ABSTRACTS

"Glycans and Glycosylation in SARS-COV2 Infection" session at the XVII Advanced School in Carbohydrate Chemistry, Italian Chemical Society. July 4th -7th 2021, Pontignano (Si), Italy

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The novel pathogenic SARS-coronavirus 2 (SARS-CoV-2), responsible for aworldwide pandemic, and the urgent necessity to look for, to find new drugs capable to reduce, possibly to avoid the infection is requesting new studies some of which fall into the world of glycobiology.

The virus enters into the cells by interaction between the viral protein spike and the angiotensin-converting enzyme-2 (ACE-2) receptor. Spike is a glycoprotein but the information on the specific role of its glycans for the interaction and fusion processes are very scant. In addition, it is claimed that a specific role in modulating both the processes is played by the ACE-2 receptor surrounding membrane gangliosides. A better knowledge of all this would generate new ideas for the production of vaccines and therapeutic monoclonal antibodies. Anti-virus antibodies must be correctly glycosylated and could be the best therapeutic opportunity in the next years, but the increase the fucosyl-transferase activity is necessary to obtain their correct neutralizing property.

On the other hands, like several other viruses, SARS-CoV-2 displays membrane components similar to those of human membranes and therefore show immunogenic properties. New cases of peripheral neurodegeneration diseases, such as cases of Guillan-Barrè and Miller-Fisher syndromes have been reported. It is well known that these syndromes are due to glycans structures associated to both virus and human membranes.

The Italian Chemical Society (SCI) organizes every two years, in the beautiful location of the Pontignano Charterhouse, near the city of Siena, the Advanced School on Carbohydrate Chemistry.

In the 2021 edition a stimulating session on "Glycans and Glycosylation in SARS-COV2 Infection" has been organized, chaired by **Francesco Nicotra** and **Domenico Garozzo**, to stimulate young glyco-scientists to start new researches in the field. The session was opened by **Rino Rappuoli** (GSK Vaccines Srl, Siena), an internationally recognized authority in the field of vaccines. Further outstanding contributions were presented by **Manfred Wuhrer** (Leiden University, The Netherlands), **Elisa Fadda** (Maynooth University, Ireland), **Antonella Bisio** (IRCB-Ronzoni, Milan) and **Fabrizio Chiodo** (CNR ICB-Pozzuoli).

Dr. **Rappuoli**, who was awarded by the SCI with the Francesco Berti medal for his contributions in vaccine research, illustrated the various advancements in the field that led to the capacity to produce anti SARS-COV2 vaccines after only 10 months while, previously, it took at least 10 years.

Dr. **Wurher** described his research on IgG glycosylation in COVID19 patients and showing that there is a difference in fucosylation between patients in need of hospitalization and those who have been treated at home (Science, 2021, 371.6532). A review on the Antibody glycosylation in COVID-19 is published in Glycoconjugate Journal special issue.

Dr **Fadda** presented a contribution on the role of the glycan shield's nature and topology in the SARS-CoV2 structure and dynamics. Like all interventions in the session, these studies have a major impact on research against SARS-COV2, in particular the study by Dr. Fadda was also reported in The New York Times (Oct.09.2020).

Dr **Bisio** illustrated the contrasting roles of heparin and heparan sulfate in COVID 19, a still very hot topic.

Finally, Dr. **Chiodo** presented the role of glycans in the design of a vaccine. It goes without saying that Dr. Chiodo participates in the team of scientists who designed and put into production Soberana 2, one of the vaccines made in Cuba.

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Really a session full of stimulating lectures a small summary of which are reported in the following abstracts.

Vaccines: past and future impact

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Vaccination is the most cost-effective way to control infectious diseases caused by bacteria and viruses.

Glycans, ranging from monosaccharides to polysaccharides, are key players in bacterial and viral surface envelopes and on binding receptors. They are with functional and structural biomolecules and are naturally present as pure carbohydrates or linked to proteins or lipids to form glycoproteins (i.e. glycoconjugates) or glycolipids (e.g. lipopolysaccharides). For capsulated bacteria (e.g. *Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Salmonella typhi* Vi), the capsular polysaccharide (CPS) is the primary virulence factor and has been selected as vaccine target. Since the first conjugate vaccine (CPS antigen chemically coupled to a carrier protein) licensed in the 1980's to protect humans against *Haemophilus influenzae* type b infection, many other CPS-protein conjugate vaccines have been developed and this technology still represents one of the most used approaches [1].

For viruses, the glycosylation of envelope proteins is known to be exploited by the pathogen to escape recognition by the host immune system by masking relevant protein epitopes from detection by antibodies.

However, for SARS-Cov-2 as also observed for SARS and MERS, despite its dense carbohydrate layer, areas of vulnerability to be attached by monoclonal antibodies recognizing aminoacidic epitopes have been demonstrated efficacious. For instance, very potent neutralizing antibodies recognizing the spike protein receptor-binding domain have been developed and tested in clinic [2].

Conflict of interest

This work was sponsored by GlaxoSmithKline Biologicals SA. RR and FB are employees of the GSK group of companies.

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The structure and evolution of the SARS-CoV-2 spike's glycan shield regulates activity and allows access to cell surface glycans as co-receptors

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The SARS-CoV-2 spike (S) is a type I fusion glycoprotein, responsible for initiating infection leading to COVID-19. The S architecture is the product of an intertwined trimeric fold[1,2], in which the receptor binding domain (RBD) of each protomer can be exposed in an 'open' conformation and thus accessible for binding by the angiotensin-converting enzyme 2 (ACE2). As other fusion proteins presented on the surface of enveloped viruses, the SARS-CoV-2 S is heavily glycosylated with 22 N-glycosylation sites per protomer with high degree of occupancy[3]. In recent work[4,5], we and others have shown through multi-microsecond molecular dynamics (MD) simulations that the thick glycan shield covering the S protein is not only essential for hiding the virus from immune detection, but that it also plays multiple functional roles. More specifically, the N-glycans at positions N165, N234 and N343, shown in Fig. 1 panel a, are key to support the RBD open conformation[4] and to gate the open-to-close transition[5].

Competence of different SARS-CoV-2 S glycoforms. The glycans' stabilization of the SARS-CoV-2 S active prefusion conformation is the result of dynamic interactions between protein residues and specific monosaccharide units along the glycans structures, as well as of less specific glycan-glycan interactions[4–6]. Because the glycans' 3D structure and terminal epitopes' presentation is intrinsically dependent on their sequence and branching[7–9], it is conceivable that different S glycoforms would show varying degrees of competence in exposing the RBD for ACE2 binding. This is an important point to investigate, as recent studies have shown that the nature of the SARS-CoV-2 S glycan shield varies and it is directly linked to the glycoprotein's stability[10,11]. To this end, we have built and analyzed through multi-microsecond MD simulations the equilibrium structure and dynamics of different SARS-CoV-2 S glycoforms[6], bearing significant changes in the N-glycosylation at key functional sites, namely N165, N234, and N343. Our results show that the shortening of the large oligomannose N-glycan at N234, characteristic of a

stable S constructs[3,10,11], to Man5 or even to Man3, greatly affects the stability of the open RBD, increasing its dynamics and, in the case of Man3, promoting a near-closed conformation, where large biantennary complex N-glycans at N165 and N343 can interact with residues in the highly flexible receptor binding motif (RBM)[6]. Furthermore, we find that shortening the N-glycans at N165 and N343 to Man5 prevents this ancillary support, ultimately undermining the stability of the open RBD to an even higher degree, in agreement with recent experimental evidence[12].

Evolution of the SARS-CoV-2 S glycan shield's topology: Loss of glycosylation at N370. Because of the glycan shield intrinsic involvement in the SARS-CoV-2 S mechanism of action, it is reasonable to think that the evolution of the sites of glycosylation may be intertwined with the evolution of the overall protein sequence to affect optimal activity. In this context, we recently studied the effect of the recently acquired T372A mutation on the stability and dynamics of the open and closed SARS-CoV-2 S RBDs[6]. This mutation prevents glycosylation at position N370, which is a highly conserved feature along the SARS-CoV-2 phylogeny[6], also present in S proteins from SARS-CoV, RaTG13 and pangolin CoV. Multi-microsecond MD simulations of a SARS-CoV-2 S mutant with the sequon restored and the CoV S FA2G2 N-glycan at N370 show that glycosylation at N370 is extremely effective in supporting the RBD open conformation, indicating that this glycan may have been the ancestral functional replacement of the N234 glycan, which only recently appeared in the phylogeny[6]. Interestingly, we also found that the N370 N-glycans occupy structurally conserved clefts on the adjacent closed RBDs surface, see Fig. 1 panel a, tying the S protomers down and thus likely increasing the opening energetics. Ultimately, the loss of the N370 glycosylation site in the SARS-CoV-2 S may have contributed to its higher infectivity relative to CoV and other variants carrying the sequon at N370, in agreement with recent experimental work[13].
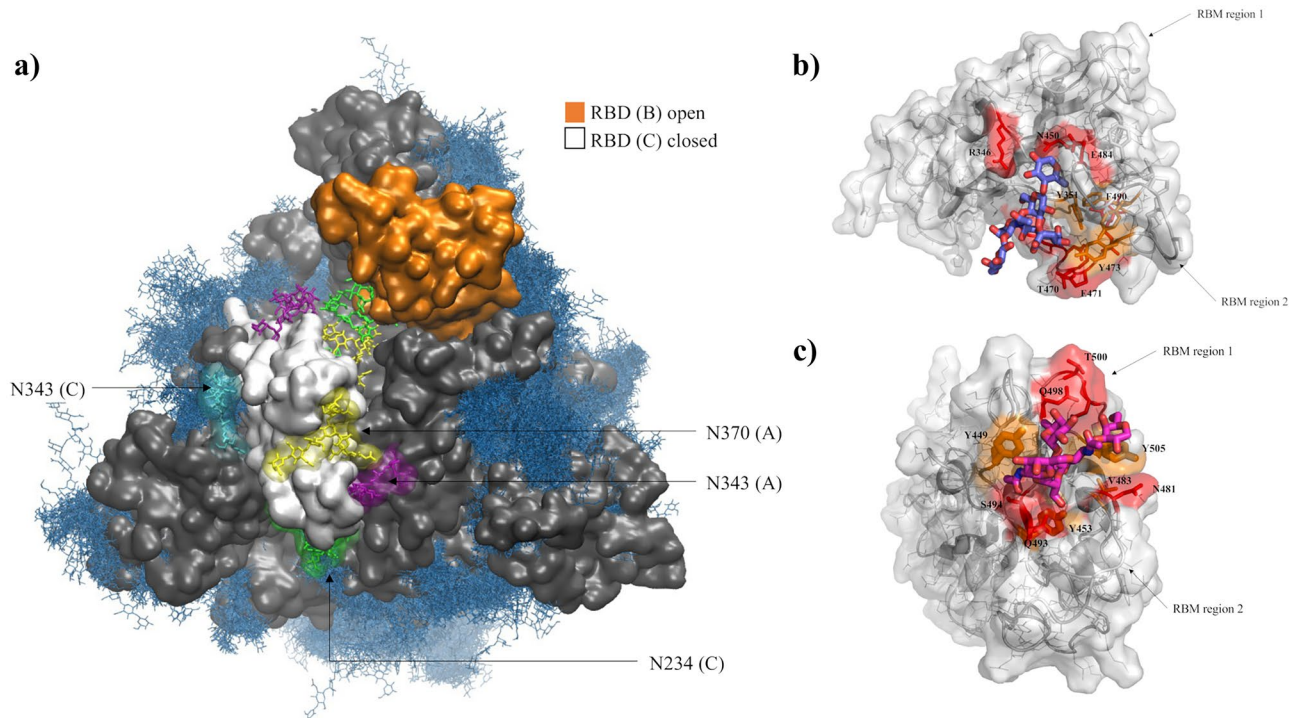
The SARS-CoV-2 S RBD glycan-binding cleft is accessible to cell-surface glycans. A recent study provides clear evidence that the SARS-CoV-2 S protein interacts with cell-surface heparan sulfate (HS) and that this interaction is essential for infection, contributing to its binding to the main ACE2 receptor in a trimeric complex[14]. An even more recent work shows that a broad range of charged and neutral glycans also bind the SARS-CoV-2 S RBD, with monosialylated gangliosides binding with comparable affinity to glycosaminoglycans (GAGs)[15]. This insight poses a formidable challenge in the identification the binding site(s) for such a diverse set of glycans, notably because of the relatively small size of the RBD (30 kDa with glycans at N343 and N331), and because approximately half of the

RBD protein surface is not accessible when in a closed conformation. We tested the ability of the N370 glycan-binding cleft available in SARS-CoV-2 S, to host other glycans by building complexes via structural alignment to the FA2G2 N370 glycan (and/or docking) of selected epitopes, namely GM1 and blood group A type 1 antigen (t1A). The stability of all the starting complexes was assessed by multiple independent runs of conventional MD simulations of up to a microsecond in length. Despite none of the starting poses being conserved, stable complexes were found in all cases, see Fig. 1 panels b and c. In the RBD-GM1 complex, shown in Fig. 1 panel b, the glycan epitope occupies the position of the terminal residues of the N370 FA2G2 (1–6) arm, with the sialic acid hosted in a deep pocket (100% occupancy over 700 ns). Meanwhile, in the RBD-t1A complex, shown in Fig. 1 panel c, the glycan epitope recurrently visits the same binding pose through binding and unbinding events in the region near where the N370 FA2G2 (1–3) arm is bound (38% total occupancy over 1 μs). These preliminary results suggest that, while the architecture of the RBD N370 glycan-binding cleft has likely evolved to host a complex N-glycan, in its absence, i.e. in SARS-CoV-2 S, it could potentially host other glycan epitopes, provided that these fit the electrostatic and steric requirements imposed by the structure of the cleft. Furthermore, because the location of the cleft on the RBD surface spans the two regions of the highly flexible RBM, directly engaged in ACE2 binding, interactions with other glycans can indeed contribute to the binding affinity, either directly or by pre-structuring the disordered loops in conformations amenable to ACE2 binding. Ultimately, because the cell surface is heavily glycosylated, the ability of the SARS-CoV-2 S to interact with a broad range of glycans in the glycocalix would enhance cell surface localization and thus contribute to increase viral infectivity.

Figure 1. Panel a) Top view of the SARS-CoV-2 S A372T mutant from a snapshot from MD simulation[6]. The N370 FA2G2 N-glycan is shown in yellow, bound to the RBD cleft in a closed protomer (chain C, shown in white) and inside the pocket left vacant by the opening of RBD from chain B (shown in orange). Chain A and all other domains are shown in grey. Chain numbering corresponds to PDBid 6VYB used to build the model. Man9 at N234 is shown in green, FA2B at N343 in purple and A2B at N165 in cyan. All other glycans are shown in blue as an overlay of snapshots collected every 10 frames during the 1 μs MD trajectory. Rendering done with VMD (www.ks.uiuc.edu/Research/vmd/). **Panel b)** Complex of the RBD (white) with GM1. The GM1 epitope is shown with a 'sticks' representation, with C atoms in dark purple. Hydrophilic contacts are highlighted in red, hydrophobic contacts in orange. **Panel c)** Complex of the RBD (white) with t1A. The t1A epitope is shown with a

'sticks' representation, with C atoms in light purple. Hydrophilic contacts are highlighted in red, hydrophobic contacts in orange. Rendering done with pyMol (www.pymol.org)

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Conflict of interest

Authors declare no conflict of interest.

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More than just an analogue: the contrasting roles of heparin and heparan sulfate in viral infections

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Many pathogens exploit heparan sulfate (HS), a glycosaminoglycan (GAG) ubiquitously expressed on the surface and in the extracellular matrix of virtually all cell types, as a means of adhering to, and accessing, host cells by taking advantage of its key role in regulating homeostasis through interactions with hundreds of extracellular proteins. The binding specificity of HS-protein interactions largely depends on the complementarity of the spatial arrangement of basic groups on the protein and of sulfate and carboxylic groups on HS chains. Heparin, the structurally related GAG well-known as anticoagulant drug, is widely used as a model of HS for in vitro studies. Starting from a common biosynthetic precursor they undergo the same numerous post-synthetic modifications (N-deacetylation, glucuronic acid epimerization, N-, 2-O, 6-O, and 3-O sulfation) but to different extent, so that heparin can be regarded as a more extensively modified version of HS, with an even higher degree of structural heterogeneity. Accordingly, HS products as well as heparin and its derivatives, have turned out to be involved, and are effective in preventing infection by a range of viruses, including coronaviruses [1] such as SARS-CoV-2, responsible for the current COVID-19 pandemic. In particular, interaction of the ectodomain of the SARS-CoV-2 spike (S) glycoprotein with cell surface HS has been reported to favor the binding of S glycoprotein with a second receptor, the angiotensin-converting enzyme 2 (ACE2), thereby promoting virus entry into the cell [2]. In parallel, by using a cell model, exogenous heparin was found to be capable of competing with HS for the SARS-CoV-2 S protein ectodomain and of inhibiting viral growth to a significant extent (80%), much higher than for SARS-CoV (Fig. 2) [3]. Moreover, the receptor binding domain (RBD) of SARS-CoV-2 glycoprotein was proved to bind heparin as well as its derivative low-molecular-weight heparin, enoxaparin, in both cases eliciting a conformational change in the protein. An hexasaccharide turned out to be the shortest effective oligosaccharide. The analysis of the 3D structure of the RBD in accord with a bioinformatic search of the sequence analysis suggest at least three putative heparin

binding sites that are solvent accessible and not shielded by glycosylated sites. (Fig. 3) [3].

Together with the documented role of heparin and enoxaparin in modulating the cytokine storm, and in the prevention and treatment of the thromboembolic complications of COVID-19 [4], all of the available evidence supports the repurposing of heparin and LMWHs and the application of non-anticoagulant heparin derivatives as antiviral agents. Despite vaccines represent the main defense against viruses, these GAGs promise to be additional weapons in the fight against both current and emerging viral diseases.

Figure 1. The heparin mediated inhibition of SARS-CoV-2 viral invasion of Vero cells. Effect of heparin addition before the infection of Vero cells with 50 plaque forming units (PFU) of SARS-CoV-2 or SARS-CoV. The results are expressed as the number of PFU per well and represent the mean ± SD of quadruplicate cultures
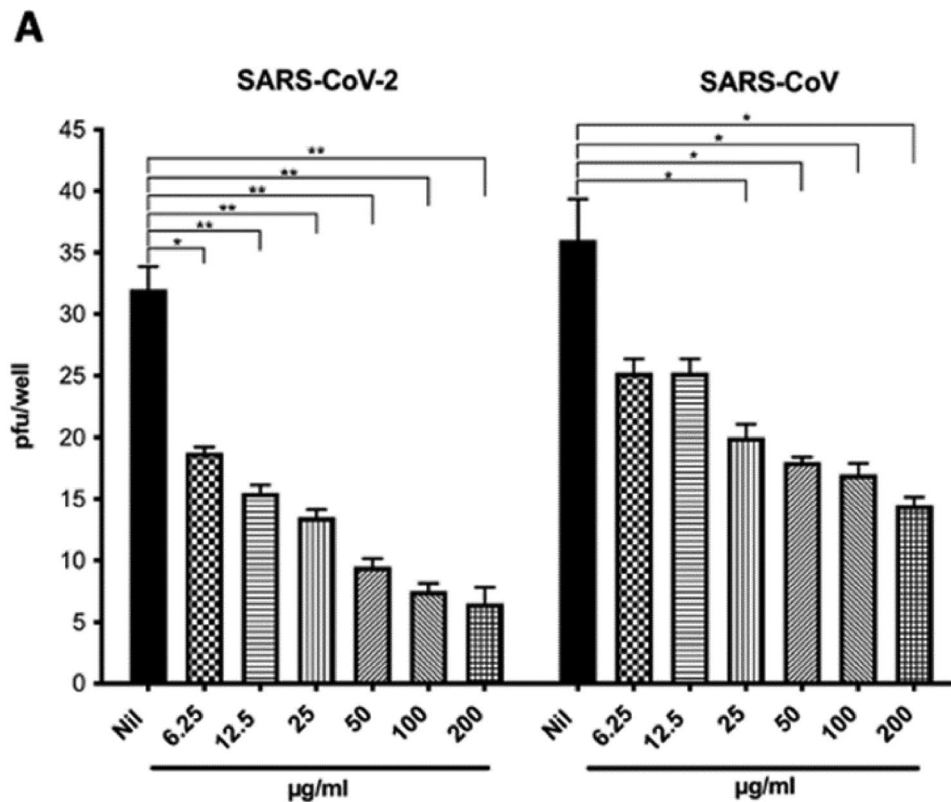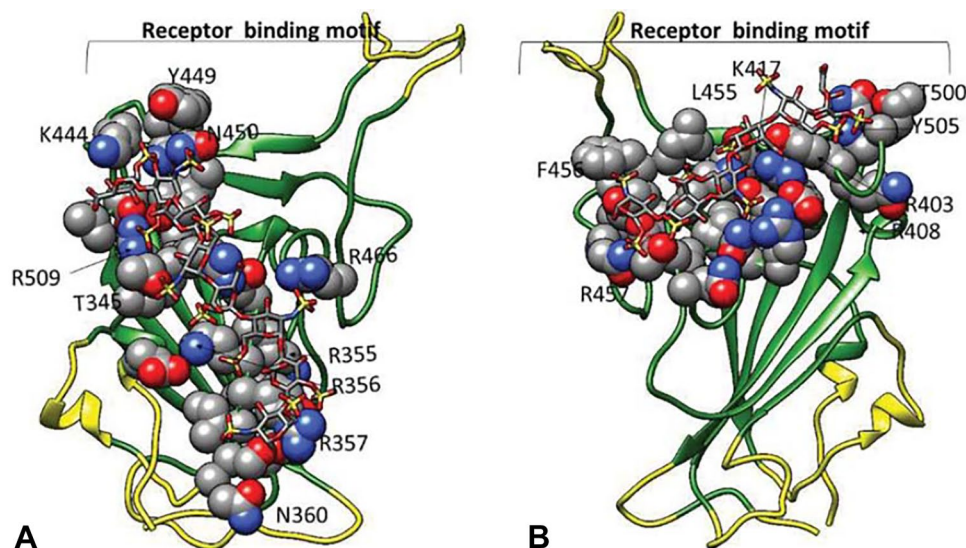
Figure 2. Snapshots [3] taken from MD simulations of SARS-CoV-2-RBD in the presence of either a heparin octasaccharide (A) or a heparin hexasaccharide (B). The heparin oligosaccharides are shown as sticks whereas amino acids of the RBD are shown as spheres. The residues are colored as per elements. Hydrogen atoms are not shown for clarity. Adapted from [3]

The traditional mechanism of action of conjugate-vaccines considers the peptides generated from the immunogenic carrier proteins (toxoids etc) to be responsible of T-cells activation, B-cells maturation and antibodies production. However, in conjugate-vaccines, the carbohydrates could play a two-faced role: they are the antigens able to elicit specific anti-carbohydrate immune responses, and at the same time they could work as an immune-potentiator to enhance the immune responses to themselves. We demonstrated the active involvement of some *S. pneumoniae* polysaccharides in determining a series of *in vitro* innate immune responses in human. Peripheral blood mononuclear cells were stimulated with approved and in clinical use *S. pneumoniae* Cuban carbohydrate-based vaccine coupled to the carrier tetanus toxoid and different read-out have been measured. We proved that some *S. pneumoniae* polysaccharides, especially the ones containing rare deoxy-amino monosaccharides, are able to "potentiate" immune responses, adding extra information for the design and development of a new generation of *S. pneumoniae* vaccines.

In addition, we also recently applied a rational "glyco-approach" to design different subunit SARS-CoV-2 vaccines recently approved for emergency use in Cuba. The receptor-binding domain (RBD) for the viral spike protein has been selected as antigen for the vaccines. The glycosylated RBD is the most accessible spike protein surface while the spike trimer is opening itself during the preliminary contacts with the host ACE-2 receptor. Glycans are also structurally involved in these interactions. In parallel, we have studied different lectins-mediated interactions comparing the binding of the spike and the RBD. The RBD was not recognized by the mannose binding lectin (MBL) avoiding an hypothetical immunological "clearance" MBL-mediated. For these structural and immunological reasons, we have decided to

Conflict of interest

Authors declare no conflict of interest.

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Carbohydrate-Mediated "Innate" Considerations in Designing Vaccine-Candidates

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Carbohydrates play key immunological roles at the host-pathogens interface, and these interactions are extremely important while designing vaccine-candidates against infectious diseases. We have described two examples were the rational design of vaccines has taken into consideration carbohydrates-mediated interactions.

use as main antigen the RBD expressed in mammalian cells. The Cuban vaccine Soberana 02 (RBD-conjugated to tetanus toxoid) plus the product Soberna Plus (RBD-dimer) have demonstrated a phase-3 efficacy vs placebo against symptomatic COVID-19 of 92%.

In conclusion, our results aim at considering carbohydrate-mediated "innate" interactions while designing vaccine-candidates against infectious diseases.

Conflict of interest

Authors declare no conflict of interest.