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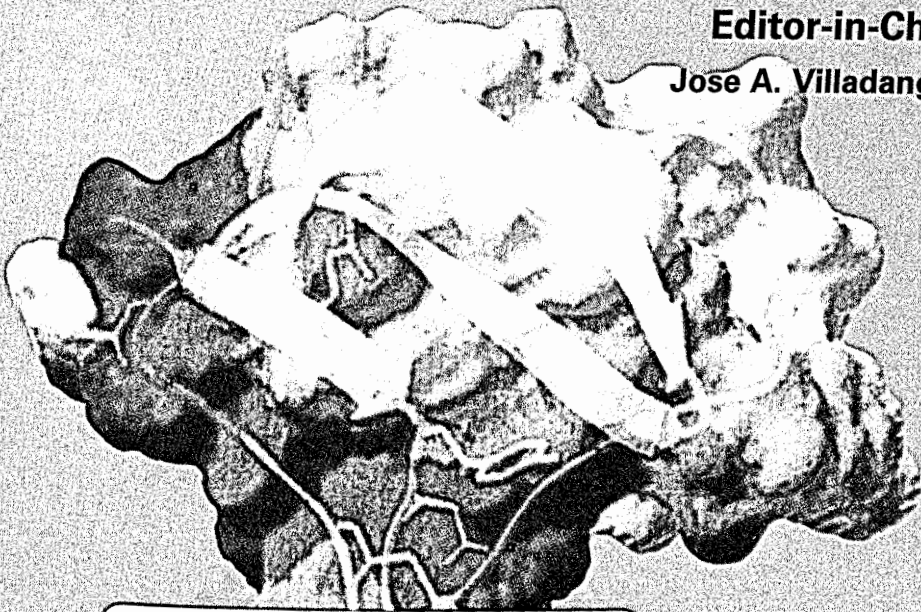
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The cover illustration shows a ribbon representation of complement component C3 and its accessible surface with each of the 13 domains coloured differently. The X-ray structure of C3 was initially published by Janssen et al. in *Nature*, Vol. 437 (2005) 505-511.

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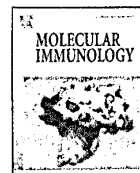
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## Short communication

## C1 Inhibitor as a glycoprotein: The influence of polysaccharides on its function and autoantibody target



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## ABSTRACT

C1 Inhibitor (C1Inh), a member of the Serine proteinase inhibitor family, is the most heavily glycosylated plasma protein. This work investigated the impact of C1Inh glycosylation on its function regarding protease targets and autoantibody binding. C1Inh deglycosylation was found to affect its function with O-linked polysaccharides, but not with N-linked polysaccharides, in controlling the contact phase but not C1s target, thus indicating the N-terminal domain's involvement in C1Inh function. Instructive samples demonstrated that O-deglycosylation strongly suppressed autoantibody binding, suggesting the polysaccharide motif is an antibody target. The autoantibodies did not directly affect C1Inh function.

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## 1. Introduction

C1 Inhibitor (C1Inh) is a multi-functional Serine protease inhibitor (serpin), which operates by inactivating various Serine proteases in different plasmatic cascades, including the complement (classical pathway C1r and C1s; lectin pathway MASP1 and MASP2), contact (Factor XII and kallikrein), coagulation (Factor XI and thrombin), and fibrinolytic (tPA and plasmin) systems. C1Inh is the primary control of contact phase, i.e., the kinin forming system and its deficiency is well known as the cause of a rare genetic disorder, namely hereditary angioedema (HAE). Its primary disease manifestation consists of swelling caused by fluid leaking from the blood vessels into connective tissue. C1Inh is a glycoprotein of 478 amino acid residues which undergoes extensive post-translational modification, with 45% polysaccharides by weight bearing six N-linked carbohydrates and 14 potential O-glycosylation sites, seven of which have been verified by carbohydrate analysis. C1Inh is thus

one of the most heavily glycosylated plasma proteins. Most of its sugars are located in the N-terminal non-serpin domain (residues 1–120) (Bock et al., 1986) and only three N-linked oligosaccharides are attached to the serpin domain, through Asn<sup>216</sup>, Asn<sup>231</sup>, and Asn<sup>330</sup> (Koles et al., 2004; Beinrohr et al., 2007).

The precise function of these carbohydrate groups on C1Inh interaction with target proteases, except C1s, has not yet been investigated. Previous findings indicated that the LPS-binding site of C1Inh was located within its N-terminal 97 amino acid residues (Liu et al., 2003) and that this binding process was dependent on the presence of N-linked carbohydrate at Asn<sup>3</sup>, Asn<sup>47</sup>, and Asn<sup>59</sup>, not the residues in the serpin domain (Liu et al., 2004).

Our study sought to investigate whether O- or N-deglycosylation affects (1) C1Inh function towards contact-phase or C1s targets and (2) anti-C1Inh autoantibody binding.

## 2. Methods

## 2.1. Patients

Serum samples from adult patients who had confirmed angioedema diagnosis (acquired angioedema, AAE; n=9). All procedures were performed according to the principles of the Helsinki

**Abbreviations:** AAE, acquired angioedema; C1Inh, C1 Inhibitor; C1Inh-HAE, hereditary angioedema with C1 Inhibitor deficiency; FXII, Factor XII; FXIIa, activated Factor XII; HAE, hereditary angioedema; HK, high-molecular-weight kininogen.

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declaration and French ethical policies governing the use of the biological sample collection (Ministry of Health authorization: DC-2008-634), with all patients having provided informed consent to participate in the investigation using biological assays. All blood donors (healthy controls) answered a standard questionnaire. All data was anonymously processed.

## 2.2. C1Inh deglycosylation

In order to investigate the impact of the polysaccharides on C1Inh function, 1 mg/ml of purified C1Inh (Berinert®; CSL Behring GmbH, Paris, France) was treated with a combination of 12.5 U/ml *N*-glycanase (PNGase F, New England Biolabs, Évry, France) which removes Asparagine-*N*-glycans, 1 U/ml *O*-glycanase (Roche Diagnostics GmbH, Mannheim, Germany) which hydrolyses Serine- or Threonine-*O*-glycans, and 0.1 U/ml neuraminidase (Roche Diagnostics GmbH, Mannheim, Germany) which hydrolyses neuraminic acid residues overnight at 37°C in a buffer containing 10 mM sodium phosphate, pH 6. Deglycosylated C1Inh was subjected to Western blot analysis, as described below.

## 2.3. C1Inh function assays

- To control contact phase proteases: C1Inh function was assessed via titration using the residual amidase activity (Pro-Phe-Arg-pNA) of the contact phase target (Ghannam et al., 2015).
- To control C1s target: C1Inh function was assessed via titration using the residual esterase activity (benzoyl-Arg ethyl ester) (Drouet et al., 1988).

## 2.4. Immunological assays

### 2.4.1. Complex-formation assay

A C1Inh–KK complex-formation assay was used to determine whether C1Inh deglycosylation interfered with its control to contact phase proteases: C1Inh species (10 µg) were incubated with reconstituted contact phase proteases (equimolar amounts) at 37°C for 30 min in 150 mM NaCl, 25 mM Tris-HCl pH 7.8, resulting in a complex with KK, then the samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Contact-phase proteins FXIIa, pKK and HK were obtained from (Enzyme Research, Swansea, UK).

The same procedure was followed for C1Inh–C1s complex-formation assay to determine whether C1Inh deglycosylation interfered with the proteinase-inhibitory function of C1Inh. C1Inh species (10 µg) were incubated with C1s protease (10 µg) at 37°C for 30 min in 20 mM Tris-HCl, 41 mM glycine pH 8.5 buffer. Samples were then subjected to SDS-PAGE.

### 2.4.2. C1Inh immunoblot

C1Inh species and C1Inh complexes were submitted to 8% polyacrylamide SDS-PAGE followed by transfer onto nitrocellulose membrane (Hybond, GE Healthcare, Chalfont St. Giles, UK), saturation with 5% non-fat-dry milk in TBS-T buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 0.01% Tween20). The blot was probed with anti-C1Inh (Binding Site, Birmingham, UK) in TBS-T buffer, using enhanced chemiluminescence (Clarity Western ECL substrate, Bio-rad, Hercules, USA).

### 2.4.3. Binding of anti-C1Inh autoantibody to C1Inh species

C1Inh species were incubated with patient serum samples in order to detect autoantibody binding, using a direct Enzyme-linked immunosorbent assay (ELISA) slightly modified from the technique used in Alsenz et al. (1987). Briefly, 96-well plates (Dominique Dutscher, Brumath France) were coated overnight with purified C1Inh species (0.25 µg/well). After washing with

PBS/0.1% Tween buffer and blocking with PBS/BSA 2%, plates were incubated for 1 h at 37°C with patient's serum. After washing, plates were incubated for 1 h at 37°C with HRP-conjugated anti-human immunoglobulins (Abliance, Compiègne, France). The assay was developed with Tetramethylbenzidine (Sigma-Aldrich, Saint Quentin Fallavier, France) as a chromogenic substrate.

## 3. Results and discussion

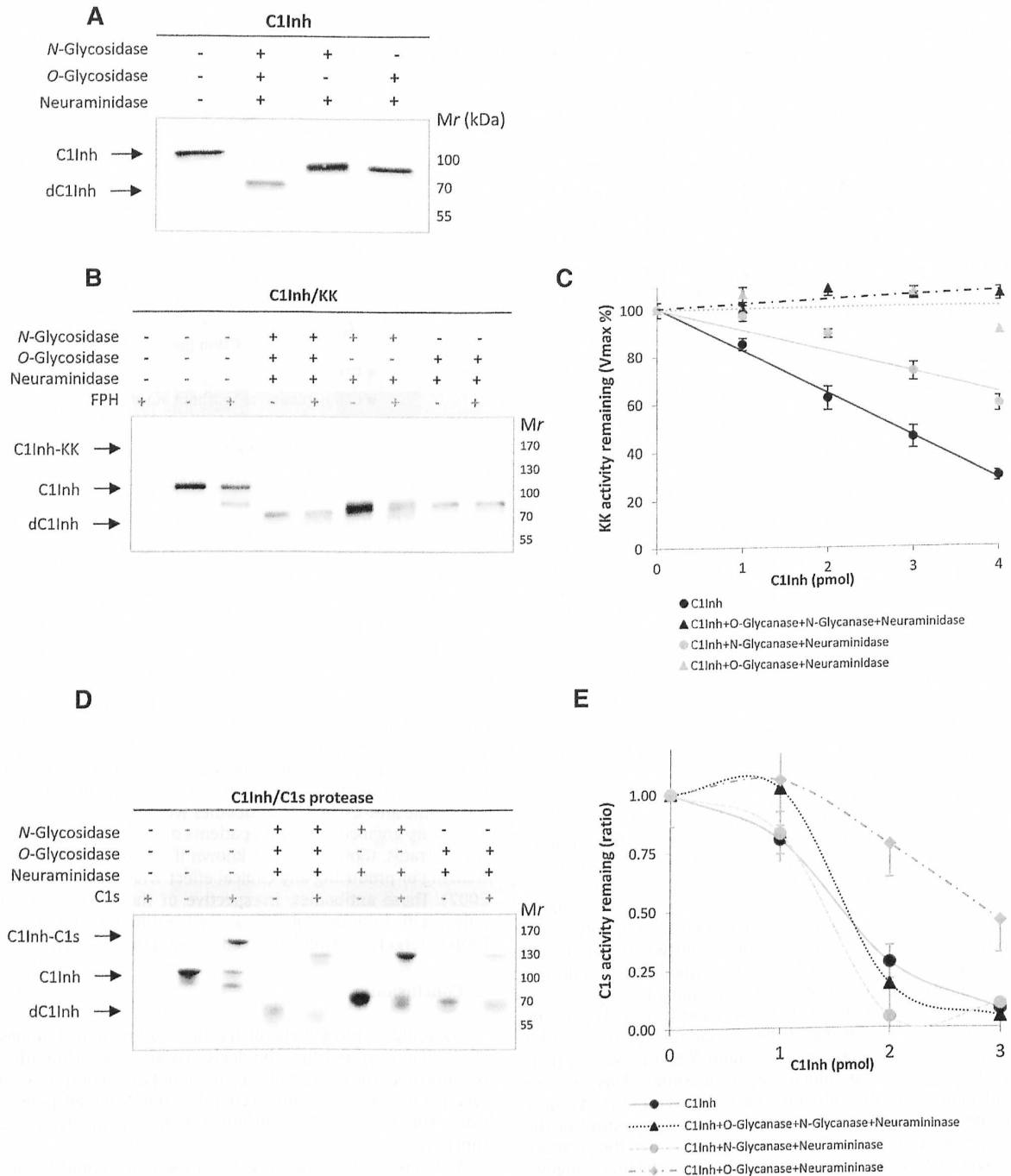
### 3.1. O-glycosylated structures play a role in KK control by C1Inh

We added a combination of glycosidase enzymes to C1Inh, then conducted SDS-PAGE to validate the decreased relative molecular mass ( $M_r$ ) (~70 to ~90 kDa) of the deglycosylated proteins (Fig. 1A). C1Inh function was then evaluated in terms of its control of contact phase. C1Inh digested by combined neuraminidase and *N*-glycanase, exhibited partially diminished control compared to that of native C1Inh. *O*-glycanase was then added, combined with either neuraminidase or neuraminidase and *N*-glycanase, causing complete loss of C1Inh control of contact phase proteases (Fig. 1B). *O*-glycanase was used to release the Gal β(1–3) GalNAc unit from *O*-glycans. Bonds to Serine as well as to Threonine are hydrolyzed. Substituents on the disaccharide, e.g. sialic acid in the great majority of soluble glycoproteins, prevent the hydrolysis and therefore must be removed enzymatically using neuraminidase.

To strengthen these results, purified contact phase components were incubated with the different deglycosylated C1Inh species. SDS-PAGE analysis revealed that the native C1Inh, but not the fully-deglycosylated species, formed a complex with kallikrein (KK). Neuraminidase- and *N*-glycanase-treated C1Inh also formed a complex with KK. However, when *O*-glycanase was used, C1Inh was not able to form a complex with KK (Fig. 1C, lanes 5 and 9). These results suggest *O*-glycosylated structures play a role in C1Inh control of KK. Our results are in line with a previous study by the Patston group in 2004 (Ravindran et al., 2004), where C1Inh lost control of KK when truncated at its *N*-terminus, in the setting of endothelial cells.

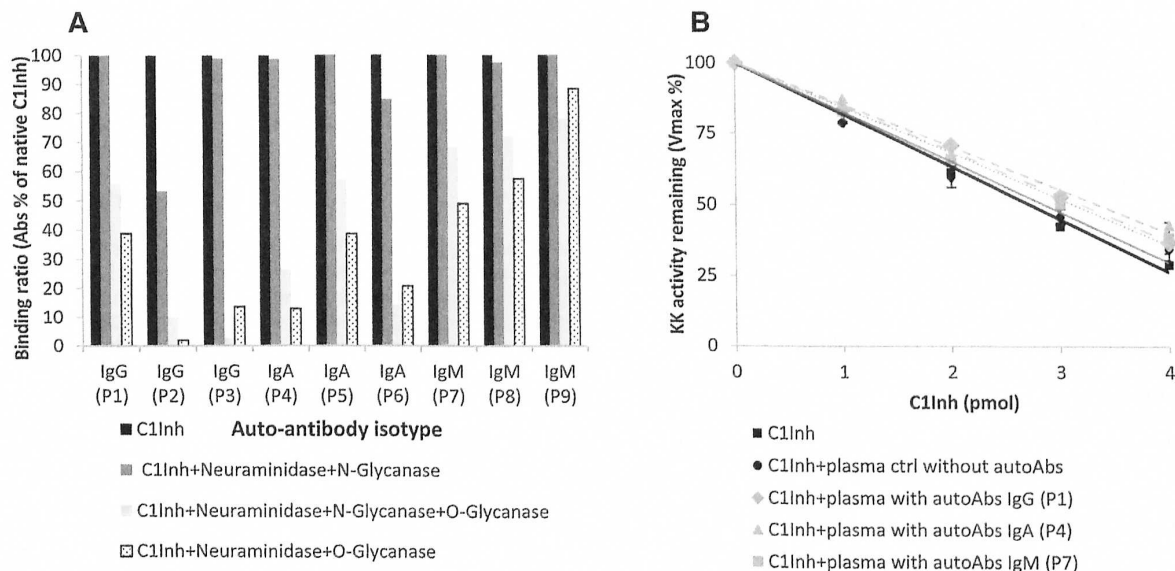
We next investigated the impact of C1Inh deglycosylation on C1s protease control. The different C1Inh species were incubated with C1s protease and analyzed by means of SDS-PAGE. In all analyses, C1Inh formed a complex with C1s (Fig. 1D). After testing C1Inh function using chromogenic substrate (Drouet et al., 1988), digested C1Inh kept control of C1s, though its capacity was diminished when *O*-glycanase was used (Fig. 1E). These results suggest that deglycosylated C1Inh retains both its ability to form complexes with C1s and its inhibitory function, as reported in earlier reports (Liu et al., 2004; Reboul et al., 1987).

Previous studies have shown that post-translational modifications of serpins, i.e. glycosylation, affect their protease specificity. A good example for this is in the case of the Protein C Inhibitor (PCI) Serpin, for which glycan trees have been proposed to regulate the association with the protease, as demonstrated by the accelerated reaction of the deglycosylated PCI with thrombin (Sun et al., 2008). Our observations support these authors' findings that the significant glycosylation impact has in modifying serpin-protease interaction. However, deglycosylated C1Inh species exhibited decreased serpin function towards contact phase proteases. This is congruent with the strongly decreased inhibition of thrombin and plasma KK due to PCI following complete removal of *N*-acetylneuraminic acid and galactose, as well as the trimannosyl core from biantennary *N*-glycans (Izutani et al., 2001). As C1Inh interaction with the C1s protease was not affected by polysaccharides, unlike its interaction with KK, this effect could be protease-specific. The *O*-linked amino acid residues are all found within the non-serpin *N*-terminal domain. Given that *O*-glycanase digestion altered C1Inh–KK interaction, it could be hypothesized



**Fig. 1.** Deglycosylation of C1Inh: effect on reactivity with contact phase and C1s proteases.

(A) SDS-PAGE analysis of deglycosylated C1Inh (dC1Inh): C1Inh was treated with a combination of neuraminidase with N-glycanase and/or O-glycanase overnight at 37 °C. C1Inh species were analyzed by SDS-PAGE. The different relative molecular mass ( $M_r$ ) is consistent with previously published data (Liu et al., 2004; Reboul et al., 1987). (B) Effect of deglycosylated C1Inh on the formation of C1Inh–KK complexes: C1Inh species were incubated with equimolar amounts of reconstituted contact phase proteases (prekallikrein: pKK; activated Factor XII: FXIIa; high molecular weight kininogen: HK; this mixture is identified FPH in the figure) at 37 °C for 30 min, resulting in C1Inh–KK complex, the samples were then subjected to SDS-PAGE analysis. (C) Effect of deglycosylated C1Inh on its function to control contact phase proteases. The different species of C1Inh, prepared as outlined above, were incubated with contact phase purified proteins, i.e. the mixture FPH, and C1Inh function was then evaluated as described by Ghannam et al. (Ghannam et al., 2015). (D) Effect of deglycosylated C1Inh on the formation of C1Inh–C1s complex: C1Inh species (10  $\mu$ g) were incubated with C1s protease (10  $\mu$ g) at 37 °C for 30 min, following which the samples were submitted for SDS-PAGE analysis. (E) Effect of deglycosylated C1Inh on its function using C1s protease as target and chromogenic substrate, data are expressed as ratio as described in Drouet et al. (1988).



**Fig. 2.** Anti-C1 Inhibitor (C1Inh) autoantibodies: C1Inh polysaccharide moiety as target and impact on C1Inh function.

(A) Binding of anti-C1Inh autoantibody on native and deglycosylated C1Inh species. C1Inh species were incubated with acquired angioedema patient serum samples in order to detect autoantibody binding using a direct ELISA. Deglycosylation was examined using native C1Inh as a reference. Nine patient samples (P1–P9) were tested as illustrative examples, and antibody binding was expressed as binding ratio. Serum samples from healthy donors exhibit values of binding ratio <5 for the three isotypes. (B) Effect of anti-C1Inh autoantibodies on purified C1Inh function. Three patient plasma samples (one illustrative patient per isotype) were incubated with increased concentrations of purified C1Inh; C1Inh function was tested using the conditions described above (Ghannam et al., 2015).

that the non-serpin domain of C1Inh could contribute to its function, in addition to its already established role in the clearance of serpin from the blood (Minta, 1981).

### 3.2. Polysaccharides are targets for anti-C1Inh autoantibodies in acquired angioedema (AAE) patients

Rare cases of acquired angioedema (AAE), i.e., not hereditary, are primarily associated with lymphoproliferative or autoimmune disorders. Anti-C1Inh antibodies have been detected in approximately 40% of these patients. In the rare cases where samples have been tested, C1Inh control of contact phase (Ghannam et al., 2015) was found decreased in patient plasma samples with anti-C1Inh antibody-associated AAE. The question arises whether polysaccharides could be a target for the autoantibodies by analyzing AAE patient plasma samples with different autoantibody isotypes ( $n=9$ ). Autoantibody binding to C1Inh species was tested by means of ELISA. Antibody binding was strongly decreased following C1Inh digestion by neuraminidase and *O*- or/and *N*-glycanases, particularly with *O*-glycanase combined enzyme digestions. This decrease was independent of the autoantibody isotype (Fig. 2A). We next tested the impact of the autoantibodies on C1Inh control of the contact phase target. Purified C1Inh was incubated in the presence of normal or AAE patient samples. Regardless of the immunoglobulin isotype, C1Inh function was not affected by the autoantibody (Fig. 2B). These results indicate that the polysaccharides represent a target for the autoantibodies which have been tested, but that the antibody did not directly affect the function of supplemented C1Inh.

*O*-linked carbohydrate removal may result in alteration of C1Inh conformation, with a subsequent partial alteration of contact phase protease interaction, potentially due to masking of the binding site. *O*-linked glycosylation plays a role in maintaining secondary, tertiary, and quaternary structures of fully-folded proteins. An example of this is mucins, in which peptide regions with *O*-linked glycan attachments adopt a “bottle brush”-like structure (Carraway

and Hull, 1991; Gowda and Davidson, 1994). Such altered conformation following *O*-linked deglycosylation could also account for the decreased autoantibody interaction.

The autoantibody-polysaccharide interaction could affect the efficiency of substitutive treatment using plasma-derived C1Inh concentrate, as observed in some rare cases with the standard dose of 20 UI/Kg of body weight (Gobert D et al., manuscript submitted). Some anti-C1Inh IgM antibodies were found in the plasma of hereditary angioedema (HAE) patients during application of C1Inh concentrates, though it is not known if the antibodies were neutralizing or producing any clinical effect whatsoever (Varga et al., 2007). These antibodies, irrespective of their isotype, could be polyspecific immunoglobulins against common membrane glycoprotein targets, on circulating cells or endothelial cells.

## 4. Conclusion

By applying protein chemistry and enzymatic techniques, our study demonstrated that the deglycosylation of C1Inh affects its function over the contact phase target, but over not the C1s target, when using *O*-linked polysaccharides, not *N*-linked polysaccharides. This suggests the *N*-terminal domain is involved in C1Inh function.

We showed that the polysaccharide motif could be an anti-C1Inh autoantibody target and that these autoantibodies did not directly affect C1Inh function.

The significance of *O*-glycosylation sites for C1Inh function should be further investigated using modified forms to decipher the C1Inh mechanism of action on protease control for potential applications of C1Inh concentrates in AAE management.

### Author contributions

AG and CD were responsible for planning and conducting the experiments, data analysis, and manuscript preparation; PS developed the experimental work; LM and OF identified AAE patients;

DP qualified the patient sample selection. All authors read and approved the final manuscript.

### Conflict of interest

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