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## GlycoForum - Technical Note

# Are sialic acids involved in COVID-19 pathogenesis?

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We read with much interest the recent mini-review by Sun on the theoretically possible roles of cell surface sialic acids (Sias) in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection (Sun 2021). Given the magnitude of this coronavirus disease 2019 (COVID-19) pandemic (Lu et al. 2020; Zhu et al. 2020) this question is not just of importance to the glycobiology community, but also to other scientists across many disciplines related to this crisis.

Numerous viruses are known to have hemagglutinin proteins that engage sialylated glycans as primary or secondary targets for infection, and many of them also display a "receptor-destroying enzyme"—either a neuraminidase (sialidase) that removes Sias, or an esterase that removes Sia *O*-acetyl groups required for virus binding (de Groot 2006; Lang et al. 2020). The latter types of viruses include some coronaviruses that cause mild infections and selectively recognize different kinds of Sias via hemagglutinin-esterase (HE) proteins (de Groot 2006; Huang et al. 2015; Tortorici et al. 2019; Lang et al. 2020).

Unlike many viruses that generally conserve their sialoglycan-recognizing properties or undergo subtle changes in binding preference, the coronaviruses seem to be involved in rapid evolution of binding specificity via convergent and divergent evolution, especially, with regard to their preferred ligands. Some coronaviruses have eliminated the esterase activity or even the entire HE protein and switched to sialic acid (Sia)-binding via a spike protein (Huang et al. 2015; Hulswit et al. 2019; Qing et al. 2020). A few appear to have evolved further to preferentially bind to very specific host proteins such as ACE2 for the SARS viruses (Li et al. 2005). Recently, some coronaviruses including SARS-CoV-2 have also been shown to bind heparan sulfate (Clausen et al. 2020; Kim et al. 2020) an interaction that appears necessary for infecting ACE2-positive cells.

In the initial sequencing of the SARS-Co-2 viral genome, it was noted that amino acid residues involved in Sia-based interactions were missing or modified (Wu et al. 2020). Another study showed that mutations at the putative Sia-binding sites did not completely abolish binding but led to reduced binding (Peng et al. 2012). Consistent with this finding, initial studies did not show evidence for sialoglycan recognition. However, a few recent papers have suggested that SARS-CoV-2 spike protein may also weakly recognize Sias, with a preference for glycolipids (Awasthi et al. 2020; Baker et al. 2020; Engin et al. 2020). Despite these and multiple other papers and reviews (Evans and Liu 2021; Sun 2021) discussing the potential role of Sias in binding by the SARS-CoV-2 spike protein, relatively little is known about which types of sialosides it binds to.

To address this question, we probed a sialoglycan microarray presenting 139 glycans representing common terminal structures on vertebrate glycans, with a recombinant, soluble form of the SARS-CoV-2 spike protein (entire external domain) stabilized by six proline residues (HexaPro spike protein) (Hsieh et al. 2020) and secondary antibody StrepMAB Classic, anti-Twin-Strep-tag MoAb (IBA Life Sciences). The first experiment using the protein produced in the UT Austin lab of Jason McLellan showed low levels of binding to some of the sialoglycans on the microarray (data not shown). However, expression of the identical construct in our laboratory at UC San Diego gave a protein that did not bind to the microarray. We noticed that a major difference between the two preparations was that the UT Austin protein was produced in the presence of kifunensine (Elbein et al. 1990), an alkaloid that blocks N-linked glycan processing and thus prevents addition of Sias to the spike protein glycans, as a result of being a potent and specific mannosidase I inhibitor. In contrast the UC San Diego protein was generated in HEK293 cells (no kifunensine) that would add Sias to the spike protein glycans (Shajahan et al. 2021). A repeat experiment with the fresh batch of the UT Austin protein once again showed some binding. The most likely explanation is that the Sias on the heavily glycosylated spike protein are inhibiting the binding to the microarray due to *cis* inhibition. This type of "masking" has been known for a long time, e.g., with CD22
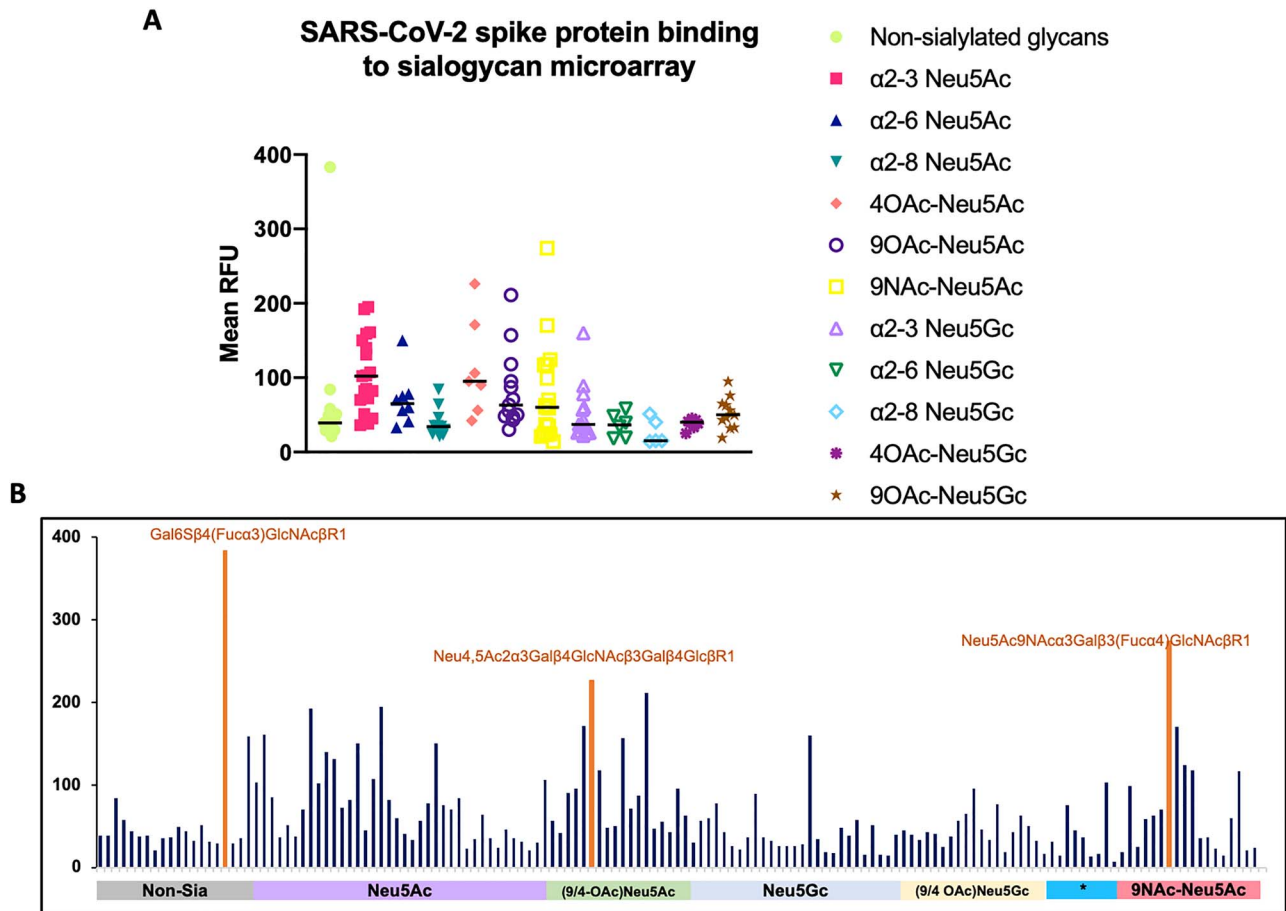
**Figure 1.** SARS-CoV-2 spike protein (25 μg/mL) binding on the sialoglycan microarray. (A) Binding to Neu5Ac- versus Neu5Gc-containing glycans. Mean RFU values were grouped into non-sialosides and sialosides based on the terminal sialic acid, linkage and acetylation status to identify outlier glycans as well as median binding tendency. Outlier binding was seen in Neu5Ac-containing glycans, *N*-acetyl Neu5Ac-glycans and non-sialylated glycans. (B) Binding pattern on complete sialoglycan microarray. Individual bars on the X-axis represent individual glycans. Some of the strongest binding glycans are labeled [Gal6Sβ4(Fucα3)GlcNAcβR1, Neu5Ac9NAcα3Galβ3(Fucα4)GlcNAcβR1 and Neu4,5Ac₂α3Galβ4GlcNAcβ3Galβ4GlcβR1]. ∗Asterisked group represent ganglioside type sialoglycans.

(Siglec-2) on B cells (Razi and Varki 1998) and also other immune cell types expressing other Siglecs (Razi and Varki 1999).

There are well-known species level differences in cell surface Sias that can affect pathogen binding (Dhar et al. 2019). While no clear pattern emerged regarding the underlying structure of the sialoglycans, there was a tendency toward preferential binding to Neu5Ac-containing glycans compared to Neu5Gc-containing glycans (Figure 1A). This binding preference was particularly noticed in glycans with α2-3-linked Neu5Ac (Figure 1A). There also was some tendency for binding to Neu5Ac-sialoglycans containing *O*-acetyl groups (particularly 4-*O*-acetylated ones) as has been hypothesized (Kim 2020). Additionally, there was preference for binding to sialoglycans synthetically replacing the *O*-acetyl group at C-9 position with an *N*-acetyl group. There appeared to be some binding to asialoglycans as well, particularly to Gal6Sβ4(Fucα3)GlcNAcβR1, an oligosaccharide that may be present in the airway mucus (Figure 1B shows the full microarray with the top three binding glycans labeled).

Looking at limited literature regarding SARS-CoV-2 infection of various animals suggests a tendency for more severe/lethal disease in those that do not express Neu5Gc-containing glycans and instead have an excess of Neu5Ac due to genomic mutations (Altman and

Gagneux 2019) inactivating *CMAH* e.g., humans, ferrets (Stout et al. 2021), minks (Oude Munnink et al. 2021) and white-tailed deer (Palmer et al. 2021). Notably, the bat species that are thought to be the origin of this virus are also deficient in Cmah (Cagliani et al. 2020).

It is currently unclear whether this weak binding to sialoglycans is important in actual SARS-CoV-2 infections. But considering that the first contact of incoming viruses is likely to be with heavily sialylated mucins, even such interactions in the form of viral "surfing" (Seyran et al. 2020) could be very important and determine the outcome. The same may be true for other viruses that are being spread by small aerosol particles (Greenhalgh et al. 2021). There is also a possibility that other variations of Sias found in the humans (e.g., 9-*O*-lactyl sialic acids) (Corfield et al. 1993) serve as potential targets. Yet another factor to be considered is the presence of sialylated glycans on the ACE2 glycoprotein receptor (Pruimboom 2020; Allen et al. 2021; Mehdipour and Hummer 2021).

We recognize that our glycan array does not mimic the in vivo situation with regard to underlying glycan structures nor the glycoproteins/glycolipids as the aglycone entities. Indeed, a novel cell-based glycan array recently confirmed (Narimatsu et al. 2019) an earlier prediction of higher order binding of microbial adhesins to "clustered saccharide patches" of sialylated O-glycans, organized by

their presentation on proteins (Cohen and Varki 2014). The array also does not take into account the complex organization of the glycocalyx and secretory glycoproteins, especially mucins. Additionally, sialylation of spike protein (dependent on cell type producing the virus) might affect binding as discussed earlier. Further studies are needed to confirm and expand these results, and comparison of binding to heparan sulfate is needed. The possibility must be considered that the individual variations in sialoglycan complexities might contribute to the extreme range of differences in severity and lethality in individual humans (Silva-Filho et al. 2020; Jiang et al. 2021). Furthermore, pathogen evolution may also be altering Sia binding tendency. While an early paper suggested that the SARS-CoV-2 spike protein lacked the amino acid residues needed for Sia recognition (Wu et al. 2020), it remains to be seen if some of the more recent infectious variants have regained these residues. There may also be other effects of Sias such as on immune recognition given that sialoglycans serve as self-associated molecular patters (SAMPs) (Varki 2011) and/or in COVID-19-associated inflammation (Siddiqui et al. 2021).

Another unexpected link to sialic acids is the alarmin molecule HMGB1, a major pathogenic and prognostic factor in severe sepsis (Andersson and Tracey, 2011), which is also elevated in COVID-19 sepsis (Chen et al., 2020; Andersson et al., 2020). Low serum zinc is another well-known risk factor for increased severity of sepsis (Hoeger et al., 2017), and hypozincemia was noted in patients with poor clinical outcomes from COVID (Yasui et al., 2020). While prospective trials of zinc supplementation are underway (Perera et al., 2020), existing retrospective meta-analyses have given inconsistent results (Fromonot et al., 2021; Dubourg et al., 2021; Wessels et al., 2021; Szarpak et al., 2021). We recently discovered that HMGB1 can be functionally sequestered away from its activating receptors by plasma sialoglycoproteins in a zinc-dependent manner, a protective effect that is lost when blood pH falls due to lactic acidosis (Siddiqui et al., 2021). Current trials independently studying zinc supplementation, HMGB1 inhibition, or pH normalization may be more successful if these approaches are combined and perhaps supplemented by infusions of heavily sialylated forms of glycoproteins like CD52 (Shathili et al., 2019).

Finally, it is notable that this virus appears to be best transmitted by very small long-lived aerosol particles generated by the human upper airways (Greenhalgh et al. 2021). It is possible that the weak but highly multivalent Sia-binding properties of the virus contribute to the formation of these particles.

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## Conflict of interest statement

None declared.

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