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Acquired drivers of C3 glomerulopathy

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ABSTRACT

C3 glomerulopathy (C3G) is a group of heterogeneous ultrarare kidney diseases characterized by dysregulated activation of the complement alternative pathway (AP) leading to excessive C3 cleavage. Diagnosis relies on kidney biopsy showing predominant C3 deposition in the glomerular basement membrane, with electron microscopy differentiating between dense deposit disease (DDD) and C3 glomerulonephritis (C3GN). The main drivers of AP dysregulation in C3G are acquired rather than genetic and consist primarily of autoantibodies called nephritic factors (C3Nefs, C4Nefs and C5Nefs) that bind to and stabilize complement convertases, causing complement overactivation. Current therapies are largely supportive, and existing complement-targeting treatments, such as eculizumab, demonstrate limited efficacy. Challenges in studying C3G include variability in autoantibody detection and a lack of standardized assays, which complicates clinical interpretation. Comprehensive assessment involving autoantibody panels, complement biomarkers, functional assays and genetic testing provides a more complete understanding of disease dynamics; however, key knowledge gaps remain regarding Nef origins, mechanisms and their pathogenic role. In this review we discuss acquired drivers of C3G with an emphasis on C3Nefs and C5Nefs and suggest areas of interest that might benefit from future research.

Keywords: complement, C3 glomerulopathy, nephritic factor, autoimmune disease, C3 convertase

INTRODUCTION

C3 glomerulopathy (C3G) is a descriptive term used to encompass a group of ultrarare kidney diseases of heterogeneous aetiology that share a common pathophysiology. While the salient phenotypic features have been known for decades, the term 'C3G' is relatively new and is based on consensus diagnostic criteria established in 2013 [1, 2]. The pathogenesis of C3G is driven by complement dysregulation, initiated through the classical, lectin, and alternative pathways, all of which converge on the formation of the C3bBb complex—the highly dynamic and tightly regulated C3 convertase of the AP. In C3G, dysregulation of this convertase primarily leads to uncontrolled C3 cleavage and subsequent deposition of C3 fragments in the glomerular basement membrane [3]. The diagnosis of C3G requires a kidney biopsy demonstrating a 2-fold predominance of complement C3 deposition over other immunoreactants in glomeruli [4]; electron microscopy findings are required to further subdivide C3G into dense deposit disease (DDD) or the more common C3 glomerulonephritis (C3GN) [5, 6].

The causes of AP dysregulation that drive C3G are broadly categorized as idiopathic, genetic or acquired [7]. Given that acquired drivers occur most frequently in C3G, a comprehensive understanding of their pathogenesis is essential and will therefore be the focus of this review. Acquired drivers of C3G consist primarily of oligoclonal autoantibodies that bind to a variety of individual complement proteins or multimolecular complement convertases *in vitro* and are associated with a dysregulated complement milieu *in vivo* [8–11]. The most frequently observed autoantibodies in C3G are termed 'nephritic factors', or Nefs. The two most studied types of Nefs are C3 and C5 nephritic factors (C3Nefs/C5Nefs) that bind to and stabilize C3 or C5 convertases,

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respectively. Occurring less frequently, but still pathologically relevant, are autoantibodies targeting the classical pathway convertase C4b2a (i.e. 'C4Nefs'), as well as autoantibodies directed towards complement regulatory proteins [factor H autoantibodies (FHAAs)] or individual convertase components [factor B autoantibodies (FBAAs) and C3b autoantibodies (C3bAAs)] [12].

The goal of this review is to present a brief overview of the current thinking around acquired drivers of C3G, with a focus on Nefs, to spotlight conceptual gaps and to suggest areas of future investigation that may prove fruitful as the field moves forward.

AUTOANTIBODY TESTING IN C3G

Historically, multiple challenges have arisen when attempting to study autoantibodies in C3G, making it difficult to perform traditional autoantibody titres or to confirm autoantibody function. Some of these challenges include difficulty in detection due to autoantibody/Nef heterogeneity; for Nefs, the lack of a known epitope and the extremely short half-life (≈90 s) of their antigenic convertase targets; a lack of standardized consensus assays; variability in testing methodology, including assay sensitivity and specificity; changes in autoantibody levels in patients over time; the presence of complement-binding autoantibodies in healthy individuals at low frequency and a lack of robust correlations with clinical outcome due to low disease prevalence paired with the previously noted challenges. Some autoantibodies in C3G, such as FHAAs and FBAAs, bind single proteins that are more stable, thus making the study and detection of these autoantibodies relatively straightforward [13, 14]. As a consequence, the mechanisms-of-action of FHAAs are better understood in spite of their low frequency occurrence in C3G patients [11, 15]. Nefs, on the other hand, are the most common driver of C3G and bind labile and dynamic multimolecular complexes that make their detection and quantification far more challenging [16–20].

Traditionally, Nefs are identified and/or categorized based on their ability to bind to fully formed complement convertases as commonly measured by enzyme-linked immunosorbent assay, surface plasmon resonance or biolayer interferometry [21–23]; cause consumption of complement proteins in fluid phase as measured by C3 and/or C5 cleavage product detection [24, 25]; stabilize the normally labile C3/C4/C5 convertases as measured in various haemolytic assays [18, 26–31] and, most recently, increase C3 convertase formation as measured by a Luminex assay [32]. Numerous variations of these overarching testing methodologies have been documented. Although haemolytic-based stability assays are currently considered the gold standard for Nef detection, each assay has its own unique strengths and limitations, and no consensus has been reached as to what classifies true 'Nef positivity'. Consequently, Nef detection remains a major challenge, requires significant expertise and is typically performed in a small number of specialized laboratories [33].

Clinical interpretation of autoantibody tests in C3G is inherently complex and requires a comprehensive approach (Fig. 1). Whenever possible, an autoantibody panel to detect the trio of C3Nefs, C4Nefs and C5Nefs, as well as FBAAs and FHAAs, should be performed in parallel with assays to measure plasma levels of complement proteins (C3, factor B, etc.), their activation products (C3c, Ba, Bb, sC5b-9, etc.) as well as overall pathway integrity (CH50, APFA, etc.). Finally, complement genetic testing, combined with clinical information enhances the overall evaluation. These four diagnostic pillars (autoantibodies, complement biomarkers, functional assays, genetics; Fig. 1) offer a detailed overview of fluid-phase complement dynamics [34–37]. By examining these findings in aggregate, the correlation between autoantibody positivity and *in vivo* complement activity can be more accurately understood.

The many challenges, nuances and advancements of Nef testing are beyond the scope of this article, and we direct the reader to an excellent review by Michels *et al.* [38]. We propose that for an autoantibody to be classified as a true C3G 'nephritic factor' the following *in vitro* criteria should be met: the autoantibody binds to an active multimolecular convertase (e.g. C3Nefs should bind to C3bBb), as compared with individual convertase components, such as factor B only; and the binding results in increased convertase stability and/or activity. Ideally, *in vitro* Nef measurements should be quantitated, compared with controls and be highly reproducible. Conceptually, we would add that a true Nef, by definition, functionally drives the C3G phenotype, although at present this criterion is nearly impossible to test directly in human or animal models.

C3Nefs

Of the known disease drivers in C3G, C3Nefs (alone or in combination with other Nefs or complement autoantibodies) occur in ≥50% of cases, with some estimates as high as 80% [7, 28, 29, 39–41]. C3Nefs are present in up to 70% of DDD patients and ≈30% of C3GN patients [42].

Mechanistically, C3Nefs bind to C3bBb convertase, prolonging its half-life and/or preventing accelerated decay by interfering with regulatory proteins such as factor H, complement receptor 1 (CR1) and/or decay accelerating factor (DAF) [43, 44] (Fig. 2). In this regard, Nefs are analogous to the C3bBb stabilizing molecule properdin, although they are dysfunctional and unregulated. It remains uncertain whether Nefs and properdin share overlapping binding sites on the C3bBb complex [45, 46].

Historically, the term 'nephritic factor' was first coined in 1969 by Spitzer *et al.*, to denote an unknown substance found in sera of hypocomplementaemic glomerulonephritis that promoted C3 cleavage [47, 48]; however, it would take another 8 years before the responsible molecules were identified as oligoclonal immunoglobulins [49, 50]. Currently, it is widely accepted that Nefs are most frequently of the immunoglobulin G (IgG) isotype, and when testing for Nefs, the majority of labs enrich and examine the IgG fraction alone. While we take a similar approach, we point out that the role of non-IgG isotypes in C3G is largely absent from the literature. Thus the question of whether non-IgG Nefs exist remains somewhat open. For example, since all naïve B cells express IgM and IgD prior to class switching, the existence of IgM-positive Nefs seems possible; of note, IgM-positive Nefs have been generated *in vitro* from membranoproliferative glomerulonephritis (MPGN) peripheral blood mononuclear cells (PBMCs) stimulated with mitogen pokeweed [51]. Nonetheless, to date, all Nefs identified in patient sera have been exclusively IgG.

Within the IgG subclass, IgG1 and IgG3 are recognized as strong activators of the classical pathway; however, it is unclear whether IgG subclass plays a role in AP regulation [52, 53]. Prior efforts to characterize the predominant isotype of Nefs have been limited to a small number of patient samples. These early studies suggest an enrichment of IgG3 in the Nef-positive serum fraction of MPGN patients [50, 54–57]. Interestingly, the first reported FBAA in DDD was also IgG3 and had C3Nef-like properties [58]. Moreover, the dominant isotype of FHAAs appears to be IgG3 as well [59].

IgG3 is unique among the four IgG subclasses, as it has a longer constant chain, more flexible hinge region that aids in

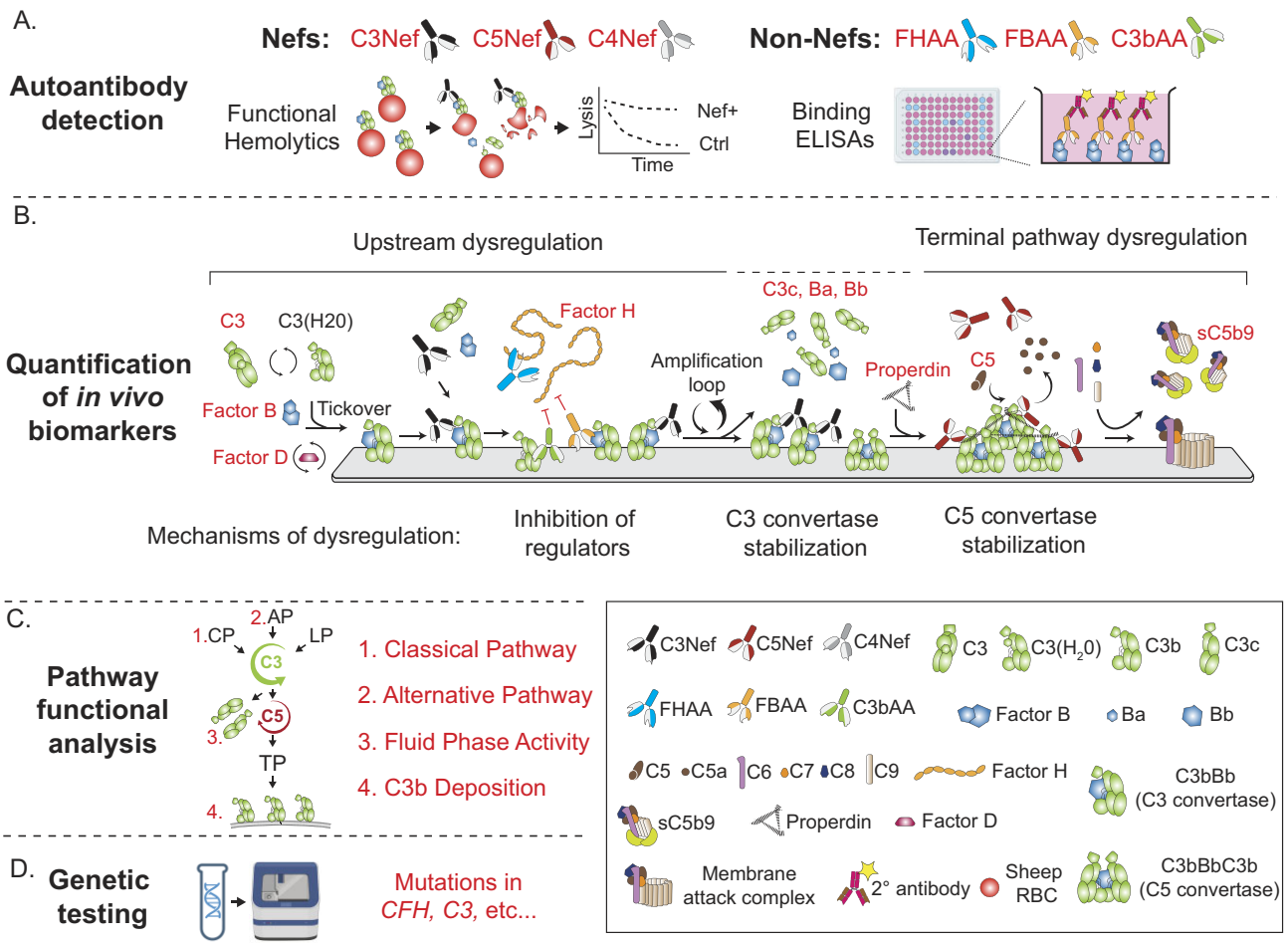

Figure 1: The four pillars complement diagnostics. For the entire figure, red font indicates all autoantibodies, biomarkers, functional assays and genetics that can be measured clinically. **(A)** Nef (left) and non-Nef (right) autoantibodies that can be clinically detected and the clinical assays most often utilized to identify them (directly below). **(B)** Quantification of *in vivo* biomarkers; diagram outlining alternative pathway flux. Clinical biomarkers commonly detected in patient samples are listed in red. Mechanisms of potential dysregulation are shown pictorially and listed below. **(C)** Pathway analysis; clinical assays commonly used to quantitate overall complement pathway activity are listed in red. **(D)** Genetic testing for known drivers of C3G. ELISA: enzyme-linked immunosorbent assay; CP: classical pathway; AP: alternative pathway; LP: lectin pathway; TP: terminal pathway; CFH: complement factor H gene; RBC: red blood cell.

classical pathway activation and greater allotypic variation [52, 60–62]. However, it remains unclear whether these features play a role in AP dysregulation, as the increased frequency of IgG3 Nefs in C3G may simply reflect a dysfunctional 'bottleneck' in the overall class-switch response. Such a trend has been observed previously in patients with low/absent C3 levels—a hallmark feature in C3G patients [63]. Moreover, complement deficiencies in general are known to have an effect on circulating isotypes [64], as are the various immunomodulating therapies commonly used to treat C3G patients [65]. Ultimately, additional studies will be necessary to determine if the increased frequency of IgG3 seen in C3G is an epiphenomenon or if IgG3 has a functional role in disease onset and/or progression.

As a testament to the difficulty in characterizing Nefs at the individual level, to date only three publications report identifying a monoclonal Nef, or Nef-like monoclonal antibody, despite their discovery more than half a century ago. In one series of articles highlighting a monoclonal Nef, PMBCs were harvested from MPGN patients, immortalized with Epstein–Barr virus, then screened for Nef activity. The authors DNA sequenced the positive hit and presented the results as a C3/C5 nephritic factor. To our knowledge, this finding has not been validated or replicated [66, 67]. In other studies, two different groups identified murine anti-C3d monoclonal antibodies that displayed C3 convertase-stabilizing properties, suggesting an unappreciated role of the TED domain (i.e. C3d) in convertase stability and Nef biology. However, both antibodies failed to prevent factor H–mediated decay [68–70]. Beyond these singular examples, the majority of evidence suggests that C3Nefs are polyclonal and quite heterogeneous in terms of their sequence, structure, binding site and function, both within a single patient as well as between patients [38].

C5Nefs

Conceptually, C5Nefs increase terminal pathway flux by causing dysregulated overactivity of the C5 convertase. However, the reality of distinguishing C3Nefs from C5Nefs through *in vitro* assay detection is a topic of spirited discussion and serves as a testament to the complexity of the complement system. While recent structural discoveries of C3b bound to factor B or Bb make it possible to speculate how, or where, an autoantibody might bind

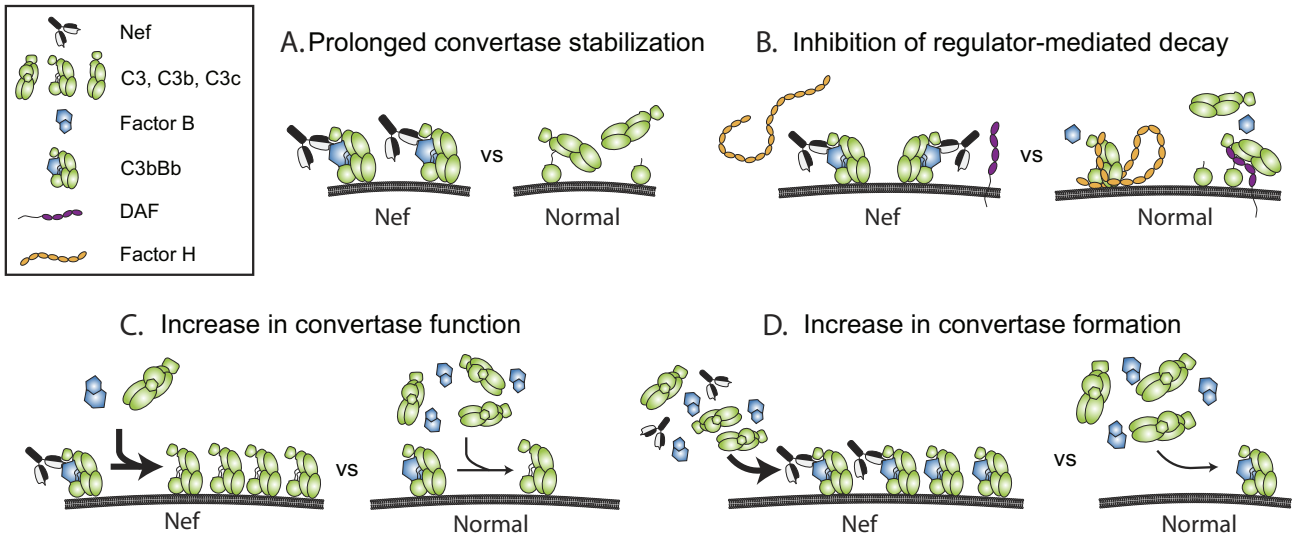

Figure 2: Diagram depicting potential mechanisms for Nef-mediated dysregulation of complement activity. Figures depict C3Nef-mediated dysregulation. **(A)** Nef-mediated prolonged stabilization of convertase. **(B)** Nef-mediated inhibition of regulators of complement activity. **(C)** Nef-mediated convertase activation and consequent increased opsonization and convertase flux. **(D)** Nef-mediated increase in the formation of convertases. DAF: decay accelerating factor.

the C3 convertase [71, 72], the lack of a definitive C5 convertase structure makes it more challenging to predict the possible binding characteristics of a C5Nef. Nevertheless, early steps to distinguish C3Nefs from C5Nefs began with the identification of Nefs whose binding and function were shown to be dependent on the presence or absence of the complement stabilizing molecule properdin [28, 45, 73–75]. While Nef binding and/or functional dependency on properdin alone is not necessarily synonymous with C5Nefs, the concept does have merit. Specifically, properdin is known to bind to C3 convertase and promote its stabilization [46]. The result of this stabilization is increased conversion of C3 to C3b, followed immediately by dramatic increases in the local density of C3b molecules. The increased density of C3b is known to be a driving factor in the switch from C3 cleavage to C5 cleavage, and hence from a C3 convertase to a C5 convertase [72, 76]. Therefore, the addition of properdin during the convertase formation step in haemolytic convertase-stabilizing assays has been used *in vitro* to differentiate the function or presence of C3Nefs versus C5Nefs [21, 28, 29, 42]. In this context, the most salient question is which of the two scenarios is necessary for C5Nef binding/activity: direct physical contact with properdin or properdin-dependent changes in C3b density and/or C3b orientation? Although currently imperfect, the ability to differentiate C3Nefs from C5Nefs has diagnostic relevance, as early data suggest C5Nefs are more prevalent in patients with C3GN than DDD, which may help distinguish the two subtypes [29].

C4Nefs

Following the identification of C3Nefs, C4Nefs were discovered as stabilizers of the classical pathway convertase (C4b2a) in patients with postinfectious glomerulonephritis, partial lipodystrophy, and systemic lupus erythematosus [77–79]. More recently, C4Nefs have been identified in small subsets of C3G cohorts, representing ≈5–15% of patients [12, 30, 80]. Like C3Nefs, C4Nefs prolong convertase half-life, although in the case of C4Nefs it is the classic convertase. Half-life is prolonged through two overlapping mechanisms: first, C4Nefs prevent the natural decay of C4b2a; and second, they block complement regulator C4 binding protein (C4BP) and CR1 to prevent its extrinsic decay [81, 82].

The stabilization of the classic convertase C4b2a leads to increased C3 cleavage into C3b. The increase in C3b, in turn, initiates formation of the AP convertase C3bBb, as well as the classic C5 convertase C4b2aC3b, both of which result in dysregulation through increased complement activation. Although not stated explicitly, many of the mechanistic concepts and gaps in knowledge that we discuss in this review regarding C3Nefs and C5Nefs reasonably apply to C4Nefs.

FHAAS, FBAAS AND C3bAAs

In addition to Nefs, a small subset of C3G patients develop autoantibodies to the complement regulatory protein factor H or to the convertase components C3b and/or factor B, which can be associated with poor outcomes [83–85]. FHAAs were first discovered in 1992 in an MPGN patient with monoclonal gammopathy of renal significance in the form of free lambda light chains [86] and much later as complete IgG in confirmed C3GN and DDD patients [87–90]. In most C3G patients, FHAAs form immune complexes with circulating FH [11, 91]. FHAAs predominantly recognize epitopes within the N-terminal short consensus repeat (SCR) domains 1–4 or the C-terminal SCRs 18–20 of factor H–regions critical for complement regulation and host recognition [59, 91]. FBAAs were first detected in 2010 in a DDD patient negative for C3Nefs [58] and shortly thereafter in two additional DDD patients who also had C3Nefs [92]. Compared with FHAAs, less is known about the epitope specificity of FBAAs and how binding might influence the degree of convertase stabilization. In general, FBAAs and C3bAAs cause aberrant activation of complement through convertase stabilization or by blocking regulators, while FHAAs prevent FH from promoting normal convertase decay.

CO-OCCURRENCE OF AUTOANTIBODIES IN C3G

One major challenge in understanding the contribution of individual Nefs to complement dysregulation is their frequent

co-occurrence with other complement-targeting autoantibodies. Current values on how many individuals co-express complement-directed autoantibodies are likely underestimates, given that most labs do not routinely test for the full panel of autoantibodies simultaneously. Nevertheless, multiple studies have identified patients carrying various combinations of complement-directed autoantibodies such as FBAAs with C3bAAs [83, 92, 93] or C4Nefs with FBAAs or FHAAs [12]. Even when focusing solely on Nefs, co-positivity remains common. In a study of 100 MPGN patients, Ohi and Yasugi identified 10 individuals co-positive for both C3Nefs and C4Nefs who had marked increases in sC5b-9 levels and worse outcomes compared with patients with C3Nefs alone or C4Nefs alone [94, 95]. More recently, two large-scale studies have provided greater insights into Nef co-positivity. First, Marinozzi *et al.* [29] observed that the largest percentage of Nef-positive patients in their cohort displayed both C3Nefs and C5Nef activity (39%), as compared to those with C3Nefs alone (29%) or C5Nefs alone (10%). In this dataset, presence of C3Nefs and C5Nefs correlated with *in vivo* C3 consumption, but only C5Nefs were correlated with terminal pathway dysregulation as demonstrated by increased sC5b-9. In a separate large-scale study by Hauer *et al.* [42], C4Nef-positive patients were found to be co-positive for C3Nefs or C5Nefs 29% or 47% of the time, respectively. In this study, co-occurrence of C3Nefs, C4Nefs and/or C5Nefs was so frequent that the authors utilized an aggregated Nef positivity score of 0–12 based on a summation of individual Nef positivity values. This dataset demonstrated that severe combined Nef activity (cumulative scores $\geq$4, regardless of the type of Nef) resulted in decreases in plasma C3 and increases in sC5b-9.

THERAPEUTIC CONSIDERATIONS FOR Nef-POSITIVE C3G PATIENTS

Currently, no therapy exists to specifically target Nefs or Nef-producing cells. Treatments in C3G are largely supportive, with immunosuppressive mycophenolic acid combined with corticosteroids often serving as a standard of care [36, 37, 96]; however, given the primary role of complement in disease pathogenesis, identifying novel complement-targeting therapies is of major importance. Additionally, a better understanding of how Nefs drive complement dysregulation might illuminate our failures and inform future therapeutic strategies. For example, recent attempts to treat the upstream dysregulation caused by C3Nefs with downstream-targeting eculizumab may explain the drug's poor efficacy, which has been limited to a small percentage of C3G patients. Similarly, the immunotherapy rituximab, when used as a single agent, has proved equally ineffective in C3G patients. This poor outcome might be due to the limited targeting window of rituximab, which binds to CD20, a surface molecule expressed on B cells but not all (auto)antibody-producing plasma cells. In this context, an interesting proof-of-principle case study was recently reported in a single patient with elevated C3Nefs and C5Nefs who failed to respond to prednisone when sequentially combined with single agents rituximab, avocopan (C5a receptor inhibitor) or danicopan (factor D inhibitor) [97]. Surprisingly, the patient significantly improved when rituximab was combined with belimumab, which blocks the molecule B cell activating factor, a strong survival signal for plasma cells. This improvement correlated with reductions in both C3Nefs and C5Nefs, suggesting a causal role for these two factors in disease phenotype and also hinting that successful Nef-reducing treatments might require targeting both B-lineage cells and plasma cells simultaneously. These insights support the notion that to improve therapeutic outcomes and select appropriate C3G patients for clinical trials, a complete understanding of the acquired drivers of C3G is mandatory.

DISCUSSION

The study of Nefs and other complement autoantibodies offers a truly unique autoimmune landscape in which dysregulation of both the innate and adaptive systems feed into each other like an ouroboros. While tremendous insights have been discovered into how acquired drivers promote C3G, we still lack a deep understanding of the underlying process. In considering Nefs alone, there are significant knowledge gaps regarding their origins, mechanisms and pathogenesis. In this discussion we address each area—focusing on Nefs—and offer a few suggestions where future research might prove fruitful.

Nef origins

Regarding origins, limited data exist on the initiating events that cause the onset of Nefs in C3G patients. Popular theories include genetic susceptibility to C3G (and autoimmunity in general) or that disease onset is the unfortunate consequence of environmental exposures or infectious agents that result in Nefs due to molecular mimicry or random chance via 'normal' stochastic adaptive mechanisms [98–101]. While the stochastic theory might explain the detection of Nef-like autoantibodies in healthy individuals, as well as the ability to generate Nef-like antibodies using pokeweed-stimulated PBMCs from normal subjects, it seems unlikely that C3G onset is purely random [51, 56, 102]. In searching for a genetic origin, most research has rightly focused on mutations in complement genes. While this approach remains valuable, it neglects the adaptive component of C3G. For example, C3G patients may have mutations in their germline immunoglobulin gene regions that predispose them to having a B cell receptor repertoire conducive to generating convertase-reactive autoantibodies. Alternatively, recent genetic studies outside of the complement orthodoxy suggest there remains much to learn by examining loci associated with autoimmune susceptibility more broadly, such as the human leucocyte antigen loci or *PTPN22* [103–105]. Molecular mimicry, as a separate means of onset, posits that antibodies generated against a foreign protein can cross-react with self-proteins [106]. For C3G, this could be a bacterial or viral protein producing an antigen that structurally mimics some portion of the C3 or C5 convertase. Here, future structural studies might provide insights into possible convertase mimics, which we discuss below. Regardless of the mechanisms, it is likely that both genes and environmental exposures play a role in disease onset, and future studies will be required to determine the contribution of each to C3G.

From onset thru development, many researchers have supported the view that Nef-positive C3G is primarily an autoimmune disease wherein the immune system mistakenly targets the complement system [8, 10, 44, 107]. We concur with this autoimmune viewpoint based on the following observations: Nefs are autoantibodies; C3G patients frequently present with multiple anticomplement autoantibodies, suggesting a more general breakdown in immune tolerance rather than a singular molecular driver; Nef-positive individuals show a genetic predisposition to other autoimmune diseases, such as type 1 diabetes [3]; autoimmune diseases lead to diverse patterns of inflammation and organ disfunction—a classic description of kidney injury in C3G; and autoimmune disease reflects the interplay of

genetic and environmental exposures, such as infection—an interplay that might help explain why Nefs have been identified in postinfectious MPGN or explain the shared disease continuum between immune complex MGPN and C3G [108–112].

The autoimmune framing of C3G (as compared with a complement-centric framing) might help us understand two significant knowledge gaps in the field: Nef heterogeneity and Nef variability across time. Regarding the first, is Nef heterogeneity driven by a single breach in B cell tolerance, followed by random somatic hypermutation and clonal expansion, resulting in Nefs with similar sequences but varying targets or affinities? Alternatively, do diverse Nefs arise independently, potentially from distinct B cells encountering different autoantigens on different convertases at different times? In either scenario, the *in vivo* milieu following an infectious trigger or inflammatory state would be ideal to promote Nef onset. In such a setting, ubiquitous C3bBb (or a mimic) could serve as an antigen for B cell receptor stimulation, paired with simultaneous viral-/bacterial-induced Toll-like receptor stimulation, thus perpetuating the (mal)adaptive immunological processes resulting in C3G autoantibodies. Regarding the second knowledge gap, few studies exist examining Nefs across time. This omission might be the easiest to address with current C3G datasets. However, the initial knowledge gained would primarily be descriptive, and an autoimmune framing might prove beneficial here as well. More specifically, forthcoming longitudinal studies will undoubtedly reveal Nef trends across time (quantity, function, etc.), which will be correlated to disease outcome; however, a true mechanistic understanding of C3G onset and progression will require knowledge of how the adaptive cellular and molecular processes break down, giving rise to such trends in the first place, and provide insights into changing trends across time.

Nef mechanisms

Decades of research have solidified the fact that Nefs are heterogeneous immunoglobulins that stabilize their respective convertase, and yet the precise mechanisms and the molecular targets of Nefs remain unclear. Furthermore, the lack of a C5 convertase structure makes it challenging to infer whether some C3Nefs and C5Nefs share a semi-homologous binding site, or the exact role properdin plays is in C3Nef versus C5Nef binding. In this regard, some of the most exciting advancements in Nef mechanistic understanding in the last 5–10 years might not have involved Nefs at all, but instead come from a series of excellent structural papers out of Denmark and The Netherlands. Although the primary focus for most of these studies was on complement inhibition and basic structural biology, the models presented offer valuable insights into Nef biology. For example, structures of complement-binding nanobodies have identified antigenic 'hot spots' on the C3 convertase [113–118]. With advances in computational modelling, these convertase regions could be screened *in silico* to identify homologous protein structures from infectious agents, providing support for the molecular mimicry hypothesis. Additionally, the nanobody–convertase models improve our understanding of where and how immunoglobulin domains bind convertases (or block other proteins from binding) and how this modulates pathway flux and other convertase dynamics [46, 119]. Using this approach, the authors have even succeeded in identifying a nanobody capable of modestly increasing complement pathway activity above baseline, similar to a Nef [120]. Furthermore, nanobodies were recently used to study the structure and function of the C3 convertase in complex with properdin, the lone activator of complement [121]. In total, these findings provide insights into potential epitopes as well as the mechanisms of convertase stabilization that could be applied to Nef biology [46, 119]. Finally, work has shown the importance of convertase density and orientation in the transition of a C3 convertase to a C5 convertase [24, 76, 122], which might be experimentally leveraged to better understand the biological difference between a C3Nef and a C5Nef.

In considering Nef targets while framing C3G as an autoimmune disease, we note the exclusive use of the terms 'neo-antigen' or 'neo-epitope' in the literature to describe the unknown Nef binding sites on the C3 or C5 convertase. Here we propose a change in terminology. Like most, we suspect that the antigenic Nef target is hidden until convertase formation or properdin binding, at which point the target is briefly exposed. As such, the terms 'neo-epitope' and 'neo-antigen' are unsuitable. Historically, 'neo-epitope' is entrenched in the jargon of cancer biology, where it is used to define a new ('neo') peptide that arises from aberrant somatic mutation, previously unseen by the immune system. In the case of C3G, we find no strong evidence to suggest the antigenic targets of Nefs are not also present in normal individuals, the most obvious support being that any *in vitro* assay that mixes Nef-positive IgG with normal C3 and factor B produces a Nef effect. Until the targets are identified, we propose the terms 'transiently exposed autoantigens', or the recently used 'confirmational epitopes' to align more closely with the current experimental evidence [35]. We raise attention to these nuances in framing and terminology not to be polemic, but instead to provoke different thinking about the onset, mechanisms and progression of C3G, and in hopes that these conceptual changes might spark new ideas and areas of investigation. Regardless of the chosen terminology, these 'function-follows-form' studies shed tremendous light on how, where and when a Nef might bind to a convertase to promote AP dysregulation.

Nef pathogenesis

Given the wide array of non-standardized Nef testing methodologies, frequent co-occurrence and predicted heterogeneity within each Nef subclass (i.e. within C3Nefs) and between Nef subclasses (i.e. between C3Nefs, C4Nefs, and C5Nefs), it is not surprising that the literature on Nef function *in vivo* is somewhat conflicting. At the most simplistic level, current models of *in vivo* Nef function view the alternative pathway as essentially bimodal in terms of its dual-convertase activity (Fig. 1B). Upstream, it is generally believed that *in vitro* detection of C3Nefs correlates with increased activation of C3 convertase, thus leading to depletion of the convertase substrate C3 *in vivo* [42]. Downstream, *in vitro* detection of C5Nefs should result in a more stable C5 convertase, thus predicting *in vivo* consumption of C5 and properdin and increased levels of the terminal pathway marker sC5b-9 [123]. However, patient data suggest this model might be overly simplistic. For example, after excluding genetic drivers of disease, the presence of C3Nefs does not guarantee reduced C3 and in ≈30% of C3Nef-positive patients, C3 levels are normal [42, 124–126]. Similarly, patients with detectable C5Nefs do not always display elevated sC5b-9. The modest correlation between *in vitro* Nef scores and corresponding *in vivo* biomarkers has been attributed to factors, such as the lack of testing for alternative genetic drivers, heterogeneity in Nef-testing methodology, disease misclassification, small sample sizes, differences in age and differences in disease progression at the time of testing. In truth, significant gaps remain in our understanding of how Nefs drive disease in C3G.

Recently, an interesting hypothesis was put forth suggesting that an equilibrium might exist within the AP between C3 and C5 convertase flux and that disease progression in C3G is less about the mere presence of Nefs, but more about the Nef-induced pathway imbalance [123, 127]. Specifically, the authors demonstrate by multivariate analysis that end-stage renal disease was more likely to occur in patients with either low C3/normal sC5b-9 or normal C3/high sC5b-9 (i.e. only one dysregulated convertase leading to imbalanced flux). On the other hand, adult-onset patients with normal C3 and normal sC5b-9 (regulated convertases with balanced flux) and, quite surprisingly, patients with low C3 and elevated sC5b-9 (i.e. dysregulated convertases but still balanced flux) tended to have better renal survival, an observation supported by an earlier finding using a cluster-based prognostic approach.

The imbalance theory is also supported by a surprising finding in *Cfh*$^{-/-}$ mice that slowly develop C3G-like disease due to unregulated complement activity. In these mice, the additional genetic deletion of properdin unexpectedly results in a worse C3G phenotype [128, 129]. The authors suggest that this deleterious change in phenotype is the consequence of AP activity being shunted toward the uncontrolled production of iC3b and C3b cleavage products. In the absence of properdin, the mouse is unable to generate sufficient C5 convertase, essentially blocking downstream flux and creating a pathway imbalance. In another interesting case study of flux imbalance, Strobel *et al*. [58] identified an FBAA in an 11-year-old DDD patient requiring dialysis. In this patient, the FBAA stabilized the C3 convertase and increased C3 consumption; however, the same autoantibody also prevented formation of C5 convertase *in vitro*, thus forcing AP dysregulation to occur in the upstream pathway only, possibly contributing to the poor outcome for the patient. Viewing C3G dysregulation through the framework of imbalanced flux may shed light on C3G patients who fail to respond to some of the promising new therapeutics such as eculizumab, iptacopan and pegcetacoplan, among others [130, 131]. For example, a patient with highly active C3Nefs (and subsequent C3 convertase overactivity) might do worse if given a C5 convertase inhibitor that, in theory, would promote increased imbalance via the build-up of upstream activation products, thus leading to a poor phenotype (as seen in the *Cfh*$^{-/-}$*Cfp*$^{-/-}$ mouse model). In such instances, it might be better to treat patients with whatever therapy would be most likely to restore their pathway flux balance, even if convertases remain dysregulated.

Our knowledge of how acquired drivers contribute to C3G pathophysiology has improved significantly since a consensus report defined the disease a decade ago [1]. While there is still much to learn, we expect many of the questions put forth in this review will be addressed by a combination of structural, functional and clinical studies. Some areas of research we find exciting include study of the adaptive branch of C3G, which might help identify cellular sources of complement-directed autoantibodies and provide insights into therapeutic strategies. We also look forward to the eventual development of a C3G mouse model that successfully incorporates adaptive dysregulation and autoantibody-driven components. Such a model will be necessary to answer some of the most challenging autoantibody questions, perhaps the most salient being whether a Nef is sufficient to cause C3G *in vivo*. On the mechanistic front, recent advancements in detection methodology, such as the Luminex-based assay, are improving our ability to adequately identify and characterize Nefs in patient samples. Structurally, advances in computational modelling and cryogenic electron microscopy have rapidly improved our ability, over traditional crystallography, to capture the multimolecular and membrane-bound complement complexes. These models, as much as anything, will inform our mechanistic understanding of how both autoimmune drivers and therapeutic inhibitors function or can be harnessed to modulate the complement system. Lastly, we are optimistic about how our improved ability to diagnose and categorize patients will improve cohort specificity, leading to better clinical trials. The resulting robust patient populations and accompanying datasets will be indispensable to our understanding of this complex and heterogeneous disease.

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DATA AVAILABILITY STATEMENT

No new data were generated or analysed in support of this research.

CONFLICT OF INTEREST STATEMENT

None declared.

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