Complement C1s as a diagnostic marker and therapeutic target: Progress and prospective

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The molecules of the complement system connect the effectors of innate and adaptive immunity and play critical roles in maintaining homeostasis. Among them, the C1 complex, composed of C1q, C1r, and C1s (C1qr2s2), is the initiator of the classical complement activation pathway. While deficiency of C1s is associated with early-onset systemic lupus erythematosus and increased susceptibility to bacteria infections, the gain-of-function variants of C1r and C1s may lead to periodontal Ehlers Danlos syndrome. As C1s is activated under various pathological conditions and associated with inflammation, autoimmunity, and cancer development, it is becoming an informative biomarker for the diagnosis and treatment of a variety of diseases. Thus, more sensitive and convenient methods for assessing the level as well as activity of C1s in clinic samples are highly desirable. Meanwhile, a number of small molecules, peptides, and monoclonal antibodies targeting C1s have been developed. Some of them are being evaluated in clinical trials and one of the antibodies has been approved by US FDA for the treatment of cold agglutinin disease, an autoimmune hemolytic anemia. In this review, we will summarize the biological properties of C1s, its association with development and diagnosis of diseases, and recent progress in developing drugs targeting C1s. These progress illustrate that the C1s molecule is an effective biomarker and promising drug target.

KEYWORDS
complement, C1s, biomarker, protein, immunity
Introduction

The complement system consists of more than 30 proteins found in soluble form or attached to cell membranes. Their biological functions include cell lysis, opsonization, degranulation of mast cells and basophils, activation of B lymphocytes, and clearance of immune complex and apoptotic cells. With its components mostly existing as precursor zymogens in the plasma, the system can be activated through the classical pathway (CP), alternative pathway (AP), and the lectin pathway (LP). Of note, the CP is typically activated by the immune complex formed by specific antibodies and antigens such as microbes, allo- and auto-antigens. Through the activation, the antibodies promote the formation of membrane attack complex that often lyse target cell directly, generate complement component-mediated opsonization, and augment inflammatory response. Thus, the classical pathway activation of the complement is involved in the initiation, progression, and prognosis of many bacterial and viral infections-induced responses, autoimmune diseases, and cancers (1–5).

Conversely, inherited deficiency and hypofunction of the complement system are associated with primary immunodeficiencies as well as systemic lupus erythematos and hereditary angioedema (6–8).

At molecular level, C1 is a complex composed of one C1q, two C1r, and two C1s subunits (C1qr2s2). In the classical pathway, antibodies complexed with antigens bind C1q and change its conformation, leading to activation of the protease activity of C1r, which in turn cleaves and activates C1s. C1q can also be activated through binding to C-reactive protein and the surface of pathogens (9, 10). The activated C1s subsequently cleaves substrates C4 and C2, resulting in the formation of C3-convertase (a complex of C4b and C2b) that splits C3 into C3a and C3b, which then cleaves C5 and triggers the formation of so-called membrane attack complex (MAC) consisting of C5b, C6, C7, C8, and polymeric C9 (Figure 1-1). Thus, monitoring C1s activity and targeting C1s with small molecular inhibitors and monoclonal antibodies have been the focus of many studies in recent years (11–14).
The C1s gene, located on the short arm of chromosome 12 (12p13.31), contains 12 exons and encodes a precursor C1s protein of 688 amino acids (15, 16). At its N-terminal region, the protein harbors CUB2 and CUB1 domains connected through an epidermal growth factor (EGF)-like domain, which are followed by complement control protein (CCP) modules CCP1 and CCP2. The C-terminal region of C1s has a serine protease (SP) domain (residues 423-688) that contains an activation peptide-like fragment (residues 423-437). During its activation, C1s is cleaved into a heavy chain of 422 amino acids (A-chain) and a light chain of 251 amino acids (B-chain). Of note, C1s is glycosylated at a number of sites and its serine protease activity shows trypsin-like specificity and cleaves arginyl bonds in substrate proteins.

Interestingly, besides C4 and C2, activated C1s also acts on many cellular proteins and exerts multiple regulatory actions. It has been found that activated C1s cleaves major histocompatibility complex I (MHC I) from the cell surface and hydrolyzes 2 microglobin, affecting T cell-mediated immune response (17, 18). It also cleaves insulin-like growth factor binding protein 5 (IGFBP5) from cultured fibroblast, whereas C1q activates the Wnt signaling pathway (17–20), which may affect cell growth as well as neuronal connectivity (21, 22). In addition, low-density lipoprotein receptor-related protein (LRP6), nucleophosmin 1 (NPM1) and nucleolin (NCL) are all substrates of C1s (20, 23). Interestingly, C1s can cleave high-mobility group box 1 (HMGB1) protein, a notable auto-antigen in autoimmune diseases (24). Although these proteolytic activities are far less efficient than the canonical C4 and C2 cleavages leading to complement activation, it is conceivable that they take part in tissue renewal process that reduces the immunogenicity of tissue debris and decreases the likelihood of autoimmunity induced by auto-antigens and danger associated molecular patterns (DAMPs). Thus, C1 activation affects physiological and pathological processes through multiple mechanisms (Figure 1-1).

**C1s and human diseases**

The critical roles of the complement system and C1s in maintaining homeostasis make their dysfunction associating with a variety of disease. While mutation of C1s is associated with rare genetic diseases and susceptibility to infections and autoimmune disorders, ongoing studies have indicated that the aberrant activation of C1s contributes to the development of autoimmune and infectious diseases and cancer, and serves as an informative biomarker and therapeutic target (Figure 1-2).

**Loss and gain function mutation of C1s**

It has been shown that deficiency of the classical pathway proteins of the complement system is strongly associated with the development of early-onset systemic lupus erythematosus (SLE), an autoimmune disease characterized by auto-antibody against multiple tissues and organs (3). The likely mechanisms include the lack of activated proteases, such as C1r and C1s, to digest the potential auto-antigens form dying cells and immune complexes and lack of modulation of the immune response set-point by activated complement components. Of note, studies in mouse model indicated that C1r/C1s deficiency alone is insufficient in inducing SLE (25), suggesting that the development of the disease is affected by multiple factors. Further analyses found that the appearance of stop codon (Y204X) in exon 6 that leads to premature protein translation termination is a commonly identified C1s mutation in SLE patients (26). It has also been reported in a family study that the propositus contains homozygous nonsense mutation R534X, and both parents and 4 family members in 2 generations are heterozygous of the mutation (27). Interestingly, it was found in an extended Japanese family that C1s deficiency may result from heterogeneous combination of different mutations, including a 4-bp TTTG deletion in exon 10 that results in a frame shift mutation (28), a stop codon mutation (ES97X) in exon 12 that encodes a C1s missing 80 amino acids at the C-terminal (29), and a missense mutation G630Q (30).

Periodontal Ehlers Danlos syndrome (pEDS) is a rare disorder characterized mostly by early-onset periodontitis. It has been shown that this subtype of Ehlers Danlos syndrome is caused by mutations in the genes of C1r and C1s (31). The mutations include heterozygous missense, in frame insertion, and deletion in C1r (15 families) or C1s (2 families). A recent study also identified multiple heterozygous C1s mutations (G962C, T961G, and T961A in an extended family as pathogenic variants (32). Interestingly, it was shown that the mutated C1s Val316del and Cys294Arg were produced in the cells as truncated proteins that lost their N-terminal domain (33). It is evident that these mutations led to constitutive activation of C1r and C1s and the development of pEDS. Structural analyses showed that C1r and C1s interact via an extensive interface encompassing the N-terminal regions of both proteins (34). Therefore, these mutations might disrupt the binding between C1r and C1s and facilitate aberrant C1s activation (Figure 1-1). It is also conceivable that the mutation break the interaction of C1r-C1s and C1q, uncoupling C1s activation from C1 complex binding.
Autoimmune diseases

As an important system involved both in innate and adaptive immunity, the aberrant activation of the complement system is present in many autoimmune diseases, including cold agglutinin disease (CAD), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) (13, 35). CAD is a rare autoimmune hemolytic anemia due to the production of IgM auto-antibody (cold agglutinin) against surface antigens of red blood cells (RBCs). The auto-antibodies bound to RBCs activate the complement CP, leading to formation of MAC and hemolysis that manifested as anemia, severe hemolytic crisis, and even death (36, 37). The success of anti-C1s mAbs in the treatment of CAD further demonstrated the critical role of C1 activation in the pathogenesis (38). Additionally, neuromyelitis optica spectrum disorder, characterized by central nervous system inflammation and demyelination, is mediated by anti-aquaporin-4 (anti-AQP4) auto-antibodies targeting astrocytes and subsequent complement activation (39). Clinical studies revealed that anti-C5 antibody eculizumab effectively prevented the disease development (40). It is interesting to further determine whether blocking C1 activation can also alleviate the tissue damage and the autoimmune disease.

Rheumatoid arthritis (RA) is an autoimmune disease of the joints, which results in inflammation and thickening of the joint capsule, and loss of underlying cartilage and bone. C1s activation has been found in the degenerative osteoarthritic cartilage, but not in normal articular cartilage. The activated C1s was mostly restricted to the severely degraded part of cartilage in osteoarthritis, indicating the participation of C1s in the inflammation and the destroy of cartilage and bone (41). It has also been found that tumour necrosis factor alpha (TNF-α) increased the production of C1s in cultured chondrocytes, suggesting that the inflammatory and destructive roles of C1s may be amplified in the inflammatory joint.

Paradoxically, the roles of C1s in SLE is complicated. While C1r and C1s deficiency predisposes to lupus (42–44), C1s levels in plasma from SLE patients are significantly higher than that in normal subjects, likely reflecting that the increased auto-antibodies activate C1 (45). It has been shown that auto-antibodies against C1s were detected in 7 out of 15 patients with SLE, and may activate C1 and enhance C4 and C2 cleavage (46). However, the positive rate of anti-C1s auto-antibody was markedly lower (6.9%) in a study of a larger group of patients with lupus nephritis, and the presence of anti-C1s and anti-C1r auto-antibodies did not correlate with the clinical parameters of the disease (47). It is worth noting that C1q is frequently targeted by auto-antibodies (anti-C1q), which correlates best with active renal disease in SLE patients (48). It is interesting to further explore how the anti-C1q auto-antibodies affect the complement system and the disease progress. Further, the auto-antibody against C1q could be induced by antigen-derived from Epstein-Barr virus, a well-known trigger of SLE (49). Thus, there are complicated interactions between C1 activation and the development and progress of SLE. It is worth to further explore whether the C1 components may be utilized as diagnostic markers for disease initiation and progress, and as targets of therapeutic intervention at different stages of the disease.

Cancer

Given the close association of complement with immunity, it is not unexpected that activation of C1 is associated with the development, progression, metastasis, and treatment of a variety of cancers (50, 51). In clear cell renal cell carcinoma, local production and activation of C1s drove tumor progression and was associated with poor prognosis (52). Further explorations suggested that C1s facilitates the cancer progression by triggering complement activation, and by modulating the tumor cell phenotype and tumor microenvironment in a complement cascade independent manner (53). One of the cascade independent function may be mediated by the C1 receptor on monocytes, whose engagement drives the cells to migrate into tissues, differentiate into macrophages or dendritic cells (DCs), and initiate adaptive immunity (54). It has also been found that macrophage produced C1q and tumor cell-derived C1r, C1s were assembled in clear cell renal cell carcinoma and resulted in an immunosuppressive microenvironment that promotes tumor progress (55). Therefore, C1s and CP of the complement system can act as an effector of anti-tumor immunity as well as an promoter of the tumorigenic microenvironment in tumor.

Infectious diseases

It is well-known that complement can be activated through CP, LP, and AP on the surface of microbes, leading to the generation of MAC that kills bacteria and parasites via forming transmembrane pores (56). The complement may also mark bacteria for phagocytosis and processing by antigen-presenting cells to stimulate adaptive immune cells. While bacteria may utilize a variety of strategies to evade the action of complement, deficiency in the complement system results in susceptibility to infections of various microbes, especially encapsulated bacteria (57). Therefore, activation of complement is a hallmark of bacterial infection. For example, it has been reported that the level of C1s was increased in children with acute pneumococcal otitis media (58). Further, the elevated complement complexes detected in the sera of children with recurrent otitis media contained C1r and C1s, and were able to kill the bacteria (58). Complement activation is also involved in virus infection. Of
note, it was shown that up-regulation of C1s led to host cell damage via the classical pathway in a model of SARS-CoV-2 infection (59). Clinical investigation found that the mean 50% hemolytic complement (CH50) level in COVID-19 patients was significantly lower compared to that in healthy individuals, likely due to sustained CP activation in these SARS-CoV-2 infected patients (60). Further studies found that antibody-mediated increase of CP activation was highly associated to COVID-19 disease severity (61), although the downstream effects of this activation may differ depending on the disease status of the individual and on the specific antigen targeted (62). Interestingly, the C1 esterase inhibitor (C1INH), which inhibits C1r and C1s activation, significantly reduced fever and inflammation in patients with COVID-19 (63). Due to the large inhibiting spectrum of C1INH, these findings suggest, but did not prove that activated C1s is an effector, biomarker, and potential therapeutic target of various infectious diseases.

**C1s as a therapeutic target**

The critical role of the complement system in innate and acquired immunity and various diseases prompted various efforts to develop specific therapeutic agents (Figure 1-3). C1s is an attractive target since its inhibition blocks the system at an early stage of the complement cascade. While peptide that binds C1q and blocks C1r and C1s activation have been developed (64), finding selective small molecular inhibitors for the serine protease is challenging. A number of reported molecules did not exhibit high specificity and adequate pharmacokinetics yet (65). A large machine learning-based virtual screening was carried out and found a series of potential valuable inhibitors (66). Further combination of *in silico* and *in vitro* approaches identified hit compounds with new chemo-types and high potency in inhibiting C1s (67). It is interesting to determine whether these inhibitors can interfere the activity of C1s under physiological and pathological conditions.

C1INH is a natural plasma protein whose level is further increased upon stress and inflammation. It has a characteristic serpin domain and irreversibly binds to and inactivates C1r and C1s proteases (68). Of note, C1INH also prevents complement activation through the lectin pathway and exhibits inhibition on multiple proteases, notably kallikrein, FXa, and FXIIa (69), and therefore modulating the kinin, coagulation, and fibrinolytic systems. It has been proposed that the protein may be utilized in a variety of diseases, including septic shock, reperfusion injury, hyperacute transplant rejection, traumatic and hemorrhagic shock. Currently, C1INH prepared from human plasma (Cinryze™) has been approved for the treatment of hereditary angioedema, a rare autosomal dominant disease manifested by recurrent acute attacks of edema that results from C1INH deficiency (70).

Monoclonal and engineered antibodies have been the most advanced agents for blocking complement activation, including antibodies against C1s (Table 1) and C5 (79). In particular, anti-C1s antibody sutimlimab significantly inhibited Ig-induced activation of B cells derived from patients with rheumatoid arthritis, indicating that targeting C1s may not only block complement-mediated tissue damage, but also suppress the activation of autoimmune B cells, which is a critical pathogenic factor in many autoimmune diseases (13). For example, bullous pemphigoid (BP) is a potentially life-threatening skin disease characterized by blister formation resulting from auto-antibody-mediated complement action and subsequently inflammation and tissue damage. In an *ex vivo* human skin cryosection assay, anti-C1s antibody TNT003 effectively prevented complement activation induced by the bullous pemphigoid auto-antibodies (80, 81). In patients with thrombotic purpura, auto-antibody against platelet membrane protein leads to activation of complement and destruction and reduction of platelet. Sutimlimab inhibited the CP of complement activation and significantly decreased plasma deposition of C3b and C5b-9 in these patients. Meanwhile, the direct damaging and reticuloendothelial system clearance of platelets were markedly reduced (14). Similar studies found

| Disease                                  | Medicine | Clinical staging | NCT NO          |
|------------------------------------------|----------|-----------------|-----------------|
| Bullous Pemphigoid                        | BIV009   | 1               | NCT02502903 (71) |
| Cold Agglutinin Disease                   |          |                 |                 |
| Warm Antibody Type Autoimmune Hemolytic Anemia |         |                 |                 |
| End stage renal disease                   |          |                 |                 |
| Cold Agglutinin Disease                   | sutimlimab (BIV009) | 3         | NCT03347422 (72) |
| Cold Agglutinin Disease                   | sutimlimab | 3               | NCT03347396 (73) |
| Idiopathic Thrombocytopenic Purpura       | sutimlimab (BIV009) | 1         | NCT03275454 (74) |
| Idiopathic Thrombocytopenic Purpura       | BIV020   | 2               | NCT04669600 (75) |
| Autoimmune hemolytic anemia               | BIV020   | 1               | NCT04802057 (76) |
| Autoimmune hemolytic anemia               | BIV020   | 1               | NCT04269551 (77) |
| Chronic Inflammatory Demyelinating Polyradiculoneuropathy | BIV020 | 2               | NCT04658472 (78) |
that anti-C1s antibodies were effective in blocking complement activation induced by auto-antibodies from patients with several types of autoimmune hemolytic anemia, such as paroxysmal nocturnal hemoglobinuria and CAD (14).

Antibody-mediated rejection (AMR) in solid organ transplantation is caused by donor-specific antibodies. It is characterized by the activation of the complement system and infiltration of macrophages (CD4+ and CD68+). In a study with aortic endothelial cells, it was demonstrated that the activation of the complement by anti-MHC antibodies facilitated the recruitment of monocytes. The anti-C1s antibodies TNT003 and TNT009 inhibited the antibody-induced complement activation and blocked C3d deposition (82), which are critical for the microvascular inflammation during AMR (83). The inhibitory effects of these antibodies on CP activation have also been demonstrated in patients (84). Whether they can reduce the microvascular inflammation during AMR and prolong the survival of transplants remain to be further explored. Interestingly, it was found that C1s-specific monoclonal antibody TNT005 did not abolish the therapeutic effects of anti-Neisseria meningitidis and Streptococcus pneumoniae antibodies, whereas simultaneous inhibition of CP and AP blocked the killing function of the anti-bacterial antibodies (85). Thus, targeting C1s may specifically prevent CP activation without abating the alternative and lectin pathways of complement activation and their associated immunological effector functions, and might also reduce the production of auto-antibodies.

There have been a number of registered clinical trials that target C1s with mAbs for the treatment of hereditary angioedema (HAE), chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), cold agglutinin disease (CAD), thrombocytopenic purpura, and autoimmune hemolysis (Table 1). Sutimlimab (also known as TNT003, BIVV-009) is an humanized IgG4 mAb against C1s. It was reported that 7 of 10 CAD patients treated with sutimlimab achieved remission, and all 6 patients with a history of blood transfusion became transfusion-free during treatment (11). In a named patient program, 7 CAD patients responded to retreatment, and sutimlimab increased hemoglobin from a median initial level of 7.7 g/dL to a median peak of 12.5 g/dL (P = 0.016). All patients remained transfusion free while receiving sutimlimab, and there were no treatment-related serious adverse events. In another trial with sutimlimab, 13 out of 24 (54%) CAD patients reached the primary end point. Of the 11 patients that did not meet the predefined criteria, 6 of them showed a therapeutic response (12). These and further clinical trials led the recent approval of sutimlimab for the treatment of CAD by the US FDA (86). Of note, another anti-C1s antibody BIVV020, whose tolerability safety study has been completed in patients with CAD, is being actively tried in clinic for the treatment of CAD, immune thrombocytopenia, and antibody-mediated transplant rejection (Table 1). It is conceivable that these antibodies may be proved as an effective therapeutics for additional forms of autoimmune hemolytic anemia in not-so-distant future.

### C1s determination in biological samples

Various methods have been developed to detect the complement in clinical samples. Traditionally, the total complement activity of CP is quantified by hemolysis assay in serum, based on the dilution achieving 50% of hemolysis (CH50). Functional ELISAs using coated IgM and colorimetric substrate have also been developed to detect the activity of CP in serum (87, 88). However, these assays only evaluate the overall CP complement activity, but not the levels of individual complement components. The C1 assay through capture of C1q in serum may represent a better measurement of C1s (89). It has also been shown that the analyses of CP components, such as C1q and C1r could be achieved quantitatively or semi-quantitatively by immunodiffusion, ELISA, and nephelometry assays (90). However, these methods only detect the immuno-reactivities of target complement components, and do not necessarily reflect their activity states. For the studies of congenital complement deficiency or for the study of genetic polymorphisms in populations, next-generation sequencing (NGS) and qPCR are often deployed to analyze particular components (91). Therefore, there are no validated C1s quantification assays available in clinical practice currently, and the quantification of C1s in research is also difficult due to limited reliable antibodies and lack of standardized assays.

The level of the total C1s protein can be detected by bilateral diffusion (92), ELISA (27), gelatin zymography (45), and LC-MS/MS (93) (Table 2). As the protein is normally present in blood and tissues, it is important and highly desirable to assess activated C1s to understand its exact roles under physiological and pathological conditions. It has been shown that the activity of C1s can be measured by the cleavage C2 and C4 (94). However, the assay is only semi-quantitative, and C2 and C4 can also be cleaved by other serum proteases such as MASP2. Interestingly, antibodies recognized activated C1s have been reported, which make it possible to determine the protein through western blot. It has been shown that such antibodies detected activated C1s in degenerative cartilage matrix of RA through immunohistochemistry (96). To investigate the effects of C1NH on C1s (97), a synthetic chromogenic tripeptide-p-nitroanilide substrate S-2314 (DTNB) was utilized to determine the level of active C1s. However, the substrate can also be cleaved by activated C1r and other enzymes, such as granzyme H, which prevented its use in clinic for measuring active C1s in blood (95). Thus, it is conceivable that the ideal novel assay should be highly specific and convenient by combining the specific antibody,
catalytic and C2/C4 binding specificity of C1s and making use of labeling technologies, such as the fluorescence labeling.

### Conclusions and future directions

The critical roles of the complement system in innate and acquired immunity make the activation of C1s an informative biomarker for a variety of diseases, particularly the inflammatory, autoimmune diseases and cancers. With the success of specific antibody preparation, C1s has been validated as an effective target of therapeutic intervention. It is conceivable that more small molecular, peptide/protein, and antibodies targeting C1s for the treatment of cancer, autoimmune, and infectious diseases will be developed. Thus, the complement component is a unique target for both diagnostic and therapeutic actions. Further, ongoing clinical trials and the emerging of novel therapeutics against C1s will likely bring new and better drugs into clinic in the not-so-distant future.

It has been shown recently that the expression of C1s and another two other genes is associated with Age-related macular degeneration (AMD), a progressive neurodegenerative disease of the central retina and a leading cause of vision loss in older adults worldwide (98). Mechanistically, it is likely due to their correlation with NK cell infiltration, CD4 memory T cell activation, and macrophage polarization in AMD. Thus, the expression level of the C1s gene might be utilized as a prognostic biomarker for early diagnosis and treatment of AMD. While measuring the level of C1s molecule and its activation states in clinical specimen are of great value, it remains a significant challenge to develop convenient, specific, and sensitive assays to fulfill the need, particularly determining the kinetic of activated C1s and C1s-mediated enzymatic reaction in the serum/plasma. It is conceivable that the successful and practical clinical evaluation of C1s will enable wide applications of the biomarker in pathogenesis, diagnosis, prognosis of diseases, and for individualized therapies targeting C1s.

### Author contributions

JY and SX designed the project, JY wrote and SX revised the manuscript, PY and YY participated in the project and revised the manuscript. All authors contributed to the article and approved the submitted version.

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### Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Inhibition of complement C1s improves severe hemolytic anemia in cold

275:37638

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