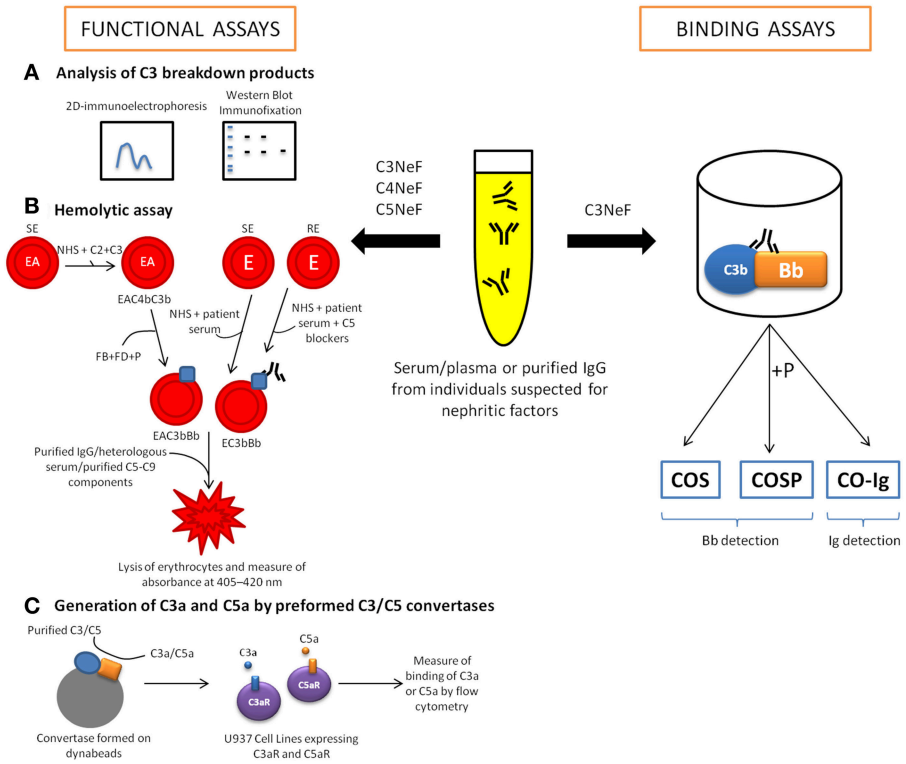


**FIGURE 3 |** Diagnostic tools for the detection of nephritic factors (NFs). The functional activity of C3NeF can be determined through quantifying complement activation products (mostly C3 fragments) by two-dimensional immunoelectrophoresis, immunofixation electrophoresis or western blotting **(A)**. However, the main tools for the detection of NFs activity are the hemolytic assays, which measure the lysis of sheep (SE)/rabbit erythrocytes (RE) **(B)**. In these assays, purified complement components are added to perform convertases on the surface SE sensitized with anti-sheep antibodies (EA). Later, purified FB, FD and P are added to form specifically alternative pathway convertase. Other assays are developed using whole serum (patient's serum and normal human serum (NHS), in ratio 1: 1) to generate convertases on the SE or RE surface. Considering that RE could be lysed by human serum, terminal pathway needs to be blocked using C5 blockers (OmCI or eculizumab). Another option to measure NF activity is through the quantification of anaphylatoxins, C3a and C5a **(C)**. There are binding assays to detect C3NeF based on enzyme-linked immunosorbent assays (ELISA) that detect immunoglobulins bound to the preformed convertase complex (CO-Ig). Other versions of the ELISA-based binding assay are the so-called C3NeF stabilization assays, which use a polyclonal antibody to detect plate-bound Bb fragment generated either in the absence (COS assay) or in the presence (COS-P assay) of P.

are very heterogeneous with regard to binding sites and that P might play a role in AP convertase stabilization by C3NeF.

## Functional Assays

### Analysis of C3 Breakdown Products

As mentioned earlier, the first functional assays relied on mixing control and NF-suspected sera, and further analysis of complement activation products (mostly C3 fragments) by two-dimensional immunoelectrophoresis (55), immunofixation electrophoresis (56) or western blotting (25) (**Figure 3**). The idea behind these assays is that the spontaneous breakdown of C3 that normally initiates the AP is augmented by convertase stabilization by NFs. Therefore, 3 h of incubation at 37°C is enough time to observe substantial C3 cleavage in samples containing C3NeF in contrast to samples containing normal serum only. Of note, all samples found positive in assays measuring C3 breakdown products were also positive in COS-P binding assays (however, COS-P assays detected more positive samples) but some of them were negative in CO-Ig binding assays (25). A possible explanation is that COS and COS-P assays can be considered quasi-functional assays that detect protein fragments resulting from convertase activity. The same applies to assays performed with surface plasmon resonance techniques, which monitor the association and dissociation of convertase complexes.

### Hemolytic Assays

Many functional convertase assays measuring the effect of enzymatic activity use red blood cells because they provide an easy readout: the release of hemoglobin, which can be easily quantified by measuring absorbance at 405–420 nm (**Figure 3**). The general rule is that after deposition of active convertases, the addition of a heterologous serum (e.g., guinea pig serum, which is capable of MAC formation on human convertases but offers limited activity of regulatory proteins) or purified terminal pathway components diluted in ethylene-diamine-tetraacetic acid (EDTA)-gelatin veronal buffer will generate lytic sites. Chelating of magnesium and calcium by EDTA avoids assembly

of new convertases but does not influence MAC formation on a platform of already assembled convertases. Depending on the version, these functional assays can analyze purified components (e.g., protein A/protein G-purified Ig fraction from individuals suspected for NFs, which is added during or just after the convertase formation step) or whole serum. The use of whole serum may introduce confounding factors like soluble convertase inhibitors, which can be present at different concentration in particular individuals. Nonetheless, the presence of NF usually prevents activity of such inhibitors and should be manifested in prolonged convertase half-life. Therefore, the assay performed with whole serum as an analyte is a rather quick screening method only showing probability of NF presence, which should be further confirmed by testing of purified Ig fraction.

### *Assays using C3/C5 convertases assembled from purified components*

The stepwise building of convertase complex on the surface of sheep erythrocytes enables the analysis of CP/LP and AP enzymes. In the first step, C1 complex is deposited followed by C4, which binds covalently to the membrane. Then, C2 together with the test sample is added, and after incubation the mixture is washed and replaced by guinea pig or rat serum (fully compatible with human serum but of higher terminal pathway efficacy) in EDTA-buffer (39, 46, 57, 58). This experimental design enables the measurement of CP/LP C3 convertase activity, whereas the addition of C3 molecules together with C2 enables the formation of C5 convertase. CP/LP C5 convertase is also formed to deposit AP convertases. Covalent binding of C3b is followed by the removal of noncovalently bound C1 and C2 by prolonged incubation in 10 mM EDTA-buffer. Later, FB, FD, and P are added, thus forming AP convertases (57, 58). After this step, purified Igs purified from patients are used to evaluate NF capacity. This method cannot ensure a clear distinction between C3 and C5 AP convertase activity, but such attempts can be performed either by titrating C3 and thus manipulating C3b density (59) or by developing lytic sites by mixing purified C5-C9 components ± C3 (57). An alternative method of C3b deposition is incubation of erythrocytes with C3 and trypsin at 22°C (59, 60).

### *Functional assays with whole serum*

The stepwise formation of convertases from isolated complement components allows the analysis of the purified Ig fraction from an NF-suspected individual, but it also presents certain limitations. A major drawback is the inability to reproduce the physiological serum context, where many proteins are potentially capable of influencing convertases' activity. Therefore, convertase-interacting components identified in assays based on purified complement proteins might not accurately reflect physiological interactions. Convertase assays performed with whole serum are based on a similar design, i.e., convertase formation on erythrocytes as the first step, and generation of lytic sites by addition of EDTA-serum in the second step. Sheep erythrocytes sensitized with anti-sheep antibodies (e.g., amboceptor) offer the possibility of forming CP/LP C3 convertase (C4b2a) when erythrocytes are incubated in C3-depleted serum (61). The complement cascade proceeds up to the step of C3 convertase but no further. Similarly, incubation in C5-depleted serum results in the formation of C5 CP/LP convertase C4b2aC3b (61). The high efficacy of CP/LP convertase formation on sensitized sheep erythrocytes enables the application of low serum concentrations (<1%), excluding the possibility of AP convertase formation, which demands a higher (2%) serum content. Rabbit erythrocytes, which spontaneously activate AP when incubated with human serum, are used for assays of AP convertase. However, this method is unable to distinguish between C3 and C5 convertases, given that both of them contain C3b. Carrying out the reaction in ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)-buffer excludes the activation of CP/LP, which demands calcium and magnesium, whereas AP only requires magnesium (not affected by EGTA).

The idea of blocking the complement cascade at the level of C5 gives rise to another version of functional convertase assays that can be performed in whole serum, e.g., collected directly from the patient. To this end, C5 inhibitors such as eculizumab or *Ornithodoros moubata* complement inhibitor (OmCI) have been successfully applied (49). Testing whole serum excludes a precise diagnosis of NFs, but it is a useful screening test for searching any components that interfere with convertase activity and/or stability. Recently, this assay was successfully validated in a cohort of C3G patients. The results revealed that the combined analysis of patient's serum and purified Ig fraction properly distinguished patients with convertase-stabilizing activity localized in the Ig fraction (with positive readouts in serum and Ig fraction) from those with familial aHUS caused by gain-of-function mutations in complement components (positive readout only in serum) (62). This method was also successfully applied to diagnose C4NeF in a C3G patient who did not present any other abnormalities in complement proteins and was negative for C3NeF and other autoantibodies against AP components (40). Technically, convertase assays with whole patient's serum demand mixing of patient's sample with normal human serum, usually in 1:1 ratio. Since NF presence is often associated with hypocomplementemia, low content of complement components in unmixed patient's serum results in low amplitude and delayed peak of convertase activity, which may mask convertase-stabilizing effect. However, addition of normal human serum as a source of complement components to NF-containing, hypocomplementemic serum, allows readouts for convertases of prolonged / enhanced activity (49). It is worth mentioning that the first hemolysis-based, two-step assay intended to diagnose C3NeF in patient serum was described more than 30 years ago (63) and was later modified (64, 65). The so-called Rother assay is still in use in some diagnostic laboratories but it often gives inconclusive results. This test uses sheep erythrocytes, which are not an optimal target for AP activation. The kinetics of this process are slower than for analogous AP activation on rabbit erythrocytes, and therefore it is theoretically possible to extract a time point when convertases are already formed but MAC is not yet assembled. In practice, choosing such a time point is arbitrary and will not necessarily work for every individual serum tested. Indeed, many samples

cause hemolysis in the first step of this assay, and thus preclude a reliable diagnosis.

An emerging problem for functional convertase assays in detecting NFs is the lack of widely available standards. The most commonly used strategy is to compare results obtained with the results of a reference serum (a highly-positive sample), which has limited availability. This problem is combined with the batch-to-batch differences in erythrocytes, giving rise to high inter-assay variability, and the inability to strictly compare the values obtained in two different laboratories. A possible solution is to use gain-of-function mutants of convertase-forming proteins, which mimic the effect of NFs. For example, a recombinant FB K323E mutant has been proposed as a reference for C3NeF detection (50).

### Generation of C3a and C5a by Preformed C3/C5 Convertases

Another option for measuring convertase activity is to quantify the anaphylatoxins C3a and C5a, which are products of C3 and C5 cleavage by the C3 and C5 convertases, respectively. In this assays, the formation of convertases from purified complement components takes place on the surface of beads. Thereafter, the substrates (C3 or C5) are added together with the tested sample, and the supernatant containing anaphylatoxins is transferred to a culture of reporter cells, previously transfected with C3aR or C5aR. The intensity of the readout in calcium mobilization assays is proportional to the amount of C3a and C5a generated; therefore, this assay enables to distinguish between C3 and C5 convertase activities (66). Such method, not yet demonstrated usefulness for NF detection would be compatible with purified Ig fraction as an analyte.

## CLINICAL ASSOCIATIONS

### C3 Glomerulopathy/Membranoproliferative Glomerulonephritis

C3G is a recently adopted term to refer to a few rare kidney diseases caused by dysregulation of the AP, and characterized by significant C3 deposition in the glomeruli with minimal or no presence of immunoglobulins (67–69). Although there is no a specific histological pattern, most of the C3G cases correspond to some types of the previously denominated MPGN. C3G is classified into Dense Deposit Disease (DDD) and C3 glomerulonephritis (C3GN) on the basis of the electron microscopy findings. Dense Deposit Disease is characterized by distinctive highly electron-dense intermembranous deposits whereas C3GN is characterized by mesangial, intramembranous, subendotelial and sometimes subepithelial deposits (67–72).

C3NeF has been detected mainly in patients with immune complex-associated-MPGN (IC-MPGN) (40–50%) and in patients with C3G (40–80%) (7). Approximately 80% of patients with DDD and in 40%-50% of patients with C3GN (51, 72) are positive for C3NeF. Recently, Donadelli et al. published a study providing an interesting method to detect and characterize C3NeF in patients with C3G and IC-MPGN (29). The authors reported that most of the patients with DDD had C3NeF that stabilized C3 convertase in the absence of P, while in the remaining patients (C3GN and IC-MPGN) C3NeF was P-dependent (C5NeF), resulting in both C3 and C5 convertase dysregulation (29). These results are reliable with the previous observation that C3NeF targets both C3 and C5 convertases in 67% of patients with C3GN (24), and that terminal pathway dysregulation is significantly higher in C3GN than in DDD (24, 29). Moreover, our group documented that a subset of C3G patients with low P levels (most of them C3GN) was characterized by C5 consumption and high sC5b-9 levels in plasma (33). However, although our results are consistent with the presence of C5NeF, we could not prove it due to the lack of a specific assay to detect this autoantibody.

The pathogenic role of C3NeFs in C3G has been debated for a long time. Although C3NeFs are closely linked with C3G, they have been detected in healthy individuals, suggesting that this autoantibody is part of the normal immune repertoire (73, 74). Therefore, a fundamental unanswered question is whether C3NeF is the cause of the disease, or whether C3NeF is a consequence of the disease process that then acts exacerbating the disease pathology (17, 74, 75). Moreover, plasma C3 consumption and disease severity do not always correlate with the presence and activity of C3NeF (76–78). Autoantibodies related to complement components, such as anti-FB, anti-C3b, anti-FH or other autoantibodies (e.g., ANAs, ANCAs or cryoglobulins) have been detected in smaller percentages of patients (79). Overall, in the series from Mayo Clinic, 13.4% of C3G patients were positive for autoantibodies other than C3NeF (80).

C4NeFs autoantibodies have been mainly found in patients with MPGN (38, 44, 45, 81). A study in 100 patients with hypocomplementemic MPGN showed that as many as 19 patients had C4NeF (alone or in combination with C3NeF) (38). The 10 patients having both C3NeF and C4NeF presented low C3, C5, and terminal complement component (C6–C9) levels, as well as massive C3 deposits in the kidneys by immunofluorescence microscopy; moreover, they presented therapy-resistant hypocomplementemia, a higher incidence of nephritic syndrome and a poorer prognosis than patients positive for only C3NeF or C4NeF. These 10 patients would have been diagnosed of C3G according to current criteria (70). Another interesting observation in this study is that one patient was subsequently C3NeF positive, C3NeF negative, and C4NeF positive, suggesting than autoantibody specificity can change along disease evolution.

More recently, a C4NeF that reduced decay of the CP C3 and C5 convertases was detected in 1 out of 13 C3G patients (40). The patient had reduced C3 levels, but C4 and FB levels were normal. C4NeFs have been also found in 4/100 C3GN patients, and in 1/68 DDD patients, representing 3% of the 168 C3G patients included in the study (39); 1 of these patients also had anti-FH autoantibodies, and another patient had C3NeF. The low frequency of C4NeF-positive patients (3%) in comparison with C3NeF-positive patients (52%) in this large and well documented C3G series suggests that the actual contribution

of C4NeF to the pathogenic mechanism is less relevant than the contribution of C3NeF, and it could be associated with an infectious trigger. This possibility is in line with the authors' observation that kidney biopsies from their C4NeF-positive patients are suggestive of post-infectious glomerulonephritis which evolved into C3G.

### Acquired Partial Lipodystrophy (APL)

APL or Barraquer-Simons syndrome (ORPHA:79087) is an ultra-rare disease characterized by progressive loss of adipose tissue starting from the face and then extend to the neck, shoulders, upper extremities and then to the thorax. Patients may accumulate fat excess in the lower extremities after puberty, especially female patients (36). APL is more frequent in females than males (4:1) (82). Metabolic syndrome and its comorbidities are absent or infrequent in this syndrome (83). However, autoimmune diseases, particularly SLE and dermatomyositis, have been reported in patients with APL (36, 84).

Adipsin is an adipokine produced by mature adipocytes (85). In 1992, White et al. shown that human adipsin was identical to complement FD and that it major source was located in the adipose tissue (86). Moreover, adipocytes also express C3, FB and complement regulators such as P and FH, and consequently complement is connected to adipocyte biology (87). Indeed, complement system functions on adipose tissue, as preadipocyte differentiation and triacylglyceride synthesis, have been described (88).

C3 hypocomplementemia in relation with the presence of C3NeF have been reported in almost all patients with APL (82–84, 89–103). Therefore, as a consequence of the presence of C3NeF, approximately 20% of patients with APL develop C3G in a period of 8 years after the onset of lipodystrophy, and some of them evolve to end-stage renal disease requiring renal transplantation (83). Although complement dysregulation is a common mechanism in C3G and APL, the exact mechanism of fat loss remains unclear (87). There is one experimental study which described complement-mediated lysis of adipocytes *in vitro* by C3NeF (87, 104). However, the proposal fails to answer the key question of not all patients with C3NeF develop lipodystrophy.

### SLE and Infections

C3NeF have been described in patients with SLE, associated in most cases with partial lipodystrophy and/or DDD (90, 95, 96, 105–111). However, there are several studies describing patients with lupus nephritis, complement abnormalities and the presence of autoantibodies against AP proteins in absence of C3NeF (112–114). For example, our group published a case of lupus nephritis with autoantibodies against C3, FB and P, which was negative for C3NeF (112). Other authors reported that approximately 30% of patients with lupus nephritis were positive for anti-C3 autoantibodies (113). These autoantibodies have a similar effect as C3NeF, which should be taken into consideration during the diagnosis.

C3NeFs have been detected in patients with meningococcal infections, without renal impairment (115–121). By contrast, there are several cases series of post-streptococcal acute glomerulonephritis associated with C3NeF activity (122–125).

C4NeFs have also been found in a few SLE patients (42), and occasionally following streptococcal (41) or meningococcal (46) infections. The latter is a peculiar case of a C4NeF autoantibody that did not provoke a renal phenotype, but it was found in a patient with severe meningococcal disease. This C4NeF autoantibody stabilized the C3 and C5 convertases from the CP and generated C3 deficiency by increased consumption (46).

## DIAGNOSTIC APPROACH TO IDENTIFY NEPHRITIC FACTORS

Several renal diseases are caused by complement dysregulation, and while the most common causes of impaired complement regulation are pathogenic mutations or anti-FH autoantibodies in atypical hemolytic uremic syndrome (aHUS), in C3G NFs are the most frequent finding (34, 69). Although definitive diagnosis should be based on light and electron microscopy findings of kidney biopsy, some laboratory tests may support the diagnosis.

After suspicion or definitive diagnosis of C3G, C3NeFs have to be screened in the first steps, together with serum C3 levels. Other parameters included in the current laboratory diagnostic protocol are commented below.

Serum C3 and C4 levels can be measured fast and easily by automated methods, as nephelometry or turbidimetry, but normal C3 levels do not exclude NF presence. Complement activation can also be assessed by measuring C3d, FB, P and sC5b9, not only to demonstrate AP dysregulation but to differentiate between C3/C4NeFs and C5NeF (33, 51, 126).

Serum from C3G patients should be screened for autoantibodies (16), including anti-FH (55, 127, 128), anti-FB (31, 129), and anti-C3 (32), all of them causing alternative pathway dysregulation, but the presence of any of these autoantibodies do not exclude NF existence, as anti-FH autoantibodies are frequently associated with C3NeF in pediatric C3G patients (130). FH deficiency is associated with DDD and C3GN (131, 132), as it leads to totally uncontrolled AP activation. Besides serum FH levels, a genetic study is necessary because single point mutations in FH gene causing C3GN have been described (102, 133).

Genetic screening of AP regulators and convertase components should be undertaken in a complete complement study of C3G patients. Common and pathogenic variants associated with C3G have been described in FH, FI, FB, CD46, C3 and thrombomodulin genes, influencing on long-term outcome (5, 34, 35). Again, the presence of any mutation or variant do not exclude NF existence, as it's shown in a French C3G series, where C3NeF was present in 50% of patients carrying mutations in complement genes (34).

Defective complement regulation has been demonstrated in *C3* and *CFB* mutations associated with C3G; these mutations impair C3b inactivation by FI (134) increase convertase

resistance to dissociation by FH (135), or promote high–affinity binding of C3 to FB (136).

A genetic and protein approach to study the Factor H-Related (FHR) proteins is also recommended, as mutations and gene rearrangements causing FH deregulation have been reported over the last years in C3G patients (137–143).

## MANAGEMENT AND THERAPEUTIC PERSPECTIVES FOR PATIENTS WITH NEPHRITIC FACTORS

In the last years, there has been an important progress in the knowledge about treatments for autoimmune diseases. The presence of NFs should be considered the main autoimmune cause of diseases as C3G and IC-MPGN. In most of consensus reports, the experts indicate that treatment is directed to improve clinical parameters. However, a specific disease-directed treatment for patients with NFs has not been established. There are no randomized trials able to generate therapeutic decisions, so all current recommendations are based on low-quality evidence since the patients' series include a heterogeneous and limited number of cases. Therapies are divided into common treatments indicated to all autoimmune diseases and specific complement blocking drugs:

- Plasma Exchange Therapy/Plasmapheresis: In order to remove NFs, plasma exchange has been used with irregular efficacy. A combined treatment based on plasma exchange and immunosuppressants has proved useful in patients with C3G positive for C3NeF (144). However, with these results it was difficult to evaluate the effect of plasma exchange since the treatments were administered all together. An interesting work showed that using long-term plasmapheresis removed C3NeF activity from the serum of patients with DDD (145).
- Immunosuppressive Therapy: These treatments are focused to limit and/or reduce the production of NFs, and they have mainly been proved in patients with C3G and MPGN. The benefit of long-term alternate-day steroid therapy for idiopathic MPGN has been suggested; however, these studies include a combination of patients with different types of MPGN, limiting possible conclusions (75, 146, 147). An interesting report from the Spanish Group for Glomerular Disease Study showed the effectiveness of the combination of mycophenolate mofetil (MMF) and glucocorticoids in patients with C3GN (148). This study compared the clinical evolution of three groups of C3GN patients: one without glucocorticoids treatment, one with glucocorticoids but no MMF and another one based on MMF plus glucocorticoids. The results showed that eight out of ten patients with C3NeF (80%) were more likely to achieve remission with this treatment. However, this clinical improvement could not be attributed to C3NeF disappearance because it determination was not performed in all patients during the whole course of the treatment. A similar although less impressive response was described in a large cohort from the Mayo Clinic (80).
- Monoclonal Antibody Therapy: Antibody-producing B cell-targeted therapies should be effective in patients with C3NeF, but the data using a monoclonal anti-CD20 antibody (rituximab) have not been consistent regarding C3NeF activity and clinical response (149–151). The experience on the use of rituximab in MPGN, C3GN, and DDD with or without NFs is limited to case reports and retrospective case series. Patients with IC-MPGN who were treated with rituximab showed partial and complete responses in the majorities of cases, while treatment was not effective in most C3GN and DDD cases (152).
- Complement Directed Therapies: Complement blocking agents such as eculizumab (Soliris) have been also tried in patients with NFs as the only pathogenic cause or in combination with mutations in complement components (153, 154). Almost all authors coincide in that eculizumab therapy should be considered after the failure of immunosuppressive and plasma exchange treatments to improve renal function. A large series using eculizumab in C3G have been published by Le Quintrec et al. from France (154). Briefly, 23% of their patients had a global clinical response, while 23% had a partial response and the remaining 54% were nonresponders. The patients with a good clinical response had lower glomerular filtration, a more rapidly progressive course and more extracapillary proliferation as determined by kidney biopsy (154). Parameters including age, extent of renal fibrosis, nephrotic syndrome, C3 levels, C3NeF activity, sC5b-9 concentration or pathogenic complement gene variants did not differ between responders and nonresponders (154). There are several reports that show that eculizumab may be a specific and useful treatment in C3NeF-related DDD (103, 151). A potential benefit of eculizumab treatment was suggested for the five C4NeF-positive patients described by Zhang et al. (39), who presented elevated plasma levels of sC5b-9.

New complement modulators targeting C3 convertase activity, such as soluble CR1 (sCR1), have been proved in sporadic cases of C3G (155). The *in vitro* activity of sCR1 prevents the hemolysis of rabbit erythrocytes caused by C3NeF with greater effectiveness than FH. The administration of sCR1 to Cfh−/−/huCR1-Tg mouse resulted in markedly reduction of C3 deposits along the glomerulus. However, C3 staining not changed before and after treatment in an 8-year-old girl positive for C3NeF with biopsy-proven DDD although the authors observed a transient improvement in C3 serum levels and a decreased of sC5b-9.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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# Rare Functional Variants in Complement Genes and Anti-FH Autoantibodies-Associated aHUS

*Elisabetta Valoti[1], Marta Alberti[1], Paraskevas Iatropoulos[1], Rossella Piras[1], Caterina Mele[1], Matteo Breno[1], Alessandra Cremaschi[1], Elena Bresin[1], Roberta Donadelli[1], Silvia Alizzi[2], Antonio Amoroso[2], Ariela Benigni[1], Giuseppe Remuzzi[1,3] and Marina Noris[1]**

[1] Clinical Research Center for Rare Diseases 'Aldo e Cele Daccò', Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Bergamo, Italy, [2] Azienda Ospedaliera–Universitaria, Città della Salute e della Scienza and Department of Medical Sciences, University of Turin, Turin, Italy, [3] 'L. Sacco' Department of Biomedical and Clinical Sciences, University of Milan, Milan, Italy

Atypical hemolytic uremic syndrome (aHUS) is a rare disease characterized by microangiopathic hemolytic anemia, thrombocytopenia and renal failure. It is caused by genetic or acquired defects of the complement alternative pathway. Factor H autoantibodies (anti-FHs) have been reported in 10% of aHUS patients and are associated with the deficiency of factor H-related 1 (FHR1). However, FHR1 deficiency is not enough to cause aHUS, since it is also present in about 5% of Caucasian healthy subjects. In this study we evaluated the prevalence of genetic variants in *CFH*, *CD46*, *CFI*, *CFB*, *C3*, and *THBD* in aHUS patients with anti-FHs, using healthy subjects with FHR1 deficiency, here defined "supercontrols," as a reference group. "Supercontrols" are more informative than general population because they share at least one risk factor (FHR1 deficiency) with aHUS patients. We analyzed anti-FHs in 305 patients and 30 were positive. The large majority were children (median age: 7.7 [IQR, 6.6–9.9] years) and 83% lacked FHR1 ($n = 25$, cases) due to the homozygous *CFHR3-CFHR1* deletion ($n = 20$), or the compound heterozygous *CFHR3-CFHR1* and *CFHR1-CFHR4* deletions ($n = 4$), or the heterozygous *CFHR3-CFHR1* deletion combined with a frameshift mutation in *CFHR1* that generates a premature stop codon ($n = 1$). Of the 960 healthy adult subjects 48 had the FHR1 deficiency ("supercontrols"). Rare likely pathogenetic variants in *CFH*, *THBD*, and *C3* were found in 24% of cases ($n = 6$) compared to 2.1% of the "supercontrols" ($P$-value = 0.005). We also found that the *CFH* H3 and the $CD46_{GGAAC}$ haplotypes are not associated with anti-FHs aHUS, whereas these haplotypes are enriched in aHUS patients without anti-FHs, which highlights the differences in the genetic basis of the two forms of the disease. Finally, we confirm that common infections are environmental factors that contribute to the development of anti-FHs aHUS in genetically predisposed individuals, which fits with the sharp peak of incidence during scholar-age. Further studies are needed to fully elucidate the complex genetic and environmental factors underlying anti-FHs aHUS and to establish whether the combination of anti-FHs with likely pathogenetic variants or other risk factors influences disease outcome and response to therapies.

**Keywords: autoantibodies, atypical hemolytic uremic syndrome, factor H, factor H related 1, complement, genetic variants, supercontrols**

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***Correspondence:**
*Marina Noris*
*marina.noris@marionegri.it*

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## INTRODUCTION

Atypical hemolytic uremic syndrome (aHUS) is a rare disease characterized by microangiopathic hemolytic anemia, thrombocytopenia and renal failure (1, 2). It is mainly caused by genetic or acquired defects of the alternative pathway (AP) of the complement system (3). Heterozygous likely pathogenic variants (LPVs) in genes encoding complement regulators, such as factor H (FH), factor I and CD46 (3–7), or components of the AP C3 convertase (C3 and factor B) (3, 8–11) were found in about half of aHUS patients. Moreover, genetic abnormalities in thrombomodulin (*THBD),* an endothelial anticoagulant protein that also regulates complement, were reported in 3–5% of aHUS cases (12).

Incomplete penetrance was observed in carriers of genetic abnormalities, indicating that although LPVs predispose to the development of aHUS, additional genetic and/or environmental hits are necessary for the disease to manifest (13). Indeed, in a large multinational study, 25% of patients with LPVs in *CD46* or *CFI*, and 8–10% of those with LPVs in *CFH*, *C3*, and *CFB*, carried combined genetic abnormalities in other complement genes (13). Common variants and haplotypes in *CFH* and *CD46* have been associated with aHUS and were reported to increase the penetrance of the disease in subjects carrying LPVs (14–16).

Factor H autoantibodies (anti-FHs) have been described in about 10% of aHUS patients and are strongly associated with the deficiency of factor H-related 1 (FHR1) (17–19). In most cases, the lack of FHR1 is due to the polymorphic homozygous deletion of the factor H related 3 and 1 genes (*CFHR3-CFHR1*) (20). In about 15% of patients the FHR1 deficiency is caused by compound heterozygous deletion of *CFHR3-CFHR1* and *CFHR1-CFHR4*, while homozygous *CFHR1-CFHR4* deletion is rare (21–23).

The pathogenic mechanism that links the FHR1 deficiency with the risk of the development of anti-FHs has not been clarified yet. Anti-FHs mainly target the C-terminal region of FH, and also cross react with FHR1 short consensus repeats (SCRs) 4–5, which have very high amino acid sequence identity with SCRs 19–20 of FH (24–27). It has recently been hypothesized that binding of certain proteins expressed by pathogens to the SCRs 19–20 of FH induces the exposure of a neoepitope in FH that is conformationally similar to that in SCRs 4–5 of FHR1, resulting in an autoimmune response in subjects with FHR1 deficiency (26).

Despite the above evidence, FHR1 deficiency is not enough to cause anti-FH associated aHUS, which is an ultra-rare disease with an estimated prevalence of 1:1,000,000 (1, 28). Indeed, the *CFHR3-CFHR1* deletion is a rather common polymorphism, and is present in homozygosity in 3–10% of healthy European populations and in 7–30% of African populations, whereas it is very rare in Asia and South America (29). The combined *CFHR3-CFHR1* and *CFHR1-CFHR4* deletion is present in 0.9% of healthy European controls (22).

Autoimmune diseases are typically complex disorders in which multiple susceptibility genetic variants and environmental factors are involved (30, 31). The contribution of each genetic variant is usually small and only the presence of multiple variants favors the development of the disease (31). Notably, each single risk variant may be found in healthy subjects, although at a lower frequency than in patients, but it is the combination of multiple risk variants that clusters with the disease (30).

Previous studies reported that about 14.7% of aHUS patients with anti-FHs also carry LPVs in complement genes (18, 19, 21–23, 32); however the prevalence of LPVs in patients with anti-FHs should be compared to that in the general population or, even better, in a control group with FHR1 deficiency. This would support the hypothesis that LPVs have a role in anti-FHs aHUS.

In this study we investigated the genetic determinants of anti-FH-associated aHUS by comparing patients with anti-FHs and FHR1 deficiency (defined here as "cases") and adult healthy subjects carrying the homozygous *CFHR1* deletion (defined here as "supercontrols") as a homogeneous, special control group, to optimize the power to detect differences. We hypothesized that the "supercontrols" were depleted for risk variants or even enriched in protective variants, since they evaded the development of aHUS despite carrying the known risk factor represented by the FHR1 deficiency.

Specifically, we compared the prevalence of rare LPVs in complement genes between cases and "supercontrols." We also investigated the association with anti-FHs aHUS of common variants in complement genes, which in previous studies were reported to increase the risk of aHUS or of other immune-mediated glomerulopathies (33–38).

We found that rare LPVs but not common complement gene variants are enriched to a significant extent in cases compared to "supercontrols," suggesting they are involved in the development of anti-FHs aHUS.

## METHODS

### Patients and Controls

In all patients, atypical HUS was diagnosed based on microangiopathic hemolytic anemia and thrombocytopenia defined as hematocrit <30%, hemoglobin level <10 g/dl, serum lactate dehydrogenase level above 500 U/l, undetectable haptoglobin level, fragmented erythrocytes in peripheral blood smear, and platelets below $150 \times 10^3/\mu l$, associated with acute renal failure (serum creatinine >1.3 mg/dl for adults, >0.5 mg/dl for children under 5 years of age and >0.8 mg/dl for children aged 5–10 years old; and/or urinary protein/creatinine ratio >200 mg/g; or an increase of serum creatinine or urinary protein/creatinine ratio >15% compared to baseline levels). All aHUS patients ($n = 305$) were recruited through the International Registry of HUS/TTP (Ranica, Bergamo, Italy).

Healthy adult subjects ($n = 960$) were from internal donors and from the Piedmont Regional Registry of the Italian Bone Marrow Donors Registry (Azienda Ospedaliera–Universitaria, Città della Salute e della Scienza, Turin, Italy). Patient and healthy subject data were handled with respect for confidentiality and anonymity. All subjects provided informed written consent in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of Azienda Sanitaria Locale, Bergamo, Italy.

## Anti-FH IgG ELISA

The presence of anti-FH IgGs was evaluated through an Enzyme-Linked Immunosorbent Assay (ELISA). Microtiter plates were coated with 100 μl of a solution of 1 μg/ml of purified human FH (0.1 μg, Calbiochem). After overnight incubation at 4°C, the plate was washed with Phosphate-Buffered Saline (PBS), 0.1% Tween20, and 0.2 M NaCl and blocked with PBS, 0.1% Tween 20, and 0.3% milk powder. After washes, 50 μl of plasma/serum samples at 1:100 dilution were added to duplicated wells, and incubated for 40 min. A parallel plate was set up in the absence of FH coating, in which only blocking solution was added to evaluate the presence of non-FH specific background, according to published recommendations (39). After washes, goat anti-human IgG antibody conjugated with horseradish peroxidase (HRP, 1:250, Sigma-Aldrich) was added and incubated for 1 h. Enzymatic activity was revealed using the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. A positive control kindly gifted by Dr. Marie-Agnes Dragon-Durey, with 2,000 AU/ml titer was used at 1:100 dilution. The positive threshold was set up at the mean titer +2 standard deviations (SD) found in plasma samples of 98 healthy controls with 1 or 2 copies of *CFHR1* (56 AU/ml). The sample concentrations expressed in arbitrary units/ml (AU/ml) were extrapolated from a sigmoidal curve and the background derived from wells without FH coating was subtracted. The ELISA was repeated in positive samples, adding 15 μg of FH into each sample to verify antibody specificity. Samples showing at least a 50% decrease of the ELISA absorbance in the assay performed in the presence of exogenous FH were considered as true positive.

This ELISA protocol was also used to evaluate the binding of autoantibodies to SCRs 1–5 and 15–20 FH fragments obtained by baculovirus transfection of *Spodoptera frugiperda* cells as reported (40). The plate was coated with molar equivalents of FH SCRs 1–5 and 15–20 and the results were expressed as absorbance at 450 nm. In a selected experiment, additional wells were coated with full length FH (positive control) or bovine serum albumin (BSA, negative control).

## HMEC-1

Serum-induced C5b-9 deposition on a human microvascular endothelial cell line (HMEC-1) was analyzed as previously described, with minor modifications (41). HMEC-1 were plated on glass coverslips and used when confluent. Cells were activated with 10 μM ADP for 10 min and incubated for 4 h with serum from patients (ID: 18 and 20) or healthy controls diluted 1:2 with test medium (Hank's Balanced Salt Solution—HBSS +0.5% BSA). HMEC-1 were fixed and stained with rabbit anti-human C5b-9 antibody, followed by FITC-conjugated antibody. Fluorescent staining on cell surface was acquired in 15 fields through confocal microscopy and the staining area was evaluated using Image J software. Results were expressed as the percentage of staining in relation to a pooled control sera ($n = 10$ subjects) run in parallel. The sera from additional 35 controls were analyzed separately and the percentages of C5b-9 deposits vs. control serum pool were calculated to establish the normal range (mean ± 2SD of % C5b-9 deposits of the control sera vs. control serum pool: 60–149%).

## FH ELISA

FH levels were evaluated by ELISA. Microtiter plates were coated with 0.15 μg of purified sheep polyclonal anti-human factor H antibody (Abcam). After three washes with PBS, 0.05% Tween20, the plate was blocked with 200 μl of PBS, 1% BSA. After four washes with PBS, 0.05% Tween20, 100 μl of plasma samples at 1:10,000 dilution were added to duplicated wells, and incubated for 2 h. After the washes, 100 μl of mouse monoclonal anti-human factor H (OX-23, LifeSpan BioSciences), which recognizes the SCRs 1–4 of the FH N-terminal domain, were added at a 1:10,000 dilution in blocking solution. Notably, this anti-human factor H antibody also detected the FH-like 1 (FHL1) which shares the N-terminal domain with FH. The plate was washed and 100 μl of goat anti-mouse antibody conjugated with HRP (1:2,000, Thermo Fisher) were added and incubated for 1 h. Enzymatic activity was revealed using the TMB substrate.

## SDS-PAGE and Western Blotting

FHR1 was studied by Western blotting in the serum of all patients with anti-FHs with at least one copy of *CFHR1*. Sera were diluted 1:40 in loading buffer (4X Laemmli Sample Buffer, Bio-Rad) in non-reducing conditions and analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Mini-Protean TGX Precast Gels, Bio-Rad). Proteins were transferred to polyvinylidene difluoride membrane (PVDF, Trans-Blot Turbo Midi PVDF Transfer; Bio-Rad), and blocked with 4% skim milk and 1% BSA. Mouse anti-human FHR1 IgGs (1:1,000, kindly given by Prof. Peter Zipfel) (42), which recognizes the N-terminal domains of FHR1, were added for 1 h. The binding of the antibody was detected by 1 h incubation with HRP goat anti-mouse IgG (1:5,000), followed by enhanced chemiluminescence (ECL) substrate (Amersham).

## Measurement of *CFH* and *CFHRs* Copy Number

Multiplex-ligation dependent probe amplification (SALSA MLPA P236-A3 ARMD, MCR-Holland, Netherlands) was used to evaluate the presence of copy number variations (CNVs) in *CFH, CFHR3, CFHR1, CFHR2, CFHR5* genes in the 30 aHUS patients with anti-FHs. The *CFHR4* CNV was evaluated in aHUS patients with anti-FHs by a multiplex PCR, as reported by Moore et al. (22). To evaluate the presence of the homozygous deletion of *CFHR1* in all 305 aHUS patients, and in the 960 healthy adult controls a multiplex PCR was set up that amplifies a 133 bp fragment in intron 3 of *CFHR1* and a 83 bp fragment in the promoter of *RNAseP*.

Primers: *CFHR1*-For 5′-ATCACTACACATGGACCTGAAA-3′; *CFHR1*-Rev 5′-GATGTGGAAAAATAAAAGAAAATAAGTC-3′; *RNAseP*-For 5′-TAGATACCGTGTGCGTGCAT-3′; *RNaseP*-Rev 5′-GGGGTTCCAATTCCCAACTA-3′.

## Genetic Screening

Through next-generation sequencing we performed genetic screening of all exons and flanking regions of *CFH*, *CD46*, *CFI*, *CFB*, *C3,* and *THBD* by highly multiplex PCR using the

Ion AmpliSeq™ Library Kit on an Ion PGM Sequencer (Life Technologies) (34).

LPVs were defined as genetic variants with Minor Allelic Frequency (MAF) in the ExAC database ≤0.001 and a Combined Annotation Dependent Depletion (CADD) pathogenic score ≥20 (43). Considering the complexity of aHUS and the very low penetrance we also considered as likely pathogenic, variants with a MAF ≤0.01 for which there was evidence of functional effects in literature. In addition, we included as likely pathogenic, genetic variants with MAF ≤0.01 for which an association with aHUS was reported (*P*-value <0.001, vs. "ExAC all" in the database of complement gene variants, www.complement-DB.org).

The *CFH* promoter and the coding and flanking regions of *CFHR1* were analyzed by direct sequencing.

## Statistical Analyses

All statistical tests were executed using MedCalc software. The Chi-square test or the Fisher's exact test were used to compare the allele frequencies between cases and "supercontrols," as appropriate. The expectation maximization algorithm by Haploview software was used to estimate each *CFH* haplotype frequency. Findings were considered statistically significant at *P*-values <0.05 after Bonferroni correction. Odds ratio (OR) was reported with the 95% confidence interval. ANOVA or Kruskal-Wallis tests were used to compare the mean or the median of variables, as appropriate.

# RESULTS

## Factor H Autoantibodies

The presence of anti-FHs was assessed in 305 consecutive aHUS patients of the International Registry of HUS for whom serum or plasma was available. Thirty patients (9.8%) were positive for anti-FHs (anti-FHs titer threshold >56 AU/ml); in all of them the specificity of the result was confirmed in a replicated assay, in which an excess of FH was added to the serum/plasma sample and competed for the anti-FHs with FH coated on the well (**Figure 1**). The mean ± SD of anti-FH titers was 1,164.7 ± 1,189.5 AU/ml in samples collected during the acute phase of the disease ($n = 4$) and 521.8 ± 504.3 AU/ml in samples collected during remission ($n = 26$; ANOVA, *P*-value = 0.062).

## FHR1 Deficiency

In all 30 patients with anti-FHs aHUS, copy numbers were evaluated by MLPA for *CFH*, *CFHR3*, *CFHR1*, *CFHR2*, and *CFHR5* and by a multiplex PCR for *CFHR4*. The homozygous *CFHR3-CFHR1* deletion was observed in 20 patients (66.7%) and the heterozygous *CFHR3-CFHR1* deletion combined with the heterozygous *CFHR1-CFHR4* deletion was present in 4 patients (13.3%, **Table 1**). Overall, 24 patients had zero copies of *CFHR1* (80%, **Table 1**). Four patients showed the heterozygous deletion of *CFHR3* and *CFHR1* (13.3%) and in two patients no CNV in *CFHR* genes were identified (6.7%, **Table 1**).

Direct sequencing of *CFHR1* was performed in the 6 patients with anti-FHs and at least one copy of *CFHR1*. In patient 23, who

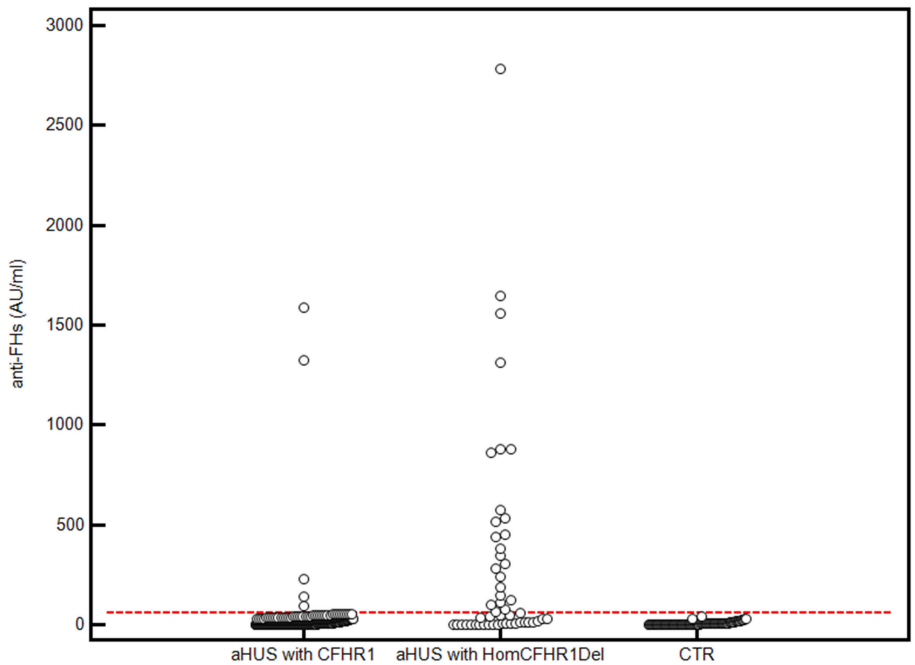


**FIGURE 1 |** Anti-FHs in aHUS patients and healthy controls. 305 aHUS patients were analyzed for anti-FHs through ELISA: 254 were carriers of 1 or 2 copies of *CFHR1* (aHUS with *CFHR1*), and 51 had 0 copies of *CFHR1* (aHUS with Hom*CFHR1*Del). Patient 23, carrying the heterozygous *CFHR1*Del and a frameshift variant in *CFHR1* exon 2 (c.104delAfsX), which resulted in a complete deficiency of FHR1 has been also included in the group of patients defined as "aHUS with Hom*CFHR1*Del." Ninety-eight healthy controls with 1 or 2 copies of *CFHR1* were also analyzed for anti-FHs (CTR). The positive threshold was set at mean +2SD of values recorded in the 98 controls (56 AU/mL) and is shown with the horizontal red dashed line.

**TABLE 1 |** Copy numbers of *CFHR3, CFHR1*, and *CFHR4* and LPVs in complement genes observed in aHUS patients with anti-FHs.

| Patient ID | Number of copies | | | LPVs | MAF (ExAC) | CADD | Functional effect | Association with aHUS (complement-db.org) |
|---|---|---|---|---|---|---|---|---|
| | CFHR3 | CFHR1 | CFHR4 | | | | | |
| 1 | 0 | 0 | 2 | nd | | | | |
| 2 | 0 | 0 | 2 | *CFH* c.2850G>T, p.Gln950His | 0.004 | 21.4 | Yes | No |
| 3 | 0 | 0 | 2 | nd | | | | |
| 4 | 1 | 0 | 1 | nd | | | | |
| 5 | 0 | 0 | 2 | nd | | | | |
| 6 | 0 | 0 | 2 | *C3* c.1909G>C, p.Gly637Arg | 0.0002 | 23.5 | No | No |
| 7 | 0 | 0 | 2 | *THBD* c.1693 G>T, p.Asp486Tyr | 0.006 | 6.12 | Yes | No |
| 8 | 0 | 0 | 2 | nd | | | | |
| 9 | 0 | 0 | 2 | nd | | | | |
| 10 | 0 | 0 | 2 | nd | | | | |
| 11 | 0 | 0 | 2 | nd | | | | |
| 12 | 0 | 0 | 2 | nd | | | | |
| 13 | 1 | 1 | 2 | *CD46* c.762delT, p.Leu254fsX43 | na | 17.57 | Yes | No |
| 14 | 0 | 0 | 2 | nd | | | | |
| 15 | 0 | 0 | 2 | nd | | | | |
| 16 | 1 | 0 | 1 | nd | | | | |
| 17 | 0 | 0 | 2 | nd | | | | |
| 18 | 0 | 0 | 2 | nd | | | | |
| 19 | 1 | 1 | 2 | *THBD* c.1502C>T, p.Pro501Leu | 0.002 | 24.7 | Yes | Yes |
| 20 | 0 | 0 | 2 | nd | | | | |
| 21 | 2 | 2 | 2 | *C3* c.1774C>T, p.Arg592Trp | $8 \times 10^{-6}$ | 34 | Yes | Yes |
| 22 | 0 | 0 | 2 | nd | | | | |
| 23 | 1 | 1* | 2 | nd | | | | |
| 24 | 0 | 0 | 2 | *CFH* c.2758T>C, p.Trp920Arg | na | 27 | No | Yes |
| 25 | 0 | 0 | 2 | nd | | | | |
| 26 | 1 | 1 | 2 | nd | | | | |
| 27 | 0 | 0 | 2 | *CFH* c.2776T>C, p.Cys926Arg | na | 28.4 | No | No |
| 28 | 1 | 0 | 1 | nd | | | | |
| 29 | 1 | 0 | 1 | *THBD* c. 241G>A, p.Val81Ile | $9 \times 10^{-6}$ | 8.28 | Yes | Yes |
| 30 | 2 | 2 | 2 | nd | | | | |

*The Minor Allelic Frequency (MAF) of LPVs from the ExAC database, the Combined Annotation Dependent Depletion (CADD) score, the availability of published data on functional effects, and the association with aHUS in the database of complement gene variants (complement-db.org) are also shown. Nd, not detected; na, not available. *The CFHR1 c.104delAfsX, p.D35fsX36 was observed in this patient.*

carried the heterozygous *CFHR1* deletion, a frameshift variant in *CFHR1* exon 2 (c.104delAfsX) that resulted in a premature stop codon (p.D35fsX36) was observed. In all six patients, the presence of FHR1 in serum was verified by Western blot (**Figure 2**). The normal pattern of two FHR1 bands was observed in patients 13, 19, and 26 (all with one copy of *CFHR1*) and patients 21 and 30 (both with two copies of *CFHR1*). At variance, patient 23, who had one copy of *CFHR1* plus the c.104delAfsX frameshift mutation did not have any FHR1 band, confirming that the mutation resulted in a non-secreted truncated protein, as expected. Thus, in our cohort, 25/30 (83.3%) patients with anti-FHs showed a complete deficiency of FHR1 and are defined as cases here.

We also evaluated the prevalence of the acidic and basic FHR1 isoforms in patients with anti-FHs and with at least one copy of *CFHR1*. In the basic FHR1 isoform that has been associated with the risk of aHUS (21), the amino acids 157His, 159Leu, and 175Glu are replaced by 157Tyr, 159Val, and 175Gln, which make the SCR3 of FHR1 identical to the SCR18 of FH. In patients with the heterozygous *CFHR3-CFHR1* deletion, one exhibited the basic isoform and two the acidic isoform. Both patients with two copies of *CFHR3* and *CFHR1* were compound heterozygous for the two FHR1 isoforms. Thus, we did not find an enrichment of a specific FHR1 isoform in our aHUS patients with anti-FHs.

A multiplex PCR was used to identify subjects with the *CFHR1* homozygous deletion in all 305 unrelated aHUS patients analyzed for anti-FHs and in a large cohort of healthy subjects ($n = 960$). Overall, 49 aHUS patients (16%) and 48 healthy subjects (5%) were found to be homozygous for the *CFHR1* deletion (OR [95%CI] = 3.6 [2.4–5.5], *P*-value = $5.5 \times 10^{-10}$, **Table 2**). The association of the homozygous *CFHR1* deletion with aHUS strongly increased in the subgroup of patients with anti-FHs vs.

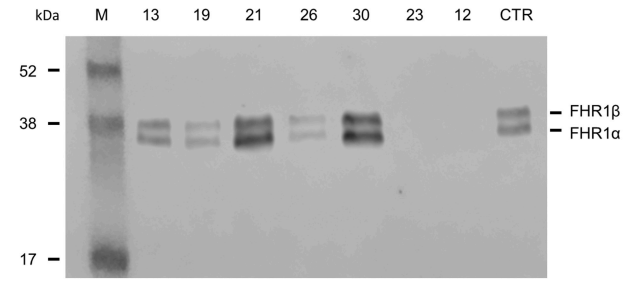


**FIGURE 2 |** Detection of FHR1 by Western blotting in aHUS patients with anti-FHs and at least one copy of *CFHR1*. ID numbers 13, 19 and 26: patients with 1 copy of *CFHR1*; ID numbers 21 and 30: patients with 2 copies of *CFHR1*; ID number 23: patient with 1 copy of *CFHR1* and a frameshift mutation in exon 2 (c.104delAfsX); ID number 12: patient with 0 copies of *CFHR1*; CTR: an healthy control with two copies of *CFHR1*.

**TABLE 2 |** Prevalence of homozygous *CFHR1* deletion in aHUS patients ($n = 305$) and healthy controls ($n = 960$).

| | Hom*CFHR1*Δ | No Hom*CFHR1*Δ | OR [95%CI] | *P*-value |
|---|---|---|---|---|
| aHUS ($n = 305$) | 49 (16%) | 256 (84%) | 3.6 [2.4–5.5] | $5.5 \times 10^{-10}$ |
| Controls ($n = 960$) | 48 (5%) | 912 (95%) | | |
| aHUS anti-FH positive ($n = 30$) | 24 (80%) | 6 (20%) | 76 [29.7–194.6] | $3 \times 10^{-52}$ |
| Controls ($n = 960$) | 48 (5%) | 912 (95%) | | |

healthy controls (OR [95%CI] = 76 [29.7–194.6], *P*-value = $3 \times 10^{-52}$, **Table 2**).

## Binding of Anti-FHs to FH Fragments and Effect of Patient Sera on C5b-9 Deposition on HMEC-1

To discern which FH epitopes were recognized by anti-FHs, anti-FH ELISA was repeated using wells coated with N-terminal FH (SCRs 1–5) and C-terminal FH (SCRs 15–20) fragments (kindly given by Prof. Peter Zipfel). The results of samples from five patients with 1 or 2 copies of *CFHR1* (patients 19, 13, 30, 21, and 26) and from seven patients carrying the homozygous *CFHR1* deletion (patients 16, 14, 15, 10, 25, 18, and 8) are shown in **Figure 3**. Antibodies from patients 13, 19, and 30 (with 1, 1 and 2 copies of *CFHR1,* respectively) and from patients 8, 10, 14, 15, and 16 (with 0 copies of *CFHR1)* strongly bound to C-terminus but not to N-terminus of FH. In patient 25 with the homozygous deletion of *CFHR1*, we observed a less intense but selective binding to FH C-terminal fragment. Antibodies from patients 18 (with the homozygous deletion of *CFHR1*) and 21 (with two copies of *CFHR1*) bound weakly to both FH SCRs 1–5 and FH SCRs 15–20 and finally, antibodies from patient 26 (with one copy of *CFHR1*) did not specifically bind any FH fragments. It is possible that autoantibodies from patient 26 recognize either central FH epitopes or epitopes exposed only in the native, full length FH, as previously reported for other aHUS patients (22).

An *ex-vivo* test to evaluate complement activation at the endothelial cell level showed that serum from two patients (ID: 18 and 20, without LPVs and with homozygous *CFHR1* deletion) taken after aHUS remission induced higher than normal (>149%) C5b-9 deposits on cultured ADP-activated human microvascular endothelial cells (**Figure 4**).

## Complement Likely Pathogenic Variants (LPVs)

*CFH*, *CD46*, *CFI*, *CFB*, *C3*, and *THBD* were sequenced in all patients with anti-FHs and 9 were found to be carriers of LPVs as defined in methods (30%, **Table 1**).

Of the 25 patients with anti-FHs and the FHR1 deficiency (cases), 6 had LPVs (24%, **Table 1**). Three cases carried LPVs in *CFH* (12%). Two Italian cases showed the heterozygous *CFH* LPV c.2850G>T, p.Gln950His in SCR16 (rs149474608, MAF (ExAC) = 0.004, CADD = 21.4) and the c.2758T>C, p.Trp920Arg (CADD = 27) in SCR15, respectively. These two patients had been previously reported by our group (3). Moreover, the p.Gln950His variant was previously described in another aHUS patient with anti-FHs (22), in other aHUS patients without anti-FHs (44–46), and in a patient who had developed TMA after renal transplantation (47). The third case was a Jewish patient who showed the heterozygous *CFH* c.2776C>T, p.Cys926Arg (CADD = 28.4) in SCR15. This variant is not present in public databases and has not been described in aHUS patients before. Notably, nuclear magnetic resonance studies (48) showed that the 3 LPVs involve amino acids that located close to each other in the three-dimensional structure of SCRs 15 and 16 of FH (**Figure 5**).

In an Italian case we found the C3 heterozygous c.1909G>C, p.Gly637Arg (rs149850773, MAF (ExAC) = 0.0002, CADD = 23.5), which is located in the Linker domain of the C3 molecule and has not been reported in patients with aHUS before.

We identified two *THBD* heterozygous LPVs: the c.1693G>T, p.Asp486Tyr (rs41348347, MAF (ExAC) = 0.006, CADD = 6.12) was identified in an Italian case and the c.241G>A, p.Val81Ile (rs772288987, MAF (ExAC) = $9 \times 10^{-6}$, CADD = 8.28) was found in a case from Yemen. Both *THBD* variants have already been reported in patients with aHUS (3, 49).

Of the 48 adult healthy "supercontrols" with the homozygous deletion of *CFHR1*, only one carried a LPV, namely the heterozygous *CFI* c.949 G>A, p.Arg317Trp [rs121964917, MAF (ExAC) = $9.9 \times 10^{-5}$, CADD = 16.27]. The FI 317Trp variant has previously been reported to have 30% C3b and C4b cofactor activity compared to wild type FI, but conflicting results have been found by other authors (50, 51).

Overall, in cases the prevalence of LPVs was significantly higher than in "supercontrols" (24 vs. 2.1%, Fisher's exact test, *P*-value = 0.005).

## Common Complement Susceptibility Variants

We then investigated whether common genetic variants in *CFH* contribute to determining susceptibility to anti-FHs mediated aHUS by comparing the prevalence of *CFH* c.1-332C>T (rs3753394), c.184G>A p.Val62Ile (rs800292), c.1204T>C

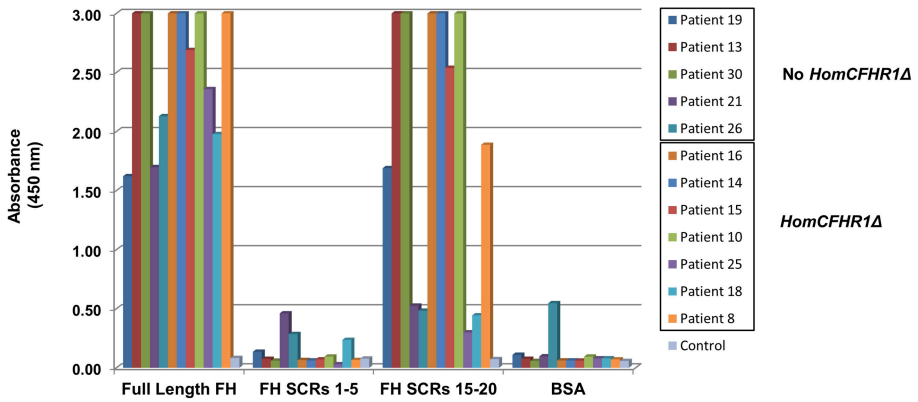


**FIGURE 3 |** Binding site localization of anti-FHs detected in aHUS patients. Anti-FH binding to FH N-terminal fragment (SCRs 1–5) and FH C-terminal fragment (SCRs 15–20) evaluated in 12 aHUS patients. Seven patients were carriers of the homozygous *CFHR1* deletion (Hom*CFHR1* Δ) while 5 patients presented at least one copy of *CFHR1* (No Hom*CFHR1* Δ). BSA coating was used as negative control and full length FH coating as positive control. Absorbance of serum from a healthy subject was used as an additional control. The absorbance is shown on the ordinate axis. Data are representative of three experiments.

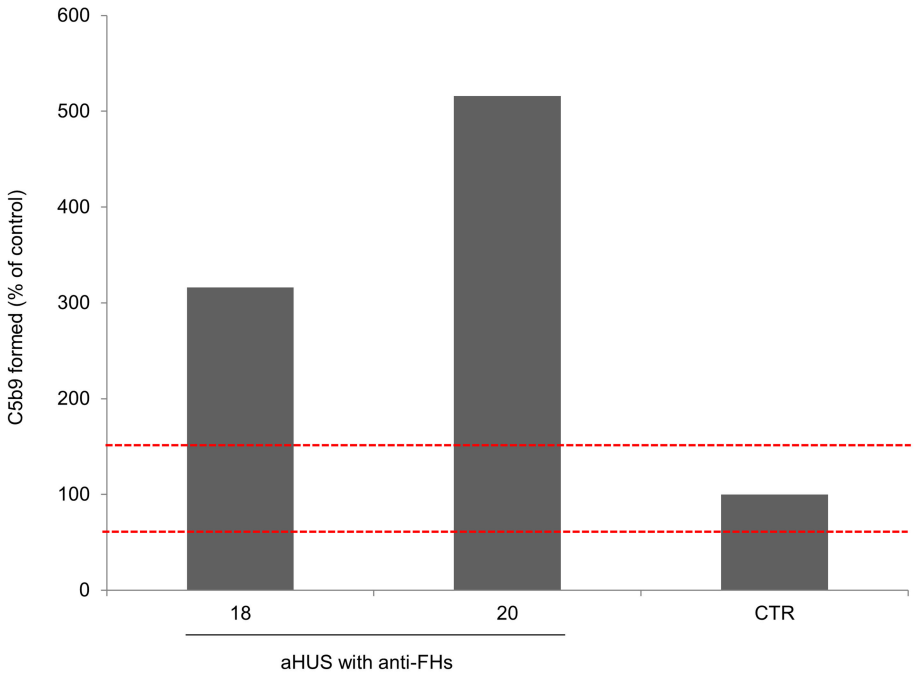


**FIGURE 4 |** Complement activation on endothelial cells. Endothelial surface area covered by C5b-9 staining after incubation of ADP-activated HMEC-1 with serum from aHUS patients with anti-FHs studied at remission (patients 18 and 20). For each sample, values were expressed as the percentage of C5b-9 deposits induced by a pool of sera from 10 healthy controls run in parallel (reference 100%). The red dashed lines indicated the normal range (60–149%) determined by testing single sera from 35 different healthy controls.

p.Tyr402His (rs1061170), c.2016A>G Gln572Gln (rs3753396), c.2237-543G>A (rs1410996), c.2808G>T Glu936Asp (rs1065489) in the 25 cases and in the 48 "supercontrols." As shown in **Table 3**, allele frequencies did not differ between cases and "supercontrols."

*CFH* haplotypes were also studied in cases and "supercontrols." The minimal informative SNPs within the *CFH* gene considered for this analysis were rs3753394 (promoter), rs800292 (exon 2), rs1061170 (exon 9), rs3753396 (exon 14), rs1410996 (intron 15), and rs1065489 (exon 19). The most frequent *CFH* haplotype was the CGTAAG (H4a haplotype), with a frequency of 0.492 in cases and 0.638 in "supercontrols," followed by the TGTAAG (H4b haplotype, **Table 4**), with no difference between cases (0.338) and "supercontrols" (0.279). On the contrary, the frequency of the H3 *CFH* haplotype (TGTGGT), which had previously been associated with aHUS

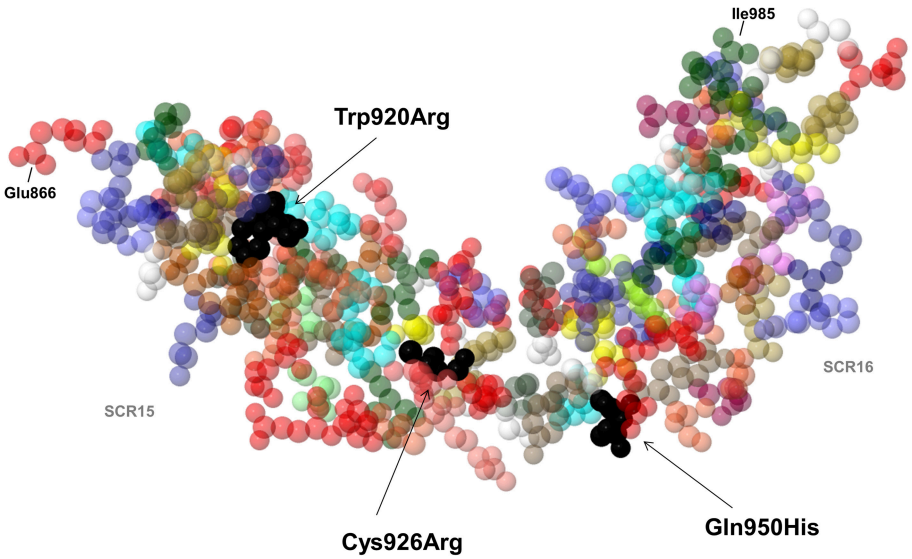


**FIGURE 5 |** 3D structure by nuclear magnetic resonance of SCR15 and SCR16 of FH (PDB code: 1HFH). Black arrows indicate the FH amino acid residues Trp920, Cys926 (SCR15), and Gln950 (SCR16) (colored in black) which are substituted in three aHUS patients with anti-FHs due to the Trp920Arg, Cys926Arg, and Gln950His heterozygous mutations. Each amino acid is represented with a specific color. The first amino acid of SCR15 (Glu866) and the last amino acid of SCR16 (Ile985) are also indicated. Cysteine residues are shown in yellow color. Created by Jmol, an open-source Java viewer for chemical structures in 3D (http://www.jmol.org).

(15) was very low and estimated to be 0.024 in cases and 0.01 in "supercontrols" ($P$-value = 0.04, **Table 4**). Notably, the frequency of the *CFH* H3 haplotype in aHUS patients without anti-FHs of our cohort ($n = 275$) was 0.292 and was significantly higher compared to cases ($P$-value = 0.0002), "supercontrols" ($P$-value = $5.6 \times 10^{-8}$) as well as the general population (H3 haplotype frequency = 0.203, among 2,504 controls from 1,000 genomes project, $P$-value = $1.85 \times 10^{-6}$).

In summary, no significant association has been found between *CFH* haplotypes and anti-FHs mediated aHUS, using "supercontrols" as the reference group.

In our cohort of aHUS patients with anti-FHs, the H4a and H4b *CFH* haplotypes were in strong LD with the *CFHR3-CFHR1* deletion (**Table 5**), which is consistent with published data (16), while the H5 and H3 haplotypes were associated with the *CFHR1-CFHR4* deletion (**Table 5**).

The frequency of the *CD46* c.*783C allele (rs7144) that tags the $CD46_{GGAAC}$ aHUS risk haplotype (52), did not differ between cases (0.38) and "supercontrols" (0.35, **Table 3**). On the contrary, the frequency of the C allele was significantly higher in our aHUS patients without anti-FHs (0.45) as compared with the 2,504 controls (allele frequency: 0.35) from the 1,000 genomes project ($P$-value = 0.001).

Moreover, we studied the prevalence of common polymorphisms in *C3* c.941C>T, p.Pro314Leu (rs1047286), *C3* c.304C>G, p.Arg102Gly (rs2230199), *CFB* c.94C>T, p.Arg32Trp (rs12614), *CFB* c.95G>A, p.Arg32Gln (rs641153) and *THBD* c.1418C>T, p.Ala473Val (rs1042579), which were previously reported in association with other immune-mediated diseases, including C3 glomerulopathy (33, 34), IgA nephropathy (35), and age-related macular degeneration (36–38). No significant difference was found with regard to allele frequencies of any of the above single nucleotide polymorphisms (SNPs) between cases and "supercontrols" (**Table 3**).

Finally, we analyzed the combination of the three variants FH Val62, FB Arg32, and C3 102Gly, which in functional studies (53, 54) have been associated with higher C3 convertase activity (risk complotype), and we did not find any difference in the prevalence of the risk complotype between cases (0.24) and "supercontrols" (0.17, $P$-value = 0.39).

## Genetic Screening of *CFH* Promoter

We then wondered whether rare variants in the *CFH* promoter that could affect FH expression in the thymus may predispose to a lack of central tolerance toward FH and to the development of anti-FHs HUS. The analysis through Matinspector software (Genomatix) revealed the presence of two sequences predicted with a good score as consensus motifs for the "autoimmune regulator" transcription factor (AIRE, **Figure 6**), a DNA binding molecule that is involved in central thymic tolerance by promoting the expression of tissue-specific antigens in medullary thymic epithelial cells. We sequenced the c.1-1070 region upstream the *CFH* gene, including the two predicted AIRE consensus regions, in our aHUS patients with anti-FHs.

We did not find any rare variant (with MAF $\leq$0.01) in the two DNA sequences predicted as AIRE consensus regions or in the entire sequenced *CFH* promoter region.

## Clinical and Biochemical Features at Disease Onset

Clinical and biochemical features at onset for all the 30 patients with anti-FHs are reported in **Table 6**.

The large majority of patients with anti-FHs in our cohort were Caucasian (53% males, **Table 6**). All but one developed

**TABLE 3 |** Prevalence of common variants in *CFH*, *CD46*, *CFB*, *C3*, and *THBD* in cases ($n = 25$) and "supercontrols" ($n = 48$).

| | Genotype | | | Sum | Allele frequencies | | *P*-value |
|---|---|---|---|---|---|---|---|
| | Hom WT | Het | Hom Var | | WT | Var | |
| ***CFH* c.1-332C>T (rs3753394)** | | | | | | | |
| Cases | 7 | 13 | 5 | 25 | 0.54 | 0.46 | ns |
| Supercontrols | 19 | 26 | 3 | 48 | 0.67 | 0.33 | |
| ***CFH* c.184G>A Val62Ile (rs800292)** | | | | | | | |
| Cases | 20 | 5 | 0 | 25 | 0.90 | 0.10 | ns |
| Supercontrols | 42 | 6 | 0 | 48 | 0.94 | 0.06 | |
| ***CFH* c.1204T>C Tyr402His (rs1061170)** | | | | | | | |
| Cases | 22 | 2 | 0 | 24 | 0.96 | 0.04 | ns |
| Supercontrols | 47 | 1 | 0 | 48 | 0.99 | 0.01 | |
| ***CFH* c.2016A>G Gln572Gln (rs3753396)** | | | | | | | |
| Cases | 22 | 3 | 0 | 25 | 0.94 | 0.06 | ns |
| Supercontrols | 46 | 2 | 0 | 48 | 0.98 | 0.02 | |
| ***CFH* c.2237-543G>A (rs1410996)** | | | | | | | |
| Cases | 0 | 4 | 21 | 25 | 0.08 | 0.92 | ns |
| Supercontrols | 0 | 2 | 46 | 48 | 0.02 | 0.98 | |
| ***CFH* c.2808G>T Glu936Asp (rs1065489)** | | | | | | | |
| Cases | 22 | 3 | 0 | 25 | 0.94 | 0.06 | ns |
| Supercontrols | 47 | 1 | 0 | 48 | 0.99 | 0.01 | |
| ***CD46* c.*783T>C (rs7144)** | | | | | | | |
| Cases | 10 | 11 | 4 | 25 | 0.62 | 0.38 | ns |
| Supercontrols | 19 | 24 | 5 | 48 | 0.65 | 0.35 | |
| ***CFB* Arg32Trp/Gln (rs12614, rs641153)** | | | | | | | |
| Cases | 15 | 9 | 1 | 25 | 0.78 | 0.22 | ns |
| Supercontrols | 33 | 15 | 0 | 48 | 0.84 | 0.16 | |
| ***C3* c.941C>T Pro314Leu (rs1047286)** | | | | | | | |
| Cases | 17 | 8 | 0 | 25 | 0.84 | 0.16 | ns |
| Supercontrols | 33 | 13 | 2 | 48 | 0.82 | 0.18 | |
| ***C3* c.304C>G Arg102Gly (rs2230199)** | | | | | | | |
| Cases | 14 | 11 | 0 | 25 | 0.78 | 0.22 | ns |
| Supercontrols | 33 | 13 | 2 | 48 | 0.82 | 0.18 | |
| ***THBD* c.1418C>T Ala473Val (rs1042579)** | | | | | | | |
| Cases | 16 | 9 | 0 | 25 | 0.82 | 0.18 | ns |
| Supercontrols | 37 | 11 | 0 | 48 | 0.89 | 0.11 | |

*Hom, homozygous; WT, wild type; Het, heterozygous; Var, variant; ns, not significant. For the CFB Arg32Trp/Gln triallelic SNP "var" includes both Trp and Gln variants. Cases: aHUS patients with anti-FHs and FHR1 deficiency; "supercontrols": adult healthy subjects with homozygous CFHR1 deletion.*

**TABLE 4 |** Estimated *CFH* haplotypes in the 25 cases and the 48 "supercontrols."

| Estimated *CFH* haplotype | Haplotype reference number | Haplotype frequency | | *P*-value |
|---|---|---|---|---|
| | | Cases | Supercontrols | |
| CGTAAG | H4a | 0.492 | 0.638 | ns |
| TGTAAG | H4b | 0.338 | 0.279 | ns |
| CATAAG | H2 | 0.07 | 0.019 | ns |
| TGCAGG | H5 | 0.042 | 0 | ns |
| TGTGGT | H3 | 0.024 | 0.01 | 0.04 |

*The estimated allele frequencies of each CFH haplotype in cases (aHUS patients with anti-FHs and FHR1 deficiency) and "supercontrols" (adult healthy subjects with homozygous CFHR1 deletion) were shown. The minimal informative SNPs within CFH gene considered for this analysis were rs3753394, rs800292, rs1061170, rs3753396, rs1410996, and rs1065489.*

**TABLE 5 |** Linkage disequilibrium between *CFH* haplotypes and *CFHR3-CFHR1* or *CFHR1-CFHR4* deletions in the 30 aHUS patients with anti-FHs.

| Estimated *CFH* haplotype | Haplotype reference number | *CFHR3-CFHR1* deletion | *CFHR1-CFHR4* deletion | Frequency mean |
|---|---|---|---|---|
| CGTAAG | H4a | Yes | No | 0.504 |
| TGTAAG | H4b | Yes | No | 0.329 |
| CATAAG | H2 | Yes | No | 0.059 |
| TGCAGG | H5 | No | Yes | 0.042 |
| TGTGGT | H3 | No | Yes | 0.021 |

*Yes, deleted allele; No, normal allele. The last right column shows the estimated frequencies of the extended haplotypes including CFH and the CFHR3-CFHR1-CFHR4 region.*

aHUS during childhood or adolescence (96.7%). The median age of disease onset was 7.6 [6.6–9.9] years (**Table 6**). The outliers were a woman who developed anti-FHs aHUS at 30 years of age after a cesarean delivery and a child who manifested aHUS at 16 months (he also carried a *THBD* LPV). The distribution of the age at disease onset in patients with anti-FHs was unimodal, with a sharp incidence peak around 8 years of age (**Figure 7A**), which was significantly different from aHUS patients negative for anti-FHs who exhibited a bimodal distribution, with an early peak before 4 years of age, and a late peak during adulthood (**Figure 7B**). Similar bimodal distribution was observed when we considered only patients negative for anti-FHs and carrying complement gene abnormalities (in *CFH*, *MCP*, *CFI*, *THBD*, *C3*, and *CFB;* **Figure 7C**).

Through logistic regression we estimated that the homozygous deletion of *CFHR1* and age at disease onset between 4 and 12 years were strongly associated with the risk for an aHUS patient to present anti-FHs. Twenty-four out of 30 aHUS patients with anti-FHs (80%) vs. 25 out of 275 aHUS patients without anti-FHs (9%) had the homozygous deletion of *CFHR1* (OR [95% CI] = 40 [14.9–107.1], *P*-value = $1.4 \times 10^{-22}$, **Table 7**). Twenty-four out of 30 aHUS patients with anti-FHs (80%) vs. 27 out of 275 aHUS patients without anti-FHs (9.8%) developed the disease between 4 and 12 years of age (OR [95% CI] = 37 [13.8–97.9], *P*-value = $1.7 \times 10^{-21}$, **Table 7**). The combination of the homozygous *CFHR1* deletion and age at disease onset between 4 and 12 years further increased the risk to have anti-FHs aHUS (OR [95% CI] = 108 [33.7–346.5], *P*-value = $6.9 \times 10^{-33}$, **Table 7**).

Prodromal signs were observed in most (70%) anti-FH positive patients: twelve patients (40%) exhibited gastrointestinal symptoms (four of them with fever), and nine patients (30%) had upper respiratory tract infections (including flu like symptoms). One patient developed aHUS post-partum and another while on oral contraception. Five patients did not have any prodromal signs or triggers and for two patients trigger data were not available (**Table 6**). At disease onset, all patients showed anemia, thrombocytopenia, high LDH levels, and high serum creatinine.


**FIGURE 6 |** *CFH* promoter sequence. Exon 1 (yellow), basal promoter (light gray), proximal promoter (dark gray), and two AIRE consensus sequences predicted by Matinspector software–Genomatix (underlined) are shown.

Twenty-three (77%) patients developed kidney failure requiring dialysis (**Table 6**).

Among patients with anti-FHs, we did not find any significant difference in the age of onset and in clinical complement parameters between cases with FHR1 deficiency and patients with one or two copies of *CFHR1* (**Table 6**), as well as between patients with or without LPVs (**Table 6**).

## Complement Profile

Data on serum C3 and C4 levels at the time of anti-FH antibody assay were available for 28 patients (**Table 8**). Fourteen patients (46.7%) had lower than normal serum C3 levels, in most of them C3 was measured during convalescence (**Table 8**). C4 levels, on the contrary, were normal in all patients (**Table 8**). Plasma FH plus FHL1 levels were in the normal range in all patients (**Table 8**) with the only exception being patient 14 (studied in remission and with a high antibody titer, 1,557.3 AU/ml) who had 80 mg/l FH (**Table 8**). Plasma FH plus FHL1 levels did not differ between patients studied during the acute episode and patients studied in remission (acute: 340.3 ± 49.3 mg/l $n = 3$; vs. remission: 344.7 ± 117.1 mg/l, $n = 21$; ANOVA, $P$-value = 0.951) and did not correlate with the antibody titer ($r^2 = 0.0085$, $P$-value = ns). The latter finding would indicate that the antibodies used in our FH ELISA assay were detecting total FH, including free FH and immune-complexed FH (55), which is consistent with previously published studies (17, 56).

## Clinical Outcome

During a median of 36 months follow-up (IQR = 12–72 months), 11 patients with anti-FHs experienced relapses of the disease (37%): 9 had the FHR1 deficiency, one the FHR1 deficiency plus a LPV in *CFH* and one a LPV in *CD46* and 1 copy of *CFHR1*. Overall, 15 patients (50%) developed end stage renal disease (ESRD): 8 had the FHR1 deficiency, 2 the FHR1 deficiency plus a LPV in *C3* and *CFH*, respectively and 2 a LPV in *THBD* (and 1 copy of *CFHR1)* and *C3* (and 2 copies of *CFHR1)*, respectively. The prevalence of relapses or ESRD during the follow-up did not differ statistically between patients with or without LPVs (relapses: patients with LPVs 22%, without LPVs 43%; $P$-value = 0.42, ESRD: patients with LPVs 44%, without LPVs 52%; $P$-value = 1.0).

Four patients received kidney transplantation and aHUS recurrence was observed in one of them, who also carried the C3 heterozygous LPV p.Arg592Trp. Another patient died following the transplant due to clinical complications unrelated to aHUS.

# DISCUSSION

Here we report the results of a retrospective study in a large cohort of patients with aHUS in which we described the clinical and genetic features of 30 patients with anti-FHs.

Through combined CNV analysis and *CFHR1* sequencing we confirm that the FHR1 deficiency is strongly associated with anti-FHs aHUS. Moreover, through an approach based on the inclusion of "supercontrols," we demonstrate that patients with anti-FHs are enriched in complement gene LPVs, while common complement gene variants known to increase the risk of aHUS or other immune -mediated diseases do not significantly contribute to anti-FHs aHUS.

The prevalence of anti-FHs in our cohort of aHUS patients (10%) is consistent with data from other European cohorts (5–13%) (28), but is lower than that observed in an Indian aHUS cohort in which the percentage of anti-FH positive patients was dramatically higher up to 56.1% (57).

**TABLE 6 |** Clinical and biological data at onset of the 30 patients with anti-FH associated aHUS.

| ID | Sex | Ethnicity | Population | Disease onset | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Age (years) | Disease triggers | Hb (14–18 g/dl) | LDH (266–500 U/L) | Platelets (150–400 $\times 10^3$/ul) | Creatinine (0.5–0.8 mg/dl)* | Dialysis (yes/no) |
| 1 | F | Caucasian (Eu) | Italian | 7.0 | Vomiting | 4.5 | na | 38,000 | 2.7 | Yes |
| 2 | M | Caucasian (Eu) | Italian | 7.7 | Flu like | 6.3 | 4,185 | 64,000 | 1.6 | No |
| 3 | F | Caucasian (Eu) | Portuguese | 7.6 | Resp. infection | 8.5 | 538 | 78,000 | 4.9 | Yes |
| 4 | F | Caucasian (Eu) | German | 8.0 | None | 6 | 2,464 | 16,000 | 1.56 | Yes |
| 5 | M | Caucasian (Eu) | Italian | 8.8 | Vomiting | 6 | na | 167,000 | 9.4 | Yes |
| 6 | M | Caucasian (Eu) | Italian | 6.2 | Flu like | 6.9 | na | 30,000 | 6.9 | Yes |
| 7 | M | Caucasian (Eu) | Italian | 5.7 | None | 6.5 | 3,650 | 58,000 | 2.4 | No |
| 8 | M | Caucasian (USA) | American | 6.7 | na | na | na | na | na | Yes |
| 9 | F | Caucasian (Eu) | Serbian | 7.6 | Vomiting | 10.4 | 5,120 | 48,000 | 7 | Yes |
| 10 | F | Caucasian (Eu) | Serbian | 6.6 | Resp. infection | 6 | 2,022 | 124,000 | 1.25 | No |
| 11 | F | Caucasian (Eu) | Italian | 5.4 | Vomiting | 7 | 2,930 | 42,000 | 2.8 | Yes |
| 12 | M | Caucasian (Eu) | Italian | 7.8 | Vomiting | 6.8 | 2,866 | 96,000 | 1.9 | Yes |
| 13 | F | Caucasian (Eu) | Turkish | 12.8 | None | 10.6 | 1,767 | 12,000 | 2.6 | No |
| 14 | F | Caucasian (Eu) | Bulgarian | 15.5 | Vomiting, fever | 9 | 3,597 | 79,000 | 3.7 | Yes |
| 15 | M | Caucasian (Eu) | Polish | 11.0 | Vomiting | 7.8 | 1,908 | 43,000 | 4.3 | Yes |
| 16 | F | Caucasian (USA) | American | 10.5 | Vomiting, fever | 8 | na | 29,000 | 11 | Yes |
| 17 | F | Caucasian (USA) | American | 5.3 | Flu like | 8 | 1,422 | 120,000 | 3 | Yes |
| 18 | M | Caucasian (Eu) | Italian | 10.1 | Vomiting | 5.2 | 3,770 | 70,000 | 7 | No |
| 19 | F | Caucasian (Eu) | Italian | 8.8 | na | 7.2 | na | 60,000 | 4 | Yes |
| 20 | F | Caucasian (Eu) | Dutch | 30.2 | Cesarean | 9.5 | 3,362 | 40,000 | 1.8 | No |
| 21 | F | Hispanic | Argentine | 17.6 | Oral contraceptive | na | na | na | na | Yes |
| 22 | M | Caucasian (Eu) | Polish | 7.6 | Resp. infection | 9 | na | na | 9 | Yes |
| 23 | M | Asian | Japanese | 9.2 | Vomiting, fever | 6.5 | 2,569 | 15,000 | 2 | Yes |
| 24 | M | Jewish | Israeli | 5 | None | 4.8 | 1,091 | 76,000 | 1.5 | Yes |
| 25 | M | Persian | Iranian | 15.0 | Resp. infection | 8 | 7,752 | 23,000 | 5.7 | Yes |
| 26 | M | Hispanic | Argentine | 6.5 | None | 9 | na | 18,000 | 3.5 | Yes |
| 27 | M | Caucasian (Eu) | Italian | 7.1 | Resp. infection | 6.6 | na | 52,000 | 4.7 | Yes |
| 28 | F | Caucasian (Eu) | Italian | 8.1 | Vomiting, fever | na | na | na | na | Yes |
| 29 | M | African-Arab | Yemenian | 1.3 | Resp. infection | 5 | 1,916 | 150,000 | 0.6 | No |
| 30 | M | Caucasian (Eu) | Belorussian | 6.9 | Vomiting | 5.8 | 5,619 | 62,000 | 2.33 | Yes |
| | | | | **Median [IQR]** | | **Mean ± SD** | **Mean ± SD** | **Mean ± SD** | **Mean ± SD** | **% Yes** |
| | **Overall** | | | 7.6 [6.6–9.9] | | 7.3 ± 1.4 | 3,081.5 ± 1,736 | 61.923 ± 41,218 | 5.7 ± 3.3 | 77 |
| | **FHR1 deficiency** | Yes | | 7.6 [6.6–9.2] | | 7.1 ± 1.3 | 3,010 ± 1,696 | 66,272 ± 42,332 | 6.5 ± 3.4 | 76 |
| | | No | | 8.8 [6.9–12.8] | | 8.2 ± 2.1 | 3,693.0 ± 2,723.8 | 38,000 ± 26,683 | 3.3 ± 1 | 80 |
| | | *P*-value | | 0.4 | | 0.23 | 0.61 | 0.21 | 0.4 | 0.7 |
| | **LPVs** | Yes | | 7.1 [5.7–8.8] | | 5.6 ± 0.9 | 2,521.8 ± 1,325.0 | 62,750 ± 40,679 | 3.3 ± 1 | 56 |
| | | No | | 7.8 [6.9–10.1] | | 7.6 ± 1.3 | 3,281.4 ± 1,832.7 | 61,555 ± 42,621 | 6.5 ± 3.4 | 86 |
| | | *P*-value | | 0.32 | | 0.34 | 0.42 | 0.95 | 0.38 | 0.18 |

*The Patient ID, the sex (F, female; M, male), the ethnicity, the population of origin, the age at the disease onset, the observed triggers, hemoglobin (Hb), lactate dehydrogenase (LDH), platelet count, serum creatinine and the need for dialysis are shown. na, not available. Normal values are in brackets. *Normal values for children between 5 and 10 years. For children <1–5 years, 0.3–0.5 mg/dl; for children >10 years/adults, 0.5–1.2 mg/dl.*

Finding that 83% of our aHUS patients with anti-FHs had a complete FHR1 deficiency due to the homozygous *CFHR3-CFHR1* deletion or, more rarely, to compound heterozygous *CFHR3-CFHR1* and *CFHR1-CFHR4* deletions is consistent with earlier published studies in other cohorts (21, 22). Interestingly, we describe a FHR1-deficient patient carrying the *CFHR1* deletion in one allele and a frameshift *CFHR1* mutation causing an early termination of protein translation in the other allele. To the best of our knowledge only one other aHUS patient with anti-FHs and a truncating *CFHR1* mutation has been described in a published study (21). However, we found 5 patients with anti-FHs carrying at least one normal copy of *CFHR1,* indicating that autoantibodies against FH can also be formed in the presence of FHR1.

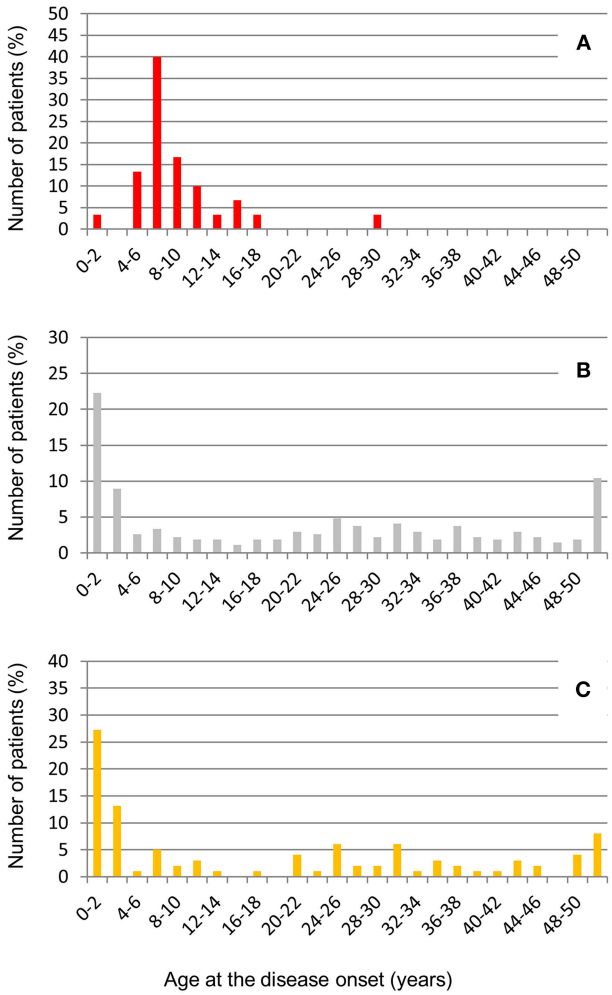


**FIGURE 7 |** Distribution of age at disease onset in aHUS patients analyzed for anti-FHs. **(A)** Patients with anti-FHs ($n = 30$); **(B)** Patients without anti-FHs ($n = 275$), and **(C)** Patients without anti-FHs and carrying LPVs in complement genes ($n = 99$).

In agreement with previous reports (24–27, 58), anti-FHs from 92% of our patients predominantly targeted the C terminus of FH, which is a mutational hot spot in aHUS and a crucial domain involved in the regulation of the complement AP on cell surface (59). Our results, showing that serum from two patients with anti-FHs and no LPVs in complement genes induced higher than normal C5b-9 deposits on cultured endothelial cells, support a pathogenetic role of anti-FHs in causing complement dysregulation on endothelium (58, 60). This finding is in line with previous studies showing that aHUS-associated anti-FH autoantibodies impaired FH capability to protect rabbit or sheep erythrocytes from complement-mediated lysis (25, 61).

The mechanisms that link the lack of FHR1 to the development of anti-FHs have not been clarified yet. Evidence that the FH C-terminal domain is homologous to FHR1 C-terminus (62) and that most anti-FHs cross-react with SCRs 4–5 of FHR1 (22, 26) suggested that the absence of FHR1 plays a role in the loss of tolerance to FH (28). In the *CFH* promoter region we identified two sequences predicted as possible consensus site for the binding of AIRE, a transcription factor that is crucial for the expression of tissue-specific antigens in the thymus and the maintenance of central tolerance (63–65). However, the failure to find any variant in the *CFH* promoter in our patients with anti-FHs aHUS does not support the hypothesis that there is a defect of central tolerance related to AIRE in anti-FHs development.

Of relevance to understand the immunologic basis of anti-FHs, are data that the large majority of patients with anti-FHs of our cohort had prodromal infectious illnesses, most commonly upper respiratory tract and gastrointestinal infections, and developed the disease between 4 and 12 years of age, which corresponds to the peak of incidence of common infections.

**TABLE 7 |** Estimate of the risk of an aHUS patient having anti-FHs in the presence of *CFHR1* homozygous deletion and/or age at disease onset between 4 and 12 years.

| | | Anti-FHs | | Sensitivity | Specificity | OR [CI 95%] | *P*-value |
|---|---|---|---|---|---|---|---|
| | | Positive | Negative | | | | |
| Hom *CFHR1*Δ | Yes | 24 | 25 | 0.80 | 0.91 | 40 [14.9–107.1] | $1.4 \times 10^{-22}$ |
| | No | 6 | 250 | | | | |
| Onset 4–12 years | | 24 | 27 | 0.80 | 0.90 | 37 [13.8–97.9] | $1.7 \times 10^{-21}$ |
| Onset <4 or ≥13 years | | 6 | 248 | | | | |
| Hom *CFHR1*Δ and onset at 4–12 years | Yes | 20 | 5 | 0.67 | 0.98 | 108 [33.7–346.5] | $6.9 \times 10^{-33}$ |
| | No | 10 | 270 | | | | |

*OR, odds ratio.*

**TABLE 8 |** Serum C3, C4, and plasma FH + FHL1 levels evaluated at the time of anti-FH measurement.

| Patient ID | Biochemical complement parameters | | | | |
|---|---|---|---|---|---|
| | Disease phase | Anti-FH titer (AU/mL) | C3 level (90–180 mg/mL) | C4 level (10–40 mg/mL) | FH+FHL1 level (172–507 mg/L) |
| 1 | Rem | 453.4 | 30 | 12 | na |
| 2 | Rem | 381.6 | 141 | 27 | na |
| 3 | Rem | 280.8 | 112 | 29 | na |
| 4 | Rem | 513.2 | na | na | na |
| 5 | Rem | 146.6 | 85 | 25 | 349 |
| 6 | Rem | 243.0 | 81 | 26 | 309 |
| 7 | Rem | 572.0 | 94 | 17 | 312 |
| 8 | Rem | 1,646.1 | 77 | 31 | 283 |
| 9 | Rem | 876.7 | 70 | 19 | 302 |
| 10 | Acute | 1,314.9 | 103 | 25 | 397 |
| 11 | Acute | 2,781.0 | 96 | 28 | na |
| 12 | Rem | 342.9 | 185 | 62 | 307 |
| 13 | Rem | 1,590.4 | 118 | 22 | 686 |
| 14 | Rem | 1,557.3 | 35 | 27 | 80 |
| 15 | Rem | 862.3 | 100 | 30 | 438 |
| 16 | Rem | 877.4 | na | na | 308 |
| 17 | Rem | 189.15 | 106 | 70 | 446 |
| 18 | Rem | 531.2 | 75 | 14 | 463 |
| 19 | Rem | 226.6 | 78 | 20 | 374 |
| 20 | Rem | 111.8 | 149 | 38 | 432 |
| 21 | Rem | 96.6 | 39 | 25 | 244 |
| 22 | Rem | 102.1 | 89 | 39 | 362 |
| 23 | Acute | 123.1 | 54 | 29 | 307 |
| 24 | Rem | 77.6 | 126 | 35 | 226 |
| 25 | Acute | 440.15 | 95 | 27 | 317 |
| 26 | Rem | 141.2 | 96 | 27 | 362 |
| 27 | Rem | 65.2 | 65 | 14 | 246 |
| 28 | Rem | 57.1 | 99 | 22 | 372 |
| 29 | Rem | 303.9 | 68 | 11 | na |
| 30 | Rem | 1,321.7 | 63 | 31 | 337 |
| | | **Mean ± SD** | **Mean ± SD** | **Mean ± SD** | **Mean ± SD** |
| | Acute ($n = 4$) | 1,164.8 ± 1,189.5 | 87 ± 22.3 | 27.2 ± 1.7 | 340.3 ± 49.3 |
| | Rem ($n = 26$) | 521.8 ± 504.3 | 90.9 ± 36.3 | 28 ± 14 | 344.7 ± 117.1 |

*The phase of the disease when the samples were collected (Rem, remission phase; Acute, acute phase), the anti-FH titer, and serum C3, C4 levels and plasma FH + FHL1 levels are shown. Normal ranges are in brackets; na, not available.*

These findings, together with published reports showing a high prevalence of respiratory tract infections (19, 66), and gastrointestinal pathogens in aHUS patients with anti-FHs (67), would support a "two-hit" model according to which the autoimmunity toward FH could develop as a result of an infection in subjects with genetic predisposing background (28). Bhattacharjee et al. (26) identified an anti-FH autoantibody epitope cluster within SCR20, which includes amino acids used by microbes to bind FH and evade the immune system (68–70). The authors proposed that binding of this domain with certain microbes induces a conformation change in FH SCR20 generating a neoepitope similar to FHR1, which might predispose to the development of the autoantibodies against FH in subjects with the FHR1 deficiency.

Anti-FHs aHUS is an ultra-rare disease with a prevalence of about 1/1,000,000 people in Europe and the USA (1, 28) which does not fit with the ∼5% prevalence of FHR1 deficiency in European Caucasian populations (29), and with bacterial infections that commonly occur during school-age (67). From these observations we infer that multiple genetic and/or environmental risk factors are required for the disease to manifest, as reported for other autoimmune disorders (71).

Recently, as a new strategy to identify genetic variants associated with complex traits, patients were compared with centenarians, as supercontrols, who have evaded all the common diseases associated with aging and are expected to be depleted in disease predisposing genetic variants or enriched in protective variants (72, 73). Inspired by these reports, to identify other anti-FHs aHUS genetic determinants, here we adopted a similar approach using as "supercontrols" adults subjects with the homozygous *CFHR1* deletion who did not get anti-FHs aHUS despite carrying the known genetic risk factor for the disease (i.e., FHR1 deficiency) and likely having been exposed to common infections during childhood. We hypothesized that the comparison of our patients with anti-FHs aHUS and FHR1 deficiency (cases) with "supercontrols" could allow us detecting genetic variants with clinical value at relatively small sample size. With this approach we demonstrate that cases are significantly enriched in *CFH*, *THBD* and *C3* LPVs compared to "supercontrols." Other authors have screened patients with anti-FHs aHUS for complement gene defects, but there was no comparison with a control group and the results were conflicting (18, 19, 21–23, 32). Our data support the hypothesis that rare complement gene variants are involved in predisposing to anti-FHs aHUS. *CFH* LPVs are the most abundant in our cases, two of which are not reported in public control databases and are predicted to be strongly damaging *in silico*. The third *CFH* LPV, the p.Gln950His, was also found in 4 patients without anti-FHs of our aHUS cohort and in other published patients (22, 44–46). The functional relevance of this variant was documented by Mohlin and colleagues (44) who showed that the 950His variant is less effective in inhibiting complement-mediated sheep erythrocyte lysis than the wild type Gln950. As for the *THBD* variants, both have been found to be less effective in promoting C3b inactivation to iC3b on the cell surface (12). Finally, the *C3* p.Gly637Arg has not been described in patients with aHUS, it is predicted to be strongly damaging by *in silico* analysis but functional data are not available. We would speculate that complement LPVs contribute to aHUS in subjects with FHR1 deficiency by synergizing with anti-FHs in inducing AP activation on cell surfaces. Since complement activation products can enhance the adaptive immune response (74), the possibility that AP dysregulation associated with LPVs *per se* could favor the proliferation of the self-reactive T cell clones and the formation of anti-FHs is worth investigating. However, further studies are required

to clarify the mechanisms through which LPVs predispose to anti-FHs aHUS.

We found only one LPV in the "supercontrol" group, namely the p.Arg317Trp in FI. This variant was previously reported to have a lower cofactor activity compared to wild type FI (50). However, later on Nilsson and colleagues found that the activity of the 317Trp variant was not impaired in any functional assay, rather this variant was more efficient than wild type FI in C3b cleavage on the surface of endothelial cells (51).

At variance with LPVs, common genetic variants in complement genes predisposing to aHUS or to other immune-mediated diseases (as C3 glomerulopathy, age-related macular degeneration, and IgA nephropathy) (33–38) were not more abundant in our cases compared to "supercontrols." Specifically, the *CFH* H3 haplotype, which has been strongly associated with an increased risk of aHUS (15), was rare both in cases and in "supercontrols," although it was slightly more prevalent in cases. As expected, this haplotype was enriched in our patients without anti-FHs. These results do not surprise, because most cases and "supercontrols" share the *CFH* H4 haplotypes that are in LD with the *CFHR3-CFHR1* deletion. As for the frequency of the *CD46* c.*783C allele (rs7144) variant that tags the $CD46_{GGAAC}$ haplotype, described in strong association with aHUS (52), we did not observe any difference between cases and "supercontrols," whereas the frequency of this allele was significantly enriched in our aHUS patients without anti-FHs. Altogether our data demonstrate that the *CFH* H3 and the $CD46_{GGAAC}$ haplotypes are risk factors for aHUS without anti-FHs but do not contribute to development of anti-FHs aHUS.

The marked difference we observed in the age of onset between patients with anti-FHs and those negative for anti-FHs further supports the hypothesis that diverse predisposing factors underlying the two forms of the disease are involved.

Importantly, within the entire aHUS cohort reported here we have shown that a disease onset between ages 4 and 12, identifies the presence of anti-FHs with a specificity of 90%. Patients with the above age of onset should be screened carefully for anti-FHs. Prompt identification of anti-FHs is of great clinical relevance since anti-FHs can be removed by successfully plasma exchange, which also supplements free FH and provides FHR1, which might act as a decoy for the antibodies (75). Patients should be followed up carefully long-term to monitor the re-emergence of anti-FHs, since this form of aHUS is associated with a high prevalence of relapses, as documented by published (28) and present data. Relapses can be prevented by maintenance therapy with immunosuppressive agents that inhibit the further production of anti-FH antibodies (28), but these drugs may have serious side effects particularly in children.

In conclusion, we have confirmed in our cohort of patients, the strong association between FHR1 deficiency and aHUS with anti-FH autoantibodies. Through an innovative approach based on the comparison with "supercontrols" carrying the homozygous *CFHR1* deletion, identified by screening a large number of healthy adult subjects, we have documented that patients with anti-FHs aHUS are enriched in complement gene LPVs. This observation indicates that the pathogenesis of anti-FHs aHUS is complex and multiple "hits" are required for its clinical manifestation. We also document that the *CFH* H3 and the $CD46_{GGAAC}$ haplotypes are not associated with anti-FHs aHUS, whereas these haplotypes are enriched in aHUS patients without anti-FHs, which highlights the differences in the genetic basis of the two forms of the disease. Finally, we confirm the role of common infections as environmental factors that contribute to the development of anti-FHs aHUS in genetically predisposed individuals. The latter finding fits with the sharp peak of disease onset during scholar-age. Further studies are needed to fully elucidate the genetic and environmental factors underlying anti-FHs aHUS and to establish whether the combination of anti-FHs with LPVs or other risk factors influences the course of the disease and the response to therapies.

## ETHICS STATEMENT

All subjects provided informed written consent in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of Azienda Sanitaria Locale, Bergamo, Italy.

## AUTHOR CONTRIBUTIONS

EV, MN, and GR designed research, interpreted data, and wrote the paper. EV, MA, PI, RP, CM, MB, AC, RD, and SA performed the research and analyzed the data. EB provided detailed clinical information of patients. AB and AA analyzed the data and critically revised the manuscript.

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# C3-Glomerulopathy Autoantibodies Mediate Distinct Effects on Complement C3- and C5-Convertases

*Fei Zhao[1], Sara Afonso[1], Susanne Lindner[1], Andrea Hartmann[1], Ina Löschmann[1], Bo Nilsson[2], Kristina N. Ekdahl[3], Lutz T. Weber[4], Sandra Habbig[4], Gesa Schalk[4], Michael Kirschfink[5], Peter F. Zipfel[1,6]* and Christine Skerka[1]**

[1] Deparment of Infection Biology, Leibniz Institute for Natural Product Research and Infection Biology, Jena, Germany, [2] Department of Immunology, Genetics and Pathology, University Uppsala, Uppsala, Sweden, [3] Linneaus Center for Bomaterials Chemistry, Linnaeus University, Kalmar, Sweden, [4] Children's and Adolescents' Hospital Cologne, University Hospital of Cologne, Cologne, Germany, [5] Institute for Immunology, University Heidelberg, Heidelberg, Germany, [6] Faculty of Life Sciences, Friedrich Schiller University Jena, Jena, Germany

C3 glomerulopathy (C3G) is a severe kidney disease, which is caused by defective regulation of the alternative complement pathway. Disease pathogenesis is heterogeneous and is caused by both autoimmune and genetic factors. Here we characterized IgG autoantibodies derived from 33 patients with autoimmune C3 glomerulopathy. Serum antibodies from all 33 patients as well as purified IgGs bound to the *in vitro* assembled C3-convertase. Noteworthy, two groups of antibodies were identified: group 1 with strong (12 patients) and group 2 with weak binding C3-convertase autoantibodies (22 patients). C3Nef, as evaluated in a standard C3Nef assay, was identified in serum from 19 patients, which included patients from group 1 as well as group 2. The C3-convertase binding profile was independent of C3Nef. Group 1 antibodies, but not the group 2 antibodies stabilized the C3-convertase, and protected the enzyme from dissociation by Factor H. Also, only group 1 antibodies induced C3a release. However, both group 1 and group 2 autoantibodies bound to the C5-convertase and induced C5a generation, which was inhibited by monoclonal anti-C5 antibody Eculizumab *in vitro*. In summary, group 1 antibodies are composed of C3Nef and C5Nef antibodies and likely over-activate the complement system, as seen in hemolytic assays. Group 2 antibodies show predominantly C5Nef like activities and stabilize the C5 but not the C3-convertase. Altogether, these different profiles not only reveal a heterogeneity of the autoimmune forms of C3G (MPGN), they also show that in diagnosis of C3G not all autoimmune forms are identified and thus more vigorous autoantibody testing should be performed.

**Keywords: C3 glomerulopathy, C3NeF, C5Nef, complement, Eculizumab**

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*Veronique Fremeaux-Bacchi, Assistance Publique Hopitaux De Paris (AP-HP), France*
*Josh Thurman, University of Colorado Denver, United States*

****Correspondence:***
*Christine Skerka*
*christine.skerka@leibniz-hki.de*
*Peter F. Zipfel*
*peter.zipfel@leibniz-hki.de*

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## INTRODUCTION

C3 Glomerulopathy (C3G) is based on glomerular changes associated with deposition of C3 cleavage fragments and the absence of immunoglobulins (1–4). C3G represents a spectrum of related kidney disorders with autoimmune and genetic causes. A defective complement control and in particular defective fluid phase regulation of the alternative pathway (AP) C3-convertase is considered relevant for this severe kidney disorder. C3G is divided in two major subtypes, dense deposit disease (DDD), which in some cases is called membranoproliferative glomerulonephritis type II (MPGN II) and C3 glomerulonephritis (C3GN) (4–7). DDD shows specific intramembranous dense deposits and C3GN includes cases with glomerular lesions with C3 accumulation and no or little immunoglobulin staining plus mesangial, discontinuous subepithelial-, and subendothelial lesions (6, 8, 9). Immune-complex mediated MPGN is defined by glomerular IgG and C3 deposits. This form derives from the deposition of immune complexes that form in the context of infections, autoimmune diseases and malignancies which trigger the classical complement pathway (10). Multiple genetic and autoimmune causes are associated with C3G (5–7). The genetic causes include complement genes, coding for the components of the C3-convertase, C3, Factor B and for the regulator Factor H (11–17) More recently mutations in the *CFHR5* gene as well as copy number variations in the *CFHR* gene cluster were identified in C3G patients (18–24). Affected *CFHR* genes can result in hybrid FHR proteins, such as FHR1::FHR1, FHR2::FHR4, FHR2::FHR5, FHR1::FHR5, together with altered FHR plasma levels. These genetic causes in the *CFHR* gene cluster are identified in patients with MPGN I, MPGN II, DDD and C3G. In those cases with FHR hybrid proteins the disease develops in context of an intact Factor H molecule.

The diagnosis of C3G and the related disorders is primarily based on histopathology, immunohistology and identified morphological changes, C3b deposits and dense deposit formation (5–7). Defective alternative complement action either in fluid phase, in plasma or on the surface of glomerular cells and the glomerular basement membrane results in stronger C3-convertase action and in C3b deposition. Continuous C3b deposition, C3a-, C5a release, and TCC deposition ultimately results in glomerular cell proliferation and thickening of the glomerular basement membrane. In a single case of DDD the lectin pathway was associated and C4 activation and complement products were massively found in the kidney (10).

Autoimmune C3G forms with C3 Nephritic factor (C3Nef) were identified in 1969. C3Nef represent serum autoantibodies that bind to neoepitopes of the assembled alternative pathway C3-convertase, C3bBb (25–28). C3Nef does not bind to the individual components of the C3-convertase, but stabilizes the enzymatic C3-convertase (C3bBb) and extends the half-life of this central complement enzyme from a few seconds to minutes or even hours (26, 29–31). C3Nef causes continuous alternative pathway activation in plasma. In addition, to such stabilizing effects, C3Nef bound to the convertase inhibits not only the access of the inhibitor Factor H, but also of CR1 and DAF and thereby blocks the dissociation of the convertase (32, 33). As a consequence, a C3Nef-stabilized C3-convertase is continuously active in fluid phase and/or on surfaces, cleaves plasma C3 continuously, subsequenty driving complement activation. This continuous action often but not always results in C3 consumption and low C3 plasma levels, in inflammation and proliferation.

The frequency of C3Nef in C3G varies between 50 and 80%, depending on the study cohort. Variations are also influenced by age and differ between juvenile and adult patients and by the methodology used for measurement (15, 25, 34). C3Nef is also identified in patients with antiphospholipid syndrome and even in healthy individuals (35–38). In addition to C3Nef, also C4Nef and C5Nef were reported in the literature (36, 39–42). However, C3Nef assays are not standardized and the relative small number of specialized laboratories around the world use different tests.

Apparently C3Nef and properdin have related C3-convertase binding activities, and properdin binds to the assembled convertase and prolongs the half-life of the surface bound enzyme (33, 43–45). However, in contrast to C3Nef the properdin stabilized C3-convertase remains accessible for regulators and can still be dissociated by Factor H and CR1.

Recently additional autoimmune forms have been described in C3G, with autoantibodies to Factor B and C3 and for another patient with autoantibodies to Factor H. C3-convertase antibodies have been described in patients with C3G or C3G with DDD pattern (46). Importantly, the patients with these autoantibodies did not score positive in standard, functional C3Nef assays.

As autoimmune antibodies, in addition to and independent of C3Nef were reported in several C3G patients we aimed to identify and characterize these additional autoimmune forms and components in C3G and to study the effect of these autoantibodies in C3- and C5 convertase regulation. To this end, we screened the Jena C3G-registry for autoimmune C3G autoantibodies. In addition we analyzed autoantibody positive serum samples and purified IgG preparations on C3-convertase formation, stabilization and protection from the inhibitor Factor H. This approach identified 33 patients with autoantibodies, revealed differences in C3 and C5-convertase binding and action. Ca 50% of the autoantibody positive sera scored positive in standard C3Nef assays, indicating that the identification of autoimmune forms in C3G is underrepresented.

## MATERIALS AND METHODS

### Patient's Samples

Sera from 33 patients (30y ±13; 12 female; 13 male) (**Table 1**) presenting with histological and/or clinical evidence of C3G were collected during the years 2009–2013 from clinics in Germany and Italy. The study was approved by the ethical board of the Medical Faculty of the Friedrich Schiller University, Jena Germany. In addition, sera from 7 healthy individuals were collected, pooled and used as controls or in case of the antibody screening 44 blood samples from healthy individuals were used to determine the background fluorescence in the assay. For purification of IgGs, serum was applied to a Protein G column (GE Healthcare) which was equilibrated with sodium phosphate

**TABLE 1 |** Patients ($n = 33$) with autoimmune C3G.

| Patient | Patient code | Diagnose | Gender | Age at onset | FHRs MLPA | C3NeF |
|---|---|---|---|---|---|---|
| **GROUP 1** | | | | | | |
| A | #748 | MPGN; I; II | f | 22 | CFHR1/3 het | Positive |
| B | #893 | MPGN II | f | 27 | – | Positive |
| C | #1149 | C3G | nk | nk | nd | Positive |
| D | #1725 | C3G | nk | nk | – | Positive |
| E | #1742 | C3G | nk | nk | | Positive |
| F | #607 | MPGN II | f | 18 | CFHR1/3 het | Negative |
| G | #1392 | MPGN II | f | 16 | – | Negative |
| H | #2123 | MPGN II | m | 22 | – | Negative |
| I | #2169 | MPGN II | f | 16 | – | Negative |
| J | #2266 | MPGN II | f | 6 | – | Negative |
| K | #2317 | MPGN II | m | 11 | – | Negative |
| L | #2488 | MPGN | m | 15 | CFHR1/3 het | Negative |
| **GROUP 2** | | | | | | |
| a | #328 | MPGN | f | 17 | nd | Positive |
| b | #329 | MPGN | f | 22 | nd | Positive |
| c | #633 | C3G | f | 25 | – | Positive |
| d | #722 | C3G | m | 24 | 3 × CFHR1/3 | Positive |
| e | #F864 | MPGN | nk | nk | nd | Positive |
| f | #F951 | MPGN | nk | nk | – | Positive |
| g | #1304 | IC-MPGN | nk | nk | – | Positive |
| h | #1549 | C3G | f | 13 | – | Positive |
| i | #1710 | IC-MPGN | nk | nk | – | Positive |
| j | #1741 | C3G | nk | nk | – | Positive |
| k | #750 | MPGN II | m | 28 | – | Negative |
| l | #971 | MPGN | f | 27 | CFHR1/3 het | Negative |
| m | #2048 | MPGN I | m | 24 | – | Negative |
| n | #2144 | MPGN I | f | 15 | CFHR1/3 het | Negative |
| o | #2146 | MPGN II | m | 54 | – | Negative |
| p | #2224 | MPGNII; | f | 81 | – | Negative |
| q | #2315 | MPGN | m | 50 | – | Negative |
| r | #2367 | TTP; MPGN | f | 15 | – | Negative |
| s | #2390 | MPGN I | | 40 | – | Negative |
| t | #2461 | MPGN II | m | 24 | CFHR1/3 het | Negative |
| u | #2540 | C3GN | m | 21 | CFHR1/3 het | Negative |

*Integration of patients in group1 or group 2 is based on the binding of the autoantibodies to the C3-convertase. Initial diagnosis is shown in column 3 and diseases onset, status of FHR1 and FHR3 genomic situation and scoring in the standard C3Nef assay. CFHR1/CFHR3 het, heterozygous deficiency of CFHR1 and CFHR3; –, no CFHR1/CFHR3 deficiency or multipiclation; Nd, not determine; nk, not known; MPGN, membranoprolefirative gloemerulonehpritis; C3G, C3-glomerulopathy; IC-MPGN, immune complex MPGN.*

buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.0). Bound IgGs were eluted with elution buffer (100 mM glycine-HCl, pH 2.7) and dialyzed to PBS. Control IgGs were purified from combined control sera (NHS pool). C3Nef was determined as previously described (29).

## Autoantibody Binding to the C3 Convertase and C5 Convertase

For binding of either serum IgGs or purified patient IgGs to an *in vitro* assembled C3-convertase, C3b (5 ug/ml, CompTech) was coated onto an ELISA plate and convertases were formed by addition of Factor B (1 ug/ml, CompTech), Factor D (0.5 ug/ml, CompTech) and properdin (1 ug/ml, CompTech) in binding buffer (PBS/2% bovine serum albumin, 2 mM $MgCl_2$, 2 mM $NiCl_2$, 0.05% Tween 20) for 1 h at 37°C. In addition, control wells were treated with PBS. The wells were blocked and following washing patient sera (1:200 diluted in binding buffer) or purified IgGs (20 µg/ml in binding buffer) were added and the mixture was incubated at room temperature for 2 h. Upon washing bound IgGs were detected with a peroxidase-labeled anti-human IgG (1:5,500, Sigma Aldrich) and the absorbance was measured at 450 nm. Serum derived from patient # F was used as autoantibody positive sample for calculations. In order to allow comparison between the various assays, the absorbance was measured at 450 nm, and each fraction of bound IgGs was given as calculated arbitrary units ($A_{450}$ test sample/$A_{450}$ patient

# F x 100, i.e., % binding as compared to control serum/IgGs # F. To test binding of patient IgGs to a preformed C5-convertase, ELISA plates were coated with C3b (5 ug/ml) and C5-convertases were formed by addition of Factor B (1 ug/ml), Factor D (0.5 ug/ml), properdin (1 ug/ml), and C3 (10 ug/ml, all CompTech) for 30 min at 37°C. Control wells were treated with PBS. Plates were blocked and purified patient IgGs (20 μg/ml in binding buffer see above) or patient sera (1:200 diluted in binding buffer) were added. Following incubation for 1 h at room temperature bound IgGs were detected with a peroxidase-labeled anti-human IgG (1:5,500, Sigma Aldrich). Absorbance was measured at 450 nm. C3Nef activity was measured by hemolytic assay as described (29).

## Plasma C3 and sC5b-9 Levels

C3 and sC5b9 concentrations were determined by ELISA as previously described (20).

## Alternative Pathway C3 Convertase Activity

The influence of patient IgGs on C3 convertase assembly or stability was investigated using a solid-phase C3-convertase assay. The C3-convertase, C3bBb, was assembled by coating C3b (5 μg/ml, CompTech) onto microtiter plates. Then, Factor B (1 μg/ml), Factor D (0.5 μg/ml, both CompTech), together with purified patient IgG (20 ug/ml) were added and the mixture was incubated at 37°C for 15, 30 or 45 min. Properdin (1 μg/ml, CompTech) was used as positive control. Following incubation and washing, the intensity of the formed C3 convertase was evaluated by quantitating Bb attached to the convertase with a Factor B reacting polyclonal antiserum (1:4,000, CompTech).

In addition, the stabilizing effect of the autoimmune IgGs was tested. C3-convertase was assembled in the presence of patients IgGs for 45 min. Then Factor H (20 ug/ml, CompTech) was added and the reaction was incubated at 37°C. At the indicated time points, 15, 30 or 45 min, convertase-bound Bb was detected by Factor B polyclonal antiserum (1:4,000, CompTech). The OD values were recorded at 450 nm.

## Biolayer Interferometry

The interaction of purified IgGs to the surface assembled C3-convertase was evaluated by bio-layer interferometry using a single channel BLITZ system (Forte Bio, Menlo Park, CA) and the binding affinity was determined. Ni(II)-NTA biosensors were hydrated for at least 10 min in DPBS with gelatin (0.01%) and loaded with biotinylated C3b. Then Factor B, Factor D and Properdin were added similar as described in the ELISA to assemble the C3-convertase. After washing the tip briefly (30 s) to remove unbound proteins the various IgGs were added and analyte binding was followed for 280 s. For determining the apparent kD, serial dilutions of IgGs at 2,500, 1,250, 650, 312, and 150 nM were analyzed. For each concentration, complex formation and dissociation was followed over 280 s. In addition,

**TABLE 2 |** Binding of selected purified antibodies to the AP C3-convertase.

| | C3NeF | Patient | $K_D$ (nM) | $k_a$ (nM) | $k_d$ (1/s) |
|---|---|---|---|---|---|
| Group 1 | pos | #A 748 | 416 | 4.8 | $2.0 \times 10^{-3}$ |
| | neg | #J 2266 | 911 | 2.3 | $2.1 \times 10^{-3}$ |
| Group 2 | pos | #a 328 | 559 | 4.5 | $2.5 \times 10^{-3}$ |
| | neg | #r 2315 | 854 | 4.5 | $3.8 \times 10^{-3}$ |
| NHS | neg | | 2,513 | 1.9 | $5.0 \times 10^{-3}$ |
| DEAP-HUS | neg | #1552 | 3,799 | 1.4 | $5.4 \times 10^{-3}$ |

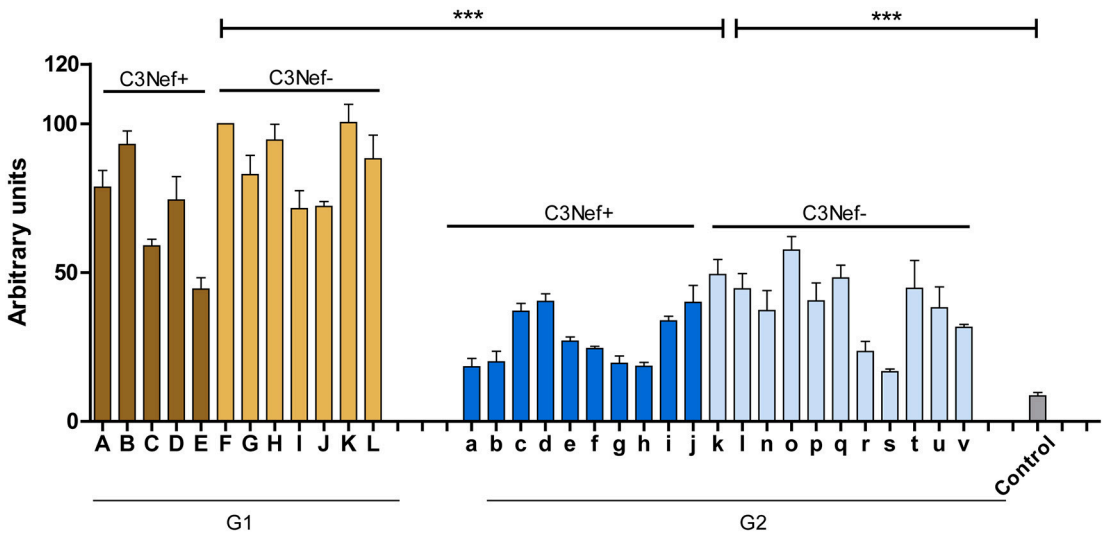


**FIGURE 1 |** C3G patients presenting with autoantibodies to the C3-convertase. Thirty-three autoimmune patients with C3G with autoantibodies that bind to the *in vitro* assembled C3 convertase were identified in the Jena C3G cohort. Based on the binding intensity of autoantibodies to the *in vitro* assembled C3-convertase patients with high and low binding intensities are identified. Separated in group 1 with high/more C3-convertase binding antibodies and group 2 with weak/less binding antibodies. Each group includes patients who score positive in a C3Nef assay as well as C3Nef negative patients. Cut off was set at arbitrary units of 8.5 (average background binding level of 44 control sera). G1 to G2 and G2 to controls (***$p < 0.001$).

IgGs purified from the NHS pool and from DEAP-HUS patient #1552 who has a high titer of Factor H binding antibodies and who lacks C3-convertase reacting antibodies were used as controls. For data evaluation, values obtained from buffer controls were subtracted and the interaction affinity $K_D$s were determined by fitting the data to a 1:1 model algorithm using BLITZ software. The non-linear association and dissociation curves were plotted using Graphad5 software.

## Activity of the Alternative Pathway C3-Convertase

The influence of patient IgG on C3-convertase activity was determined by measuring C3a generation. C3-convertase were assembled in the fluid phase by mixing C3b (5 ug/ml), Factor B (5 ug/ml), Factor D (1 ug/ml, all CompTech) together with purified IgGs of patients (20, 60, or 100 ug/ml) and subsequent incubation at 37°C for 8 min in MgEGTA buffer (20 mM HEPES, 144 mM NaCl, 10 mM EGTA, 7 mM $MgCl_2$, pH 7.4). Then native C3 (50 ug/ml, CompTech) was added. In inhibition experiments Factor H (20 ug/ml, Comptech) was added to the mixture. The samples were incubated at 37°C, and after 40 min C3a generated in the supernatant was detected using a C3a immunoassay (QUIDEL).

## Activity of the Alternative Pathway C5-Convertase

The role of patient derived IgGs on the alternative pathway C5-convertase was monitored by following C5a generation. Purified patient derived IgGs (7.5 µg) alone or together with Eculizumab (50 nM) were added to NHS (25 µl, 10% diluted in Mg-EGTA buffer) and the mixture was incubated at 37°C. After 45 min generated C5a was detected using the C5a Plus Enzyme immunoassay (QUIDEL).

## C3b, C5b-9 Surface Deposition

Microtiter plates were coated with LPS (10 µg/ml, Sigma-Aldrich) overnight at 4°C and blocked for 1 h with BSA (2%, AppliChem Panreac). Purified patient's IgGs (10, 20, 30, or 40 µg) were added to NHS (25 µl; 20% in MgEGTA buffer) and the mixture was incubated at 37°C for 15 min. Thereafter the mixture

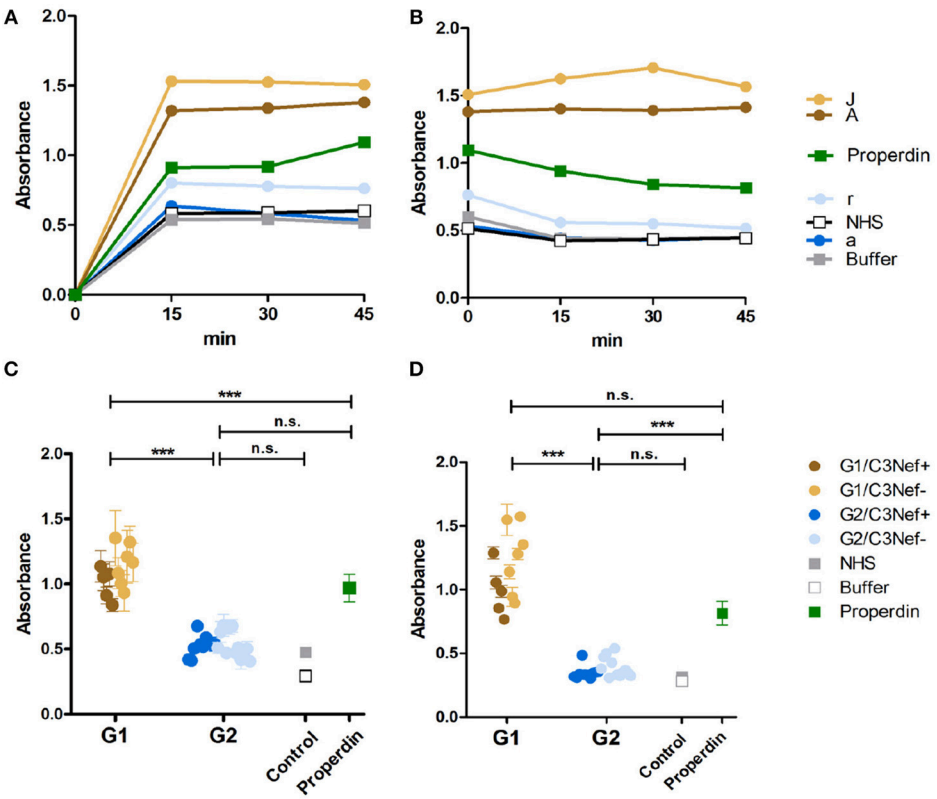


**FIGURE 2 |** C3G autoantibodies of group 1 and group 2 influence C3 convertase assembly and dissociation by Factor H. **(A)** Two group 1 autoantibodies from patients #A and #J with high C3 convertase binding IgGs enhance C3-convertase formation. This enhancing effect is even stronger than the C3 convertase stabilizer and complement activator properdin. In contrast, group 2 autoantibodies from patients #a and# r do not enhance C3 convertase formation. Their effect is comparable to IgGs derived from NHS or from a DEAP-HUS patient with autoantibodies to Factor H or to buffer. **(B)** The same group1 antibodies when bound to the 3 convertase stabilize the enzyme and enhances resistance to factor H mediated dissociation. C3-convertase assembly *in vitro* and stabilization by autoantibodies are measured over 45 min by detection of convertase Bb using ELISA. **(C)** The same experiments as outlined in panel **(A)** are performed with all 33 patients derived autoantibody fractions and bound IgGs are identified after 45 min. All 12 group 1 antibodies enhance C3-convertase formation and again all group 2 antibodies have no or rather low effects (***$p < 0.001$). **(D)** All group 1 autoantibodies stabilize the C3 convertase from dissociation by factor H and all group 2 antibodies lack this activity (***$p < 0.001$).

was added to the LPS coated wells and the reaction was incubated further at 37°C for 1 h. Following washing surface deposited C3b or C5b-9 were quantified by ELISA at 450 nm using C3b mAb (1:1,000, Fitzgerald) and anti C5b-9neo (1:1,000, Comptech) and secondary anti-mouse (1:1,000, Dako) or anti-goat antiserum (1:1,000, Dako).

## Hemolytic Assay

Purified patient derived IgGs (10, 30, or 40 ug) were added to NHS (10%) and the mixtures incubated for 20 min at 37°C with agitation (550 rpm). Rabbit erythrocytes ($2*10^8$/ml) were washed, suspended in GVB** buffer (Comptech), added to IgGs-NHS and the mixture was then incubated for additional 30 min at 37°C with agitation (450 rpm). Cells were centrifuged and erythrocyte lysis was evaluated by measuring the absorbance in supernatant at 414 nm.

## Statistical Analysis

Significant differences between two groups were analyzed using the unpaired two tailed Student's *t*-test of GraphPad Prism 5 for Windows. Values of $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ were considered as statistically significant.

# RESULTS

## Identification of C3G Patients With Autoantibodies

To identify and characterize autoimmune C3G patients in the European/Jena cohort in more detail, serum samples of 250 nephritis patients were analyzed for autoantibodies which bind to the *in vitro* assembled alternative pathway C3-convertase. Thirty-three C3G patients were identified having C3bBb binding antibodies. Twelve sera showed strong binding (arbitrary units: $80 \pm 4.8$) and 21 patients showed weak binding (arbitrary units: $34 \pm 2.6$) as compared to IgGs from 44 NHS control samples (arbitrary units: $8.5 \pm 1.2$, $p < 0.001$) (**Figure 1**). Based on the different binding intensities two groups were separated, patient with high binding-, termed group 1 and patients with low binding antibodies, as group 2. The demographic data of the patients in these subgroups were rather homogenous for age and gender (**Table 1**).

All 33 sera were further analyzed using a standard assay for C3 Nephritic factor. Fifteen sera (42%) were positive for C3Nef which were identified in both group 1 and group 2 patients. Thus, in group 1 sera from seven individuals were negative and five sera were positive for C3Nef. Among group 2, 11 sera were negative and 10 were positive for C3Nef (**Figure 1** and **Table 1**).

In DEAP-HUS the presence of Factor H antibodies is associated with homozygous *CFHR1-CFHR3* deficiency. Copy number variations in the *CFHR* gene cluster were determined for patients with C3-convertase antibodies. In particular no homozygous *CFHR3/CFHR1* deficiency was detected; seven patients showed heterozygous *CFHR3-CFHR1* deficiency (**Table 1**). Thus, autoimmune antibodies in C3G patients develop independently of *CFHR3/CFHR1* homozygous deficiency.

## Estimating Binding Affinity of Autoantibodies

All here tested autoantibodies bound to the assembled C3-convertase, but with different intensity. These differences can be explained either by higher autoantibody titers in group 1 sera, or by a higher binding affinity to the C3-convertase. To find out if the autoantibodies bind stronger or weaker, binding of representative IgG preparations of group 1 or group 2 with a C3Nef negative and C3Nef positive variant of the C3-convertase was followed in real time and the apparent affinity was evaluated. The C3-convertase was assembled on the surface of a Ni(II)-NTA coated biosensor and purified IgGs from group 1 patients (#A C3Nef pos and #J C3Nef neg) of group 2 patients (#a C3Nef pos and #r C3Nef neg) were added as analyte. Association and dissociation was followed for 320 s. IgG fractions isolated from the patients harbored autoantibodies that bound to the assembled convertase. They showed a strong association and a slow dissociation upon removal of the analyte. In contrast, IgGs isolated from the NHS pool, from DEAP-HUS patient #1552, and also BSA did not bind to the C3 convertase. To compare the binding strength and to define an apparent $K_D$, serial dilutions ranging from 25 to 75 nM were tested. Assuming that 10% of total IgG isolation represent C3-convertase binding autoantibodies the apparent $K_D$ values were calculated and identified for group 1 as 911 and 853 nM and for group 2 as 416 and 559 nM (**Table 2**). Control IgGs prepared from the NHS pool or from DEAP-HUS patient #1552 showed no, or background binding ($K_D > 2.5\,\mu M$). The results demonstrate that autoantibodies from representative patients from both groups bind with rather similar affinity and binding under the assumption of 10% C3 convertase antibodies is in the nanomolar range. The differences in binding could be explained by the recognition of different epitopes.

## Group 1 but Not Group 2 Autoantibodies Enhance C3-Convertase Formation and Block Dissociation by Factor H

To analyze whether all C3-convertase binding autoantibodies have a stabilizing and activating effect we analyzed if the autoantibodies influence C3-convertase formation. First, purified IgGs from representative sera of group 1 (#A and #J) and group 2 (#a and #r) patients were tested in a functional kinetic assay. Again, the C3-convertases were assembled in presence of purified IgGs and convertase formation was followed by measuring attached Bb. IgGs from the two group 1 patients (#A and #J) enhanced C3-convertase assembly within the first 15 min and over time more C3-convertases were formed. The effect was more pronounced as that of properdin, the known C3-convertase stabilizer. In contrast, IgGs from the two group 2 patients (#a and #r) did not affect convertase formation in a significant manner. Their effect was lower than that of properdin and was almost comparable to NHS or buffer control (**Figure 2A**). Thus, group 1 IgGs allow faster C3-convertase formation and the formation of more enzyme (**Figure 2A**).

Next we asked if the IgG autoantibodies influence convertase dissociation by the inhibitor Factor H. To this

end, C3-convertases were assembled in presence of the autoantibodies and Factor H. In this setting both group 1 autoantibodies (#A and #J) inhibited convertase dissociation by Factor H and the stabilizing effect on the assembled C3-convertase was even stronger than that of properdin (**Figure 2B**). In contrast, both group 2 antibodies (#a and #r) did not or weakly block C3-convertase dissociation by factor H (**Figure 2B**).

To evaluate if these differences are characteristic for the two groups, all IgGs were evaluated. All twelve group 1 autoantibodies efficiently stabilized the C3-convertase as shown by Bb binding (mean absorbance group 1: 1.1, $p < 0.001$, compared to NHS pool) (**Figure 2C**) even in the presence of Factor H (**Figure 2D**). All group 2 autoantibodies, had a rather low or no stabilizing effect on the C3-convertase (mean absorbance group 2: 0.5, n.s. difference to NHS pool) (**Figure 2C**) and no major influence on Factor H stabilization (**Figure 2D**). The effect by group 2 autoantibodies was comparable to IgGs derived from the control NHS pool or buffer. In summary, group 1 but not group 2 autoantibodies increased C3-convertase formation and stabilized the enzyme from decay by Factor H. A significant difference exists between group 1 and group 2 antibodies. IgGs from group 1, but not the group 2 group stabilized the C3-convertase. In each group, no significant difference was detectable between the C3Nef positive or the C3Nef negative samples. The effect of all group 1 autoantibodies was stronger than that of properdin, the known C3-convertase stabilizing protein and complement enhancer. This may explain the pathologic role of the antibodies.

## Effect of Autoantibodies on C3-Convertase Activity

As group 1 autoantibodies enhanced C3-convertase formation, stabilized the enzyme and blocked the dissociation, we asked

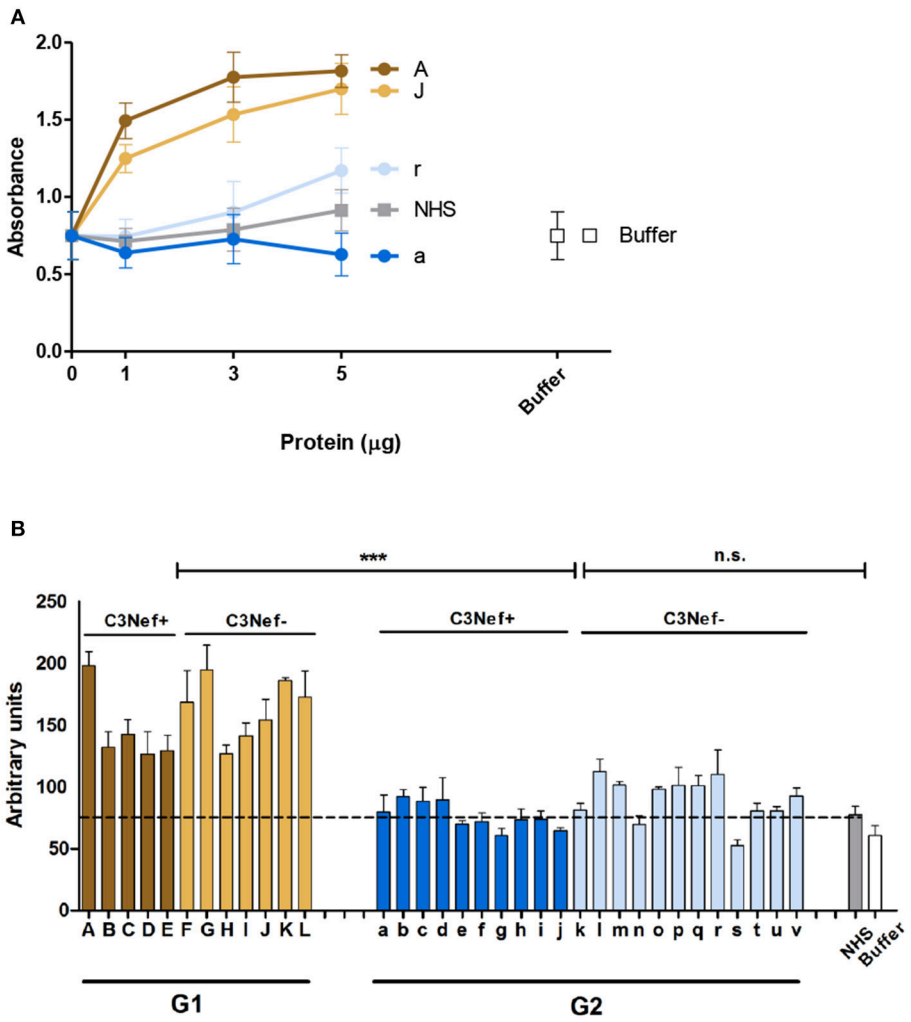


**FIGURE 3 |** Group 1 but not group 2 antibodies enhance C3a generation. Fluid phase C3-convertase is assembled in presence of autoantibodies followed by addition of substrate C3. Following incubation for 45 min, C3a generation is monitored by ELISA. **(A)** Group 1 autoantibodies increase C3a generation and the effect is dose dependent. In contrast, group 2 antibodies do not enhance C3a formation. **(B)** When all autoantibodies were evaluated group 1 but not group 2 autoantibodies enhance C3a generation (***$p < 0.001$). C3a generation by NHS is shown as a stippled line.

if the IgG autoantibodies influence C3-convertase activity. Therefore, C3-convertases were assembled in fluid phase in presence of the autoimmune IgGs, and after 30 min incubation generated C3a was quantitated by ELISA. The selected group 1 antibodies (i.e., #A and #J), but not the two group 2 antibodies (#a and #r) enhanced C3a generation, and the effect was dose dependent (**Figure 3A**). IgGs from C3Nef negative patient #r, had some activating activity at higher concentrations, but IgGs prepared from the C3Nef pos patient #a lacked any activity (**Figure 3A**).

Similar results were obtained when IgGs from all patients were analyzed. All high binding C3-convertase group 1-IgGs enhanced C3a formation (mean absorbance G1: 156, mean absorbance G2: 84; $p < 0.0001$ Group 1 compared to group 2). The group 2 IgGs had minor or no effects on C3a release ($p =$ n.s. compared to NHS pool) (**Figure 3B**). Again, no difference between C3Nef positive and C3Nef negative samples was detectable. Thus, the high binding group 1 antibodies enhanced C3-convertase action also in the fluid phase which supports their role in pathology even further. Identified C3Nef in group 1 but not C3Nef in group 2 did activate the C3-convertase. These results are in agreement with C3 concentrations in patients' samples. G1 samples showed very low C3 amounts (103 ± 17 μg/ml) compared to G2 samples (331 ± 80 μg/ml; $p < 0.0001$).

## Both Group 1 and Group 2 Autoantibodies Bind to the C5-Convertase

Group 1 and group 2 antibodies both bind to neoepitopes of the C3-convertase. As the AP C5-convertase, C3bBbC3b, with one additional C3b attached has a related structure or composition, we asked if the autoantibodies also bind to this enzyme. Therefore, autoantibody binding to AP C5-convertases assembled on the surface of a microtiter plate was evaluated by ELISA. Autoantibodies derived from all patients of the cohort bound to the assembled C5-convertase. Also for C5-convertase interaction, the high C3-convertase binding group 1-autoantibodies bound also more to the alternative pathway C5-convertase than group 2 antibodies (mean absorbance group 1: 1.2; mean absorbance group 2: 0.8; $p < 0.01$ group 1 compared to group 2) (**Figure 4**). In this set up both group 1 and group 2 antibodies bound with significantly higher intensity to the C5-convertase as NHS derived IgGs ($p < 0.01$).

## Most Autoantibodies Activate the C5-Convertase

Based on binding to the C5-convertase we asked if the autoantibodies affect C5-convertase activity. First, IgGs from the four selected patients were added to NHS and after 45 min incubation newly generated C5a was determined in the supernatant by ELISA. All four antibody IgG preparations (#A, #J, #a, and #r) increased C5a generation ($p < 0.05$ to 0.001 as compared to NHS-IgGs) (**Figure 5A**). When IgGs from all patients were tested all, except one sample (# F) from group 1 and two samples from group 2 (#b and #s) increased C5a generation. The IgGs from both groups generated rather similar C5a levels, however, the range was higher for group 1 autoantibodies (mean absorbance group 1: 58, mean absorbance group 2: 48, G1 to G2 $p =$ n.s.; G1 to buffer $p < 0.05$; G2 to buffer $p < 0.005$) (**Figure 5B**). Again, this effect was independent and did not correlate with the presence or absence of C3Nef.

## IgG Antibodies Induce C3b Deposition

Next the effect of the autoantibodies on fluid phase C3-convertase mediated C3b opsonization was evaluated. Purified patients IgGs were added to NHS and after 30 min surface deposited C3b was quantitated by ELISA. Both group 1 antibodies (#A and #J) reduced C3b surface deposition and this inhibitory effect was already detected with 10 μg/ml IgG (**Figure 6A**). In contrast,

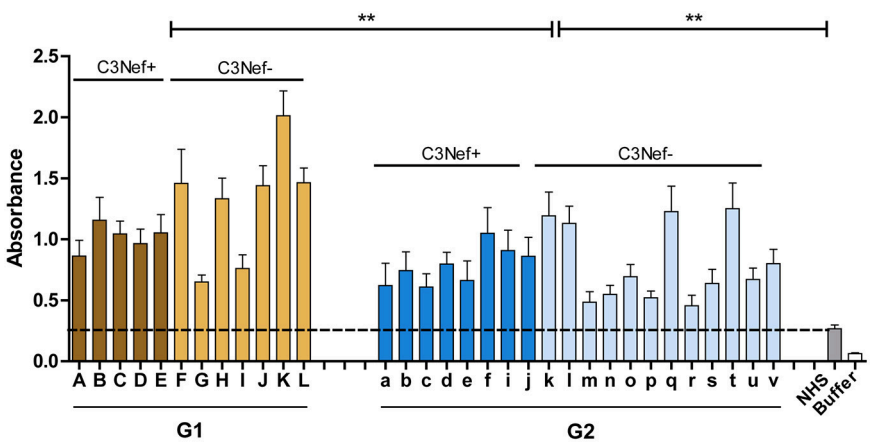


**FIGURE 4 |** C5 Convertase binding. Autoantibody binding to the *in vitro* assembled C3 convertase was evaluated. In this case, group 1 and also group 2 antibodies bind to the assembled C5-convertase (**$p < 0.01$). IgGs prepared from NHS show background binding (stippled line). In addition, more group 1 antibodies bind to the C5-convertase as compared to group 2 antibodies (**$p < 0.01$). The C5-convertase was assembled on an ELISA plate by immobilizing C3b (10 μg/ml) and adding Factor B (1 μg/ml), Factor D (0.5 μg/ml), properdin (1 μg/ml), and C3 (10 μg/ml).

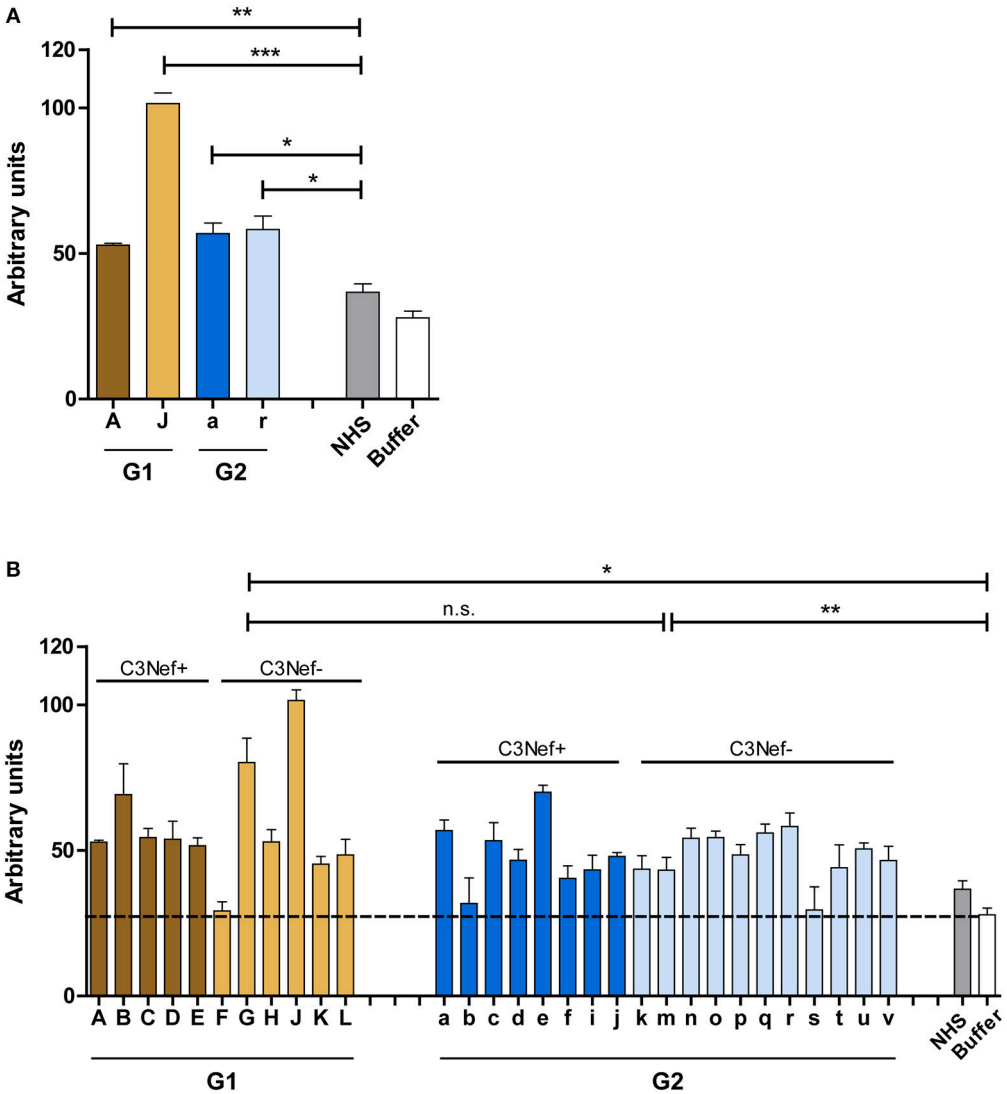


**FIGURE 5 |** Autoantibodies modulate C5a generation. **(A)** Representative patient's autoantibodies from group 1 (#A and #J) and group 2 (#a and #r) increase C5a generation significantly when compared to IgGs prepared from NHS compared to control (**$p < 0.01$, ***$p < 0.001$). **(B)** IgGs from all patients except # 607 (group 1), # 328, #2367 (both group 2), enhance C5a generation in a significant manner (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Purified IgGs from each patient was added to complement active NHS at 37°C and the mixture was incubated for 45 min. C5a generation was monitored by ELISA and compared to C5a generation in NHS (stippled line).

both group 2 antibodies (#*a* and #r), as well as NHS-derived IgGs did not affect C3b deposition (**Figure 6A**).

The inhibitory effects were specific for group 1. All group 1 antibodies, but no group 2 antibody blocked C3b deposition (mean absorbance G1: 0.2, mean absorbance G2: 2.2; $p < 0.001$ group 1 vs. group 2) (**Figure 6B**). These results reveal a functional difference between group 1- and group 2 autoantibodies in C3b deposition.

## Autoantibodies Influence TCC Deposition

The effect of the autoantibodies on TCC formation was evaluated by following C5b-9 deposition and using a hemolytic assay. First, the representative antibodies from group 1 (#A and #J) and group 2 (#a and #r) were evaluated. The IgGs were added to NHS and the deposition of C5b-9 was measured by ELISA. IgGs derived from the group patients (#A and #J) decreased C5b-9 deposition in a dose dependent manner, whereas group 2 autoantibodies (#a and #r) enhanced C5b-9 deposition when compared to NHS derived IgGs (**Figure 7A**). sC5b-9 concentration in the samples ranged from 3.4 ±1.3 μg/ml in group 1 to 7.4 ± 2.3 μg/ml in group 2.

The differences between group 1 and group 2 antibodies was confirmed for all autoantibodies using a hemolysis assay. The IgGs derived from the patients were added to NHS and then rabbit erythrocytes which represent activators of human complement were challenged with the supplemented serum and after 20 min incubation lysis of erythrocytes was followed. All group 1, but no group 2 antibody blocked hemolysis (mean

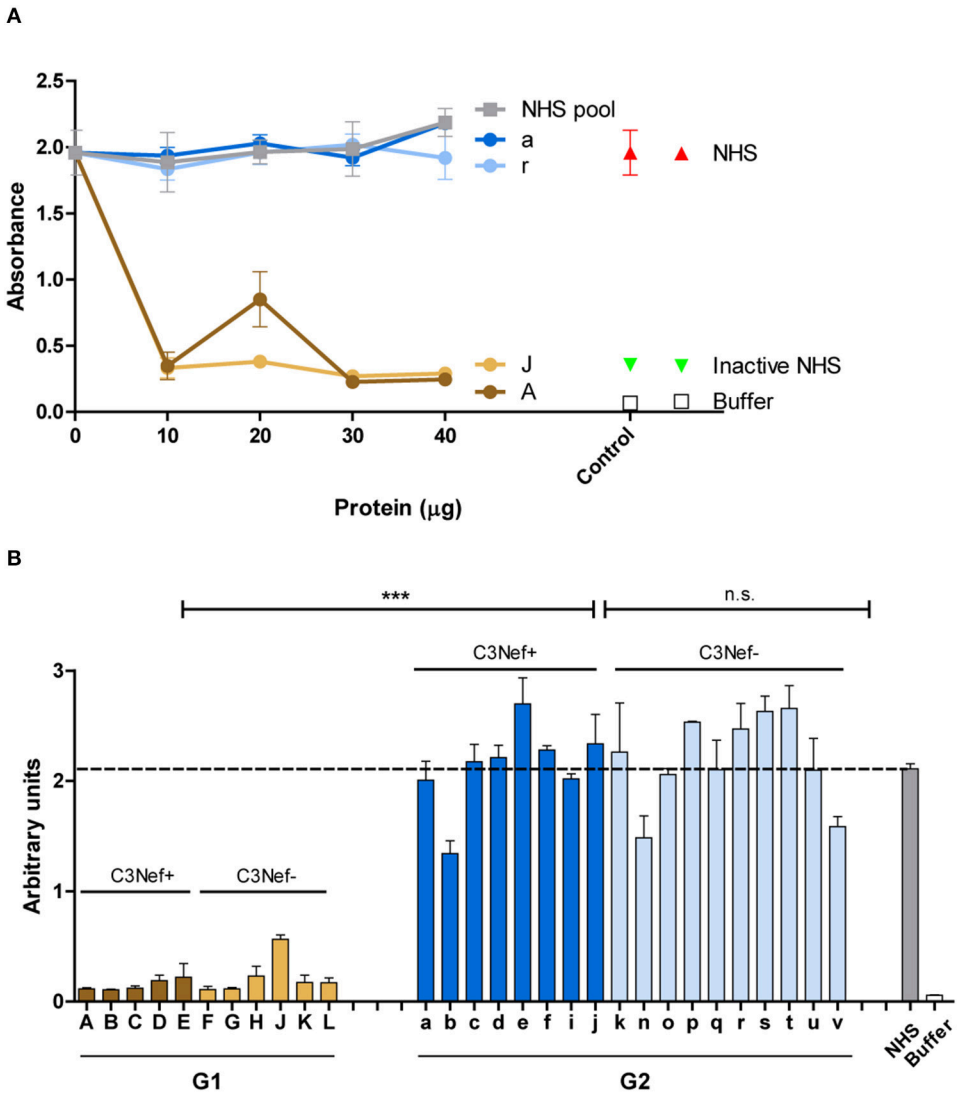


**FIGURE 6 |** Group 1 but not group 2 antibodies affect surface C3b deposition. **(A)** Two representative group 1 autoantibodies (#J and #A) inhibit C3b deposition and the effect is dose dependent. In contrast, both group 2 antibodies (#a and #r) fail to reduce C3b deposition. C3b deposition was followed with adding increasing amounts of IgGs (10–40 μg) to complement active NHS and after surface deposited C3b was quantitated by ELISA **(B)** Autoantibodies (each 30 μg) from all patients were tested in the same set up. Again, all group 1, but not group 2 antibodies block C3b surface deposition (***$p < 0.001$). Purified IgG in **(A,B)** were added to NHS, and the supplemented serum was added to a LPS-coated microtiter plate. After 1 h C3b deposition was assayed by ELISA. Hemolysis by NHS is marked by a stippled line.

absorbance group 1: 0.3, mean absorbance group 2: 1.0, $p < 0.001$ group 1 compared to group 2; $p =$ not significant group 2 to NHS pool) (**Figure 7B**). This clear difference between group I and group II antibodies was again independent of the presence or absence of C3Nef. Thus, a clear functional difference between group 1 and group 2 antibodies in autoimmune C3G affects complement mediated TCC deposition and hemolysis.

## Eculizumab Inhibits C5a Generation in All Probes

Given that all C3G autoantibodies activate the C5-convertase we asked whether the C3G autoantibodies influence the action of Eculizumab, the therapeutic C5 binding complement inhibitor. Fluid phase C3-convertases were assembled in presence of autoantibodies or in presence of autoantibodies and Eculizumab. After addition of C3 to the mixture and 45 min incubation, C3a as well as C5a, generated in fluid phase, was quantitated by ELISA. As expected, Eculizumab did not influence C3a release (**Figure 8A**). However, Eculizumab blocked C5a release in presence of both group 1 as well as group 2 autoantibodies (**Figure 8B**). Thus, C5 binding Eculizumab blocks the action of a C5-convertase which has the C3G autoantibody attached. Eculizumab and autoantibodies bind to different proteins. Eculizumab binds to the substrate

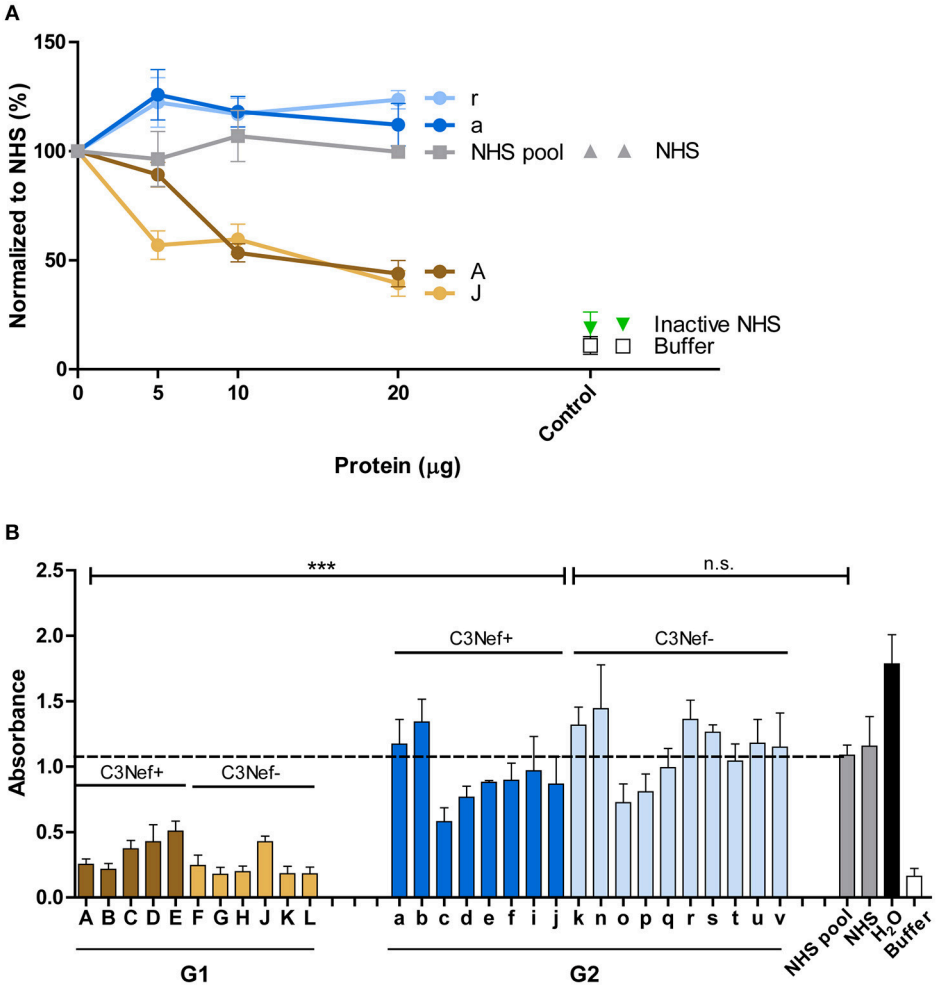


**FIGURE 7 |** Group 1 but not group 2-antibodies reduce C5b-9 surface deposition and prevent hemolysis. **(A)** Group 1 autoantibodies (#J and #A) inhibit C5b-9 deposition by about 50% and the effect is dose dependent. In contrast, group 2 antibodies (#a and #r) enhance C5b-9 deposition as compared to NHS derived IgGs. C5-9 deposition was followed upon addition of increasing amounts of IgG fractions (5–20 µg) to NHS. **(B)** Autoantibody fractions derived from group 1 patients (each 30 µg) inhibit lysis of rabbit erythrocytes (***$p < 0.001$). Purified IgGs were added to NHS, then this mixture was combined with rabbit erythrocytes. Hemolysis of erythrocytes was assayed by measuring the absorbance. Lysis of erythrocytes in NHS is marked as stipled line.

C5 and the autoantibodies bind to different neoepitopes of the C5-convertase.

## DISCUSSION

Here we identified and characterized autoimmune forms of the kidney disease C3G. A cohort of 33 C3G patients displayed autoantibodies which bound to both the C3- and the C5-convertase of complement. All autoantibody isolates activated the C5-convertase (C5Nef), but only half of them activated in addition the C3-convertase (C3Nef). IgG fractions with C3Nef plus C5Nef (group 1 antibodies) had stronger complement activating functions in *in vitro* assays compared to fractions with C3Nef alone (group 2 antibodies). Group 1 antibodies induced a fast consumption of the complement components C3 and C5 in hemolytic assays. Eculizumab, the therapeutical C5 monoclonal antibody inhibits C5 cleavage and C5a generation triggered by each group 1 or group 2 antibody fraction, but as expected has no effect on enhanced C3 cleavage.

About 45% of all autoantibodies in our cohort were identified as C3Nef using the classical hemolytic assay. This assay identifies autoantibodies that bind to a neoepitope formed in the C3-convertase. However, additional autoimmune antibodies than C3Nef add to C3G, which are not identified by the classical C3Nef assay. These antibodies bind to the assembled C3-convertase or components thereof. In addition, several C3Nef antibodies bound to the C3-convertase but did not lead to the activation of the enzyme *in vitro*. In contrast, initially identified C3Nef antibodies bound/activated the C5-convertase.

Based on the reactivity to the assembled C3-convertase, two groups of autoantibodies were identified. Group 1 and group 2 antibodies, which both had high affinities in binding to the

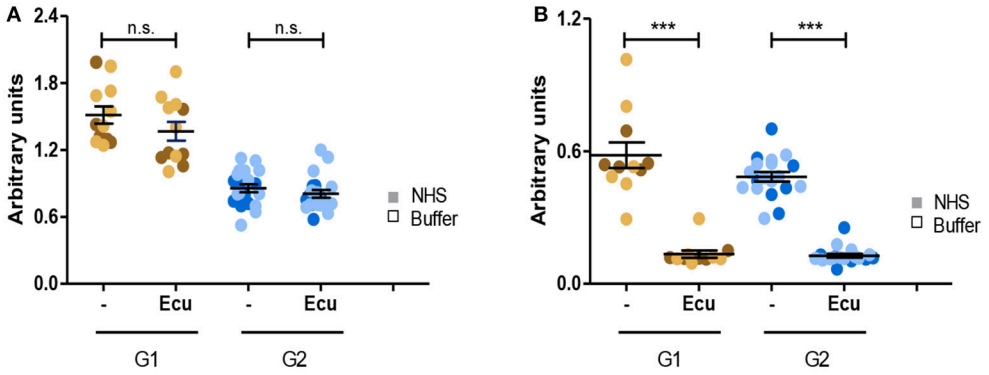


**FIGURE 8 |** C5 inhibitor Eculizumab reduces C5a but not C3a generation of convertases with autoantibodies attached. **(A)** Eculizumab does not affect C3a generation. Fluid phase C3 convertases were assembled in presence of group 1 or group 2 antibodies and C3a release was monitored by ELISA. In the absence of Eculizumab group 1 but not group 2 antibodies induced C3a release (panels 1, 3). C3a generation was not enhanced in group 2 (panels 2, 4). **(B)** Eculizumab blocks C5a generation of C5-convertases with either group 1 or group 2 antibodies attached (***$p < 0.001$). Eculizumab was added to NHS together with group 1 or group 2 antibodies (each 30 μg). Following incubation C5a generation was measured by ELISA.

assembled C3-convertase, but exclusively group 1 antibodies also activated the C3-convertase. As the binding affinities of IgGs to the C3-convertase were very similar in both groups, the different functional effects between the groups are explained by either a higher titer of the autoantibodies in each isolated IgG fraction of group 1 patients or by a higher C3-convertase stabilizing effect by group 1 antibodies compared to group 2. Different antibody titers between the two groups were excluded, as dilutions of group 1 antibody preparations did neither influence nor diminish the stabilizing effect or the resistance to convertase decay by Factor H. Thus, the functional differences between group 1 and group 2 antibodies are likely independent of the autoantibody titers and rely on the recognition of different epitopes which are recognized in the C3- and C5-convertases. All autoantibody fractions from the patients bound to the C5-convertase and as most of them enhanced the activity, they are likely similar to previously defined C5Nef (46). This is surprising as group 1 and group 2 autoantibodies contained C3Nef (according the C3Nef assay) which stabilize the C3-convertase but not the C5-convertase. Therefore, we conclude that two different types of antibody carriers were identified in this cohort of C3G patients: C3Nef+/C5Nef+ (stabilizing the C3-convertase and also the C5-convertase) and C3Nef–/C5Nef+ (stabilizing the C5 convertase) patients. A similar classification was found in a cohort study described by Donadelli et al. (47), who in addition identified patients with exclusively C3Nef antibodies. Thus, C3G patients are heterogeneous and include patients with C3Nef or C5Nef or combinations thereof. This heterogeneity results in presence of nephritic factors with different functional activities and consequences *in vivo* for the patient. In the Jena cohort, nearly all patients were C5Nef positive and show that a large and substantial fraction of autoimmune C3G patients are not identified by only a standard C3Nef assay.

Genetic copy number analyses of the *CFHR* gene cluster revealed for all 33 autoimmune C3G patients no homozygous deletion in the *CFHR gene* cluster. This lack of correlation with CFHR copy number variations is in clear contrast to DEAP-HUS patients, i.e., patients who present with the autoimmune form of hemolytic uremic syndrome. DEAP-HUS patients who developed autoantibodies to Factor H present frequently with a homozygous deletion of the *CFHR1-CFHR3* segment or alternatively but rarely by a compound heterozygous *CFHR1-CFHR3*, together with *CFHR3-CFHR4* deletions (48).

There is no clear therapy regiment for C3G today. As serum derived autoantibodies, as well as purified IgGs in our cohort bound to the *in vitro* assembled C5-convertase and enhanced fluid phase C5a generation, the question arose whether inhibition of C5 by Eculizumab is a therapeutic option. Off-label use of Eculizumab in C3G revealed promising results in some, but not all patients (49–51). *In vitro* assays in our cohort confirmed inhibition of C5 by Eculizumab and reduced hemolysis. However, the inhibitory effect was exclusively present in group 2, with C5Nef only. In group G1 carriers with C3Nef plus C5Nef, complement activation is likely too strong to be restricted by Eculizumab alone. Whether these patients benefit or profit from a stratified immunosuppressive therapy needs to be determined in the future in clinical practice.

## ETHICS STATEMENT

The study was approved by the ethics committee of the University Hospital of Jena, Germany.

## AUTHOR CONTRIBUTIONS

FZ, MK, SA, SL, IL, and AH performed experiments and analyzed the data. BN and KE helped in C3Nef assays. LW, SH, and GS provided patient's samples. All authors read the manuscript and discussed the data. PZ and CS designed the study, analyzed data, and wrote the manuscript.

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# Clinical and Immunological Profile of Anti-factor H Antibody Associated Atypical Hemolytic Uremic Syndrome: A Nationwide Database

*Mamta Puraswani[1†], Priyanka Khandelwal[1†], Himanshi Saini[1], Savita Saini[1], Bahadur Singh Gurjar[2], Aditi Sinha[1], Rajashri Pramod Shende[3], Tushar Kanti Maiti[4], Abhishek Kumar Singh[4], Uma Kanga[5], Uma Ali[6], Indira Agarwal[7], Kanav Anand[8], Narayan Prasad[9], Padmaraj Rajendran[10], Rajiv Sinha[11], Anil Vasudevan[12], Anita Saxena[13], Sanjay Agarwal[14], Pankaj Hari[1], Arvind Sahu[3], Satyajit Rath[3,15] and Arvind Bagga[1*]*

[1] Division of Nephrology, Department of Pediatrics, All India Institute of Medical Sciences, New Delhi, India, [2] Immuno Biology Laboratory II, National Institute of Immunology, New Delhi, India, [3] National Centre for Cell Science, Pune, India, [4] Regional Center for Biotechnology, Faridabad, India, [5] Department of Transplant Immunology and Immunogenetics, All India Institute of Medical Sciences, New Delhi, India, [6] Department of Pediatrics, BJ Wadia Hospital for Children, Mumbai, India, [7] Department of Pediatrics, Christian Medical College, Vellore, India, [8] Division of Pediatric Nephrology, Sir Ganga Ram Hospital, New Delhi, India, [9] Department of Nephrology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India, [10] Department of Pediatric Nephrology, Institute of Child Health and Hospital for Children, Madras Medical College, Chennai, India, [11] Department of Pediatrics, Institute of Child Health, Kolkata, India, [12] Department of Pediatric Nephrology, St. Johns Medical College and Hospital, Bengaluru, India, [13] Department of Cardiology, All India Institute of Medical Sciences, New Delhi, India, [14] Department of Nephrology, All India Institute of Medical Sciences, New Delhi, India, [15] Translational Health Science and Technology Institute, Faridabad, India

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**Edited by:**
Bradley Patton Dixon,
Children's Hospital Colorado,
United States

**Reviewed by:**
Christoph Licht,
Hospital for Sick Children, Canada
Mihály Józsi,
Eötvös Loránd University, Hungary
Pilar Sánchez-Corral,
University Hospital La Paz Research Institute (IdiPAZ), Spain

***Correspondence:**
Arvind Bagga
arvindbagga@hotmail.com

[†]These authors have contributed equally to this work

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**Background:** Atypical hemolytic uremic syndrome (aHUS), an important cause of acute kidney injury (AKI), is characterized by dysregulation of the alternative complement pathway. Autoantibodies to factor H (FH), a chief regulator of this pathway, account for a distinct subgroup. While high anti-FH titers predict relapse, they do not correlate well with disease activity and their functional characterization is required.

**Methods:** Of 781 patients <18-year-old of aHUS in the nationwide database from 2007 to 2018, 436 (55.8%) had anti-FH antibodies. Clinical features and outcome of patients managed in the last 6-year ($n = 317$) were compared to before ($n = 119$). In plasma samples of 44 patients, levels of serial circulating FH immune complexes (CIC), free FH, soluble terminal complement complex (sC5b-9), sheep red blood cell (SRBC) lysis and epitope specificity ($n = 8$) were examined. Functional renal reserve, ambulatory hypertension, left ventricular hypertrophy (LVH), and proteinuria were evaluated in a subset.

**Results:** Patients presented with markedly elevated anti-FH titers (10,633.2 ± 998.5 AU/ml). Management varied by center, comprising plasma exchange (PEX; 77.5%) and immunosuppression (73.9%). Patients managed in the last 6-year showed better renal survival at mean 28.5 ± 27.3 months (log rank $P = 0.022$). Mean anti-FH titers stayed 700–1,164 AU/ml during prolonged follow-up, correlating with CIC. Patients with relapse had lower free-FH during remission [Generalized estimating equations (GEE), $P = 0.001$];

anti-FH levels ≥1,330 AU/ml and free FH ≤440 mg/l predicted relapse (hazards ratio, HR 6.3; $P = 0.018$). Epitope specificity was similar during onset, remission and relapse. Antibody titer ≥8,000 AU/ml (HR 2.23; $P = 0.024$), time to PEX ≥14 days (HR 2.09; $P = 0.071$) and PEX for <14 days (HR 2.60; $P = 0.017$) predicted adverse renal outcomes. Combined PEX and immunosuppression improved long-term outcomes (HR 0.37; $P = 0.026$); maintenance therapy reduced risk of relapses (HR 0.11; $P < 0.001$). At 4.4±2.5 year, median renal reserve was 15.9%; severe ambulatory, masked and pre-hypertension were found in 38, 30, and 18%, respectively. Proteinuria and LVH occurred in 58 and 28% patients, respectively.

**Conclusion:** Prompt recognition and therapy with PEX and immunosuppression, is associated with satisfactory outcomes. Free-FH predicts early relapses in patients with high anti-FH titers. A significant proportion of impaired functional reserve, ambulatory hypertension, proteinuria and LVH highlight the need for vigilant long-term follow-up.

**Keywords: atypical hemolytic uremic syndrome, factor H, plasma exchange, renal reserve, thrombotic microangiopathy**

## INTRODUCTION

Hemolytic uremic syndrome (HUS) is an important cause of acute kidney injury (AKI) in children (1, 2). While the majority of patients follow gastrointestinal infection with Shiga toxin associated organisms, abnormalities in the complement and coagulation pathways are associated with atypical hemolytic uremic syndrome (aHUS) (1, 3). Although 5–25% patients in European cohorts show antibodies to factor H (FH) (4–6), this subset of illness is common in India accounting for ∼50% cases (7). Recent data from the global aHUS registry, from centers in Europe, North America and Australia, confirm the presence of anti-FH antibodies in 24% children and 19% adults (8).

The pathogenesis of anti-FH associated aHUS and reasons for its high frequency in south Asia are unclear. While more than 80% patients show a homozygous deletion in the gene encoding FH related protein 1 (*CFHR1*), the deletion is present in 5–10% healthy people across the world. High levels of antibodies at disease onset or relapse are believed to induce functional deficiency of FH; their decline in response to plasmapheresis is associated with disease remission (7, 9, 10). The antibodies bind chiefly to the C-terminus of FH, inhibiting its cell surface regulatory functions (11, 12). A dose-response relationship is not established as many patients show high antibody levels even during remission, emphasizing the need to evaluate other markers of complement activation. Studies relating antibody titers to functional assays of FH inhibition, such as level of sheep red blood cell (SRBC) lysis, free FH, soluble terminal complement complex (sC5b-9) and epitope specificity of antibodies are limited (9, 13, 14).

We report the clinical features and outcomes of a large nationwide database of patients with anti-FH associated HUS. We also examined the functional implications of anti-FH antibodies and biomarkers that might enable prediction of a relapse.

## METHODS

Since March 2007, 781 patients younger than 18-year-old with aHUS have been enrolled in a prospective multicenter nationwide database at the All India Institute of Medical Sciences (AIIMS). Of these, 436 patients with anti-FH antibody associated aHUS, diagnosed in presence of microangiopathic hemolytic anemia (hemoglobin <10 g/dl, schistocytes ≥2%, lactate dehydrogenase >450 U/l), thrombocytopenia (platelets <150,000/μl), AKI and anti-FH antibody titers >150 AU/ml (15) were included. Clinical features of patients enrolled until February 2013 have been reported earlier (7). Patients with septicemia, disseminated intravascular coagulation, and thrombotic microangiopathy secondary to medications, lupus, HIV infection, and following bone marrow transplantation were excluded. Institute ethics committee approval was obtained and informed written consent was taken prior to enrolment.

### Investigations

Anti-FH antibodies were screened in plasma samples by enzyme linked immunosorbent assay (ELISA) (7). Antibody titer, determined at serial dilutions, was expressed as arbitrary units (AU)/ml at 1:50 dilution; values >150 AU/ml were considered abnormal (7). Investigations included urinalysis, blood levels of complement C3, antinuclear antibody, and antineutrophil cytoplasmic antibody. Leptospirosis, dengue, malaria, and rickettsia might rarely mimic clinical and laboratory features of HUS; we therefore screened for these infections. Levels of anti-FH antibodies, creatinine and urinalysis were estimated every 3–6 months. Studies for functional effects of anti-FH antibodies, including circulating FH immune complexes (CIC), free FH, sC5b-9, and SRBC lysis, were performed in 44 consecutive patients managed at AIIMS. Epitope specificity of FH-antibodies was determined during onset, remission, and relapse, in a subset of eight patients.

### Circulating FH Immune Complexes (CIC)

Briefly, 96-well plates (Nunc-Immuno Micro Well, Sigma-Aldrich, MO), coated with sheep anti-FH polyclonal antibody (AbD Serotec, Hercules, CA) diluted 1:10,000 in 0.5 M carbonate buffer (pH 9.6), were incubated overnight at 4°C (13). After washing, plates were blocked with 1% non-fat milk (Sigma-Aldrich) for 2-h at 37°C. Serial dilutions of plasma samples and controls were incubated for 1-hr, followed by incubation with goat anti-human IgG conjugated with horse radish peroxidase (HRP; Sigma-Aldrich) and titers reported at 1:100 dilution. For color development, $H_2O_2$ substrate and 3,3′,5,5′-tetramethylbenzidine were added; optical density was read at 450 nm. Titers of immune complexes, expressed as AU/ml, were calculated based on reference plasma (courtesy Marie Agnès Dragon-Durey). Screening of 100 healthy donors was used to establish the positive threshold of 110 AU/ml, corresponding to mean + 2 SD; intra- and inter-assay coefficient of variation was <10%.

### Free Factor H

Plasma samples diluted to 1:100 were incubated with Protein G-coated beads (10:1; Sigma-Aldrich) for 1-h at room temperature, followed by centrifugation. The supernatant was used for CIC ELISA to confirm removal of all IgG-bound FH. ELISA plates, coated with sheep anti-FH polyclonal antibody (AbD Serotec) and blocked with 1% bovine serum albumin (BSA), were incubated for 1-h at room temperature with the supernatant diluted to 1:5000. The plate was washed and monoclonal anti-FH antibody (AbD Serotec) added, followed by washing and addition of anti-mouse IgG labeled with HRP with color development as described above. Purified FH (Calbiochem, Meudon, France) was used as the calibrator.

### Sheep Red Blood Cell (SRBC) Hemolysis

Patient plasma was serially diluted to 1:1, 1:2, and 1:4 with buffer (20 mM Hepes, 7 mM $MgCl_2$, 10 mM EGTA, 144 mM NaCl, 1% BSA; pH 7.4). Diluted plasma (10 μl) was incubated with 10 μl sheep erythrocytes ($10^8$ cells/ml) at 37°C for 45 min [modified from (11)]. Following addition of 280 μl normal saline, the samples were centrifuged and absorbance read at 414 nm. Complete hemolysis with water was considered as 100%. Hemolysis was calculated as follows:

$$\text{SRBC lysis} = \frac{(\text{OD of patient sample - OD of blank}) \times 100}{\text{OD of well labeled 100\%}} \quad (1)$$

Mean SRBC lysis in 20 healthy controls was 16.9±2.1%; the positive threshold was 21%.

### Soluble Terminal Complement Complex (sC5b-9)

sC5b-9 was quantitated by ELISA using the MicroVue kit (Quidel Corp, San Diego, CA). Diluted plasma specimens were added to 96-well plate pre-coated with monoclonal antibody against C9 ring and incubated at room temperature for 1-hr. After addition of biotinylated antibody specific to sC5b-9, HRP-conjugated streptavidin and tetramethylbenzidine, optical density was read at 450 nm. sC5b-9 levels were calculated from a standard curve, limits of detection being 3.7–170 ng/ml.

### Production of FH Fragments

Short consensus repeats (SCR) 1–4, 5–8, 9–12, 13–16, and 17–20 were PCR amplified with specific primers from the FH cDNA (OriGene, Rockville, MD) and cloned in pET28 and pET29 expression vectors (16). The clones were verified by DNA sequencing (ABI 3730 DNA analyzer; Applied Biosystems), products were transformed in *E. coli BL21* and expressed in inclusion bodies generated after induction with 1 mM isopropyl-1-thio-b-D-galactopyranoside and purified using Ni-NTA resin. The eluted fragments were purified by gel filtration using Superdex75 columns (GE Healthcare) and their concentration determined by Bradford assay (Bio-Rad).

### Epitope Specificity

Plasma samples were incubated with FH fragments for 1-h and added to ELISA plates pre-coated with purified FH (Calbiochem) and blocked with 1% BSA. Bound antibodies were detected by HRP conjugated goat anti-human IgG with color development, as above.

## Therapy and Outcomes

The management of patients across centers was at the discretion of treating physicians, depending on their experience and available facilities. Renal replacement therapy was provided, when required. Specific management varied across centers and included plasma exchanges (PEX) and/or immunosuppressive therapies (9). Hematological remission was defined as platelet count >100,000/$mm^3$, schistocytes <2% and LDH less than upper limit of normal on two consecutive days. Disease relapse was considered when there was a new episode of illness after the patient had achieved remission for ≥2 weeks. Outcomes at 3-months and last follow-up were assessed, in terms of estimated glomerular filtration rate (eGFR) (17), hypertension (18), dipstick proteinuria and relapses. Adverse outcome was defined as eGFR <30 mL/min/1.73 $m^2$ on follow-up or patient death.

## Renal and Cardiovascular Outcomes

Long term outcomes were evaluated in 50 consecutive patients with eGFR more than 60 ml/min/1.73 $m^2$ and two or more years' follow-up. Blood pressure was recorded and classified using standard guidelines (18). First morning urine protein-to-creatinine ratio >0.15 mg/mg was considered abnormal (19). On echocardiography, left ventricular mass index (LVMI) was defined as ratio of left ventricular mass to $height^{2.7}$; LVH was LVMI >95th centile for age and sex (20). Dyslipidemia was defined as fasting total cholesterol >170 mg/dl, LDL cholesterol >110 mg/dl, non-LDL cholesterol >120 mg/dl, triglycerides >75 mg/dl or HDL cholesterol <40 mg/dl (21).

### Ambulatory Blood Pressure Monitoring (ABPM)

Ambulatory blood pressure was recorded by oscillometry (90207, Spacelabs Medical, Redmond, WA). Data was reported as standard deviation scores (SDS) based on height and sex specific normative data (22). Patients were classified as having ambulatory hypertension if they showed clinic hypertension (blood pressure ≥95th percentile), mean ambulatory systolic

or diastolic blood pressure exceeding 95th percentile for sex and height, and systolic or diastolic load >25% (severe if load >50%) (22). Masked hypertension was defined as ambulatory hypertension with normal clinic blood pressure (22).

### Functional Renal Reserve

Functional renal reserve was evaluated following administration of oral trimethoprim (10 mg/kg/day) for 5 days (23), discontinuation of ACE-inhibitors, and a vegetarian diet for 48-h (24). Patients voided completely at 7:00 am on the day of test; residual urine, estimated by ultrasonography, was required to be below 10 ml. They were instructed to drink 5 ml/kg water every 30 min throughout the study period. An accurately timed urine collection of 2-h duration was obtained and residual urine rechecked; serum creatinine was estimated at its midpoint. A protein meal (1 g/kg; RiteBite Max bar containing 20 g casein and whey protein) was ingested. Following complete voiding, 40 min later, similar 2-h urine collection and measurement of serum creatinine were done. Renal clearance of creatinine (CrCl) and functional renal reserve were calculated:

$$\text{CrCl}=\frac{\text{UCr}\times\text{V}}{\text{sCr}\times\text{t}}\times\frac{1.73}{\text{BSA}} \quad (2)$$

UCr urine creatinine, V urine volume over 2-h, *t* duration of collection (minutes), BSA body surface area, $m^2$

$$\text{Functional renal reserve}=\frac{(\text{CrCl after protein load - Baseline CrCl})}{\text{Baseline CrCl}}\times 100 \quad (3)$$

## Statistical Analysis

Six-year cohorts of March 2007 to December 2012 and January 2013 to August 2018 were compared in terms of clinical features and outcomes. Data is presented as proportions, and median (interquartile range, IQR) or mean ± SD, based on distribution and analyzed using Stata version 14.0 (Stata Corp, College Station, TX). Anti-FH antibodies were expressed as mean ± SEM. Tests for significance included *t*-test, Wilcoxon signed rank and rank sum tests, and chi-square test; correlation was measured by Spearman coefficient. Anti-FH and free FH levels were log transformed to satisfy normality. Repeated measures analyses by generalized estimating equations (GEE) approach was used to compare serial anti-FH and free FH concentrations in sustained remission or subsequent relapse; receiver operator characteristic (ROC) curves were used for threshold of relapse. Determinants of adverse outcome and relapse were estimated as odds and hazards ratios, by univariate and multivariable analyses. Functional renal reserve was normalized by Box-Cox transformation. Linear regression analyses were used to evaluate predictors of renal reserve, LVMI and proteinuria; two tailed $P < 0.050$ was considered significant.

# RESULTS

From March 2007 to August 2018, 436 (55.8%) of 781 patients from 30 centers in the nationwide database were diagnosed as having anti-FH associated aHUS (**Supplementary Figure 1**). Proportion of patients with anti-FH antibodies younger than 4-year, between 4–11 and 11–18 year at presentation were 20.8, 73.8, and 52.0%, respectively; five patients presented in infancy (**Supplementary Figure 2**). Patients between 4 and 11 year had higher antibody titers (11,127 ± 1,170 AU/ml vs. 8,870 ± 1,890 AU/ml; $P = 0.025$). There was seasonal variation, with peak between December and April (**Supplementary Figure 3**). Prodromal illness included fever (54.6%), upper respiratory tract infection (10.3%), and diarrhea (6.7%). Eight of 282 patients (2.8%) showed antinuclear antibodies, 3 of 219 (1.4%) had antineutrophil cytoplasmic antibodies. Three of 197 (1.5%) showed antibodies to leptospira; vivax or falciparum malaria was present in fifteen.

Five adult patients (aged 22–48 years) also showed anti-FH antibodies (854–60,032 AU/ml) and presented with similar clinical features, chiefly following a febrile illness. One patient each with systemic lupus erythematosus and following bone marrow transplant also showed elevated levels (852 and 4,264 AU/ml, respectively). These seven-patients have not

**TABLE 1 |** Clinical and biochemical features in patients with anti-FH associated hemolytic uremic syndrome in two 6-year cohorts.

| Variable | 2007-12 (*n* = 119) | 2013-18 (*n* = 317) | Whole cohort (*n* = 436) | *P* |
|---|---|---|---|---|
| Boys | 91 (76.5) | 211 (66.6) | 302 (69.3) | 0.048 |
| Age, years | 7.9 ± 3.6 | 7.6 ± 3.2 | 7.7 ± 3.3 | 0.26 |
| Time to presentation, days[a] | 18.0 ± 18.2 | 12.4 ± 12.1 | 13.8 ± 14.1 | 0.001 |
| Duration of oligoanuria, days | 11.7 ± 11.2 | 6.7 ± 9.9 | 8.0 ± 4.5 | <0.001 |
| Anuria | 52 (43.7) | 79 (24.9) | 131 (30.0) | <0.001 |
| Prodromal illness | | | | |
| Febrile illness | 63 (52.9) | 175 (55.2) | 238 (54.6) | 0.74 |
| Diarrhea, dysentery | 10 (8.4) | 19 (6.0) | 29 (6.7) | 0.24 |
| Upper respiratory tract infection | 6 (5.0) | 39 (12.3) | 45 (10.3) | 0.032 |
| Jaundice, elevated transaminases | 24 (20.2) | 138 (43.5) | 162 (37.2) | <0.001 |
| Seizures | 46 (38.7) | 71 (22.4) | 117 (26.8) | <0.001 |
| Stage 2 hypertension | 84 (70.6) | 154 (48.6) | 238 (54.6) | <0.001 |
| Hemoglobin, g/dl | 5.5 ± 1.3 | 5.2 ± 1.3 | 5.3 ± 1.3 | 0.67 |
| Platelet count, $\times 10^3/mm^3$ | 63.9 ± 39.3 | 58.5 ± 39.1 | 59.9 ± 39.1 | 0.57 |
| Reticulocyte count, % | 11.0 ± 9.5 | 8.7 ± 6.6 | 9.2 ± 7.3 | 0.10 |
| Nephrotic range proteinuria | 36 (30.3) | 211 (66.6) | 247 (56.7) | <0.001 |
| Blood creatinine, mg/dl | 5.84 ± 2.67 | 5.46 ± 3.08 | 5.56 ± 2.98 | 0.28 |
| Lactate dehydrogenase, IU/L | 3042.0 ± 2701.4 | 3582.5 ± 2873.1 | 3,447 ± 2837.4 | 0.005 |
| Complement C3, mg/dl | 70.0 ± 28.6 | 71.2 ± 27.9 | 70.9 ± 28.1 | 0.84 |
| Anti-FH antibody, AU/ml* | 7330.5 ± 2017.4 | 11847.4 ± 1,140.1 | 10,633.2 ± 998.5 | 0.005 |

*Normal range: hemoglobin 11.0–15.8 g/dl, platelet count 190–590 × $10^3/mm^3$, reticulocyte count 3.6–11%, blood creatinine: 0.17–1.01 mg/dl, lactate dehydrogenase 195–314 IU/L, C3 90–130 mg/dl.*

[a]*Days between disease onset and evaluation.*

*Data shown as N (%), mean ± standard deviation or *mean ± standard error of mean; AU arbitrary units; FH factor H.*

been included to maintain homogeneity within a pediatric aHUS population.

**Table 1** shows that patients presented earlier during the illness in the last 6-year compared to before 2013; stage 2 hypertension and seizures were also fewer in the latter cohort. Sixteen (3.6%) patients did not have thrombocytopenia, including six with mildly deranged renal function. Neurological features (31.3%) comprised seizures and/or hypertensive encephalopathy (12.4%). Imaging showed posterior reversible encephalopathy ($n = 15$), infarcts ($n = 7$) and intracranial hemorrhage ($n = 2$). Other features included cardiogenic shock ($n = 16$), pulmonary edema and hemorrhage ($n = 8$), and pancreatitis, mesenteric ischemia and peripheral gangrene ($n = 3$, each).

## Anti-FH Titers and Their Functional Characterization

Anti-FH titers at onset negatively correlated with serum C3 ($P < 0.001$), platelets ($P = 0.013$), and hemoglobin level ($P = 0.057$), and positively with LDH ($P = 0.010$; **Supplementary Figure 4**).

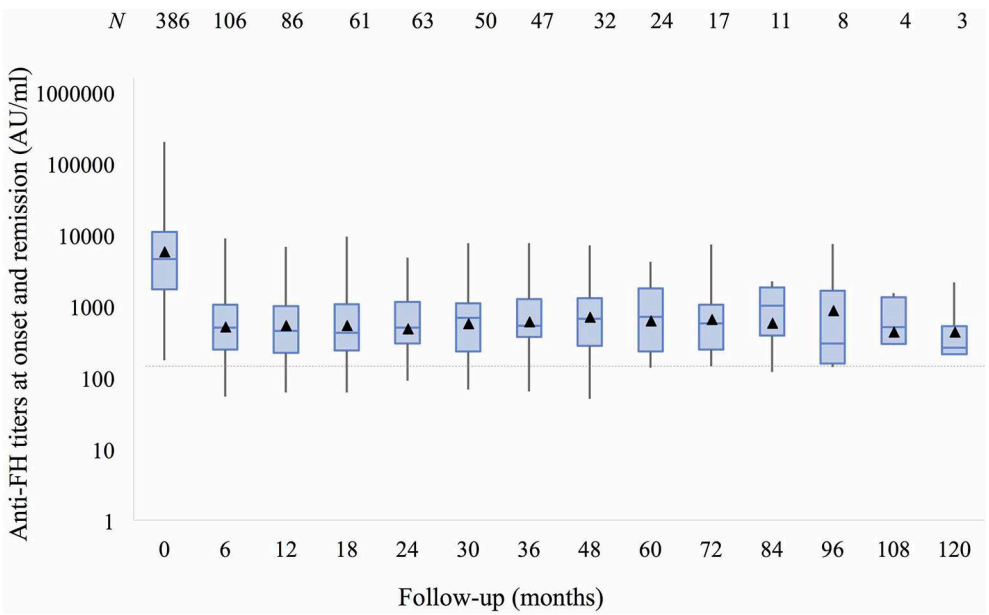


**FIGURE 1 |** Anti-factor H antibodies (shown as mean titers, ▲) at onset and 6–12 monthly follow-up over 10 years remain detectable (>150 AU/ml) despite remission. Boxes depict median and interquartile range; whiskers show range of antibody titers at each follow-up visit.

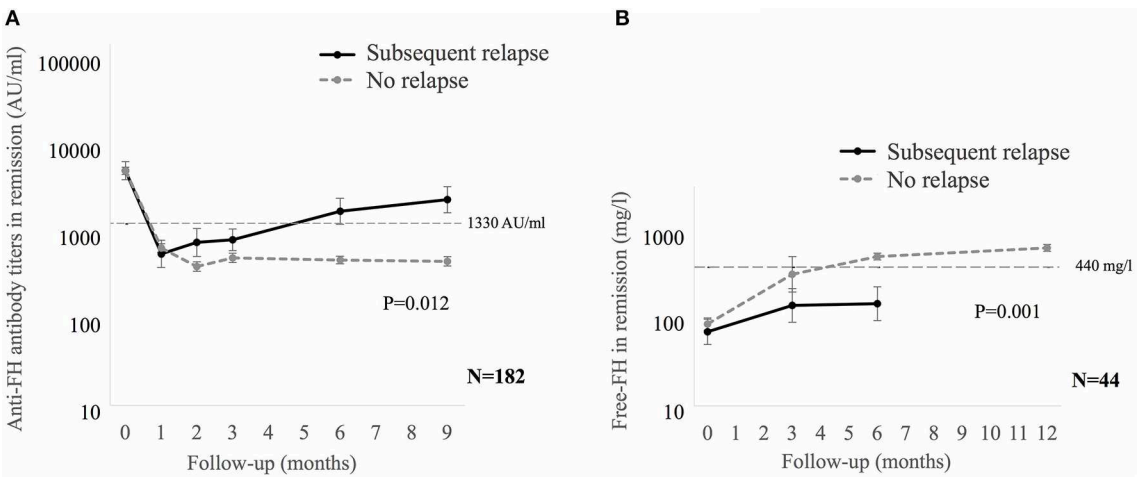


**FIGURE 2 | (A)** Mean anti-factor H (FH) antibody titers, and **(B)** Free FH during remission in patients with or without subsequent relapse. Relapses were seen in 12 of 182 **(A)** and 10 of 44 **(B)** patients. Generalized estimating equations approach was used on log transformed data. In patients with relapse, the last anti-FH titer and free FH was estimated at median of 1 (0.7–2) months and 5 (2–6.9) months before relapse, respectively. Anti-FH antibody titers ≥1,330 AU/ml or free FH ≤440 mg/l predict later relapse.

**TABLE 2 |** Functional characterization of anti-factor H antibodies at onset and during remission during first year of follow-up.

| | Onset (*N* = 44) | Remission | | |
|---|---|---|---|---|
| | | 3-months (*N* = 42) | 6-months (*N* = 37) | 12-months (*N* = 23) |
| Anti- FH antibody, AU/ml | 5,000 (2,123–163,829) | 409 (254–861) | 277 (154–893.6) | 408 (262–691.8) |
| Circulating FH immune complexes, AU/ml | 20,000 (7,168–44,480) | 806 (289–1,328) | 710 (244–1,681) | 1,004.5 (397–1,663) |
| Sheep red blood cell hemolysis, % | 72.8 (57.2–88.7) | 16.9 (13.5–22.6) | 14.6 (13–19.1) | 13 (5.4–20) |
| Free FH, mg/l | 64 (34–106) | – | 553 (376–630) | 779 (571–1,071) |
| Soluble terminal complement complex (sC5b-9), ng/ml | 1,510 (832–2,220) | – | 351 (260–720) | 355.5 (232.5–642.5) |
| Complement C3, mg/dl | 60.7 (48.7–82.6) | – | 108.9 (95.5–129.9) | 121.9 (94.4–130.2) |

*P < 0.001 for all comparisons from onset.*
*Values are median (interquartile range); AU, arbitrary units; FH, factor H.*
*Normal ranges: Anti-FH antibody <150 AU/ml; circulating FH-immune complexes <110 AU/ml; sheep red blood cell hemolysis 16.9 ± 2.1%; median free FH 720 (459–810) mg/l; median sC5b-9 195.3 (151.1–292.5) ng/ml; C3 90–130 mg/dl.*

Patients requiring dialysis had higher mean antibody titers than those not dialyzed (11,287 ± 1,173 vs. 8,198 ± 2,017 AU/ml; $P = 0.015$). **Figure 1** shows that anti-FH titers remained detectable during remission, with mean titers varying between 700 and 1,164 AU/ml on follow-up. Mean anti-FH titers during remission in patients with or without subsequent relapse are shown in **Figure 2A**. Using GEE approach, we found that patients with relapses had significantly higher antibody titers 1 month preceding a relapse compared to those in sustained remission (ß = 0.18; $P = 0.023$). ROC curves showed that titers ≥1,330 AU/ml at 6-months predicted the occurrence of a relapse (sensitivity 75%, specificity 81.4%; area under curve 0.86). However, 27 (15.8%) patients with sustained remission had antibody levels above this cut-off.

**Table 2** shows serial levels of CIC, free FH, C3, sC5b-9 and SRBC lysis from onset to 12-months in 44 patients. While median CIC declined from 20,000 AU/ml at onset to 806 AU/ml at remission ($P < 0.001$), it remained detectable and correlated with antibody titers at 6- and 12-months ($r = 0.44$ and 0.40; $P = 0.007$ and 0.067, respectively). SRBC lysis reduced from 72.8% at onset to 16.9% during remission, and levels of free FH increased from 64 to 553 mg/l. SRBC lysis and free FH correlated with CIC ($r = 0.68$ and $r = -0.63$, $P < 0.001$) and anti-FH titers ($r = 0.60$ and $r = -0.55$, $P < 0.001$) at all times points. While blood levels of sC5b-9 declined significantly during remission compared to onset (**Table 2**), they were high compared to controls ($P < 0.001$).

Since anti-FH titers ≥1,330 AU/ml were a predictor of relapse, we examined levels of free FH, sC5b-9 and CIC in patients with sustained remission but having anti-FH titers persistently above ($n = 11$) or below ($n = 33$) this cut-off. Median sC5b-9 and CIC were 709 ng/ml and 2,196 AU/ml in the former vs. 329.9 ng/ml and 594 AU/ml ($P = 0.067$ and $P = 0.060$, respectively); levels of free FH were also similar ($P = 0.66$).

We also examined serial free FH levels during remission in 44 patients, including 10 who later relapsed (**Figure 2B**). Free FH levels were significantly lower at 6-months in patients who relapsed compared to those in sustained remission (ß = 0.29; $P = 0.001$). ROC curves showed that free FH ≤440 mg/l at 6-months predicted occurrence of relapse (sensitivity 70%, specificity 100%; area under curve 0.91). Among patients with anti-FH levels ≥1,330 AU/ml, free FH ≤440 mg/l at 6-months discriminated between patients with a relapse and those with sustained remission, with sensitivity of 75%, positive predictive value of 79% and negative predictive value of 91%; area under curve = 0.91; hazards 6.3, 95% CI 1.7–23.8 ($P = 0.018$).

Epitope specificity ($n = 8$ patients) showed similar pattern of antibody binding during onset, remission and relapse. Antibodies

**TABLE 3 |** Therapy and outcomes in patients with anti-factor H associated hemolytic uremic syndrome.

| Variable | 2007-12 (*n* = 119) | 2013-18 (*n* = 317) | Whole cohort (*n* = 436) | *P* |
|---|---|---|---|---|
| Dialysis requirement (%) | 101 (84.9) | 236 (74.4) | 337 (77.3) | 0.021 |
| Duration of dialysis, days | 26 (10–57) | 13 (5.8–30.3) | 15 (6–36) | <0.001 |
| Plasma exchange (PEX, %) | 91 (76.5) | 247 (77.9) | 338 (77.5) | 0.75 |
| Days to PEX | 17 (7–32) | 11 (6–22) | 12 (6–24) | 0.011 |
| Induction immunosuppression (%) | 79 (66.4) | 243 (76.7) | 322 (73.9) | 0.029 |
| Maintenance immunosuppression (%) | 50 (42) | 139 (43.8) | 189 (43.3) | 0.73 |
| Days to immunosuppression | 34 (20–53) | 21 (11–31.8) | 27 (12–35) | <0.001 |
| Onset to hematological remission, days | 38 (22.5–54.3) | 24 (16–35) | 27 (17–41) | <0.001 |
| **Outcome at 3-months** | ***N* = 105** | ***N* = 251** | ***N* = 356** | |
| Stage 2 HTN or proteinuria ≥2+ | 48 (45.7) | 104 (41.4) | 152 (42.7) | 0.46 |
| CKD stages 2–3 | 18 (17.1) | 46 (18.3) | 64 (18.0) | 0.79 |
| Adverse outcome CKD stage 4–5; death | 35 (33.3) | 46 (18.3) | 81 (22.8) | 0.002 |
| **Outcome at last follow-up** | ***N* = 105** | ***N* = 251** | ***N* = 356** | |
| Stage 2 HTN or proteinuria ≥2+ | 31 (26.1) | 64 (20.2) | 95 (26.7) | 0.43 |
| CKD stages 2–3 | 12 (10.1) | 27 (8.5) | 39 (11.0) | 0.86 |
| Adverse outcome CKD stage 4–5; death | 38 (36.2) | 53 (21.1) | 91 (25.6) | 0.003 |
| Relapse | 26 (24.7) | 35 (13.9) | 61 (17.1) | 0.003 |

*Data shown as median (interquartile range) or N (%); CKD, chronic kidney disease; HTN, hypertension; PEX, plasma exchange.*

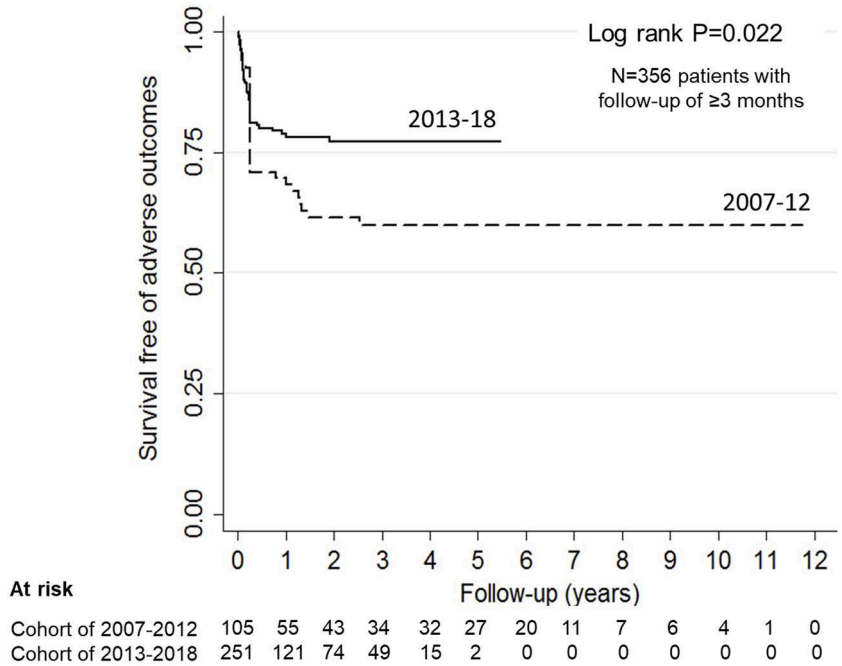


**FIGURE 3 |** Probability of renal survival in patients with anti-factor H antibody associated HUS. Patients diagnosed and managed from 2007 to 12 showed 70.8, 59.8, and 59.8% renal survival at 1-, 5-years and at last follow up (interrupted line). Corresponding renal survival in patients managed during 2013-18 (continuous line) was 78.7, 77.1, and 77.1% (log rank $P = 0.022$).

demonstrated strongest binding to SCR 17–20 ($n = 8$), moderate binding to SCR 9–12 and SCR 13–16 ($n = 7$, each), and also to SCR 1–4 ($n = 3$), and SCR 5–8 ($n = 4$; **Supplementary Table 1**).

## Therapy

PEX was initiated earlier (median 11 vs. 17 days from onset of illness) and dialysis requirement was briefer (median 13 vs. 26 days) in the last 6-year (**Table 3**). PEX were done for at least 14 days in 72.7%; 42 patients received plasma infusions only. Based on center preference, initial immunosuppression comprised prednisolone with IV cyclophosphamide ($n = 171$) or rituximab ($n = 43$). Maintenance immunosuppression included prednisone alone (16), and with mycophenolate mofetil (133) or azathioprine (40).

## Outcome

Information on outcome at 3-months or longer was available for 356 (81.7%) patients. During the last 6-year, patients achieved earlier hematological remission and showed better outcomes at 3-months (**Table 3**) and last follow-up (**Figure 3**). In the subset of 196 patients, managed with PEX and immunosuppression, adverse outcomes occurred in 12.2% at 3-months (**Supplementary Table 2**); survival free of adverse outcomes was 86, 86, and 78% at 1-year, 5-year and last-follow up at mean 28.5 ± 27.3 months, respectively. Sixty-one (17.1%) patients relapsed at mean 11.0 ± 12.9 months from onset; relapse free survival was 86.4, 73.5, and 68.3% at 1-year, 5-year and last follow up, respectively. Relapses were early, with 46% occurring within the first 6-months and 95% within 2-years.

## Determinants of Outcome

Mean antibody titer was 22,801 ± 6,712 AU/ml in 19 patients who died in the first month of illness compared to 9,851 ± 1,058 AU/ml in those who survived ($P = 0.002$). On multivariable analysis, antibody titer at onset ≥8,000 AU/ml (OR 6.1, 95% CI 0.88–43.0; $P = 0.066$) predicted mortality within 30 days of onset, while daily PEX for 7 days (OR 0.14, 95% CI 0.02–0.95; $P = 0.044$) and concomitant immunosuppression (OR 0.07, 95% CI 0.01–0.71; $P = 0.024$) were protective (data not shown). Independent predictors of adverse outcome were antibody titer ≥8,000 AU/ml (HR 2.23, 95% CI 1.11–4.48; $P = 0.024$), longer time (≥14 days) between onset of illness and initiation of PEX (HR 2.09, 95% CI 0.93–4.69; $P = 0.071$), and short duration PEX <14 days (HR 2.60, 95% CI 1.19–5.67; $P = 0.017$). Therapy with PEX and induction therapy (HR 0.37; 95% CI 0.16–0.89; $P = 0.026$) and maintenance immunosuppression (HR 0.02; 95% CI 0.001–0.413; $P = 0.011$) protected against adverse outcomes (**Supplementary Table 3**). Maintenance immunosuppression was associated with relapse free survival (HR 0.11; 95% CI 0.05–0.27; $P < 0.001$).

## Renal, Cardiovascular Outcomes

Fifty consecutive patients with eGFR 100.2 ± 21.1 (range 67–156) ml/min/1.73 $m^2$ were screened for renal and cardiovascular outcomes at mean 4.4 ± 2.5 (range 2–15) year from onset. Seventeen patients had CKD stage 2 and proteinuria was present in 29. Despite 37 patients receiving antihypertensive agents, 24 had clinic hypertension. ABPM showed normal blood pressure (14%), severe ambulatory hypertension (38%), masked

hypertension (30%), pre-hypertension (18%), and abnormal nocturnal dipping (76%). Mean systolic and diastolic blood pressures were at 77th and 87th percentile with mean load 43 ± 26 and 40 ± 26%, respectively. LVH was present in 28% and 5 patients had dyslipidemia.

Median renal reserve was 15.9 (6.3, 28.5)% in 41 patients (**Supplementary Figure 5**). Proportion of patients with renal reserve below 20, 10, and 5% were 49, 27, and 15%, respectively. Renal reserve was 10.3 (−5.3, 31.5)% in patients with stage 2 CKD compared to 27.9 (16.9, 40.4)% with eGFR >90 ml/min/1.73 $m^2$ ($P = 0.010$). Factors independently and inversely associated with renal reserve were mean systolic ambulatory pressure (ß = −0.49, 95% CI −0.85,−0.12; $P = 0.010$), number of relapses (ß = −0.65, 95% CI −1.23, −0.07; $P = 0.030$), urine protein-to-creatinine ratio >1 (ß = −0.9, 95% CI −0.1, −1.71; $P = 0.031$) and increased LVMI (ß = −0.04, 95% CI −0.08, −0.001; $P = 0.044$). Proteinuria was associated with ambulatory systolic (ß = 0.19, 95% CI 0.07, 0.32; $P = 0.003$) and diastolic hypertension (ß = 0.16, 95% CI 0.03, 0.3; $P = 0.021$).

## DISCUSSION

We report the clinical features and outcomes of a large, prospective multicenter cohort of 436 children with anti-FH associated aHUS managed across the country over a period of 12-year. Although the management was heterogeneous and based on center practices and physician preference, patients managed in the recent years show overall better renal survival, with decline in proportion of adverse outcomes from 36.2 to 21.1%. Prompt PEX, performed for at least 2-weeks, combined with immunosuppressive medications resulted in renal survival of 86% at 5-year. Serial examination showed that anti-FH titers and circulating FH immune complexes persisted well above the normal range during remission. Among patients with high anti-FH level exceeding 1,330 AU/ml, level of free FH below 440 mg/l predicted a 6.3-fold risk of later relapse. On long-term follow-up, patients show significant sequelae in terms of severe and masked ambulatory hypertension, left ventricular hypertrophy and proteinuria (summarized in **Table 4**).

While 24% children and 19% adults in the global aHUS Registry had anti-FH antibodies (8), these autoantibodies were present in 55.8% patient in the current cohort, confirming the increased frequency of this condition in India (7). In conformity with previous reports, predominantly children between 4 and 11-years were affected (4, 8, 25). The reason for high prevalence of anti-FH antibody associated HUS in Indian children is not clear. The population frequency of homozygous *CFHR1* deletion is similar in India (9.5%) compared to elsewhere (2–10%) (7, 9). The high prevalence of the illness in school going children, predilection for the cold weather, and associated prodromal symptoms indicate a possible infectious trigger. While a gastrointestinal prodrome is reported by others (1, 25), the chief preceding illness in the present patients was low-grade fever (55%) or a respiratory tract infection. A previous study from this center, using multiplex polymerase chain reaction on stool specimens, showed multiple gastrointestinal pathogens in 35 patients predominantly in patients with anti-FH antibodies (26).

Anti-FH titers were related to disease severity as evidenced by relationship with platelet count, hemoglobin, blood levels of LDH, dialysis requirement, mortality, and renal outcome. As shown previously, we found that CIC and SRBC lysis were markedly elevated during active disease compared to remission (12, 13, 27–29); the decline in CIC during remission was more than that of free antibodies, perhaps due to change in avidity of antibodies for free FH (13). While our finding of persistently high antibodies during remission has been reported (4, 25), the present report emphasizes that levels of CIC continued to be high during follow-up. Other authors suggest that CIC correlate better with disease activity, than do antibody titers (13).

The present analysis confirms our previous findings on the association of high antibody titers (≥1,330 AU/ml at 6-months) with subsequent relapse (30). However, 15.8% patients with quiescent disease had persistently high titers, suggesting that elevated levels are not always pathogenic. While free FH has been measured in few patients (31, 32), we determined serial levels in a larger cohort. Among patients with high anti-FH titer, reduced free FH concentration (≤440 mg/l) predicted a 6.3-fold higher risk of subsequent relapse with a negative predictive value of 91%. These findings suggest that formation of CIC reduce availability of free FH, impairing cell surface protection. This was shown previously *in vitro* as dose-dependent reduction in SRBC lysis on addition of FH to sera of anti-FH positive patients (11). Our findings suggest that estimation of free FH is therefore promising for indicating the risk of relapses.

Previous studies on levels of sC5b-9, which assesses activity of the soluble terminal complement pathway, show variable levels during active disease and remission (29, 33–36). While all the present patients had elevated sC5b-9 at onset that declined during remission, the levels were higher than normal, as shown previously in some but not all studies (29, 36). Serial estimation of blood levels of sC5b-9 did not therefore predict relapses, limiting its utility as a biomarker of disease activity. In contrast, free FH levels were normal in patients with sustained remission but having high anti-FH antibody titers, suggesting its potential role as a biomarker.

While free FH is presently a research tool and not available widely, close monitoring of anti-FH antibody titers is required with more careful assessment if elevated >1,300 AU/ml during the first 12–24 months (37). Relapses usually follow minor infectious illnesses during which close clinical and biochemical monitoring is required.

Using FH fragments that were generated in *E. coli* and purified by gel filtration, Gurjar et al. used inhibition ELISA to determine epitope specificity in 21 patients of this cohort with anti-FH associated HUS (16). Antibodies showed strong binding to SCR 17–20; binding with lower affinity was present to SCR 5–8 (16). We extended this work to examine whether there was altered epitope specificity to FH at onset of the illness, remission and during relapse in eight more patients. Similar to the previous work, we found binding to SCR 17–20 in all patients at onset; binding to SCR 9–12 and SCR 13–16 was also present in most patients. There were no significant changes in

**TABLE 4 |** Summary of key findings in patients with anti-factor H (FH) associated hemolytic uremic syndrome (HUS).

| Objective, *N* | Result | Conclusion |
|---|---|---|
| Demographic features, $N = 436$ | Of 781 patients <18-years-old, 55.8% had anti-FH antibodies. Cases peak between December and April; prodrome: fever (54.6%), upper respiratory tract infection (10.3%), diarrhea (6.7%) | Seasonal predilection and prodromal symptoms indicate possible infectious trigger |
| Cohorts: 2007-12 ($N = 119$); 2013-18 ($N = 317$) | Earlier diagnosis and initiation of therapy in cohort of 2013-18; better outcomes at 3-months (33.3 vs. 18.3%) and at last follow-up ($P = 0.022$) in recent years | Prompt recognition and appropriate management improves outcomes |
| Anti-FH antibody titer and impact on course | Anti-FH titers at onset negatively correlate with serum C3, platelets and hemoglobin level; positive correlation with LDH levels and need for dialysis.<br>Mean anti-FH titers 700-1164 AU/ml over 10-year follow up<br>Anti-FH titers ≥1,330 AU/ml at 6-months predicts relapse (sensitivity 75%, specificity 81.4%; AUC 0.86); 15.8% patients in remission show antibody levels >1,330 AU/ml | Anti-FH antibody titer correlates with disease severity at onset. Titers high in remission; need biomarkers to predict relapse. Patients with anti-FH titers >1,330 AU/ml at risk of relapse—require careful clinical monitoring. |
| Functional characterization of antibodies, $N = 44$ | Circulating FH immune complexes (CIC) decline but correlate with anti-FH titers during remission. During remission, median soluble terminal complement complex (sC5b-9) levels were 329.9 ng/ml and 594 ng/ml in patients with high or low titers, respectively. | CIC and sC5b-9 elevated even during remission; unsatisfactory biomarkers of disease |
| | Free FH ≤440 mg/l at 6-months predicts relapse (sensitivity 70%, specificity 100%; AUC 0.91). Presence of free FH ≤440 mg/l *and* antibody ≥1,330 AU/ml associated with 6.3-fold risk of relapse | Low levels of free FH predict relapse; requires examination in a larger cohort |
| FH epitope specificity, $N = 8$ | Similar binding during onset, relapse, remission. Strong binding to SCR 17–20; also to others | Binding at multiple epitopes on FH |
| Outcome, $N = 356$ | Independent predictors of adverse outcome: Anti-FH ≥8,000 AU/ml, long time to begin PEX (>14 days from onset) and short duration PEX (<14 days); combined PEX and immunosuppression were protective. Maintenance immunosuppression reduces risk of relapses. | Antibody titers at onset predict early mortality and outcomes. Adequate PEX with immunosuppression improve outcomes. |
| Outcomes at $4.4 \pm 2.5$ year from onset, $N = 50$ | eGFR $100.2 \pm 21.1$ ml/min/1.73 $m^2$; proteinuria (58%), severe ambulatory hypertension (38%), masked (30%), prehypertension (18%), left ventricular hypertrophy (28%), and dyslipidemia (10%). | More than one-third patients show renal and cardiovascular sequelae |
| Renal reserve, $N = 41$ | Median renal reserve 15.9%. Inverse association with mean systolic pressure, number of relapses, urine protein-to-creatinine ratio, and increased left ventricular mass index. | Suggest long term assessment for proteinuria, ambulatory hypertension, cardiovascular outcomes |

*AUC, area under the curve; eGFR, estimated glomerular filtration rate; FH, factor H; LDH, lactate dehydrogenase; PEX, plasma exchange; SCR, short consensus repeats; sC5b-9, soluble terminal complement complex.*

epitope binding between onset, remission and relapse, as shown previously (14). The small number of patients studied limits conclusions regarding epitope specificity. **Table 5** summarizes findings from various reports on epitope specificity of anti-FH antibodies, emphasizing predominant binding to the C-terminal, and also other domains on FH (5, 11, 13, 14, 16, 25, 38–40).

Education and dissemination of management protocols through scientific meetings and efforts for consensus guidelines (37) has resulted in prompt recognition, early referral, and protocol based management, improving patient outcomes. While PEX and immunosuppression are considered primary therapies for patients with anti-FH associated HUS, the duration of therapy is empirical (41). The present report suggests that prompt and adequate duration of PEX was associated with decreased mortality and improved renal outcomes. Since most relapses occurred in the initial 2 years, immunosuppression is recommended for this period. We did not find increased rates of infections with use of immunosuppressive agents, as was a concern in a previous study (25). Our findings also suggest that strategies like PEX with corticosteroids alone, or immunosuppression with/without plasma infusions had limited benefit on long-term outcome.

An audit on safety of PEX from centers in Europe and North America showed procedure related complications and hypersensitivity to plasma in one-third patients, limiting the safety of this procedure in children (42). A similar audit of 2024 PEX sessions in 109 patients in New Delhi showed chiefly self-limiting adverse events (9.1%), including chills, vomiting, abdominal pain, and urticaria; hypotension (1.6%), hypocalcemia, tachycardia, seizures (0.2%, each), and hemorrhage (0.1%) were rare and catheter-related adverse events comprised only bloodstream infection (1.45/1,000 catheter-days). Hematological remission was achieved in 93.4% of patients within a fortnight of initiating PEX, with 80% and 90% patients discontinuing dialysis by 1 and 3 months, respectively (43). PEX was therefore overall safe and effective with satisfactory short-term outcomes.

Inhibition of the complement pathway with eculizumab is the standard of care for aHUS in developed countries (15). Most patients with anti-FH associated illness are treated similarly (25), although international pediatric and KDIGO guidelines suggest the use of PEX and immunosuppressive therapy for this disorder (15, 44). However, eculizumab does not impact generation of antibodies and additional immunosuppression might still be required. On the other hand, present findings show that despite PEX and immunosuppressive therapy almost one-quarter of all patients with anti-FH associated HUS had an adverse outcome. Some

**TABLE 5 |** Epitope specificity of anti-factor H (FH) antibodies to short consensus repeats (SCR) of FH.

| References | SCR 1–4 | SCR 1–7 | SCR 7 | SCR 5–8 | SCR 8–14 | SCR 9–12 | SCR 11–14 | SCR 13–16 | SCR 15–20 | SCR 17–20 | SCR 19–20 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Blanc et al. (13) | 13/14 | 17/18 | | | 5/18 | | | | 18/18 | | 8/17 |
| Bhattacharjee et al. (38) | | | | | | | | | 10/10 | | 10/10 |
| Moore et al. (39) | 1/12 | | | | | | | | | | 7/12 |
| Jozsi et al. (11) | | 0/5 | | | 1/5 | | | | 5/5 | | 5/5 |
| Jozsi et al. (5) | | 0/16 | | | 4/16 | | | | 16/16 | | 16/16 |
| Nozal et al. (14) | 1/14 | 1/14 | | | 2/17 | | | | 12/14 | | |
| Guo et al. (40) | 4/36 | | 6/36 | | | | 4/36 | | | | 12/36 |
| Gurjar et al. (16) | | | | 21/21 | | | | | | 21/21 | |
| Brocklebank et al. (25) | | 5/17 | | | 1/17[#] | | | | 1/17* | | 15/17 |
| Present study | 3/8 | | | 4/8 | | 7/8 | | 7/8 | | 8/8 | |
| Total (%) | 35.4% (81/229) | | | | 24.8% (31/125) | | | | 73.9% (164/222) | | |
| | N-terminal of FH | | | | Mid-portion of FH | | | | C-terminal of FH | | |

[#]*Short consensus repeats (SCR) 8–15;* [*]*SCR 16–18.*

of these patients might have benefited from prompt use of eculizumab, especially if hematological remission was delayed beyond 7–10 days. There is need for a prospective study examining the efficacy and safety of eculizumab in this specific condition.

The rates of persistent proteinuria (15–30%) and ambulatory hypertension (10–46%) following Shiga toxin HUS are similar to the present cohort (45–48). Masked hypertension and abnormal dipping of blood pressure are proposed to be associated with adverse cardiovascular outcomes and microalbuminuria (49, 50). The findings of abnormal dipping in three-quarter of all patients, and masked hypertension, LVM in one-third are therefore important. We also found reduced renal functional reserve in one-third patients, similar to 24–65% in patients with Shiga toxin HUS (51–54). Conforming to previous reports, functional reserve was inversely associated with proteinuria and ambulatory hypertension (53, 55–57). Since we assessed functional reserve in patients with eGFR >60 ml/min/1.73 $m^2$, its overall magnitude is perhaps higher. The present findings emphasize that patients recovering from anti-FH associated HUS require long-term assessment for cardiovascular and renal outcomes.

## DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

Institute ethics committee approval was obtained from All India Institute of Medical Sciences, New Delhi and informed written consent was taken prior to enrolment.

## AUTHOR CONTRIBUTIONS

MP: performed experiments, manuscript preparation. PK: patient care, data collection and analysis, manuscript preparation. HS, BG, RS, TM, AKS, and SS: laboratory work for the study. AnS, SA, AdS, and PH: patient care, critical review of manuscript. UA, IA, KA, NP, PR, RS, and AV: patient care. ArS, SR, UK, and AB: supervision of experiments. AB: study design, patient care, manuscript preparation and also is the guarantor for this paper. All authors approved the manuscript before it was submitted.

## CONTRIBUTING CENTERS

Indira Agarwal, Christian Medical College, Vellore; Vinay Aggarwal, BL Kapoor Hospital, New Delhi; Uma Ali, Bai Jerbai Wadia Hospital for Children, Mumbai; Kanav Anand, Sir Ganga Ram Hospital, New Delhi; Arvind Bagga, All India Institute of Medical Sciences, New Delhi; Chinmay Behera, Kalinga Institute of Medical Sciences, Bhubaneswar; Gurdeep Singh Dhooria, Dayanand Medical College & Hospital, Ludhiana; Sanjeev Gulati, Fortis Escorts Heart Institute, New Delhi; NK Hase, King Edward Memorial Hospital, Mumbai; Madhuri Kanitkar, Armed Forces Medical College, Pune; Sriram Krishnamurthy, Jawaharlal Institute of Post-graduate Medical Education & Research, Puducherry; Manish Kumar, Chacha Nehru Bal Chikitsalaya, Delhi; Amarjeet Mehta, Sawai Man Singh Hospital, Jaipur; Mahantesh Patil, KLES Dr. Prabhakar Kore Hospital & Medical Research Center, Karnataka; Saroj. K. Patnaik, Army Hospital Research and Referral, New Delhi; Ravindra Prabhu, Kasturba Medical College, Manipal; Narayan Prasad, Sanjay Gandhi Post-Graduate Institute of Medical Sciences, Lucknow; Padmaraj Rajendran, Institute of Child Health, Chennai; Abhijeet Saha, Lady Hardinge Medical College, New Delhi; VK Sairam, Kanchi Kamacoti Child Trust Hospital, Chennai; T. Saravanan, PSG Coimbatore;

Sidharth Kumar Sethi, Medanta The Medicity, Gurugram; Fagun Shah, Child's Kidney Care Center, Surat; Rajiv Sinha, Institute of Child Health, Kolkata; Deepti Suri, Post-Graduate Institute of Medical Education and Research, Chandigarh; Nilam Thaker, Ahmedabad; AS Vasudev, Indraprastha Apollo Hospital, New Delhi; Anil Vasudevan, St. John's Medical College and Hospital, Bangalore; M Vijayakumar (late), Mehta Children Hospital, Chennai; Susan Uthup, Trivandrum Medical College, Thiruvananthapuram.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01282/full#supplementary-material

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REVIEW
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# Anti-double Stranded DNA Antibodies: Origin, Pathogenicity, and Targeted Therapies

*Xiaoyu Wang and Yumin Xia**

*Department of Dermatology, The Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, China*

Systemic lupus erythematosus (SLE) is characterized by high-titer serological autoantibodies, including antibodies that bind to double-stranded DNA (dsDNA). The origin, specificity, and pathogenicity of anti-dsDNA antibodies have been studied from a wider perspective. These autoantibodies have been suggested to contribute to multiple end-organ injuries, especially to lupus nephritis, in patients with SLE. Moreover, serum levels of anti-DNA antibodies fluctuate with disease activity in patients with SLE. By directly binding to self-antigens or indirectly forming immune complexes, anti-dsDNA antibodies can accumulate in the glomerular and tubular basement membrane. These autoantibodies can also trigger the complement cascade, penetrate into living cells, modulate gene expression, and even induce profibrotic phenotypes of renal cells. In addition, the expression of suppressor of cytokine signaling 1 is reduced by anti-DNA antibodies simultaneously with upregulation of profibrotic genes. Anti-dsDNA antibodies may even participate in the pathogenesis of SLE by catalyzing hydrolysis of certain DNA molecules or peptides in cells. Recently, anti-dsDNA antibodies have been explored in greater depth as a therapeutic target in the management of SLE. A substantial amount of data indicates that blockade of pathogenic anti-dsDNA antibodies can prevent or even reverse organ damage in murine models of SLE. This review focuses on the recent research advances regarding the origin, specificity, classification, and pathogenicity of anti-dsDNA antibodies and highlights the emerging therapies associated with them.

**Keywords: anti-dsDNA antibody, catalysis, lupus nephritis, peptide, suppressor of cytokine signaling 1 (SOCS1), systemic lupus erythematosus (SLE)**

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***Correspondence:**
*Yumin Xia*
*xiayumin1202@163.com;*
*yuminxia@mail.xjtu.edu.cn*

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## INTRODUCTION

Systemic lupus erythematosus (SLE) is a severe disease that involves dysregulation of the immune system, excessive production of pathogenic autoantibodies (and their upregulation in serum), and multiple immune-system-mediated injuries. Anti-double-stranded (ds)DNA antibodies have been some of the classic diagnostic and nosological criteria of SLE since 1982. In 2012, the high titer of anti-dsDNA antibodies in serum accompanied by biopsy-proven lupus nephritis (LN) was accepted as an independent classification criterion for SLE by the Systemic Lupus International Collaborating Clinics (1). However, the wide spectrum of molecular antibody specificity and complex contexts of antibody generation, as well as the diverse antigen structures to which these antibodies bind, make anti-dsDNA antibodies difficult to accept without further distinction as a classification criterion for SLE (1). Pathogenic anti-dsDNA autoantibodies react with DNA but are

not strictly specific to it. Multiple self-antigens can be recognized by anti-dsDNA antibodies, subsequently triggering apoptosis, inflammatory responses, and tissue fibrosis. Recently, synthesized peptides that mimic a molecular DNA structure were reported to specifically recognize and interact with the anti-dsDNA antibodies, thus pointing to a novel therapeutic opportunity via inhibition of the antigen recognition of anti-dsDNA antibodies (2, 3). This review sheds light on the involvement of anti-dsDNA antibodies in the progression of SLE and provides new insights into the therapies for SLE.

## THE ORIGIN OF ANTI-DSDNA ANTIBODIES

Normally, nuclear antigens, such as dsDNA, are not accessible to the immune system because they are restricted to the nucleus and mitochondria and are quickly degraded by DNases in the cytoplasm and endosomes. However, nuclear materials can be released from apoptotic cells after exposure to ultraviolet light, infection, and drugs. Cells undergo death via different processes. During NETosis, cells extrude DNA and neutrophil extracellular traps (NETs), in which DNA is covered by anti-bacterial substances (4, 5). In apoptosis, DNA cleavage leads to the formation of apoptotic bodies, which contain DNA-bound microparticles (6). In necrosis, high-molecular-weight DNA is liberated after cell lysis.

The released DNA can be recognized by anti-DNA antibodies and compose immune complexes. Extracellular nucleic acids containing immune complexes can be captured by immature dendritic cells (iDCs) via FcγRs; these cells subsequently get activated and migrate from peripheral tissues to lymphatic organs, where iDCs undergo maturation (7). Activated DCs induce the over-representation of costimulatory molecules, such as CD80, CD86, and PD-L1 (8, 9). Furthermore, these DCs trigger redistribution of the MHC II molecule and production of proinflammatory cytokines, such as interferon α (IFN-α), tumor necrosis factor (TNF)-α, and interleukin 6 (IL-6), which are interrelated with the activation of B cells and T cells (10, 11). The immune complexes act as an antigen to stimulate B cells by activating the recognition receptors, such as Toll-like receptors (TLRs). TLR7 and TLR9 are the key receptors for the recognition of self-DNA or immune complexes and trigger the production of IFN-1 and inflammatory responses (12, 13). There are also some intracellular sensors in B cells and in other immune cells that can identify foreign DNA or self-DNA, including TLR9 and cyclic guanosine monophosphate–adenosine monophosphate, contributing to the overproduction of IFN-1 (14–16). Multiple signaling pathways are involved in the IFN-1 production process, which are activated after the interaction between sensors and DNA or another nucleic acid, such as pathways dependent on TLR9 and on stimulator of interferon genes (16, 17). As a key factor in the regulation of the innate immune response, IFN-1 can upregulate B cell-activating factor, promote B-cell differentiation, and suppress regulatory T cell (Treg) function (18, 19). With the help of IFN-1, autoreactive B cells undergo amplification, somatic mutation of immunoglobulin variable region genes, and class switch recombination, resulting in high-affinity immunoglobulin G (IgG) (20). In addition to suppressing Treg cells, IFN-1 can promote Th17 differentiation and increase the number of activated T cells (21). The insufficiency of Treg function contributes to the loss of immune tolerance in SLE via the TLR pathway (22). TLR9 can recognize dsDNA with a CpG motif and is dynamically upregulated in B cells (23). After a knockdown of TLR9, B cells produce fewer anti-dsDNA antibodies, and SLE syndrome is ameliorated in mice (24).

Moreover, autophagy is associated with the immune regulation and is essential for the homeostasis in immune cells. LC3-associated phagocytosis is an autophagy pathway that participates in the endocytosis of DNA or immune complexes by immune cells, especially plasmacytoid DCs. LC3 induces production of IFN-α via the TLR9 pathway in plasmacytoid DCs, whereas beclin-1, another member of the autophagy pathway, inhibits the production of IFN-α (25, 26). Beclin-1 knockdown macrophages can remarkably reduce antibody production and deposition of renal immune complexes, suggesting that autophagy may be an additional mechanism for the production of these antibodies (27).

Anti-dsDNA antibodies recognize diverse DNA structures, including single-stranded (ss)DNA, Z-DNA, bent or elongated dsDNA, DNA:RNA hybrids, locked-nucleic acids, and peptide: nucleic acid hybrids (28). Anti-dsDNA antibodies can bind to a complex of native dsDNA or modified DNA that contains a thymine dimer (generated during DNA photodamage). The chemically modified dsDNA may acquire a higher affinity for autoantibodies and form more stable complexes (29). Otherwise, antibodies can react with many non-DNA antigens in target cells and tissues: annexin II, α-actinin, laminin, collagen III, collagen IV, entactin, complement receptor type 1 (C1q), N-methyl-d-aspartate receptor (NMDAR), ribosomal P proteins, heparan sulfate, and others (30–39) (**Table 1**). Although there are no obvious similarities between DNA and potential cross-reactive antigens, some peptides can bind to both murine and human anti-dsDNA antibodies by mimicking a molecular DNA structure (2, 3).

Anti-dsDNA antibodies have different subclasses, including IgA, IgE, IgG, and IgM. Nonetheless, not all of them contribute equally to tissue injuries in SLE. It is IgG and IgA but not IgM that correlate with disease activity in humans. Most pathogenic antibodies are affiliated with the IgG class in SLE patients (47). IgM antibodies seem to be protective by enhancing the elimination of apoptotic material and via immunomodulatory effects, thereby alleviating renal dysfunction (48). The class switch and somatic mutations of the IgG variable chain can increase the risk of pathological changes. Activation-induced deaminase (AID) in B cells is a key enzyme required for such processes, as confirmed in transgenic or non-transgenic murine models, where a loss of tolerance is accompanied by high production of class-switched IgG (49–51). When lacking AID, lupus-prone mice produce more anti-dsDNA IgM, but not the high-affinity IgG, and show substantial attenuation

**TABLE 1 |** Antigenic recognition of anti-dsDNA antibodies.

| Self-antigen | Cells/Tissue | Contribution to SLE | References |
|---|---|---|---|
| Annexin II | • Mesangial cells<br>• Epithelial cells | • Activate p38 MAPK, JNK, and AKT<br>• Induce secretion of IL-6 | (30) |
| Alpha-actinin | • Mesangial cells | • Change cell shape and migration<br>• Increase production of anti-chromatin antibody<br>• Induce glomerular IgG deposition | (38) |
| Laminin | • Glomerular matrix | • Trigger inflammation<br>• Damage the structure of ECM<br>• Inhibit formation of the capillary structure | (40) |
| Collagen III/ IV | • Glomerular basement membrane<br>• Glomerular matrix<br>• Keratinocytes | • Exacerbate inflammatory infiltration<br>• Activate the Fn14 and SOCS1 pathways | (33, 41, 42) |
| C1q | • Sera | • Induce immune complex deposition<br>• Activate FcR-bearing cells<br>• Induce production of anti-C1q antibodies | (36) |
| NMDAR | • Neuronal cells | • Apoptosis of neuronal cells<br>• Enhance expression of proinflammatory factor<br>• Damage blood-brain barrier | (43–45) |
| Entactin | • Glomerular basement membrane | • Increase production of anti-entactin antibodies<br>• Promote the damage to glomerular basement membrane structure | (39) |
| Ribosomal P | • Hepatocytes<br>• Lymphocytes<br>• Mesangial cells | • Induce production of anti-Rib-P antibody<br>• Enhance secretion of IFN-α and IL-10<br>• Induce T helper 1 responses | (37) |
| Heparan sulfate | • Glomerular basement membrane<br>• Mesangial matrix | • Mediate penetration of living cells<br>• Induce cell apoptosis and inflammation | (35, 46) |

of glomerulonephritis and longer survival than do the wild-type mice (52). In SLE, DNA emerges as an inducer of an antigen-driven selection of B cells and is essential for a somatic mutation. During this process, the insertion of positively charged amino acids (for example, asparagine, arginine, and lysine residues) into complementarity-determining regions (CDRs) is critical for the binding of high-affinity dsDNA to the negatively charged phosphodiester backbone (53, 54). Among almost all IgG antibodies, anti-DNA IgG may have the potential for recognizing somatically produced idiotypes (53). Besides, the IgA and IgE subclasses of anti-DNA antibodies are implicated, independently or in combination with IgG, in lupus nephritis (LN) (47, 55, 56).

## THE DETECTION OF ANTI-DSDNA ANTIBODIES

Tests for anti-DNA antibodies can be positive at least 2 years before clinical diagnosis of SLE, and a surge in serum levels can present as a predictor for a severe SLE flare-up in the following 6 months (57). Subsequently, patients may undergo a course of relapsing–remitting with differences in the possibility of a flare between individuals. Anti-dsDNA antibodies emerge as a heterogeneous population in SLE, whereas they can be detected in some non-SLE patients with rheumatic symptoms, indicating that the employment of anti-dsDNA antibodies as a criterion for diagnosis and classification without subdivision may result in misdiagnosis and misclassifying of SLE (58, 59).

There are a variety of tests for anti-dsDNA antibody detection, such as the Farr radioimmunoassay (FARR-RIA), Crithidia luciliae indirect immunofluorescence test (CLIFT), and enzyme-linked immunosorbent assays (ELISA) (**Table 2**). The former two assays have been well-demonstrated for diagnosis and prognosis, whereas ELISAs are more valuable for detecting high-avidity anti-dsDNA antibodies in clinical laboratories and most closely associated with SLE activity (60). However, the materials used in assays limit their utilization, and researchers have been working on developing new suitable assays with high sensitivity and specificity. Panza et al. explored a novel assay that uses complexed histone peptides (PK201/CAT plasmid) with a fragment from Crithidia luciliae, which has more simple procedures (61). We utilized trypoanosoma equiperdum (TE) that containing uniformed dsDNA but no histone in the cell nucleus or kinetonucleus, which is much easier for purification with a simpler structure (62). Poulsen et al. developed a flow-induced dispersion analysis, offering a more sequence-specific antibody characterization via utilizing short and well-defined dsDNA sequences (63). It also exhibits the advantages of shorter analysis time and less sample volume consumption. Although the association between high levels of anti-DNA antibodies and disease activity has been widely appreciated, no signal-detecting approach can reliably evaluate disease activity in SLE. The current assays are not sufficient for detecting all of the antibodies in sera, especially the low-level antibodies, or the immune complex. Thus, at least two assays should be used for better evaluation and higher accuracy.

## THE PATHOGENICITY OF ANTI-DSDNA ANTIBODIES

### Anti-dsDNA IgG Correlates Closely With LN

Among the affected organs, renal involvement indicates major internal damage in SLE patients. Anti-dsDNA antibodies are present in serum in nearly 80% of patients with LN. Anti-dsDNA antibodies directly or indirectly interact with renal antigens, thus producing immune complexes (32) Nevertheless, renal damage is not initiated solely by the complexes of

**TABLE 2 |** Detection methods with strengths and weaknesses.

| Methods | Strengths | Weaknesses |
|---|---|---|
| FARR-RIA | • Detect high-avidity antibodies<br>• High specificity<br>• Qualitative assay | • Low sensitivity<br>• Use radioactive material |
| CLIFT | • Detect high-avidity antibodies<br>• Qualitative assay<br>• High sensitivity<br>• High specificity | • Only score the kinetoplast fluorescence since nuclei always contain many antigens other than DNA<br>• Lack of accurate serum titer |
| ELISA | • Detect low and high avidity antibodies<br>• Quantitative assays<br>• High sensitivity | • Low specificity<br>• False-positive results due to binding of immune complexes (with negatively charged moieties) |

**TABLE 3 |** Effects on renal cell types.

| Cell type | Proliferation | Inflammation | Fibrogenesis |
|---|---|---|---|
| Mesangial cells | Increase/Decrease? | • ↑ IL-1β, IL-8, IL-6, TNF-α, hyaluronan, lipocalin-2, iNOS, MCP-1 | • ↑ PKC, TGF-β1/MAPK, JAK2/STAT1<br>• ↑ TGF-β1, F-actin<br>• ↓ SOCS1 |
| PTECs | Increase | • ↑ IL-6, IL-8, TNF-α, MCP-1, (NF)-κB, IP-10, MIP-1a, ICAM-1, VCAM-1 | • ↑ TWEAK/Fn14<br>• ↑ MAPK, EMT, c-JNK, ERK, p38, Akt, JAK2/STAT1<br>• ↓ SOCS1 |
| Endothelial cells | Increase/Decrease? | • ↑ IL-1β, IL-8, IL-6, TNF-α | • ↑ JAK2/STAT1<br>↓ SOCS1 |

a chromatin fragment and IgG depositing in the mesangial matrix or glomerular basement membranes. In some studies, enrichment of anti-dsDNA antibodies was not present in all extracted samples from the patients with proliferative LN and accounted for <10% of all antibodies, where overexpression of antibodies to C1q, Sm, SSA, and SSB was also responsible for the process (64). Similarly, researchers also discovered that a mouse strain (NZM.C57Lc4) with genetic modification has a severe renal disorder and the serological testing for anti-dsDNA antibodies yielded negative results (65). Moreover, anti-dsDNA IgG does not exert nephritogenic effects without the exposure of chromatin fragments in glomerular membranes and matrices (66). These results confirm that the initiation of LN is promoted by multiple autoantibodies, not by their single type. Reasonable factors that can explain this discrepancy including the subtypes of antibodies with differences in reactive specificity and affinity that have been described previously, differences in mouse strains utilized for the creation of animal models and other factors need to be illustrated in the future. Despite the doubts about their nephritogenic role in launching LN, the pivotal role of anti-dsDNA IgG and immune complexes in the acceleration of the LN process has been underscored by a wealth of evidence, where their effects on renal resident cells are well-documented (**Table 3**).

Most immunoglobulins are unable to penetrate into living cells (67). However, anti-dsDNA antibodies can penetrate into cells and engage in an interplay with targets. Glomerular cells, hepatocytes, monocytes, fibroblasts, and neuronal cells are vulnerable to penetration by anti-DNA antibodies, mirroring the findings in the liver, spleen, and skin after treatment with penetrating antibodies *in vivo* (68, 69). The cellular penetration of antibodies is assisted by $F(ab)'2$ fragments with the mediation of the antigen-antibody binding region, which is also temperature-dependent and energy-consuming (70). Although the Fc fragment of an anti-DNA antibody contributes to its binding activity with monogamous bivalency, in which both Fab combining sites come into contact with DNA, the binding is not inhibited by the blockage of Fc receptor in mesangial cells (71, 72). In addition, the existence of manifold charged amino acids in CDRs is a specific feature of penetrating antibodies, which is explained by the energy-independent electrostatic interactions of arginine residues in the CDR2 and CDR3 with the negatively charged sulfated polysaccharides on the cell surface (68, 73), suggesting that cell penetration is an intrinsic property of anti-dsDNA antibodies. The DNA–histone complexes binding to purified cell-penetrating antibodies extracellularly can significantly enhance their subsequent cell entry depending on the environment (70). Besides, a wide variety of cognate nuclear antigens can function as endocytic receptors and promote subsequent internalization of antibodies into the cell, e.g., ribosomal P proteins (37), C1q (36), annexin II (30), α-actinin (38), and heparan sulfate (35, 46), further confirmed that the antigen-antibody mediation is crucial for the penetration mechanism. Although it is still unclear how anti-dsDNA antibodies actually cross the cell membrane, these data provide novel insights into antibody-binding endocytic receptors and elucidate their pathogenic role in SLE.

The penetrating anti-DNA antibodies can affect the proliferation and apoptosis of resident cells in LN, but the exact effect remains controversial. The anti-DNA IgG purified from the serum of LN patients has been shown to downregulate miR-10a and to trigger the proliferation of mesangial cells by targeting HOXA1, KLF4, and CREB1. Concomitantly, the expression of IL-6 is also enhanced, which is a crucial cytokine for promotion of the processes of cell proliferation and inflammation (74). Similar effects on cell proliferation are also seen in proximal renal tubular epithelial cells (PTECs) after exposure to the human anti-DNA antibodies; this treatment is associated with upregulated secretion of TNF-α, IL-1β, and IL-6 (75). These findings indicate that such antibodies can promote the proliferation of renal cells mainly by modulating the relevant inflammatory cytokines. These findings are also supported by the characteristic biopsy lesions of LN with deposition of immune complexes, hypercellularity of mesangial cells and epithelial cells,

and infiltration by inflammatory cells. However, monoclonal and polyclonal anti-dsDNA antibodies show the ability to induce apoptosis, either by reacting with antigens on the cellular membrane or by entering the cell (76). Apoptosis can also be induced by these autoantibodies via upregulation of extracellular regulated kinase (ERK) activation and Bcl-2 production in murine mesangial cells. Endoplasmic-reticulum stress is upregulated in human mesangial cells after stimulation by anti-dsDNA antibodies, with activation of nuclear factor-kappa B (NF-κB) and increased expression of relevant inflammatory cytokines (72). This phenomenon may account for the apoptosis of mesangial cells, because enhanced endoplasmic-reticulum stress can cause cell death by changing the expression of multiple factors, including CHOP and Bcl-2, as discussed by Iurlaro (77). This discrepancy may be a result of the differences in the methods or circumstances of *in vitro* studies, but the diffuse proliferation can be observed in renal biopsy specimens from LN patients. Upregulation of apoptosis can manifest itself as DNA cleavage in inflammatory lesions as well as increased lysis of renal proximal tubular cells in the presence of complement (78). The apoptosis of podocytes leads to the destruction of glomerular basement membranes, and this event is accompanied by the aggravated progression of inflammation and fibrogenesis in renal tissues with the proliferation of mesangial cells and PTECs.

It is well-known that accumulation of inflammatory cytokines is sufficient for accelerating the recruitment of immune cells and the induction of inflammatory and fibrogenic processes promoting kidney damage. A wealth of proinflammatory factors, including monocyte chemotactic protein 1 (MCP-1), TNF-α, IL-1β, IL-6, IL-8, hyaluronan, lipocalin-2, and inducible nitric oxide synthase, are overexpressed in both human and murine mesangial cells upon stimulation with anti-dsDNA antibodies (72, 79, 80). Consistently with this evidence, the amounts of these cytokines have been shown to increase in human PTECs under the influence of anti-dsDNA antibody-mediated activation of the mitogen-activated protein kinase (MAPK) pathway; these factors further trigger the infiltration of monocytes and macrophages (81). These cytokines not only augment the infiltration by inflammatory cells but also increase the synthesis of fibronectin, eventually reproducing the features of tubulointerstitial fibrosis (41). Anti-dsDNA antibodies also sustain endoplasmic-reticulum stress and activated NF-κB accompanied by overexpression of cytokines in mesangial cells, resulting in a chronic inflammatory response and renal tissue damage (72). The high-titer anti-dsDNA antibodies are always accompanied by overexpression of TLR9 in podocytes, leading to increased production of TNF-α, IL-6, and IFN-γ (82). Besides, a multitude of signaling pathways are activated or involved in the autoantibody-mediated proinflammatory response. Excessive or persistent activation of tumor necrosis factor-like weak inducer of apoptosis (TWEAK)/fibroblast growth factor-inducible 14 (Fn14) signaling cascade positively correlates with the progression of LN. Anti-DNA IgG enhances the expression of Fn14 in keratinocytes through recognizing and binding to self-antigen, such as collagen III, indicating a relation between anti-DNA antibodies and the TWEAK/Fn14 signals (33). The mRNA expression levels of TWEAK and Fn14 increase in the glomeruli and tubulointerstitium in both LN patients and in a mouse model and act as an inducer of constitutive nuclear factor (NF-κB) activation, which mediates the expression of MCP-1, interferon γ-induced protein 10 (IP-10), macrophage inflammatory protein 1a, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1 (83, 84). Additionally, MAPK, epithelial-to-mesenchymal transition (EMT), c-Jun N-terminal kinase, ERK, p38, and serine/threonine kinase Akt are also involved in TWEAK/Fn14 activation and downstream inflammatory and fibrotic responses (85, 86). Although the precise mechanisms of interaction of anti-DNA IgG and TWEAK/Fn14 have not been directly confirmed, anti-TWEAK antibodies, as well as Fn14 deficiency, are known to ameliorate renal damage induced by anti-dsDNA IgG in a murine model. Inhibition of TWEAK/Fn14 decreases renal IgG deposition without influencing serum anti-DNA IgG levels by relieving inflammation and protecting the filtration barrier, further confirming the crucial participation of the TWEAK/Fn14 signaling pathway in anti-DNA IgG-mediated LN (87–89).

A persistent chronic inflammatory response is always followed by fibrogenesis when there is no effective intervention, thereby leading to abnormal structure and dysfunction of kidneys with characteristic oversecretion of profibrogenic chemokines. Fibronectin is critical for fibrotic progression in glomerulonephritis and is distributed widely in the mesangial matrix, glomerular, and tubular basement membranes, and Bowman's capsule. The formation of glomerular fibronectin is enhanced both in patients and mice with active LN (90, 91). We have demonstrated that anti-dsDNA IgG contributes to renal fibrosis by downregulating suppressor of cytokine signaling 1 (SOCS1) and activating Janus kinase/signal transducer and activator of transcription 1 signals, which modulate the expression of profibrotic genes, transforming growth factor beta 1 (TGF-β1), platelet-derived growth factor B, and connective tissue growth factor (92, 93). In addition to SOCS1, protein kinase C signaling is also simulated by anti-dsDNA antibodies with increased secretion of TGF-β1 (94). As a profibrotic growth factor, TGF-β1 is responsible for the cellular myofibroblast-like phenotype switch, in which the assembly of the actin cytoskeleton induces the synthesis of plasminogen activator inhibitor 1 through the TGF-β1/MAPK pathway in mesangial cells, with an accumulation of the extracellular matrix (95–97). Moreover, pathogenic anti-dsDNA antibodies cross-react with alpha-actinin 4; these data account for the promotion of myofibroblast-like phenotypic changes via upregulation of fibrogenic factors in mesangial cells, including F-actin and TGF-β1, as confirmed both *in vitro* and *in vivo* (38, 98). Anti-DNA antibodies can also enhance the phenotypic changes or EMT in cultured PTECs. The activation of EMT with increased mesenchymal markers contributes to the interstitial infiltration by leukocytes and to tubule-interstitial fibrosis (75). Therefore, we can conclude that anti-dsDNA antibodies play a crucial part in the inflammatory and fibrogenic mechanisms of LN (**Figure 1**); however, the complicated network of inflammatory cytokines and intricate signaling pathways should be investigated further.

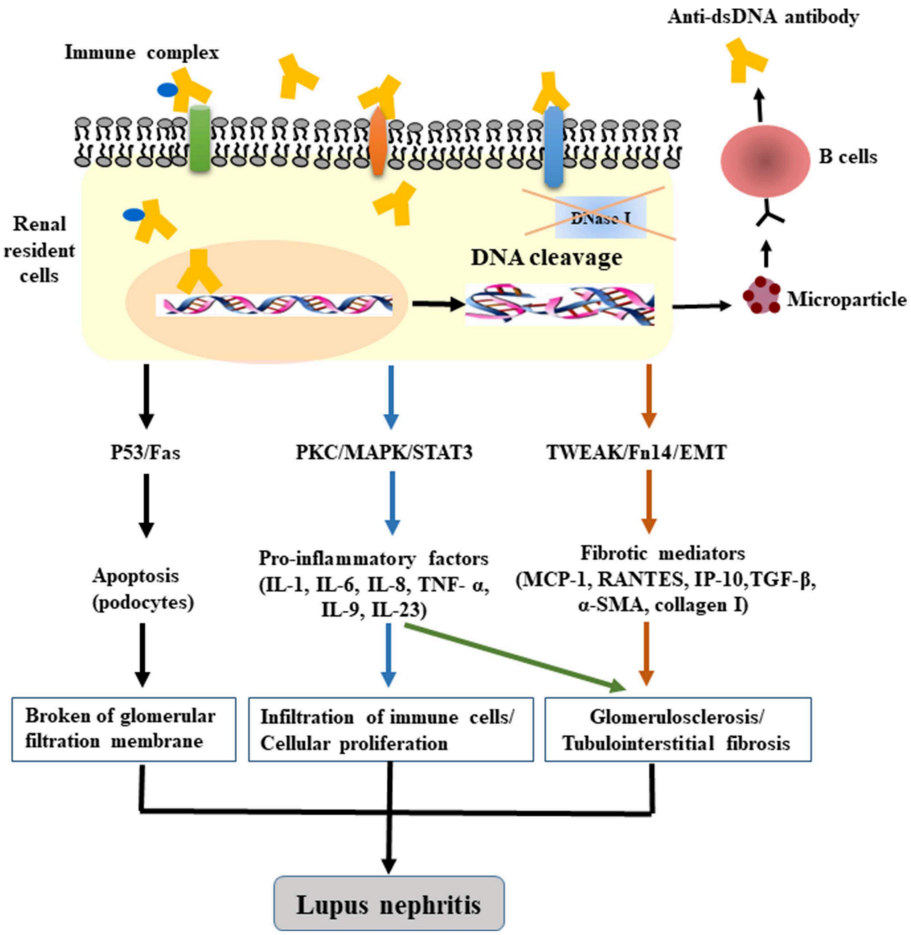


**FIGURE 1 |** The pathogenic mechanism of anti-dsDNA antibodies in LN. After binding to DNA and non-DNA antigens, the penetrating anti-dsDNA antibodies relocate to the cytosol and cell nucleus, cause DNA fragmentation (accompanied by dysfunction of DNase), and induce apoptosis by regulating the gene expression of p53, Fas, or c-myc. The internalized anti-dsDNA antibodies enhance the expression of IL-6, IL-1β, TNF-α, and TGF-β1, activate the PKC, MAPK, TWEAK/Fn14, and EMT signaling pathways, and trigger the fibrotic process. Local deposition of anti-dsDNA IgG—in combination with the secretion of inflammatory or profibrogenic cytokines as well as the recruitment of immune cells—is sufficient for the initiation of renal fibrosis in LN.

## Anti-dsDNA IgG Participates in Other Injuries

Skin is frequently affected by SLE and can be the only affected organ. The serum positivity for an anti-dsDNA antibody is observed in only a subgroup of patients with cutaneous lupus erythematosus. The binding specificity of anti-DNA IgG to skin sections and keratinocytes has been observed *in vitro*, which can be promoted by ultraviolet light irradiation, resulting in keratinocyte apoptosis via antibody-dependent and cell-mediated cytotoxicity (33, 99). The deposition of an immune complex at the dermoepidermal junction has been confirmed in both murine models and patients with serological positivity for anti-DNA IgG (33). Actually, the mild inflammatory response in combination with the presence of autoantibodies is necessary for further cross-reactions with collagen III, collagen IV, and SOCS-1 and−8 for induction of antibody accumulation in skin tissue (42, 100). In addition to LN, the role of TWEAK/Fn14 signals in cutaneous lupus erythematosus has been discovered in murine lupus models (87, 101–103). Ultraviolet B irradiation promotes the anti-DNA IgG binding to keratinocytes as well as the expression of Fn14, which engages in an interplay with TWEAK and subsequently enriches the expression of proinflammatory factors and induces apoptosis (33, 103). These induced factors, including IL-6, IL-8, MCP-1, and RANTES (regulated upon activation, normal T cell expressed and secreted), further amplify the inflammatory responses by attracting immune cells (101).

Central nervous system (CNS) diseases are associated with a poor prognosis among patients with SLE and have been increasingly reported during the past decade. Neuropsychiatric complications occur in 30–40% of patients with SLE and may constitute the initial presentation or a flare (89). Anti-phospholipid, anti-N-methyl-D-aspartate receptor (NMDAR), anti-ribosomal-P, and anti-dsDNA antibodies account for the progression of CNS disease (43). Anti-dsDNA antibodies can cross-react with NMDAR on neurons. An extracellular epitope has been identified near amino acid residue 369 of GlyN1 in

NMDAR and decreases the density of the surface receptor, causing further neuronal disorders, such as abnormalities of cognition and memory (43, 44). Enriched serum anti-dsDNA and anti-NMDAR antibodies are associated with the emergence of neuropsychiatric lupus symptoms (45). Nevertheless, an increased titer of an anti-DNA antibody is not always consistent with neurological dysfunctions (104). The inconsistency may be due to the differences in cognitive assessments applied in diverse studies and the samples tested. The anti-NMDAR/dsDNA antibodies can generate and gain access to the brain tissue after the destruction of the blood-brain barrier, leading to neuronal cell death and abnormalities in mice (45). Injection of mouse brains with an anti-DNA antibody (R4A) causes apoptosis of hippocampal cells (105). Besides, we observed enhanced expression of proinflammatory factors as well as abnormal cognition and behavior in wild-type mice, while the cognition and integrity of the blood-brain barrier are attenuated in Fn14-deficient mice, indicating the amplification of TWEAK/Fn14 signals in the pathogenesis of CNS diseases (106).

## The Constant Region Contributes to the Antigenic Specificity and Pathogenicity of Anti-DNA Antibodies

Classically, the variable region is the only part of an antibody that is credited with the antigenic specificity, whereas the functions of antibodies are determined by the constant region. These two domains are believed to be structurally and functionally independent. However, the specificity and affinity of antibodies are believed to be determined by both regions but not solely by the variable one (107). The variety of isotypes of anti-DNA antibodies is generated through the isotype switch recombination in the sequence from IgM to IgG, whose variable regions are identical to those of the initial antibody. Particular subclasses of anti-DNA antibodies are more closely associated with a pathogenic potential (47). In lupus patients, serum anti-DNA IgG1 is always elevated before the occurrence of renal relapse, while IgG2a, IgG2b, and IgG3 are more frequently eluted from kidneys with active nephritis (78, 108). In addition, different IgG subclasses with similar specificity for DNA show a remarkable difference in the binding properties for basement membranes, the formation of immune-complex deposits, and the severity of the induced proteinuria (109).

To validate the association between subclasses and pathogenicity, we found that different isotypes of anti-dsDNA antibodies differ from each other in the recognition specificity of nuclear and renal antigens when we generated a panel of murine anti-DNA antibodies (IgM, IgG1, IgG2a, IgG2b, and IgG3), which contain identical variable-joining-diversity (VDJ) regions, suggesting that the effect of the constant region on antibody binding is directly associated with autoimmunity (34). In fact, the factors responsible for the pathogenicity of anti-DNA antibodies are diverse, including their binding avidity for DNA, cross-reactivity for self-antigens, characteristics of the idiotype, and localization of specific amino acid residues in CDRs (110). Additionally, the diverse subclasses of autoantibodies that determine the complement fixation and Fc binding are crucial in LN. The isotypes that form the immune complex can fix complement—in a process involving IgG1 and IgG3 in humans and IgG2a and IgG2b in mice—upregulated in the renal glomerulus (111, 112). IgG2a and IgG2b manifest the most robust complement activity and binding affinity for triggering Fc receptors III and IV and are the most potent autoantibodies during the mediation of hemolytic anemia *in vivo* (108). Although IgG2a is the predominant subclass among IgG-reactive IgGs and is present as the most pathological subclass in murine lupus models (113), IgG3 derived from MRL/lpr mice is crucial for the spontaneous glomerular disruption and dysfunction (109). Besides, the constant region of antibodies affects their binding ability toward DNA and histones not only by varying the environments of their paratopes but also by altering their secondary structures (34, 114). Nevertheless, the prognostic value of these subclasses of anti-dsDNA antibodies remains to be illustrated.

## The Catalytic Properties of Anti-dsDNA IgG

The first IgG antibody with DNA-related catalytic activity in SLE was found in 1992 (115). Antibodies binding to nucleoprotein complexes, to DNA, and enzymes that account for nucleic acid metabolism may acquire the feature of the primary antigen-catalytic activity. The amino acid residues of catalytic anti-dsDNA antibodies and DNA enzymes may have similarities because anti-dsDNA antibodies in patients with SLE have been shown to hydrolyze plasmid DNA (116–118). The catalytic activity of anti-DNA antibodies is associated with the binding of magnesium and calcium ions, and their cytotoxicity results from the hydrolysis of nuclear matrix proteins (**Figure 2**) (116). Moreover, the variety of light chains of anti-DNA IgG, the tolerance of temperature, optimum pH, and hydrolysis rate are thought to be responsible for distinct activities of anti-DNA antibodies and their capacity for DNA hydrolysis (119).

In the sequence analysis of anti-DNA antibodies from humans and mice, there is a high frequency of somatic mutations in the VH and VL sequences of anti-dsDNA IgG with high affinity (53). Such somatic mutations result in a higher proportion of certain amino acids, especially arginine, asparagine, lysine, and tyrosine in the CDRs; this phenomenon promotes the formation of electrostatic interactions and hydrogen bonds between these amino acids and DNA (53). In our study, anti-dsDNA antibodies of various isotypes (PL9-11 clone) that share identical variable regions but different constant regions could cleave not only dsDNA but also peptide antigens depending on the isotype, e.g., the ALWPPNLHAWVP peptide, indicating that the catalytic cleavage of DNA can be regarded as a natural property of these antibodies (120). The specific affinity of anti-DNA antibodies to antigens probably determines the possibility of catalysis, but not the catalytic efficiency of the reaction. Although antibodies derived from various sources contain similar variable-chain sequences, these antibodies have different catalytic activities. In addition, in the context of this specific autoantibody system, a specific mutation results in the reduction of DNA binding as well as weakened catalytic activity (121). The DNA binding and hydrolytic activities of anti-DNA antibodies are well-conserved

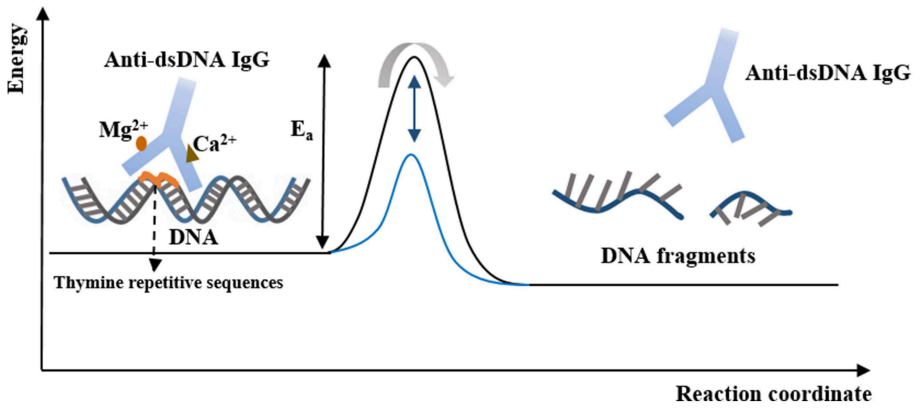


**FIGURE 2 |** The catalytic properties of anti-dsDNA IgG. Anti-dsDNA IgG binds to DNA at the thymine repetitive sequences via tyrosine side chains within a hydrophobic pocket. Hydrolysis of DNA is an energy-intensive process and can be activated by the binding of $Ca^{2+}$ and $Mg^{2+}$. After binding to DNA, the active site of IgG is converted to a transition state, and the DNA fragments are produced and released. At this point, the free IgG binds to another DNA molecule and begins its new cycle, in which IgG stabilizes the transition state of the reaction and lowers the activation energy, and thereby increases the rate of the reaction.

in both VH and VL. The catalytic characteristics of anti-DNA antibodies are isotype-dependent, and constant regions have an influence on the autoantibody-antigen interaction presumably by modifying the secondary structure of antibodies (34, 114, 120).

## THE BLOCKADE OF PATHOGENIC ANTI-DSDNA IGG

### An iDC Vaccine Suppresses the Production of Anti-dsDNA IgG

This therapeutic strategy against SLE is focused on eliminating or controlling active B cells that offer limited benefits but hamper the advancement of treatment for SLE (122, 123). Recently, the significance of iDCs in the induction of clonal anergy and immune tolerance was highlighted. iDCs participate in the anergy of T cells by inducing the apoptosis and promoting the differentiation of Treg cells because the expression of costimulatory factors and MHC molecules on the surface of iDCs decrease considerably (124). As a key cytokine for inhibiting excessive immune responses, IL-10 can reduce the production of relevant cytokines (such as TNF-α and IL-1β,) as well as increase production of inhibitory factors (such as programmed death ligand 1) in DCs, thereby inhibiting Treg responses and the function of effector T cells (125–127). Among these factors, programmed death ligand 1 in combination with TGF-β is particularly important for promoting the conversion of CD4+ T cells to Foxp3+ CD4+ Treg cells, whereas blockade of programmed death ligand 1 can reduce anti-dsDNA IgG production and immune-complex formation in lupus-prone mice by suppressing the synthesis of IL-10 in CD4+ Treg and B cells, thus further validating the therapeutic potential of iDCs (128, 129). After the induction of clonal anergy, T cells fail to bind to cognate antigens that are presented by DCs.

The iDCs exert weaker activating effects on T cells by expressing lower amounts of costimulatory cytokines, including IL-6 and IL-12 in patients with SLE; this observation is consistent with iDCs' effects in lupus-like mice where iDCs lower the responses of Th1/Th2 cells and thus inhibit the secretion of IL-2, IL-4, IL-12, and interferon γ and the formation of anti-dsDNA IgG (130–132). Besides, iDCs from hemopoietic stem cells can be loaded with nuclear antigens, thereby serving as a live cell vaccine (131, 132). Thus, iDCs are emerging as promising immunomodulators in SLE. Nonetheless, there are many challenges regarding the appropriate production protocols and administration route and timing of an iDC vaccine. Moreover, the exact mechanism by which iDCs interact with Treg cells, B cells, and other cells, as well as the possible side effects are largely unclear. Therefore, the translational application of the iDC vaccine in clinical settings requires multidisciplinary efforts.

### DNA-Mimicking Peptides Block the Binding of Anti-dsDNA IgG

Based on the interactions between self-antigens and anti-dsDNA antibodies, novel therapeutic peptides that bind to autoantibodies have attracted increasing attention. These peptides can be synthesized by combining desired amino acid residues or by chemical modification of certain sequences, to ensure higher activity and specificity. To date, therapeutic peptides have proven to be effective against experimental autoimmune diseases, for example, rheumatoid arthritis and multiple sclerosis (133). Moreover, some therapeutic peptides have been demonstrated to prevent anti-dsDNA antibodies from binding and reacting with target antigens and tissues in the studies on SLE.

Peptide DWEYS (D/E W D/E Y S/G), a part of NMDAR expressed on neurons, has been shown to bind to anti-dsDNA antibodies, thereby disrupting the neurotoxicity of anti-DNA antibodies through the NMDAR-activated pathway, in which circulating antibodies penetrate the hippocampal pyramidal cells followed by impairment of spatial cognition (134). In addition, peptide DWEYS can prevent the proliferation of both B cells and T cells and ameliorate the production of

anti-DNA IgG and proteinuria in lupus mice by selectively suppressing the autoreactive B lymphocytes via cross-reaction with the immunoglobulin receptors on B cells (135). Although the DWEYS peptide is certainly beneficial for therapeutic applications owing to the small molecular weight and non-immunogenicity, it is not highly useful due to its short half-life.

FISLE-412, an analog of a reduced protease inhibitor for the human immunodeficiency virus, has the potential to block DNA recognition of anti-dsDNA antibodies (136). FISLE-412 can reduce glomerular deposition of IgG, protect kidneys from damage, and suppress the onset of SLE in murine models. Moreover, the FISLE-12-bound anti-dsDNA antibodies exert no neurotoxic effects on C57BL/6 mice; this finding shows a greater neuroprotective effect than that of the DWEYS peptide (136). As compared with DWEYS, FISLE-412 has higher affinity and a more stable structure and is more suitable for oral administration (136). The study on the structure-activity relationship of several synthesized analogs of FISLE-412 has revealed that the hydrophobic portion is the key region for this therapeutic function (137). In addition, the quinaldic region contributes to the binding activity of FISLE-412 (137).

ASPVTARVLWKASHV is a 15-mer peptide selected by purified polyclonal anti-dsDNA IgG in lupus patients and can inhibit antigen-IgG binding (138). ALW (ALWPPNLHAWVP) is a 12-mer peptide mimic originating from a panel of murine anti-DNA IgG isotypes; it can bind to all four IgG isotypes and prevent IgG binding to DNA or glomerular antigens (3). ALW is physiologically stable and resistant to oxidation, cyclization, and degradation because it lacks a sequence of certain amino acid residues (3). Although ALW and its mimics are receiving increasing attention as therapeutic molecules with less toxicity for lupus patients, a combination of different peptides should be taken into consideration because of the variability and cross-reactivity of anti-DNA antibodies. In addition, peptides have low toxicity when administered orally or subcutaneously and would not be expected to be immunogenic.

pCons (FIEWNKLRFRQGLEW) is a 15-mer peptide derived from murine anti-dsDNA antibodies; it exerts therapeutic effects by preventing antibody-antigen binding (139). pCons significantly enhances the survival rate and alleviates glomerulonephritis in NZB/W lupus mice; this beneficial action may be mediated by the regulation of T-cell autoreactivity (140, 141). The protective effects have been validated in lupus-prone mice treated with a B lymphocyte gene vaccine that codes for IgG1 Fc-pCons, where early and repeated administration of the vaccine delayed proteinuria and enhanced survival remarkably as well as the expansion of Foxp3+ CD4+ Treg cells (142).

hCDR1 (GYYWSWIRQPPGKGEEWIG) is a 19-mer peptide that is based on CDR1 sequences from human anti-DNA antibodies. hCDR1 has suppressive effects on T cells by reducing apoptosis of T cells with less secretion of interferon-γ and upregulation of IL-10 in lupus-prone mice (143). The effects of hCDR1 on B cells are also associated with downregulated levels of anti-DNA antibodies in lupus-prone mice (144). In an early clinical study, weekly subcutaneous administration of different doses of hCDR1 (Edratide)-—0.5, 1.0, and 2.5 mg—was found to be effective in ameliorating the manifestations and in downregulating the production of proinflammatory and proapoptotic cytokines in SLE patients; the affected proapoptotic proteins include IL-1β, TNF-α, IFN-γ, IL-10, caspase 3, and caspase 8 (145). The efficacy of hCDR1 in the treatment of SLE was questioned because of the limited sample size (9 patients) and disease severity from mild to moderate among the patients. In a later clinical phase II trial, the injection of hCDR1 once a week at a dose of 0.5 mg in SLE patients with a larger sample size (340 patients) was found to be safe and effective, with a decreased SLE disease activity index (SLEDAI-2K). However, there was no remarkable difference between the hCDR1 group and placebo group. In addition, the shortest course of efficacy proven by this trial was nearly 1 year, and the primary endpoint was not reached; this factor may be responsible for the insignificance of the therapeutic effects (146). Thus, a longer-term and appropriate endpoint should be scheduled in later studies on hCDR1.

## MAIN CONCLUSIONS AND FURTHER INVESTIGATIONS

Anti-dsDNA antibodies, the hallmark of SLE, constitute a potent parameter for classifying and diagnosing SLE. Their contribution to damage to the kidneys, skin, and brain in SLE has been well-documented. The production of anti-dsDNA antibodies results from combining multiple factors, including abnormalities of dendritic cells, B cells, or T cells and deficiency of a DNase that leads to failure of cleaning released nuclear materials; however, it still need further studies. The effects of penetrating autoantibodies on triggering a complicated inflammatory and fibrotic process underlie the role of anti-DNA antibodies in damaging target cells and organs. The pathogenicity of antibodies highlights the promising therapeutic potential of DNA-mimicking peptides that react with these autoantibodies in SLE, which can ameliorate the manifestations of SLE in murine models. However, the clinical trials progress slowly in which the timing, dosages, route of administration, alteration of bioactivity, and possible adverse effects of such peptides should be taken into account for developing more efficient therapies for SLE.

## AUTHOR CONTRIBUTIONS

YX conceived this paper. XW wrote this manuscript. XW and YX read and approved the final manuscript.

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# Immunological Pathogenesis of Membranous Nephropathy: Focus on PLA2R1 and Its Role

*Wenbin Liu[1,2], Chang Gao[1,3], Haoran Dai[4], Yang Zheng[1], Zhaocheng Dong[1,3], Yu Gao[1,2], Fei Liu[3], Zihan Zhang[3], Zhiyuan Liu[5], Weijing Liu[6], Baoli Liu[1*†], Qingquan Liu[1*†] and Jialan Shi[7*†]*

[1] Beijing Hospital of Traditional Chinese Medicine, Capital Medical University, Beijing, China, [2] Capital Medical University, Beijing, China, [3] Beijing University of Chinese Medicine, Beijing, China, [4] Shunyi Branch, Beijing Hospital of Traditional Chinese Medicine, Beijing, China, [5] Taishan Medical University, Shandong, China, [6] Key Laboratory of Chinese Internal Medicine of Ministry of Education, Beijing, China, [7] Departments of Medicine, VA Boston Healthcare System, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, United States

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**Edited by:**
*Marie-Agnes Dragon-Durey, Université Paris Descartes, France*

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***Correspondence:**
*Baoli Liu*
*liubaoli@bjzhongyi.com*
*Qingquan Liu*
*liuqingquan2003@126.com*
*Jialan Shi*
*jialan_shi@hms.harvard.edu*

*[†]These authors have contributed equally to this work*

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Membranous nephropathy (MN) is the major cause of nephrotic syndrome with special pathological features, caused by the formation of immune complexes in the space between podocytes and the glomerular basement membrane. In idiopathic membranous nephropathy (IMN) the immune complexes are formed by circulating antibodies binding mainly to one of two naturally-expressed podocyte antigens: the M-type receptor for secretory phospholipase A2 (PLA2R1) and the Thrombospondin type-1 domain-containing 7A (THSD7A). Formation of antibodies against PLA2R1 is much more common, accounting for 70–80% of IMN. However, the mechanism of anti-podocyte antibody production in IMN is still unclear. In this review, we emphasize that the exposure of PLA2R1 is critical for triggering the pathogenesis of PLA2R1-associated MN, and propose the potential association between inflammation, pollution and PLA2R1. Our review aims to clarify the current research of these precipitating factors in a way that may suggest future directions for discovering the pathogenesis of MN, leading to additional therapeutic targets and strategies for the prevention and early treatment of MN.

**Keywords: membranous nephropathy, immunological pathogenesis, PLA2R1, PM2.5, lung, kidney**

## INTRODUCTION

Membranous nephropathy (MN) is an immune-mediated glomerular disease that affects 12 new cases out of every million adults every year globally (1, 2). MN is characterized by a pathological change in the glomerular basement membrane (GBM) caused by the accumulation of immune complexes which appear as granular deposits of immunoglobulin (Ig)G when imaged with immunofluorescence and as electron-dense deposits under electron microscopy. These immune deposits in the space between podocytes and GBM contain the complement membrane attack complex (C5b-9). MN can be either idiopathic (idiopathic membranous nephropathy or IMN) or caused partially by clinical disease such as hepatitis B, systemic lupus erythematosus, cancer, or drug side-effect (secondary membranous nephropathy or SMN) (1). However, it is not clear whether these clinical diseases are direct causes of MN or merely triggers, such as the potential association between cancer and MN (3–5). In addition to the typical GBM changes, SMN exhibits mesangial deposits, "full-house" immunostaining (IgG, IgA, IgM, C3, and C1q, mesangial deposits, and

reticular aggregates) in membranous lupus nephritis, and amyloid or fibrillary glomerulonephritis which are seen as fibrils under electron microscopy (**Table 1**) (6). The deposition of immune complexes in SMN may be caused by exogenous antigens "pre-planted" between podocytes and the GBM which bind to circulating antibodies, such as in hepatitis B virus-associated MN (7). The formation of glomeruli subepithelial immune complex deposits in IMN is now believed to be mediated by specific intrinsic podocyte antigens and their corresponding autoantibodies in humans, such as neutral endopeptidase (NEP), M-type receptor for secretory phospholipase A2 (PLA2R1), and Thrombospondin type-1 domain-containing 7A (THSD7A) (8–10). PLA2R1 (70–80% of IMN) and THSD7A (3–5% of IMN), the two major podocyte antigens identified in adult IMN, can be detected through both direct immunofluorescence staining of renal tissue and detection of their autoantibodies in serum for diagnosis and prognosis (1).

In recent years in China, the morbidity of MN has been gradually increasing possibly due to long-term exposure to air pollution, mainly fine particulate matter of $<2.5\,\mu m$ (PM2.5). Each 10 $mg/m^3$ increase in PM2.5 concentration over 70 $mg/m^3$ is associated with 14% higher odds for MN (11). However, the association between the air pollution and the pathogenesis of MN remains unclear. It is worth noting that PLA2R1 and THSD7A are naturally expressed in other parts of the human body, such as the lungs (3). In this review, we have focused on the possible mechanisms of anti-podocyte antibody production, particularly in PLA2R1-associated MN, and proposed several hypotheses, which may be beneficial to further exploration of the pathogenesis of IMN.

## THE DEFINITION AND PATHOLOGY OF MEMBRANOUS NEPHROPATHY

Membranous nephropathy, originally called membranous glomerulonephritis (MGN), was first described in 1946 by Bell as a type of glomerular disease, which manifests pathologically as a thickening of the GBM and is clinically characterized by marked proteinuria and edema (12). In the later half of the 1950s, understanding of the pathology of MN developed rapidly, driven by several outstanding pioneers. In 1956, Mellors and Orgeta discovered immunoglobulins containing in the glomeruli deposits using immunofluorescence. In 1957, Jones demonstrated the presence of silver-positive rods projecting from the GBM using periodic acid silver methenamine stain. And in 1959, Movat defined the causal relationship between thickening of the GBM and protein deposition between the GBM and the podocyte using electron microscopy (12). In this way, these pioneers identified the basic features of glomerular lesions in MN, consisting of changes in GBM structure caused by subepithelial electron-dense deposits (**Figure 1A**). Moreover, in 1968, Ehrenreich used repeated renal biopsy to describe the four stages of glomerular lesions in MN: Stage I consists of only a few small subepithelial deposits such that the GBM may appear normal or be slightly thickened under light microscopy; in stage II spikes protruding from the GBM can be observed using appropriate staining; in stage III the deposits are incorporated within the GBM; in stage IV the GBM appears to be irregularly thickened by reabsorbed deposits, and in complete clinical remission the deposits may disappear and leave some areas lucent or the GBM may return to normal (13) (**Figure 1A**). These pathological discoveries in the middle of the last century not only defined MN as a unique type of renal pathology, but also guided the development of clinical practices that are still in use today (6).

## ENLIGHTENMENT BROUGHT BY HEYMANN NEPHRITIS

### Megalin, the Rat Podocyte Antigen

In 1959, Heymann et al. established an animal model that develops pathological manifestations similar to human MN by immunizing rats with tissue components of the proximal tubular brush border (Heymann nephritis or HN) (**Figure 1B**) (14). This animal model relies on the active immune response of the rat and therefore is often referred to as active Heymann nephritis (AHN). In passive Heymann nephritis (PHN), an analogous lesion can be induced more rapidly by administering heterologous antibody to tubular brush border (anti-Fx1A antibody) (15). At the time, it was thought that the deposition of subepithelial immune complex was caused by circulating immune complexes because Fx1A and its antibody are simultaneously present in glomerular deposits and circulation in HN (16, 17), and the development of immune complexes in circulation was found to be coincident with the glomerular deposits (18). However, in 1978, Couser et al. and Hoedemaeker et al. simultaneously induced HN pathological changes in isolated rat kidneys using anti-Fx1A antibodies suggesting that IMN is not caused by the deposition of circulating immune complexes, but in instead by *in situ* immune complex formed by the autoantibodies binding to antigens located on podocytes (19, 20). In the same year, Makker et al. further confirmed that the podocyte antigen in HN is a mannose-containing glycoprotein (21). And then in 1982, Kerjaschki et al. identified megalin as an antigen on the rat podocyte membrane and tubular brush border (22) (**Table 2**). Consistent with previous studies, the pathogenicity of megalin is related to glycosylation (23), and anti-megalin autoantibodies fail to react with megalin in Western blots run under reducing conditions, indicating that autoantibodies recognize the spatial conformation of their epitopes (24). As the rat-specific podocyte target antigen in HN, megalin contains multiple epitopes (25–27), and demonstrates intramolecular epitope spreading during the progression of HN proteinuria (28). Intermolecular epitope spreading has also been observed with the development of autoantibodies to plasminogen, a known ligand of megalin (24). Although megalin is not the pathogenic antigen in human MN, this series of animal studies reveals the possible pathogenesis of human MN, suggesting that autoantibodies bind to podocyte intrinsic antigens to form *in situ* immune complexes in the glomerular subepithelial. Thus, the search for human podocyte-specific target antigens has become the focus of further studies (29).

**TABLE 1 |** Pathogenic factors, histopathological features, and clinical outcomes of secondary and idiopathic membranous nephropathy.

| | Secondary MN | Idiopathic MN |
|---|---|---|
| Pathogenic factors | **Immune diseases:** Systemic lupus erythematosus, rheumatoid arthritis, hashimoto thyroiditis, sjögren's syndrome, psoriasis, sarcoidosis, mixed connective tissue disease, IgG4-related disease. | **Genetic predisposition:** Two risk alleles (HLA-DQA1 and PLA2R1) were identified in patients with IMN using the genome wide association study of single nucleotide polymorphism (SNP) genes. |
| | **Infections:** Hepatitis B virus, hepatitis C virus, syphilis, schistosomiasis, HIV, helicobacter pylori, streptococcal infection, malaria, leprosy. | |
| | **Drugs and toxins:** Penicillamine, tiopronin, gold, mercury, lithium, formaldehyde, NSAIDs, captopril, clopidogrel. | |
| | **Tumors:** Various solid tumors and lymphomas. | |
| | **Miscellaneous:** Diabetes mellitus, renal transplantation, sickle cell disease, haematopoietic stem cells transplantation. | |
| Histopathological features | **Light microscopy:** Progressive homogeneous thickening of the capillary wall and significant mesangial proliferation. | **Light microscopy:** Progressive homogeneous thickening of the capillary wall. |
| | **Immunofluorescence:** Positive staining for IgG1 or IgG3, IgA, IgM; positive staining for C1q, C3, C4; subepithelial and mesangial immunofluorescence deposition. | **Immunofluorescence:** Positive staining for Mostly IgG4, relatively little IgG1; positive staining for C3, C4, rarely C1q; subepithelial immunofluorescence deposition. |
| | **Electron microscopy:** Electron-dense deposits in subepithelial, intramembranous, mesangial and tubuloreticular inclusions. | **Electron microscopy:** Electron-dense deposits in subepithelial and intramembranous. |
| Clinical outcome | Clinically induced by drugs or toxins, usually followed by spontaneous remission after pathogen withdrawal. Therefore, detailed understanding of the patient's medical history is important. | Spontaneous remission occurs in up to 30–40% of cases; the remaining two-thirds of the patients present with persistent proteinuria, and ∼40% of those will progress to ESRD within 10 years. |

*MN, Membranous nephropathy; HIV, Human Immunodeficiency Virus; HLA-DQA1, The gene encoding HLA complex class II HLA-DQ alpha chain 1(SNP rs2187668); PLA2R1, The gene encoding M-type phospholipase A2 receptor (SNP rs4664308); SNP, Single-nucleotide polymorphisms; IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M; C1q, C3, and C4 are all complement components; ESRD, End-stage renal disease.*

## Human Podocyte Antigens

The results from animal model studies have since been validated in humans (**Figure 1C**). In 2002, Ronco et al. found that the pathogenic antigen in three families of neonatal MN was neutral endopeptidase (NEP) located on the foot process membrane of the podocytes and the brush border of the renal tubules, while the pathogenic antibody derived from NEP deficiency in the mother. This was the first confirmation that the immune complex antigen of human MN could be an intrinsic component of the podocyte membrane (8, 30). More importantly, in 2009 Beck et al. discovered PLA2R1 and its circulating autoantibodies, the first podocyte-targeted antigen-antibody system found in adult IMN (9) (**Table 2**). The presence of circulating anti-PLA2R1 antibodies can be detected in 70–80% of IMN (31–33). As with megalin, PLA2R1 has multiple antigenic epitopes (34–36), and the epitopes spread as the disease progresses (36). The second target podocyte antigen, THSD7A, was discovered in 2014 by Tomas et al. (10) (**Table 2**). These findings not only demonstrate that human MN immune complexes form by the same mechanism as seen in HN, but also gives us reason to believe that there are still undiscovered pathogenic podocyte antigen-autoantibody pairs in IMN (37). Other proteins postulated to serve as autoantigens in adult MN include superoxide dismutase (SOD2), aldose reductase (AR), and alfa-enolase. While these proteins typically localize inside the cell, AR, and SOD2 are also expressed on the plasma membrane of podocytes in patients with MN (24, 38, 39). However, the clinical significance and pathogenic role of these cytoplasmic antigens, and the differences between them and the podocyte membrane antigens (PLA2R1 or THSD7A) in pathogenic autoimmune responses has yet to be clearly demonstrated.

## Complement Activation and Podocyte Injury

Another important area of research regarding MN is the mechanism of kidney injury following the formation of immune complexes (**Figure 1B**). In 1980, Salant et al. found that C3-deficient rats treated with cobra venom factor did not produce proteinuria within 5 days after PHN was induced with sheep anti-rat Fx1A antibody injection (40). Similar results were obtained when HN was established in C6 and C8-deficient rats (41, 42). These findings suggest that complement activation to produce C5b-9 is a key factor in the development of proteinuria in HN. Subsequent studies on experimental MN have further clarified that the pathogenic role of C5b-9 is mainly on podocytes because of its non-dissolving activity. The mechanisms involved include: (1) Inducing podocytes to produce oxygen free radicals; (2) Stimulating podocytes to produce various proteases to cause GBM damage; (3) Influencing a microfilament skeleton structure in podocytes, by separating and redistributing the proteins nephrin and podocin, which are major components of the membrane; (4) Upregulating cyclooxygenase 2 (COX-2) in podocytes, causing damage to the endoplasmic reticulum; (5) Increasing the extracellular matrix by promoting the production of TGF-β by podocytes, leading to GBM thickening and glomerular sclerosis; (6) Promoting podocyte apoptosis and shedding from GBM (43). In addition to the above findings from experimental MN, C5b-9 is also found in the glomeruli and urine of patients with MN, with levels of urine C5b-9 correlating with disease severity and patient prognosis (44, 45), indicating that complement activation is involved in the pathogenesis of human MN. The complement system mainly consists of three early activation pathways (classical pathway, alternative pathway,

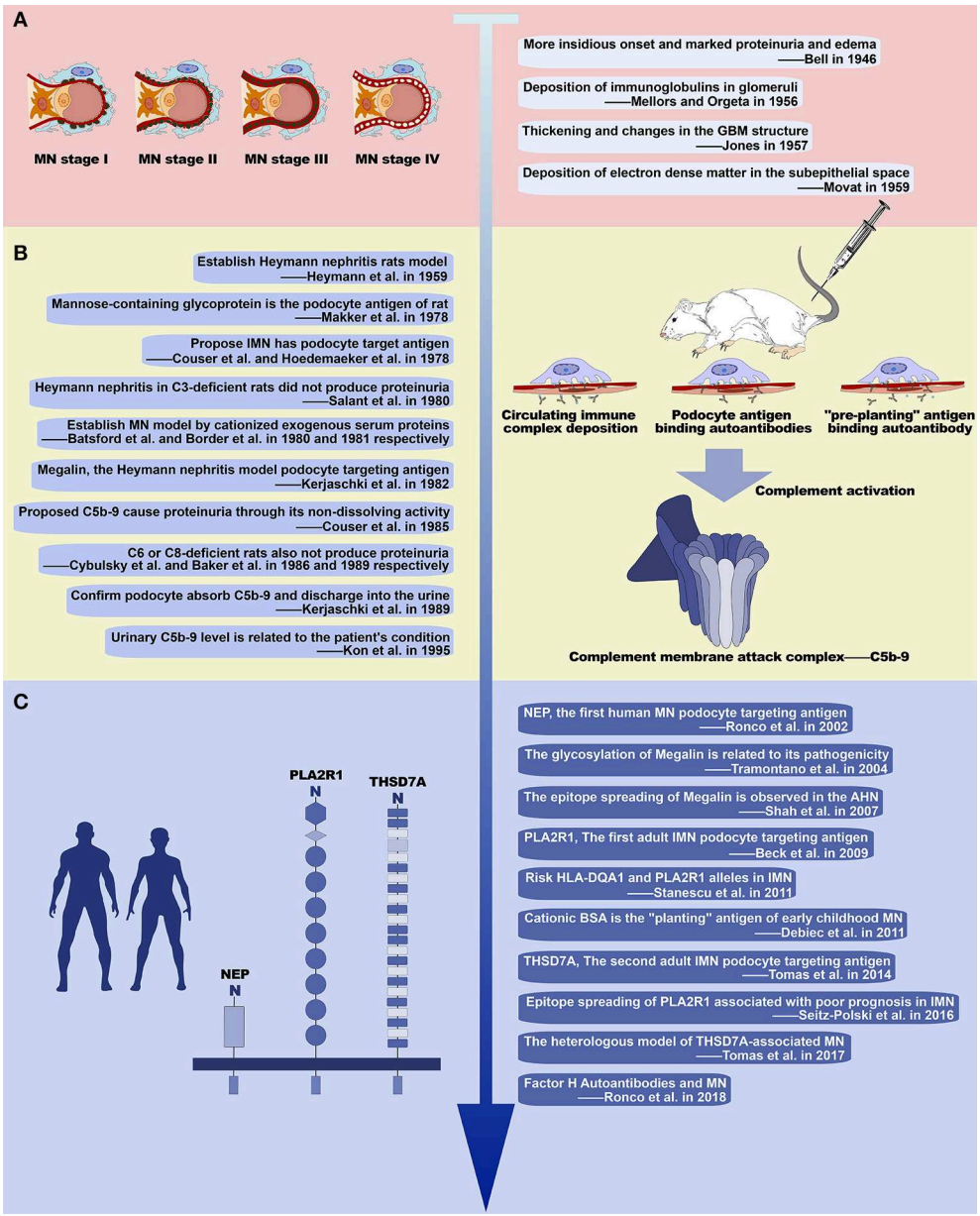


**FIGURE 1 |** History of the study of membranous nephropathy. **(A)** Basic pathological features of MN. Illustration of the progression of glomerular lesions in MN (left) with four early studies describing its basic pathological features (right). **(B)** Studies of experimental MN and important results. Ten seminal studies of experimental MN (left) and the major discoveries relating to development of MN, including immune complex deposition and complement activation (right). **(C)** Recent advances in our understanding of MN. Three major podocyte antigens found in human MN (left), and other major work on MN since 2000 (right).

and lectin pathway), terminal pathway and regulatory system. The formation of C5b-9 is the final step of complement activation (46). Activation of the classical pathway starts with the binding of antibodies to C1q, which can generally be visualized in SMN, and especially lupus-associated MN, by immunofluorescence. However, the subepithelial deposition of immune complexes in IMN mainly consists of IgG4, whose ability to bind C1q is very weak. Indeed, the amount of C1q is very low or undetectable in immune deposits of IMN, indicating that activation of the classical pathway is not the main pathogenic mechanism in IMN (1). Aside from the fact that the presence of IgG1 in early deposits could activate the classical pathway (47), some evidence suggests that the alternative pathway and/or the lectin pathway may be more crucial in the pathogenesis of IMN. Hayashi et al. detected the deposition of mannose-binding lectin (MBL) in glomeruli in IMN, and found that the staining intensity of MBL correlated with the IgG4 staining intensity (48). Bally et al. reported five patients with IMN who had complete MBL deficiency and the

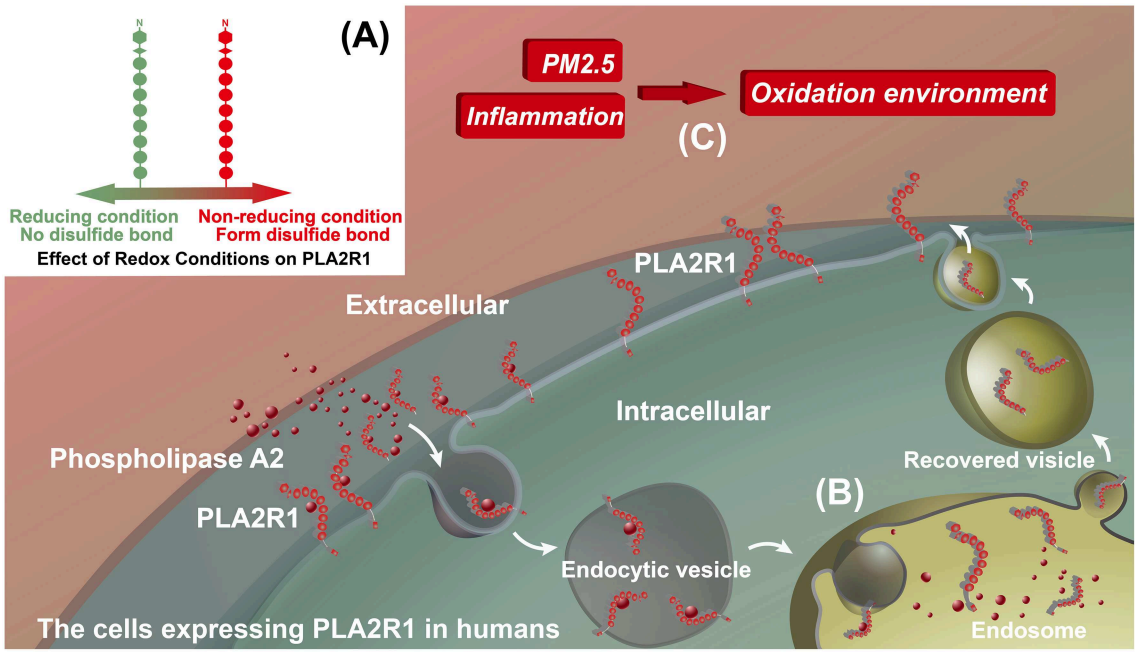


**FIGURE 2 |** Hypothetical model for the effect of inflammation on PLA2R1. **(A)** Effect of redox conditions on PLA2R1. PLA2R1 without disulfide bond under reducing condition (Green) and with disulfide bond under non-reducing condition (Red). **(B)** The potential endocytic property of human PLA2R1. We hypothesize that the extracellular domain of PLA2R1 binds to phospholipase A2 and is transported into the cell by receptor-mediated endocytosis. This model has yet to be supported by any direct evidence. **(C)** Inflammation, PM2.5, and oxidative microenvironments. We hypothesize that inflammation, including that associated with PM2.5, alters the microenvironment of PLA2R1-expressing cells. PLA2R1 exposed to this oxidative microenvironment may form or retain disulfide bonds resulting the long-term expression of pathogenic epitopes.

complement activation was mainly due to the activity of the alternative pathway (49). Moreover, Luo et al. found that factor B-null mice with established MN did not develop albuminuria or exhibit glomerular deposition of C3c and C5b-9, which suggests that the alternative pathway is necessary in the pathogenicity induced by glomerular subepithelial immune complexes (50).

## REVELATION FROM OTHER EXPERIMENTAL MEMBRANOUS NEPHROPATHY

### Exogenous "Planting" Antigen

There is another possibility for the formation of *in situ* immune complexes (**Figure 1B**). In 1980 and 1981, Batsford et al. and Border et al. created an animal model with the typical pathological lesion of MN by intravenous infusion of cationized exogenous serum proteins (51, 52) (**Table 2**). The glomerular subepithelial deposition of IgG and C3 can only occur after immunization with a cationized antigen. After immunization with an anionic or neutral antigen, deposition occurs simultaneously in the mesangial area. Furthermore, the proteinuria of an animal that is immunized with cationized antigen is more severe (53, 54). This is due to the negative charge of the glomerular capillary wall, which interacts electrostatically with the cationized antigen resulting in the "planting" of the exogenous antigen. This exogenous antigen then binds to circulating antibodies *in situ* to form the immune complex. In humans, cationic bovine serum albumin (cBSA) is most often the exogenous "planting" antigen found in early childhood MN (55) when the diet of the children is mainly based on milk products. This suggests that some cases of MN may be related to dietary and environmental factors. Moreover, the formation of subepithelial immune complexes in SMN, such as hepatitis B virus-associated MN, is similar (7).

### Other Causes of Podocyte Injury

Complement activation is the crucial mechanism of MN podocyte injury, but it is not the only one. After Beck et al. discovered PLA2R1 in 2009, Tomas et al. found THSD7A, the second adult podocyte antigen, in 2014 (9, 10). THSD7A and PLA2R1 are large transmembrane proteins with multiple domains which are both expressed on podocyte membranes. However, in contrast to PLA2R1, THSD7A is also expressed in mouse podocytes (56). Human anti-THSD7A containing sera can be used to immune-precipitate THSD7A from mouse glomeruli *in vitro* and *in vivo* injections of human anti-THSD7A can specifically bind to murine THSD7A on podocyte foot processes, inducing proteinuria and initiating a histopathological pattern that is typical of MN (57) (**Table 2**). However, no C3 deposition has been found in the renal tissue of mice shortly following immunization with rabbit anti-THSD7A antibodies, similar to the absence of complement deposition after the injection of purified human anti-THSD7A antibodies (56, 57). These findings

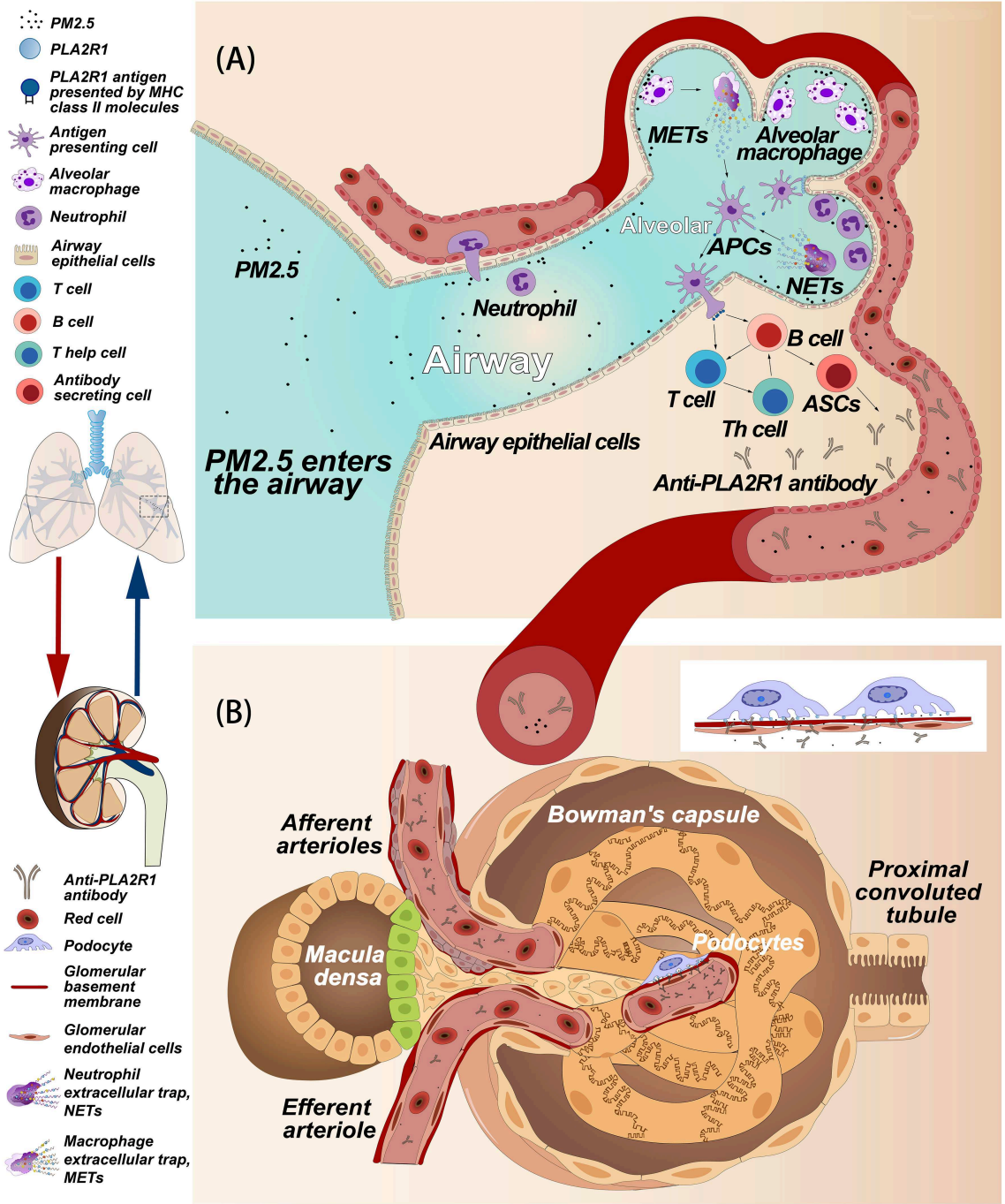


**FIGURE 3 |** Hypothetical model of the relationship between PM2.5 and membranous nephropathy. **(A)** Hypothesis of how PM2.5 induces anti-PLA2R1 antibody production. PM2.5 in the airways and alveoli causes an inflammatory response involving neutrophils, alveolar macrophages, and airway epithelial cells. We hypothesize that these cells may express PLA2R1, that due to oxidative stress associated with inflammation, may assume a conformation that includes pathogenic epitopes that contribute to the formation of autoantibodies. Alternatively, PLA2R1 may be released into the inflammatory space during the release of extracellular traps. PLA2R1 may then be bound by antigen presenting cells, triggering the humoral immune response, and producing anti-PLA2R1 antibodies. **(B)** The hypothetical process of subepithelial immune complex deposition caused by the anti-PLA2R1 antibody exogenous to glomeruli. Both PM2.5 and anti-PLA2R1 antibodies enter blood vessel and circulate into the glomerular capillaries. The anti-PLA2R1 antibodies penetrate the endothelial cells and glomerular basement membrane (GBM), recognize and bind to naturally-expressed PLA2R1 on podocytes to form the immune complex. These complexes then deposit into the space between podocytes and GBM.

indicate that complement activation is not vital in the initiation of podocyte injury and proteinuria in the THSD7A-dependent mouse model of MN. And further, they demonstrate that human and rabbit anti-THSD7A antibodies are directly pathogenic by altering the architecture of podocytes (57). Furthermore, proteinuria also develops in C6-deficient rats with established Heymann nephritis and in C3-deficient mice with established anti-podocyte glomerulonephritis (58–60). In conclusion, the podocyte injury of MN is the result of a complex multifactorial process, especially in IMN where IgG4 deposition predominates, and the mechanism of podocyte injury beyond complement activation needs to be further explored.

# PRODUCTION AND PATHOGENESIS OF ANTI-PLA2R1 ANTIBODY

## PLA2R1 and Its Epitopes

PLA2R1 is a type I transmembrane receptor that is a member of the mannose receptor family (61, 62). In general, the mannose receptors have endocytic properties and circulate continuously between the plasma membrane and the endosome, with ∼70% of the receptors located inside the cell at steady state (61, 63). PLA2R1 is the specific receptor that promotes the internalization of phospholipase A2 (sPLA2), as has been demonstrated in rat vascular smooth muscle cells, rabbit skeletal muscle cells, and human embryonic kidney (HEK 293) cells transfected with rabbit PLA2R1 and human PLA2R1 (64–66). The exact mechanism by which PLA2R1 mediates PLA2 removal has yet to be determined. Therefore, it may act either as a clearance receptor that inhibits, inactivates and removes sPLA2s from the extracellular milieu, or as a signaling receptor that transduces a sPLA2 cellular signal in a manner independent of the catalytic activity of sPLA2 (62). Several studies have shown that, the physiological role of human PLA2R1 in its expression cells is related to sPLA2s (67–69). However, the interaction of the human PLA2R1 to sPLA2 appears very different from that of other mammals (65), and the endocytic properties of human PLA2R1 has yet to be validated by direct experimental evidence. Thus, we can only propose that, hypothetically, the extracellular domain of human PLA2R1 can bind to sPLA2s and transport it into the cell. In this hypothesis, human PLA2R1 is expressed both intra- and extracellularly (**Figure 2B**). The higher pH value for extracellular conditions relative to the endosome, vesicle or intracellular environment may result in a more extended conformation of human PLA2R1 (70, 71), which may contribute to the exposure of the human PLA2R1 epitope. The observed epitope spreading may relate to the neutralization of antibodies that bind to the initial epitope (72), which may causes human PLA2R1 to lose its hypothetic endocytic activity and become exposed to the extracellular environment long-term. The PH-dependent conformational change of human PLA2R1 may lead to the exposure of internal domains, which would otherwise not be recognized by the immune system, triggering autoimmune responses to different epitopes. Interestingly, similar to megalin, the binding of IMN patient serum anti-PLA2R1 antibodies to PLA2R1 *in vitro* needs to be carried out under non-reducing conditions, because addition of a reducing agent eliminates the binding of these antibodies (9, 34–36). These facts demonstrate that the epitope(s) to which anti-PLA2R1 antibodies bind is spatial and requires the presence of disulfide bonds in PLA2R1 (**Figure 2A**). The oxidative extracellular environment may cause PLA2R1 to form or preserve disulfide bonds, resulting in long-term expression of pathogenic epitopes that specifically bind to the anti-PLA2R1 antibodies circulating in the peripheral blood of a patient with IMN (**Figure 2C**).

## PLA2R1 Exposure

In humans, PLA2R1 is expressed not only in podocytes (9), but also in neutrophils (67), pulmonary macrophages (73), airway epithelial cells, and submucosal epithelial cells (69) (**Figure 3A**). Podocytes are highly differentiated glomerular capillary epithelial cells whose big cell bodies swell into the crude urine space while the foot processes adhere to the GBM, which is interlaced with the foot processes of adjacent podocytes (74). Normally, the filtration barrier, which is composed of endothelial cells, GBMs and podocytes, only allows water and small molecules to pass freely, while retaining larger molecules such as plasma proteins (75). Even if the glomerulus is easily damaged by the immune system, antigen-presenting cells such as macrophages or dendritic cells remain inaccessible to podocytes if endothelial cells and GBMs are not damaged (75). We hypothesize that podocytes or other cells expressing PLA2R1 may become damaged and release extracellular vesicles (EVs), which can be measured in the urinary tract (76, 77), leading to the onset of autoimmune activity and the development of MN. EVs are small vesicles with membrane proteins on their surface derived from the source cells. Though it has not yet been proved experimentally, cells expressing PLA2R1 may release EVs carrying PLA2R1 on the surface after stimulation. In order to meet the precise specificity required for antigen-antibody binding (78), the epitopes exposed on these non-podocyte cells must be consistent with the PLA2R1 epitope expressed in podocytes, theoretically in a non-reducing environment.

## Inflammation, Oxidative Stress, and PLA2R1

Both neutrophils and pulmonary macrophages are inflammatory cells that can accumulate at the site of inflammation (79, 80). In an inflammatory environment, reactive oxygen species (ROS) are produced in an amount that exceeds the ability of the cell to detoxify itself, leading to an oxidative stress state (81). Under this circumstance, the intracellular environment becomes strongly oxidizing, which results in disulfide bond formation in cytoplasmic proteins (81, 82). Interestingly, there is a significant correlation between the morbidity of MN patients in China and the concentration of PM2.5 (11). Indeed, respirable particulate matter (PM) in polluted air is also associated with various inflammatory lungs diseases (83–85). The occurrence of pulmonary inflammation caused by PM is associated with the production of ROS and oxidative stress (86). Polycyclic aromatic hydrocarbons and transition metals in the PM can directly produce ROS (87, 88). In addition, target cells in the lungs, such as airway epithelial cells, macrophages and neutrophils,

**TABLE 2 |** Morphological and serological connections between animal and human membranous nephropathy.

| | Human IMN | Heymann nephritis | Cationic bovine serum albumin model | THSD7A-associated Heterologous model |
|---|---|---|---|---|
| Immunoglobulin deposition | Mostly IgG4 | IgG from rabbit or other sources | Rat or rabbit IgG | IgG from human or rabbit |
| Complement deposition | C3, C4, and C5b-9 | C3, C5b-9 | C3, C5b-9 | No early complement deposition |
| Pathogenic antigen | PLA2R1, THSD7A | Megalin | Exogenous cationic bovine serum albumin | THSD7A |
| Identification in humans | – | None | Identification in early childhood MN | 3–5% of IMN |
| Antibodies in peripheral blood | Anti-PLA2R1 antibody, anti-THSD7A antibody | Anti-Megalin antibody | Rat or rabbit IgG | Anti-THSD7A antibody |

*C3, C4, and C5b-9 are all complement components; PLA2R1, The M-type receptor for secretory phospholipase A2; THSD7A, The Thrombospondin type-1 domain-containing 7A.*

**TABLE 3 |** Known and unknown about the pathogenesis of membranous nephropathy.

| | Known | Unknown |
|---|---|---|
| Membranous nephropathy | Changes to the GBM caused by IC deposition. | In addition to complement activation, what are the causes of proteinuria? |
| | (1) Circulating ICs deposition; (2) *in situ* ICs deposition: the podocyte antigen or the foreign "pre-planted" antigen. | What are the physiological functions of PLA2R1 and THSD7A? |
| | IC-associated complement activation leads to proteinuria. | How is complement activated in IMN? |
| | HLA-DQA1 and PLA2R1 are risk alleles in IMN. | What role do risk alleles play in pathogenesis? |
| | The incidence of MN is related to environmental and diet. | How do environment and diet affect MN patient population? |
| PLA2R1-associated MN | PLA2R1 is expressed in multiple places in the human body. | Where the PLA2R1 expose? How and the relevant influencing factors? |
| | The anti-PLA2R1 antibody is the serum marker. | How is the humoral immune response initiated? |
| | PLA2R1 epitope spread as the disease progresses. | How does epitope spreading occur? Why is it associated with disease progression? |
| THSD7A-associated MN | THSD7A-associated MN is significantly associated with malignancies. | What is the role of THSD7A in membranous nephropathy and malignancies? |

*GBM, glomerular basement membrane; ICs, Immune complexes.*

can also produce ROS when exposed to PM (87–90). In theory, these cells could aggregate in the airway or alveolar and express PLA2R1 with pathogenic epitopes in response to PM and oxidative stress. PLA2R1 could also then be discharged into the inflammatory space when the neutrophils or macrophages release extracellular traps (91, 92) (**Figure 3A**). Interestingly, rat PLA2R1 expressed on lymphocytes and granulocytes can be up-regulated by interleukin-1β *in vitro* (93). However, it is unclear whether this is the case with human PLA2R1. Most of the cells involved with inflammation are also capable of presenting antigens (94). Therefore, in theory, PLA2R1 in an inflammatory environment may be more easily recognized by the immune system, thus triggering an autoimmune response (**Figure 3A**). In addition, PM2.5 exposure in lung could also be considered as a danger signal that induce activation of autoreactive T cells, which also may a trigger be required for induce autoimmune response. However, further studies are needed to explore these hypotheses.

## Risk Genes and Anti-PLA2R1 Antibodies

Two risk alleles (HLA-DQA1 and PLA2R1) were identified in French, Dutch, and British patients with IMN using the genome-wide association study of single nucleotide polymorphism (SNP) Genes (95). Moreover, there is likewise a correlation between HLA-DQA1 and PLA2R1 risk alleles in patients with IMN in the Spanish and Chinese populations (96, 97). Interestingly, anti-PLA2R1 antibodies were detectable in 73% of patients with both PLA2R1 and HLA-DQA1 high-risk genotypes and in none of the patients with both low-risk genotypes (98). HLA-DQA1 produces a receptor protein on antigen presenting cells as part of the major histocompatibility complex, and PLA2R1 produces the specific podocyte target antigen of IMN patients, both of which are related to the triggering of autoimmune responses. Gupta et al. proposed that after PLA2R1 protein has been processed and displayed on the surface of antigen presenting cells as peptides bound to the class II receptor (DQA1) groove, the genetics of DQA1 will shape the amino acid structure of its receptor groove to fit with the peptide sequences available from PLA2R1. Additionally, the genetics of PLA2R1 may control the fragmentation of protein or the level of transcript due to a change in the number of positions of enzyme cut sites, leading to higher levels of peptide (98). The two risk alleles of IMN may be involved in the formation of anti-PLA2R1 antibodies. However, the SNPs identified are common and thus can only partially explain the onset of MN in an individual, even in an individual with multiple risk factors. Therefore, further studies are needed to identifying the triggers or environmental factors that can contribute to MN. It would be especially useful to compare IMN patients with a control population that carries the risk allele (98).

## Pathogenesis of Anti-PLA2R1 Antibodies

In the case where non-podocyte cells initiate the autoimmune response, the process by which the circulating autoantibodies

locate, recognize, and bind the target antigens on podocytes is similar to the process seen in the PHN animal model. The exogenous antibodies can penetrate endothelial cells and the GBM to bind with the podocyte target antigen (15) (**Figure 3B**). However, just as megalin glycosylation and spatial conformation determine its pathogenicity in HN (23), antigenicity of PLA2R1 also requires some additional conditions. When the anti-PLA2R1 antibodies are produced by non-podocyte cell sources in and or enter the circulation, the antibodies will only bind to the podocyte PLA2R1 under non-reducing conditions (9, 34–36). As a cellular transmembrane receptor (61, 62), the extracellular domain of PLA2R1 is in contact with the extracellular environment and thus is most likely to be in the naturally expressed spatial conformation (**Figure 2B**). PM2.5 also causes early kidney damage through oxidative stress or inflammation (99), which may contribute to the development of non-reducing conditions in the renal microenvironment. Autoantibodies binding to podocyte antigens *in situ* form immune complexes, which result in podocyte injury (1). However, since the immunoglobulins deposited in IMN are mainly IgG4 whose ability to bind C1q is weak, complement may be activated from non-classical pathways (Please refer to the paragraph of "Complement activation and podocyte injury"). Beyond that, antibody binding can also inhibit the normal function of the antigenic protein (72). PLA2R1 may be involved in the adhesion of podocytes to GBM and the serum anti-PLA2R1 antibodies may interfere with adhesion by binding to PLA2R (100), suggesting that serum antibodies binding to podocyte PLA2R1 may cause kidney damage through more than just complement activation.

## CONCLUSIONS

Although research on MN has greatly improved quality of life in the patients, there are still many unsolved mysteries (**Table 3**). In this review, we have mainly discussed the pathogenesis of PLA2R1-associated MN, and proposed some hypotheses based on the available research. We believe that further research into these questions will be beneficial to the clinical treatment of IMN patients by further revealing mechanisms behind the development of pathogenic antigens and antibodies and searching for treatments that prevent or inhibit the resultant kidney damage.

## AUTHOR CONTRIBUTIONS

WenL collected most of the material for reviewing and wrote the main part of the review. CG and HD collected the rest of the material for reviewing. YZ, ZD, and YG wrote the rest part of the review. FL, ZZ, and ZL made the figures and tables. WeiL, JS, QL, and BL discusses and modifies the content of the review article.

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