UNIVERSITÀ POLITECNICA DELLE MARCHE

FACOLTA' DI MEDICINA E CHIRURGIA

Ph.D. Course

Biomedical sciences

RELATIONSHIP BETWEEN VIRAL VARIABILITY AND ANTIBODY RESPONSE IN SARS-CoV-2 INFECTION

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XXXVI cycle

A chi c'è sempre stato

e a chi se ne è andato.

A chi ha creduto in me dall'inizio alla fine,

e a chi non ha mai lasciato la mia mano.

Che questo percorso ti possa ricordare di fissare sempre l'occhio del ciclone come fosse arte,

di continuare a sembrare la persona strana che continua a testa alta a cercare la parte migliore di sé.

A tutti voi che siete nel mio cuore,

e a quel fiore contro il diluvio che non ha mai mollato.

Credici, sempre.

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1. INTRODUCTION

1.1 SARS-CoV-2 pandemic

In December 2019, Chinese health authorities reported several clusters of pneumonia of unknown cause in Wuhan, Hubei Province, China and all early cases were related to the exposure at Wuhan's South China Seafood City market.

The novel pathogen was a new type of coronavirus, in particular it was a *betacoronavirus*, originally called 2019-nCoV by international health authorities and finally named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2); whereas the respiratory disease was officially named Coronavirus Disease 2019 (COVID-19).

This virus was called in this way because, based on phylogeny and taxonomy, is formally associated with Severe Acute Respiratory Syndrome coronavirus, (SARS-CoV), sharing with it 79.5% sequence identity. [2]

Together with MERS and SARS-CoV, since the beginning of the present century, represents the third spillover of an animal coronavirus to humans.

Presently, the pandemic results under control with decreasing number of confirmed cases in most of countries: globally, in June 2023, the number of new cases is diminished of 30%, and the number of deaths is diminished of 39%. [3]

2. SARS-CoV-2 STRUCTURE

SARS-CoV-2 is an enveloped virus with single-stranded, positive-sense RNA (+ssRNA). It is roughly spherical and exhibits a moderate degree of pleomorphism. The size is approximately 100 nm in diameter.

From outside to inside SARS-CoV-2 virions consists in (Fig.1):

- **Envelope**: is the virus' most external portions. It is a phospholipidic double layer of cellular origin, and it is associated with three structural proteins: membrane protein (M), spike protein (S) and envelope protein (E). [2]
- **Nucleocapsid**: made of nucleoproteins (N) which enclose and protect the viral genome.

One of the features of SARS-CoV-2 consists of the presence of several Spike proteins (S) that emerge from the virion surface giving it a characteristic bulb-like shape that resembles a solar corona. For this reason, scientists have coined the name "Coronavirus". [12]

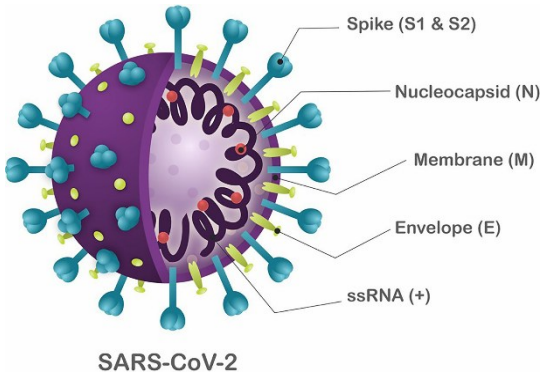

Fig. 1: De Andrade Santos I., et al., Schematic structure of SARS-CoV-2. From "Antivirals Against Coronaviruses: Candidate Drugs for SARS-CoV-2 Treatment?". *Frontiers in Microbiology*. Volume 11 – 2020.

2.1 Genome structure

The novel Coronavirus' RNA is approximately 30 kb (Fig.2), which is currently the largest known genome size for an RNA virus. [13]

Similar to all other Coronaviruses, SARS-CoV-2 genome is comprehensive of a 5' terminal Cap and a 3' poly-A tail, thus being immediately available for translation in host cells.

The RNA genome has six open reading frames (ORFs). The first one, ORF1ab, is about two-thirds of the whole genome and is located at the 5' end. It encodes the RNA-dependent-RNA-polymerase, along with secondary proteins involved in replication mechanisms. Into the target cell cytoplasm, the RNA genome translates into pp1a and pp1b further processed into 16 non-structural proteins (NSPs), and forms a replication-transcription complex (RTC).

The ORFs located on 3' end encodes four structural proteins: S protein that recognizes host cell receptors, E protein for virions assembly and release and nucleocapsid protein (N) for packaging of the RNA genome and pathogenicity of the virus as an interferon (IFN) inhibitor.

In addition, species-specific ORF3a, ORF3d, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF14 and ORF10 genes encode nine accessory proteins.

Each structural and accessory gene is preceded by transcriptional regulatory sequences (TRSs), a prerequisite for their functional expression.

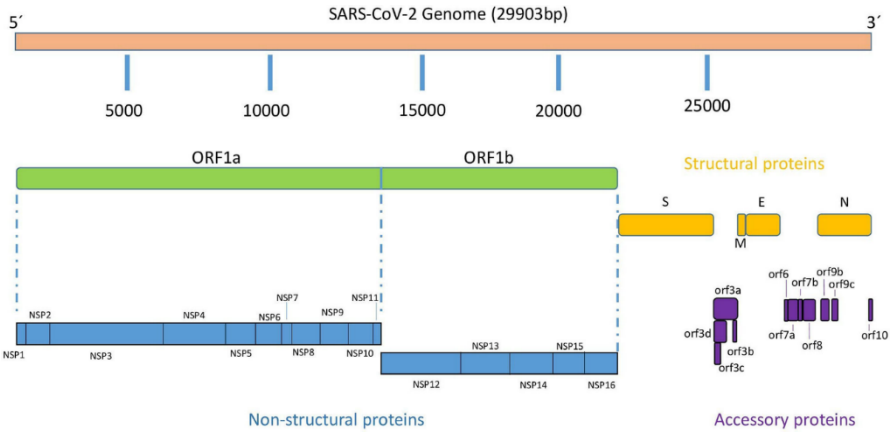

Fig.2: Representation of SARS-CoV-2 genome organization. Redondo N., et al., SARS-CoV-2 Accessory Proteins in Viral Pathogenesis: Knowns and Unknowns. *Frontiers in immunology*. Volume 12 – 2021.

2.2 Structural protein

SARS-CoV-2 has 4 main structural proteins:

1) **Spike protein (S)**: is 200 nm long and it is located on the surface of the viral membrane. It is a trimeric, bulb-like glycoprotein involved in host tropism, receptor binding and subsequent membrane fusion to facilitate viral entry into the host cell. [14, 15]

   Each monomer is made of two portions (Fig.3): S1 and S2. The first one acts as a major surface antigen. It contains two subunits, N-terminal domain (NTD) and C-terminal domain (CTD). This one acts as a receptor-binding domain (RBD), that interacts with the 18 residues of ACE-2. The S2 domain is a membrane fusion subunit. It contains the fusion peptide (FP), heptad repeat 1 and 2 (HR1, HR2), central helix (CH), connector domain (CD) and trans-membrane domain (TM). HR1 and HR2 domains interact with each other to form an antiparallel six-helix bundle (6-HB)

structure known as fusion core. This allosteric process begins immediately after RBD-ACE2 binding and S2 cleavage, leading to the viral-cellular membrane fusion. The activation of the S protein is a complex process which requires proteolytic cleavages at two different sites: S1/S2, that contains a furin cleavage site (RRAR), and a second site in S2 (S2'), located immediately upstream of the furoin peptide (FP). [7] In particular, cleavage at S1/S2 of the spike protein is essential for efficient viral entry into its target cells. The furin site is cleaved by host proteases primarily during maturation, and S2' site cleavage can occur upon binding to the surface of the host cell by the TMPRSS2 enzyme.

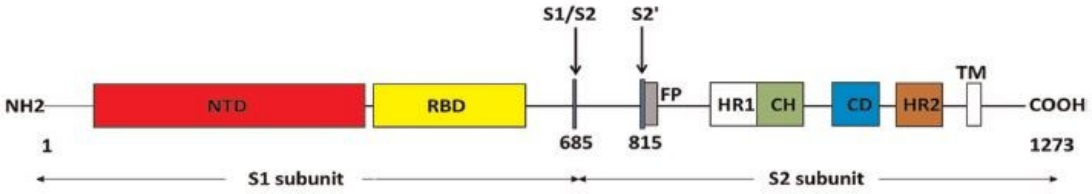

Fig.3: SARS-CoV-2 Spike protein. Kadam S.B., et al., SARS-CoV-2, the pandemic coronavirus: molecular and structural insights. *Journal of basic Microbiology*. 2021;61:180-202.

2) **E protein**: has an important role in virion assembly and escape from the host cells. Is also known to contribute to pathogenesis: is responsible for a condition known as unfolded-protein response (UPR), that may ultimately lead to apoptosis. [7, 14, 15]

3) **M protein**: defines the shape of the viral envelope and it acts as a "bridge" between the nucleocapsid and the lipid bilayer. [7, 14, 15]

4) **N protein**: its main function is to bind to the RNA genome in a beads-on-a-string configuration [12] keeping it in an ordered conformation for replication and transcription and packaging it into a long nucleocapsid, which is known as ribonucleoprotein. [7, 14, 15]

2.3 Non-structural proteins

SARS-CoV-2 ORF1a and ORF1b encode 16 non-structural proteins (nsps), which contain multiple enzymatic functions and are involved in several phases of the viral replication (Fig.4). They are:

- **Nsp1**: it can inhibit innate immune responses by binding to the 40S ribosome of the host cell and accelerate the degradation of mRNA, causing a reduction in cellular antiviral protein synthesis and promotes virus survival. [7, 15]

- **Nsp2** and **Nsp3:** interact to act as proteases and cleave the product of ORF1a [16]

- **Nsp4:** has a significant role in viral replication and the formation of the reticulovesicular network, assembling the viral double-membrane vesicles. [7]

- **Nsp5:** is a cysteine protease, also known as the main protease (Mpro) that processes ORF1a and ORF1b to generate 12 functional proteins. [15]

- **Nsp6:** locates to the endoplasmic reticulum (ER), generates autophagosomes and may lead to changes in host antiviral defenses. [15]

- **Nsp7** and **Nsp8:** are cofactors of Nsp12 because they catalyze the synthesis of RNA primers for the primer-dependent primase. [15]

- **Nsp9:** is able to bind the RNA in complex with Nsp8. [16]

- **Nsp10:** interacts with Nsp14 and Nsp16 as a cofactor to stimulate their respective activities of 3'-5' exoribonuclease and 2'-O-methyltransferase for 5'-capping. [15]

- **Nsp11:** has been shown to be essential in viral replication, although its specific functions are still unknown. [16]

- **Nsp12:** is a key component of the SARS-CoV-2 replication and transcription machinery. It catalyzes the synthesis of viral RNA with the assistance of nsp7-nsp8. [15]

- **Nsp13:** works as a helicase during viral RNA replication, and it is also involved in 5'-mRNA capping. [14, 16]

- **Nsp14:** is a 3'-5' exonuclease that corrects error and can replace the incorrect nucleotide with the correct one, leading to an increase in RNA syntesis's fidelity. It is also involved in the RNA 5' Cap formation. [14, 16]

- **Nsp15:** is an endonuclease that cleaves RNA at polyuridylate sites. [7]

- **Nsp16:** forms a heterodimer with the Nsp10 cofactor and enhances the activity of 2'-O-MTase during 5' capping. [14]

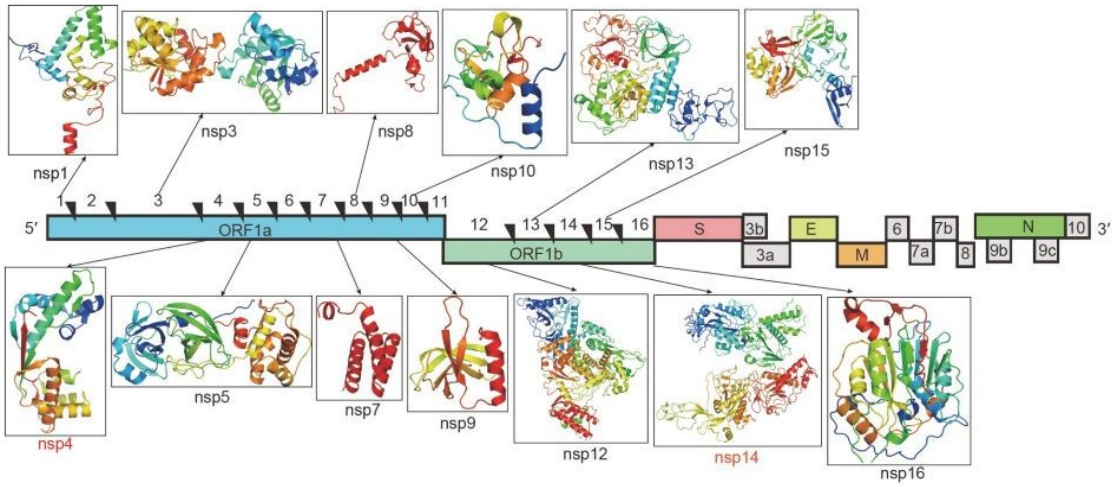

Fig.4: Structure of non-structural proteins. Bai.C., et al., Overview of SARS-CoV-2 genome-encoded proteins. *Life sciences-Science China Press*. (2022) Vol.65 N°.2: 280-294

3. SARS-CoV-2 LIFE CYCLE

3.1 Entry into cells

The first step of the SARS-CoV-2 life cycle is the virus entry into the host cell. This happens when virus binds the host receptor angiotensin-converting enzyme 2 (ACE-2). In particular, ACE-2 recognition is mediated by the Receptor Binding Domain (RBD) sequence located in the viral S protein. In more detail the RBD contains five antiparallel β sheets that assembly to form the core. It contains the Receptor Binding Motif (RBM), that is a crucial element in the overall structure of the RBD, as it comprises most of the contacting residues that bind to ACE-2. [2, 20]

In particular, when virus particles come close to the host cell, a pre-activation of the viral S protein by a proteolytic cleavage occurs in a multibasic site (Arg-Arg-Ala-Arg) in the S1-S2 junction, which is cleaved by Furin. [21]

This enzymatic cleavage leads to a conformational change in the RBD position, that switches from “down” to “up”-position and binds the host receptor. Virus binding to ACE-2 induces conformational changes in the S1 subunit and exposes the S2’ cleavage site in the S2 subunit, that is cleaved by the transmembrane protease serine 2 (TMPRSS2). The last step mentioned induces the exposure of the fusion peptide (FP), that goes forward into the target membrane initiating membrane fusion and the viral RNA is released into the host cell cytoplasm for uncoating and replication.

Alternatively, if the target cell expresses insufficient TMPRSS2 or if a virus-ACE-2 complex does not encounter TMPRSS2, the virus-ACE-2 complex is internalized via clathrin-mediated endocytosis into the endolysosomes, where S2’ cleavage is performed by cathepsin. (Fig.5)

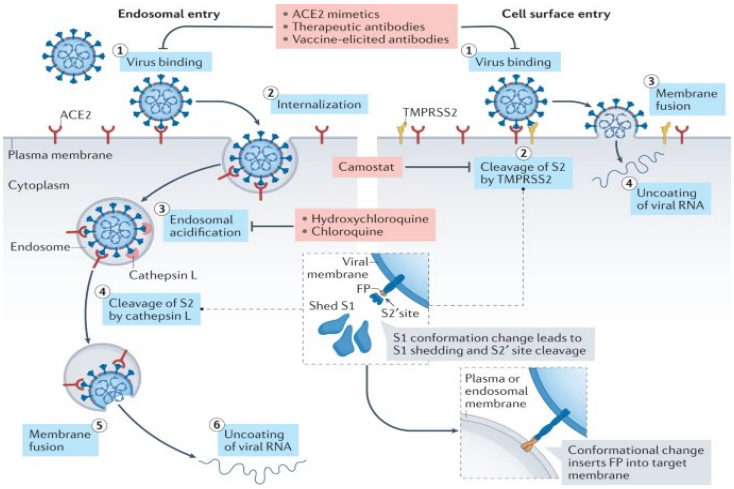

Fig.5: Two distinct SARS-CoV-2 entry pathways. Jackson C. B., et al., Mechanism of SARS-CoV-2 entry into cells. (January 2022). *Nature Reviews | Molecular cell biology*. Vol.23.

3.2 Viral RNA replication and expression

Coronaviruses carry out their genome replication inside a reticulo-vesicular network that integrates convoluted membranes (CM), several interconnected double-membrane vesicles (DMVs) and vesicles packets (VPs), which are assembled in close connection to the ER. This counteracts the host innate immunity. (Fig.6) [23,24]

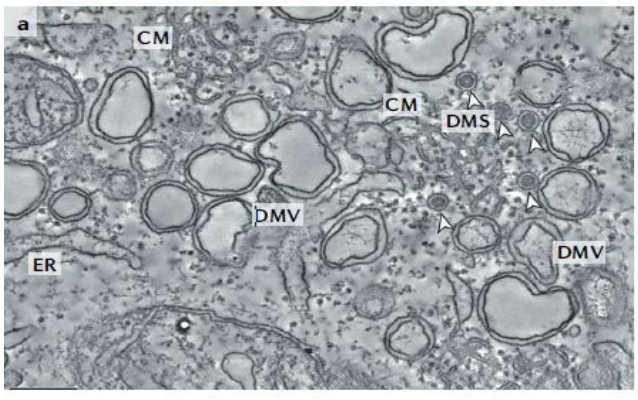

Fig.6: Coronavirus replication organelles. Malone B., et al., Structures and functions of coronavirus replication-transcription complexes and their relevance for SARS-CoV-2 drug design. Nature Reviews | Molecular cell biology. Vol.23. January 2022.

The viral genome is a ssRNA+ so, after the entry process into host cytoplasm, when a ribosome meets it starts to translate its two large replicase ORFs, ORF1a and ORF1b. Production of pp1ab polypeptide depends on the occurrence of a -1 programmed ribosomal frameshift just upstream of the ORF1 termination codon, thus extending pp1a with the ORF1b-encoded polyprotein.

Then, sixteen mature nsps are released from pp1a and pp1ab following 15 proteolytic cleavages performed by the virus encoded papalin-like protease (PL^{pro}) in nsp3 and chymotrypsin-like or main protease (M^{pro}) in nsp5. In this manner, pp1a yields nsp1 to nsp11, whereas pp1ab is cleaved into nsp1 to nsp10 and nsp12 to nsp16. [22]

These ones are able to grab onto a ribosome and occupy it in such a way that the ribosome can only read viral RNA and not the host cell's own RNA. This means that the virus takes over the cell's protein production machinery, turning the host cell into a virus building factory.

Some nsps interacts to form the replication and transcription complex (RTC), where the RNA-dependent-RNA-polymerase (RdRP) with its cofactors nsp7 and nsp8 forms the RNA replication unit. [7] The newly formed RTC synthesizes, inside DMVs, new genomic RNA (gRNA) and a set of subgenomic mRNAs that include open reading frames (ORFs) 2-9b, which encode for viral structural and accessory proteins. Newly made gRNAs can be translated to yield additional non-structural proteins, serve as a template for further RNA synthesis or be packaged into new virions. [23,24]

3.3 Assembly and release

When all viral proteins and genomes are produced, they can assemble to form new viral particles.

The translated structural and accessory proteins are released in endoplasmic reticulum (ER). A specialized smooth-walled Golgi intermediate compartment (ERGIC) carries these viral particles across the secretary pathways. [7]

In the first step N protein associates with newly made viral RNA genomes forming cytoplasmic nucleocapsids.

The viral structural proteins (S, E and M) are synthetized on ER-associated ribosomes and, at ER exit sites (ERES), packaged into transport vesicles for delivery to the ERGIC or the intermediate compartment (IC). In this region, nucleocapsids combine with envelope components to form virions, which undergo on budding process. This one is suggested to take place at vacuolar domains of the IC, which are large enough to accommodate the virus particles and may undergo further dilatation. Moreover, budding is probably largely based on specific interactions between the viral structural proteins. Then, the saccular IC elements develop into mobile transport carriers and virus particles are ready to move towards the plasma membrane (PM). Here virions are released by exocytosis. (Fig.7) [25]

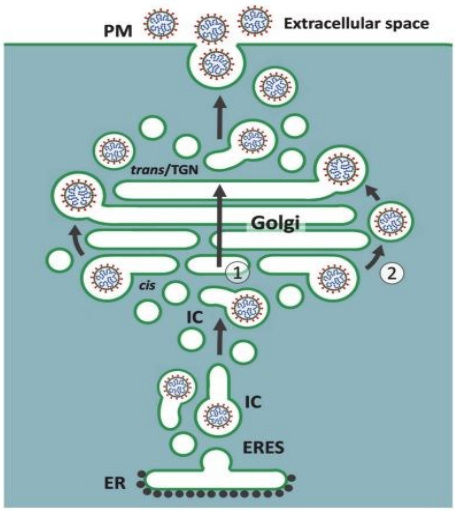

Fig.7: Virus assembly process. Saraste J., et al., Assembly and cellular exit of coronaviruses: hijacking an unconventional secretory pathway from the pre-Golgi intermediate compartment via the Golgi ribbon to the extracellular space. Cells. 2021, 10, 503.

4. SARS-CoV-2 PATHOGENESIS AND IMMUNE RESPONSES

SARS-CoV-2 is responsible for Coronavirus disease 2019 (COVID-19). The infection begins when viral S protein binds host receptor ACE-2. This one is a main component of the renin-angiotensin-aldosterone system (RAAS) which maintains fluid and salt balance, as well as blood pressure homeostasis. In the RAAS system, the angiotensin-converting enzyme (ACE) generates angiotensin II (ANGII), an effector peptide able to stimulate vasoconstriction. ANGII, then, is converted in ANG(1-7) by a proteolytic cleavage carried out by the angiotensin-converting enzyme 2 (ACE-2), therefore promoting vasodilation. [26]

Overactivation of RAAS has been implicated in the pathophysiology of atherosclerosis, heart failure, hypertension, diabetes, renovascular disorders, pulmonary hypertension, pneumonia, fibrosis and sepsis. In fact, patients with this type of pathology are associated with high-risk severe COVID-19. [26, 27]

Furthermore, the viral entry factors in the host cell are the presence of ACE-2 receptor (as mentioned above) and the presence of the TMPRSS2. These ones are expressed at high levels in nasal epithelial cells and lungs, explaining why this region is the SARS-CoV-2 infection first site. [28]

4.1 Pathophysiology

Depending on immune response, COVID-19 can give different type of clinical syndromes: the most of patients exhibit mild symptoms, some subjects are asymptomatic and other can exhibit severe clinical manifestations. In particular, it can result fatal for some people because of the direct and indirect

damage on the endothelial cells that cover blood vessels, especially in the lung.

SARS-CoV-2 infects first nasopharynx cells. When it enters into the hosts, it begins its replication cycle. Meanwhile host immune system activates to contrast and block viral replication by inducing an antiviral cellular state [31]. If innate and prompt adaptive responses are not adequate, the virus can spread to the low respiratory tract, by progressive dissemination through the tracheo-bronchial tree. [31] When it arrives in lungs, it infects endothelial cells of blood capillaries that cover pulmonary alveoli, including type II pneumocytes, damaging them. These mechanisms cause white cells accumulation in the lungs and the release of signaling molecules such as Interleukins. The increase of local blood pressure and the weakness of the cellular junctions occur, leading to the exposure of the basal membranes, triggering clotting factors and platelets and causing an uncontrolled coagulation. [32]

An inflammatory state is created, with the cytokine storm, and pulmonary alveoli are damaged leading to an accumulation of fluid around them that avoid gas exchange. Clinically patients show respiratory difficulties defined Acute Respiratory Distress Syndrome (ARDS). [31]

Because of severe COVID-19 is often characterized by this vascular damage, patients with vascular problems, diabetes, obesity, hypertension and other comorbidities are classified as at-risk subjects due to their already compromised blood vessels. [32]

Sometimes subjects who have been infected exhibit a multisystemic condition characterized by debilitating sequelae, and this condition can last for several weeks. [42] The most common symptoms are weakness (41%), general

malaise (33%), fatigue (31%), concentration disorders and brain fog (26%) and breathlessness (25%) [41]

4.2 Clinical features

SARS-CoV-2 infects host cells by binding the viral Spike (S) protein to the host angiotensin-converting enzyme 2 (ACE2). Viral load is highest in the upper respiratory tract (nasopharynx and oropharynx) early in disease and then increases in the lower respiratory tract (sputum), suggesting that the first one is the usual initial site of viral replication, with subsequently descending infection. [17]

So, the dominant route of transmission of SARS-CoV-2 is respiratory and takes place through droplet production (and possibly aerosol). These are liquid particles dispersed in the air when a person talks, sneezes or coughs and contain particles like SARS-CoV-2. In particular, droplets can be more than of 5-10 µm or less than 5 µm in diameter. The former lay on surfaces because of their size and can infect the subject in close contact (1 m) with the infected person, causing conjunctival or mucosal infection. The latter are dispersed hundreds of meters in the air and can remain airborne for a long time making a small, enclosed place potentially contagious. For these reasons the use of a face mask decreases the risk of viral infection. [18]

Transmission can occur also through direct contact between infected and non-infected subjects, or because of contact with objects, fomites, contaminated surfaces. [17]

The incubation period can take 2-8 days, but often subjects have symptoms in 3 days after their contact with infected person.

In naïve subjects, SARS-CoV-2 causes mild disease in at least 80% of subjects infected, severe disease in 14% and critical disease in 6% of them. Persons who have SARS-CoV-2 with or without symptoms can transmit it. The latter may be either presymptomatic or they may remain asymptomatic. [17]

Mild disease means flu-like illness, and includes symptoms like cough, headache, fever, sore throat, muscle pain. Sometimes COVID-19 affects also the gastrointestinal tract, with vomiting and diarrhea, and the central nervous system with loss of taste and smell. Mostly, the disease spontaneously resolves in 7-14 days with a total recovery of the subjects. Sometimes, in subjects with severe and critical disease (with respiratory and renal failure, respiratory distress, etc..) the recovery can be partial, or can lead to the death of the patient.

In general, COVID-19 clinical severity increases with age, therefore older people are more likely to develop severe respiratory syndrome compared to children and young adults. This happens because of children and young adults have powerful innate immunity than older people, so they can overcome the virus. By contrast, in elderly subjects' innate immunity is less effective, so SARS-CoV-2 can replicate and go down to the lower respiratory tract, leading to the appearance of more severe respiratory symptoms. In addition, as mentioned above, the disease outcome is strongly connected to pre-existing co-morbidity: underlying medical conditions such as diabetes, heart disease, chronic lung inflammation, etc. can increase the risk for severe illness, even in young people. [19]

4.3 Diagnosis

Since the beginning of the pandemic, early and accurate viral detection has been essential to isolate infected patients and prevent contagion. The SARS-CoV-2 diagnosis can be done by direct and indirect tests.

4.3.1 Direct tests

This type of test is used with the aim to detect directly the pathogen inside clinical samples. These can be collected from the upper respiratory tract, such as nasopharynx, oropharynx and saliva, or lower respiratory tract, for example sputum, tracheal aspirate or bronchoalveolar lavage (BAL). This clinical samples are processed mainly by two types of tests:

- **RT-PCR**: it is a real-time reverse transcriptase polymerase chain reaction, and for now it is the international "gold standard". Before performing this test, viral RNA must be extracted from clinical samples by automatic or manual extraction, and it is subjected to RT-PCR: viral RNA is first converted into the complementary DNA (cDNA) and then it is amplified by a thermostable DNA polymerase - DNA dependent. In general, the Polymerase chain reaction is a technique for rapidly producing (amplifying) millions to billions of copies of a specific segment of DNA, and the Real-rime PCR can also be performed as a quantitative PCR. In fact, it is used mainly to quantify the pathogen's genome.

- **Antigenic tests**: have the purpose of detecting SARS-CoV-2 antigen, such as Spike protein, by binding it (if present) to a specific antibody. This type of test is performed in 1 hour or less, depending on the format (rapid or instrumental) of the assay. The disadvantage is the poor sensitivity, especially in case of low viral load.

4.3.2 Indirect tests

Indirect tests, or serological tests, detect if the patients have been exposed to SARS-CoV-2 by detecting the presence of specific antibodies against viral Nucleoprotein (N) or Spike protein (S) and may estimate previous or recent viral infections. In fact, IgM are the first immunoglobulin to appear, but their levels seem to decrease two weeks after SARS-CoV-2 infection and are not always detectable, so they are used to evaluate a recent infection. IgG appear later and persist for months or years, and are used as a marker of late or previous infections. [33]

Several automated methods have been developed since the pandemic began, such as IgG and IgM detection based on chemiluminescent immunoenzymatically reactions, or lateral-flow immunoassays.

4.4 Human immune system

The host can cope with the threat posed by microbes by three strategies: avoidance, resistance, and tolerance. Avoidance mechanisms prevent exposure to microbes, and include both anatomic barriers, neutralizing antibodies and behavioral modifications. If an infection is established, resistance is aimed at reducing or eliminating pathogens. To defend against the great variety of microbes, the

immune system has multiple molecular and cellular functions, suited to resist different categories of pathogens. [34]

The immune system is a large network of organs, white blood cells, proteins (antibodies) and chemicals and this system has the task of protecting against germs (bacteria, viruses, parasites, and fungi) and foreign substances that cause infection, illness and disease. [35]

There are two types of immunity: innate (general) and adaptive (specialized) immune system. They work closely together and take on different tasks. (Fig.8)

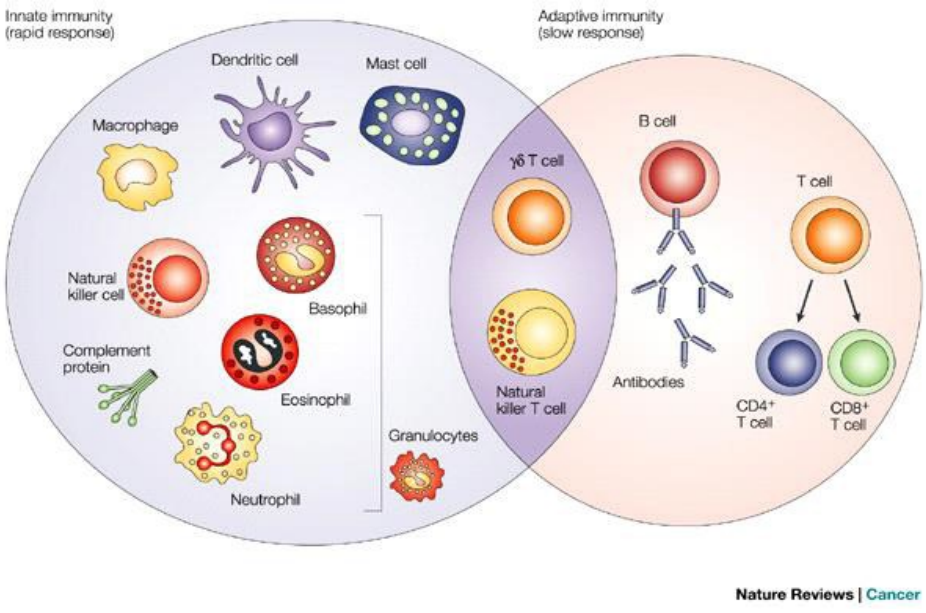

Fig.8: Innate and adaptive immune system's components. Nature Reviews Cancer, 4, 11-22

4.4.1 Innate immunity

It is also called natural immunity and is the first defense line present in the body. Innate immunity is called in this way because it is composed of readily available defense mechanisms possessed by birth, which are immediately activated. [34]

The innate immune system consists of: [35]

- **Chemical-physical barriers:** impede germs entry in the host cells and can be of different nature:

    - *Mechanical defenses:* for example, skin and mucous membrane that cover respiratory, gastrointestinal and urinary tract, mucus production. They act like barriers that physically avoid the entry of the pathogen.

    - *Chemical defenses*: such as antimicrobial molecules. These last ones include different type of enzyme: lysozymes (in tears and saliva), gastric pepsin, pH (stomach acidity and vaginal secretions) and antimicrobial peptide, such as “defensins” and “catelicidine” and prevent bacteria and viruses from gaining a foothold.

    - *Microbiological defenses*: microbiota competes for nutrients and for attack to the epithelium, also producing substances.

- **Inflammation or phlogosis**, is a local reaction and produces an attraction of phagocytes and other immune cells, like mastocytes, with

the aim to remove what caused the cell or tissue damage and start the reparative process.

- **Fever** consists in an increase in temperature to accelerate metabolism, enhance the action of the immune system in fighting infections and reduce the growth rate of microorganisms, because most of them suffer at temperatures near 40°C.
- **Defense cells**, that are activated when germs get past the previews barriers and enter the body. They include:
    - *Phagocytes*. They encompass *granulocytes* (i.e., neutrophils, eosinophils, basophils, and mast cells), *monocytes/macrophages*, and *dendritic cells*. Neutrophils and macrophages' role consist in identifying, ingesting (by phagocytosis) and eliminating pathogens.

    - *Natural killer* (NK) cells are lymphocytes that identify and kill infected or dysfunctional cells by releasing proteins which disrupt plasmatic membrane (perforins and granzymes). NK cells also secrete inflammatory cytokines, for example IFN-γ that activates the macrophages' microbicidal activity.

- **Defense proteins**, such as:
    - ***Cytokines***: recruit and activate leukocytes. The main sources of cytokines are macrophages, neutrophils and NK cells, but a lot of them are produced by endothelial cells and some epithelial cells, such as keratinocytes. Cytokines include:
        - Interleukins (IL): produced by white blood cells, with the function of local and systemic messengers. For example, IL-

1 actives the thermoregulation center in the hypothalamus inducing fever during infections. Together with IL-6, it stimulates the production of tumor necrosis factor from white blood cells. Further, IL-2 is important for lymphocytes clonal expansion.

- Tumor necrosis factor (TNF): proteins involved in the struggle against tumoral or infected cells and in the inflammatory reaction, by inducing the increasing production of complement proteins, fever and vasodilatation in the acute phase of the inflammation. There are two types of TNF: α and β. The first one is produced by monocytes and lymphocytes, the second one is produced by activated T lymphocytes.
- Interferon (IFN): they can inhibit viral replication in infected cells, enhance the activity of immunity cells and inhibit the tumoral cells' growth.

- ***The complement system*** consists of about 20 plasma proteins and are activated by microorganisms. The complement system determines the recruitment of phagocytic cells and granulocytes in infection sites, the opsonization of pathogens and their lysis. [35]

4.4.2 Adaptive immunity

It is also called specific or acquired immunity and is initiated when a pathogen overwhelms the innate defense mechanisms. Thanks to specific receptors the adaptive immunity can, by discriminating against different epitope's

structure, bind only specific antigens: this is fundamental to avoid an immune reaction toward self-antigens. Furthermore, this type of immunity is able to adapt against each pathogen, leading to a rapidly reaction to each exposure to it.

. There are different lymphocytes sub-population:

- **B lymphocytes**. They are the only one that can produce antibodies and do this after their contact with non-self-antigens and their differentiation in "*plasmacells*".

- **T lymphocytes**. They recognize peptidic antigens associated with proteins exposed on the host cell surface. Further, this type of lymphocytes can be functionally distinguished in sub-populations:

- T helper lymphocytes. Express CD4 membrane protein. They are able to produce cytokines, that stimulate the proliferation and differentiation of T and B lymphocytes, macrophages and other leucocytes.

- Cytotoxic T lymphocytes (CTL). Express CD8 membrane protein. They have the task of killing infected cells or enhancing their innate responses.

- Natural killer lymphocytes (NK): Kill infected cells and produce IFN-γ to activate phagocytes.

Antigen presenting cells (APC) are fundamental for antigen presentation to helper lymphocyte, the most specialized ones are dendritic cells, that capture germs antigens and present it to the naïve T lymphocytes with the aim to start cellular and humoral immune response.

The last component of adaptive immunity is effector cells. They are, for example, activated T lymphocytes, mononuclear phagocytes and other leucocytes and have the task of participating in the antigen clearance.

4.4.3 Defense mechanism

The first type of immunity that intervenes is innate immunity: the skin acts like a barrier to impede the entry of the pathogens, but when they can cross it find macrophages. These cells have different functions: they can phagocytize and destroy it with reactive oxygen species (ROS) and lysosomal enzymes, and can produce cytokines, that have the role of recalling leucocytes from blood to the inflammation site. In most cases, these events are sufficient for pathogens clearance, but when this doesn't happen germs enter the bloodstream. Here, they are recognized by complement proteins that activate when they come into contact with them.

When innate immunity can't stop the infection, adaptive immunity comes into play. Dendritic cells, that act as sentinel on epithelium and connective tissue, when find pathogens internalize them by endocytosis. They process their antigen to produce peptides that are expressed on membrane cells with the aim to presenting the antigen to B and T naïve lymphocytes, leading to their proliferation and differentiation.

The T lymphocytes activation leads to the activation of cellular immunity: $CD8^+$ T lymphocytes' differentiation results in cytotoxic cells production and the lysis of infected cell, to avoid the infection's dissemination. Instead, $CD4^+$ T lymphocytes mature and produce cytokines, such as interleukins-2 (IL-2) and interferon-γ (IFN-γ). The first one stimulates activated lymphocytes proliferation, leading to the production of effector cells able to produce different types of cytokines. Effector cells migrate towards the infection site to eliminate microorganisms. Whereas the second one promotes the production of substances with antimicrobial activity in macrophages, to help them in elimination of phagocytized pathogens. Further, some cytokines produce Immunoglobulin E. They activate eosinophils leukocytes with the aim to kill pathogen that are too big to be phagocytized. Some activated T lymphocytes migrate in bloodstream, instead some activated $CD4^+$ T lymphocytes remain in the lymphoid organ with the aim to help B lymphocytes to differentiate in plasmacells. (Fig.9)

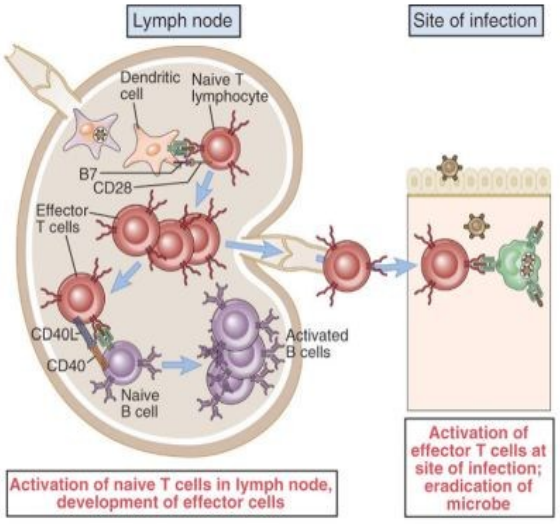

Fig.9: naïve T lymphocytes activation. Abbas A., Lichtman H., Pillai S., Immunolgia cellulare e molecolare. 2010. 6th ed. Elsevier.

The B lymphocytes activation leads to the activation of humoral immunity: when B lymphocytes' membrane antibodies recognize the specific antigen presented to them, proliferate and their differentiation leads to the production of plasmacells, that produce specific antibodies. T helper lymphocytes stimulate, also, the production of antibodies with major affinity against antigen and this process is called *affinity maturation*.

There is another important process named *immunological memory*: naïve lymphocyte activation generates long-lived cells, that can survive for years after infection and are more effective than naïve lymphocytes because they are antigen specific. They are called memory cells. (Fig.10)

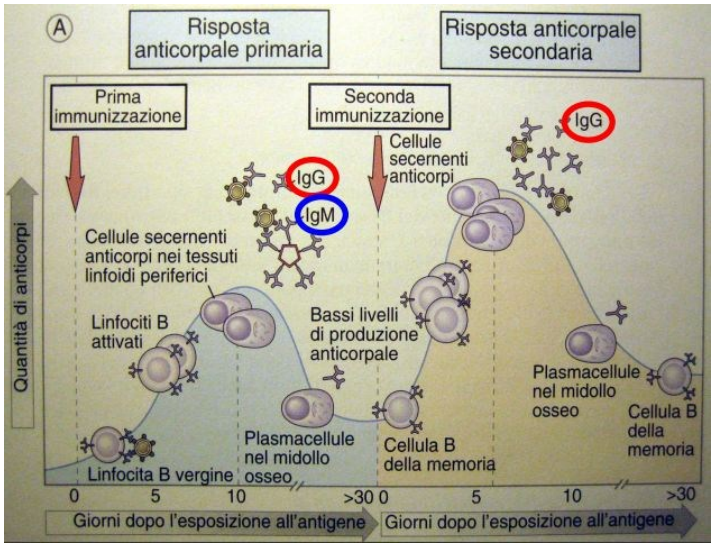

Fig.10: naïve B lymphocytes activation. Abbas A., et al., Immunolgia cellulare e molecolare. 2010. 6th ed. Elsevier.

Additionally, antibodies bind pathogen's antigen, avoiding the binding of the last one to host cells. This allows to prevent infection, and for this reason vaccination is recommended to produce antibodies.

4.4.4 Antibodies

Antibodies are a large, Y-shaped proteins used by the immune system to identify and neutralize proteins and microorganisms such as bacteria and viruses. They have two heavy chains (H) and two light chains (L), which contain several globular subunits called Ig domains. Each H and L chain consists of N-terminal variable regions (V_L for that of the light chain, V_H for that of the heavy chain) and C-terminal constant region (C_L and C_H). The first one contains the "hypervariable" or "complementary determining region" (CDR), that is responsible for the antigen binding. Instead, the second one doesn't interact with antigens, but interacts with effector molecules and cells of the immune system. Furthermore, antibodies are flexible proteins thanks to disulfide bonds between cysteine residues of heavy and light chains. [35]

In mammals, antibodies occur in a few "isotypes": IgA, IgD, IgE, IgG, and IgM, (Fig.11) and the production of different types of antibodies gives plasticity to immune responses allowing it to play different functions. [35]

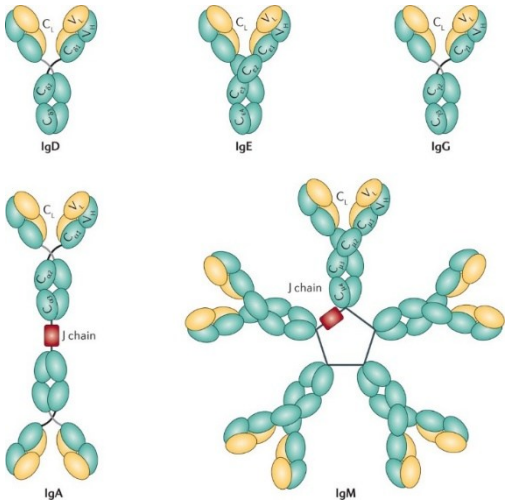

Fig.11: Antibodies isotypes. Duarte J.H. Functional switching. *Nature Immunology*. Volume 17, pageS12 (2016).

Immunoglobins play distinct roles:

- IgA is involved in the immune responses in mucous membranes.

- IgD makes up the proteins in the plasma membranes of naïve B lymphocytes, where it is usually co-expressed with another cell surface antibody called IgM.

- IgE is thought to be an important part of the immune response against infection by certain parasitic worms, protozoan parasites and may have evolved as a defense to protect against venoms. IgE also plays a pivotal role in responses to allergens…

- IgG represents about 75% of serum antibodies in humans. IgG acts through different mechanisms: opsonization, complement activation, antibody-dependent cellular cytotoxicity, neonatal immunity, B cells' inhibitory feedback.

- IgM is the largest antibody, and it is the first to appear in response to initial exposure to an antigen with its multiple-binding capacity they offer a first emergency response.

4.4.5 Neutralizing antibodies

Neutralizing antibodies are an important specific defense against viral invaders and they bind to a virus in a manner that blocks infection. A neutralizing antibody might block interactions with the receptor or might bind to a viral capsid in a manner that inhibits the uncoating of the genome. [37] This prevents the absorption and invasion of viruses in the host cells. [36] Neutralizing antibodies are generally detectable, using *in vitro* biological assays, between 7 to 15 days after the disease onset; then an increase occurs during days 14-22 before reaching a plateau and decline over period of six weeks.

In the case of infection by SARS-CoV-2, the processes that lead to the development of memory B cell clones and serum neutralizing activity with higher potency and breadth against different viral variants is as follows (Fig.12): exposure to viral antigen activates B cells and they differentiate into short-lived antibody-secreting plasma cells or are recruited into germinal centers, where they interact with antigen-presenting follicular dendritic cells and T follicular helper cells to undergo isotype-switch recombination and gain antigen affinity by acquiring somatic hypermutation, to increase SARS-CoV-2 neutralizing activity. Affinity-matured germinal center B cells can remain in the germinal center to acquire higher levels of somatic mutations or differentiate into memory B cells or long-lived antibody-secreting plasma cells. Then, re-exposure to SARS-CoV-2 can induce differentiation of evolved SARS-CoV-2-reactive memory B cells into antibody-secreting plasma cells, can elicit further B cell evolution and acquisition of mutations, and can induce expansion of novel memory B cell clones. [38]

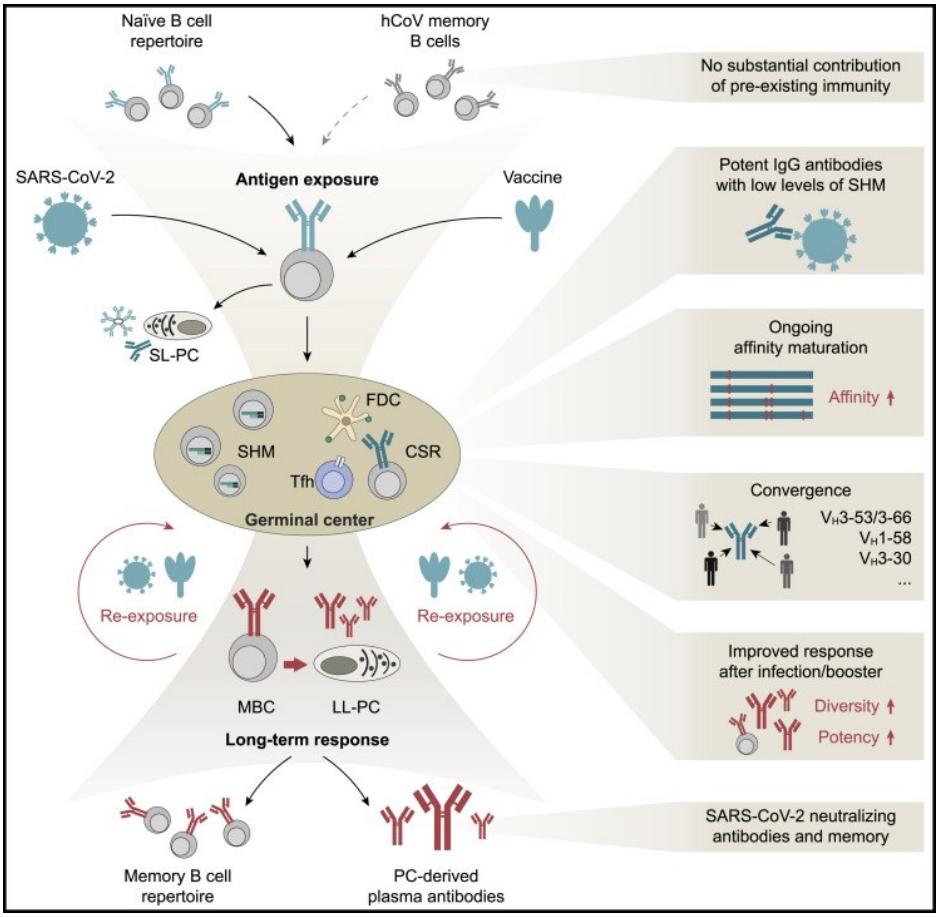

Fig.12: Development of SARS-CoV-2 neutralizing antibodies. Gruell H., et al., Antibody-mediated neutralization of SARS-CoV-2. Immunity CellPress. 55, 2022.

SARS-CoV-2 neutralizing antibodies target the viral spike glycoprotein, in particular RBD of the S1 subunit, the NTD, stem helix (SH) and fusion peptide (FP) regions in the S2 subunit. (Fig.13-14) [39]

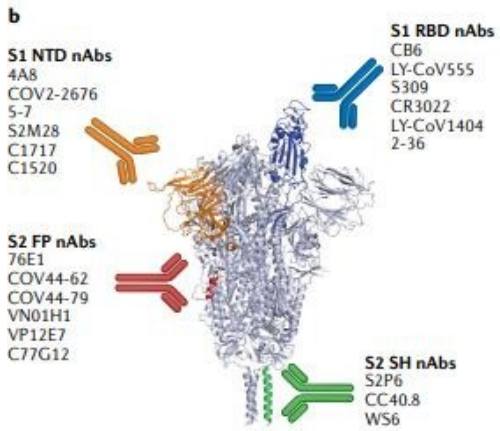

Fig.13: Neutralizing antibodies directed against the SARS-CoV-2 spike. Chen Y., et al., Broadly neutralizing antibodies to SARS-CoV-2 and other human coronaviruses. Nature Reviews Immunology. Vol. 23. 189-199. 2023

The precise mechanism of NTD-targeted neutralizing activity has not yet been elucidated, but it has been postulated to include interference with conformational changes required for membrane fusion after ACE2 attachment. [38]

4A8 is one of the earliest identified neutralizing antibodies that binds NTD region. [39]. Compared to the other regions of the spike protein, the NTD has a relatively high glycan density that limits accessibility. However, a site more vulnerable to neutralization has been identified and called NTD supersite. Many naturally circulating viral variants carry mutations within this site, which could damp the neutralization activities of these NTD supersite-recognition neutralizing antibodies. For example, a deletion of NTD amino acid residues 242-244 made 4A8 lose their neutralizing activity for SARS-CoV-2 Beta variant. [38,39]

The neutralizing epitopes in the S2 subunit are more conserved than those in the S1 one, therefore this type of antibodies would have a greater probability of being broad-spectrum to SARS-CoV-2. One of them is S2P6, that recognizes an epitope located in the S2 SH region that spans 14 residues. It can inhibit viral infection by preventing S protein-mediated fusion with cellular membrane. However, S2P6 does not show as great neutralizing potency as some RBD-targeting antibodies. [39]

Also, S2 FP is highly conserved, suggesting that broad-spectrum antibodies could be found by targeting these epitopes. These antibodies don't compete with S2P6 for binding to the SARS-CoV-2 S protein, suggesting the possibility of combining S2SH and S2 FP recognition in a bispecific antibody.

Most of the SARS-CoV-2 neutralizing antibodies discovered target the RBD region, and based on a structural analyses, functional characteristics, and

antigenic mapping, has been proposed a classification system where this type of antibodies is grouped in four classes [38,39,40]:

- Class 1: overlaps with the receptor-binding motif (RBM) in the RBD preventing ACE2 from binding to the S protein. They are encoded by VH3-53 and VH3-66 germ lines and recognize only the “up” RBD conformation.

- Class 2: bind to the RBM where the RBD interacts with ACE2, but can bind both “up” and “down” conformations of the S protein. Further, the orientation of this type of antibodies can block adjacent RBDs in the pre-fusion trimer from adopting the “up” ACE2-binding-competent conformations.

- Class 3: bind across adjacent RBDs in the trimeric SARS-CoV-2 S and sterically block the RBD up conformation of adjacent RBDs. Some of them only block the up conformation of the RBD, while others sterically block ACE2 binding, and some have both neutralization mechanisms.

- Class 4: bind to an RBD surface comprising a helix-strand-helix motif (residues 366-389) that is buried in the trimeric S, even when the RBD is in the up conformation.

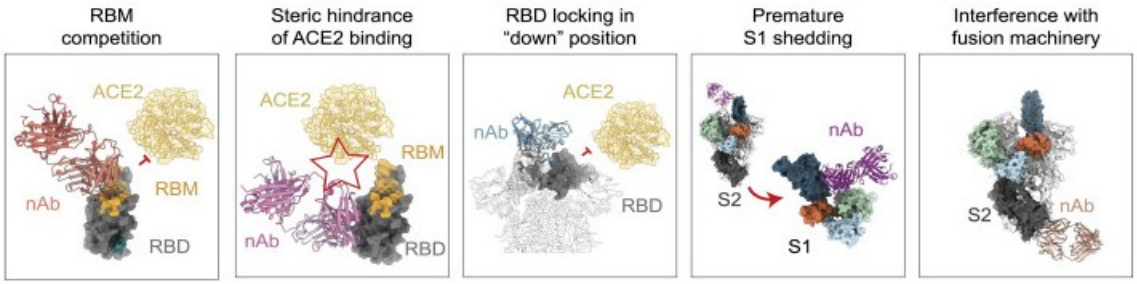

Fig.14: Example for mechanisms of SARS-CoV-2 neutralization. Gruell H., et al., Antibody-mediated neutralization of SARS-CoV-2. Immunity CellPress. 55, 2022.

4.5 Human immune system against SARS-CoV-2

When SARS-CoV-2 enters the upper respiratory tract, it binds the cellular receptor to start its replicative cycle. In this phase the host innate immune system can detect it and activate the inflammatory signaling pathways, cytokines production and cell death. [43] In addition, it has 2 important tasks: blocking viral replication and trigger the adaptive immune responses. [44] This occurs, for example, thanks to TLR signaling. TLRs transduce signals using two molecules: TRIF and MyD88. The former activates IRF3 and induce IFNI and IFN III expression. The latter activates the nuclear factor (NF)-kB, mitogen-activated protein kinases (MAPKs) and IFN regulatory factors. These migrate into the cellular nucleus and activate the transcription of some pro-inflammatory cytokines (such as TNF, IL-6, IL-1 and IFN) and NLRP3, an inflammasome sensor that lead to the activation of caspase-1, the production and release of IL-1β and IL-18, and the cleavage of gasdermin D. The latter event results in the formation of pores on membrane cells and their rupture. Pro-inflammatory cytokines help to clear the infection and keep the cellular homeostasis. [43]

SARS-CoV-2 can count on different proteins counteract the innate immunity to enhance replication in the first phase of the infection.

Congenital or acquired defects in innate immunity (for example in older people) contribute to viral replication enhancement in this phase, that can lead to a greater expansion of the infection in the lower respiratory tract. As a consequence, the activation of adaptive immunity is also considerably increased. [44]

This leads to the loss of cytokine release control, determining the cytokine storm. [43]

Events downstream of IFN activation are so crucial. Class-1 IFNs deficiency can be determined by two main causes: presence of genetic mutations on gene that coding for class-1 IFNs and surface receptors IFNARs, or production of antibodies anti-class-1 IFNs. In particular, in normal condition, B cells that produce this type of antibodies are selectively deleted, but this doesn't occur when there are faulty B-cells-tolerance-checkpoints. [47]

So, the IFN-1 deficiencies just described lead to an uncontrolled SARS-CoV-2 replication, which causes an overactivation of the immune system with the consequent appearance of an hyperinflammatory-state. [46,47]

The cytokine storm, defined as an hyperinflammatory condition that develops when there is a deregulated immune response. In a healthy organism the balanced production of cytokine pro- and anti-inflammatory occurs, so inflammatory reactions can be limited and attenuated when pathogens are erased. [46] But when this balance is disrupted, the production of pro-inflammatory cytokine is no longer under control, and the inflammatory reaction reaches a systemic scale. Patients are characterized by a high level of cytokines like IL-1β, IL-2, IL-6, IL-10, IFN-γ, TNF-α, IP-10, GM-CSF (granulocyte macrophage-colony stimulating factor) and MCP-1 (monocyte chemoattractant protein-1). From the clinical point of view, patients exhibit firstly flu-like symptoms, and then multi-organ failure and coagulopathy, leading to severe/fatal COVID-19. [45,46]

As for the humoral response, activated B cells can immediately differentiate in short-lived plasma cells to produce quickly antibodies to struggle the virus, and produce memory B cells. Subsequently, B cells can take affinity-increasing immunoglobin gene mutations and can differentiate in durable memory B cells and long-lived plasma cells that secrete high affinity neutralizing antibodies. [48]

In addition, antibodies have a crucial role thanks to different mechanisms that involve their Fab and Fc portions. [49] The first one is important for neutralizing activities, to avoid the binding between viral S protein and ACE-2 receptor. [50] The second one is involved in the complement activation, cytotoxicity and phagocytosis. [49].

Antibodies responses are affected by patients' characteristics, like sex (males have got more IgG, IgA and IgM in serum), age (young people have less antibodies titer in serum and high levels of IgA in mucose). [49]

Mucosal responses are dominated by IgA and IgG antibodies and systemic responses are characterized by initial production of IgM and IgA antibodies, subsequently of IgG antibodies. [49] Anti-SARS-CoV-2 antibodies decrease gradually in patient's serum, but neutralizing antibodies and memory B cells remain longer. [49] Several studies had observed an important quantitative difference in humoral response: patients with severe COVID-19 had more antibodies than that with mild disease, and they persisted for long time. [49, 50, 51] Further, asymptomatic subjects had durable viral shedding compared to symptomatic ones, but had lower levels of antibodies. [51] This implies that cell-mediated and humoral responses are stronger in the second ones, and such high levels of antibodies indicate an uncontrolled adaptive response that may lead to an additional cytokine release and the aggravation of the disease. [52]

4.6 Vaccines

As mentioned above SARS-CoV-2 can elicit different form of COVID-19 and some subjects, such elder people or those with comorbidities, can exhibit a severe form of this pathology. This happens, for example, due to the poor effectiveness of innate immune responses and allows the virus to replicate uncontrolled and reach the low respiratory tract. For this reason, the first objective of scientific communities was to immunize these subjects to accelerate viral clearance and avoid the severe form. Different vaccines have been introduced since December 2020, with the aim to prevent infection by wild-type variant of SARS-CoV-2 and protect against severe forms of COVID-19. [59]

Several companies were engaged in the development of a vaccine, such as Pfizer and BioNTech, Sanofi-GSK, Curevac, AstraZeneca and the University of Oxford, Johnson & Johnson and Moderna. [60] The choice on the type of vaccine was influenced by the fact that they had to be produced as soon as possible, for this reason classical strategies, such as virus attenuated vaccines, used throughout vaccine history were excluded because of the long testing time due to the long cell culturing processes to achieve attenuated strains [60].

The main vaccines used during these years are based on the presence of mRNA encoding the viral S protein, or are vaccines based on a viral vector. [61] The first technology, is based on the fact that mRNA can be complexed in lipid nanoparticles that protect it and allow entry in the cell. When the latter event occurs, mRNA is released and is translated by the ribosome as the Spike proteins. These are exposed on the surface of the cell and the immune system recognizes them as non-self-proteins, synthesizing specific antibodies and activating T cells, and preparing the subject to a possible future exposure to

the virus. This type of vaccines (e.g. BioNTech/Pfizer Comirnaty BNT162b2 and Moderna (NIAID) Spikevax mRNA-1273) are administered in at least 2 doses and any booster doses.

Instead, vaccines like Oxford Astrazeneca ChAdOx1 nCov-19 (AZD1222), Sputnik V and Johnson & Johnson Janssen Ad26.CoV2.S, are based on a viral vector. This technology uses a modified (defective) version of a chimpanzee's adenovirus, that can't replicate, but is transgenic for the Spike gene of SARS-CoV-2. In particular, the first two vaccines need 2 doses, the last was formulated in only one dose.

The new emerging VOCs were associated to a decreased efficacy of vaccines in blocking infection, even if the effectiveness in avoiding the onset of serious illness was conserved. [65] At the beginning of vaccination programs, vaccines' effectiveness was 83% against infection, 92% against hospitalization and 91% against mortality, but it was observed that the first rapidly decreased as of new viral variants emerged. [66] For this reason, booster doses were recommended.

By contrast, cell-mediated immunity is not affected by this phenomenon: the relatively low number of mutations identified, mostly concentrated in the S gene (compared to the relatively stable rest of the viral genome) can't act so negatively with this type of immunity, because T cell receptors recognize a score of conserved SARS-CoV-2 epitopes. For this reason, cell-mediate immunity is important, because allows to preserve this part of the protection conferred by vaccines. [67]

Another feature observed was the mRNA vaccines' major effectiveness compared to adenoviral vector vaccines. In addition, because of a peculiar serious collateral effect of the latter, adenoviral vaccines were rapidly replaced. In fact, boosted doses were made by only mRNA vaccines. [66]

As the virus continues to evolve in different variants, which reduce vaccine efficacy by immune escape, the FDA and the European Medicine Agency (EMA) have authorized the use of updated versions of Pfizer-BioNTech and Moderna COVID-19 vaccines for the Omicron's sub-variants BA.4 and BA.5 in 2022-2023, and for the recent Omicron's sub-variant XBB.1.5 in 2023-2024.

5. SARS-CoV-2 IMMUNE ESCAPE STRATEGIES AND VARIANTS

Coronaviruses are masters in counteracting the effects of intracellular "antiviral state", mainly based on the interferon system. They can do this through different strategies, for example they can mask its RNA by capping its 5' termini thank the work of nsp13, nsp12, nsp14, nsp16 and nsp10. Furthermore -ssRNA and dsRNa that generates during viral replication are placed within replication organelles (ROs) that are associated with ER to prevent their host detection. When viral replication intensified and new viral RNA accumulates outside ROs, N protein binds to it to hide it. [54] Another immune escape strategy consists in blocking host signaling pathways such as that of interferons, or blocking nuclear transport avoiding the export of the host mRNA and its translation [54]. In addition, SARS-CoV-2 has shown an extraordinary capability of evading humoral immune response. During these years of pandemic, the whole world population was immunized several times by natural infections or vaccines. Despite this, SARS-CoV-2 still manages to cause millions of new infections worldwide. This happens because of its

capacity of escaping the neutralizing activity of humoral immune response. [57]

In fact, despite the proofreading activity by its exoribonuclease enzyme, which has the task of attenuating mutagenesis introduced by the replication process SARS-CoV-2 has already evolved in a score of variants. The mutation rate of the SARS-CoV-2 is estimated at $1x10^{6}$-$2x10^{6}$ substitution per base in each transmission event (the frequency of new mutations per generation in an organism or a population) [62]. The huge number of replication cycles generated during the infections of billions of people have indeed created the opportunity of generating and selecting a variety of biologically relevant mutations. In addition, due to the discontinuous polymerization of viral sub genomic RNAs [55, 56], there is a high rate of recombination that introduces other chances of variability [55]. Indeed, the success of the different viral variants was driven by natural selection. For example, the first mutation occurred on wild-type form of SARS-CoV-2 was D614G, in Spike protein sequence. It conferred a better transmissibility to the virus and this allowed it to prevail on the wild-type. Since October 2020, different viral variants have appeared, characterized by different non-synonymous mutations mostly on spike protein. [55]

The World Health Organization has classified these variants in Variants Of Interest (VOIs) and Variants Of Concern (VOCs). The first include variants with limited global distribution, but with mutations that may hamper diagnostic tests. They are characterized by high transmissibility and the increase in the amount of cases. [58] The latter include variants with high transmissibility, with a consequent impact on the number of cases (challenging the capacity of healthcare systems to provide assistance to COVID-19 patients), high virulence and decreased efficacy of diagnostic tests, vaccines and therapies.

Each new VOC sowed a greater transmissibility than the previous one, due to the capability of escaping herd immunity, and this allowed the rapid turnover of variants. [58]

From the beginning of the pandemic the Centre of Disease/Control and Prevention has identified five VOCs:

- Alpha (B.1.1.7), formerly known as English variant, identified in September 2020. It bears 22 mutations, 9 of which on S gene.

- Beta (B.1.351), isolated for the first time in South Africa in December 2020, and it bears 16 mutations, 8 on S gene.

- Gamma (P.1), identified for the first time in Brazil in December 2020. It bears 23 mutations, 12 on S gene.

- Delta (B.1.617.2), identified in India in October 2020. It bears 29 mutations, 8 on S gene.

- Omicron (B.1.1.529). also originated in South Africa in November 2021, it bears 39 mutations on the S protein, 27 of which are new.

Presently, different omicron sub-lineages have been identified: BA.1, BA.2, BA.4, BA.5, and the recombinant sublineages.

Most of this variability occurs in gene S gene, that codes for viral spike protein, whereas the genes encoding for Non-Structural Proteins (NSPs) are less subject to change. [62] This happens probably because enzymes need a conservative evolution, especially those involved in viral replication. For this reason, this portion of the viral genome, being highly conserved, should be considered a potential target for diagnostic tests, antiviral therapies and vaccines. [62]

Concerning the S gene, mutations occur in both RBD and NTD regions. [63] Most of the mutations that occur in first are located at the RBD-ACE2 interface. Examples are: D614G, N439K, Y453F, N501Y, E484K, E484Q and

E484P. The selective pressure which led to their appearance is mostly linked to the evasion of neutralizing immunity, however, some of these mutations led to an increase of H bonds that rise the binding affinity between RBD and ACE-2 receptor. For example: [64]

-N501Y: present in the Alpha (B.1.1.7), Beta (B.1.351) and Gamma (P.1) variants. It is involved in the increase of the binding affinity with cellular receptor and has immune escape properties;

-E484K: present in the Beta and Gamma variants, significantly decrease neutralizing efficacy;

-L452R and T478L: present in delta variant. In particular, the first one increases S protein's stability, infectivity, replication and immune evasion. The second one increases the entry in the host cells, but sensitizes the virus to neutralization;

-L452Q, F490S and R346K: present in Lambda (C.37) and Mu (B.1.621) variants. They lead to the increase of neutralization escape;

Omicron subvariants bear more than 30 mutations in the S gene, 15 of which in RBD region. For example, G339D, S371L, S375F, N440K, G446S, E484A, Q493K and G496S, and all of these have hampered neutralization. In particular, BA.4 and BA.5 Omicron sub-lineages have 3 special mutation that distinguish them from other Omicron sub-lineages: L452R, F486V and R493Q. Their importance is due to the fact that they may have a major impact on immune escape, because of significant antigenic property changing.

Mutation in NTD domain also occur frequently, such as insertions, acquisition of additional glycosylation motifs and deletions. Again, these have the ability to change the NTD's antigenicity, leading to an immune escape. [63] Several studies claim that this property is due to the overlap of the N3 loop and N5 loop with the 4A8 antibody epitope. [64]

In summary, mutations in S gene are essential to the virus because they allowed it to increase the binding affinity between spike protein and ACE-2 receptor, and their continuous variability allows the evasion of neutralizing immunity. This is of vital to the virus in order to increase its reproductive success (fitness) in a population that over time enhances and refines its herd immunity. New mutations will appear, with generation of new variants. Monitoring these mutations is important to understand how they impact act on viral phenotype: infectivity, transmissibility, virulence and antigenicity pattern. [63]

6. MATERIALS AND METHODS

6.1 Variant surveillance

Once clinical samples resulted positive for SARS-CoV-2, they were subjected to genotypic analysis to identify the viral variant. The first step was viral RNA extraction from nasopharyngeal swabs using either a manual procedure (QIAamp^{R} Viral RNA Minikit (250), QIAGEN, Hilden, Germany), or automatic procedures, such as those performed on Abbott m2000sp (Abbott Laboratories, Illinois, U.S.A.), QIAsymphony-QIAGEN (QIAGEN, Hilden, Germany). Viral RNA was subjected to Reverse transcription Polymerase chain reaction (RT-PCR) to obtain several copies of spike protein cDNA. The gene is nearly 4000 base long, to achieve a better efficiency it was divided in three fragments with three pairs of primers. Different primer sequences were used, according to the variants circulating at the time.

Initially, in November 2020, internal surveillance was carried out based on a viral variant named 20A.EU1 that had originated from Spain. Only the first fragment of the spike protein was amplified, with the following primers (Tab.1):

Primers	Sequence (5'-3')
NheSpike1F	5'-GATCCGCTAGCGCCGCCACCATGTTTGTTTTTCTTGTTTTATTGCCACTAG-3'
Spike1nrunheR	5'-ATCTGCTAGCTCGCGACTTCCTAAACAATCTATACAG-3'

Tab.1: Primers sequences used for RT-PCR assay.

The kit SuperScript^{TM}IV One-Step RT-PCR System (Invitrogen, by ThermoFisher Scientific. Vilnius. Lithuania) was used to perform the RT-PCR according to the protocol illustrated in Tab 2:

Components	Volume per reaction
Primer F (10 µM)	2.5 µl
Primer R (10 µM)	2.5 µl
Buffer 2X	25 µl
RNase inhibitor	1 µl
Taq/RT Polymerase	0.5 µl
H_2O	13.5 µl
Viral RNA	5 µl

Thermal profile	Time (min)	Cycles
50°C	10:00	1
98°C	2:00	1
98°C	0:15	35
55°C	0:30	
72°C	1:30	
72°C	5:00	1

Tab.2: RT-PCR assay mix and thermal profile.

The amplified products were subsequently subjected to Sanger sequencing on an automated platform (Applied Biosystems 3130).

From mid-December 2020, additional primers were used to identify the Alpha variant (B.1.1.7) and to obtain the whole Spike gene sequence. The following primers were used, with identical protocols (Tab.3):

Primers	Sequence (5'-3')
NheSpike1F	5'-GATCCGCTAGCGCCGCCACCATGTTTGTTTTTCTTGTTTTATTGCCACTAG-3'
Spike1nrunheR	5'-ATCTGCTAGCTCGCGACTTCCTAAACAATCTATACAG-3'
Spike2F	5'-AATCTCAAACCTTTTGAGAGAGATATTTC-3'
Spike2afeR	5'-CAGCGCTGAAGTGTATTGAGCAAT-3'
Spike3F	5'-ACTGTTAGCGGGTACAATCACTTC-3'
Spike3R	5'-CTATGTGTAATGTAATTTGACTC-3'

Tab.3: Primers sequences used for RT-PCR assay and sequencing, to search Alpha variant.

The sequence of the first Italian positive sample of Alpha variant was submitted to the GISAiD database with the name EPI_ISL_778869.

In order to screen large numbers of clinical samples, a method that allowed direct genotyping by variant specific real time RT-PCR assay was developed. (Tab.4) The assay was based on variant specific primers/probe combinations (the probe that was specific for the 69-70 deletion). The SuperScript^{TM}III Reverse Transcriptase (ThermoFisher Scientific, Waltham, Massachusetts, USA) kit was used for reverse transcription.

Primers	**Sequence (5'-3')**
AS 2	5'-TTTTGTTGTTTTTGTGGTAAAC-3'
S 3	5'-CTTGGTTCCATGCTATCTC-3'
Probe	**Sequence (5'-3')**
VIP	5'-/5Cy5/GTCTAACATTAOAATAAGAGGCTGG/3IAbRQSp/-3'

Components	**Volume per reaction**
Primer AS 2 (10 µM)	1 µl
Primer S 3 (10 µM)	1 µl
2X Master Mix	12.5 µl
$MgSO_4$ (50 µM)	0.4 µl
Superscript III	1 µl
H_2O	4.1 µl
Sample	5 µl

Thermal profile	**Time (min)**	**Cycles**
55°C	20:00	1
94°C	3:00	1
94°C	0:20	45
60°C	0:10	
72°C	0:20	

Tab.4: Primers and probe sequences, reaction mix and thermal profile used for the genotyping RT-PCR assay.

Positive samples were, subsequently subjected to Sanger sequencing of the spike gene.

In addition to the surveillance performed on local positive samples (Ancona and surroundings), a periodical survey on clinical samples from the other provinces of Marche region was also implemented, updating the primers/probes combinations of the genotyping test to detect variants most recently isolated throughout the world.

By these methods, the first Gamma (P.1) (EPI_ISL_1118260) and Beta (B.1.351) (EPI_ISL_1118258) variants were detected.

The following tables show the different primers/probes combinations (and corresponding protocols) sequentially used as different variants spread in the world.

For the Beta and Gamma variants (Tab.5):

Primers	**Sequence (5'-3')**
501 s	5'-TAGCACACCTTGTAATGGT-3'
501 as	5'-GGACAGCATCAGTAGTGT-3'

Probes	**Sequence (5'-3')**
V501N	5'-/56-JOEN/TATGGTGTT/ZEN/GGTTACC/3IABkFQ/ -3'
V501F	5'-/56-FAM-AATGGTGTT/ZEN/GGTTACC/3IABkFQ/-3'

Components	Volume per reaction
Buffer 2X	12.5 µl
Primer VisS3	1.25 µl
Primer VIS AS2	1.25 µl
Probe VIP	0.25 µl
Primer 501S	1.25 µl
Primer 501AS	1.25 µl
Probe V501F (Fam)	0.25 µl
Prove V501N (Joe)	0.5 µl
Taq SuperScript III	0.75 µl
H_2O	0.35 µl
$MgSO_4$	0.4 µl
Sample	5 µl

Thermal profile	Time (min)	Cycles
55°C	10:00	1
94°C	3:00	1
94°C	0:15	45
62°C	0:40	

Tab.5: Reaction mix and primers/probes sequences used in the Real-time PCR for Beta and Gamma variants.

Since June 2021, to detect the Delta variant (B.1.617.2), the Real-time PCR was modified: (Tab.6)

Probes	Sequence (5'-3')
Probe 1	5'-6FAM-CTGAAATCTATCAGGCCGGTAG-QSY-3'

Primers	Sequence (5'-3')
452s	5'-TTGGTGGTAATTATAATTACCG-3'
478as	5'-CTTCAACACCATTACAAGGTT-3'

Tab.6: Primers/probes sequences used in the Real-time PCR for Delta variant.

Later, with the appearance of Omicron BA.1 variant, the real-time PCR was modified by replacing primer AS2 with primer VO AS (Tab.7a). Whereas, with the appearance of Omicron BA.2 variant, another two primer and one probe were added to the real-time PCR: primers “omicron 2F” and “spike1intR” and probe “SAp”. (Tab.7b)

a)

Primers	Sequence (5’-3’)
VO AS	5’-TTTTGTTGTTTTTGTGGTCCA-3’

b)

Probes	Sequence (5’-3’)
SAp	5’-GATTCTTCTTCAGGTTGGA-3’

Primers	Sequence (5’-3’)
Omicron2F	5’-CACACGCCTATTAATTTAGG-3’
Spike1intR	5’-ACCCACATAATAAGCTGCAGCAC-3’

Tab.7: **a)** New primer used for Delta/BA.1 Real-Time PCR.

b) New primers and probe used for BA.1/BA.2 real-time PCR

Since January 2022 all surveys were carried out by selecting Next Generation Sequencing (NGS), performed on selected samples.

6.2 Sequencing

Sanger sequencing. Clinical samples, from all Marche region’s provinces, with high viral load were selected to perform the periodically viral variant survey with the purpose of identify the viral variant circulating in that territory: viral RNA was extracted from clinical samples and the spike protein gene was amplified in three contiguous fragments by a RT-PCR. The amplified products were later, subjected to cycle sequencing reactions by the use of the Big Dye™

Terminator v1.1 Cycle Sequencing kit (Applied biosystems by Thermo Fisher Scientific. Vilnius. Lithuania), according to the following protocol: (Tab.8)

Components	Volume per reaction
Bid Dye^{R}Terminator v1.1, v3.1 5X sequencing Buffer	2 µl
Big Dye	1 µl
Primer	1 µl
H_2O	5.5 µl
Sample	0.5 µl

Thermal profile	Time (min)	Cycles
96°C	0:10	25
50°C	0:05	
60°C	2:00	

Tab.8: reaction sequence reaction mix component and thermal profile.

Where, primers that have been used were the same used for the previews RT-PCR. The reactions were subsequently loaded onto a 96-well plate as follow: in each well, was loaded 8 µl of H_2O, 2 µl of sample and 55 µl of resin (Big Dye^{R}xTerminator^{TM} Purification kit. Applied biosystems by life technologies. Bedford, USA) and the whole was subjected to vortex and centrifugation to allow the resin to purified the well's contents. The 3130 Genetic Analyzer (Applied BioSystems) was used for automated capillary electrophoresis and reading of the sequence (Fig. 15):

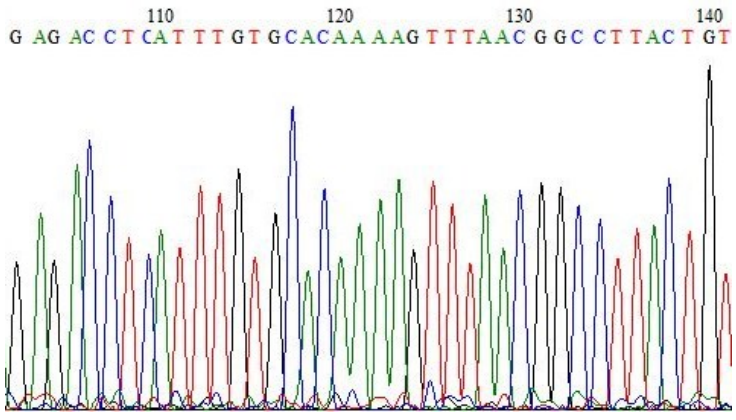

Fig.15: example of an electropherogram from Sanger sequencing.

Next Generation Sequencing (NGS): 3 different NGS platforms were compared for an initial evaluation, the Ion-Torrent (Thermo Fisher), the MinIon (Oxford nanopore) and the Illumina technologies, which was finally chosen as the default method for its advantages in the automation of the entire process. In this process, viral RNA was extracted from clinical samples using the instrument Elite InGenius (ELITechGroup MDx, Italia). RNA extracts were subsequently processed to create the libraries: the viral genome was amplified with an array of primer couples to obtain several overlapping fragments of 300-500 bp in length. This was performed using the EasySeq Nimagen SARS-CoV-2 WGS Library Prep kit and the procedure was automated on the liquid handler instrument Ep Motion 5075. Bridge amplification and sequencing were performed on the MiSeq platform (Illumina Inc., California).

Sequences obtained were read using the IRIDA-ARIES (Integrated Rapid Infectious Disease Analysis - Advanced Research Infrastructure for Experimentation in GenomicS) for I-Co-Gen project, that represents an Italian platform designed for the collection and analysis of the data obtained from the surveillance.

6.3 Vero E6 cell cultures

To perform *in vitro* studies on live SARS-CoV-2, the VERO E6 cell line was used. These cells derive from the kidney of an African green monkey named *Cercopithecus aethiops*. They are permissive for viral infection because of the presence of ACE-2 receptor and the TMPRSS2 enzyme. Further, they have a defective interferon production allowing abundant viral replication. [68]

Cells were maintained in *Dulbecco's Modified Eagle Medium* (DMEM, Euroclone Spa, Italy) completed with 10% fetal bovine serum (Euroclone Spa,

Italy), 5 ml of an antibiotic/antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B, Euroclone Spa, Milano, Italy), 5 ml of non-essential amino acids (Gibco, Fisher Scientific, Italy) and 12.5 ml of a buffer solution named HEPES (Signa Aldrich). VERO E6 were cultured in 75 cm^2 flasks (CELLSTAR, Greiner Bio-One, Frickenhausen, Germany) at 37°C, 5% of CO_2 and 95% of humidity. VERO E6 grow in adherence, forming a monolayer until confluency, then they undergo contact inhibition and being to die.

To select the best cells, it is important to avoid cell confluency and contact inhibition, which requires daily monitoring and frequent sub-culturing. This was performed twice a week, splitting them with a dilution of 1:5 or 1:10 (based on the amount of cells into the flask) after trypsin treatment (1X Trypsin-EDTA, Gibco, Thermo Fisher Scientific), harvesting and washing with 1.5 ml of Dulbecco's Phosphate Buffered Saline (PBS, Euroclone, Milano, Italy).

6.4 Viral isolation and stock preparation

Several viral variants have been isolated on VERO E6 cell cultures. SARS-CoV-2 replication in these cells is very evident and appears as the formation of syncytia, cell death and detachment from the flask bottom.

Each new variant identified in the laboratory was isolated. This was performed in two steps: primary and secondary isolation:

1) an aliquot of clinical sample (500 µl) was combined with $2*10^6$ VERO E6 cell suspension into 2 ml of complete growth medium, and left to

incubate for an hour at 37°C and 5% CO_2. Subsequently, 4 ml of growth medium were added to the suspension, which was transferred into a 25 cm^2 surface's tissue culture flask, and incubated as mentioned above. As the cytopathic effect appeared in the next days (at least 80%), the primary isolate underwent another passage and one aliquot was stored at -80°C. 2).

2) 2 ml of primary isolate were included into the cell monolayer in 75 cm^2 flasks and incubated for 1 h at 37°C and 5% CO_2 to allow the viral adhesion. Subsequently, 8 ml of complete growth medium were added and the cell culture flask was incubated at the same condition. When 80% of cytopathic effect appeared, the entire solution was centrifugated to remove cellular debris, and the supernatant (containing the virus) was filtered using 0.2 µm filter, aliquoted and stored at -80°C.

6.5 Titration of the viral stock

Before performing neutralization tests, virus stocks had to be titrated in terms of TCID50/ml (Tissue Culture Infectious Dose 50: the viral dose that is able to infect the 50% of cells in culture).

The titration was performed in a 96-well plate, and each new viral stock was tested along with an another one of previously known concentration as a control.

$2.6*10^4$ cells were seeded in 100 µl of growth medium in each well, and the day after 8 ten-fold serial dilution of each viral stock were prepared in 1.5 ml tubes. The cells were incubated with 50 µl of each stock dilution at 37°C and 5% CO_2 for 2 hours. At the end of the incubation, the inoculum was removed, cells were washed and new growth medium was added. Subsequently, plates were incubated for 72 hours, at the end of which the cytopathic effect (CPE) was evaluated. Finally, the Reed-Muench method [81] was used to assess the TCID50/ml of each viral stock.

6.6 Microneutralization assay

This assay determines the neutralizing antibodies' titer in human serum (IC50) as the highest serum dilution capable of inhibiting the infection of the 50% of the challenged cell cultures *in vitro*.

Vero E6 cells were seeded in 96-well plates, at $2.6*10^4$ cells each well, 24 hours before the infection. [69] The day after, both patient sera (decomplemented at 56°C for 30 minutes) and viral stock dilutions were prepared. Each viral stock (e.g. B.1, Alpha, Beta, Gamma, Delta, Iota, Omicron BA.1, BA.2, BA.4, BA.5) was diluted to reach a standard 100 TCID50 in 50 µl. Different fourfold dilutions of human serum (1:10 to 1:640) were tested in three replicates in U-shaped-bottom-plates. Plates were incubated at 37°C for 1 hour, to allow the binding between antibodies (if present) and virus. Subsequently the mixture was transferred on Vero E6 cells, after medium removal.

After 72 hours, supernatants were discarded and cells were fixed using a solution containing 20% acetic acid and 80% methanol. And colored with a solution containing 70% of water, 20% methanol and 10% methylene blue 0.1M. CPE was evaluated on the colored palate (Fig.16)

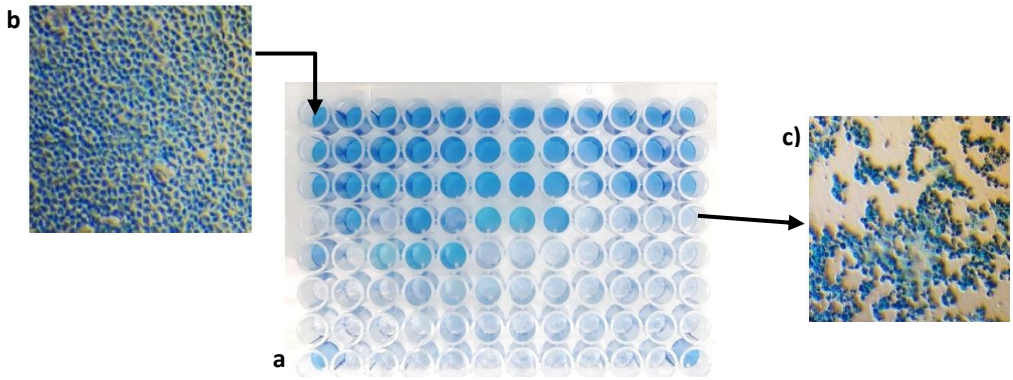

Fig.16: a) Final result of microneutralization assay. b) Vero E6 without CPE. c) Vero E6 with CPE.

Neutralizing titers were calculated, by interpolating the frequency of replicate wells where a CPE was observed in an exponential curve, to establish the virtual dilution inhibiting infection in 50% of wells.

6.7 Study subjects and sera samples

Volunteer healthcare workers from the University Hospital "AOU delle Marche" were enrolled in this study and were grouped according to number and type of immunological stimulations of SARS-CoV-2 spike they had received.

Blood samples were drawn according to the following schedule: 20 days, 4 and 6 months and 1 year after the last immunological stimulus.

Tab. 9 shows how patients enrolled in this study were divided:

	Name of group	B.1 wave	Alpha wave	Alpha wave	Alpha wave	Delta wave	BA.1/2 wave	BA.4/5 wave	BA.4/5 wave
	No. of stimulations	1 NI	1 NI or 1 VD	2VD or 1VD + 1NI	2VD + 1NI	3VD	3VD + 1NI	4VD or 3VD + 1NI	4VD + 1NI
	Subjects	33	35 = 20VD + 15NI	68 = 55(2VD) + 13(1VD+1NI)	26	26	36	40 = 6(4VD) + 34(3VD+1NI)	8
Sex no. (%)	Males	90,90%	51,40%	27,94%	26,90%	23,10%	33,33%	22,50%	50%
	Females	9,10%	48,60%	72,06%	73,10%	76,9	66,66%	77,50%	50%
Age	Mean ± SD	47,1	49,1	47,1	42,3	47,1	46,5	45,9	59,4
	Median (range)	52	49	50	42	51	47	48	62
Sampling days post the last immunological stimulation	Mean ± SD	117,5	34,8	228,6	158,1	-	23,03	15,3	121,9
	Median (range)	110	34	142	81,5	-	21	20,5	113,5
Anti-spike IgG II (BAU/ml)	Mean ± SD	352,6	582,3	2537,9	3425,8	6095,1	6979,4	6482,7	6941,1
	Median (range)	270,9	221,5	2069,2	2303	3863,3	4847,2	4532,4	3773,9
Anti-nucleocapsid Ig tot	Median (range)	Reactive	Reactive and non reactive	Reactive and non reactive	Reactive and non reactive	Non reactive	Reactive and non reactive	Reactive and non reactive	Reactive and non reactive

Tab.9: Patients and any relevant COVID-19 history pertaining to the groups, where: NI= natural infection and VD= vaccine doses

6.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0.1. The non-parametric test Wilcoxon matched pair signed rank and the Mann-Whitney test were applied to data analysis. Statistical significance was placed at $p < 0.05$.

6.9 Development of a plasmid vector carrying viral spike protein

Amplification of the Spike protein gene

Because of the size of this gene, it was amplified as 3 contiguous fragments using the following primers: (Tab.9)

Primers	Sequence (5'-3')
Spike1F tris	5'-CCATGTTTGTTTTCTTGTTTTATTG-3'
Spike1R tris	5'-CATCAATATTCTTAAACACAAATTC-3'
Spike2F tris	5'-AAGCACACGCCTATTAATTTAGTG-3'
Spike2R tris	5'-GGTAATATTTGTGAAAAATTAAAACC-3'
Spike3F tris	5'-GTCATTTATTGAAGATCTACTTTTCA-3'
Spike3R tris	5'-TTATGTGTAATGTAATTTGACTCCTT-3'

Tab.9: Primers sequences used to amplify spike protein.

The protocol was performed using the kit SuperScript^{TM}IV One-Step RT-PCR System (Invitrogen, by ThermoFisher Scientific. Vilnius. Lithuania): (Tab.10)

Components	Volume per reaction
Primer F (10 µM)	2.5 µl
Primer R (10 µM)	2.5 µl
Buffer 25x	25 µl
RNase inhibitor	1 µl
RT/Taq	0.5 µl
H_2O	13.5 µl
Sample	5 µl

Thermal profile	Time (min)	Cycles
50°C	10:00	1
98°C	2:00	1
98°C	0:15	45
58°C	0:30	
72°C	1:30	
72°C	5:00	1

Tab.10: Reaction mix and thermal profile used to amplify spike protein.

Once obtained the amplified products, each of them was further amplified with a specific enzyme called Pwo polymerase, with the aim to create blunt ends. (Tab.11)

Components	Volume per reaction
Primer F (10 µM)	2.5 µl
Primer R (10 µM)	2.5 µl
dNTP (10 µM)	1 µl
Buffer 10X	5 µl
Pwo	0.5 µl
H_2O	37.5 µl
Sample	1 µl

Thermal profile	Time (min)	Cycles
94°C	3:00	1
94°C	0:30	25
58°C	0:30	
72°C	1:00	
72°C	3:00	1

Tab.11: Reaction mix and thermal profile used for create spike protein with blunt ends.

Subsequently, electrophoresis gel and sequencing were performed to check presence and quality of the amplified products before cloning.

Cloning strategy

Plasmid pF4K CMV Flexi^{R}Vector plasmid (Promega corporation. Madison, USA) was used for the cloning of a dedicated polyliker, to be used for the subsequent cloning of each of the 3 contiguous Spike gene fragments. The first step was to insert a DNA fragment (polylinker) that contained 3 restriction sites for different enzymes: (Tab.12)

Restriction enzymes	Sequence (5'-3')
AfeI	5'-AGC|GCT -3'
NruI	5'-TCG|CGA-3'
Eco53KI	5'-GAG|CTC -3'

Tab.12: restriction sites introduced in the polylinker and their sequences.

In particular, the polylinker was inserted into the plasmid digested with SwaI and SmaI restriction enzymes (both a blunt-end enzymes) in order to delete the barnase gene. Subsequently, the restriction site AfeI was used to insert the first fragment of spike protein, the NruI site was used for the second and the Eco53kI site was used for the last one. All these enzymes generated blunt-end cuts.

Ligation

T4 DNA Ligase (Promega corporation, Madison, USA), was used along its specific buffer in the following reaction (Tab.13), composed by 18 µl of water and 2 µl of specific buffer.

The ligation reaction was performed overnight, at 20°C.

Components	Volume per reaction
Linearized plasmid	0.5 µl
Insert	1.5 µl
DNA ligase	0.5 µl
Buffer 10X	1 µl
Solution	1 µl
H_2O	5.5 µl
Sample	1 µl
Plasmid digestion's enzyme	0.2 µl

Tab.13: ligation reaction mix.

Transformation

JM109 competent cells (Promega corporation, Madison, USA) were transformed by the ligation products, according to the manufacturer's instructions: cells were incubated at 42°C for 40 seconds and then placed in ice for 2 minutes.

Subsequently, cells were plated on Petri plates containing LB + kanamycin solid culture medium. The presence of the antibiotic was important to allow the selective growth of cells transformed by the plasmid, equipped with the kanamycin resistance gene.

Screening

The day after transformation, colonies appearing on the plates were screened to identify which of them contained the insert in the correct orientation, necessary after blunt-end cloning. For this purpose, colonies were subjected to a PCR amplification with DreamTaq™ DNA Polymerase kit (Thermoscientific. Vilnius. Lithuania) which involved the use of one primer

recognizing a sequence immediately upstream of the insert and the other recognizing a sequence in the insert sequence. This allows amplification only of clones containing the insert in the correct orientation. The screening protocol was (Tab.14):

Components	Volume per reaction
Primer F (10 µM)	2.5 µl
Primer R (10 µM)	2.5 µl
dNTP (10 µM)	1 µl
Buffer DreamTaq 10X	5 µl
DreamTaq	0.2 µl
H_2O	38.8 µl

Thermal profile	Time (min)	Cycles
94°C	3:00	1
94°C	0:30	25
55°C	0:30	
72°C	1:00	
72°C	3:00	1

Tab.14: screening reaction protocol.

Plasmid extraction and sequencing

Once positive clones were identified, their plasmid was extracted using the PureYield™ Plasmid Miniprep System (Promega corporation, Madison, USA), and checked by Sanger sequencing. If the sequence resulted free of mutations, the plasmid(s) could be used to insert the next Spike protein gene fragment.

7. AIM OF THE STUDY

SARS-CoV-2 was responsible for the devastating pandemic declared in 2020 by the WHO. During these years, it proved capable of persisting indefinitely in the human population as its variability has circumvented the strong herd immunity generated in the human population. Indeed, despite the presence of a viral exonuclease, which curbs the number of misincorporations that can occur during viral replication, SARS-CoV-2 has shown a great ability to change in selected, crucial residues, mostly in the Spike gene. The huge number of human hosts and replication cycles have helped the emergence of viral variants, while the observed mutations have been subjected to strong selective pressures. Some of this pressure might have been exerted by tropism (for example the binding affinity to the ACE-2 receptor, the use of different proteases). However, most of the variability observed in crucial Spike protein domains was selected as it allowed the virus to evade neutralization by antibodies, and infect humans despite strong neutralizing activity in their sera, elicited by vaccinations and former infections. This led to a reproductive advantage of each new variant, capable of substituting the preceding one(s), which gradually lost their infectiousness to herd immunity. However, as the first variants swept in subsequent epidemic waves through the human population, apparently unaffected by herd immunity (by preceding infections or vaccinations) in 2023 epidemic waves are apparently decreasing in frequency and magnitude.

Possibly, the growing herd immunity confers increasing protection not only against the pulmonary spread of the virus and severe infection, but also against infection itself by new emerging variants. Hence, as neutralizing antibodies represent the first line of defense against infection it is possible that the variability in the spike protein is becoming insufficient to massively

evade neutralizing responses consolidated by multiple rounds of immunizations.

Therefore, the aim of this study was to analyze the relationships between viral variability and neutralizing power of the antibody responses, and how it evolves through multiple rounds of immunizations. In particular, this study will integrate regional molecular epidemiology data and data on the neutralizing activity of sera at different time points during the pandemic years, in a group of healthy subjects, to focus on the variant-specific antibody response, the determinants of cross-neutralizing efficacy of these responses and their duration in time. Part of the study will also take advantage of *in vitro* mutagenesis studies to investigate the phenotypic aspects of selected mutations/variants.

The results should help us to outline a possible scenario of how COVID 19 could affect the human population in the near future.

8. RESULTS

8.1 Epidemiological studies

During the COVID-19 pandemic the Virology laboratory of the University Hospital “Ospedali Riuniti di Ancona” was involved in the national molecular epidemiology surveillance program, with the aim of monitoring circulating SARS-CoV-2 variants in the Marche region. For that purpose, clinical samples with high viral load were periodically sampled from different provinces of the region and either the spike gene or the whole genome sequences were obtained from them.

In particular, in 2021 were analyzed 1012 sequences (69 whole genomes and 943 from Sanger sequencing), in 2022 were analyzed 954 sequences (424 whole genomes and 530 from Sanger sequencing) and in 2023 were analyzed 419 sequences (372 whole genomes and 47 from Sanger sequencing).

As shown in Figure 17, since its start, through august 2023, the pandemic was characterized by subsequent waves of infections.

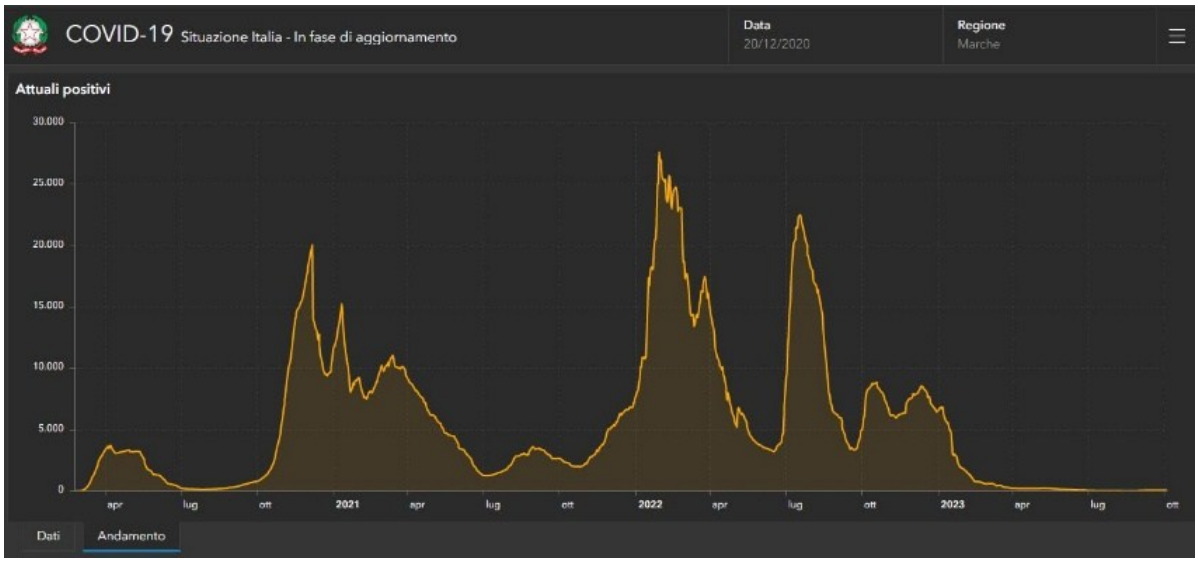

Fig. 17: trend of new COVID-19 cases in Marche region. https://opendatamds.maps.arcgis.com/

During the same observation period and in the same geographical area, different clades became predominant, as reported in figure 18.

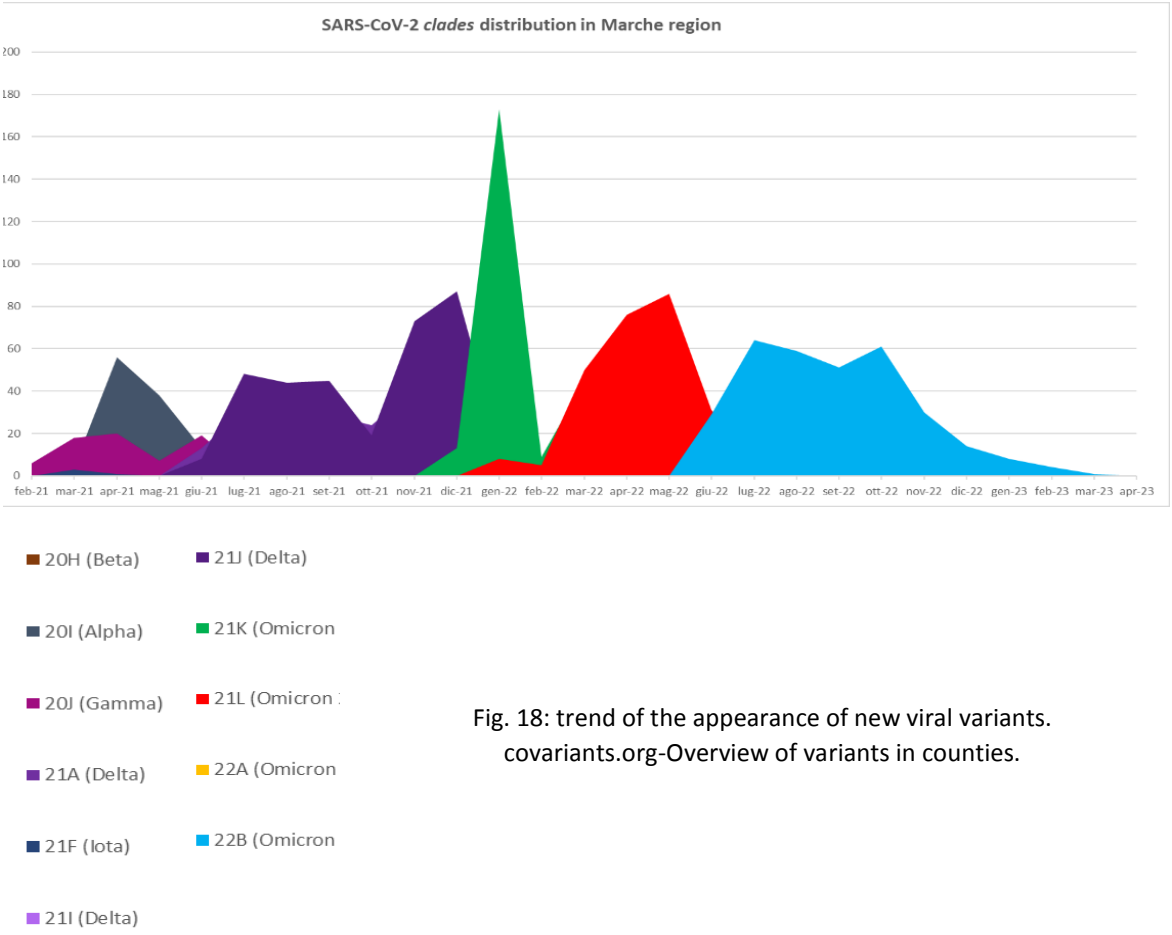

Fig. 18: trend of the appearance of new viral variants. covariants.org-Overview of variants in counties.

By comparing these 2 charts, it clearly emerges that each increase in the number of total cases corresponded to the emergence of a new SARS-CoV-2 variant. In particular, the first increase of total cases in April 2020 corresponded to the appearance of the Wild-type virus (B.1 lineage). Instead, at the end of 2020 the number of infections increased because of the appearance of the Alpha (B.1.1.7, 20I), Beta (B.1.351, 20H) and Gamma (P.1, 20J) lineages, in July 2021 appeared the Delta (B.1.617.2, 21J) variant, from

January 2022 arrived the Omicron BA.1, and the following waves corresponded to the appearance of BA.2, BA.4 and BA.5 Omicron variants.
This analysis suggests that the emergence of new viral variants determined the increase in the number of total cases because of their new ability to circumvent a somewhat lineage-specific herd immunity. Apparently, pre-existing antibodies in a human population immunized towards the latest variant could not block the infection. Subsequently, as the population went on to produce new variant-specific antibodies, more effective in curbing transmission at a population level, the number of cases decreased. Further on, the next new SARS-CoV-2's lineages (emerged by selection somewhere in the word) determined the subsequent wave, as other studies confirmed this concept. [71, 72] Moreover, it was possible to observe (Fig. 17 and 18) that the last waves following the Omicron BA.1 variant were characterized by lower peaks, meaning a lower total number of cases. This can possibly be explained as a consequence that Omicron BA.1 variant infected a larger portion of the population due to its a combined effect of 1) its increased transmission potential and 2) the relaxation of social distancing measure as a consequence of extensive vaccination and mortality reduction. Therefore, since 2022 the population had been more extensively immunized, both as a result of vaccination and natural infection.

8.2 Neutralization assays

To gain more insight into this hypothesis, the neutralizing power of sera drown from patients during different times in the last 3 years of pandemic was analyzed. In particular, microneutralization assays were performed on sera

from volunteering healthcare workers employed at the university hospital "Ospedali Riuniti di Ancona".

This cohort was subjected to periodic blood sampling: 20 days, 4 and 6 months and 1 year after the last immunological stimulation, this being either vaccination by Pfizer BioNTech BTN162b2 or natural infection. Each serum was tested against 10 viral variants that were isolated from previous clinical samples and titrated to obtain standardized 50% Infectious Doses (ID50). The following lineages were included in the study: B.1 (the first viral variant arrived from Wuhan, called Wild-type or WT), B.1.1.7 (Alpha), P.1 (Gamma), B.1.351 (Beta), B.1.526 (Iota), B.1.617.2 (Delta) and BA.1, BA.2, BA.4 and BA.5 (Omicron).

The patients' sera were grouped according to the type and the number of immunological stimulations.

Firstly, in order to investigate the lineage specificity of the neutralizing humoral response, unvaccinated patients with a single immunological stimulation, represented by a natural infection of different lineages were analyzed. Their sera were challenged in microneutralization assays against both the specific viral variant which they were infected with and the others. In particular, the following patients /groups were analyzed: 33 patients infected with the WT virus, 13 patients infected with Alpha variant, 1 patient infected with Gamma variant and 1 patient infected with the Beta variant.

As clearly shown in figure 19, all patient groups displayed a significantly higher neutralization titer against the variant they were infected with: for example those infected with WT strain showed an increase of 5.2-fold compared to Alpha and Gamma strains and 16-fold compared to Beta variant; patients infected with Alpha strain had a higher neutralizing titer compared to WT strain (1.5-fold), Gamma (4.6-fold), Beta (11.4-fold), Delta (5.7-fold) and

Omicron BA.1/2 (24.7-fold); patients infected with Gamma strain showed an increase compared to WT strain (1.8-fold), Alpha (3.9-fold), Beta (6.6-fold), Delta (7.8-fold), Omicron BA.1 (73-fold) and Omicron BA.2 (23.7-fold).

This did not occur with those infected by the Beta variant, which showed similar neutralization titers also against the WT strain. This indeed could imply that neutralizing antibodies in infected subjects were not as efficient in contrasting the diffusion of novel variants at a population level. In countries such as Italy, where before vaccination infections were limited due to strict prophylactic measures, this phenomenon was not as evident as in countries where prophylactic measures were only sporadically applied, such as in Africa or in other developing countries.

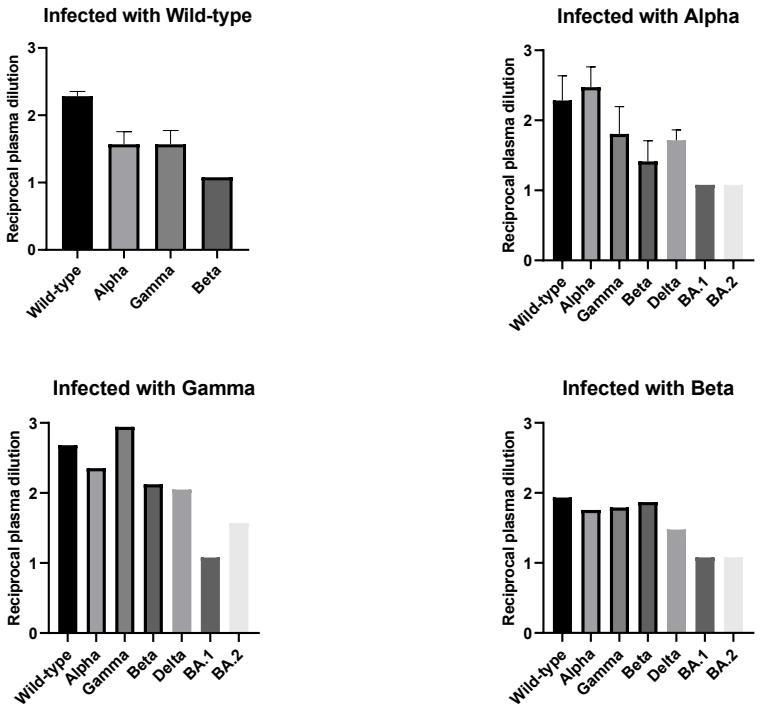

Fig. 19: neutralization activity in patients with only one infection (no vaccination).

In order to compare the neutralizing and cross-neutralizing power in sera of subjects who received a growing number of immunological challenge by the

SARS-CoV-2 Spike protein, the study cohort was divided in groups according to the different epidemic waves, according to the notion that the number of average immunological challenges in the population kept growing with time:

- WT virus wave included the period of time from March 2020 to September 2020 and 33 patients
- Alpha wave from December 2020 to June 2021. It was divided into three groups according to the number of immunological stimulations: the first one included 35 patients, the second one 68 and the third one 26 patients
- Delta wave from June 2021 to December 2021, included 26 patients
- Omicron BA.1 from December 2021 to February 2022, included 36 patients
- Omicron BA.2 from March 2022 to June 2022, included 40 patients
- Omicron BA.4/BA.5 from July 2022 until today, included 8 patients.

Samples were subjected to microneutralization tests against the different viral variants to analyze how the growing number of immunizations could affect neutralizing power in general and the ability to cross-neutralize unrelated variants. (Fig.20)

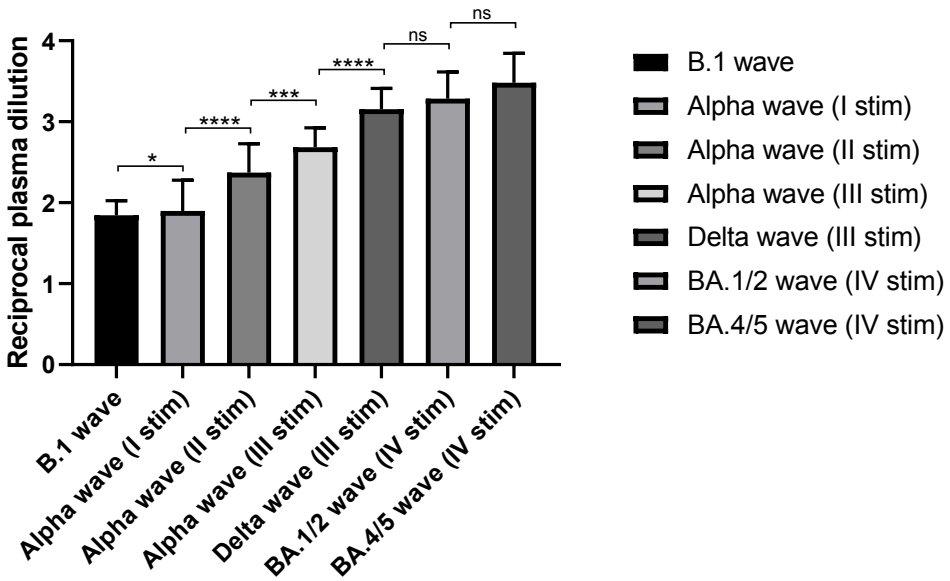

Fig. 20: mean neutralizing activity after an increasing number of immunizations. Statistical difference was assessed by the Mann-Whitney rank test. Statistical significance: ns = $p > 0.05$, * = $p < 0.1$, *** = $p < 0.001$, **** = $p < 0.0001$.

As shown in figure 20, the average neutralizing power rose significantly as the first epidemic waves hit the population, not only for the contribution of vaccination but also as a consequence of subsequent infections. For example patients included into “BA.4/5 wave (IV stim)” group showed an increase in neutralization titer compared with the other groups, such as Omicron BA.1/2 wave (1.1-fold), Delta wave (1.2-fold), Alpha wave III stim (2.9-fold), Alpha wave II stim (11.8-fold), Alpha wave I stim (89-fold) and B.1 wave (22.4-fold).
In order to investigate whether this improvement in neutralizing power was simply quantitative or also conferred greater cross-neutralizing capabilities, the response against 2 different variants was compared: the ancestral variant B1, against which all study subjects were immunized by vaccination, and the Beta variant, an intrinsically difficult-to-neutralize variant against which all the study subjects (but one) were naïve. In particular, the difference between the neutralizing titer (rNT) against the ancestral variant (B1) and against the Beta variant, standardized to the former titer, was assessed for each time point, according to the following calculation: $(nRT_{B1}\text{-}nRT_{Beta})/nRT_{B1}$ (fig.21). This parameter can be considered a selectivity index of neutralizing power, hence inversely proportional to cross-neutralizing power. Indeed, this parameter consistently decreased after the third immunization, indicating a significant increase in cross-neutralizing power. In fact the “Alpha wave (III stim)” group was 1.3-fold compared to the “Delta wave (III stim)”, 1.4-fold compared to the “BA.1/2 wave (IV stim)” one and 1.5-fold compared to the “BA.4/5 wave (IV stim)” group.

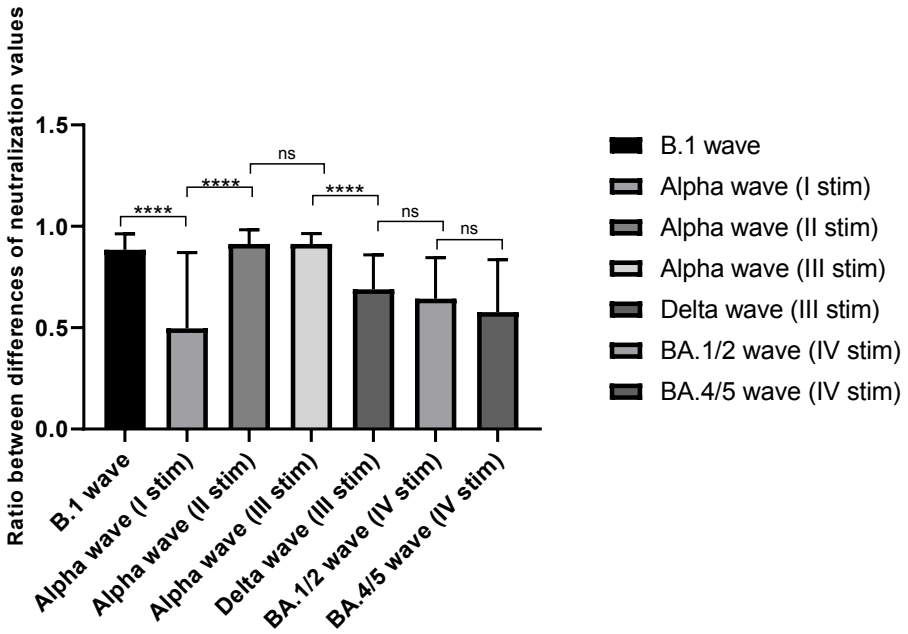

Fig. 21: selectivity index of neutralizing responses after an increasing number of immunizations. Statistical difference was assessed by the Mann-Whitney rank test. Statistical significance: ns = $p > 0.05$, **** = $p < 0.0001$.

Furthermore, as shown in figure 22, multiple immunizations clearly enhanced neutralization power against all variants, as neutralization power kept growing constantly from the earlier trough the latest samples. For example, comparing the first and the last groups (B.1 wave and BA.4/5 wave), it was possible to observe that the neutralization titer against the same strains was higher in the last group: 15.6-fold for the WT strain neutralization, 56.6-fold for Alpha and Gamma and 62.5-fold for Beta neutralization.

Neutralizing capability against unrelated variants tended to increase in relation to the number of natural infection/vaccine doses, including the most recent ones (not circulating at the time of sera sampling) independently of the variant(s) that generated the immunologic stimuli.

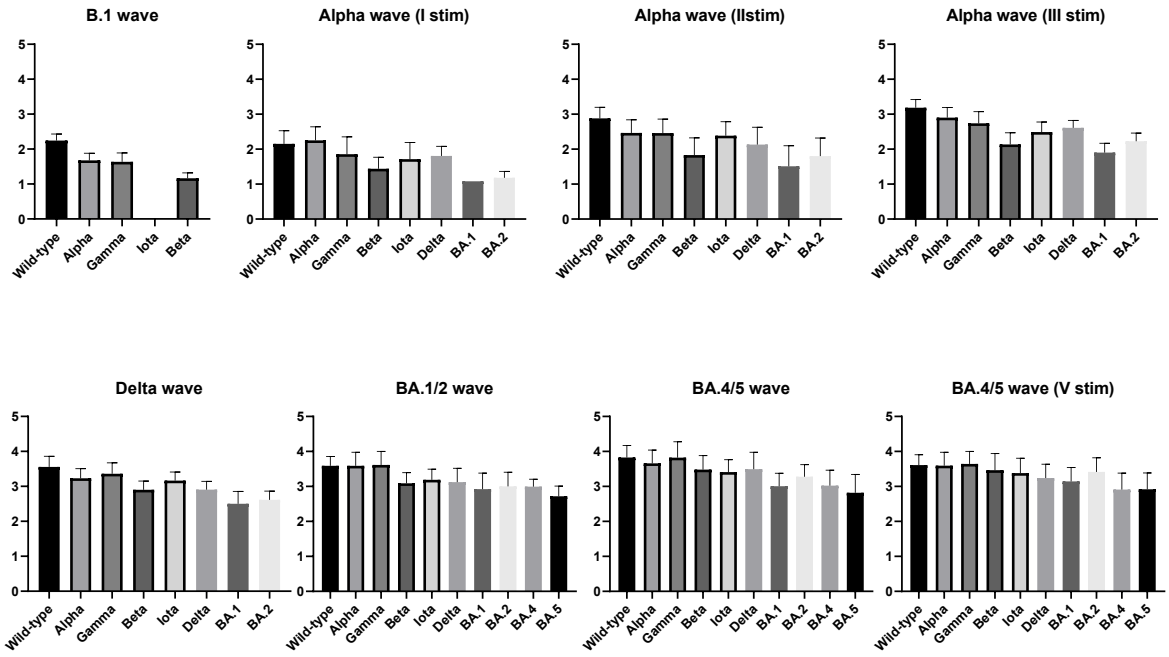

Fig. 22: neutralizing activity elicited by different viral variants.

These data show that multiple immunological stimuli make the humoral response stronger and more proficient in neutralizing SARS-CoV-2, in particular, as a possible effect of affinity maturation through multiple stimuli, it confers a broader neutralizing capability, which is possibly the reason for the reduction in the number of infections globally reported in each following epidemic wave since Omicron BA.1. In order to assess the advantages of repeated immunological stimuli on the duration of the response, neutralizing power was analyzed in sera collected 20-120-180 days after infection or vaccination. The results showed that this phenomenon affects also the durability of the response, including neutralizing response. In particular, Fig.23 displays the results of two groups of patients: one represents 20 patients who received three vaccine doses (dotted lines) and the other represents 54

subjects who, in addition to the vaccine doses, become naturally infected during the omicron wave (solid lines).

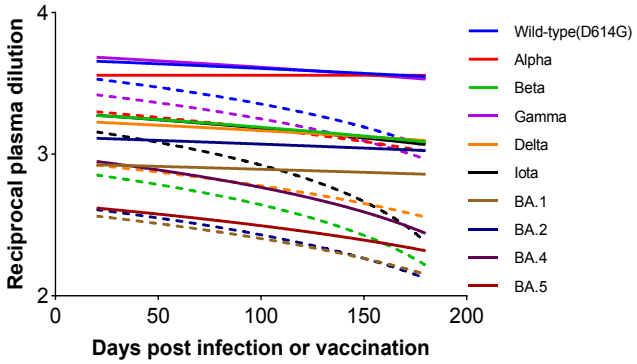

Fig. 23: neutralizing activity over time.

subjects with vaccine doses as the only immunological stimulus, despite initially valid titers, suffered a faster decay of the neutralization activity over time whereas, those who become infected were able to maintain a good neutralizing activity longer.

8.3 Development of plasmid vector for mutagenesis studies

Neutralizing response in sera from immunized subjects is a composite result which derives from the summary of single antibody-antigen interactions, involving multiple epitopes throughout the spike protein. Mutations impact on this phenomenon by changing crucial residues in epitopes recognized by neutralizing antibodies. Such epitopes have been identified in several regions of the protein such the NTD, the proteolytic cleavage site, and of course in the RBD, however, in order to identify the biological significance of any novel mutation, in terms of antibody binding, neutralization or tropism, a method for performing *in vitro* mutagenesis was designed and crafted, with the aim to analyze the phenotypic aspects of mutations *in vitro*.

The pF4K CMV Flexi^{R}Vector (Promega) was used as plasmid backbone, which contains a CMV immediate early enhancer/promoter plus a chimeric intron for mammalian expression of the protein-coding region and a T7 promoter for *in vitro* expression of the protein-coding mRNA. It also contains the barnase and kanamycin-resistance genes for selection. This vector is equipped with SgfI/PmeI encompassing the barnase gene, with the aim to allow the straightforward replacement of that gene with any another of interest. (Fig. 24)

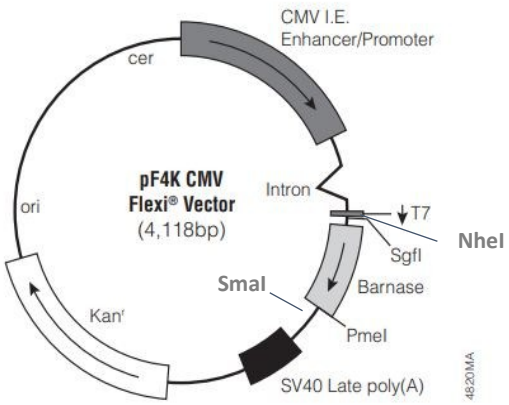

Fig. 24: pF4K CMV Flexi^{R}Vector plasmid. https://ita.promega.com

The aim of the first part of this approach was to create a cloning vector capable of secreting a soluble form of SARS-CoV-2 spike protein, easily modified by *in vitro* mutagenesis, to be used in antibody binding assays. For this purpose, the leader sequence of the human albumin was cloned downstream of the promoter, immediately followed by the sequence of the spike protein in the same frame. Such plasmid vector will be used to infect cell cultures so that they will produce the secreted form of the spike protein in the supernatants, facilitating its purification for further experiments.

Before cloning, synonymous mutations were introduced in the amplified human albumin leader in order to introduce the SmaI restriction site. The NheI restriction site placed downstream of the promoter and upstream of the barnase gene was used to clone this leader sequence. Subsequently, the novel SmaI (blunt end) restriction site was used together with the one downstream the barnase gene in order to delete it from the vector. (Fig.25)

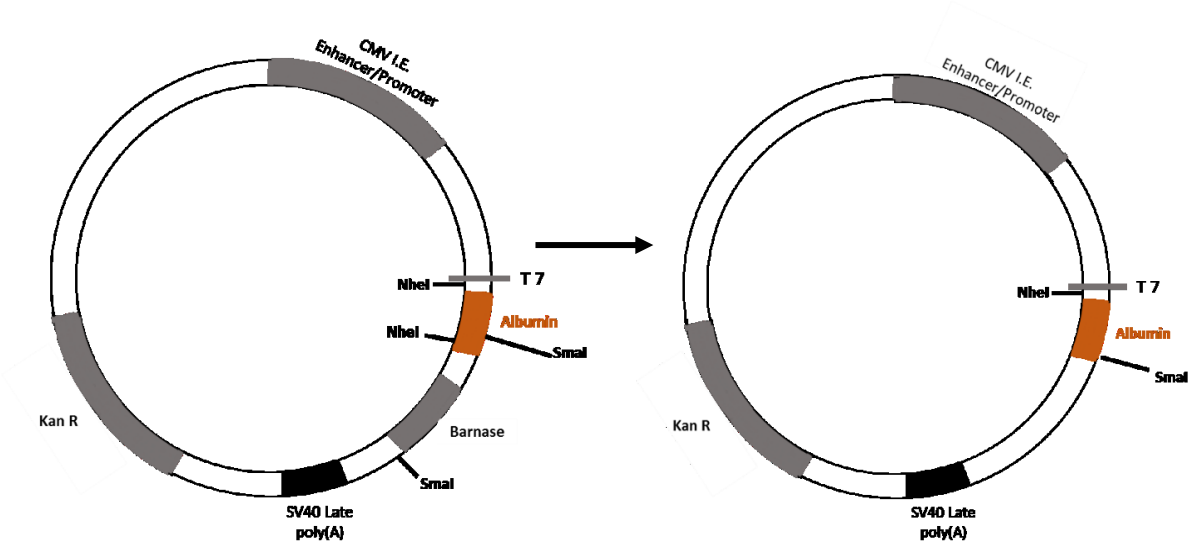

Fig. 25: Albumin insertion and barnase removal from cloning vector.

A synthetic polylinker sequence (based on synthetic oligonucleotides) was introduced in the remaining SmaI site (Fig.26). The synthetic polylinker was equipped with SwaI (blunt end) restriction sites in order to delete the SmaI sites from the vector after cloning (Fig.27).

Fig. 26: Polylinker with restriction enzymes.

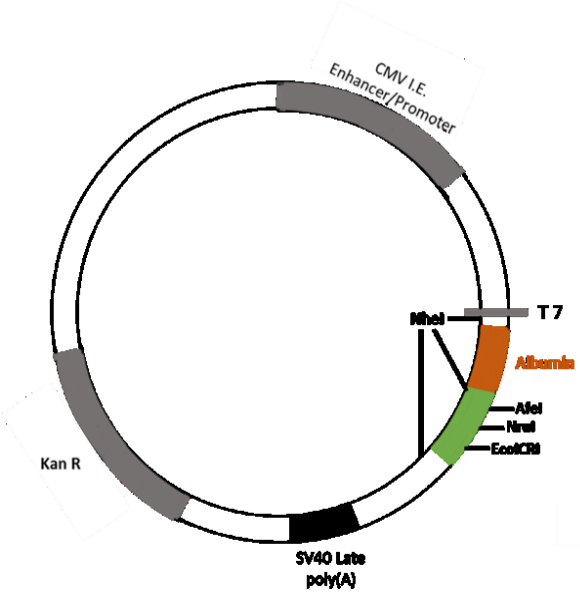

Fig. 27: Insertion of polylinker fragment (green region).

The polylinker was inserted with the purpose of introducing a multiple cloning site (MCS) into the plasmid vector to allow the cloning of the spike protein in 3 independent portions (all as amplified products). In particular, the restriction site for AfeI was used to insert the first fragment, the NruI site for

the second and the EcoICRI site for the third. These 3 fragments can be inserted independently of each other and when the procedure is complete the entire sequence of the spike gene is cloned in frame downstream of the albumin leader (Fig. 28). This procedure allows the independent mutagenesis in any part of the spike protein with the advantage of requiring the cloning of relatively small fragments (<1300 bp), easier to amplify and to clone.

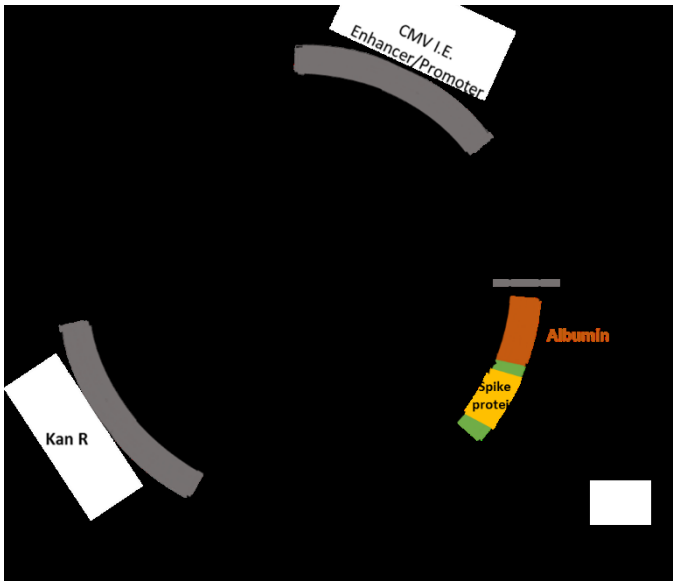

Fig. 28: Insertion of spike protein (yellow region) inside the MCS.

9. DISCUSSION

SARS-CoV-2 represents the third spillover event of an animal coronavirus occurred since the begin of the present century, and was responsible of the pandemic state declared in March 2020.

Since the beginning of the pandemic, this virus showed its great transmissibility and its ability to select advantageous mutations. In fact, during these years SARS-CoV-2 generated several viral variants associated to the appearance of mutations into the whole viral genome, and mostly in the Spike protein sequence. Although relatively limited in number, these mutations demonstrated a great impact on the viral phenotype, influencing its transmissibility, the ability to bind cellular receptor(s), the immune escape and affecting neutralizing activity exerted by the host antibodies.

At the beginning of the pandemic, SARS-CoV-2 caused lots of new infections and deaths in the immunologically naïve human population, for this reason the scientific communities have focused on the development of new vaccines to immunize the population. Big pharmaceutical companies became soon involved in the vaccine race, and those whose products were firstly approved and distributed in the population were: BioNTech/Pfizer, Moderna, Johnson & Johnson, Gamaleya, Astra Zeneca, Novavax, Sinopharm and SinoVac.

The vaccination strategy, was based on the assumption that the immunized population could produce specific antibodies and other immune cells against SARS-CoV-2 capable of reducing the severity of symptoms during COVID-19.

As the proportion of immunized subjects rose in the population as a function of infections and vaccinations, it became clear that the virus could circumvent herd immunity by modifying a remarkably limited number of residues in the Spike protein. Further on into the pandemic, as several subsequent epidemic waves swept through humanity, the virus kept modifying its Spike sequence,

upscaling the incidence of new mutations (with the possible contribution of passages in other animal populations such as rodents), in an effort to overcome the ever-growing immune competence of the human population

This study investigates several features of the pandemic in order to shed some light on the relationship between viral variability (in the Spike protein) and the evolution of the neutralizing immune response in the population. For this purpose, the trend of the incidence of SARS-CoV-2 infections in Marche region, since the beginning of the pandemic, was analyzed and the results were compared to the molecular epidemiology data obtained in the same period and population. It was clearly shown that incidence varied in a periodical peak/valley pattern generating typical infection waves each of which corresponded to the appearance of a new viral variant. This occurred probably because the novel variants bore mutations that allowed immune escape and the loss of neutralizing activity, reducing its ability to block the infection and leading to an increase in the number of new cases. This finding was perfectly in agreement with previous work by Beesley and colleagues claiming that new waves were determined by the appearance of new viral variants due to their increased transmissibility and infectivity. [73]

Thus, each epidemic wave subsided as, with the exposure of the population to the new variant, new specific antibodies were produced, capable of coping with that SARS-CoV-2 variant, and determining the reduction in the incidence of total cases.

The loss of neutralization activity was investigated by other research groups, for example Thakur V. [75] observed that vaccines lost their efficacy against viral variants because of mutation inside the spike protein's RBD region that affect its recognition by human antibodies. In addition, said that viral variants containing E484K mutation tended to avoid neutralization so, for example,

Alpha, Beta and Gamma variants were not neutralized. The latter observation was also confirmed by Nonaka C.K.V and colleagues [74] and El-Shabasy R.M. and their research group. [76]

An interesting phenomenon that was observed during the present study was that the most recent waves were more limited, and characterized by a reduced number of cases, despite the growing number of mutations displayed by the latest variants. This study has demonstrated that this is probably related to a better immunization in the general population that allows a substantial neutralizing activity also against new variants.

The main determinant of this improvement has been identified in the growing cross-reactivity against the all viral variants displayed during the follow-up of the study subjects, meaning that subjects immunized against a specific lineage could neutralize also the others. For these reason, recent new viral variants have accumulated a disproportionate number of new mutations, compared to older variants, to obtain meager results in terms of incidence and epidemic wave magnitude.

The impact of repeated immunizations on cross-neutralization has been described also by Luczkowiak J. and his group, that observed that the increasing of immunological stimuli determines the appearance of cross-neutralization. In particular they observed that, for example, the third vaccine dose determined the restoration of the neutralizing activity that had been partially lost after a long time interval after the second one, and with the third dose they observed that there was an increase in the neutralizing activity also against other VOC, such as Alpha, Beta, Gamma and Delta variants. [77]

In our study, to gain more insight in this phenomenon, we introduced a specific surrogate marker: The Selectivity Index based on the difference between the neutralizing titers against 2 unrelated variants, and analyzed its

trend through different immunization round. This analysis confirmed the trend of cross-neutralizing power, showing its tendency to grow significantly after each round of immunization.

A few research groups, such as Luczkowiak J. et al. [77] and Gili Regev-Yochay M.D. et al.[78], claimed that the fourth immunological stimulation didn't lead to a significant increase in the levels of neutralization compared to the third (unlike the significant increase after the third dose [79,80]). However, our results, based more on an additional immunization by natural infection rather than by vaccination, not only demonstrate a significant increase in average neutralizing power against all variants, but also demonstrate an increase in cross-neutralizing power, which in experimental conditions could also involve the most recent omega variants that circulated after blood sampling in the study population. In addition, the number of immunological stimuli (both natural infections and vaccination) was also related to the rate of neutralizing activity decay. In particular, the greater the number of immunological stimuli, the lower the speed of this decay.

In conclusion, this study confirms that vaccination campaigns aiming at a complete 3 dose vaccination were essential to reduce the adverse effects of COVID-19 on the population, reducing morbidity and mortality. Furthermore, it was observed that the increasing number of immunological stimuli by natural infection after vaccination has further contributed to an increasing trend in neutralizing activity, its cross-neutralization power on unrelated viral variants and its duration. Taken together, these results suggest that after multiple immunizations, including infections, herd immunity might continue to rise, progressively curbing the incidence (in addition to the severity) of SARS-CoV-2 infections.

BIBLIOGRAFIA

[1] International outbreak of novel SARS-CoV-2 coronavirus infection. Epicentro.iss.it. 2020.

[2] Wang M., et al., SARS-CoV-2: Structure, Biology, and Structure-Based Therapeutics Development. Frontiers in Cellular and Infection Microbiology. Volume 10. (2020)

[3] WHO Coronavirus (COVID-19) Dashboard. World Health Organization.

[4] Antonelli G., Clementi M., Pozzi G., Rossolini G. M., Principi di microbiologia medica (3rd ed.). Casa editrice Ambrosiana. (2017)

[5] Stadler, K., et al. (2003). SARS – beginning to understand a new virus, Nature Reviews Microbiology, 1, 209-218

[6] Middle East respiratory syndrome coronavirus (MERS-CoV). World Health Organization.

[7] Kadam S. B., et al., SARS-CoV-2, the pandemic coronavirus: Molecular and structural insights. Journal of Basic Microbiology. 2021; 61:180-2021.

[8] Su Q., et al., The biological characteristics of SARS-CoV-2 spike protein Pro330-Leu650. Vaccine. 2020;38:5071-5.

[9] Wan Y., et al., Receptor recognition by the novel coronavirus from Wuhan. J Virol. 2020;94:e00127-20.

[10] Zhang Y., et al., A Genomic Perspective on the Origin and Emergence of SARS-CoV-2. Cell. 2020.

[11] Pagani I., et al., Origin and evolution of SARS-CoV-2. The European Physical Journal Plus. 2023;138:157

[12] Knipe, D. M., & Howley, P. M. (2013). Fields Virology (6th ed.). Lippincott Williams & Wilkins

[13] Chen B., et al., Overview of lethal human coronaviruses. Signal Transduction and Targeted Therapy. 2020;5:89.

[14] Kakavandi S., et al., Structural and non-structural proteins in SARS-CoV-2: potential aspects to COVID-19 treatment or prevention of progression of related disease. Cell Communication and Signaling. (2023) 21:110

[15] Bai.C., et al., Overview of SARS-CoV-2 genome-encoded proteins. Life sciences-Science China Press. (2022) Vol.65 N°.2: 280-294.

[16] Raj, R. 2021. Analysis of non-structural proteins, NSPs of SARS-CoV-2 as targets for computational drug designing. Biochemistry and Biophysics Reports, 25, 100847.

[17] Meyerowitz, M.D., et al., Transmission of SARS-CoV-2: a review of viral, host, and environmental factors. Annals of Internal Medicine. (2020)

[18] Tabatabaeizadeh S. Airborne transmission of COVID-19 and the role of face mask to prevent it: a systematic review and meta-analysis. European Journal of Medical Research. (2021); 26:1.

[19] COVID-19 Overview and infections Prevention and Control Priorities in non-US Healthcare Settings. (2021, February 26). Centers for Disease Control and Prevention.

[20] Lan J., et al., Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE-2 receptor. Nature. 2020; 581, 215-220

[21] Jackson C. B., et al., Mechanism of SARS-CoV-2 entry into cells. (January 2022). Nature Reviews | Molecular cell biology. Vol.23.

[22] Malone B., et al., Structures and functions of coronavirus replication-transcription complexes and their relevance for SARS-CoV-2 drug design. Nature Reviews | Molecular cell biology. Vol.23. January 2022.

[23] Perlman S., et al., Coronaviruses post-SARS: update on replication and pathogenesis. Nature Reviews Microbiology, 7. 2009.

[24] Brant A. C., et al., SARS-CoV-2: from its discovery to genome structure, transcription, and replication. Cell & Bioscience. 2021. 11:136.

[25] Saraste J., et al., Assembly and cellular exit of coronaviruses: hijacking an unconventional secretory pathway from the pre-Golgi intermediate compartment via the Golgi ribbon to the extracellular space. Cells. 2021, 10, 503.

[26] Trougakos I. P., et al., Insights to SARS-CoV-2 life cycle, pathophysiology, and rationalized treatments that target COVID-19 clinical complications. Journal of Biomedical Science. 2021. 28:9.

[27] Tang D., et al., The hallmarks of COVID-19 disease. PLoS Pathog. 2020;16:ee1008536

[28] Ravi V., et al., Basic virology of SARS-CoV-2. Indian Journal of Medical Microbiology. 2020. 182-186

[29] Yeon Joo Jeong M.D., et al., Current and emerging knowledge in COVID-19. Radiology. 2023; 306(2):e222462

[30] Monteil V., et al., Inhibition of SARS-CoV-2 infections in engineered human tissues using clinical-grade soluble human ACE2. Cell. 2020;181:905-13. E7.

[31] Lamers M.M., et al., SARS-CoV-2 pathogenesis. Nature. 20, 270-284 (2022).

[32] Matacic C., Blood vessel attack could trigger coronavirus fatal 'second phase'. Science. June 2020.

[33] Kevadiya B.D., et al., Diagnostics for SARS-CoV-2 infections. Nature Materials, 20, 593-605.

[34] Murphy K., Weaver C., (2017). Janeway's Immunobiology. 9th ed. Garland Science.

[35] Abbas A., Lichtman H., Pillai S., Immunologia cellulare e molecolare. 2010. 6th ed. Elsevier.

[36] Chen H., et al., The Role of serum-specific-SARS-CoV-2 antibody in COVID-19 patients. International Immunopharmacology 91 (2021) 107325.

[37] Payne S., Viruses from understanding to an investigation. Academic Press. 2017. Pages 61-71.

[38] Gruell H., et al., Antibody-mediated neutralization of SARS-CoV-2. Immunity CellPress. 55, 2022.

[39] Chen Y., et al., Broadly neutralizing antibodies to SARS-CoV-2 and other human coronaviruses. Nature Reviews Immunology. Vol. 23. 189-199. 2023

[40] Deshpande A., et al., Epitope Classification and RBD Binding Properties of Neutralizing Antibodies Against SARS-CoV-2 Variants of Concern. Front. Immunol. Vol. 12. 2021.

[41] Altmann D.M., et al., The immunology of long COVID. Nature. July 2023.

[42] Davis E.H., et al., Long COVID: major findings mechanisms and recommendations. Nature. January 2023.

[43] Diamond M.S., et al., Innate immunity: the first line of defense against SARS-CoV-2. Nature. Vol 23. 165-176. 2022

[44] Sette A., et al., Adaptive immunity to SARS-CoV-2 and COVID-19. CellPress. 2021.

[45] Hu B., et al., The cytokine storm and COVID-19. JMed Virology. 2021; 93:250-256.

[46] Zanza C., et al., Cytokine storm in COVID-19: immunopathogenesis and therapy. Medicina. 2022, 58, 144.

[47] Meffre E., et al., Interferon deficiency can lead to severe COVID-19. Nature. 587, 374-376. 2020.

[48] Qi H., et al., The humoral response and antibodies against SARS-CoV-2 infection. Nature immunology. Vol 23, 1008-1020. 2022.

[49] Yaugel-Novoa M., et al., Role of the humoral immune response during COVID-19: guilty or not guilty?. Nature. Vol 15, 1170-1180. 2022

[50] Lee E., et al., Humoral immunity against SARS-CoV-2 and the impact on COVID-19 pathogenesis. Molecules and cells. 44(6); 392-400. 2021.

[51] Zheng J., et al., Characterization of SARS-CoV-2-specific humoral immunity and its potential applications and therapeutic prospects. Nature. Vol 19, 150-157. 2022.

[52] Jordan S.C., et al., Innate and adaptive immunity to SARS-CoV-2. British society for immunology. Vol 204, 310-320. 2021.

[53] Maemura T., et al., Antibody-dependent enhancement of SARS-CoV-2 infection is mediated by the IgG receptors FcγRIIA and FCγRIIIA but does not contribute to aberrant cytokine production by macrophages. American society for microbiology. Vol 12. 2021

[54] Minkoff J.M., et al., Innate immune evasion strategies of SARS-CoV-2. Nature. Vol 21, 178-194. 2023

[55] Carabelli A.M., et al., SARS-CoV-2 variant biology: immune escape, transmission and fitness. Nature. Vol 21, 162-177. 2023.

[56] Peacock T., et al., SARS-CoV-2 one year on: evidence for ongoing viral adaptation. Journal of general virology. Vol 102. 2021.

[57] Zhang S., et al., The battle between host and SARS-CoV-2: innate immunity and viral evasion strategies. Molecular therapy. Vol 30. 2022.

[58] SARS-CoV-2 Variant Classifications and Definitions. CDC: Centers for Disease Control and Prevention. September 2023.

[59] Barnes E., et al., SARS-CoV-2 specific immune responses and clinical outcomes after COVID-19 vaccination in patients with immune-suppressive disease. Nature. Vol 29, 1760-1774. 2023.

[60] Kyriakidis N. C., et al., SARS-CoV-2 vaccines strategies: a comprehensive review of phase 3 candidates. Nature. Vol 6. 2021.

[61] Fiolet T., et al., Comparing COVID-19 vaccines for their characteristics, efficacy and effectiveness against SARS-CoV-2 and variant of concern: a narrative review. Elsevier|Clinical microbiology and infection. Vol. 28, 202-221. 2022.

[62] Abbasian M. H., et al., Global landscape of SARS-CoV-2 mutations and conserved regions. Journal of translational medicine. (2023) 21:152.

[63] Harvey W.T., et al., SARS-CoV-2 variants, spike mutations and immune escape. Nature. Vol 19, 409-424. 2021.

[64] Wang Q., et al., Key mutations in the spike protein of SARS-CoV-2 affecting neutralization resistance and viral internalization. J Med Virol. 95(1):e28407. 2023.

[65] Hogan A.B., et al., Estimating long-term vaccine effectiveness against SARS-CoV-2 variants: a model-based approach. Nature. Vol 14. 2023.

[66] Beasley R., et al., COVID-19 vaccine effectiveness and evolving variants: understanding the immunological footprint. The Lancet. Vol 11. 2023.

[67] Dhawan M., et al., Updated insights into the T cell-mediated immune response against SARS-CoV-2: a step towards efficiency and reliable vaccines. Vaccines. 11(1), 101. 2023.

[68] Emeny J.M. & Morgan M.J., (1979). Regulation of the interferon system: evidence that Vero cells have a genetic defect in interferon production. Journal of General Virology, 43, 247-252.

[69] Caucci S., et al., Weak Cross-Lineage Neutralization by Anti SARS-CoV-2 Spike
Antibodies after Natural Infection or Vaccination Is Rescued by
Repeated Immunological Stimulation. Vaccines. 2021, 9, 1124.

[70] Markov P.V., et al., The evolution of SARS-CoV-2. Nature. 21, 361-379. 2023.

[71] Yoshikura H. Synchronization of epidemic curves of COVID-19 among nearby counties. Epidemiology International Journal. Vol.6, issue 1. 2022.

[72] Lai A., et al., Genomic epidemiology and phylogenetics applied to the study of SARs-CoV-2 pandemic. New microbiologica, 46, 1, 1-8, 2023.

[73] Beesley L.J., et al., SARS-CoV-2 variant transition dynamics are associated with vaccination rates number of co-circulating variants, and convalescent immunity. The Lancet. Vol. 91. 2023.

[74] Nonaka C.K.V., et al., Genomic evidence of SARS-CoV-2 reinfection involving E484K spike mutation. Emerg. Infect. Dis. 27:1522-1524. 2021.

[75] Thakur V., et al., Waves and variants of SARS-CoV-2: understanding the causes and effect of the COVID-19 catastrophe. Infection. 50: 209-325. 2022.

[76] El-Shabasy R.M., et al., Three waves changes, new variant strains, and vaccination effect against COVID-19 pandemic. Int. J. Biol. Macromol. 204: 161-168. 2022.

[77] Luczkowiak J., et al., Cross neutralization of SARS-CoV-2 Omicron subvariants after repeated doses of COVID-19 mRNA vaccines. J med Virol. 2023. 95(1): e28268.

[78] Gili Regev-Yochay M.D., et al., Efficacy of a fourth dose of COVID-19 mRNA vaccine against omicron. The New England Journal of Medicine. 2022.

[79] Lusting Y., et al., Superior immunogenicity and effectiveness of the 3rd BNT162b2 vaccine dose. 2021.

[80] Nemet I., et al., Third BNT162b2 vaccination neutralization of SARS-CoV-2 Omicron infection. The New England Journal of Medicine. 2022; 386:492-494.

[81] Diagnosi virologica–principi e metodi. J.M. Hoskins. Ambrosiana, 1975.