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Coordinator: Prof. Antonio Toscano

GENOTYPING OF SARS-COV-2 VARIANTS IN A SOUTH EAST SICILIAN POPULATION

PhD Thesis:

Dr. Ilenia Cappadona

Tutor:

Prof. Alessandra Bitto

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1. INTRODUCTION

1.1. THE COVID-19 PANDEMIC

On December 2019, in the city of Wuhan (Cina), an outbreak of violent pneumonia with unknown etiology appeared. The patients showed also mild symptoms like fever, chest pain and cough, in severe cases they had dyspnea and lung infiltrations [Zhu et al, 2020].

A little bit after, on January 2020, the Chinese Center for Disease Control and Prevention identified the cause of this pneumonia cases from bronchoalveolar lavage fluid samples that were submitted to metagenomic RNA sequencing.

The "bad guy" was a new Coronavirus, temporarily named 2019 n-CoV, for which inter-human transmission was confirmed. On the 10th of January 2020 was published the genome sequence of this new Coronavirus. The International Committee on Taxonomy of Viruses named this new virus as SARS-CoV-2, due to its similarity to previous Coronaviruses SARS and MERS [Wu F. et al, 2020]. The SARS-CoV-2 started to spread across China, reaching 34 cities before the end of January 2020. The National Health Commission of China declared that during February 2020 the infection rate was about 3000 new cases per day. Despite the control measures adopted in China, the Covid-19 started to spread across the world, maybe through travelers [Fisher and Heymann, 2020].

Its rapid spreading led the World Health Organization to declare that we were facing a really big emergency in the international public health. The WHO itself, on the 11th of February 2020 announced that this respiratory disease was named officially as Covid-19 and a month later, March 11th 2020, declared the pandemic, because of the rapid spreading of this virus for all over the world [WHO]. Covid-19 was the sixth public health emergency, after H1N1 in 2009, polio in 2014, Ebola in West Africa in 2014, Zika in 2016 and Ebola in the Democratic Republic of Congo in 2019 [Yoo, 2020].

Since 2020 to nowadays, the confirmed cases in all the world are 770.085.713 and 6.956.173 deaths. On May 5th 2023 the WHO general director Tedros Adhanom Ghebreyesus declared the end of the Covid-19 global emergency. This pandemic persisted for over three years [WHO].

1.1.1. EPIDEMIOLOGY

The first SARS-CoV-2 transmission in Wuhan in December 2019 was detected at the Huanan Seafood Wholesale Market, that is believed to be the "point zero" where the first patient was infected, but possibly it might started before that moment [Li Q. et al, 2020]. A retrospective study done by Wu and McGoogan individuates the possible "zero case" on the 8th December 2020 [Wu and McGoogan, 2020]. Actually, among the first cases, there's people that had no connection with the seafood market, suggesting that maybe the real first point of infection could be another one [Li Q. et al, 2020].

The need to isolate infected patients became compelling when the major contagion events occurred within common and populated places, such as homes or workplaces [Yu et al, 2020].

Bats seems to be the natural reservoir of SARS-CoV-2 [Zou et al, 2020]. Some studies show the genetic similarity between SARS-CoV-2 and a pangolin coronavirus in Malaysia. The similarity between the genes that encode for structural proteins is up to 90%, suggesting that pangolin species could be the intermediate host for animal to human transmission [Xiao et al, 2020]. Other animals as dogs, ducks, pigs and chicken can't be infected, even if they're in close contacts with infected humans [Shi et al, 2020].

SARS-CoV-2 demonstrated to be more transmissible than the other Coronaviruses that humanity knew during last years, like SARS-CoV and MERS-CoV [Li R. et al, 2020]. This is likely due to its unique virological characteristics. The viral load is yet really high during the first week of infection, in particular in the cells of upper respiratory tract, thus promoting the spread of the virus [Zou et al, 2020].

SARS-CoV-2 is transmitted by droplets during a non-protected contact with an infected person. These droplets can get to hands or mouth, nose and eye or they can remain in the air for a relatively long time and so enter into someone else's respiratory system, even if this tract is not the only one involved in transmission [Stadnytskyi et al, 2020].

The aerodynamic nature of SARS-CoV-2 was studied at the beginning in Wuhan hospitals, measuring viral RNA in aerosols. That study showed the real potential of air transmission, that inside the hospital was due to aerosols generated by medical procedures and so spread by the patients, but outside became the main transmission mechanism [Meselson, 2020].

Other kinds of samples where viral material was found are urine and feces, letting think that this systems could be injured by the virus and also be a transmission mechanism. But the viral load in this kind of samples is really low, above all if compared with the nasopharyngeal ones [Jones et al, 2020].

Anyway, some pediatric patients were tested for Covid-19, but the nasopharyngeal swabs were negative, while rectal swabs were highly positive [Xu et al, 2020]. According to some studies, SARS-CoV-2 could effectively replicate in intestinal epithelium and in human intestinal organoids. So there's an actual potential to spread through this system. There are also some studies about the intestinal cells of bats where the cells resulted infected [Lamers et al, 2020].

The major factor of transmission is the spreading by asymptomatic patients. For symptomatic patients, the viral load is yet higher 1 or 2 days before symptoms onset, becoming lower after the early stages of the infection. This means that before the symptoms onset, the transmission possibility is higher [Sunjaya and Jenkins, 2020]. The person-to-person transmission could also happen during incubation period [Yu et al, 2020].

1.1.2. SYMPTOMATOLOGY

Covid-19 infection could be asymptomatic or symptomatic. The incubation period is between 1 and 14 days, with an onset of symptoms around the 5th day [Wu and McGoogan, 2020].

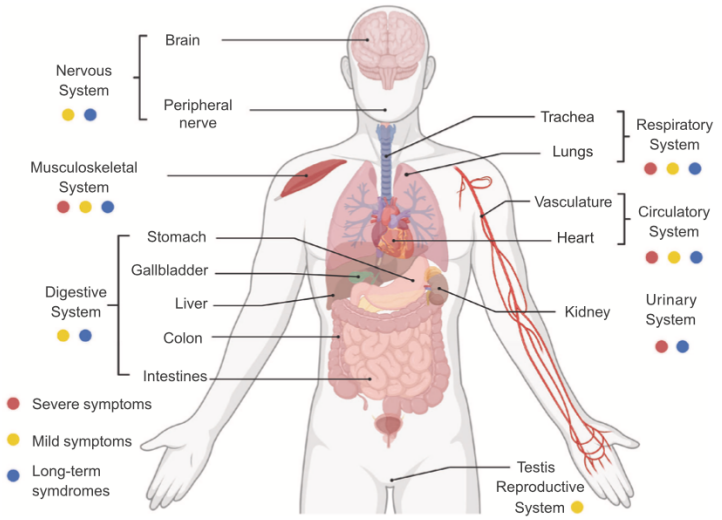
The death rate decreased across the pandemic, at the beginning was really high, around the 50% or more. Thanks to natural selection, vaccines and changes into virus biology, the survival rate reached over the 80% [Dennis et al, 2021].

The most common symptoms of the Covid-19 are fever, dry cough, dyspnea, fatigue and changes in smell and/or taste. Fever is the most variable symptom, it has different duration and it could be present just at the beginning or during the all infection. Even the temperature changes from person to person, with a media of 37 °C [Peyrony et al, 2020].

Less common symptoms are diarrhea, nausea, vomiting, chest pain, headache, hemoptysis, sometimes also taste and olfactory disorders during early stages of the disease [Guan et al, 2020]. Not ordinary symptoms that are reported in infected patients are erythematous rashes and urticaria [Recalcati, 2020].

There's not a susceptibility defined by age, the thing that changes are the symptoms, that differ with age. Young people develop just mild disease or they're totally asymptomatic. Instead, people over 60 years with co-morbidities tend to develop severe respiratory disease sometimes with hospitalization or death. In these patients were found high levels of blood urea nitrogen or other inflammatory markers, together with bilateral lung damage. During the first studies done in China, people between 70-80 years or older had major chance to die, in particular if they were affected yet by pathologies like cancer, hypertension, diabetes, cardiovascular or respiratory diseases [Wu and McGoogan, 2020]. Children are less prone to develop symptoms or severe disease than the adults, but there were a few cases of multisystem inflammatory syndrome (MIS-C) [Mahase, 2020].

During a symptomatic infection, Covid-19 shows three different levels of severity in the disease: mild, severe and critical. Mild disease is the most widespread, the common symptoms are: fever, cough, mild-pneumonia (not in all the cases). Severe disease symptoms are: dyspnea, low oxygen saturation, high respiratory frequency, lung infiltrations and hospitalization need. Critical level of disease shows respiratory failure, septic shock, multiple organ dysfunction or failure, coagulation dysfunction [Hu et al, 2020]. Critical patients reach very quickly acute respiratory syndrome, together with metabolic acidosis, in their serum is also detectable viral RNA [Huang et al, 2020].



System	Acute symptoms		Long-term symptoms		
	Severe	Mild	Symptoms	Reported occurrence	Occurrence time
General	Septic shock Electrolyte disturbance	Fever Fatigue	Fatigue Effort-intolerance	32(22-44) 19(7-35)	3-6m 3-6m
Respiratory system	Pneumonia ARDS Respiratory failure	Dry cough Dyspnea Shortness of Breath	Cough Dyspnea	15(10-21) 25(17-34)	3-6m 3-6m
Circulatory and blood system	Heart failure Acute cardiac injury Coagulation disorder Arrhythmia	Chest pain	Palpitations Chest pain	14(5-25) 11(6-16)	3-6m 3-6m
Digestive system		Diarrhea Nausea Abdominal pain Loss of appetite	Diarrhea Nausea/vomiting	10(2-21) 8(0-25)	3-6m 3-6m
Musculo-skeletal system	Rhabdomyolysis	Myalgias	Myalgia Joint pain	12(4-22) 14(4-27)	3-6m 3-6m
Nervous system	Stroke Encephalitis	Dizziness Headache	Cognitive disorder Headache Loss of smell Loss of taste Brain structure change	14(3-31) 12(5-20) 9(4-17) 8(3-15)	3-6m 3-6m 3-6m 3-6m
Reproductive system		Testicle pain			

Figure 1. *Overview of Sars-CoV-2 symptoms* (Cong et al, 2022).

After respiratory illness, a severe Covid-19 disease could lead to myocardial damage, arrhythmic instability [Bansal, 2020], neurological complications like intracranial hemorrhage or stroke [Paybast et al, 2020]. In case of heart damage, the patients showed hypoxic injury, coronary damage, hypercoagulability and atherosclerotic plaques. This can lead to acute coronary occlusion and so myocardial infarction [Ackermann et al, 2020].

Myocardial damage is present in the 20% of the patients hospitalized in intensive care. The major demonstration of this kind of damage are: myocarditis, dysrhythmias, heart failure and acute coronary syndrome. Between the dysrhythmias, tachycardia and bradycardia are the most common, but there was detected also a QT prolongation, possibly secondary to electrolyte imbalance in cases with symptoms like diarrhea or due to inflammation or preexisting cardiac diseases [Madjid et al, 2020].

Additionally, in the 80% patients were observed neurological manifestations, mild or severe, like cerebrovascular disease, headache or altered conscious state [Mao et al, 2020]. The cerebrovascular disease manifests through ictus episodes, but also through cerebral ischemia, encephalopathy or convulsions. The stroke is a rare consequence, showed by the 6% of severe cases. Instead, mental alterations can be present in old patients with comorbidities or bad prognosis, or in psychiatric patients. Older patients show symptoms like delirium. For psychiatric patients the symptoms are more severe, like psychosis, anxiety, insomnia or mood disorders [Taquet et al, 2021].

Patients hospitalized in intensive care unit showed leucopenia and lymphopenia, with high levels of IL2, IL7, IL10, GSCF, IP10, MCP1, MIP1A, and TNFα [Huang et al, 2020].

Another kind of damage could be secondary infections, generally bacterial of fungal co-infections, that can cause even death. The most involved bacteria are Heamophilus Influenzae, Streptococcus pneumoniae and Klebsiella pneumoniae, but there were cases of pulmonary aspergillosis and mucormycosis [Zhou et al, 2020].

There are other patients who did not die for Covid-19, but they manifested long-term consequences, as anomalies to heart functionality or olfactory disfunction. The olfactory disfunction has not clear origins, because how the virus damages olfactory cells is still not known completely [Luers et al, 2020]. Some autopsy studies showed that in severe cases, patients present abnormal consequences like necrosis of hilar lymph nodes and kidney hemorrhage, liver with infiltration of inflammatory cells, splenic atrophy and neuronal degeneration in brain [Yao et al, 2020]. Other studies like these, showed that patients with pulmonary embolism had also deep venous thrombosis that lead to death [Wichmann et al, 2020].

There are lots of studies focused on the different categories of ages or co-morbidities. Generally, a worst prognosis seems to be associated with some risk factors, like age over 75/80 years, cancer, hypertension, pulmonary diseases, diabetes, immune diseases or cardiac diseases, even obesity is a risk factor [Williamson et al, 2020].

In a study, by Zhang and coworkers, about the SARS-CoV-2 infection in smokers, they observed a lower rate of infection, but a more severe disease [Zhang et al, 2020]. Also obesity could be a risk factor to develop severe disease ending in a hospitalization [Lighter et al, 2020].

For pregnant women, there isn't a really high risk. Even the transplacental transmission happened a few times, according to literature [Chen H. et al, 2020]. There were rare cases of mothers infected during the last trimester of pregnancy, with consequences for the newborns like neurological compromission or high cytokines and SARS-CoV-2 IgM antibodies [Dong et al, 2020]. Actually, is not well known about exposition during the first and second trimester of pregnancy [Parazzini et al, 2020]. The last known coronaviruses, like SARS or MERS, were more violent during infection of pregnant women, causing even miscarriage [Fan et al, 2020].

1.2. INSIDE SARS-COV-2: MOLECULAR BASIS

1.2.1. FAMILY AND STRUCTURE

The Coronaviridae family, in particular Nidovirales order, are classified in four families: α, β, γ e δ. Sars-CoV-2 is a β-Coronavirus, that is one of the three genera that infects mammalians [Wu A. et al, 2020]. The Coronaviridae Study Group of the International Committee on Taxonomy of Viruses studied the phylogenesis of the Sars-CoV-2 genome. They found that Sars-CoV-2 is clustered with SARS-related CoVs found in bats, in the subgenus Sarbecovirus of the β-Coronavirus. Even if it's phylogenetically related, Sars-CoV-2 is different from the other CoVs in bats or similar species [ITCV, 2020].
These are enveloped viruses, with positive single-stranded RNA genome, and most of them are pathogenic [Xu et al, 2020]. Before Sars-CoV-2, two other coronaviruses infected humans: the Sars-CoV in 2002 and the MERS-CoV in 2013. These two were less pathogenic than the new form that caused the pandemic [Chen et al, 2020].

1.2.2. GENOME STRUCTURE

Sars-CoV-2 genome is composed by a single-stranded positive-sense RNA and is ~29.9 Kb in size. The open reading frames are between 13 and 15 containing ~30,000 nucleotides, 12 of which are functional. The GC content is about 38%, the protein coding genes are 11, with 12 expressed proteins. The genomic identity is shared for the 89% with other CoVs; Specifically, it is really similar, for genome organization also, to Sars-CoV and MERS-CoV. Indeed, Sars-CoV and Sars-CoV-2 have a large amino acid similarity (about the 90%) in their structural protein, differing only for the S gene [Lu et al, 2020].

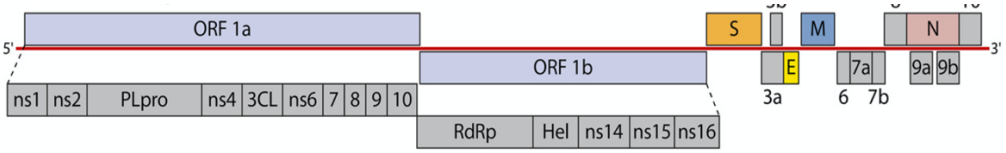


Figure 2. *Sars-Cov-2 genome* (Safiabadi Tali et al, 2021).

Starting from the 5'-end, two thirds of this genome contain the replicase gene, that encodes for two open reading frames, named 1a and 1b [Zhou et al, 2020]. The rest of genome contains, for other ORFs, encoding for structural proteins like Spike, Membrane, Envelope and Nucleocapsid. There are other minor ORFs, that encode for accessory proteins, but their function is not well known [Poran et al, 2020]. OFR1a and ORF1b encode respectively for replicase polyprotein 1a (ppla) and replicase polyprotein 1ab (pp1ab). ORF1a, at its 3'-end, encodes also for a −1 ribosomal frameshift, that helps ORF1b translation for pp1ab production [Chiara et al, 2021]. There's a knot-like slippery sequence at the 3' (UUUAAAC) named frameshift stimulation element (FSE). This FSE stimulates the −1 ribosomal frameshift, so the translation doesn't end and a bigger protein with around 2700 added is generated. The ribosomal frameshift doesn't always happen; it occurs with a frequency of 0,25-0,75 in this site. When it doesn't happen, the stop codon at stem 1 causes translation termination [Bhatt et al, 2021]. The two polyproteins are cleaved into functional non-structural proteins (Nsps), respectively the pp1a is cleaved into 11 Nsps and the pp1ab into 15 Nsps [Gupta et al, 2021]. ORF3a, 7a and 7b encode for transmembrane proteins. Going into detail, ORF3a encodes for a homodimer that forms an ion channel into the host membrane [Kern et al, 2021]. ORF7a encodes for a type I transmembrane protein that is an immunomodulatory factor for cell binding and inflammatory responses. ORF7b was found into the host Golgi, that could mean an increase of virulence [McBride et al, 2012]. ORF8 seems to be a new gene, really far from other coronaviruses. This protein interacts with major histocompatibility complexes I and stimulates their degradation. This is one of the immune escape mechanisms of the virus [Zhang et al, 2021]. Another one of these mechanisms

is done by ORF9b. It inhibits the Interaction between Hsp90 and TOM70, this suppresses interferon response [Gao et al, 2021]. Even ORF10 encodes for a small protein, but its role is unknown and seems not to be so important. ORF10 with other ORFs like 3b, 6 and 9c, haven't been characterized so well [Pancer et al, 2020].

1.2.3. VIRAL STRUCTURE

Sars-CoV-2 contains four structural proteins: spike (S), membrane (M), envelope (E) and nucleocapsid (N) [Lau et al, 2005].
The Non-Structural Proteins have multiple functions. Some of them are more important because they are involved in replication and transcription; others have minor functions.

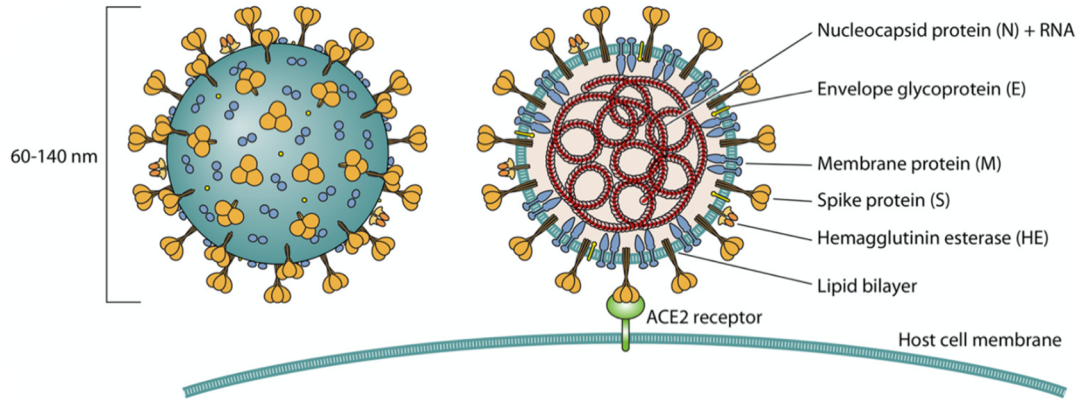


Figure 3. *Sars-CoV-2 structure* (Safiabadi Tali et al, 2021).

Non-Structural Proteins

The sixteen Non-Structural Proteins are called Nsp from 1 to 16, with the following roles:

- Nsp1: it is the N-terminal product of ORF1ab cleavage; it mediates RNA processing and replication, it's the main host translation inhibitor and it's involved in mRNA degradation [Almeida et al, 2007]. It also interacts with the host ribosome to stop its protein synthesis, in particular its C-terminus binds to the host 40S ribosomal subunit, accelerating mRNA degradation [Kamitani et al, 2006].
- Nsp2: it's a replicase product that modulates the survival signaling pathway into the host cell, interacting with host PHB and PHB2 [Harcourt et al, 2004]. Possibly, it interacts with ribosome and replication-transcription complexes [Gupta et al, 2021].
- Nsp3: it's a papain-like proteinase that separates the translated proteins [Snijder et al, 2003]. It cleaves polyproteins forming Nsp1-3 [Shin et al, 2020]. It contains other domains, named ubiquitin-like, X, nucleic acid-binding, SARS coronavirus-unique, transmembrane ad Y1-3 [Lei et al, 2018]. The transmembrane one works with Nsp4 and Nsp6 to modify the ER membranes [Oostra et al, 2008].
- Nsp4: it's a membrane-spanning protein that contains transmembrane domain 2 and probably modifies ER membranes [Manolaridis et al, 2009]. It works with Nsp3 and Nsp6, leading to a curvature of the ER membrane that helps virus replication [Oostra et al, 2008].
- Nsp5: it's a 3C-like proteinase, named main proteinase that takes part to the replication [Anand et al, 2002]. It also cleaves the polyproteins pp1a and pp1ab, generating Nsp4-16 [Dai et al, 2020].
- Nsp6: it's putative transmembrane domain that takes part in the autophagosomes induction from the host endoplasmic reticulum [Anand et al, 2002].

- Nsp7: it's the RNA-dependent RNA polymerase that forms a hexadecameric complex with Nsp8 implicated in replication [Peti et al, 2005]. Nsp7 and 8 are assembled with Nsp12, this enhances the polymerase activity [Yan et al, 2021].
- Nsp 8: it's a multimeric RNA polymerase and replicase that forms a hexadecameric complex with Nsp7 implicated in replication [Zhai et al, 2005].
- Nsp9: it's an ssRNA-binding protein that takes part in viral replication [Egloff et al, 2004]. It inhibits the nucleotidyltransferase of Nsp12 [Yan et al, 2021].
- Nsp10: it's a growth-factor-like protein with two zinc-binding motifs, it stimulates Nsp14 and Nsp16 in viral transcription and is critical for the cap methylation of viral mRNAs [Joseph et al, 2006].
- Nsp11: it's composed by 13 aminoacids, and it's identical to the first segment of Nsp12; its function is unknown.
- Nsp12: it's part of the replication/transcription complex, as it contains the RdRp (RNA-dependent RNA polymerase) and it has also nucleotidyltransferase activity so, it's fundamental in replication and transcription processes [Zanotto et al, 1996].
- Nsp13: it's a RNA 5'-triphosphatase, it has a zinc-binding domain that is involved in replication and transcription and a NTPase/helicase core domain that binds ATP [Van Dinten et al, 2000].
- Nsp14: it has a proofreading endoribonuclease domain that acts in 3' to 5' direction and a N7-guanine methyltransferase activity [Lin et al, 2021]
- Nsp15: is and endoRNAse with Mn^{2+}-dependent activity [Naqvi et al, 2020].
- Nsp16: it's a 2'-O-ribose methyltransferase for viral mRNAs capping [Von Grotthuss et al, 2003].

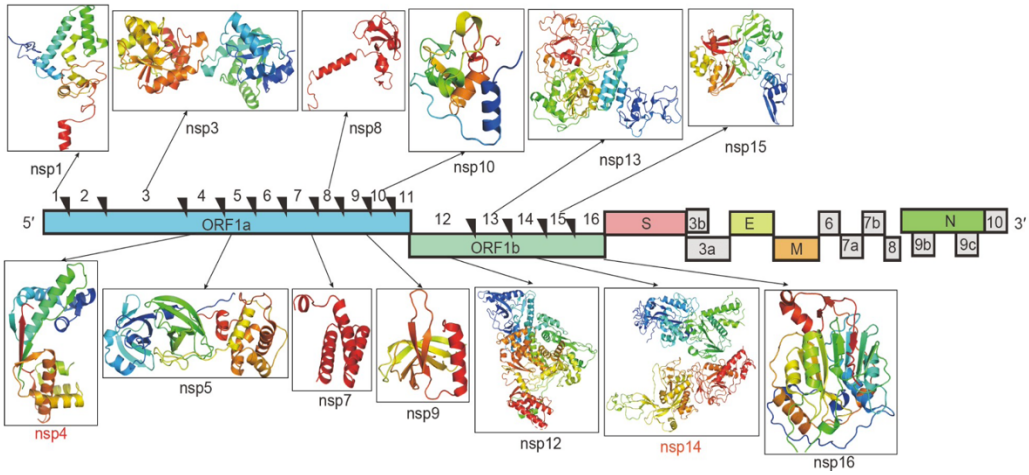


Figure 4. *Structures of SARS-CoV-2 Non-Structural proteins* (Bai et al, 2022).

Membrane Protein

Membrane protein is about 222 amino acids long, which are conserved for the major part, and is involved in RNA packaging. Membrane proteins are the most abundant in the virus conformation [Tang et al, 2020]. It's a type III glycoprotein and defines the viral envelope shape [De Haan et al, 2000].

Envelope Protein

Envelope membrane is formed by small proteins with the function of assembly and release the virions. The protein size is small, around 75-109 amino acids, with a single α-helical transmembrane domain [Parthasarathy et al, 2008]. These little proteins act as viroporins that form protein-lipidic pores into the host membrane after the virus entry. These pores will be involved in ion transport [Yuan et al, 2020]. Their role is to regulate viral lysis and viral genome release [Weiss and Navas-Martin, 2005]. They consist of hydrophobic residues, except for the N-terminal pore, that is like an entrance site. The N-terminal region of the protein is inside the membrane, in the ER and Golgi region specifically; instead, the C-terminal is in the cytoplasm. This localization allows the protein to pump Ca^{2+} out to the ER, so that it may

activate the host inflammasome [Mandala et al, 2020]. These proteins are abundantly expressed inside infected cells during replication: thus, at the end of the process, just a small portion of them emerges from the viral envelope [Venkatagopalan et al, 2015].

Nucleocapsid Protein

Nucleocapsid protein is highly conserved in sequence, stable and immunogenic. Its role is to interact with viral genome and M protein to help viral RNA packaging. N proteins have an RNA-binding domain in their core that binds viral RNA. This domain is about 140 amino acids long [Neuman et al, 2016]. The bond with the RNA creates a ribonucleoprotein complex, that keeps RNA in an ordered conformation for transcription and replication [Masters et al, 1990]. N proteins is kind of a molecular chaperone for regulation of cellular processes and viral synthesis [Zuniga et al, 2010].
It's composed by a N-terminal domain, a C-terminal domain and three disordered regions, named N-arm, central linker and C-tail [Kang et al, 2020]. The N-terminal domain is a monomer and has a subdomain core region, with a four-stranded antiparallel β-sheet. Some loops protrude from this core; they have a positive charge and they're supposed to bind the RNA. The C-terminal is a homodimer with a rectangular shape formed by some protomers. Each protomer has two β-hairpin structures that form four antiparallel β-strands. In its all conformation, N protein shows a various charge distribution that probably leads the RNA binding [Peng et al, 2020].

1.2.4. SPIKE GLICOPROTEIN

Spike glycoprotein is the main character of the entry process into host cells; it is responsible of receptor recognition and viral attachment. Like the other spike glycoproteins, the Sars-CoV-2 one is transmembrane and creates homotrimers with a particular crown-like halo form, that protrude from the viral surface [Walls et al, 2020]. This trimer is the unit used by the virus for binding the receptor [Walls et al, 2020].

The polysaccharides that coat the S protein hide the virus from the host immune system [Watanabe et al, 2020].

It is a trimeric TM glycoprotein of class I and present in various viruses, like CoVs, influenza, Ebola, HIV or paramyxovirus [Weissenhorn et al, 1999]

The entire protein is composed by an extracellular N-terminal domain (that contains a signal peptide), a transmembrane domain and an intracellular C-terminal domain [Bosch et al, 2003]. It is about 180-200 kDa in size and it's composed by 1273 amino acid residues, that form three subunits: S1, S2 and S2' [Hoffmann et al, 2020].

S1 subunit

S1 subunit is composed by the N-terminal domain (NTD) and the receptor binding domain (RBD). S1 subunit interacts with the host cell membrane through the contact taken with the receptor angiotensin-converting enzyme 2 (ACE2). The S1 subunit recognizes ACE2 on host cells via the receptor binding domain (RBD), that is the most variable part of the whole protein [Yan et al, 2020]. ACE2 is a homolog of ACE, so its role is to convert angiotensin I to angiotensin 1-9. It is distributed in lung, intestine, heart, kidney. The cells that express it for the most are alveolar epithelial type II ones [Zhang et al, 2020]

RBD domain contains two structural domains: the core and the external subdomain. The core subdomain is composed of five β-strands arranged in antiparallel manner, connected by loops and short helices, and a disulfide

bond between two β-strands. This is a highly conserved domain. The external subdomain has a loop stabilized by a disulfide bond [Wang Q. et al, 2020].
RBD has two different conformations: up and down. During the down conformation, the virus can't recognize the ACE2 on the host cell. RDB goes through conformational transitions that hide or expose portions of spike protein in order to engage host receptor [Lan et al, 2020].
RBD has a long receptor binding motif (RBM), around 90 amino acids, that facilitates the bond to the host: there are some critical residues for binding ACE2, in particular: Leu455, Phe486, Gln493, Ser494, Asn501, and Tyr50 [Andersen et al, 2020]. The receptor-binding motif (RBM) is done by loops, α helices and short β strands. In RBD there are nine Cysteine residues, eight of them form four pairs of disulfide bonds. Three of these bonds (C336-C361, C379-C432 and C391-C525) are in RBD core, enhancing the stabilization of the β sheet. The remaining disulfide bond (C480- C488) promotes the connections between the loops in RBM. Two lobes of RBM and ACE2 form the binding site, that is contained within the ACE2 N-terminal domain. RBM binds ACE2 on the small lobe on its bottom side, thanks to its slightly concave inward that creates a sort a chamber for ACE2 [Lan et al, 2020].
There are two S protein forms: closed and open. In the close state the three recognition motifs do not protrude from the interface; instead, in the open state, the RBD domain is the one that protrudes and that is fundamental for the fusion of the virus to the host cell membrane and so for entering into the host cell [Walls et al, 2020].

Sars-CoV-2 has a higher affinity for ACE2 than other coronaviruses, due to some ACE2 residues, some examples are:

- at the F486/L472 position, SARS-CoV-2 F486 interacts with ACE2 Q24, L79, M82, and Y83
- at the Q493/N479 position, SARS-CoV-2 Q493 interacts with ACE2 K31, E35, and H34 (with a hydrogen bond between Q493 and E35)

- on SARS-CoV-2 RBM, there is a salt bridge between ACE2 D30 and SARS-CoV-2 K417 [Lan et al, 2020].
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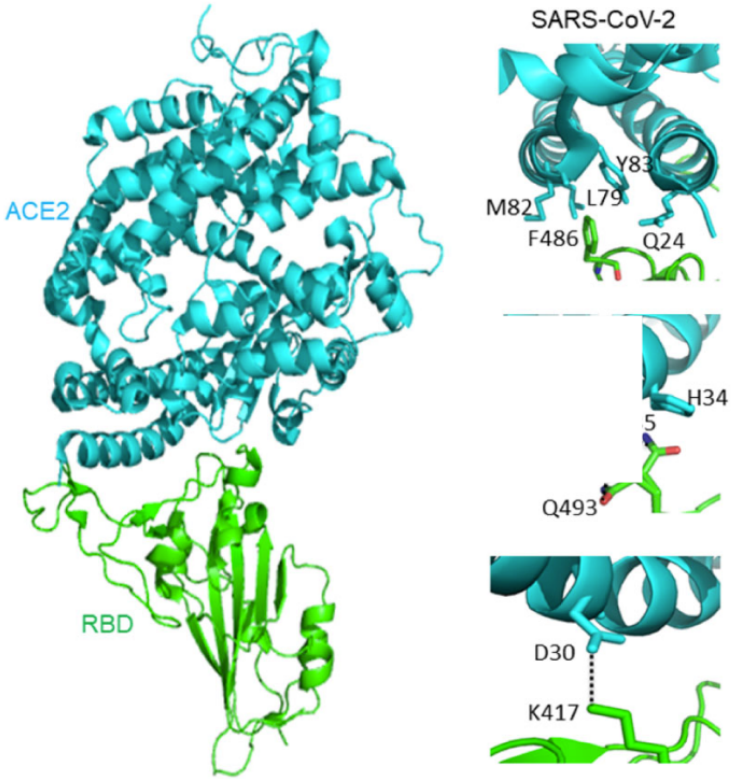


Figure 5. *The overall structure of SARS-CoV-2 RBD bound with ACE2* (Wang M.Y. et al, 2020).

S2 subunit

S2 subunit is the fusion protein that merges with the cell membrane. During this fusion process, it changes in three conformational states: pre-fusion native, pre-hairpin intermediate and post-fusion hairpin [Walls et al, 2020].

It is composed by different domains: fusion peptide (FP), heptad repeat 1 (HR1), central helix (CH), connector domain (CD), heptad repeat 2 (HR2), transmembrane domain (TM), and cytoplasmic tail (CT). The function of this subunit is to fuse the membranes of viruses and host cells [Xia, Zhu et al 2020].

The fusion peptide is 15-20 amino acids long, totally preserved, consisting of hydrophobic residues for the most part, like glycine or alanine, that are useful

for the anchoring to the target membrane when the S protein is in pre-hairpin conformation. Its role is to disrupt and connect lipid bilayers of the host cell membrane to favorite the membrane fusion [Millet et al, 2018].
Heptad repeat 1 (HR1) and heptad repeat 2 (HR2) are composed by a repetitive heptapeptide, which is formed by hydrophobic or bulky residues, polar or hydrophilic residues, and other charged residues [Chambers et al, 1990]. HR1 is at the C-terminus of the fusion protein (FP) and HR2 at N-terminus of the transmembrane domain (TM) [Robson B, 2020].
They form a six-helical bundle that is critical for membrane fusion and entry of S2 [Liu et al, 2004]. This creates the HR1-L6-HR2 complex, that contains most parts of HR1 and HR2 domain and a linker. This fusion protein exhibits a stick-like shape, where three HR1 domains form a spiral-like trimer in a parallel manner and other three HR2 domains are weaved around the spiral center in an antiparallel manner. This is possible due to hydrophobic residues on the HR2 domain that bind with the hydrophobic groove formed by HR1 helices. The main hydrogen bonds are between serine 929 in HR1 and serine 1196 in HR2, a salt bridge between lysine 933 in HR1 and asparagine 1192 in HR2, another salt bridge between aspartic acid 936 in the HR1 and arginine 1158, and a bond of serine 943 and lysine 947 with the glutamic acid 1182 in HR2 through a hydrogen bond and a salt bridge. This structure may result in increased infectivity of SARS-CoV-2 [Xia et al, 2020].
The transmembrane domain (TM) anchors the S protein to viral membrane. The cytoplasmatic tail (CT) is the ending part of the S protein [Tang et al, 2020]. The S protein is the one most susceptible to mutation during replication process. This leads to Sars-CoV-2 variants.

Accessory Proteins

Sars-CoV-2 genome encodes for some accessory proteins, whose role is to interact with the host cells, helping immune evasion. These proteins are

named ORFs, like the ORFs in the genome that encode for them, and are: ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c and ORF10.

ORF3a is well-conserved in all the β-coronavirus. It's formed by three transmembrane regions that create ion channels [Hachim et al, 2020]. It takes part to virus release, inflammasome activation and cell death [Nieva et al, 2012].

About ORF3b, we just know that it is overexpressed, probably due to the activation of some pathways, like ERK and JNK. Its structure is quite complicated and not well known [Yuan et al, 2006].

ORF6 interacts with two mRNA export proteins, RAE1 and NUP98, so maybe it inhibits cellular translation [Gordon et al, 2020].

ORF7a interacts with M, E and S, the ribosomal transport proteins HEATDR3 and MDN1, ORF3a and other accessory proteins. Probably, these viral proteins create complexes in infected cells [McBride and Fielding, 2012]. This protein is 122 amino acid long, it's a type I transmembrane composed by a N-terminal signal peptide, an ectodomain, a C-terminal transmembrane domain and a cytoplasmatic tail [Huang et al, 2006].

ORF8 has a core of 60 amino acids and probably interferes with immune response, due to its high mutation frequency [Laha et al, 2020].

ORF9b is a dimeric protein with β-sheet structure, has an amphipathic surface and a hydrophobic tunnel that binds lipids. It seems to interact with ER and Golgi during virions assembly [Meier et al, 2006]. ORF9b binds to Tom70, a mitochondrial receptor, causing loss of mitochondrial import efficiency and probable mitophagy [Gordon et al, 2020b].

ORF9c probably modifies host mitochondrial activity, but is not well known [Gordon et al, 2020b].

About ORF10, it' s just known that encodes for a 38 amino acids peptide [Wu et al, 2020].

1.2.5. THE REPLICATION COMPLEX AND THE RNA-DEPENDENT RNA POLYMERASE (RDRP)

The replication process of Sars-CoV-2 is leaded by a replication/transcription complex that works with a multiunit mechanism. It is composed by non-structural proteins, like Nsp 1-5-13 and a core complex, consisting in another Nsp, the Nsp12, which contains the RdRp. RdRp is targeted for RNA synthesis due to its D623, S682, and N691 residues that interact with the 2'-OH group of the triphosphate.

Nsp12 has little activity on its own, but it also requires accessory proteins, like Nsp7 and Nsp8, which with their presence increase the interaction of Nsp12 and template-primer RNA [Gao et al, 2020]. In Nsp8 there are some α-helical extensions that interact with RNA with their positive residues [Hillen et al, 2020]. The RNA-dependent RNA extension activity starts when an RNA substrate gets inside the complex Nsp12-7-8. The association of Nsp 7-8-12 and the RNA template is the main component of the Replication-Transcription Complex [Gao et al, 2020].

Nsp12 contains a N-terminal extension domain, which is a nidovirus RdRp-associated nucleotidyltransferase (NiRAN), an interface domain and an RNA-dependent RNA polymerase in the C-terminal domain. N and C domains are connected by an interface domain. In the N-terminal extension domain there's a β-hairpin structure, between the V31-K50 residues, that is unique and highly conserved. It forms links with the NiRAN and the palm subdomain to stabilize the full structure [Yan et al, 2021].

The C-terminal domain, which contains RNA-dependent RNA polymerase, has a shape similar to a hand. Indeed, it consists in three subdomains: fingers, palm and thumb. There are also some conserved structural motifs. The Nsp7-8 heterodimer binds the thumb subdomain and stabilizes Nsp12. The second Nsp8 of the heterodimer takes contacts also with finger and interface domain [Jiang et al, 2021].

The RdRp-RNA complex contains an extended protein region in Nsp8 and a protruding RNA. When the RNA template arrives, the fingers and thumb subdomains of Nsp12 are responsible for its binding [Hillen et al, 2020].
The palm subdomain and the NiRAN domain form a clamping groove, inside which the β-hairpin structure inserts. Inside the palm subdomain, there are also some polymerase motifs (A−E) that form the active site of RdRp domain. These motifs mediate the RNA synthesis in a central cavity through four positively charged paths: the template entry path, the primer entry path, the NTP entry channel and the nascent strand exit path [Gao et al, 2020]. The Motif C of the active site, that is one of the Nsp12 motifs inside the palm subdomain, is formed by two aspartic acid residues in 760 and 761 positions. Its role is the interaction with the 3′ end of the RNA [Hillen et al, 2020]. The afore mentioned residues, D760 and D761, coordinate two Mg^{2+} ions fundamental for polymerase activity. One of them coordinates Motif C, as it is this single residue the one that binds the 3′ of the RNA template. Instead, the second Mg get into position the incoming NTP [Wang et al, 2020].
Instead, the Motifs F and G are into the fingers subdomain, and they get the RNA template in place. [Hillen et al, 2020].
The RNA product exits from the active site and extends to two Nsp8 N-terminal helices, that are positively charged and like platforms where the RNA slides [Hillen et al, 2020].
The replication/transcription complex needs some accessory factors during the elongation, because the work has to be processive, without product dissociation, efficient and with the least amount of mistakes, all inside the host cell. For the Coronavirus, it's a really hard condition to maintain, because they possess the largest RNA-positive-sense among all viruses [Choi, 2012].
One of these accessory factors is Nsp13, that works as a helicase to unroll the double strand RNA, destroying its secondary structure before it enters in the active site [Chen et al, 2020]. The RdRp translocates in 3′-5′ direction on template RNA strand; instead, Nsp13 translocates in the opposite direction, 5′-3′. Nsp13 facilitates reverse RNA translocation and perhaps it enhances the

correct action of RdRp [Malone et al, 2021]. Probably, Nsp13 is also involved in mRNA capping. Its zinc-binding, stalk and 1B domains are important for helicase activity [Ivanov and Ziebuhr, 2004].

From the newborn RNA, the mRNA is synthetized. The mRNA must be modified with capping process, as it is crucial for viral propagation and immune escape [Daffis et al, 2010]. The mRNA capping is done by RTC with NSPs and is divided in some steps:

- removal of the γ-phosphate of 5′-pppA by Nsp13
- transfer of GMP to 5′-ppA by the Nsp12 NiRAN domain with GTase activity
- methylation of N7-guanine by Nsp14, which has N7-methyltransferase activity
- methylation of the ribose 2′-O nucleotide into the final cap structure by Nsp1, which has 2′-O-methyltransferase activity

After these modifications, Nsp9 terminates the capping reaction by insertion into the NiRAN catalytic center and inhibition of the GTPase activity. Nsp9 is also an RNA-binding protein, thanks to its positive-charged residues, and together with the β-hairpin at N-terminus of Nsp12, they create an exit for the new capped mRNA [Bouvet et al, 2010].

Another important mechanism in SARS-CoV-2 replication is handling errors to preserve the genome from mutations. Nsp14 is the exonuclease with proofreading activity that has this role. After Nsp14 finds the mismatched new RNA duplex, the mismatched nucleotide comes back into the RdRP tunnel. There Nsp13 stimulates the RdRP correction activity, possibly with its helicase activity it can help RdRP during this process [Malone et al, 2021].

1.2.6. PATHOGENESIS

The N-terminal of ACE2 interacts with the RBM and triggers the fusion. In these interactions 16 residues of the RBD and 20 residues of ACE2 are involved, for a total of one salt bridge and 14 hydrogen bonds [Lan et al, 2020]. S1 and S2 subunits remain non-covalently bound in the prefusion conformation. Between the two subunits, there's the S1/S2 protease cleavage site. After RBD binds ACE2, host proteases cleave the S protein, creating the two subunits, that will remain in noncovalent form until viral fusion happens. The creation of this subunits is critical to fuse the membranes of virus and host cell, leading them through irreversible conformational changes. When we talk about "viral fusion", we refer to the fusion of viral and host membrane, after which the viral genome is released into the host cell [Tortorici et al, 2019]. Here the host serine protease TMPRSS2 is used as a protein primer that activates the S protein. This is a common mechanism for all coronaviruses [Andersen et al, 2020]. Trypsin is another host cell protease, that like TMPRSS2 cleaves S protein [Ou et al, 2020].

S1 and S2 subunits are bonded in a furin cleavage site, that is unique for Sars-CoV-2; indeed, it is not present in other coronaviruses, like Sars-CoV or SARS-CoV related group 2b betacoronaviruses, which leads to a higher virulence [Coutard et al, 2020]. This furin cleavage site includes four residues: P681, R682, R683, and A684, that are kind of a classical polybasic furin cleavage site [Li W, 2020]. After the cleavage of the S protein, the two subunits stay associated through non-covalent interactions [Wrapp et al, 2020].

When RBD binds ACE2, S1 dissociates from the structure and the RBD is locked in up conformation [Xia et al 2020]. In the meantime, S2 refolds into a stable post-fusion conformation [Wrapp et al, 2020]. This S2 change of conformation happens by inserting the FP portion into the targeted cell membrane. Here the HR1 domain exposes its pre-hairpin coiled-coil and starts the interaction between HR1 and HR2, creating the HR1-L6-HR2 complex that we were talking about before. Then, viral envelope and host cell membrane

are brought into proximity to favorite viral fusion and entry into the host [Xia et al, 2018]. So, the viral membrane binds in a really tight way the host cell membrane and the two membranes fuse [Eckert and Kim, 2001].

After the fusion, virions enter the host cell and viral RNA attaches to the host ribosomes. This way, the virus can translate its proteins; specifically, it starts with the translation of 2 co-terminal and large polyproteins, that are later processed by two proteolytic enzymes, 3CLpro and PLpro. The results are little components for the packaging of new virions [Graham et al, 2008] 3CLpro, PLpro, main protease and RdRp are the main enzymes involved in proteolysis, replication, and production of new virions [Tong, 2009].

When viral proteins are produced, every new virion is packaged with a S protein heavily N-glycosylated, M protein in dimeric conformation that maintains the virus shape and the E protein with its ion channels. The N protein binds new viral RNA and forms the nucleocapsid [Nieto-Torres et al, 2014]. At the end, the viral RNA is replicated and all the structural viral proteins are synthetized, so new virions are assembled and released from the host cells [Fehr et al, 2015].

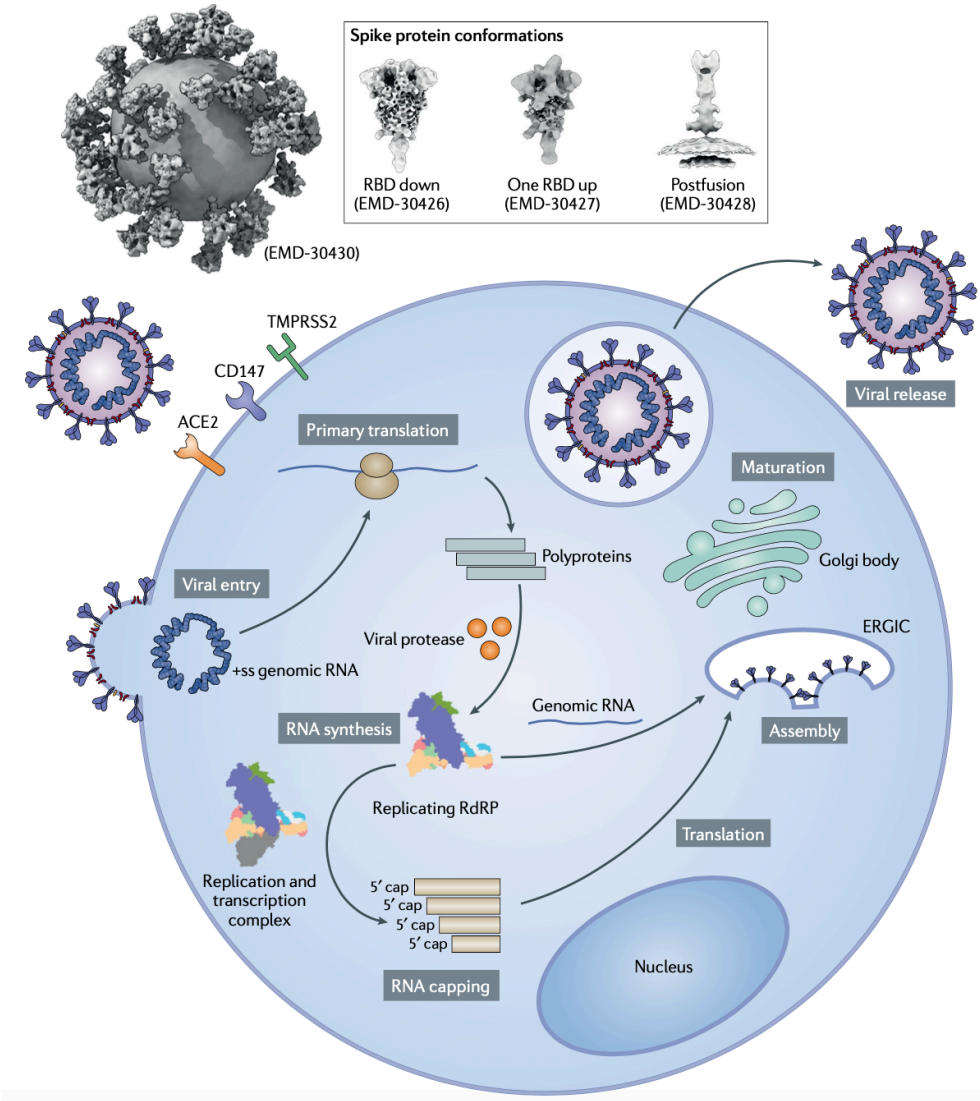


Figure 6. *The life cycle of SARS-CoV-2* (Haitao and Rao, 2021).

After bond with ACE2, this receptor is downregulated by the virus to favorite the production of AT2 (Angiotensin-2). AT-2 increases pulmonary vascular permeability, causing lung damage. The ACE2 receptor are highly distributed not just in alveolar epithelial cells, but also in other tissues like heart, kidney, endothelium, and intestine, leading to multi-organ dysfunction [Zhang et al, 2020].

Common mechanisms to all the CoVs influence various processes inside the host cell, like cytocidal activity. The infection leads to apoptosis and cell lysis. After the virus invade the host cell, there's the formation of syncytia and the formation of vesicles, which role is to create the replication complex and

provide for the disruption of Golgi. Even some Pattern Recognition Receptor (PRRs) recognize pathogen molecules or molecules released by damaged cells and modulate innate and adaptive immune response to the infection [Morse et al, 2020].

After virus replication, the virions migrate to the airways and enter alveolar epithelial cells of the lungs. This leads to a big immune response, where a cytokine storm syndrome causes acute respiratory stress and at the end respiratory failure, that causes the death of the patient [Huang C et al, 2020]. There are also evidences of multiple organ failure [Yao et al, 2020]. Viral particles were detected in the upper airway, bronchiolar epithelium and submucosal gland epithelium, as well as in type I and type II pneumocytes, alveolar macrophages and hyaline membranes in the lungs [Zeng et al, 2020]. Some histopathological analyses showed bilateral alveolar damage, pneumocytes desquamation, hyaline membrane formation and fibrin deposits, sometimes also exudative inflammation, in patients with severe infection: these are all pathological signs of Acute Respiratory Distress Syndrome [Martines et al, 2020].

Immune Response

As we said before, host immune response is dysregulated. The recognition of the RNA virus by the PRRs activates signaling cascades, with resulting cytokine storm, which is the main reason for excessive inflammation and possible death of the patient [Moens et al, 2020]. The immune response and inflammatory pathways are influenced by the action of some accessory proteins, like ORFs 8a, 9b, 7a, 6, 3a. ORF8a and ORF 9b cause cellular apoptosis and alters interferon responses by degradation of mitochondrial antiviral proteins. ORF7a activates NF-kB. ORF6 limits interferon production. ORF3a causes necrotic cell death. At the end, we can see that these ORFs take control of a lot of host pathways and in this way decide the progression of the infection [Shi et al, 2014]. The cytokine storm shows high levels of pro-inflammatory

cyotkines like IL-1, IL-2, IL-6, IL8, IL-17, G-CSF, GM-CSF and chemokines such as IP- 10 and MCP-1. This kind of response possibly generates an accumulation of immune cells in the lungs, leading to their disfunction [Shi et al, 2020]. Furthermore, IL-6 receives a negative feedback mechanism by SOCS3 (Suppressor of Cytokine Signalling 3), because at high concentration it recruits inflammatory monocytes [Okabayashi et al, 2006]. The immune cells accumulated in lungs are for the major part lymphocytes. The T-cells became over activated, so the T CD4+ release high concentrations of pro-inflammatory cytokines and TCD8+ stars to accumulate a lot of cytotoxic granules, leading to a heavy immune injury. Alveolar macrophages and epithelial cells are attacked by the virus and a sustained lung inflammatory response takes place [Li et al, 2020].

When the virus shows its clinical expression, it has already reached the peripheral blood from the lungs. The infection becomes then acute because the patient has a limited levels of T and B lymphocytes. At the end, the inflammatory cytokines are really high and so the coagulation parameters. This can lead to an excessive coagulation cascade, resulting in Disseminated Intravascular Coagulation (DIC) [Lin et al, 2020].

1.3. DIAGNOSIS AND TREATMENT

1.3.1. SARS-COV-2 DIAGNOSIS

The maneuvers of containment for the pandemic were recommended by Center for Disease Control and Prevention and WHO. The first step was the screening of symptomatic and asymptomatic patients, made on upper respiratory samples like nasopharyngeal/oropharyngeal/throat swabs for asymptomatic people and on lower respiratory samples for symptomatic patients, like sputum or bronchoalveolar lavage. In the case of a patient positive for Covid-19, both upper and lower respiratory samples will have a high viral load that will follow the disease progression [Riley et al, 2003]. Other samples analyzed are whole blood and urine or saliva. For the first two kind of samples, there isn't an approved diagnosis test that is rapid and effective. Instead, saliva is applied for surveillance and seems to have a rate of detection as good as the nasopharyngeal swabs [To et al, 2020].

Real-Time Reverse Transcriptase Polymerase Chain Reaction

RT-PCR is actually the gold standard method for SARS-CoV-2 detection, actually there are common laboratory methods approved by the WHO, the Infectious Diseases Society of American and the Centers for Disease Control and Prevention [Carpenter et al, 2020].

The clinical samples are obtained usually from nasopharyngeal swabs. The molecular kits for SARS-CoV-2 detection target the main genes of the virus, like S (Spike), N (Nucleocapsin), E (Envelope) or RdRP. The SARS-CoV-2 could be detected from a few kinds of samples, like saliva, sputum, bronchial fluid, but was demonstrated that lower tract samples have a minor viral load, so the detection is more difficult and sensitive [Zou et al, 2020].

From this samples, the viral RNA is extracted and processed with a RT-PCR. Now there are lots of extraction and RT-PCR kits approved and with CE-IVD

(In Vitro Diagnostics Certified). The RT-PCR is a sensitive assay with first a retro-transcription phase to generate cDNA from the viral RNA and then a REAL TIME PCR to amplify and detect the viral genes. Actually, is the more sensitive assay for SARS-CoV-2 detection, but it's also expensive, time-consuming and requires a good laboratory preparation by sanitary workers [Yip et all, 2020].

The sensitivity reaches the 100% when the viral RNA is about 500/5000 copies/ml. This obstacle has been overcome thanks to the new RT-PCR kits, but the timing of the test is crucial. Immediately after exposure, the test must be very sensitive to detect very low amounts of viral load, 2-3 days after the onset of symptoms, the viral load will be very high and therefore it will be easier to detect the virus [Yohe, 2021].

Sars-Cov-2 Rapid Antigenic Tests

This type of test was created to cover the need for an out-of-control number of screening tests, especially urgent ones, where the PCR takes too long to produce a result. This method detects viral proteins, after a sample collection identical to that used for molecular detection, in other words, a nasopharyngeal swab. The swab is treated with a lysis and running buffer, which is then inserted into a card that allows the detection of viral antigens, in case of positivity.

The results are available in about 15 minutes and the test shows a good sensitivity, even at low viral loads. Considering that we are talking about an antigenic test, it is clear that sensitivity will be higher in cases where the PCR sample shows a CT<30, at very high CTs sensitivity will be lower and false negatives may occur [Brümmer et al, 2021].

Radiological Tests

The main radiological tests used to control Covid-19 disease are Computed Tomography and X-Ray. A normal chest X-ray could be detected in a good percentage of Covid-19 patients, but later could change towards abnormal patterns [Wong et al, 2020].

The Computed tomography is really sensitive in detecting pulmonary anomalies and seems useful when a X-ray is negative. Even the TC could be negative and then change during the disease progression. A typical CT pattern of a Covid-19 patient shows lungs with opacity and shadows, pleural effusion lymphadenopathy and when the disease is progressed, even lesions [Pan et al, 2020].

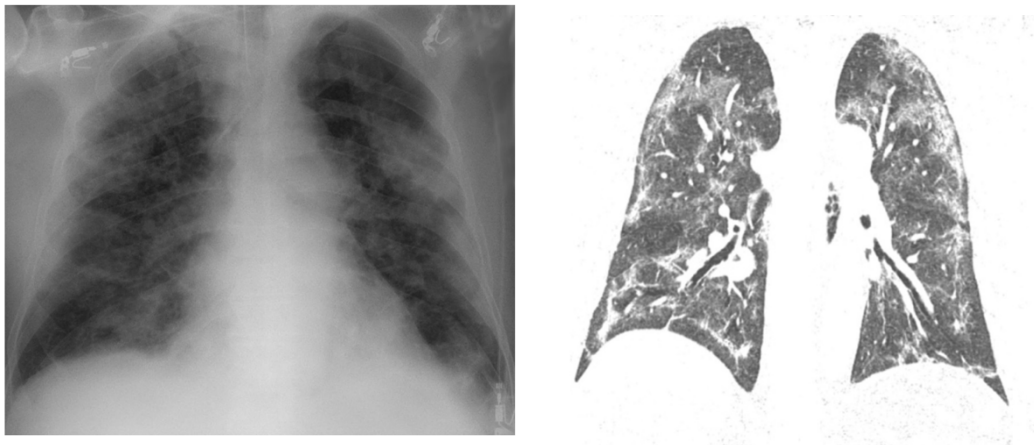


Figure 7. *Chest X-Ray and CT images of a Sars-CoV-2 patient* (Long et al, 2022).

1.3.2. THERAPEUTICS

There isn't a real drug therapy for Covid-19, on a first line, there's a symptomatic approach or ventilation assistance for severe cases. General prevention lines were diffused at the beginning of the pandemic, like use of masks, correct hand hygiene, avoid touching every. To find an effective drug, WHO created an international clinical trial as a common global platform, named SOLIDARITY. Some treatments were just tested and gave some benefits to certain categories of patients

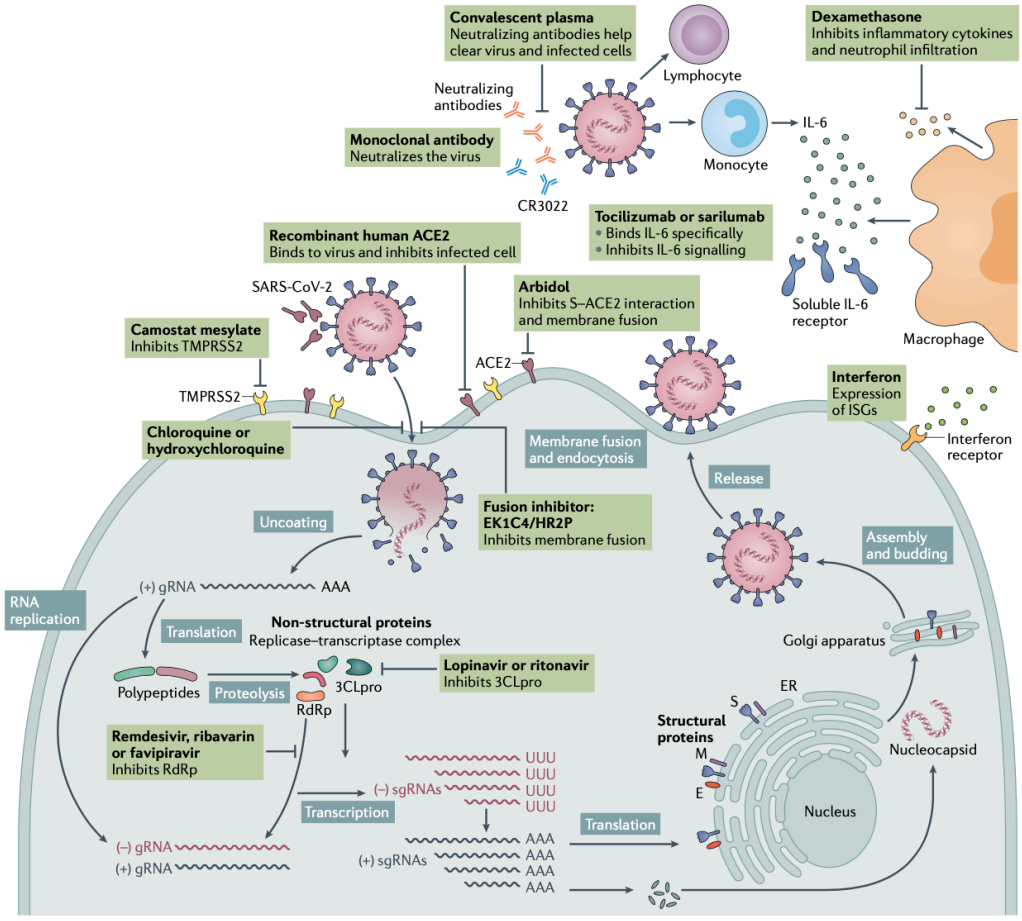


Figure 8. *SARS-CoV-2 replication and potential therapeutic targets* (Hu et al, 2021).

Inhibitors Of Virus Entry

These are drugs that interfere with the interaction between the S protein and ACE2. For example, the Umifenovir is approved in China and Russia, used for respiratory viral infections. It showed to be more effective than other drugs like Lopinavir and Ritonavir [Li Y. et al, 2020]. Chloroquine and hydroxychloroquine are used in treatment of autoimmune disease, like lupus erythematosus and rheumatoid arthritis. There were clinical studies that suggest that they could increase the risk of cardiac arrest in treated Covid-19 patients, in fact the FDA revoked their emergency use [Geleris et al, 2020].
CR3022 is an antibody discovered in SARS-CoV-2 infected patients' plasma, it can bind RBD. If used in combination with CR3014, that is a powerful SARS-CoV neutralizing antibody, neutralizes SARS-CoV-2 because the two antibodies can bind different epitopes on RBD [Tian et al, 2020]. B38 and H4 are MAbs isolated from convalescent patients that bind different epitopes on S protein RBD to compete with ACE2. They have the capability to reduce viral load in infected lungs [Wu Y et al, 2020].

Inhibitors Against Structure Or Virus Replication

Replication inhibitors used are Lopinavir and Ritonavir that inhibit 3CLPro or Remdesivir, Favilavir and Ribavirin that target RdRP [Ul Qamar et al, 2020]. The Remdesivir was authorized by FDA for emergency use in severe Covid-19 cases and approved by the European Union for treatment of pneumonia requiring supplemental oxygen. This drug is in continuous evaluation in clinical trials for Covid-19 treatment [Beigel et al, 2020]. NIH recommends another drug in combination with Remdesivir, the Baricitinib, for patients treated with mechanical ventilation. The Baricitinib is an anti-inflammatory drug that inhibits selectively JAK-1 and 2. According to some studies, it leads to a better oxygenation and to a diminution of inflammatory markers [Kalil et

al, 2020]. Favilavir is an antiviral drug created in Japan to treat influenza. In China, India and Russia is approved for Covid-19 treatment [Cai et al, 2020].

Immunomodulatory Therapy

This kind of drugs can reduce the excessive inflammatory response caused by Covid-19. One typical agent is Dexamethasone, a corticosteroid with anti-inflammatory and immunosuppressive effects [Recovery Collaborative Group et al, 2020]. General anti-inflammatory drugs, like ibuprofen or cortisone, are used to control infection or symptoms [FitzGerald, 2020]. The drugs that aren't recommended for Covid-19 treatment are the Non Steroids Anti-Inflammatory Drugs, that seem to make worst the infection because they block the immune system action. On the contrary, paracetamol was tested and didn't show a significant role in suppressing the virus [Capuano et al, 2020].
Tocilizumab and Sarilumab are two monoclonal antibodies against IL-6 receptor, that in severe Covid-19 treatment showed the capacity to reduce the cytokine storm [Xu X. et al, 2020].
An addition therapy for severe Covid-19 is convalescent plasma, for which the FDA gave some guidance. Is a plasma taken from totally recovered patients and reinfused in infected patients. This kind of treatment was used in 2014 with Ebola. The main problems are the possibly adverse effects linked to immune reactions, like allergies or an enhanced infection mediated by antibodies, or the risk of pathogen transmission. Another obstacle is the limited availability and the impossibility of amplification. Is just an adjunctive therapy [Duan et al, 2020].

Vaccines

If we consider that there isn't a therapy specific for Covid-19, vaccines are the unique kind of long prevention therapy to help the global immune system to respond to the pandemic. The strategies developed to produce vaccines

included DNA, mRNA, inactivated or live attenuated viruses, recombinant vectors, protein subunits. In October 2020, there were 177 vaccine candidates for Covid-19, some of them were developed and then used.

Pfizer/BioNTech developed a mRNA vaccine, named BNT162b2 (Comirnaty). The vaccine contains a mRNA that encodes for a SARS-CoV-2 Spike protein, in a prefusion and stabilized conformation, is membrane-anchored and full length. The complete vaccination required two doses and even if the response depends by single characteristics of a person, it showed the capacity to prevent Covid-19 at the 95%. The side effects were pain at the injection site and fever, fatigue and headache [Polack et al, 2020].

Moderna developed the mRNA-1273 vaccine, that stimulates the expression of target antigen by the injection of the mRNA that is encapsulated into nanoparticles [Amanat and Krammer, 2020]. The mRNA strand is synthetic and encodes for a spike protein in the stable prefusion form, that consents to the immune system to active an antiviral response. The nanoparticles are lipidic and synthetized without the virus, protecting the host from any type of infection risk [Jackson et al, 2020]. The mRNA-1273 went over all the clinical trial phases and actually is one of the vaccine used against Covid-19.

1.4. SARS-COV-2 VARIANTS

SARS-CoV-2, like the other viruses, evolves with continuous changes in transmissibility and virulence, trying to survive inside the hosts and to favorite the transmission. Usually, in viruses a higher transmissibility is associated with lower virulence. This means that a virus evolves becoming fatal just for susceptible individuals, because the others acquire total or partial immunity to the infection [Enard et al, 2016]. SARS-CoV-2 evolved keeping some new mutation involved in transmissibility and immune escape. Its virulence is now influenced by a few elements, like age or comorbidities, but the transmissibility became higher in mild or asymptomatic cases. Its selective advantage became the lower virulence, thanks to the choice to favorite a high rate of transmissibility and so a preference for a upper respiratory infection [Willett et al, 2022]. The mutations accumulated by the variants generate and adaptation to the populational immunity, that permitted to the SARS-CoV-2 to spread and react to therapies and vaccines [Bushman et al, 2021].

At the beginning of the pandemic, for all the 2020, SARS-CoV-2 evolved really slowly. But in December 2020, when the first variant was detected, started to change rapidly. From that moment, were distinguished some Variants of Concern and Variants of Interest.

The Variants of Concern are more dangerous in terms of virulence, symptomatology and resistance to vaccines and therapies. They are those that become established both in terms of territorial distribution and temporal permanence. The VOCs until now are: the Alpha or English variant, the Beta or African variant, the Gamma or Brazilian variant, the Delta or Indian variant and the Omicron variant with its derivates.

The Variants of Interest, on the other hand, are variants with minor mutations, which may influence the characteristics of the virus, but not incisively. The VOIs are: Lambda or C.37, Mu or B.1.621, Iota or B.1.526, Kappa or B.1.617.1 and Epsilon or B.1.427/429 [Harvey et al, 2021].

The mutations that generate the variants involve Spike protein, in particular its RBD and N-terminal domains and the furin cleavage site. The RBD mutation can enhance the affinity for ACE2 receptor. Other mutations are localized in regions bound by neutralizing antibodies, so this favorites immune escape [Greaney et al, 2021].

In the early 2020, the first mutation detected in SARS-CVoV-2 Spike protein was D614G, that is located in the S1 subunit [Chakraborty et al, 2021]. This mutation, by a clinical point of view, seems to be involved in the maintenance of the anosmia, that became the common symptom of the first variants until Omicron, where seems to compare less often in patients [Von Bartheld et al, 2021]. This mutation is shared by Alpha, Beta, Gamma, and Delta variants [Weissman et al, 2021].

Another important mutation is N501Y, that was the second significative Spike mutation showed by the variants, it was detected for the first time in England [Luan et al, 2021]. It became the most common mutation in all the variants, from Alpha to Omicron. The N501 aminoacidic residue is present on the Spike RBD and is involved in the bond with ACE2, so this mutation increases the binding affinity through the addition of another π-π packing between the 501Y of RDB and the 41Y of human ACE2. Not all the mutations detected in the variants can enhance or favorite the bond between RBD and ACE2, like K417N, that seems to compromise this link. Mutations like N501Y, D614G, L452R, and P681R enhance transmissibility and increase viral replication [Gupta et al, 2021].

HV69/70del is a mutation that increases binding affinity to ACE2 and so the fusion to the host cell. Its presence, in combination with other mutations like N501Y or D796H, can boost the infectivity level through a compensation mechanism that strengthens the link with ACE2 [Meng et al, 2021].

P681H/R is a mutation located in the furin cleavage site that enhances the fusion between virus and host. Both of the amino acidic substitutions have this result. The difference is that P681H is founded in Alpha and Theta, P681R in Delta and Kappa [Garcia-Beltran et al, 2021].

At the same time, some mutational studies have shown that certain mutations are able to elude antibody binding. K417N, E484K, N501Y and L452R have this capacity [Bates et al, 2022].

The crossover between immune escape and faster replication brought to diversity in pathogenesis. The transmissibility is the factor that increased in particular, starting with an increase between 43% and 90% for the Alpha to Delta variants and arriving at Omicron, which clearly outperformed Delta in transmissibility, reaching an increase of 95%, even in the vaccinated population. This suggests that the real advantage that has allowed the omicron variant to persist over time with the development of its sublineages is precisely its ability to evade vaccines [Dong et al, 2022].

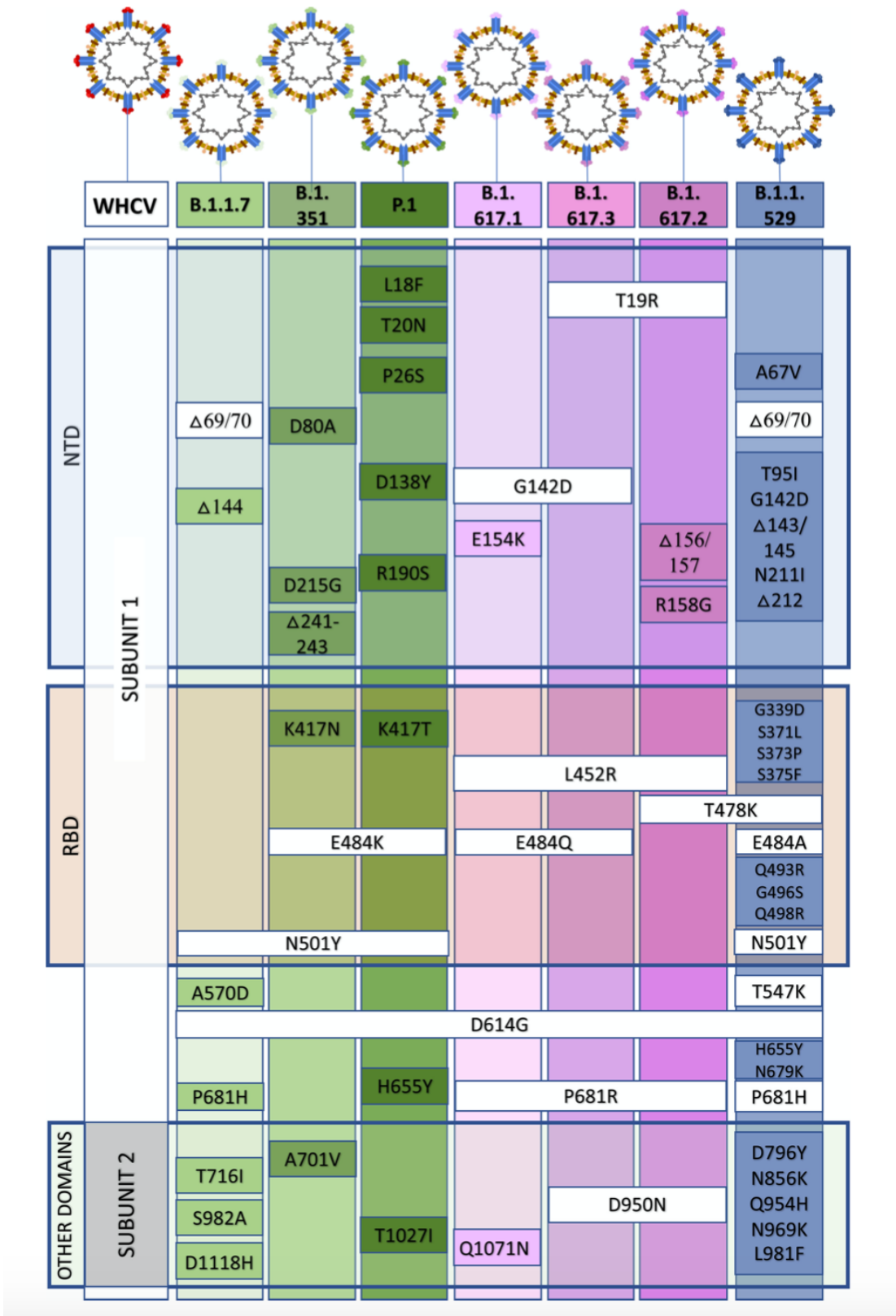


Figure 9. *Mutations in the Spike protein from the VOCs* (Hirabara et al, 2021).

1.4.1. B.1.1.7 – ALPHA VARIANT

It was detected in December 2020 in United Kingdom and it was characterized from a lot of unexpected mutations, all in one. This mutation seem to come from immunocompromised patients with chronic infection [Corey et al, 2021]. It contains 14 nonsynonymous point mutations and three deletions. Eight of these are in the Spike protein: ΔH69/ΔV70, ΔY144, A570D, P681H, T716I, S982A, N501Y and D1118H. Each one of them has a particular role for virus survival [Meng et al, 2021]. P681H is located in furin cleavage site and enhances the infectivity [Harvey et al, 2021]. From April 2021 to June 2021, the Alpha variant rate reduced in favor of newborn variants. The transmission became more efficient, while the risk of death decreased not so much but the hospitalization rate increased [Davies et al, 2021]. Instead, there isn't a good number of mutations in the region bound by neutralizing antibodies, so in this variant the immune escape isn't so much pronounced [Shen et al, 2021].

1.4.2. B.1.351 – BETA VARIANT

The Beta variant was first detected in South Africa in October 2020 and spread until April 2021. This variant showed a fast domestic spreading. It has a few mutations in Spike protein, like R264I, D80A, L18F, D215G, and A701V. Some of them are in the RBD, like K417N, E484K, and N501Y. In particular K417N, E484K and N501Y are responsible of resistance to immunity and vaccines [Harvey et al, 2021]. Even this variant decreased since April 2021, in the meantime it has spread creating new sublineages, named B.1.351.1, B.1.351.2 and B.1.351.3, everyone with other mutations accumulated. The transmissibility increased of the 50% and so the hospitalization [Tegally et al, 2021].

1.4.3. P.1 – GAMMA VARIANT

This variant was detected in January 2021, it seemed to come from Brazil. The Gamma variant presents the same mutations as Beta variant that give immune resistance, in particular, the mutation E484K is shared. In addition, there are some mutations in Spike protein unique for Gamma: L18F, T20N, P26S, D138Y, R190S, H655Y, and T1027I. Also the Gamma evolved in several sublineages, that are P.2 (Zeta) and P.3 (Theta). The Theta variant, for example, has just the E484K mutation [Faria et al, 2021]. Gamma variant was analyzed by an evolutionary point of view, that revealed how this variant is such a mixture of mutations added in subsequent infections. The place where all of this happened is the state of Amazonas in Brazil, where the sanitary intervention is really poor, this favored the increased transmissibility of the variant [Naveca et al, 2021]. The hospitalization rate increased and so the death cases [Paredes et al, 2022].

1.4.4. B.1.617.2 -DELTA VARIANT

The Delta variant was found in India at the end of the 2020, but was declared as a VOC on May 2021. In this variant, there are new mutations in the Spike protein, like T19R, L452R, T478K, D614G, P681R, and D950N. L452R mutation, together with Y453F, are the responsible for immune escape [Motozono et al, 2021].

But the different thing is the presence of multiple mutations in the N-terminal domain. It was detected in India and Turkey, but there were cases also in Vietnam, UK and Russia. Another Delta sublineage was detected in USA, the New Delta Plus Variant or B.1.617.2.1, which differences from primary Delta for the K417N mutation. This isn't the only one Delta subclade, there were some other sublineages that spread in epidemic areas, like in Asiatic region, on example is the Clade 20I [Mlcochova et al, 2021].

Delta variant has a higher rate of transmissibility, due to a faster replication and a major virus entry mediated by Spike [Challen et al, 2021]. Its particular mix of mutation, lead Delta variant to be more infective, to have better viral mechanisms of entry and replication [Motozono et al, 2021]. The pathogenicity seemed to be higher than Alpha variant, but just in terms of hospitalization, the death rate was instead lower [Twohig et al, 2021].

1.4.5. B.1.1.529 – OMICRON VARIANT

Omicron variant or B.1.1.529 was first detected in November 2021 in South Africa and it rapidly reached Hong Kong. It spread really fast until it became the major variant in the world. The spread of this variant has been particularly fast. From South Africa and Botswana, it spread to Great Britain, Denmark and Norway, Canada, France, Spain, Iceland, Ireland, India, South Korea, Singapore, Hong Kong, Mozambique and Australia by 15 December 2021. [Thakur and Ratho, 2022].

It shows significant immune escape and difficulties in disease control. The unique advantage was the decrease of the disease severity [Shuai et al, 2022].

The rate of mutation is the highest in this variant, it totalizes 18 261 mutations, 97% of which are in the coding region [Bansal et al, 2021]. Thirty mutations are in the Spike RBD, two in particular are in common with Delta variant like K417N that alters Spike structure and enhances immune evasion. Another one is T478K, that increases RBD binding affinity through steric interference and higher electrostaticity. The L452R mutation isn't present in this variant [Quarleri et al, 2021]. N501Y and Q498R generate a strong binding affinity, getting easier for the virus to access into the host [CDC, 2021]. H655Y and N679K are typical of Omicron variant and they are localized near furin clevage site, that enhances Spike cleavage and so infectivity, P681H has a similar role but is common with other variants [Hossain et al, 2020]. At the residue 484, there's a different mutation compared to Beta and Gamma variants. Here the glutamic acid is substituted with an alanine. In Beta and Gamma the E484K

mutation causes reinfection, in Omicron the substitution of a negative residue with positive one seems to alter the bond between RBD and ACE2 [Kannan et al, 2021]. Q493R, N501Y, S371L, S373P, S375F, Q498R e T478K are RBD mutations responsible of the stronger bond with ACE2 [Wang and Cheng, 2021]. Other mutations present in RBM with the role of increasing affinity for ACE2 are at positions P499, F486, A475 and L455 [Yi et al, 2020].
This high quantitative of mutation created a problem at the beginning for the PCR detection, that often gave false negatives. The obstacle was solved when the variant showed a drop out of the S gene [Ganesan et al, 2021].

From BA.1 To BA.5 – Omicron Variant Derivates

From the omicron variant, three different lineages formed simultaneously, almost immediately after the appearance of the variant. They were named BA.1, BA.2 and BA.3 [Haseltine et al, 2022]. Shortly afterwards, two more lineages were formed, named BA.4 and BA.5. BA.1 was the first lineage to spread around the world, remaining the only one for a good while. Shortly afterwards, it was overtaken by BA.2. The more recent BA.4 and BA.5 lineages were initially identified in Belgium, China, Portugal, France, Botswana, Australia and Germany, probably the Omicron lineage from which they originated appeared around November 2021. Regarding the mutation framework, BA.1 and BA.2 have several in common, except for 13 specific for BA.1 and 8 specific for BA.2. The Spike protein of BA.4 and BA.5 is very close to the amino acid composition of BA.2. However, the two most recent lineages show mutations that are not present in BA.2, such as Del69/70, L452R, F486V. The composition of the 5' region of the genome also appears to be stackable between BA.4 and BA.5, so the E gene is similar. Differences are found in the 3' region, especially at the level of the M gene [Tegally et al, 2022].
Considering that the Omicron variant has remained the only one to have become firmly established and spread with its lineages, several phylogenetic studies have been carried out to understand the evolution of this variant. The

variety of mutations present in the lineages has made it difficult to understand the evolutionary process of the variant, leading mainly to three hypotheses. According to the first one, the Omicron variant simply spread uncontrolled. The second hypothesis assesses that the lineages may have originated from immunocompromised patients affected by long-Covid, which gave the virus numerous replication opportunities, so it accumulated mutations. The last option assesses that the host that has accumulated mutations is an animal that has then retransmitted the infection to humans. Certainly, the most probable hypothesis turns out to be the second one, first of all because in the literature there are already cases of mutation accumulation in immunocompromised patients. Furthermore, Omicron originated on the African continent, where the health situation is severely compromised and the population has poor collective immunity and HIV infections are widespread [Mallapaty, 2022]. With regard to clinical outcome, even Omicron lineages showed lower risk of developing severe forms of the disease, hospitalization and death [Menni et al, 2022]. In this scenario, BA.2 causes more severe disease than BA.1 [WHO, 2022]. As a transmission capacity, the BA.2 lineage is more infectious than BA.1. The BA.4 and BA.5 lineages exceed both BA.1 and BA.2 as a transmission capacity, probably due to the presence of the F486V mutation [Tegally et al, 2022].

Several sublineages have formed from these lineages, generating an immense branch of the Omicron variant. Between July and December 2022, the BA.5 variant became dominant. From that moment further sublineages were born, so much so as to make talk of a "variant soup" [Callaway, 2022]. Over the course of one year, BA.4.6 (from BA.4), BA.2.75 (from BA.2), XBB (from the sublineages of BA.2) and BQ and BF.7 (from BA.5) were formed. The most established sublineages over time are BQ and XBB. BQ.1 and BQ.1.1 were first detected in Nigeria in June 2022, then expanded to America, France and the United Kingdom. The sublineage BQ is derived from BA.5, while XBB is derived from the recombination of BA.2 with other lineages, namely BJ.1 and BA.2.75. Defining a mutational framework now becomes difficult, as the

variants now evolve into numerous sublineages, all different from each other, even if only a few mutations. However, studies have shown that BQ.1 in its protein Spike reports the mutations K444T and N460K along with all those detected in BA.5. This mutation appears to promote resistance to neutralizing antibodies. BQ.1.1 presents this same set of mutations in Spike, with the addition of R346T. This background of mutations then again promotes infectivity and resistance to the immune system, even in vaccinated subjects [Wang et al, 2023].

XBB and XBB.1 were detected in India in August 2022 and then spread throughout Asia. The nation that was most affected, however, was the United States, precisely from the XBB.1.5 sublineage, also known as "Kraken" variant. XBB has a spike protein further studded with mutations. Basically it has the same of BA.2, to which 14 more are added. Of these 14, 5 are in the N-terminal domain and 9 in the RDB. In RBD there is a new mutation, G252V. Another mutation present in RBD is F486P in XBB.1.5, which appears to increase affinity for the ACE2 receptor. Variants of the XBB vein have shown increased levels of immune escape, especially with regard to the action of neutralizing antibodies produced by vaccinations, which appear to be very ineffective [Callaway, 2023].

1.4.6. VARIANTS OF INTEREST

This minor variants had a little distribution all over the world, enough not to cause concern.

The first one VOI was Lambda, or C.37, detected in August 2020. It presents some of the Spike mutations mentioned before, but even several ones, like D614G, T859N, L452Q, F490S, T76I, G75V, R246N, and del247/253. The T76I and the L452Q mutations are the cause of the high infectivity. The lifetime of this variant was really brief, from April to June 2021 and Lambda few cases were detected principally in South America [Kimura et al, 2021].

The Mu variant, named B.1.621, was declared as a VOI on August 2021 and was detected in more than 20 countries.

Iota variant, or B.1.526, was first detected on November 2020 in the USA. Its Spike protein shows several mutations, like E484K/S477N, D614G, A701V, T95I, D253G, L5F and S477N that is responsible for immune escape. This variant had very low diffusion. From this one have descended three sublineages, B.1.526.1, B.1.526.2, and B.1.526.3 [West et al, 2021].

Kappa variant, also named B.1.617.1, was detected for the first time in India on late 2020 and decreased in September 2021, leaving behind a very low number of cases. The mutations in its Spike protein are L452R, E484Q, D614G, and P681R. All these mutations together seem to be an accumulation due to evolution [Cherian et al, 2021].

Epsilon variant, or B.1.427/429, was first detected at the end of the 2020 and it lasted until September 2021, remaining in the USA. Here the Spike mutations are D614G, L452R, and W152C. The infectivity was a little higher than other VOIs, but pathogenicity is not really clear [Deng et al, 2021].

1.4.7. IMMUNE ESCAPE

Analyses carried out on patients vaccinated with different types of formulations have estimated the protection against infection to be as high as 50%, which is the true protection against contracting the disease compared to a non-vaccinated subject [Khoury et al, 2021].

As for the monoclonal antibodies used as therapy against SARS-CoV-2, as they bind to a single epitope they are more easily affected by the point mutations present in the different variants. An example is Bamlanivimab/LY-CoV555, whose potency has been reduced at least 100-fold in the Beta, Gamma and Delta variants. If, on the other hand, we consider the neutralizing antibodies generated by mRNA vaccination, evasion is much more complicated, especially in the case of variants such as Beta and Delta [Planas et al, 2021] .

Omicron proved to be much more resistant in this respect, managing to evade most of these neutralizing antibodies. In the sera of vaccinated patients, the neutralization titer was reduced by up to almost 12-fold [Wilhelm et al, 2021]. The only epitopes that appear to remain unaffected by variant mutations are those recognized by T lymphocytes. Consequently, the immune memory conferred by vaccines and infections is maintained and becomes the primary protection against the development of a severe form of Covid-19. This seems to justify why high rates of hospitalization or death do not occur with the Omicron variant [Bernasconi et al, 2021].

A number of surveillance studies have been carried out on the vaccinated population, especially in relation to the occurrence of variants. In a study by Tartof and colleagues, a cohort of patients vaccinated with Pfizer/NBiotech's BNT162b2 in California was analyzed. The results showed declines in efficacy five months after vaccination. In contrast, no differences were found for hospitalization rates [Tartof et al, 2021]. Similar results were presented in surveillance studies in the territory of Qatar, with the same drops in vaccination efficacy but good protection from mortality and severe forms of the disease [Chemaitelly et al, 2021]. This remained unchanged for all the first four variants from Alpha to Delta, perhaps a slight drop in efficacy was detected for the Delta variant, but not particularly significant. The result of the studies led to the inference that the virus survival and persistent spread of the infectious phenomenon could be due not to those mutations present in the variants that tended to favor immune escape, but rather to a decline in overall immunity itself [Abu-Raddad et al, 2021].

The situation is different with the onset of the Omicron variant, which is more successful in creating continuous outbreaks despite the presence of vaccination or previous infection in patients. Surveillance studies carried out in the UK showed that the risk of re-infection with the Omicron variant was significantly higher than with the last pre-existing variant, Delta [UK Health Security Agency, 2021]. Studies were also conducted on the efficacy of the most commonly used vaccines, i.e. Pfizer and AstraZeneca, which was found

to be lower. The final results of several studies confirmed that this risk of reinfection occurs despite a strong immune background given by the vaccine and previous infection, which could be alarming due to the extreme spread of the variant [Pulliam et al, 2022].

The major limitation demonstrated by the vaccines that have so far been administered to the population lies in the effectiveness of the treatment determined only by the completion of the vaccination cycle, which turns out to be with a minimum of three doses per vaccine. The doses of the vaccinations themselves were implemented with the emergence of variants such as Omicron, precisely after the results of studies demonstrating their reinfecting power [Arbel et al, 2021]. The administration of further doses became the response to the drop in antibody levels in the months following vaccination. In particular, this type of protection is aimed at protecting individuals considered 'fragile' due to their pre-existing clinical background, who in the case of Covid-19 infection would risk severe forms or even death. Another possibility considered was the use of boosters made from heterologous vaccines, so as to create broad-spectrum immunity against current and future variants. A number of studies support this thesis, such as the one carried out by Tan and colleagues, who analyzed the antibody level of individuals vaccinated with BNT162b2 and who survived SARS-CoV-1, showing that the level of neutralizing antibodies proved to be broad-spectrum against all currently present VOCs. This type of approach certainly needs to be studied in more detail, considering what types of vaccines and/or past infections might be useful and also the possible timing of vaccination boosters. Certainly in populations vaccinated with Pfizer or AstraZeneca vaccines, a longer break between the first and second dose seems to develop a more decisive antibody response [Tauzin et al, 2022].

AIM OF THE THESIS

The purpose of this thesis was to perform a genotyping of SARS-CoV-2 variants in a cohort of patients who tested positive to molecular assay for virus detection. Data on the presence or absence of symptoms were collected from the patients themselves. An assessment of the evolution of variants and their respective symptoms was then carried out over the period 2020 to 2023, when the end of the pandemic was declared.

2. MATERIALS AND METHODS

2.1. STUDY GROUP RECRUITMENT

The study group was selected from patients who tested positive to molecular test for SARS-CoV-2. At L.C. Campisi Laboratories 73077 molecular swabs were processed from 2020 to 2023, covering the entire period of the international emergency. Among them, a total of 374 patients were selected, divided as follows:

- 109 positive patients between December 2020 and November 2021
- 228 positive patients between January 2022 and December 2022
- 37 positive patients between January 2023 and May 2023

The candidate's selection parameter was influenced first by the results of molecular diagnostics in RT-PCR. At our laboratory, this analysis is carried out with the SARS-CoV-2 Assay kit by Allplex Seegene. This kit enables multiplex RT-PCR by targeting the four main genes of the virus with three couples of primers: E, N, M and RdRP/S respectively. After extensive reverberation with brand specialists, it was found that positives that could be suspected variants exhibited an unusual trend in the RdRP/S expression curve, probably due to the modification of the Spike protein in the variants. The positives in the cohort studied were therefore chosen from those that showed this expression pattern on molecular testing in RT-PCR and with an expression CT<28, to ensure quality nucleic acid extraction.

2.2. RNA EXTRACTION

RNA Extraction was performed with Norgen Total RNA Purification Kit. In this kit purification is based on spin column chromatography using a resin as separation matrix.

First, it has to be prepared a lysate from the nasal or throat swabs. The cotton tip of a swab was cut and put into an RNase-free microcentrifuge tube with

600 µL of Buffer RL (a lysis buffer). The tube was vortexed gently and incubated for 5 minutes at room temperature. 250 µL of this lysate was transferred into another RNase-free microcentrifuge tube.
An equal volume (250 µL) of 70% ethanol was added to the lysate, then the tube was vortexed to mix all.
600 µL of the lysate with the ethanol was dispensed into a column with a tube and centrifuged for 1 minute at 6,000 RPM.
The waste inside the tube was discarded and then the column was washed for three times with 400 µL of Wash Solution A and a centrifuge for 1 minute at 6,000 RPM. Every time the waste was discarded. During the third wash, after the centrifuge at 6,000 RPM, it has to be done another centrifuge at 14,000 RPM for 1 minute in order to dry the resin. Also here, the waste has to be discarded with the all tube. The column was placed into and elution tube.
50 µL of Elution Solution A was added to the column. The all tube was centrifuged for 2 minutes at 2,000 RPM, followed by 1 minute at 14,000 RPM. The final eluted volume has to be 50 µL.

2.3. REVERSE TRANSCRIPTION

On the samples containing viral RNA was performed cDNA synthesis, using ThermoFisher kit.
Each RNA sample, in a quantity between 1-15 µL, was combined 10 µL of 5X SuperScript IV VILO Master Mix and water until reaching a final volume of 50 µL. The samples were vortexed for 2–3 seconds, and then centrifuged briefly (5–10 seconds) at 1,000 x g. Then was perfomed a PCR with the following profile:

- Annealing: 25°C for 10 mins
- Polymerase extension: 50°C for 15 mins
- Polymerase inactivation: 80°C for 10 mins

2.4. SANGER SEQUENCING

ThermoFisher Scientific developed a protocol for analyzing the entire S gene by Sanger sequencing. The primer sequences used are based on those published by the Centers for Disease Control and Prevention (CDC). The obtained cDNA was used in specific regions of target amplification using tailed primers that cover the S gene. For this, we used directly the Applied Biosystems BigDye Direct Cycle Sequencing Kit and M13 sequence–tagged primer sets. The amplified sequences were then subjected to cycle sequencing using either M13-forward or M13-reverse primers provided in the BigDye Direct Cycle Sequencing Kit.

M13-tagged primers sequences are the following:

Coordinates*	Forward primer name	Forward primer sequence**	Reverse primer name	Reverse primer sequence
20990-21562	SC2M1-54_ LEFT_M13	TGTAAAACGACGGCCAGTTGATTGGTGATTGTGCAACTGTACA	SC2M1-54_ RIGHT_M13	CAGGAAACAGCTATGACCTGTTCGTTTAGTTGTTAACAAGAACATCA
21421-21916	SC2M1-55_ LEFT_M13	TGTAAAACGACGGCCAGTAGGGGTACTGCTGTTATGTCTTTAAA	SC2M1-55_ RIGHT_M13	CAGGAAACAGCTATGACCAAGTAGGGACTGGGTCTTCGAA
21775-22345	SC2M1-56_ LEFT_M13	TGTAAAACGACGGCCAGTTGGGACCAATGGTACTAAGAGGT	SC2M1-56_ RIGHT_M13	CAGGAAACAGCTATGACCACCAGCTGTCCAACCTGAAGAA
22203-22697	SC2M1-57_ LEFT_M13	TGTAAAACGACGGCCAGTGTGATCTCCCTCAGGGTTTTTCG	SC2M1-57_ RIGHT_M13	CAGGAAACAGCTATGACCACTTAAAAGTGGAAAATGATGCGGAA
22563-23128	SC2M1-58_ LEFT_M13	TGTAAAACGACGGCCAGTACTTGTGCCCTTTTGGTGAAGT	SC2M1-58_ RIGHT_M13	CAGGAAACAGCTATGACCTGCTGGTGCATGTAGAAGTTCA
22986-23519	SC2M1-59_ LEFT_M13	TGTAAAACGACGGCCAGTCCGGTAGCACACCTTGTAATGG	SC2M1-59_ RIGHT_M13	CAGGAAACAGCTATGACCCCCCTATTAAACAGCCTGCACG
23379-23876	SC2M1-60_ LEFT_M13	TGTAAAACGACGGCCAGTACCAGGTTGCTGTTCTTTATCAGG	SC2M1-60_ RIGHT_M13	CAGGAAACAGCTATGACCCAGCTATTCCAGTTAAAGCACGGT
23737-24231	SC2M1-61_ LEFT_M13	TGTAAAACGACGGCCAGTAATTCTACCAGTGTCTATGACCAAGAC	SC2M1-61_ RIGHT_M13	CAGGAAACAGCTATGACCGCACCAAAGGTCCAACCAGAAG
24095-24623	SC2M1-62_ LEFT_M13	TGTAAAACGACGGCCAGTGCTGCTAGAGACCTCATTTGTGC	SC2M1-62_ RIGHT_M13	CAGGAAACAGCTATGACCAAGCTCTGATTTCTGCAGCTCT
24493-25003	SC2M1-63_ LEFT_M13	TGTAAAACGACGGCCAGTAAATGATATCCTTTCACGTCTTGACAAA	SC2M1-63_ RIGHT_M13	CAGGAAACAGCTATGACCTGAGTCTAATTCAGGTTGCAAAGGA
24858-25369	SC2M1-64_ LEFT_M13	TGTAAAACGACGGCCAGTGCACACACTGGTTTGTAACACAA	SC2M1-64_ RIGHT_M13	CAGGAAACAGCTATGACCTTTGACTCCTTTGAGCACTGGC
25214-25790	SC2M1-65_ LEFT_M13	TGTAAAACGACGGCCAGTTAGGTTTTATAGCTGGCTTGATTGC	SC2M1-65_ RIGHT_M13	CAGGAAACAGCTATGACCCATTTCCAGCAAAGCCAAAGCC

2.4.1. PCR AMPLIFICATION OF TARGETS

The 10X sequencing amplification primer mixes were prepared with 492 µL of TE buffer and 4 µL each of both the left and right oligos of a pair. The initial PCR amplification required two identical reactions for each sample (a forward and a reverse reaction ran for each sample).

In each well of a 96-well PCR plate, was combined: 1.5 µL of 10X sequencing amplification primer mix, 5 µL of 2X BigDye Direct PCR Master Mix, 1 µL of

cDNA sample and Water to 10 µL total volume. The plate was vortexed for 2–3 seconds, and then centrifuged briefly (5–10 seconds) at 1,000 x g. In this case, the PCR thermal cycle was the following:

- Polymerase activation: 95°C for 10 mins

And the 40 cycles with the following program:

- Denaturation: 96°C for 3 secs
- Annealing: 62°C for 15 secs
- Extension: 68°C for 30 sec

2.4.2. CYCLE SEQUENCING

To the same plate used for targets amplification, were added to each well 2 µL of BigDye Direct Sequencing Master Mix and 1 µL of BigDye Direct M13 Forward or M13 Reverse primer. Also here, the plate was vortexed for 2–3 seconds and then centrifuged briefly (5–10 seconds) at 1,000 x g. This time the run parameters were the following:

- Post PCR cleanup: 37°C for 15 mins
- Post PCR inactivation: 80°C for 2 mins
- Polymerase activation: 96°C for 1 min

And then, 25 cycles with the following program:

- Denaturation: 96°C for 10 secs
- Annealing: 50°C for 5 secs
- Extension: 60°C for 75 secs

2.4.3. SEQUENCING CLEANUP

Unincorporated nucleotides and primers were next removed using the Applied Biosystems BigDye XTerminator Purification Kit. In each well was

added 45 µL of SAM Solution and 10 µL of XTerminator Solution. The reaction plate was vortexed for 40 minutes and then spinned at 1,000 x g for 2 minutes.

2.4.4. CAPILLARY ELECTROPHORESIS

The capillary electrophoresis was done with Applied Biosystems 3500 Series Genetic Analyzer, taking 15-20 µL of the purified sequences. The obtained electropherograms were analyzed with software Sequence Scanner v2.0.

2.5. HAPLOTYPE ANALYSIS

The haplotype analysis was performed using the Genious tool, which allows the alignment of the detected sequences with the reference sequences in the databases and the setting of parameters to find certain mutations in the considered sequences. For each sample, mutations were detected in the respective electropherogram and based on this, the haplotype was reconstructed and then compared with each SARS-CoV-2 variant until the corresponding one was identified.

3. FINDINGS

3.1. GENOTYPING OF SARS-COV-2 VARIANTS

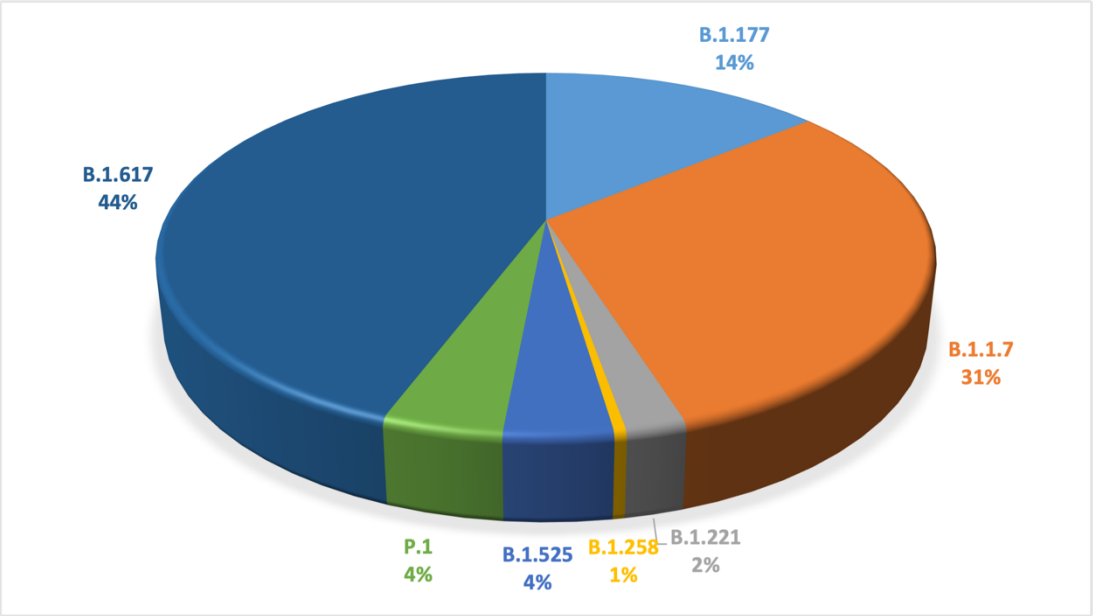
The genotyping of the SARS-CoV-2 positive samples was carried out by scanning the study according to each year in which the pandemic persisted, in order to assess the trend of variants in a final overview. The results were then divided according to the following timeframes:

- December 2020-November 2021
- January 2022-December 2022
- January 2023-May 2023

Variant Trend between December 2020 and November 2021

The cohort analyzed in this period is of 235 patients.

From what can be deduced from the graph below, the most represented variant is B.1.617 (or Delta) at 44%, followed by B.1.1.7 (or Alpha) at 31%, and by B.1.177 (Alpha2) at 14%, that is one of Alpha sublineages. There was also a slight presence of some VOIs such as B.1.525 (Eta) at 4%, and very few cases of variants B.1.221 (Netherlands) with 2% and B.1.258 (Central Europe) with 1%. Finally, P.1 (Gamma), which is a VOC but only represented at 4% in the population here genotyped.



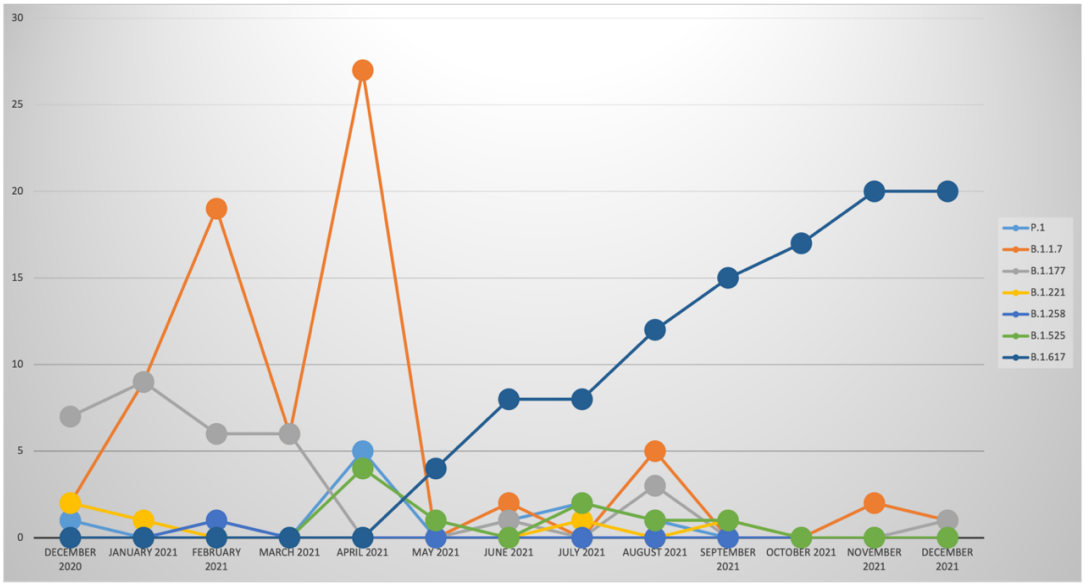
In addition to the presence of these variants and the prevalence of some of them, the trend over the months was also assessed.

The graph below shows that variant B.1.1.7 and its sublineage B.1.177 persisted consistently over time, but with a considerable decrease from May onwards. The B.1.177 sublineage was the first to show a sudden decline around April 2021, followed by B.1.1.7, which declined sharply around May 2021, to persist with only a few cases until the end of 2021. After the decrease

of these variants, B.1.617 took their place, becoming the main variant until the end of the 2021.

These data confirm what is present in literature regarding the greater infectiousness of these variants.

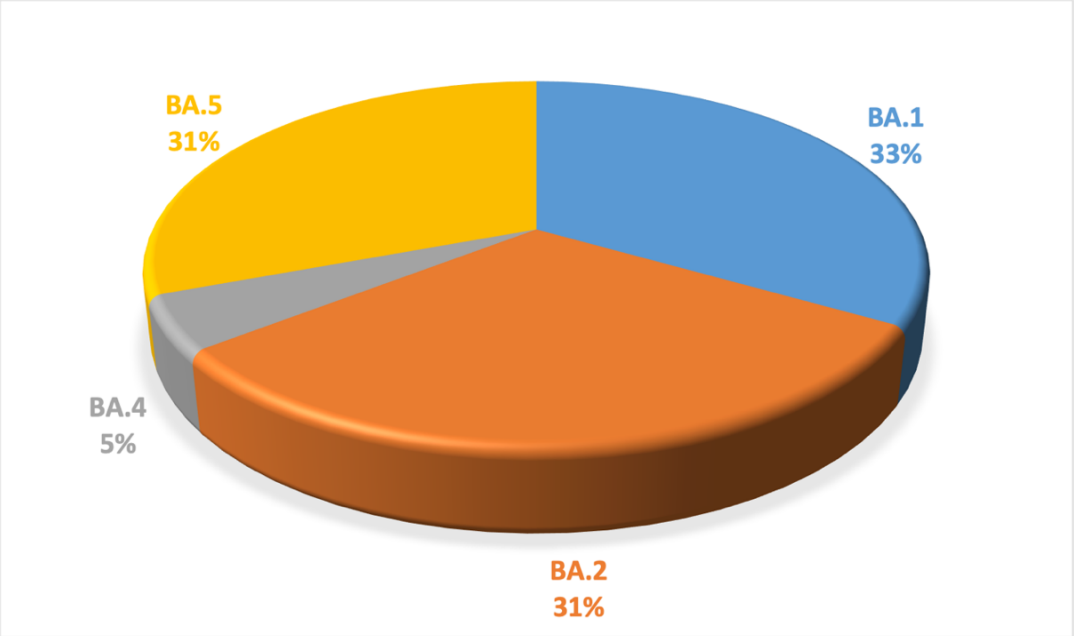
The VOIs had low representation throughout the year, with a slight presence of B.1.525 and B.1.258 during April 2021, which immediately declined in the following months. Variant P.1 maintained an almost non-existent presence throughout the time period considered.



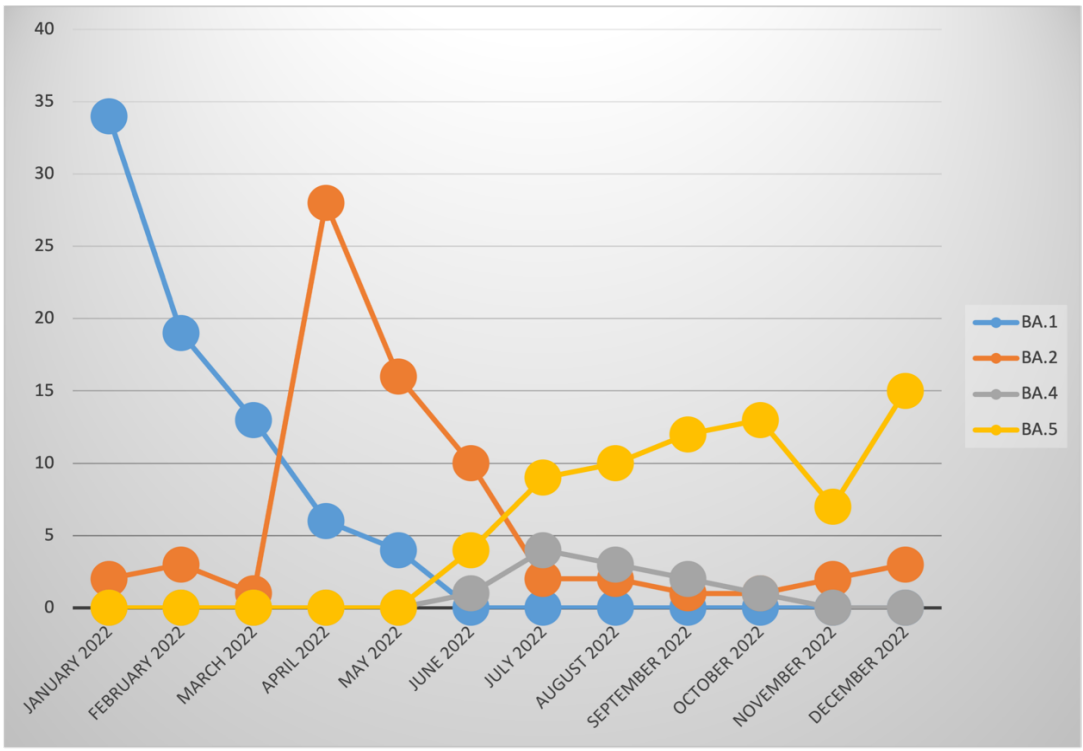
Variant Trend between January 2022 and December 2022

The cohort analyzed in this period is of 228 patients.

The result of the genotyping of this population showed the total prevalence of the Omicron variant (B.1.1.529) , with all other previous variants disappearing. The graph shows an equal representation of three of the Omicron lineages: BA.1 at 33%, BA.2 at 31% and BA.5 at 5%. The only lineage poorly represented is BA.2 at 5%.



Regarding the variants trend during 2022, it can be seen from the graph below how different lineages have established themselves over time, effectively following the chronological order of appearance. The first lineage to become established was BA.1, which remained the main variant until March. From that moment, the BA.2 lineage variants increased, holding the lead until June. From July onwards, the BA.5 lineage established itself and remained the main one until the end of 2022. The presence of the BA.4 lineage, on the other hand, is low or nil over time.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35
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25
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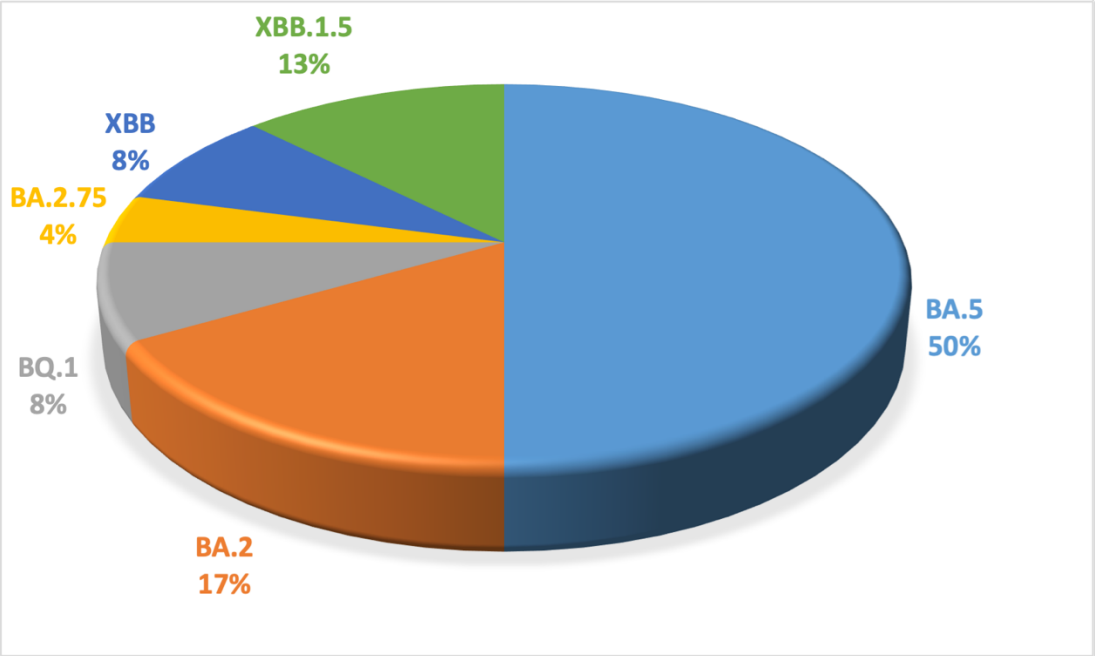
JANUARY 2022
FEBRUARY 2022
MARCH 2022
APRIL 2022
MAY 2022
JUNE 2022
JULY 2022
AUGUST 2022
SEPTEMBER 2022
OCTOBER 2022
NOVEMBER 2022
DECEMBER 2022
BA.1
BA.2
BA.4
BA.5

Variant Trend between January 2023 and May 2023

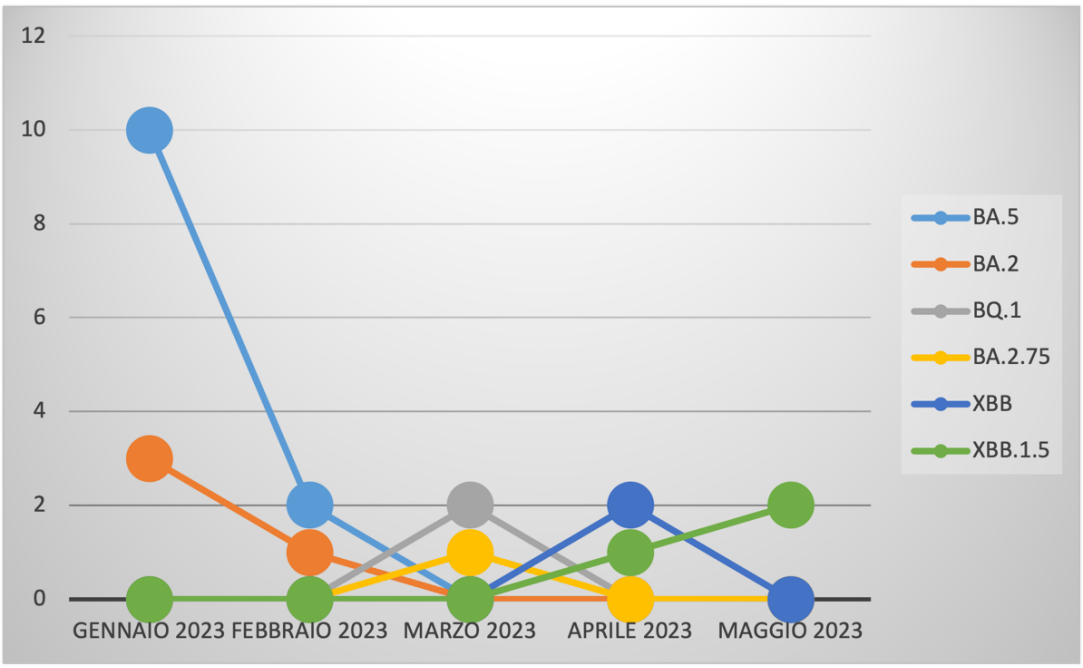
The cohort analyzed in this period is 37 patients, a much smaller number due to the decreased request for molecular analysis for SARS-CoV-2, corresponding to the period when the end of the pandemic was declared. In this study group, although small in number, there are several variants, all belonging to or derived from Omicron lineages, considering that since the beginning of 2023 the lineages have branched out, creating the so-called 'variant soup'.

From the graph below, it can be seen that the most represented lineage is BA.5 with 50%, followed by BA.2 at 17%. In smaller percentages, however, are the sub-lineages, respectively XBB.1.5 at 13%, BQ.1 and XBB with 8% and BA.2.75 with 4%.



The course of the variants in the graph below shows that the 50% of the BA.5 lineage only actually occurred in the first months of 2023, having already disappeared in March. The same course, albeit with less presence, was taken by BA.2, which also disappeared in March. The sublineages BA.2.75, BQ.1 and

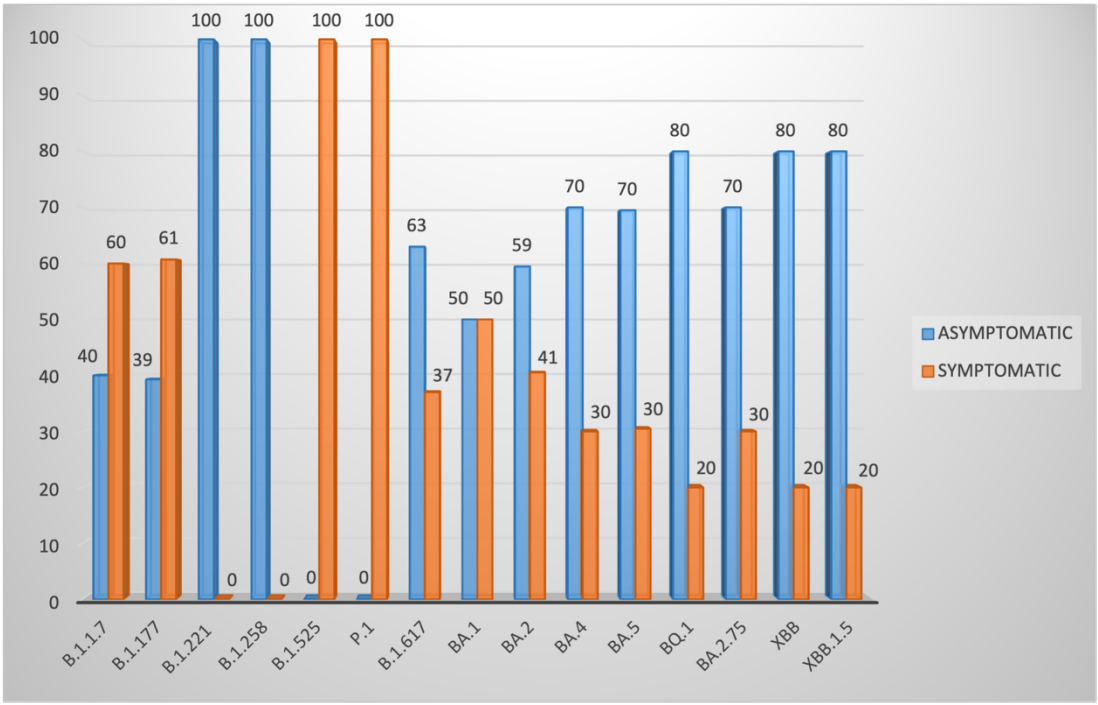
XBB are very poorly represented, all in the time span between February and April. The sublineage that is emerging for the last but remains the only one to be present is XBB.1.5, which congruently with the data reported nationally and worldwide, has become the most represented sublineage in the various territories.



3.2. SYMPTOMATOLOGY TREND

Each variant brought with its symptoms of different severity. As the mutational rate progressed and new variants appeared, the clinical outcome itself varied. In general, the variants became more infectious and less severe in terms of symptoms.

Each time a SARS-CoV-2 swab is taken in our laboratory, the patient undergoes a questionnaire, including if symptoms are present or not. The cases that later turned out to be positive and symptomatic mainly reported symptoms such as fever, sore throat, cold, taste and smell disturbances, more rarely intestinal disorders and migraines.



The graph above shows the trend in symptomatology in the populations analyzed according to the variants, rearranged in chronological order of appearance. The rate of symptomatology appears to be decreasing from the presence of the first variant detected, namely B.1.1.7, to the last, XBB.1.5. In

particular, it is noticeable that the highest rate of symptomatic cases is found in all variant families that appeared before Omicron, respectively:

- 60% in B.1.1.7, with 40% asymptomatic cases
- 61% in B.1.177, with 39% asymptomatic cases
- 0% in B.1.221, with 100% asymptomatic cases
- 0% in B.1.258, with 100% asymptomatic cases
- 100% in B.1.525, with 0% asymptomatic cases
- 100% in P.1, with 0% asymptomatic cases
- 37% in B.1.617, with 63% asymptomatic cases

The symptoms reported by patients in this period were mainly those mentioned above, often present all together, except for migraines and intestinal disorders, which were present singly or rarely together with the rest of the symptoms. During the period 2021-2022, swabs were therefore mainly taken to establish whether it was flu or not.

With the emergence of the Omicron variant, its first lineage, BA.1, is the only one with an equal rate of symptomatic and asymptomatic cases. From the BA.2 lineage up to the 'variant soup', the rate of symptomatic cases is totally decreasing, down to 20%, respectively:

- 50% in BA.1, with 50% asymptomatic cases
- 41% in BA.2, with 59% asymptomatic cases
- 30% in BA.4, with 70% asymptomatic cases
- 30% in BA.5, with 70% asymptomatic cases
- 20% in BQ.1, with 80% asymptomatic cases
- 30% in BA.2.75, with 70% asymptomatic cases
- 20% in XBB, with 80% asymptomatic cases
- 20% in XBB.1.5, with 80% asymptomatic cases

Asymptomatic cases thus increased over time, consistent with a decrease in the severity of symptoms reported by patients, which became overlapping with those of influenza. A further difference from the pre-Omicron variants was the presence of single or very few symptoms compared to the above-mentioned pool, with the loss of gastrointestinal symptoms or fever and the increase in symptoms such as sore throat and cold.

4. DISCUSSION

Covid-19 is a pandemic that has affected the world since December 2019, when several cases of very severe pneumonia of unknown etiology appeared in the city of Wuhan (China). Shortly afterwards, it was discovered that the cause was a new Coronavirus, which the International Committee on Taxonomy of Viruses named SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) because of its high overlap at the genetic and structural level with the previous coronaviruses that had generated more severe diseases in humans, namely SARS-CoV (Severe Acute Respiratory Syndrome Coronavirus) and MERS-CoV (Middle East Respiratory Syndrome coronavirus). On late January 2020, the WHO declared Covid-19 an international public emergency and in March 2020 was officially declared as a pandemic [Sun et al, 2020].

SARS-CoV-2 belongs to the Beta Coronavirus family. SARS-CoV-2 is a single-stranded RNA-enveloped virus. The genome of SARS-CoV-2 is around 30 kb on size, contains 14 open reading frames (ORFs) and encodes 29 viral proteins. At the 3′ terminus four ORFs encode for structural proteins that include the nucleocapsid (N), spike (S), membrane (M) and envelope (E), which are responsible for virion assembly and suppression of the host immune response [Chen et al, 2020]. Spike, Envelope and Membrane are present on virion membrane surfaces, with the Nucleocapsid protein that is involved in the binding and packing of the RNA genome. Spike plays an essential role in the host receptor binding and membrane fusion [Moreira et al, 2020]. M protein is associated with N protein and other viral structural proteins to facilitate the viral assembly and is involved in the pathogenesis process [Fu et al, 2021]. E protein forms an ion channel, which promotes virus assembly and pathogenesis [Nieto-Torres et al, 2014].

The S protein is a homotrimer, which protrudes from the virion and extensively decorates the viral surface like a crown. It binds to the host cell by recognizing the receptor ACE2, that is distributed mainly in the lung, intestine,

heart, and kidney, and alveolar epithelial type II cells are the major expressing cells [Yan R et al, 3030].

The SARS-CoV-2 S protein comprises ~1,200 residues and can be cleaved by a furin-like protease into two functional subunits, S1 and S2 [Walls et al, 2020]. The S protein is cleaved into two parts, the S1 subunit and S2 subunit, by host proteases, and the subunits exist in a noncovalent form until viral fusion occurs [Tortorici et al, 2019]. The S1 subunit contains the Receptor Binding Domain that acts as the binding region for ACE2 [Lan et al, 2020].

Since the emergency of SARS-CoV-2, its viral genome has been under constant and rapid mutation to adapt host system. Like the others RNA viruses, the high mutation rate leads to the creation of new variants with a significant change in viral phenotypes [Gupta A. et al, 2021].

Mutations in the spike protein of SARS-CoV-2 variants influence the structure and the conformation, furthermore influence the interaction with ACE2 or neutralizing antibodies [Barton et al, 2021].

In early 2020, the first Spike mutation emerged, D614G [Bhattacharya et al, 2021]. In December 2020, was detected the Alpha variant (B.1.1.7), harboring another Spike mutation, N501Y. Initially, it expanded in the southeast of England [Galloway et al, 2021]. Later the Beta variant (B.1.351) was found in South Africa and manifested a rapid domestic distribution to an over 80% prevalence [Tegally et al, 2021]. One month later, the Gamma variant (P.1) was reported in Brazil, and the travelers arriving in Japan from Brazil [Naveca et al, 2021]. Delta variant (B.1.617.2) was first detected in India in May 2021 and rapidly became the dominant variant worldwide by late 2021, while some sub-clade of Delta variant displayed a unique penchant in epidemic areas, such as Clade 20I (Delta) in some parts of Asia [Mlcochova et al, 2021]. Delta-dominant period lasted until Omicron variant (B.1.1.529) was detected in November 2021, which was first reported in South Africa and the in Hong Kong [Jansen et al, 2021]. Omicron rapidly became the major variant worldwide with all its lineages (BA.1, BA.2, BA.3, BA.4, BA.5) and sublineages (BQ.1, BA.2.75, XBB,

XBB.1.5), derived from the lineage with the "variant soup" phenomenon [Callaway, 2022].

In this work, a total of 374 positive samples were genotyped for the SARS-CoV-2 variants.

The study was carried out according to two principles:

- Genotyping the SARS-CoV-2 variants present in a study group belonging to the territory of South-Eastern Sicily
- Assessing the trend of the variants and their clinical outcome during the course of the pandemic

The study group was selected from molecular swabs of SARS-CoV-2 positive patients who at RT-PCR showed an unusual trend in the expression curve of the S-gene. That expression trend could recall possible mutations in virus Spike protein.

The results of variant genotyping reflected the spread of the variants across Italy at certain times. In fact, in the first time frame considered, between the end of 2020 and the end of 2021, variants B.1.1.7 and B.1.177 appear to be preponderant both in terms of presence and constancy over time until May 2021, when variant B.1.617 appears and takes its place as the main variant. These data coincide with the ISS reports, which further confirm the very poor presence in Italy of VOI B.1.221, B.1.258 and B.1.525 and of VOC P.1, which in general were not very well represented in Italy [ISS]. The high prevalence of the above mentioned variants is supported by mutations in the Spike protein that favor virus transmissibility and are dominant in each variant.

These are H69-V70del, N501Y, and P681H in B.1.1.7 and B.1.177, which increased transmissibility by 40%, and D614G and P681R in B.1.617, which increased transmissibility by 60% [Harvey et al, 2021].

This justifies in this study and in the data reported by ISS the survival of these two variants compared to the others, which did not have favorable mutations, and were found to have few cases. The trend in symptomatology also reflects the main characteristics of the variants. The B.1.1.7 (Alpha) variant in fact presented symptoms similar to those of the original virus, like rarer flu-like

symptoms together with loss of smell and taste, shortness of breath and cough, and possible vascular lesions. In particular, the Alpha variant in Italy was associated with an increased risk of muscle pain, insomnia, and brain fog. However, the increased infectiousness had cushioned the onset of symptoms, so despite the severity, a smaller proportion of the infected population was affected [Davies et al, 2021].

The landscape of clinical manifestations began to change after the appearance of the first variants, aided by the introduction of vaccines and therapies.

This combination of elements generated a decrease in hospitalizations and in the severity and frequency of symptoms, leading to variant B-1-617 (Delta) which presented the classic symptoms of a common flu, like runny nose, cough, sore throat, headache, loss of taste and smell. The duration of symptoms proved to be shorter in vaccinated persons [Challen et al, 2021]. This coincides with the data reported in this study, which show a decrease in symptomatology below 40% in Delta variant cases.

The data reported by this study support the total presence of the B.1.1.529 (Omicron) variant from the beginning of 2022 until 2023 with its different lineages, which has taken the place of all previous variants due to mutations ensuring transmissibility and immune escape, such as P681H, N501Y, D614G, K417 and T478k, or H655Y and N679K in the furin cleavage site [Wu L et al, 2021]. The Omicron lineages detected here coincide with the data collected on the Italian and Sicilian territory in the ISS reports, both in terms of the chronological order of appearance of the lineages and sublineages and the type of variants present [ISS]. The mutational background that has accumulated in this variant explains its total prevalence as a result of biological evolution of the virus itself.

Indeed, this study denotes a primary evolution of the Omicron variant in its lineages BA.1, BA.2, BA.4 and BA.5, which show few differences from one another at the mutational level, to the XBB and BQ sublineages, which have dominated the variant landscape since March 2023. They, being part of the 'variant soup', have accumulated such a high number of mutations in the Spike

protein that they spread at a very high rate and in all categories of subjects, even vaccinated ones, such as K444T, N460K or R346T in the BQ sublineages conferring infectivity and immune resistance [Wang et al, 2023] and G252V and F486P in the RBD present in the XBB sublineages increasing affinity for the ACE2 receptor [Callaway, 2023]. Changes in the clinical outcome also coincided with the development of omicron, whose main symptoms were rhinorrhea, fatigue, sore throat and headache, febrile states not exceeding a temperature of 38°, rarely nausea and diarrhea. Derivatives of the Omicron variant present similar but much milder symptoms, such as nasal congestion, sore throat and headache, cough, muscle aches and mild febrile states. The decrease in the severity of symptoms coincides with the lower onset of symptoms themselves, as reported by several studies [Wang et al, 2023].

This study also confirms this trend, with an increase in asymptomatic cases from the appearance of BA.1 to the sublineage XBB.1.5, which has 80% asymptomatic cases and 20% symptomatic cases with flu-like symptoms among those mentioned above.

Looking at the study as a whole, the small population analyzed here provides a point to follow the trend in the virus. We can hypothesize from the results that the virus has certainly become highly transmissible, even managing to overcome a possible immune coverage brought about by vaccines, but at the same time it has weakened in terms of severity of manifestations. The absence of symptoms or the mild manifestation of symptoms brings with its pros and cons.

The elements in favor are certainly the decrease in mortality or post-Covid damage, which suggests a strengthening of the population at the immune level and a kind of evolutionary 'victory' of mankind over the virus. At the same time, if the virus manifests itself with weak or absent symptoms, it becomes difficult to detect it because the asymptomatic case will certainly not test for SARS-CoV-2 and those with flu-like symptoms might mistake a Covid-19 infection for a normal flu. This, too, results in a small victory for the virus itself, which has evolved in such a way that it coexists perfectly with the host and

manages to survive with its high transmissibility. All in all, the entire pandemic has finally been overcome by mankind, which for a number of reasons is now facing a harmless virus. Surely this study, along with many others, could, however, provide a cue to continue investigating the progression and evolution of the virus, leaving many open questions, such as the variation in the characteristics of the virus itself or possible changes in the global immune system. This last open question would be a very broad and interesting branch to open in the future.

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Sitography

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