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Sparavigna, Amelia Carolina

Let us study viruses and the role of sialic acids in their first step of reproduction, the attachment to cells. We begin our study with the help of the "Earth's virology course", created by Vincent Racaniello, with David Tuller and Gertrud U. Rey. The first virus that we will study is that of influenza A. Actually, "Earth's virology course" is a proper starting point for learning the mechanisms the virus uses to attach and enter the host cells. In the case of the influenza virus, its spike protein is used to attach the virion to sialic acid receptors of the host cells. Then, we will consider coronaviruses, with the help of literature about the role of sialic acids in their attachment by means of spike proteins. In fact, there are seven human coronavirus and they use sialic acid or angiotensin-converting enzyme 2 (ACE2) or other receptors or a combination of them. Let us review the literature to understand if Sars-Cov-2 virus can use sialic acid for the attachment to cells or not. Its main target is considered the ACE2 receptor. We will see that a study exists which detected that Sars-Cov-2 protein molecules can bind to heparan sulfate glycans in a sulfation-dependent manner.

About the role of sialic acids in Sars-Cov-2 attachment: a) the study in 10.1021/acscentsci.0c00855 used a glyco-nanoparticle platform, discovering that N-acetyl neuraminic acid has affinity toward the SARS-COV-2 spike glycoprotein, that is a glycan-binding function. b) a recent study, 10.1016/j.scib.2021.01.010, which has investigated the binding of the spikes of the virus by means of micro-arrays, tells that no binding with sialic acid residues was detected. All the tested protein molecules can bind to heparan sulfate glycans (as in 10.1016/j.cell.2020.09.033) in a sulfation-dependent manner. c) A preprint published on March 8, 2021, entitled "Sialic acid-Dependent Binding and Viral Entry of SARS-CoV-2". bioRxiv 2021.03.08.434228; doi: https://doi.org/10.1101/2021.03.08.434228, gives different results,

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Keyword(s):

Virus Influenza Sialic acids NANA Sars-Cov-2 ACE2 Integrin Virion Plasma membranes Spike proteins Hemagglutinin Neuraminidase Human Coronaviruses HCoV-229E HCoV-OC43 HCoV-HKU1 HCoV-NL63 NeuAc 9-O-Ac-NeuAc NeuGc hemmaglutinin esterase Influenza A Heparan sulfate Influenza C

Communities:

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Sialic acids and viruses

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Let us study viruses and the role of sialic acids in their first step of reproduction, the attachment to cells. We begin our study with the help of the "Earth's virology course", created by Vincent Racaniello, with David Tuller and Gertrud U. Rey. The first virus that we will study is that of influenza A. Actually, "Earth's virology course" is a proper starting point for learning the mechanisms the virus uses to attach and enter the host cells. In the case of the influenza virus, its spike protein is used to attach the virion to sialic acid receptors of the host cells. Then, we will consider coronaviruses, with the help of literature about the role of sialic acids in their attachment by means of spike proteins. In fact, there are seven human coronavirus and they use sialic acid or angiotensin-converting enzyme 2 (ACE2) or other receptors or a combination of them. Let us review the literature to understand if Sars-Cov-2 virus can use sialic acid for the attachment to cells or not. Its main target is considered the ACE2 receptor. We will see that a study exists which detected that Sars-Cov-2 protein molecules can bind to heparan sulfate glycans in a sulfation-dependent manner.

Keywords: Virion, Virus, Plasma membranes, Spike proteins, Hemagglutinin, Neuraminidase, Sialic acids, Heparan sulfate, Sars-CoV-2, Human Coronaviruses, HCoV-229E, HCoV-OC43, HCoV-NL63, HCo-HKU1 , Influenza A, Influenza C

Torino, 19 March 2021

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Introduction

It exists a remarkable course that we can find in the web. It is one of the "Earth's virology course", created by Vincent Racaniello, with David Tuller and Gertrud U. Rey. The course that we use here is "Influenza 101"[1]. Let us read how an influenza virus

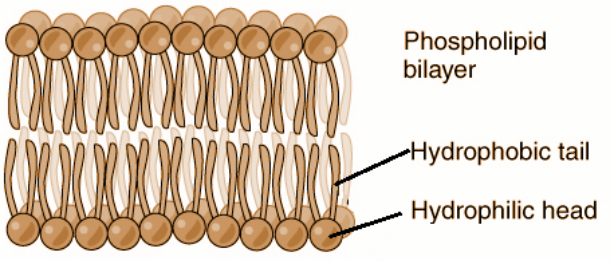
1 https://www.virology.ws/influenza-101/ archived https://archive.is/qNstu

attach to cells[2] (4 MAY 2009), and the particular proteins and acids which are involved.

First, we have to consider that viruses cannot reproduce outside of a cell, and therefore viruses are "parasites" of cells. Then, for their replication, the viruses must enter the cells. The first step of the reproduction is made by the attachment and the entry of the virion into the cell (virion is the term used by Racaniello, not virus[3]). After entering, the further steps are the translation of mRNA into protein, the genome replication, the assembly of new particles. The final process is the release of viral particles from the cell. *However, before starting the "Earth's virology course - Influenza 101" let us shortly discuss the membranes.*

1) PLASMA MEMBRANE - Cells are surrounded by a barrier, the *plasma membrane*. This membrane, also called *cell membrane*, exists in all cells. It separates the interior of the cell from the outside environment. The membrane consists of a semipermeable lipid bilayer. "The plasma membrane regulates the transport of materials entering and exiting the cell", tells NIH[4]. A lipid bilayer consists of two layers of lipid molecules, which are *phospholipids.* These molecules have *hydrophilic heads* and *hydrophobic tails*.

"The tail regions, being repelled by water and slightly attracted to each other, congregate together. This exposes the head regions to the outside, creating a barrier between two bodies of water"[5].



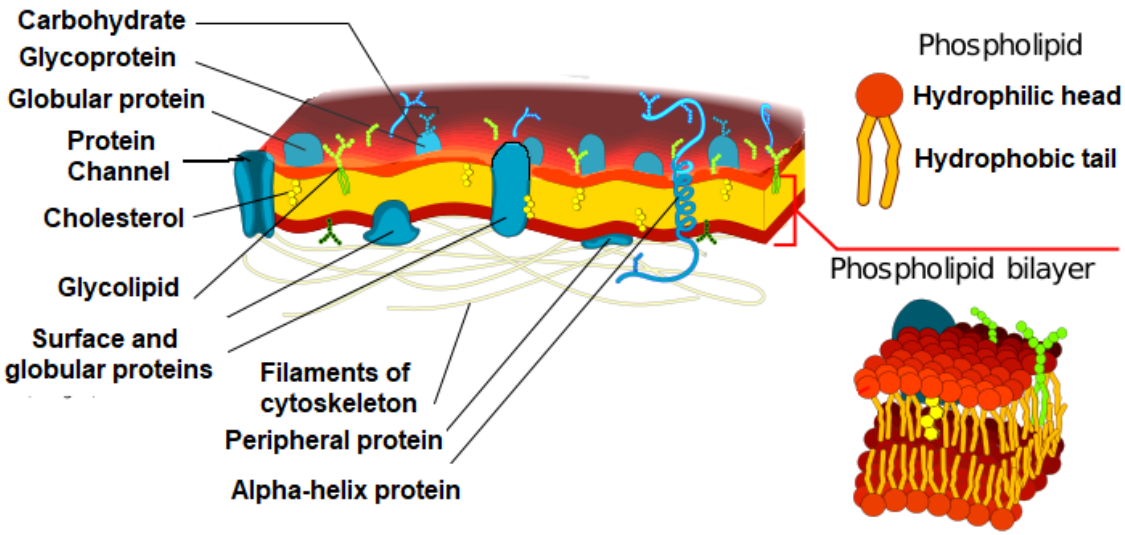
"The phospholipid bilayer consists of two adjacent sheets of phospholipids[6], arranged tail to tail. The hydrophobic tails associate with one another, forming the interior of the membrane. The polar heads contact the fluid inside and outside of the cell. Source: OpenStax - https://commons.wikimedia.org/wiki/File:0302_Phospholipid_Bilayer.jpg

2 https://www.virology.ws/2009/05/04/influenza-virus-attachment-to-cells/ archived https://archive.is/ZOJY5
3 https://www.virology.ws/2010/07/22/the-virus-and-the-virion/
4 https://www.genome.gov/genetics-glossary/Plasma-Membrane
5 https://biologydictionary.net/lipid-bilayer
6 https://en.wikipedia.org/wiki/Phospholipid

From https://en.wikipedia.org/wiki/Phospholipid - Phospholipids, also known as phosphatides, are a class of lipids whose molecule has a hydrophilic "head" containing a phosphate group, and two hydrophobic "tails" derived from fatty acids, joined by a glycerol molecule. The phosphate group can be modified with simple organic molecules such as choline, ethanolamine or serine. Phospholipids are a key component of all cell membranes. They can form lipid bilayers because of their amphiphilic[7] characteristic.

In eukaryotes, cell membranes also contain *another class of lipid*, sterol, *interspersed* among the phospholipids. The combination provides fluidity in two dimensions combined with mechanical strength against rupture. Purified phospholipids are produced commercially and have found applications in nanotechnology and materials science. The first phospholipid identified in 1847 as such in biological tissues was lecithin, or phosphatidylcholine, in the egg yolk of chickens by the French chemist and pharmacist Theodore Nicolas Gobley.

In the case of cells, the plasma membrane is not just a phospholipid membrane. This membrane is containing proteins, cholesterol, glycolipids, carbohydrates, and proteins structured in channels. The membrane must allow the connection of the cytoplasm to the extracellular fluid for the life of the cell.

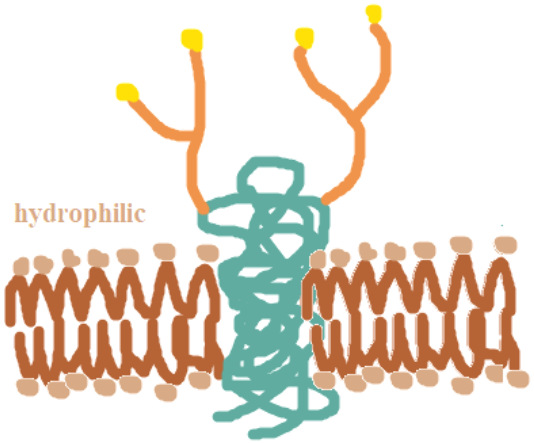


The image is adapted from a courtesy image by the web site https://en.wikiversity.org/wiki/The_Cell_Membrane

7 See please the discussion at http://www.chem.ucla.edu/~harding/IGOC/A/amphiphilic.html

From the previously given image, we can appreciate that there are many structures emerging from the membrane. Many of the ends of these structures are represented by sialic acids.

2) VIRUSES AND RECEPTORS - "Viruses have evolved different ways" to enter the barrier, the "Earth's virology course" tells. But all viruses "must first attach to a receptor on the plasma membrane in order to enter the cell". Then the virus has "a specific receptor that it attaches to, and in turn there is a particular viral protein that binds this cell receptor". Influenza virions have binding devices on their surfaces which are the "spikes". "The influenza viral spike that attaches to the cell receptor is the HA protein, the *hemagglutinin*[8]. The cell receptor is *sialic acid*, a small sugar that is attached to many different proteins on the cell surface".



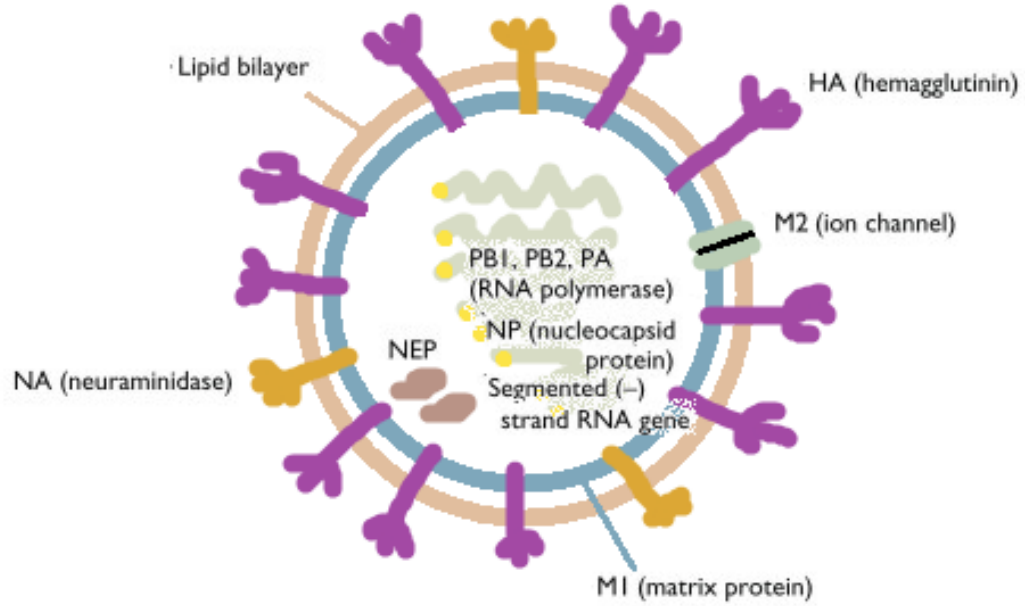
A drawing of a cell protein embedded in the plasma membrane. The cytoplasm, which is the interior of the cell is below the membrane represented in the figure. Part of the protein (pale blue) crosses the membrane, and we can see that there are also parts inside and outside the cell. The yellow spheres at the top of the branches are sugars, "attached to many proteins (protein + sugar = glycoprotein). ***Sialic acid is always the last sugar in a chain*** that is attached to a protein". "Influenza virions attach to cells when the HA grabs onto the very small sialic acid".

The sugar, represented by the yellow sphere, is small when compared to HA. The sugar "fits into a small pocket on the top of the spike. ... So far we have *docked* the influenza virion onto the surface of the cell. It is sitting there quite firmly, but is still on the outside of the cell". How do the viral RNAs get into the cell?

8 https://en.wikipedia.org/wiki/Hemagglutinin_(influenza)

3) A QUESTION - In the web page of the "Earth's virology course" we can find also questions from students. A question from duck - 4 May 2009, 3:27 pm, is the following. *So what is the function of sialic acid (apart from allowing virus infection). Is it useful to us, or just an accident? Why is it there?* - The answer is that the sialic acids are present in every cell. These acids possess many functions. One of the function is that the sialic acid-rich oligosaccharides "on the glycoconjugates found on surface membranes help keep water at the surface of cells. Since water is a polar molecule with partial positive charges on both hydrogen atoms, it is attracted to cell surfaces and membranes. This also contributes to cellular fluid uptake"[9].

It is clear that viruses are firstly using the most common and useful units of the cells. For a further answering to the question, see also Appendix - Health and Disease.



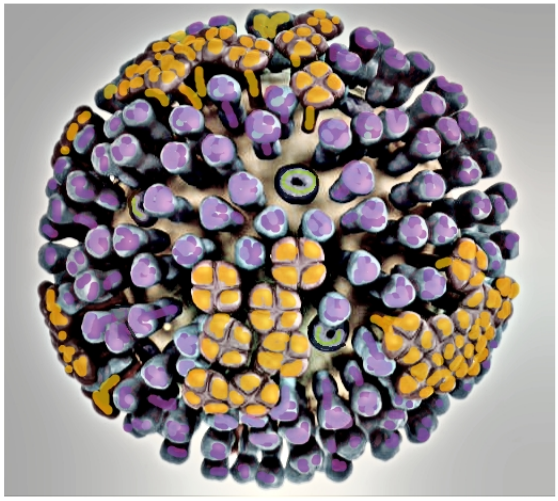
Virion of Swine flu A/Mexico/09 (H1N1)

4) HA, HEMAGGLUTININ - Adapted from https://en.wikipedia.org/wiki/Hemagglutinin. See please references therein. "Hemagglutinin" is a term coming from the Greek for “blood” + Latin term for “glue”. It is a glycoprotein which causes red blood cells to agglutinate or clump together. It mostly happens when adding influenza virus to erythrocytes, just as virologist George K. Hirst discovered in 1941, even though it can also occur with measles virus, parainfluenza virus and mumps virus, among

9 https://www.virology.ws/2009/05/04/influenza-virus-attachment-to-cells/

others. Subsequently, more related discoveries were made such as when Alfred Gottschalk proved in 1957 that hemagglutinin links virus in order to host cells by attaching sialic acids on carbohydrate side chains of cell-membrane glycoproteins and glycolipids. - Hemagglutinin has a cylindrical shape and it is a protein 13 nanometers long. It's a glycoprotein formed by three identical subunits called monomers, so we can tell that it is a homotrimer. The monomers are linked by two disulfide polypeptides: membrane-distal HA1 and the membrane-proximal HA2 which is much smaller. The trimer structure is linked to the membrane by little elastic chains.

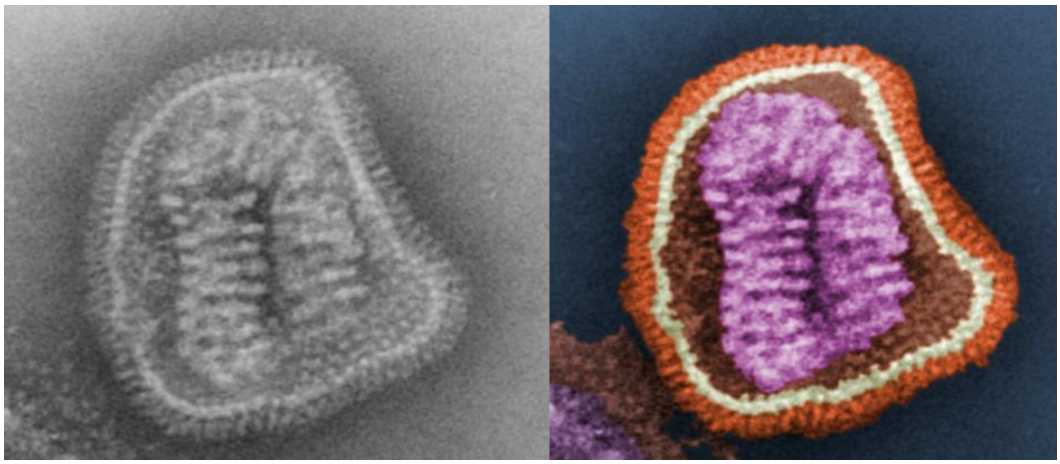
5) THE VIRION - "The influenza virion (as the infectious particle is called) is roughly spherical"[10]. This virus is an "enveloped" virus. It has an outer layer which is a lipid membrane, taken from the membrane of the host cell. Viruses multiply inside the cell, taking a part of its membrane to move in the body's environment. Protruding from the lipid membrane, there are the 'spikes' of the virus. These spikes are proteins, glycoproteins, consisting of a protein linked to sugars. We have already told of the HA protein (hemagglutinin). There is also the NA protein (neuraminidase). The "Earth's virology course" explains that these are the proteins that determine the subtype of influenza virus. HA and NA proteins are relevant for the response of the immune system against the virus. The antibodies produced by our bodies against the viruses - "Earth's virology course" explains - are responding to these spikes.



Adapted from a 3D image (Credits of 3D; Dan Higgins, Douglas Jordan, USCDCP) - NA in yellow, HA in pale violet, M2 in pale green. Lipid bilayer in pale pink.

10 https://www.virology.ws/2009/04/30/structure-of-influenza-virus/

The NA protein is the target of some antiviral drugs. Another target of antivirals is M2 protein. Beneath the lipid membrane, there is the M1 viral protein, the matrix protein. The matrix protein forms a shell which acts to reinforce the lipid envelope. Within the interior of the virion are the viral RNAs. These are the genetic material of the virus. Each *RNA segment*, as they are called, "consists of RNA joined with several proteins shown in the diagram: PB1, PB2, PA, NP. These RNA segments are the genes of influenza virus. The interior of the virion also contains another protein called NEP".



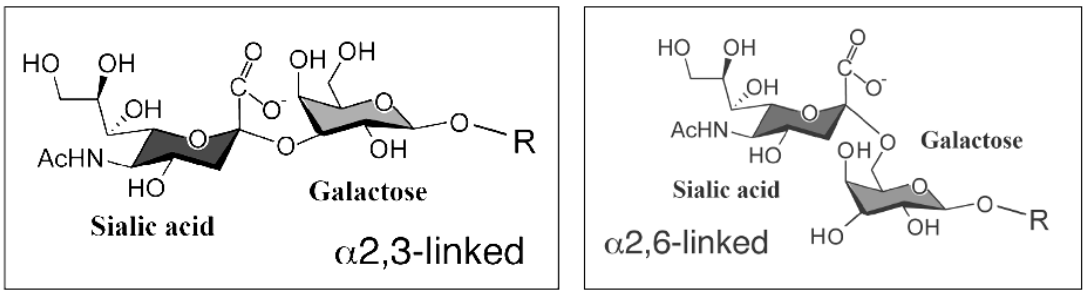
On the left - Image courtesy: https://phil.cdc.gov/Details.aspx?pid=8430 - ID#: 8430. Description: This negative-stained transmission electron microscopic (TEM) image depicts the ultrastructural details of an influenza virus particle, or virion. Content Providers(s): CDC/ Erskine. L. Palmer, Ph.D.; M. L. Martin. Creation Date: 1981. Photo Credit: Frederick Murphy. Let us note that the virion is not a perfect sphere. On the right - Image Courtesy: https://phil.cdc.gov/Details.aspx?pid=10073 - ID#: 10073. Description: The same as before digitally colorized. Content Providers(s): CDC/ Erskine. L. Palmer, Ph.D.; M. L. Martin. Photo Credit: Frederick Murphy

6) NEURAMINIDASE - From https://en.wikipedia.org/wiki/Viral_neuraminidase - Neuraminidase is an enzyme that *cleaves* sialic acid groups from glycoproteins. Neuraminidase inhibitors are antiviral agents that inhibit influenza viral neuraminidase activity and are of major importance in the control of influenza [1]. Sialic acid is a negatively charged sugar associated with the protein and lipid portions of lipoproteins.

"To infect a host cell, influenza virus attaches to the exterior cell surface using hemagglutinin, a molecule found on the surface of the virus that binds to sialic acid groups. Sialic acids are found on various glycoproteins at the host cell surface. The virus then moves from sialic acid group to sialic acid group until it finds the proper cell

surface receptor (whose identity remains unknown) [2]. Neuraminidase enables this movement by cleaving sialic acid groups that hemagglutinin was attached to. After the virus has entered the cell and has replicated, new viral particles bud from the host cell membrane. The hemagglutinin on new viral particles remains attached to sialic acid groups of glycoproteins on the external cell surface and on the surface of other viral particles; neuraminidase cleaves these groups and thereby allows the release of viral particles [3] and prevents self-aggregation [2]. Neuraminidase also facilitates the movement of virus particles in the present of mucus rich in sialic acid. [2]"

"IAVs [Influenza A Viruses] initiate the infection process by using the HA molecules on the viral envelope. Upon reaching a potential host cell, the HA receptor-binding site attaches the virus to surface glycoconjugates that contain terminal SA [Sialic Acid] residues ... IAVs *then scan the cell surface for the proper sialylated* "receptor" by using the sialidase function of NA to remove local SAs and liberate nonproductive HA associations. Currently, the "receptor's" identity remains unknown, but it is generally thought that HAs from *avian* IAVs have higher specificity for receptors with α-2,3-linked SAs that have a "linear" presentation, whereas HAs from *human* IAVs prefer an α-2,6 linkage, which results in a more "bent" presentation [See figures and references in [2]]. While these preferences correlate with SA linkages in the respective hosts, several studies have shown that matching HA receptor binding preferences with the SA linkages in a particular host is not essential for infection, but is more critical for transmission. This implies that the IAV "receptor" either displays significant cell tropism in the airways or that IAVs can potentially use more than one receptor." [2]

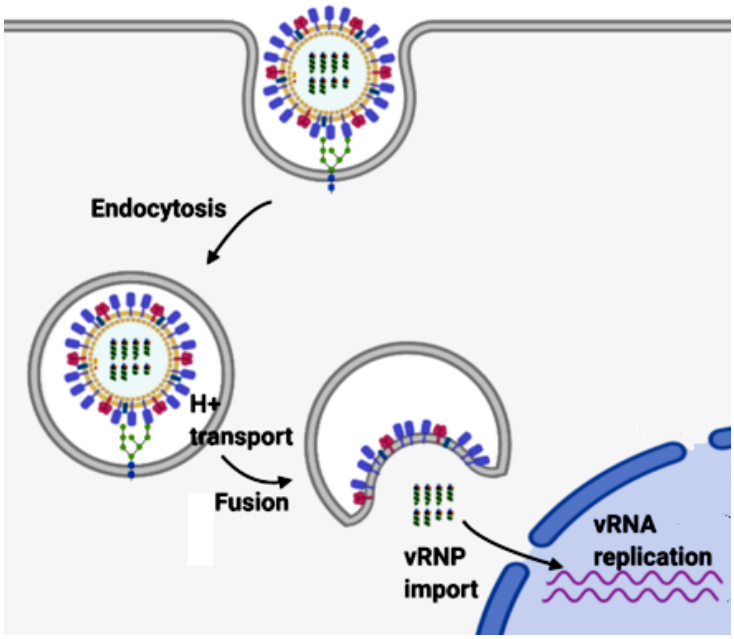


Here we can see sialic acids attached to galactose in two conformal manners: alpha(2,3) linkage and alpha(2,6) linkage.

The caption in the Figure 2 of Ref.[2] shows a diagram of a bi-antennary N-linked glycan, with the two ends with sialic acid residues displaying an α-2,3 linkage and an α-2,6 linkage. Then, one has a "linear" presentation and the other a "bent" presentations. As previously told, IAVs initiate cell entry by using the HA receptor-binding domain to associate with sialylated glycoconjugates on a host "receptor." We will consider the sialic acid and its linkages in further sections.

7) WHERE? - We have seen that the virion of influenza attaches to the cell by means of sialic acid. However, we could ask what is the specific receptor of the host cell that the virion uses to enter the cell. The answers that we can find are as told in [2]. It seems that in the case of influenza, there is not a specific receptor (that is a specific receptor like the ACE2 for Sars-Cov-19). In any case, after the attachment, the influenza virion enter the cell because the cell "swallows" it.

"Binding to the “receptor” triggers endocytosis" [2]. "The influenza virus enters the host cell by having its hemagglutinin bind to the sialic acid found on glycoproteins or glycolipid receptors of the host. The cell then endocytoses the virus. In the acidic environment of the endosomes, the virus changes shape and fuses its envelope with the endosomal membrane. This is followed by a signal to release the virus nucleocapsid into the host cytoplasm. From there, the nucleocapsid travels to the host nucleus" (Rahul Hate, https://web.stanford.edu/group/virus/1999/rahul23/replication.htm).



The virus uses HA on the host cell surface. After attachment, it enters cells via endocytosis. "After fusion of the viral envelope and endosomal membrane, vRNPs are released into the cytoplasm and translocate into the nucleus to initiate replication". Image adapted from a courtesy of Hi Eun Jung and Heung Kyu Lee for Wikipedia. See please https://www.mdpi.com/1999-4915/12/5/504/htm [4].

From Ref. [5]. "Influenza Virus Receptor Specificity". For influenza viruses, we know that the hemagglutinin (HA) "binds to and uses sialic acid-containing molecules as receptors. The use of *such abundant and ubiquitous molecules as receptors*, while providing the *apparent advantage* to the virus of allowing infection of multiple cell types and species, also results in binding to nonproductive receptors present in respiratory secretions, surfaces of dead cells, and even other virions". The influenza virus has evolved a second protein on its surface, the *neuraminidase*, which possesses a receptor-destroying enzyme that cleaves sialic acid [see Section 9 too]. In this manner, the virus can be released after binding "to sialic acid–containing molecules that do not lead to viral infection". Ref. [5] continues with a detailed discussion of the studies made by van Riel et al. [6]. Then the author, Adolfo García-Sastre, noted in 2010 that "there are still many unknowns with respect to the relationship between HA receptor specificity and influenza virus host and tissue tropism. It is, for example, quite clear now that the determinants of influenza tropism are more complex than the simplistic early view of classifying HAs by preferential binding to α2-6 and α2-3 sialic acids ... It is now clear that not only the linkage between the sialic acid and the next sugar influences binding of a specific viral and/or HA strain, but also the type of sialic acid as well as the rest of the carbohydrate." The author continued explaining other points that need investigations. "Thus, more research is required to understand how the complexity of interactions of influenza viruses with their receptor determines the outcome of viral infection and transmission. A better understanding of these processes might facilitate the design of specific antivirals that stop influenza virus transmission and infection of the lower respiratory tract".

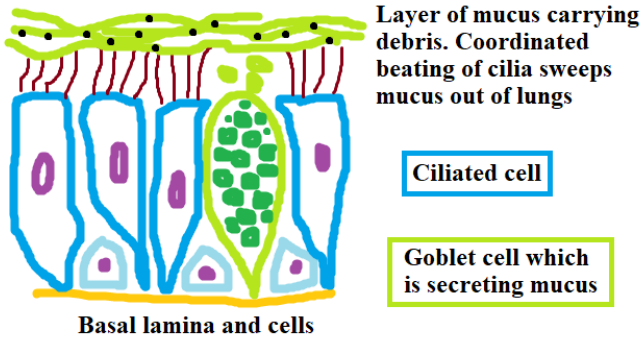
8) MUCUS - It is "a slimy substance, typically not miscible with water, secreted by the mucous membranes and glands of animals for lubrication, protection, etc." (from Oxford Languages" - "Interactions of Influenza A viruses (IAVs) with mucus were first described in the mid 20th century, and led early researchers to classify influenza as a (ortho)myxovirus[11] – a virus with affinity for mucus. IAVs must penetrate a secreted mucus layer (up to 50 μm thick) to reach target tissues in mammalian airways. Mucus is a defensive layer containing highly glycosylated mucins rich in terminal sialic acids (Sias). It has been suggested that mucus may protect against IAV infection by presenting sialylated “decoys” that mimic receptors on the cell surface. Influenza viruses bind these unproductive receptors, become trapped in the mucus layer, and can then be removed by the normal process of mucus clearance as part of the innate defence system" [7] (see references therein).

In fact, in [8], in an article of 2008, Ajit Varki wrote about avian influenza and the examples of severe human infections, that they "might be explained by the fact that these individuals inhaled a very large dose of virus, which reached the *lower* airways, where α2-3-linked sialic acids are present in humans. ... Of course, *pathogen binding*

11 Myxo- comes from the Greek mýxa, meaning “mucus” or “slime.

can also be blocked by the decoy function of soluble mucins, which are secreted into airways and carry large amounts of sialic acids".

"Mucin glycosylation and sialylation vary significantly between species, and thus could influence influenza host species specificity. Human airway and salivary mucins have been well characterized and their constituents and glycosidic linkages differ dramatically from those of other species such as chimpanzees and pigs. Humans express predominantly N-acetylneuraminic acid (Neu5Ac). Human airway sialoglycans lack the sialic acid (Sia) N-glycolylneuraminic acid (Neu5Gc), which is the predominant Sia in porcine mucus. In *the human upper respiratory tract Sias are predominantly found in α2-6 glycosidic linkage*. In contrast, these Sias are mostly α2-3-linked in chimpanzees and pigs" [7] (see references therein). We will discuss in other sections the sialic acids.



A sketch made according the Figure 22-18 (Respiratory epithelium) at the web site https://www.ncbi.nlm.nih.gov/books/NBK26875/ - The Airways and the Gut. Molecular Biology of the Cell. 4th edition. Alberts B, Johnson A, Lewis J, et al., New York: Garland Science; 2002 [9].

"The goblet cells secrete mucus, which forms a blanket over the tops of the ciliated cells. The regular, coordinated beating of the cilia sweeps the mucus up and out of the airways, carrying any debris that is stuck to it. The mechanism that coordinates the ciliary beating is a mystery, but it seems to reflect an intrinsic polarity in the epithelium." [9].

"There are two main epithelial cell types in the bronchus – ciliated cells and goblet cells that secrete mucus. Within the submucosa there are also submucous glands present. ... SAα2,6Gal [SA, sialic acid] has been reported to be present on the apical surface of ciliated cells but there have been conflicting reports about SAα2,3Gal expression on cell types. ... On the contrary, others have reported that SAα2,3Gal expression is found in

goblet cells. In addition, the patterns of glycosylation and the expression profile of SAα2,6 on cell surfaces may change during the course of developmental differentiation and following oncogenesis. For instance, SAα2,6Gal binding is weak during the glandular stage of lung development but increases as the lung matures. Furthermore, if cells are exposed to inflammation and tumour necrosis factor there may be qualitative changes in glycosylation and the glycosyltransferases that lead to sialylation. Recent publications, however, have indicated that both SAα2,3Gal and SAα2,6Gal may be present in the respiratory tract but with different distributions. The presence or absence of these SA is important as human influenza A strains have been reported previously to preferentially attach to cells with SAα2,6Gal linkages and avian strains preferentially bind SAα2,3Gal" [10].

9) A FUNCTION OF NA - A function of neuraminidase (NA) protein - "Earth's virology course" tells - is that of removing the sialic acid from glycoproteins. "The NA is therefore an enzyme that is essential for release of progeny virus particles from the surface of an infected cell. The NA protein also functions during entry of virus into the respiratory tract. The epithelial cells of the respiratory tract are bathed in mucus, a complex protective coating that contains many sialic acid-containing glycoproteins. When influenza virions enter the respiratory tract, they are trapped in mucus where they bind sialic acids". This interaction with the mucus would prevent the viruses from binding to a susceptible cell, but the viruses have "the action of the NA protein which cleaves sialic acids from glycoproteins. When the virus particle encounters a cell, it binds the sialic acid-containing receptor and is rapidly taken into the cell before the NA protein can cleave the carbohydrate from the cell surface". That is, it is also involved the time. The cell swallows the virus, before the NA protein has time to detach the virus.

"The essential nature of the NA for virus production has been exploited to develop new drugs designed to inhibit viral release. Both Tamiflu (Oseltamivir) and Relenza (Zanamivir) are structural mimics of sialic acid that bind tightly in the active site of the NA enzyme. When bound to drug, the NA cannot remove sialic acids from the cell surface, and consequently newly synthesized virus remains immobilized. The result is an inhibition of virus infection because virions cannot spread from one cell to another."

10) PERFECT PARASITES - From Ref. [11] - "Viruses are perfect parasites. ... Viral mechanisms are capable of translocating proteins and genetic material from the cell and assembling them into new virus particles." As previously told, an individual viral particle is a virion. "Virions consist of genetic material—DNA or RNA enclosed in a protein coating". Many viruses are enveloped viruses, explains Ref. [11], because they have an additional outer membrane made by material co-opted from the cell’s own membrane.

"Some types of enveloped virus fuse directly to the cell's outer (plasma) membrane, whereas others are engulfed whole by endocytosis or similar processes and then fuse their envelope with the membrane of the engulfing internal organelle (e.g., an endosome) to gain access to the interior of the cell. ... In fusion with the plasma membrane, the virus binds to a protein in the cell membrane. The function of this cellular protein (a receptor for the virus, ...) is perverted to induce a conformational change in the viral fusion protein, leading to fusion. For virus that is triggered within an endosome, the endosome's acidic conditions induce fusion. In either case, the viral genome passes through a fusion pore into cytosol, and infection is initiated" [11].

"Viral genetic material is relatively small, encoding only a few proteins. All enveloped viruses contain fusion proteins, which are the molecules responsible for fusing the envelope to a cellular membrane. These proteins are derived from the virion's genetic sequence". Each type of virus possesses its genetic material, its amino acid sequence, and its fusion proteins. "Consequently, *broad-spectrum antiviral drugs do not exist, and specific vaccines and drugs typically need to be developed for each virus type*. ... The membrane that is the skin of a cell and an enveloped virion, and is the gateway of viral entry, consists of lipids and proteins". As we have already seen, lipids spontaneously arrange themselves into a lipid bilayer. "Integral membrane proteins, such as viral fusion proteins, are inserted into the bilayer and project out from the lipid surface into the external solution-like icebergs. ... *Membranes are able to fuse to each other because they are fluid, and the lipids provide fluidity to the membrane*. Viruses initially stick to cell membranes through interactions unrelated to fusion proteins. *The virus surfs along the fluid surface of the cell and eventually the viral fusion proteins bind to receptor molecules on the cell membrane".*

Ref. [11] stresses a fundamental fact. ″ If only binding occurred, the two membranes would remain distinct. Fusion does not happen spontaneously because bilayers are stable. Fusion proteins do the work of prodding lipids from their initial bilayer configuration. *These proteins cause discontinuities in the bilayers that induce* the lipids of one membrane (e.g., the viral envelope) to connect with lipids of another (e.g., a cellular membrane), converting two bilayers into one" [11].

11) THE FUSION - Molecular dynamic simulation. Here the abstract from Ref. [12], an article entitled "Influenza hemagglutinin drives viral entry via two sequential intramembrane mechanisms". "Viral proteins that accomplish membrane fusion between the virus and a host cell do two things: draw virus and host membranes together and act within these membranes to induce fusion". The Abstract stresses that "This second property is much less well understood than the first". The authors used molecular dynamics simulations "approximating influenza virus fusion to obtain a model of how influenza proteins promote fusion within interacting membranes. This

model helps explain previous mutational data and new experiments. *Viral fusion proteins have long been thought to curve and disorder membranes*; [the authors of [12]] can now specify when and how this is accomplished. This provides a better fundamental understanding of viral entry and a basis for interpreting host defences that act on membranes to interfere with infection". The remarkable Ref. [12] is giving figures concerning the fusion, which are not simply sketches, but images based on molecular dynamics models.

The entry on the virus in a host cell is schematically given by https://en.wikipedia.org/wiki/Viral_entry - As we have seen, at first, the virion is reducing the distance from the host cell. In fact, the virion must attach to a host cell, by means of specific proteins on its surface. However, it must enter the cell to replicate itself. The cell is covered by the phospholipid bilayer barrier. The process by which this barrier is breached depends upon the virus. Types of entry are: - Membrane fusion or Hemifusion state: The cell membrane is punctured and made to further connect with the unfolding viral envelope; - Endocytosis: The host cell takes in the viral particle through the process of endocytosis, essentially engulfing the virus like it would a food particle; - Viral penetration: The viral capsid or genome is injected into the host cell's cytoplasm.

Let us return to the discussion of the role of sialic acids.

12) DIFFERENT SIALIC ACIDS - We have already seen that there are different sialic acids, when we mentioned Ref.[7]. Here, let us consider again "Earth's virology course", to discuss the role of these different acids. As stressed by the course, "there are a number of chemically different forms of sialic acids, and influenza virus strains vary in their affinity for them. These differences may determine which animal species can be infected." ... For instance, as previously told, "Avian influenza virus strains preferentially bind to sialic acids attached to galactose via an alpha(2,3) linkage. This is the major sialic acid on epithelial cells of the duck gut. In contrast, human influenza virus strains preferentially attach to sialic acids attached to galactose by an alpha(2,6) linkage. This is the major type of sialic acid present on human respiratory epithelial cells. ... This receptor specificity has implications for human infection with avian influenza virus strains. For example, highly pathogenic avian H5N1 influenza viruses undergo limited replication in the human respiratory tract due to the presence of some cells with alpha(2,3) linked sialic acids."

Let us also add that humans express predominantly N-acetylneuraminic acid (Neu5Ac). Human airway sialoglycans lack the sialic acid (Sia) N-glycolylneuraminic acid (Neu5Gc), which is the predominant Sia in porcine mucus for instance.

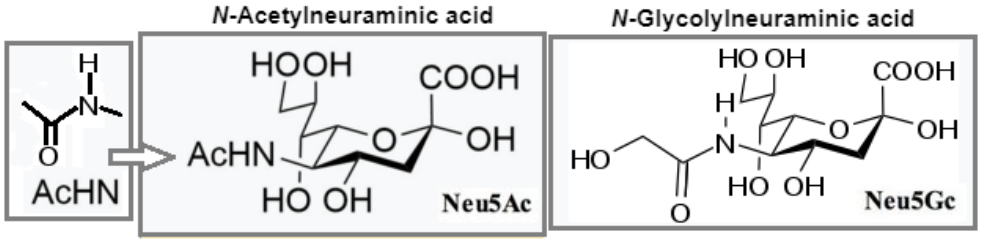
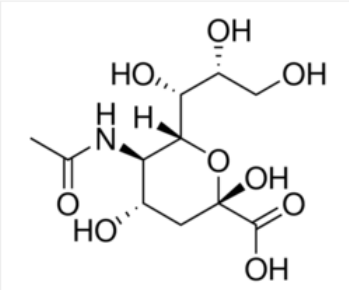


Image courtesy: Wikipedia - (see also Ref. [13]).

"Sialic acid (SA) is a generic term for nine-carbon acidic amino sugars (5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid). The amino group is always substituted with either an N-acetyl or N-glycolyl group, yielding N-acetylneuraminic (NeuAc) or N-glycolylneuraminic (NeuGc) acid, respectively, while the hydroxyl groups can be substituted by acetyl, lactoyl, methyl, sulfate, or phosphate residues. The distribution of specific SAs varies among animal species. For example, bovine, equine, and swine tissues possess both NeuAc and NeuGc, whereas human tissues possess only slight concentrations of NeuGc (less than 0.1% of total SA). Although a difference in the distribution of SAs among different animal species has been recognized for many years, its biological significance is largely unknown" [14].

"Influenza viruses differ in their ability to recognize SA-galactose (Gal) linkages, depending on the animal hosts from which they are isolated. For example, human viruses preferentially recognize SA linked to Gal by the α2,6(SAα2,6Gal) linkage, while equine viruses favor SAα2,3Gal. However, whether a difference in relative abundance of specific SA species (N-acetylneuraminic acid [NeuAc] andN-glycolylneuraminic acid [NeuGc]) among different animals affects *the replicative potential of influenza viruses is uncertain*" [14].

A0812 Sigma-Aldrich- https://www.sigmaaldrich.com/catalog/product/sigma/a0812

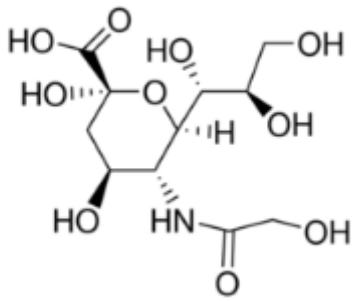


N-Acetylneuraminic acid

N-Acetylneuraminic acid - Synonym: 5-Acetamido-3,5-dideoxy-D-glycero-D-galactononulosonic acid, Lactaminic acid, NAN, NANA, Sialic acid - CAS Number 131-48-6 Empirical Formula (Hill Notation) $C_{11}H_{19}NO_9$

From [15]. Sialic acid is a generic term for a family of derivatives of neuraminic acid, an acidic sugar with a nine-carbon backbone. It is also the name for the most common member of this group, N-acetylneuraminic acid (Neu5Ac or NANA)[12],[13]

https://www.sigmaaldrich.com/catalog/substance/nglycolylneuraminicacid32527111383311



N-Glycolylneuraminic acid

N-Glycolylneuraminic acid - Synonym: Neu5Glc, NeuNGl - CAS Number: 1113-83-3 - Empirical Formula (Hill Notation): $C_{11}H_{19}NO_{10}$

13) SWEET SPOTS AND GLYCAN MICROARRAYS - We can find a review in [16], an article entitled "The sweet spot: defining virus–sialic acid interactions". The Abstract of [16] is the following. "Viral infections are initiated by attachment of the virus to host cell surface receptors, including sialic acid-containing glycans. It is now possible to rapidly identify specific glycan receptors using *glycan array screening*, to define atomic-level structures of virus–glycan complexes and to alter the glycan-binding site to determine the function of glycan *engagement* in viral disease". The Review given in [16] highlights the general principles of virus–glycan interactions. The Review is also giving specific examples of sialic acid binding by viruses "with stalk-like attachment proteins, including influenza virus, reovirus, adenovirus and rotavirus". Therefore, and the Abstract of [16] is stressing this fact, "understanding virus–glycan interactions is essential to combating viral infections and designing improved viral vectors for therapeutic applications".

12 https://en.wikipedia.org/wiki/Sialic_acid archived https://archive.is/IR0fJ
13 https://en.wikipedia.org/wiki/N-Acetylneuraminic_acid archived https://archive.is/jOjIk

Another study about sialic acids and their variants is proposed in Ref.[17], an article entitled "Viruses and sialic acids: rules of engagement". From the abstract: "Viral infections are initiated by specific attachment of a virus particle to receptors at the surface of the host cell. For many viruses, these receptors are glycans that are linked to either a protein or a lipid. Glycans terminating in sialic acid and its derivatives serve as receptors for a large number of viruses, including several human pathogens". The "structural analyses of complexes of viruses with sialylated oligosaccharides have provided insights into the parameters that underlie each interaction." The authors compared the available structural data (2011) on viral attachment proteins in complex with sialic acid and its variants.

The aim of the authors was that of defining parameters of recognition of specificity. "This information could be of use for the prediction of the location of sialic acid binding sites in viruses for which structural information is still lacking. An improved understanding of the principles that govern the recognition of sialic acid and sialylated oligosaccharides would also advance efforts to develop efficient antiviral agents". In this article, we find mentioned the *glycan microarrays*.

At the site https://www.creative-proteomics.com/services/glycan-microarray-assay.htm, it is told that "With the development of microarray technologies, glycan microarrays come to predominant for the high throughput study of protein–glycan binding events and screening glycan-binding proteins (GBPs). Glycan microarrays are presentations of multiple glycans or glycoconjugates printed on a single slide for screening with GBPs, which include lectins, antibodies, bacteria, and viruses."

"Microarray analysis techniques are used in interpreting the data generated from experiments on DNA (Gene chip analysis), RNA, and protein microarrays, which allow researchers to investigate the expression state of a large number of genes - in many cases, an organism's entire genome - in a single experiment. Such experiments can generate very large amounts of data, allowing researchers to assess the overall state of a cell or organism. Data in such large quantities is difficult - if not impossible - to analyze without the help of computer programs"[14].

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It is clear that influenza viruses have high affinity with sialic acids. However what is the situation for coronaviruses?

14) NOVEL CORONAVIRUSES CoVs - What was the knowledge about coronaviruses in 2012? Let us read from Ref. [18], an article entitled "Discovery of Seven Novel Mammalian and Avian Coronaviruses in the Genus Deltacoronavirus Supports Bat Coronaviruses as the Gene Source of Alphacoronavirus and

14 https://en.wikipedia.org/wiki/Microarray_analysis_techniques

Betacoronavirus and Avian Coronaviruses as the Gene Source of Gammacoronavirus and Deltacoronavirus".

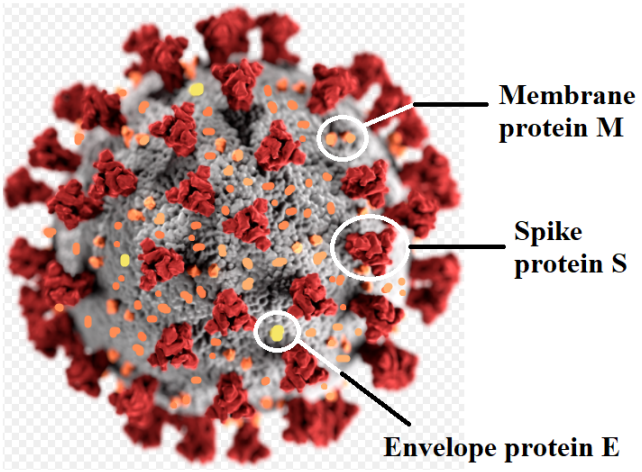
In [18] it is told that we can find coronaviruses (CoVs) in a large variety of animals. These viruses "can cause respiratory, enteric, hepatic, and neurological diseases of varying severity". CoVs were traditionally divided into three distinct groups [see [18] and references therein]. "Recently [let us stress the [18] was published in 2012], the Coronavirus Study Group of the International Committee for Taxonomy of Viruses has proposed three genera, Alphacoronavirus, Betacoronavirus, and Gammacoronavirus, to replace the traditional CoV groups 1, 2, and 3. As a result of the unique mechanism of viral replication, CoVs *have a high frequency of recombination*. Their tendency for recombination and the inherently high mutation rates in RNA virus *may allow them to adapt to new hosts* and ecological niches". See the given references in [18].

The SARS epidemic, "the discovery of SARS coronavirus (SARS-CoV), and the identification of SARS-CoV-like viruses from Himalayan palm civets and a raccoon dog from wild live markets in China have boosted interest in the discovery of novel CoVs in both humans and animals" [see the references given in [16]]. "A novel human CoV (HCoV) of the genus *Alphacoronavirus*, *human coronavirus NL63* (HCoV-NL63), was reported independently by two groups in 2004 [19], [20]. In 2005, [athe authors of Ref. [18]] also "described the discovery, complete genome sequence, clinical features, and molecular epidemiology of another novel HCoV, human coronavirus *HKU1* (HCoV-HKU1), in the genus Betacoronavirus" [[18] and references therein].

For what concerns the animal CoVs, the authors of Ref. [18] and others have described the discovery of SARS-CoV-like viruses in horseshoe bats in Hong Kong Special Administrative Region (HKSAR) and other provinces of China. Molecular surveillance studies have been made to examine the diversity of CoVs in bats of Hong Kong region and in the Guangdong province of southern China. This was the place where SARS epidemic originated. In these studies, the authors of [18] found at least nine other novel CoVs, including two novel subgroups in Betacoronavirus, subgroups C and D. Other researchers have conducted molecular surveillance studies in bats and other animals, and additional novel CoVs were discovered [see the references given in [18]].

In [18], it is stressed that birds are the reservoir, for instance, of avian influenza viruses. "As for CoVs, the number of known CoVs in birds is relatively small compared to that in bats". The authors of [18] discovered three novel CoVs "in three families of birds, named bulbul coronavirus HKU11 (BuCoV HKU11), thrush coronavirus HKU12 (ThCoV HKU12), and munia coronavirus HKU13 (MunCoV HKU13)". These CoVs formed a unique group of CoV, which probably represented a novel genus of CoV, *Deltacoronavirus*. The auhtors hypothesize that there are other previously unrecognized CoVs in this novel genus.

15) 3D MODEL OF SARS-COV-2



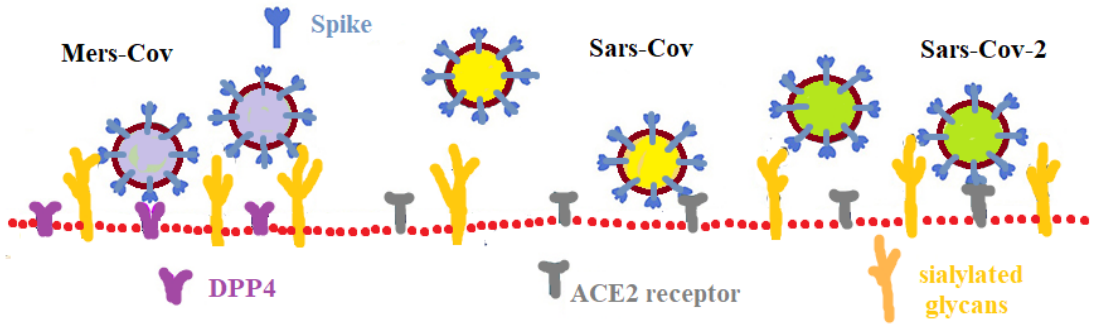
SARS-CoV-2 in a 3D image showing surface spikes of the virus Glycoprotein S (red), M-protein (orange), E-protein (yellow). Illustration based on a 3D model created at the Centers for Disease Control and Prevention (CDC) (Courtesy Alissa Eckert, Dan Higgins).

A 3D interactive model is proposed by the University of Glasgow at https://www.gla.ac.uk/news/coronavirus/headline_723737_en.html. The model tells, about the large spike protein, that it is essential for the virus to attach to and enter the hist cells. "The individual S proteins are arranged themselves into groups of three on the outer membrane, giving the coronavirus its distinctive crown". Source: https://coronavirusexplained.ukri.org

The membrane protein M is "embedded in the outer lipid membrane and is the most abundant of all the structural proteins, giving the virus particle its shape and integrity". The site of the Glasgow University is also telling that protein M "is also thought to play a role in the final stages of infection, when new virus proteins are assembled into particles before they are released and move on to infect new cells". Source: https://coronavirusexplained.ukri.org . The same source tells that the envelope (E) protein "is found in relatively low numbers in the virus particle and is thought to have several functions that contribute to virus growth and its ability to cause disease. These functions are not completely understood but include: the ability to form small pores that alter the properties of host cell membranes, preventing the M protein from clumping together and the transport and assembly of virus particles within the host cell".

16) SARS AND MERS - Therefore, we have previously reported information about influenza viruses. Now, let us consider coronaviruses and read what is told Ref. [21], an article explaining similarities and differences among these viruses. Actually, Sars-Cov-2 is a novel Coronavirus (CoV) causing the pandmic Covid-19. In [21], we find that "analogies with the previous infamous outbreaks of Severe Acute Respiratory Syndrome (SARS) in 2003 and Middle East Respiratory Syndrome (MERS) in 2012, caused by other CoV strains, have offered some insight".

"Although all CoV infections are initiated by the transmembrane spike (S) glycoprotein, a homotrimeric class I viral fusion protein, the binding site on the host cell surface differs among CoV strains". Then we have the MERS-CoV viruses which "weakly binds to non-acetylated sialoside attachment receptors on epithelial cells of the respiratory tract, promoting clustering and facilitating its binding to its receptor dipeptidyl peptidase-4 (DPP4)" [21],[22]. "The novel SARS-CoV-2, despite having evolved independently, shares with the previous SARS-CoV the cell receptor for Angiotensin Converting Enzyme 2 (ACE2)" [21],[23]. However, in the case of Sars-Cov-2, a recent study [21],[24] "*suggests* that there is an *in-silico evidence* that, in addition to ACE2, certain sialic acids on the cell surface may act as additional receptors for binding sites of the S protein of SARS-CoV-2, thus playing a role in the pathogenicity and epidemiology of the associated disease, as it has already been demonstrated for MERS-CoV. Sialic acids could therefore be used by SARS-CoV-2 as attachment receptors on the epithelium of the respiratory tract, promoting SARS-CoV-2 clustering, as already known for MERS-CoV" [21],[22]. In Ref.[21], it is told that virus-ACE2 binding could be facilitated.



In the given sketch, the situation as proposed in Ref.[21].

In [21] it is also reported another publication [25] which has investigated the properties of Sars-Cov-2 by means of computational approaches. It is told that ACE2 binding sites

are conserved among strains, "whereas the potential SARS-CoV-2-sialic acid binding domain is highly variable, as reported in MERS-CoV. This variability could result in different binding affinities of SARS-CoV-2 strains for cellular sialic acids, possibly explaining the broad range of host-immune responses in the human population" [21], [25].

This is what we can find in [21]. However, see please 21) ATTACHMENT OF SARS-COV-2 - Binding to HS, not to sialic acid residue?

For what concerns the glycoproteins, in Ref. [26], it is told that "a glyconanoparticle platform is used to discover that N-acetyl neuraminic acid has affinity toward the SARS-CoV-2 spike glycoprotein, demonstrating its glycan-binding function. Optimization of the particle size and coating enabled detection of the spike glycoprotein in lateral flow and showed selectivity over the SARS-CoV-1 spike protein. Using a virus-like particle and a pseudotyped lentivirus model, paper-based lateral flow detection was demonstrated in under 30 min, showing the potential of this system as a low-cost detection platform". "The spike-protein from SARS-CoV-2 is shown to bind sialic acids, which is exploited to assemble a lateral flow diagnostic tool, using glycans rather than antibodies, as the recognition unit".

In the conclusion of [26], the authors tell that they have "demonstrated a glycan-based lateral flow detection system that can detect the spike glycoprotein from the SARS-COV-2 virus in under 30 min. Guided by sequence alignment against other coronavirus spike proteins, it was hypothesized that sialic acids may bind this protein, to enable capture/detection. Using a nanoparticle-based biolayer interferometry platform, [the authors] demonstrated that α,N-acetyl neuraminic acid is a ligand for the spike glycoprotein. ... Finally, the observation that SARS-COV-2 can engage sialic acids found on human respiratory cells may provide insight into its zoonosis and infection pathways to help guide new interventions".

17) ACE2 RECEPTOR - adapted from https://en.wikipedia.org/wiki/Severe_acute_respiratory_syndrome_coronavirus_2 - The virion of Sars-Cov-2 is 50–200 nanometres in diameter. This coronavirus has four structural proteins. One is the spike protein (S). Then there are E (envelope), M (membrane), and N (nucleocapsid) proteins. The "N protein holds the RNA genome, and the S, E, and M proteins together create the viral envelope". The spike protein is used by the virion "to attach to and fuse with the membrane of a host cell; specifically, its S1 subunit catalyzes attachment, the S2 subunit fusion". The spike has affinity to the receptor angiotensin converting enzyme 2 (ACE2) of host cells. This is the receptor used for the cell entry.

"Initial spike protein priming by transmembrane protease, serine 2 (TMPRSS2) is essential for entry of SARS-CoV-2. After a SARS-CoV-2 virion attaches to a target cell, the cell's protease TMPRSS2 cuts open the spike protein of the virus, exposing a

fusion peptide in the S2 subunit, and the host receptor ACE2. After fusion, an endosome forms around the virion, separating it from the rest of the host cell. The virion escapes when the pH of the endosome drops or when cathepsin, a host cysteine protease, cleaves it. The virion then releases RNA into the cell and forces the cell to produce and disseminate copies of the virus, which infect more cells. SARS-CoV-2 produces at least three virulence factors that promote shedding of new virions from host cells and inhibit immune response." An illustration of the mechanism of fusion and endosome is given in [27].

From rndsystems.com/resources/articles/ace-2-sars-receptor-identified archived archive.is/qywuf. "Identification and sequencing of the virus responsible for COVID-19 (view SARS-CoV-2 protein sequence) determined that it was a novel CoV that shared 88% sequence identity with two bat-derived SARS-like CoV, suggesting it had originated in bats. Additionally, it was shown that this CoV, which was termed ... SARS-CoV-2, shared 79.5% sequence identity with SARS-CoV. The coronaviral genome encodes four major structural proteins: the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and the envelope (E) protein. The spike protein is responsible for facilitating entry of the CoV into the target cell. It is composed of a short intracellular tail, a transmembrane anchor, and a large ectodomain that consists of a receptor binding spike S1 subunit and a membrane-fusing spike S2 subunit. Sequence analysis of the SARS-CoV-2 spike protein genome showed that it was only 75% identical with the SARS-CoV spike protein. However, analysis of the receptor binding motif (RBM) in the spike protein showed that most of the amino acid residues essential for receptor binding were conserved between SARS-CoV and SARS-CoV-2, suggesting that the two CoV strains use the same host receptor for cell entry. The entry receptor utilized by SARSCoV is Angiotensin-Converting Enzyme 2 (ACE-2)". See references therein.

18) ACE2 GLYCOSYLATION - We have seen before a discussion about the "sweet point" of proteins. Now, let us read what en.wikipedia.org/wiki/Angiotensin-converting_enzyme_2 is telling about ACE2. Angiotensin-converting enzyme 2 (ACE2) is an enzyme attached to the cell membranes of cells located in the lungs, arteries, heart, kidney, and intestines. ACE2 lowers blood pressure by catalyzing the hydrolysis of angiotensin II (a vasoconstrictor peptide) into angiotensin (1–7) (a vasodilator). ACE2 serves as the main entry point into cells for some coronaviruses, including HCoV-NL63, SARS-CoV, and SARS-CoV-2. More specifically, the binding of the spike S1 protein of SARS-CoV and SARS-CoV-2 to the enzymatic domain of ACE2 on the surface of cells results in endocytosis and translocation of both the virus and the enzyme into endosomes located within cells. "This entry process also requires priming of the S protein by the host serine protease TMPRSS2, the inhibition of which is under current investigation as a potential therapeutic".

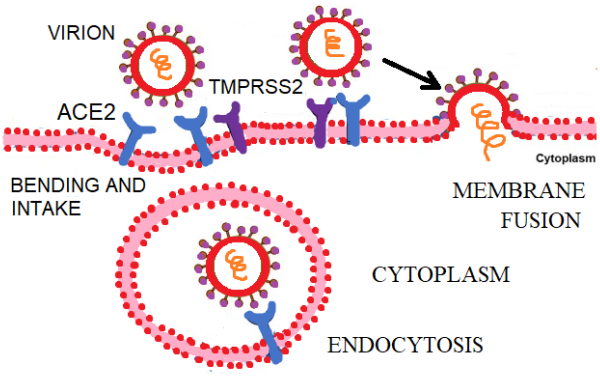
Wikipedia is giving a reference [28], to a discussion entitled "Understanding Glycans in

COVID-19 Drug Design", about the role of the disruption of S-protein glycosylation can influence the viral entry, indicating the importance of glycan-protein interactions in the process. In fact, the viral spike glycoprotein (S) of SARS-CoV-2 and the host cell receptor, angiotensin-converting enzyme 2 (ACE2) are both *densely glycosylated.* Authors of [28] hope an intensification of studies concerning the role of glycosylation. In fact, a recent publication, Ref. [29], entitled "Subtle Influence of ACE2 Glycan Processing on SARS-CoV-2 Recognition", investigated whether the glycosylation state of ACE2 impacts the interaction with SARS-CoV-2 viral spike. The authors "probed the impact of ACE2 glycosylation on S binding and revealed a subtle sensitivity with hypersialylated or oligomannose-type glycans slightly impeding the interaction. In contrast, deglycosylation of ACE2 did not influence SARS-CoV-2 binding. Overall, ACE2 glycosylation does not significantly influence viral spike binding". The authors suggest "that any role of glycosylation in the pathobiology of SARS-CoV-2 will lie beyond its immediate impact of receptor glycosylation on virus binding".

Glycosylation is the reaction in which a carbohydrate is attached to a hydroxyl or other functional group of another molecule. In biology, glycosylation mainly refers to processes that attaches glycans to proteins, or other organic molecules. Glycans serve a variety of structural and functional roles in membrane and secreted proteins.

19) TWO MECHANISMS - In Ref. [30], we find a discussion of two mechanisms the virus uses to enter the cell. First, let us report what it is told in [30], about the spike proteins. "The S protein is structurally divided into two functionally distinct subunits called the S1 and S2 subunits. While the S1 subunit is responsible for receptor binding and includes the N-terminal domain and C-terminal receptor binding region (RBD), the S2 subunit facilitates membrane fusion and anchors S into the viral membrane" (see reference therein). Then, it is told that "ACE2 as a receptor may mediate the entry of CoV into host cells in two independent ways (Fig. 2 of Ref. [30])".

The first way involves ACE2-receptor-mediated clathrin-dependent endocytosis. The RBD of the virus is recognized by the extracellular PD [peptidase domain] of ACE2 mainly through polar residues. "When CoV is connected to ACE2, the ACE2 extracellular domain controlling the catalytic effect is cleaved off by specific proteases, such as metalloproteinase ADAM17, and the transmembrane domain is internalized. Next, with the assistance of clathrin, viral particles and host cells fuse, and the intracellular structure of ACE2 aids viral transport from the cell membrane to the cytoplasm. ... The second way involves ACE2-receptor-mediated transmembrane serine protease 2 (TMPRSS2)-dependent membrane fusion. One study found that ACE2-mediated viral invasion involves TMPRSS2, which is employed for S protein priming and activation of membrane fusion processes, and TMPRSS2s priming role has recently been confirmed for 2019-nCoV . When the SARS-S protein binds to ACE2, processing by TMPRSS2 is thought to allow fusion at the cell surface or upon uptake into cellular vesicles but before virion transport into cell endosomes".



On the left, the endocytosis, on the right the membrane fusion.

From https://en.wikipedia.org/wiki/TMPRSS2 - "Some coronaviruses, e.g. SARS-CoV-1, MERS-CoV, and SARS-CoV-2 are activated by TMPRSS2 and can thus be inhibited by TMPRSS2 inhibitors. SARS-CoV-2 uses the SARS-CoV receptor ACE2 for entry and the serine protease TMPRSS2 for S protein priming. A TMPRSS2 inhibitor approved for clinical use blocked entry and might constitute a treatment option. One experimental candidate as a TMPRSS2 inhibitor for potential use against both influenza and coronavirus infections in general, including those prior to the advent of COVID-19, is the OTC (in most countries) mucolytic cough medicine bromhexine, which is also being investigated as a possible treatment for COVID-19 itself as well." [See the references given in the web page]. (OTC means Over the Counter).

https://en.wikipedia.org/wiki/Bromhexine - "Bromhexine is intended to support the body's mechanisms for clearing *mucus* from the respiratory tract. It is secretolytic, increasing the production of serous mucus in the respiratory tract, which makes the phlegm thinner and less viscous. This contributes to a secretomotoric effect, allowing the cilia to more easily transport the phlegm out of the lungs".

20) INTEGRIN BESIDES ACE2 - Adapted from https://en.wikipedia.org/wiki/Integrin (see please references therein) - Integrins are transmembrane receptors that facilitate cell-cell and cell-extracellular matrix adhesion. Upon ligand binding, integrins activate signal transduction pathways that mediate cellular signals such as regulation of the cell cycle, organization of the intracellular cytoskeleton, and movement of new receptors to the cell membrane. The presence of integrins allows rapid and flexible responses to events at the cell surface (e.g. signal platelets to initiate an interaction with coagulation

factors).

In Ref. [31], in its abstract, we can find told that "Although ACE2 (angiotensin converting enzyme 2) is considered the primary receptor for CoV-2 cell entry, recent reports suggest that alternative pathways may contribute". In [31] we can find the hypothesis "that viral binding to cell-surface integrins may contribute to the high infectivity and widespread extra-pulmonary impacts of the SARS-CoV-2 virus. This potential is suggested on the basis of the emergence of an RGD (arginine-glycine-aspartate) sequence in the receptor-binding domain of the spike protein. RGD is a motif commonly used by viruses to bind cell-surface integrins. ... Integrins on the surfaces of pneumocytes, endothelial cells and platelets may be vulnerable to CoV-2 virion binding. ... Binding of virions to integrins on endothelial cells could activate angiogenic cell signaling pathways; dysregulate integrin-mediated signaling pathways controlling developmental processes; and precipitate endothelial activation to initiate blood clotting. Such a procoagulant state, perhaps together with enhancement of platelet aggregation through virions binding to integrins on platelets, could amplify the production of microthrombi that pose the threat of pulmonary thrombosis and embolism, strokes and other thrombotic consequences". The abstract is also mentioning the highly infectious variant, B.1.1.7 (or VUI 202012/01)[15]. This variant "includes a receptor-binding domain amino acid replacement, N501Y, that could potentially provide the RGD motif with enhanced access to cell-surface integrins, with consequent clinical impacts".

Emily Arntsen, on February 8, 2021, writes in her contribution entitled "Covid-19 can affect the blood. Its spike protein may be the culprit", .https://news.northeastern.edu/2021/02/08/covid-19-can-affect-the-blood-its-spike-protein-may-be-the-culprit/ that "Makowski, who recently published [31] his hypothesis in the journal Viruses, believes the spike protein found on the surface of the virus might mimic proteins that regulate blood vessels and control the formation of blood clots, which could explain many of the non-respiratory complications of COVID-19."

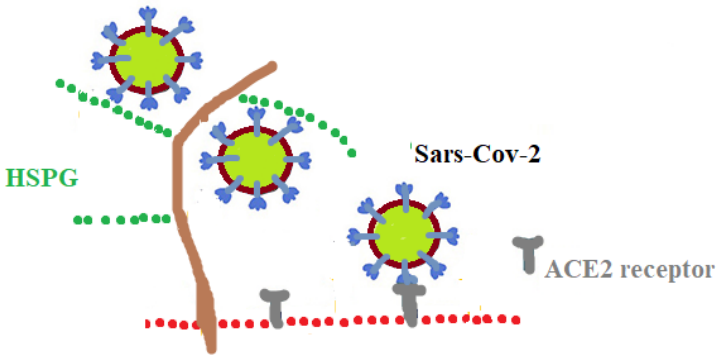
21) ATTACHMENT OF SARS-COV-2 - Binding to HS, not to sialic acid residues?

Here from Ref. [32] entitled "Binding of the SARS-CoV-2 spike protein to glycans". This article is reporting a systematic research on the "binding of the subunits and spike (S) proteins of SARS-CoV-2 and SARS-CoV, MERS-CoV to *heparan sulfate* (HS) and sialic acid-containing glycans". The study reveals "that all the tested protein molecules can bind to HS in a sulfation-dependent manner and *no binding with sialic acid residues* was detected. Overall, this work suggests that HS binding may be a general mechanism for the attachment of these coronaviruses to host cells, and supports the potential importance of HS in infection and in the development of antiviral agents against these viruses".

15 https://www.who.int/csr/don/21-december-2020-sars-cov2-variant-united-kingdom/en/

From https://en.wikipedia.org/wiki/Heparan_sulfate - "Heparan sulfate (HS) is a linear polysaccharide found in all animal tissues. It occurs as a proteoglycan (HSPG, i.e. Heparan Sulfate ProteoGlycan) in which two or three HS chains are attached in close proximity to cell surface or extracellular matrix proteins. It is in this form that HS binds to a variety of protein ligands ... and regulates a wide range of biological activities, including developmental processes, angiogenesis, blood coagulation, ... and tumour metastasis. HS has also been shown to serve as cellular receptor for a number of viruses, including the respiratory syncytial virus. [33] A recent study reports that cellular heparan sulfate has a role in SARS-CoV-2 Infection, particularly when the virus attaches with ACE2 [34]".

Ref. [32] explains that "previous studies of many other viruses suggested that SARS-CoV-2 S protein may use other molecules on host cell surface as attachment factors to facilitate binding to the high-affinity receptor ACE2" [see reference in [32]]. "Examples of such molecules include glycosaminoglycans (GAGs) and sialic acid-containing oligosaccharides". "GAGs are primarily localized at the outer surface of cells. Such a location makes them particularly suitable for acting as attachment factors to recruit viruses to cell surfaces". "HS is one of the most prevalent types of GAGs in mammals. It is a linear and sulfated polysaccharide that is abundantly expressed on the surface of almost all cell types and in the extracellular matrix". HS chains are mostly linked as side chains to core proteins to form HS proteoglycans (HSPGs).



This is a sketch made according to the Figure 1 of [32]. The caption in [32] tells that a possible mechanism for SARS-CoV-2 entry and infection is the following. "SARS-CoV-2 may first interact with the HSPGs on the surface of susceptible cells using the S protein ... This initial attachment may promote the subsequent binding of the virus to the high-affinity entry receptor ACE2". The figure caption is also telling that the transmembrane protease serine 2 (TMPRSS2) and other host cell proteases "may assist in viral entry by cleaving the S protein at the S1/S2 and/or at the S2' sites".

Authors of [32] tells that "an early study has suggested that the RBD of SARS-CoV-2 S

protein might bind heparin [35]. In order to determine if there is any preference of the SARS-CoV-2 S protein for particular HS structures", the authors investigated the binding to a HS microarray containing 24 synthetic heparan sulfate oligosaccharides. The microarray experiment was performed using a previously established standard protocol. The analysis "revealed that the SARS-CoV-2-RBD is able to bind to almost half of the molecules on the microarray, and not surprisingly, the binding is strongly affected by the sulfation level, which is a trend that has been previously noted for many HS-binding proteins".

In [32], the experimental study has been performed by means of sialylated glycan microarrays too. "In order to find out if SARS-CoV-2 can bind to sialic acid residues," the authors of [32], "carried out microarray analyses of its S protein and subunits". The microarray used contained 100 different N-glycans sites as they can be found on the surface of cells, "49 of them terminated with α2,3- and α2,6-linked sialic acid, also known as N-acetylneuraminic acid (Neu5Ac), 8 with α2,3- and α2,6-linked N-Glycolylneuraminic acid (Neu5Gc), and the rest with other glycan residues. The experiment was performed in the similar way as described for the HS microarray study". In [32], the authors report that the results displayed that SARS-CoV-2 gaves no binding signal. This suggests that the virion is "not able to interact with sialylated N-glycans or the binding signal is too low to be detected". The authors have also tested "the full-length S proteins of SARS-CoV-2, SARS-CoV, and MERS-CoV to more sialylated glycans, including sialylated N- and O-linked glycans and glycolipid glycans, but again no specific binding was detected" [32].

Here a fundamental observation written in [32]. "In general, the interactions of viruses with HS or sialic acids are responsible for the first contact with host cells. Such contact may serve to concentrate viruses on the surface of target cells, facilitate their binding to more specific high-affinity protein receptors and/or promote their entry into host cells".

However, in [36], the authors tell that they are revealing "that the receptor-binding domain (RBD) of the spike (S)-protein on SARS-CoV-2 recognizes oligosaccharides containing sialic acid (SA), with preference for the oligosaccharide of monosialylated gangliosides ... Together, these results suggest that sialylated glycans, specifically glycolipids, facilitate viral entry of SARS-CoV-2". The authors also tell that "while other members of the Coronavirus family also bind glycolipids, they do so through their NTD (N-terminal domains); therefore, our observation of binding glycolipids through the RBD is an entirely new finding. Interestingly Hao et al. did not detect any binding of S1 to the glycans of glycosphingolipids, including gangliosides, in glycan microarray screening. This absence of binding (with the array) might be due to the relatively low affinities of these interactions, or deleterious effects of glycan labeling on binding."

And the mucus?

22) HUMAN SIALOME, AGE AND SEX- As told in [15], the term "human sialome" is used for the broad variety of sialic acid compounds in the human body. It has been hypothesized that it is "the result of genomic changes occurred under the selective impulse of an alleged pandemic event, roughly 3 million years ago, provoking the so-called sialoquake" [21] . From Ref. [37] - "Whereas most vertebrates, including our closest evolutionary relatives (bonobos and chimps), express significant amounts of NeuGc as well as NeuAc ... , humans only synthesize NeuAc. The inability of humans to synthesize NeuGc is due to an exon deletion in the gene responsible for converting the N-acetyl to the N-glycolyl form, CMP-N-acetylneuraminic acid hydroxylase (CMAH). *Molecular clock comparison* of the disrupted human and intact chimpanzee genes places the insertion at *3 million years ago*. What caused the species to diverge? Since sialic acid recognition is a virulence factor for some pathogens, it has been speculated that evolutionary selection against expression of functional CMAH in the human lineage was due to a *catastrophic pandemic* by a pathogen that targeted NeuGc. Varki [38] has termed this theoretical event the “sialoquake.” His hypothesis is consistent with the glycan binding specificities of related human and non-human pathogens, and with evolutionary changes in immune system sialic acid binding proteins that postdate the loss of CMAH".

A section in [21] is discussing the antiviral protective role of the Sialome. "Sialic acid viral recognition has been long known to be a virulence factor for various pathogens". To this fact, in [21] it is stressed that the sialome exerts also a protective effect against viral infections, and this is clearly depicted in the Figure 1B in [21]. The sialylated O-linked glycans, which are covering the mucins on mucosal cell surfaces, are a defence mechanism. There is a layer of sialylated residues which is acting "as a barrier, preventing pathogens from entering the cell by offering a decoy alternative binding site" [21]. In fact, we have seen this action for the viruses of influenza A.

In the Ref.[21] it is also noted that the sialome changes with the age. It is observed an age-related accumulation of a glycosylated IgGs, related to a pro-inflammatory status. It is also observed that elderly people have a lower sialic acid level in saliva compared to children, and a general decrease of the sialylation processes in the human body with aging. In [21], it is also told that "sialome seems to be affected by the body's hormonal asset, in that estrogens upregulate antibody sialylation, determining an anti-inflammatory effect, whilst a decrease in estrogen levels, as seen in menopause, leads to lower sialylation activity" [21].

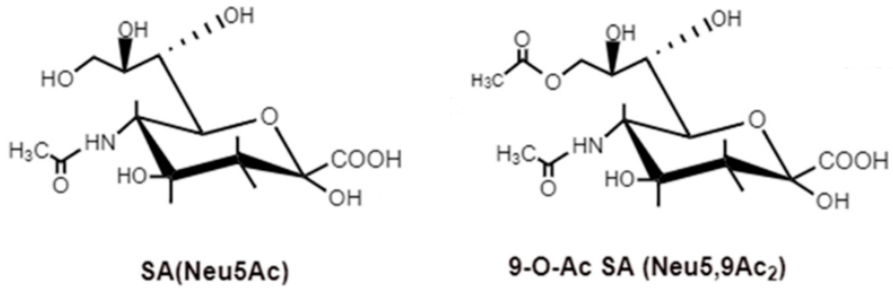
On glycosylated IgGs, see please Ref. [39]. For the loss of N-Glycolylneuraminic acid in human evolution, see please Ref. [40].

23) SUPER-SPREADERS - Vince Horiuchi, in a contribution entitled "Mucus and the coronavirus", March 31 ,2020, attheu.utah.edu/facultystaff/mucus-and-the-coronavirus/, tells that researchers do not know exactly "the role different compositions of mucus, ...

play in the transmission and infection of coronaviruses. Nor do they know why some people known as “super-spreaders” will spread the disease more than others. But University of Utah biomedical engineering assistant professor Jessica R. Kramer is now researching how mucus plays a part in transferring coronaviruses from person to person". "Understanding how different compositions of the proteins that make up mucus spread coronaviruses could help identify those who are “super-spreaders” as well as those who could be more vulnerable to becoming infected, says Kramer".

24) CORONAVIRUSES AND SIALIC ACIDS - OC43 - Coronavirus OC43 is binding a sialic acid. From https://en.wikipedia.org/wiki/ Human_coronavirus_OC43 (see references therein) - Human coronavirus OC43 (HCoV-OC43) is a member of the species Betacoronavirus 1, which infects humans and cattle. This coronavirus is an enveloped, positive-sense, single-stranded RNA virus which enters its host cell by binding to the N-acetyl-9-O-acetylneuraminic acid receptor. OC43 is one of seven known coronaviruses to infect humans. It is one of the viruses responsible for the common cold. It has, like other coronaviruses from genus Betacoronavirus, subgenus Embecovirus, an additional shorter spike protein called hemagglutinin esterase (HE).

Here the abstract of an article entitled "Structural basis for human coronavirus attachment to sialic acid receptors", published in June 2019 [41]. "Coronaviruses cause respiratory tract infections in humans and outbreaks of deadly pneumonia worldwide. Infections are initiated by the transmembrane spike (S) glycoprotein, which binds to host receptors and fuses the viral and cellular membranes. To understand the molecular basis of coronavirus attachment to oligosaccharide receptors, we determined cryo-EM structures of coronavirus OC43 S glycoprotein trimer in isolation and in complex with a 9-O-acetylated sialic acid".



This image, showing the 9-O-Ac SA compared to SA is adapted from the article entitled "SARS-CoV-2 Evolutionary Adaptation toward Host Entry and Recognition of Receptor O-Acetyl Sialylation in Virus–Host Interaction" [42].

25) HE AND HEF GLYCOPROTEINS - From Wikipedia - Hemagglutinin esterase (HEs) is a glycoprotein that certain enveloped viruses possess and use as invading mechanism. Viruses that possess HEs include *Influenza C* virus, toroviruses, and coronaviruses[16]. HEs is a transmembrane protein. The different HEs enzyme activities include: receptor binding activity, receptor hydrolysis (esterase) activity, and membrane fusion activity. The receptor binding activity involve the attachment of HEs to N-acetyl-9-O-acetylneuraminic acid (9-O-Ac-Neu5Ac) of glycolipids and glycoproteins and in turn serve as viral receptor. Receptor hydrolysis (esterase) activity allows virus particles to escape the infected cell by removing an acetyl group from the C9 position of terminal 9-O-Ac-Neu5Ac residues. Membrane fusion activity helps in incorporation viral genome into the host cell cytoplasm by enhancing the attachment between the viral envelope and host cell membrane.

In certain Influenza viruses, the cell surface consists of both Hemagglutinin (HA) and Neuraminidase (NA) proteins that encompass enzymatic activities, whereas hemagglutinin-esterase fusion (HEF) proteins have been found to be the primary single spike protein that combines all of the enzymatic activities listed above. HEF proteins have been tested to be high-temperature and low-pH resistant and are the primary source of virulence in viruses. Influenza C have been shown to have unique HEF structure proteins that enhance its ability to infect the host cell compared to Influenza A and B. Let us stress that "Influenza C virus uses 9-O-acetyl-N-acetylneuraminic acid as a high affinity receptor determinant for attachment to cells" [43].

Now, is HE protein present on Sars-Cov-2?

From https://encyclopedia.pub/1859 - O-Acetyl Sialylation, Submitted by: Cheorl-Ho Kim, archived https://archive.ph/7hZSz (May-June 2020) - "Currently, information on SARS-CoV-2 and its receptors is limited. O-acetylated SAs interact with the lectin-like spike glycoprotein of SARS CoV-2 for the initial attachment of viruses to enter into the host cells. SARS-CoV-2 hemagglutinin-esterase (HE) acts as the classical glycan-binding lectin and receptor-degrading enzyme. ..." . See also Ref. [44].

But, in [45], it is told "Similar to SARS CoV, SARS CoV-2 lacks the hemagglutinin esterase gene, which is found in human coronavirus (hCoV) HKU1, a lineage A betacoronavirus" [46]. "The 2019-nCoV genome is arranged in the order of 5′-replicase (orf1/ab)-structural proteins [Spike (S)-Envelope (E)-Membrane (M)-Nucleocapsid (N)] −3′ and lacks the hemagglutinin-esterase gene which is characteristically found in lineage A β-CoVs" [46].

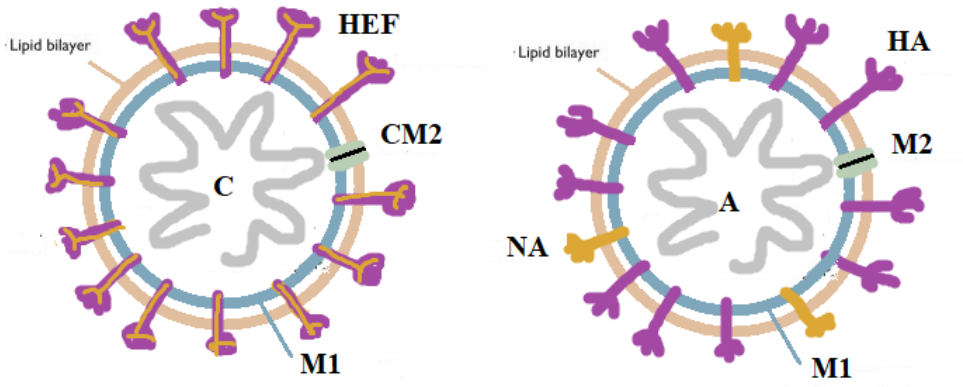
Here the protein of Sars-Cov-2 given in the Table 1 of [45]: ORF1ab polyprotein - ORF1a polyprotein - Spike protein (S protein) - ORF3a protein - Envelope protein (E protein) - Membrane protein (M protein) - ORF6 protein - ORF7a protein - ORF7b

16 Wikipedia adds the parenthesis "(but not SARS-like coronaviruses)". See 17 March 2020, archived https://archive.ph/NwXBA - We will discuss this point. further.

protein - ORF8 protein - Nucleocapsid phosphoprotein (N protein) - ORF10 protein.

"Basically, the structure of SARS-CoV-2 shares all the typical characteristics with other coronaviruses. Several recent studies considering the structure of SARS-CoV-2 were all focused on the S protein. Wrapp et al. reported a structure at 3.5 Å resolution of SARS-CoV-2 S protein. Yan et al. reported the complex structure of B°AT_1, an amino acid transporter protein, with human host cell binding receptor angiotensin-converting enzyme 2 (ACE2), which provided important insights into the molecular basis of coronavirus infection. Lan et al. reported a crystal structure of SARS-CoV-2 S protein's receptor binding domain (RBD) region bound to ACE2. The viral architecture of SARS-CoV-2 with post-fusion spike was observed by Cyro-EM, which showed the image of disassociated spikes" [47].

26) HEF AND INFLUENZA C VIRUS - From Ref. [48], entitled "Hemagglutinin-esterase-fusion (HEF) protein of influenza C virus". " Humans are the main reservoir of the virus, but it also infects pigs and dogs. Very recently, influenza C-like viruses were isolated from pigs and cattle *that differ from classical influenza C virus* and might constitute a new influenza virus genus. Influenza C virus *is unique since it contains only one spike protein*, the hemagglutinin-esterase-fusion glycoprotein HEF that possesses receptor binding, receptor destroying and membrane fusion activities, thus combining the functions of Hemagglutinin (HA) and Neuraminidase (NA) of influenza A and B viruses". "Influenza C virus was first isolated during an epidemic of respiratory illness in 1947".



On the left, the C virion and on the right the A virion.

This sketch was made according to the Figure 1 in [48], which is comparing the

influenza C virus and influenza A (and also B) virions. The influenza C virus "has only one spike protein, the hemagglutinin-esterase-fusion glycoprotein HEF that combines the functions of both hemagglutinin (HA) and neuraminidase (NA) from influenza A and B virus. PB1, PB2, P3 and PB1, PB2, PA are the polymerase proteins of influenza C virus and influenza A/B virus, respectively, that build together with the nucleoprotein NP and the viral RNA-segments the ribonucleoprotein complexes (vRNP). M1 is the matrix protein and M2 and CM2 the proton-channel" [48].

"Influenza C virus particles exhibit two morphologies, either spherical with a diameter of 80–120 nm or filamentous with the same diameter but with lengths in µm range" [see reference given in [48]]. "Already during the budding process at the plasma membrane, filamentous particles may aggregate via their long axes into 500 µm long cord-like structures, which are all covered by a layer of surface projections ... Another unique characteristic of influenza C virus particles observed by electron microscopy is a reticular hexagonal structure, which is formed by the HEF protein ...".

27) CORONAVIRUSES AND SIALIC ACIDS - HKU1 - Human coronavirus HKU1 - From Wikipedia: Human coronavirus HKU1 (HCoV-HKU1) is a species of coronavirus in humans. It causes an upper respiratory disease with symptoms of the common cold, but can advance to pneumonia and bronchiolitis. It was first discovered in January 2004 from one man in Hong Kong. Subsequent research revealed it has global distribution and earlier genesis. The virus is an enveloped, positive-sense, single-stranded RNA virus which enters its host cell by binding to the N-acetyl-9-O-acetylneuraminic acid receptor [49]

In [50], we find information about the virus attachment. Human coronavirus (hCoV) HKU1 was one of six hCoVs identified in 2015. "hCoV-HKU1 encodes a hemagglutinin-esterase (HE) protein that is unique to the group a betacoronaviruses (group 2a). The function of HKU1-HE remains largely undetermined". In [50]. the authors examined binding of the S1 domain of hCoV-HKU1 spike to a panel of cells and found that ... hCoV-HKU1 exploits O-Ac-Sia as a cellular attachment receptor determinant to initiate the infection of host cells and that its HE protein possesses the corresponding sialate-O-acetylesterase RDE [receptor-destroying enzyme] activity".

"Human coronaviruses OC43 and HKU1 are related, yet distinct respiratory pathogens, associated with common colds, but also with severe disease in the frail. Both viruses employ sialoglycanbased receptors with 9-O-acetylated sialic acid (9-O-Ac-Sia) as key component." [51]. The authors of [51] "identify the 9-O-Ac-Sia–specific receptor binding site of OC43 S and demonstrate it to be conserved and functional in HKU1. The considerable difference in receptor binding affinity between OC43 and HKU1 S, attributable to differences in local architecture and receptor-binding site accessibility, is suggestive of differences between OC43 and HKU1 in their adaptation to the human sialome" [51].

28) CORONAVIRUS HCOV-229E AND NL63 - Human coronavirus 229E (HCoV-229E) is a species of coronavirus which infects humans and bats. It is an enveloped RNA virus which enters its host cell by binding to the APN receptor. [APN, that is aminopeptidase N]. Human coronavirus NL63 (HCoV-NL63) was identified in late 2004 in a seven-month-old child with bronchiolitis in the Netherlands. The virus is an enveloped RNA virus which enters its *host cell by binding to ACE2*. Infection with the virus has been confirmed worldwide, and has an association with many common symptoms and diseases.

In [52], we can find a review about Human Coronavirus-229E, -OC43, -NL63, and -HKU1. "Seven human coronaviruses (HCoVs) have been so far identified, namely HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and the novel coronavirus (2019-nCoV, a.k.a. SARS-CoV-2). Unlike the highly pathogenic SARS-CoV, MERS-CoV, and 2019-nCoV, the four so-called common HCoVs generally cause mild upper-respiratory tract illness and contribute to 15%–30% of cases of common colds in human adults, although severe and life-threatening lower respiratory tract infections can sometimes occur in infants, elderly people, or immunocompromised patients". In [52]. the authors review the molecular virology of these common HCoVs, and summarize knowledge on HCoV-host interaction.

In [52], about the attachment it is told the following. "Coronavirus infection is initiated by binding of virions to cellular receptors. The S protein includes two functional domains: S1 (bulb) is the part binding to the receptor(s) and S2 (stalk) is responsible for fusion between virion and cell membranes. The receptor-binding domain (RBD) of S1 varies among different coronaviruses. ... Receptor binding is critical to initiate viral infection. HCoV has been shown to use either cellular proteins or carbohydrates displayed on the plasma membrane as receptors. Interestingly, all known protein receptors for HCoVs are cell surface peptidase, such as aminopeptidase N (APN) for HCoV-229E, dipeptidyl peptidase 4 (DPP4) for MERS-CoV, and angiotensin converting enzyme 2 (ACE2) for HCoV-NL63, SARS-CoV and SARS-CoV-2. On the other hand, HCoV-OC43 and HCoV-HKU1 employ glycan-based receptors carrying 9-O-acetylated sialic acid. In addition to the receptor binding by the S protein, other HCoV structural proteins may also facilitate the early stage of attachment. For example, the M protein of HCoV-NL63 binds to target cells using heparan sulfate proteoglycans as the initial attachment factors. This is followed by the engagement of the S protein with the ACE2 receptor protein".

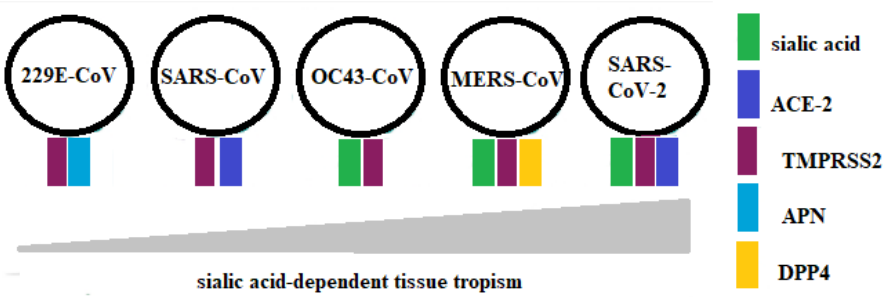
29) THE CYTOKINE STORM - The starting points of our study were the hemagglutinin and neuraminidase glycoproteins of influenza A virus, which are responsible for the surface interactions of the virion with the host [see Ref. [53]]. For Sars-Cov-2, we have studied the ACE2 receptors and other proteins. And we have seen

that there are coronaviruses which are using N-acetyl-9-O-acetylneuraminic acid to enter the cells. Therefore, it is not surprising to find an article, such as the Ref. [54], entitled "Coronaviruses: Is Sialic Acid a Gate to the Eye of Cytokine Storm? From the Entry to the Effects", which starts precisely from these acids.

Of this storm we discussed in [55]. Covid-19 is the disease resulting from the infection caused by Sars-Cov-2. This infection can have as its product "an excessive and aberrant host immune response", associated with what is defined as a "cytokine storm" [56]. This storm is characterized "by the plasma increase of many cytokines that produce long-term damage and fibrosis of lung tissue" [56]. Among the cytokines, there is the Interleukin 6 (IL-6), produced by a variety of cell types and involved in various physiological processes [56]. "Elevated tissue and serum levels of IL-6 are implicated in the pathogenesis of various inflammatory and autoimmune disorders including many forms of rheumatic diseases; they are also implicated in the cytokine release syndrome (CRS)" [56]. For the term "cytokine" and others, see [55].

As previously told, Ref. [54] is proposing in its title the question "*Is Sialic Acid a Gate to the Eye of Cytokine Storm?*". Here some sentences from the abstract proposed by authors. We have already seen that Coronaviruses (CoVs) are enveloped human and animal viruses. "The high pathogenic potential of human CoVs, including SARS-CoV, MERS-CoV and SARS-CoV-2, is closely related to the invasion mechanisms underlying the attachment and entry of viral particles to the host cells. There is increasing evidence that sialylated compounds of cellular glycocalyx can serve as an important factor in the mechanism of CoVs infection. Additionally, the sialic acid-mediated cross-reactivity with the host immune lectins is known to exert the immune response of different intensity in selected pathological stages".

The authors of [54] focus on the "last findings in the field of glycobiology in the context of the role of sialic acid in tissue tropism, viral entry kinetics and immune regulation in the CoVs infections". In fact, the title of the paper is proposing a path from the attachment of the virus to the host cells, where the sialic acid can have a role, to the worst consequence of Covid-19, that is the cytokine storm.



Ref. [54] summarizes the receptor recognition pattern during the CoVs infections in its Figure 1. "The CoVs invade host cells through the attachment, binding and entry mechanism based on the sialic acid and protein receptors. After sialic acid-mediated virus attachment and its spike protein activation by transmembrane serine protease 2 (TMPRSS2), the entry event is associated with the binding of specific protein receptor: ACE-2 (angiotensin converting enzyme type 2); DPP4 (dipeptidyl peptidase 4); APN (aminopeptidase N). The ability to recognize sialoglycans determines virus tissue tropism and clinical manifestations among infected organs."

Viral tropism is the ability of a given virus to productively infect a particular cell (cellular tropism), tissue (tissue tropism) or host species (host tropism).

Let us stress that the recent publication [32], which has investigated the binding of the spikes of the virus by means of micro-arrays. [32] tells that no binding with sialic acid residues was detected. All the tested protein molecules can bind to heparan sulfate glycans in a sulfation-dependent manner. However, we have also seen different results in [36]

Let us also add that, in [57], we can find told "that clinical isolates of HCoV-OC43 and -HKU1 preferred the cell-surface TMRRSS2 to endosomal cathepsins for cell entry, similar to HCoV-229E". Therefore, we could consider these data besides those given in [54].

30) SIALIC ACID TARGETING - From abstract of [58]. "Over the past decades, several antiviral drugs have been developed to treat a range of infections. Yet the number of treatable viral infections is still limited, and resistance to current drug regimens is an ever-growing problem. Therefore, additional strategies are needed to provide a rapid cure for infected individuals. An interesting target for antiviral drugs is the process of viral attachment and entry into the cell. Although most viruses use distinct host receptors for attachment to the target cell, some viruses share receptors, of which sialic acids are a common example. This review aims to give an update on entry inhibitors for a range of sialic-acid-targeting viruses and provides insight into the prospects for those with broad-spectrum potential" .

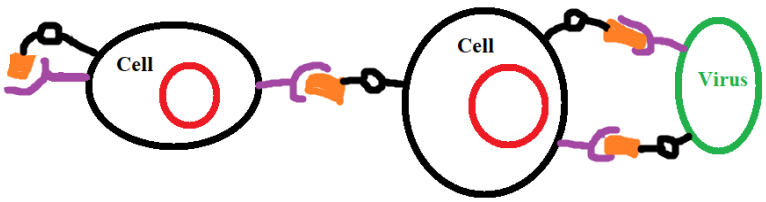
Let us stress that an old drug exists, the Oxolamine, which inhibits the formation of N-acetylneuraminic acid (see [15] and references therein).

31) THE SPILLOVER - Let us conclude with Ref. [59], a review entitled "Sialic Acid Receptors: The Key to Solving the Enigma of Zoonotic Virus Spillover". "Most of the human epidemics and pandemics were caused by the spillover of viruses from wild mammals. ... An in-depth understanding of the mechanisms of viral emergence and zoonotic spillover is still lacking. Receptors are major determinants of host

susceptibility to viruses. Animal species sharing host cell receptors that support the binding of multiple viruses can play a key role in virus spillover and the emergence of novel viruses and their variants. Sialic acids (SAs), which are linked to glycoproteins and ganglioside serve as receptors for several human and animal viruses. In particular, influenza and coronaviruses, which represent two of the most important zoonotic threats, use SAs as cellular entry receptors." Ref. [59] is a comprehensive review of current knowledge of sialic acid receptor distribution among animal species and the viruses using SAs as receptors. "SA receptor tropism and the predicted natural susceptibility to viruses can inform targeted surveillance of domestic and wild animals to prevent the future emergence of zoonotic viruses".

Appendix - Health and Disease

Suggested reading - Ref. [8], the article entitled "Sialic acids in human health and disease". Ajit Varki illustrates some biological and pathological roles of sialic acids. These acids have negative charge and hydrophilicity, and therefore they have many *structural or physical roles.* Given examples are their function in neural plasticity, glomerular filtration or blood cell charge repulsion. Sialic acids serve as ligands for *intrinsic receptors* such as Siglecs and factor H. So we have the possible interaction between sialic acids (as sialylated glycan molecules) expressed on host cells (on the same cell or different host cells, see the figure in the next page). But the sialic acids serve as components of binding sites for various pathogens and toxins, such as those listed in the Table. "In most such interactions, a pathogen-binding protein (extrinsic receptor) recognizes certain forms of sialic acids presented in specific linkages to a defined underlying sugar chain". Another class of functions is ‘molecular mimicry’, in which successful microbial pathogens decorate themselves with sialic acids, assisting in evasion of host immunity.



Intrinsic and extrinsic receptions.

Zenodo - DOI: 10.5281/zenodo.4624160

Examples of pathogens that bind to sialic acids on human cell surfaces that we have seen in the discussion, and that are reported in [8]

Pathogen	Binding protein	Sialylated target
Human Influenza A	Hemagglutinin	Siaα2-6Gal(NAc)
Avian Influenza A	Hemagglutinin	Siaα2-3Galβ1-
Human Influenza C	Hemagglutinin - esterase	9-OAc-iaα2-

APPENDIX- Types of Influenza Viruses

Suggested reading - https://www.cdc.gov/flu/about/viruses/types.htm

Influenza A viruses are classified by means of subtypes according to the properties of their hemagglutinin (H) and neuraminidase (N) surface proteins. CDC tells that there are 18 different HA subtypes and 11 different NA subtypes. Subtypes are named by combining the H and N numbers – e.g., A(H1N1), A(H3N2). The calculus of combinations tells that there are potentially 198 different influenza A subtype. Only 131 subtypes have been detected in nature. "Current subtypes of influenza A viruses that routinely circulate in people include: A(H1N1) and A(H3N2)" [CDC].

In fact, four types of influenza viruses exist: A, B, C and D. Human influenza A and B viruses cause seasonal epidemics of disease. Influenza A viruses are the only influenza viruses known to cause flu pandemics. "A pandemic can occur when a new and very different influenza A virus emerges that both infects people and has the ability to spread efficiently between people" [CDC]. "Influenza type C infections generally cause mild illness and are not thought to cause human flu epidemics. Influenza D viruses primarily affect cattle and are not known to infect or cause illness in people" [CDC]

For what concerns the name of viruses, it exists an internationally accepted naming convention by WHO in 1979 and published in February 1980 in the Bulletin of the World Health Organization, 58(4):585-591 (1980). Here the details given by CDC. The antigenic type (e.g., A, B, C, D) is a part of the name. Then we have the host of origin (e.g., swine, equine, chicken, etc.). For human-origin viruses, no host of origin designation is given. Also the geographical origin is given.

Here the CDC proposed examples: (avian case) Avian influenza A(H1N1), A/duck/Alberta/35/76 ; (Human case): seasonal influenza A(H3N2), A/Perth/16/2019. Note that we have the sequence of geographical origin, the strain number and the year of year of collection. For influenza A viruses, the hemagglutinin and neuraminidase

antigen description are provided in parentheses.

"The 2009 pandemic virus was assigned a distinct name: A(H1N1)pdm09 to distinguish it from the seasonal influenza A(H1N1) viruses that circulated prior to the pandemic. When humans are infected with influenza viruses that normally circulate in swine (pigs), these viruses are call variant viruses and are designated with a letter ‘v’ (e.g., an A(H3N2)v virus)" [CDC].

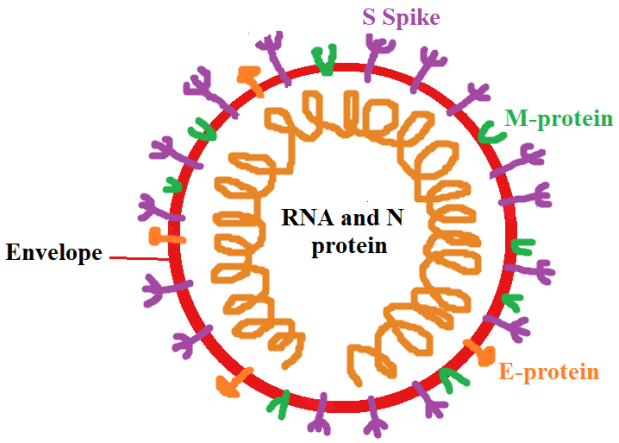
Appendix - Viruses and Reptiles

Suggested reading - Ref. [60], a review article entitled "Viruses Infecting Reptiles". Here from abstract. "A large number of viruses have been described in many different reptiles. These viruses include arboviruses that primarily infect mammals or birds as well as viruses that are specific for reptiles. Interest in arboviruses infecting reptiles has mainly focused on the role reptiles may play in the epidemiology of these viruses, especially over winter. Interest in reptile specific viruses has concentrated on both their importance for reptile medicine as well as virus taxonomy and evolution. The impact of many viral infections on reptile health is not known. Koch’s postulates have only been fulfilled for a limited number of reptilian viruses".

Appendix (in Italian) - Viaggio al centro del virus.

Si suggerisce la lettura di https://www.unisr.it/news/2020/3/viaggio-al-centro-del-virus-come-e-fatto-sars-cov-2 archived https://archive.is/p0m8R

I Coronavirus sono stati identificati a metà degli anni ’60 e sono noti per infettare l'uomo ed alcuni animali. L’illustrazione mostra schematicamente la morfologia del SARS-CoV-2. Sulla superficie vi sono le glicoproteine S-spike. Il virione è rotondo di 100-150 nm di diametro. All'esterno ci sono la Glicoproteina S (“spike”), della lunghezza di circa 20 nm. "Tre glicoproteine S unite compongono un trimero; i trimeri di questa proteina formano le strutture che, nel loro insieme, somigliano a una corona che circonda il virione. ... La glicoproteina S è quella che determina la specificità del virus per le cellule epiteliali del tratto respiratorio: il modello 3D infatti suggerisce che SARS-CoV-2 sia in grado di legare il recettore ACE2 (angiotensin converting enzyme 2), espresso dalle cellule dei capillari dei polmoni".



La proteina di membrana (M) attraversa il rivestimento (envelope), in modo da interagire con l'interno del virione dove c'è il complesso RNA-proteina. La Proteina E aiuta aiuta la S ad attaccarsi alla membrana della cellula bersaglio[17]. Si noti che l'Envelope, che è il rivestimento del virus, è costituito da una membrana che il virus prende dalla cellula ospite, quando esce da essa. RNA e proteina N costituiscono il genoma del virus. Vi è un singolo filamento RNA. L'RNA è associato alla proteina N, che ne aumenta la stabilità.

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