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VARIABILITÀ DI SARS-CoV-2: PROPRIETÀ REPLICATIVE E NEUTRALIZZAZIONE DA SIERI CONVALESCENTI E INDOTTI DA VACCINO DI DIFFERENTI VARIANTI

VARIABILITY OF SARS-CoV-2: REPLICATIVE PROPERTIES AND NEUTRALIZATION BY CONVALESCENT AND VACCINE-INDUCED SERA OF DIFFERENT LINEAGES

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A Luca

Out of the night that covers me,
Black as the pit from pole to pole,
I thank whatever gods may be
For my unconquerable soul.
In the fell clutch of circumstance
I have not winced nor cried aloud.
Under the bludgeonings of chance
My head is bloody, but unbowed.
Beyond this place of wrath and tears
Looms but the Horror of the shade,
And yet the menace of the years
Finds and shall find me unafraid.
It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate,
I am the captain of my soul.

W. E. Henley

L'11 marzo 2020 l'Organizzazione Mondiale della Sanità (OMS) ha dichiarato lo stato di pandemia da SARS-CoV-2, circa tre mesi dopo la prima identificazione dell'agente eziologico della COVID-19, riportata nella città di Wuhan in Cina alla fine del 2019.

Ad oggi, l'Italia ha riportato oltre 4 milioni di casi confermati e 126 mila morti. Nonostante le contromisure adottate a livello globale, come il distanziamento sociale e la vaccinazione di massa, il SARS-CoV-2 continua a diffondersi, con l'importante insorgenza di nuove varianti in tutto il mondo. In particolare, le Variants of Concern (VOCs) sono quelle di maggiore interesse in termini di salute pubblica, in quanto rappresentano mutanti virali potenzialmente capaci di evadere la risposta immune indotta sia da infezione naturale che da vaccinazione.

Tuttavia, non sono ancora chiare la rapidità e l'efficienza con cui SARS-CoV-2 riesca ad evolvere evadendo la risposta immune. Per tale ragione, dal momento che nuove terapie e nuovi vaccini vengono testati ed approvati con grande velocità, l'identificazione dei pattern di resistenza alla risposta anticorpale resta un argomento fondamentale nella ricerca su SARS-CoV-2.

Lo scopo della presente tesi è:

1) Studiare le proprietà replicative di cinque differenti varianti di SARS-CoV-2 (B.1 (D614G), B.1.1.7, B.1.351, P.1 e B.1.526) emerse nel corso dell'ultimo anno ed isolate nel nostro laboratorio;

2) Valutare l'attività neutralizzante di sieri da soggetti infettati naturalmente (convalescenti) e da individui vaccinati nei confronti delle suddette varianti.

Per quanto riguarda le proprietà replicative, dai risultati ottenuti si evince che:

- Tutte le varianti virali inducono la formazione di un effetto citopatico (ECP) simile sia per caratteristiche che per i tempi rapidi di comparsa, con l'unica eccezione della variante B.1.1.7, che è risultata capace di indurre la formazione di sincizi, ed è più lenta delle altre nell'indurre ECP. Anche in termini di cinetica replicativa su colture cellulari, le varianti B.1.1.7 e B.1.526 sono risultate più lente di B.1.
- Gli isolati B.1 e B.1.351 rappresentano le varianti rispettivamente più e meno efficienti nella produzione di virioni infettanti. Tuttavia, non è ancora chiaro come tali proprietà replicative *in vitro* possano essere ricondotte ad una maggiore o minore patogenesi dell'infezione virale *in vivo*.

Per quanto riguarda la seconda parte dello studio, relativamente all'attività neutralizzante:

- I titoli neutralizzanti dei sieri di individui vaccinati con due dosi del vaccino Pfizer-BioNTech sono risultati significativamente più elevati rispetto ai titoli dei sieri di individui convalescenti, esaminati contro le varianti B.1, B.1.1.7 e P.1. Tuttavia, i titoli ottenuti contro la variante B.1.351 sono risultati significativamente molto ridotti, per entrambi i gruppi di individui.
- I titoli neutralizzanti dei sieri derivati da individui vaccinati precedentemente infettati sono risultati superiori a quelli vaccinati naïve (entrambi i gruppi analizzati a 15 gg alla seconda dose) per tutte le varianti valutate. Tale evidenza suggerisce che la periodica stimolazione immunologica provoca una migliore neutralizzazione cross-lineage. Inoltre, 3 sieri ottenuti dopo la prima dose del vaccino Pfizer-BioNTech, da individui con precedente infezione naturale, hanno mostrato titoli neutralizzanti paragonabili a quelli ottenuti dopo due dosi. Questo dato conferma quanto messo in atto nell'iter di vaccinazione per soggetti già sieropositivi al SARS-CoV-2: una singola dose di vaccino è probabilmente sufficiente a garantire un livello di protezione ottimale in tali individui.

- Il siero convalescente evocato da una specifica variante mostra il maggiore potere neutralizzante contro quella variante (specificità di risposta). Tuttavia, è interessante notare come il siero convalescente di un paziente infettato dalla variante B.1.351 abbia manifestato un elevato titolo neutralizzante contro tutte le altre varianti. Pertanto, si può ipotizzare che la proteina Spike della variante B.1.351 presenti mutazioni nella sequenza amminoacidica in grado di stimolare la produzione di anticorpi neutralizzanti cross-lignaggio particolarmente efficaci.
- I titoli neutralizzanti ottenuti da 8 sieri derivati da pazienti infettati dopo la vaccinazione Pfizer-BioNTech non mostrano differenze significative rispetto ai sieri derivati dalla popolazione generale dei vaccinati, pertanto il titolo neutralizzante non rappresenta un marker sufficiente per predire inadeguati livelli di immunizzazione.
- Non sono presenti differenze significative tra i titoli anticorpali neutralizzanti ottenuti in individui infettati nel corso della prima ondata (marzo – settembre 2020) rispetto a quelli infettati nel corso della seconda ondata (ottobre – dicembre 2020), vaccinati o con vaccino Pfizer-BioNTech (sieri ottenuti dopo la seconda dose) o con vaccino AstraZeneca (sieri ottenuti dopo la prima dose). Pertanto, il potere neutralizzante degli anticorpi rilasciati durante l'infezione naturale potrebbe essere

efficacemente potenziato dalla vaccinazione, a prescindere dal periodo intercorso tra infezione e vaccinazione. Inoltre, nonostante il vaccino AstraZeneca sia meno efficiente del vaccino Pfizer-BioNTech nella stimolazione di anticorpi neutralizzanti nei vaccinati naïve, negli individui convalescenti una singola dose di vaccino AstraZeneca sembra sufficiente ad evocare una buona risposta neutralizzante.

Tutte queste evidenze, ottenute contestualmente alla pubblicazione di studi epidemiologici, strutturali, filogenetici e clinici, non solo rappresentano indicazioni rilevanti per la ricerca virologica sul SARS-CoV-2, ma possono fornire dati interessanti agli enti di salute pubblica, chiamati a prendere decisioni d'impatto sull'andamento della pandemia.

Contents

1. Introduction

1.1 COVID-19 pandemic

In December 2019, several clusters of unexplainable pneumonia have been observed in Wuhan, the most densely populated city of the Hubei province in China. Hospitalized patients showed symptoms such as fever, cough, serious lung injury and respiratory distress, but the aetiology was still to be determined. Virus isolation coupled with the innovative technique based on high-throughput sequencing called "next generation sequencing" (NGS) allowed scientists to rapidly identify the causative agent of this respiratory syndrome, which turned out to be a β-coronavirus never seen before, belonging to the *Coronaviridae* family [30].

Because of the genetic and clinical similarities with the previously identified SARS-CoV responsible for the 2003 SARS epidemy, the new viral agent has gained the name of SARS-CoV-2, which stands for “Severe Acute Respiratory Syndrome Coronavirus 2”, whereas COVID-19 is the name created to define the “Coronavirus Disease 2019” [1, 30].

At the beginning of the outbreak most cases have been epidemiologically linked to the wet animal wholesale market in Wuhan, which is still considered the very first source of infection.

In late January 2020, the World Health Organization (WHO) declared COVID-19 a “public-health emergency of international concern”, but only after a lot of cases have been reported worldwide, the global spreading of SARS-CoV-2 was finally recognised by WHO as a pandemic. This happened on 11th March 2020, more than two months from the beginning.

Up to now the virus is far from being globally under control, despite the fact that strict countermeasures, such as mass vaccination and social distancing, have been implemented by governments to prevent the hospitalization rate from raising.

To get an idea about the proportions of the current emergency, more than 140 million cases have been confirmed, among which over 3 million deaths have occurred. The most concerning number of infections has been registered in the United States (over 31 million cases and 5 thousand deaths), and India (over 27 million cases and 319 thousand deaths) [31].

Regarding the European Union, Italy, France, Spain and Germany have suffered the most severe impact. Regarding Italy, over 4 million confirmed cases have been reported, including 136 thousand cases in health-care workers (as of June 2021). Moreover, about 126 thousand of deaths were registered in patients with a mean age of 81 years [65].

1.2 Taxonomy of Coronaviruses

Regarding classification, SARS-CoV-2 belongs to the *Nidovirales* order, comprehensive of enveloped, positive-stranded RNA viruses, which can be further subdivided into three families:

- *Arteriviridae,* which infect mammalian hosts, e.g. horses, mice and chimpanzees;
- *Roniviridae*, which infect shrimps;
- *Coronaviridae*, sorted in two subfamilies:

  1. *Torovirinae,* which mainly affect mammalian hosts, e.g. horses and swine;

  2. *Conoravirinae,* which includes three genera (Fig. 1): *Alphacoronaviruses* and *Betacoronaviruses,* which only infect mammalian hosts, and *Gammacoronaviruses*, which have been isolated from avian hosts.

  HCoV-229E, one of the several viruses responsible of common cold in humans, belongs to the *Alphacoronaviruses* along with hCoV-LN63. Common cold viruses belonging to the *Betacoronavirus* genus are hCoV-OC43 and hCoV-HKU1.

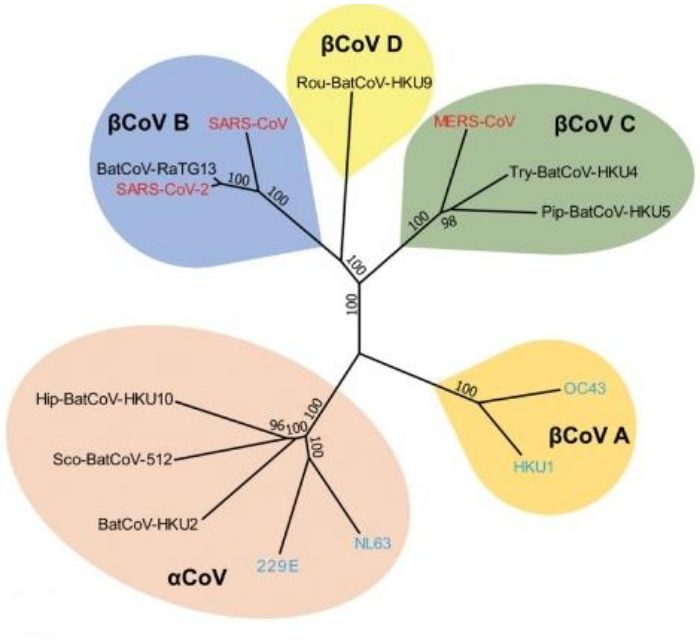


Fig. 1 Phylogenetic tree and genetic relationships of different Coronaviruses. Reprinted from "Does cross-neutralization of SARS-CoV-2 only relate to high pathogenic Coronaviruses?" Zhongren, M., et al., 2020, *Trends in Immunology*, 41(10), 851-853.

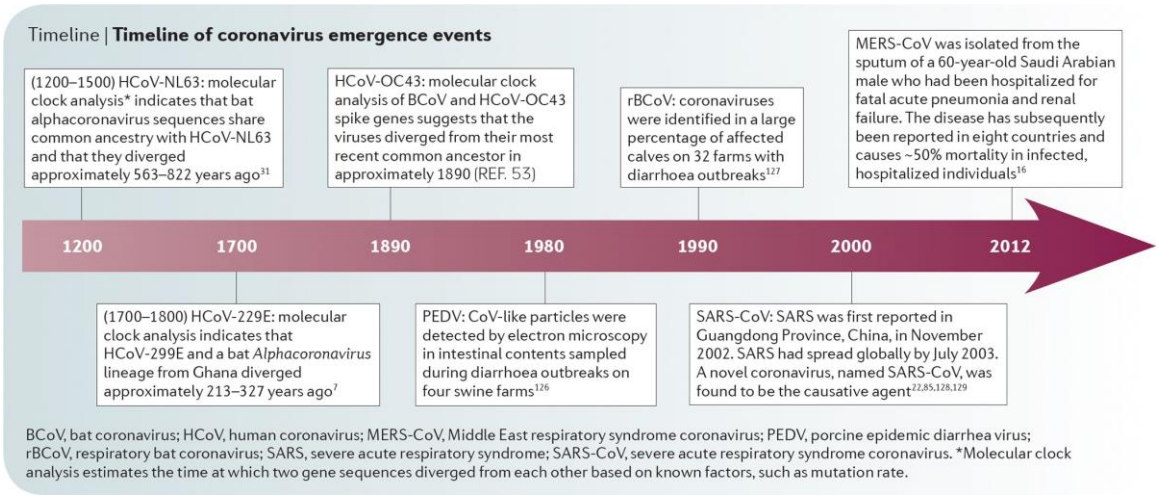


Fig. 2 Timeline of Coronaviridae emergence events. Reprinted from "A Decade after SARS: Strategies for Controlling Emerging Coronaviruses", Graham, R. L., *et al*., 2013, *Nature Reviews Microbiology*, 11(12), 836-48.

Moreover, regarding *Betacoronaviruses*, two species have emerged in the early 2000s: SARS-CoV and MERS-CoV, respectively Severe Acute Respiratory Syndrome Coronavirus and Middle East Respiratory Syndrome Coronavirus [5]. Finally, Fig. 2 shows the history of the emergence of coronaviruses up to the last SARS-CoV-2 event in December 2019.

SARS-CoV

From November 2002 to February 2003, 305 cases and 5 deaths due to atypical pneumonia appeared in the Guangdong province of southern China [44]. Later, a Chinese doctor who had been treating patients in Guangdong was responsible for the spreading of the infection outside the province, which began from the Metropole Hotel, in Hong Kong, to a total number of 29 countries worldwide between November 2002 and August 2003 (Fig. 3) [44, 45]. The SARS epidemic was mainly contained by strict quarantine measures, since no vaccines were available.

Regarding its transmissibility, SARS-CoV primarily spreads through droplets, however its efficiency of infection seems to be low, meaning that a high viral load is necessary for transmission to occur [44].

SARS-CoV epidemic has caused a total of 8.096 cases and 774 deaths [15]. Moreover, in 38% of all the reported cases, pneumonia led to acute breathing

failure and required hospitalization, whereas case fatality rate was around 10% [44].

The epidemic strain hTor02 has been shown to have a S protein with a high affinity for ACE2, which represents the SARS-CoV receptor in human cells, and shows a number critical differences with SARS-CoV-2 [5].

Indeed, regarding receptor-binding domain (RBD) of S protein, SARS-CoV RBD shows only 73% amino acid similarity with that of SARS-CoV-2, suggesting a significant difference in the S protein – receptor interaction [1].

Indeed, one of the most remarkable features of SARS-CoV-2 S protein consists in the insertion of four residues (PRRA) at the junction between subunits S1 and S2 of the S protein, which is absent in the SARS-CoV S protein [1].

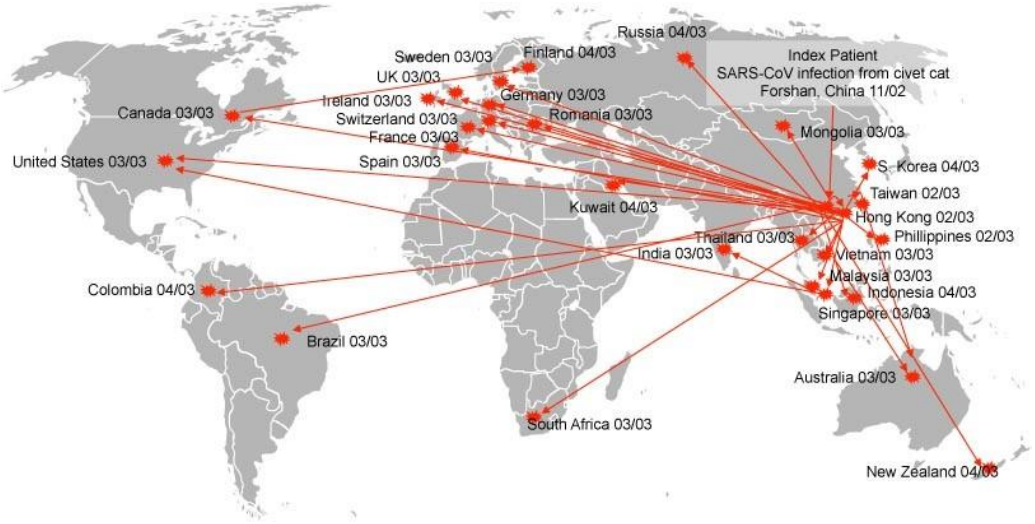


Fig. 3 World map of SARS-CoV spreading. Arrows show travel of infected people along international air travels resulting in outbreaks of SARS. Reprinted from https://www.nsf.gov/about/history/globalhealth_impacts.jsp (Accessed June 15, 2021).

MERS-CoV

In 2012, the emergence of another *Betacoronavirus,* namely MERS-CoV, has made it clear that *Betacoronavirus* pose a major threat to human health.

MERS-CoV was identified for the very first time in 2012, in a lung sample collected from a 60-years-old patient with respiratory and renal failure of unknown cause in Jeddah, Saudi Arabia [15].

Afterwards, it has spread worldwide, specifically across 27 countries, leading to 862 registered deaths and a total of 2.506 laboratory-confirmed cases (Fig. 4) [15]. Moreover, MERS-CoV has shown a higher case fatality rate (34%) compared to that of SARS-CoV [45].

Regarding its receptor usage, MERS-CoV S protein predominantly recognizes a dipeptidyl peptidase 4 (DPP4), which is mainly expressed in human alveoli, kidney, liver and small intestine. Therefore, this Coronavirus shows a broad organotropism [15]. However, DPP4 does not seem to be the only surface target, since MERS-CoV S protein has been demonstrated to effectively bind to the carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5). This surface protein seems to facilitate MERS-CoV entry when overexpressed in host cells [47].

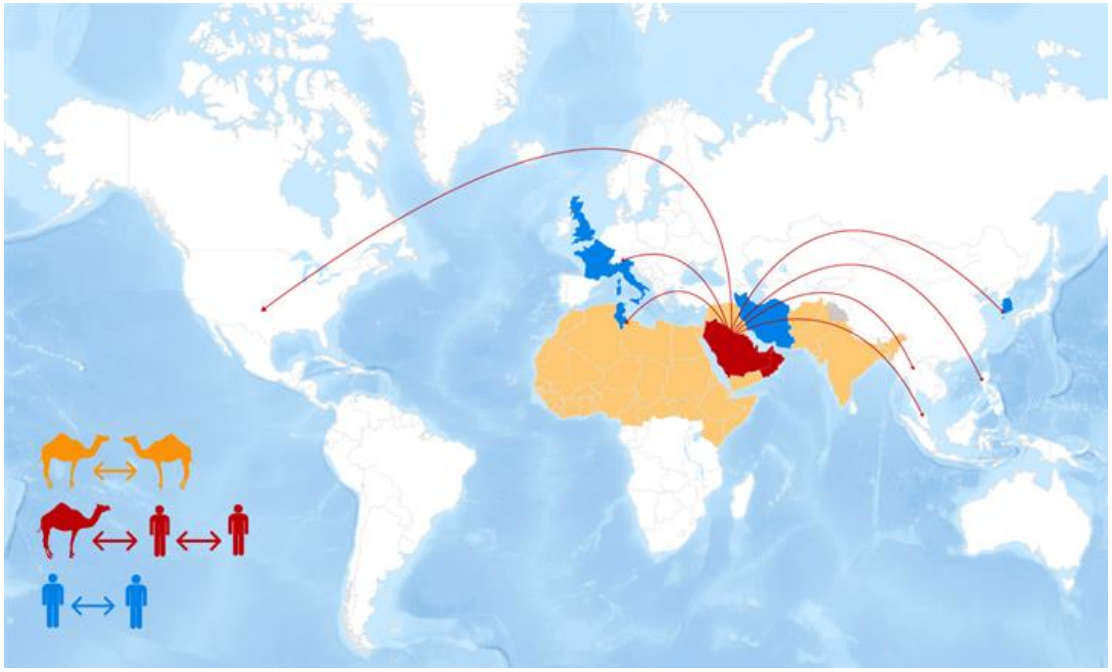


Fig. 4 MERS-CoV spreading map. Retrieved from https://www.who.int/health-topics/middle-east-respiratory-syndrome-coronavirus-mers#tab=tab_1. Copyright 2018 World Health Organization.

1.3 SARS-CoV-2 and Spillover

Phylogenetic analyses of SARS-CoV-2 have proved its belonging to the genus *Betacoronavirus*, subgenus *Sarbecovirus*, clustering with SARS-CoV and other SARS-related coronaviruses [1].

Unlike MERS-CoV and SARS-CoV, which have originated from bats and were associated respectively to dromedary camels and palm civets as intermediate hosts, at the moment the identity of both the actual natural reservoir among bat species and the intermediate host remains an incognita for SARS-CoV-2 (Fig. 5) [1, 15].

Early findings indicated that the closest relative to SARS-CoV-2, among bats coronaviruses living in South China, only shares 96,2% whole genome similarity with it [1]. It will probably be necessary to further study the bat reservoir by extending the survey to the whole Southeast Asia.

Moreover, scientists are still trying to understand whether there is an intermediate host or not. On one hand, pangolins from Guangdong province in China seem to be good candidates, because they have shown clinical manifestations after infection, unlike bats, which do not exhibit symptoms.

Furthermore, amino acids located in the receptor-binding motif (RBM) of the S protein found in pangolins are almost identical to those of SARS-CoV-2.

But, despite this, pangolins involvement in the SARS-CoV-2 zoonosis is still

uncertain and very unlikely.

However, there is no doubt that the recurrent detection of novel Coronaviruses in bats represents a constant warning for future spillover events.

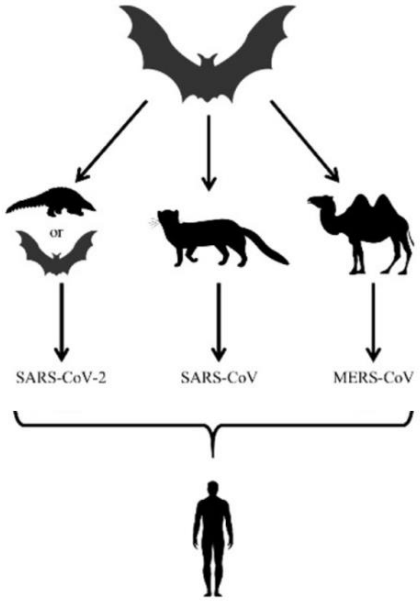


Fig. 5 Zoonotic transmission of Coronaviruses. Adapted from "Coronavirus Disease 2019: A Brief Review of the Clinical Manifestations and Pathogenesis to the Novel Management Approaches and Treatments", by Kooshkaki, O., et al., *Frontiers in Oncology*, 10, art. 572329.

1.4 Clinical features

In general, COVID-19 clinical severity increases with age, therefore older people are more likely to develop a severe respiratory syndrome compared to children and young adults, who usually show mild clinical signs or may even be asymptomatic. In addition, the disease outcome is strongly connected to pre-existing co-morbidity: underlying medical conditions such as diabetes, heart disease, chronic lung inflammation, etc. can increase the risk for severe illness, even in young people [7].

The reported symptoms range from mild ones (81% of reported cases), such as chills, fever, cough, sore throat, taste and smell loss, nausea and diarrhea to severe ones, for instance dyspnea, chest pain, acute respiratory distress syndrome (ARDS) and, in the most critical cases, multi-organ failure [1, 7].

The incubation period (1-14 days) paves the way to the disease onset, then the mild-symptomatic phase takes place (7-14 days). Later, symptoms can either disappear or get worse, for instance in patients aged >60 years, who are more likely to develop severe manifestations (14%). The disease exacerbation (5%) can occur if symptoms do not seem to decrease in 16-20 days and is usually associated with patients aged >68 years, leading to critical or, in worst cases, fatal conditions (Fig. 6) [1].

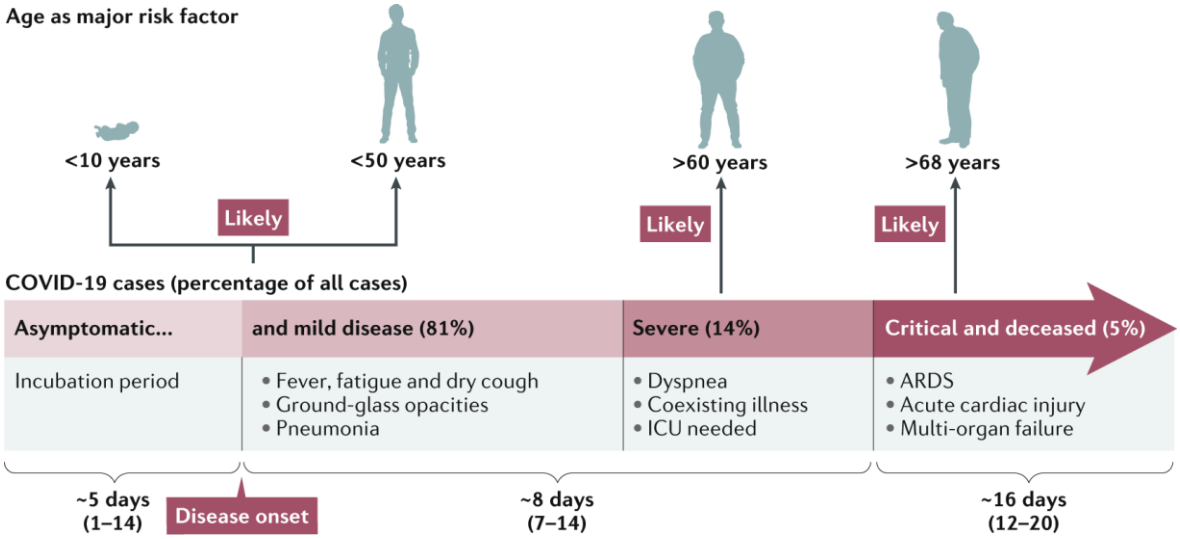


Fig. 6 Clinical features of COVID-19. Reprinted from "Characteristics of SARS-CoV-2 and COVID-19", by Hu, B., *et al*., 2021, *Nature Reviews Microbiology,* 19, 141–154.

1.5 Transmission

One of the most critical steps in managing a pandemic is to understand the modes of transmission of the pathogen: this would allow health authorities and governments to adopt proper measures to prevent, control and, at last, break chains of transmission. An infected host can either show mild symptoms or be asymptomatic. In both cases, from the very beginning of infection, SARS-CoV-2 can replicate and be released in large amounts, even when symptoms have not yet appeared.

Consequently, since the infective state precedes the illness onset, this virus is capable of spreading very easily from person to person due to the unawareness of being infected and infective [20].

As far as we know, SARS-CoV-2 reaches the upper respiratory tract in multiple ways [8]:

- **Infected secretions and droplets**: an infected person can expel respiratory secretions or particles (5-10 μm in diameter) through coughing, sneezing, or talking. Therefore, the mouth, nose or eyes of a susceptible person in close contact with a symptomatic patient can be reached by these droplets.
- **Fomites**: contaminated objects, including hands, can be carriers of SARS-CoV-2 as well. Infected surfaces may allow SARS-CoV-2 survival for a few days, depending on environmental conditions. Therefore, there is a strong likelihood that transmission may also occur in an indirect way, just like other coronaviruses and respiratory viruses.
- **Airborne transmission**: this mode of transmission consists in expelling infected aerosol particles (<5 μm in diameter) that may reach the upper respiratory tract of a recipient. This is the more efficient way of transmission.

  It has been studied in naive ferrets, which have shown positivity for viral RNA after being exposed to SARS-CoV-2 positive

faecal samples collected from infected ferrets, thus suggesting airborne transmission [32].

To date, contagion seems to occur mainly through close contact with symptomatic patients, not only in household, but also in various indoor environments, such as gyms, restaurants and, last but not least, workplace.

All these findings explain why SARS-CoV-2 has spread so rapidly worldwide.

1.6 Laboratory diagnosis

Early and accurate viral detection is essential to reduce pandemic by enabling Public Health facilities to prevent contagion from isolate cases and putting in place effective contact tracing.

SARS-CoV-2 surveillance is closely associated with important challenges, such as the necessity of high-throughput technologies that could face the rapid increase of positive cases.

1.6.1 Direct tests

Clinical samples for SARS-CoV-2 detection can be collected from the upper respiratory tract, such as nasopharynx, oropharynx and saliva, or lower respiratory tract, for instance sputum, tracheal aspirate or bronchoalveolar

lavage (BAL).

Up to now, the most sensitive and specific laboratory methods used in COVID-19 diagnosis are the molecular ones, based on SARS-CoV-2 RNA detection through a real-time reverse transcriptase polymerase chain reaction (RT-qPCR) assay, that is the international "gold standard" [9].

Real-time reverse transcriptase polymerase chain reaction

The workflow consists in extracting viral RNA and converting it into the complementary DNA (cDNA). Later, the cDNA is amplified by a thermostable DNA polymerase - DNA dependent.

Real-time PCR relies on the use of probes, which are fluorescently labelled DNA oligonucleotides properly designed to anneal to a specific target sequence. These probes are labelled with a "reporter dye". During the PCR cycling, a fluorescent signal is generated by the reporter dye and is recorded at the end of the extension phase, and as the number of gene copies increases during the reaction, so does the fluorescence. The assay can be designed in *Singleplex* and *Multiplex* formats, this latter allowing the simultaneous detection of multiple target sequences by using specific primers in combination with probes labelled with different fluorophores. The level of target sequences present in the sample is characterized by the Cycle

Threshold (CT) that indicates the PCR cycle in which the fluorescence exceeds the threshold of detection and is stably detected by the machine. The CT roughly represents a quantitative index, being inversely proportional to the amount of the target sequence.

Unfortunately, this procedure suffers from some limitations, for instance reagents contaminations, or the incapability of designed primers to anneal specific viral genome parts because of occurring mutations [10]. These abnormalities may lead, respectively, to false positive and false negative results.

Rapid antigenic tests

In addition to RT-qPCR, rapid antigenic tests are routinely performed in laboratories worldwide and consist in detecting SARS-CoV-2 antigens in about an hour or less, depending on the format (rapid or instrumental) of the assay. However, these tests do not seem to be highly sensitive, especially in case of low viral load. Therefore, in the well-founded suspicion of an acute infection, a negative result must be confirmed by an RT-qPCR test, as well as any positive results must be confirmed due to the possibility of false positives.

1.6.2 Indirect tests

Serological tests to detect antibodies against viral Nucleoprotein (N) or Spike protein (S) can also be used as diagnostic predictors to estimate previous or recent viral infections. Although IgM levels seem to decrease two weeks after SARS-CoV-2 infection and are not always detectable, they can be used as valuable parameters to estimate an early infection. IgM detection combined to RT-qPCR assay ensures a high accuracy level, both for positive and negative results. In any case, the significant diagnostic data is more correlated to the molecular assay.

On the other hand, IgG detection is possible not earlier than one week after infection and is used as a predictor for late or previous infections [10].

The accurate qualitative and quantitative assessment of antibody titre in a serum can be achieved through the "gold standard" method, the microneutralization test. This assay is based on the reduction of cytopathic effect in cell cultures challenged with a quantified viral stock pre-treated with serum. Unfortunately, this technique is very cumbersome and dramatically disadvantageous in terms of time, costs and equipment (a BSL-3 environment is required), so it is not recommended for large-scale diagnostic purposes.

Therefore, several automated methods have been developed since the

pandemic began, such as IgG and IgM detection based on chemiluminescent reactions, or lateral-flow immunoassays.

2. Virion structure

The SARS-CoV-2 virions, that is the infectious particles, can be either spherical or pleomorphic, the size being approximately 100 nm in diameter. Several Spike (S) proteins organized in trimers protrude from the envelope surface, giving the virion a characteristic bulb-like shape that resembles a solar corona – for this reason scientists have coined the name 'Coronavirus' (Fig. 7). According to the Baltimore classification, being the genome a positive sense single-stranded RNA molecule, these viruses are assigned to the Group IV. SARS-CoV-2 virion consists in an external portion named 'envelope', rich in Spike proteins, and a 'nucleocapsid', made of nucleoproteins which enclose and protect the viral genome. Notably, apart from other (+) ssRNA viruses, SARS-CoV-2 nucleocapsid shows a helical symmetry and therefore seems to be highly flexible [6].

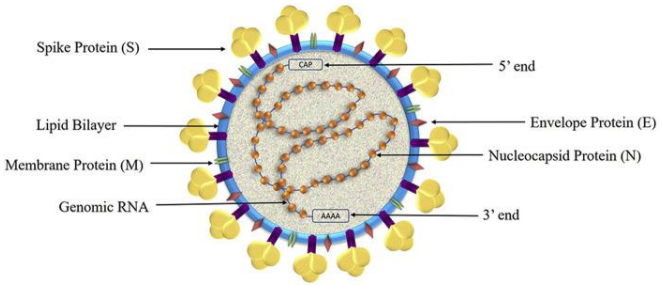


Fig. 7 Schematic representation of SARS-CoV-2 virion structure. Reprinted from "Structural proteins in Severe Acute Respiratory Syndrome Coronavirus-2", Satarker, S., *et al*., 2020, *Archives of Medical Research,* 51, 482-291.

2.1 Genome organization

As discussed above, the novel Coronavirus is a non-segmented (+) ssRNA virus, consequently its genome, which is approximately 30.000 bp long, acts both as a mRNA for translation and a template for replication.

Similarly to all other Coronaviruses, SARS-CoV-2 genome is comprehensive of a 5' terminal Cap structure and 3' a poly-A tail [6, 15], thus being immediately available to be translated in host cells.

It consists in a specific set of genes arranged in a linear way as follows: *5'-replicase-S-E-M-N-3',* the organization of which is evolutionarily conserved among other Coronaviruses (Fig. 8). Apart from the abovementioned structural genes, several "nested" non-structural coding sequences have been identified, however their function is not yet entirely understood.

In addition, four conserved sequences called transcription-regulating sequences (TRS) have been found upstream of each gene, and seem to be involved in template-switching phenomena and sub-genomic RNA generation.

The ORF1a/b (21.291 bp long) is located at the genome 5' terminus, representing the longest coding sequence and encodes the RNA-dependent RNA-polymerase, along with secondary proteins involved in replication mechanisms.

At the beginning of the viral replicative cycle ORF1a is translated, followed by ORF1b, generating two polypeptides (respectively pp1a and pp1ab) which are nothing but precursors that need to be further processed into 16 mature proteins, through multiple proteolytic cleavages.

In addition, the 3'-end of the genome encodes for S, E, M and N structural polypeptides, in parallel with eight accessory proteins generated from interspersed sequences. The S gene corresponds to the largest gene, with a total length of 3.822 bp [15].

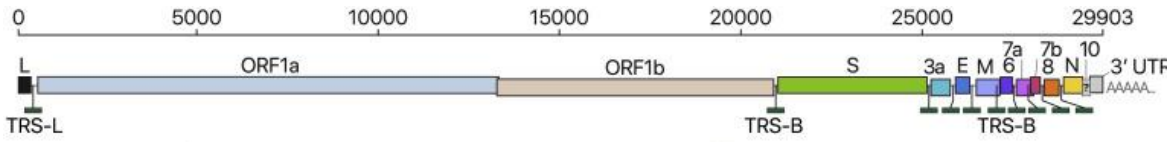


Fig. 8 Schematic representation of SARS-CoV-2 overall genome organization

2.2 Structural proteins in SARS-CoV-2

Several structural proteins cooperate to maintain functional viral particles:

- **Membrane protein (M)** is the most abundant structural protein. This 30 kDa polypeptide is a N-linked glycosylated monomer located between the internal side of the envelope and the nucleocapsid, working as a 'bridge' between the nucleocapsid and the lipid bilayer, through three transmembrane domains (TMD). The C-terminal domain, namely the endo-domain, accounts for the major part of the polypeptide and is located in the inner part of the virion (Fig. 9).

  Furthermore, the M protein exists in a "long-form", which enables Spike installation on the viral envelope and gives the virion its characteristic spherical shape [6, 39];

- **Nucleocapsid protein (N)** is a monomeric 50 kDa polypeptide and is the unique component of the helical nucleocapsid. This structural protein binds to the viral genomic RNA through a "beads-on-a-string" conformation [6]. Being the N-terminal and C-terminal domains highly basic, they represent the RNA binding domains, and are separated by a Serine and Arginine (SR) rich linker, which undergoes a phosphorylation process during viral replication. This event has been proven to increase the N protein affinity for viral RNA rather than non-viral RNA (Fig. 9) [6,

39]. Moreover, this polypeptide plays an essential role in inhibiting viral proteins degradation by cellular proteasome and blocking type 1 Interferon [39].

- **Envelope protein (E)** is an integral membrane protein and has been proved to form 75-residue "viroporins", which are crucial cation-selective channels across the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) membrane and play a role in viral assembly and virions morphogenesis [6, 39, 42]. This protein has a hydrophilic N-terminal domain followed by a hydrophobic region, and finally a hydrophilic C-terminal domain. The N-terminal contains Golgi associating elements and its structure has been proven to be essential during the virion release (Fig. 9) [6, 39];

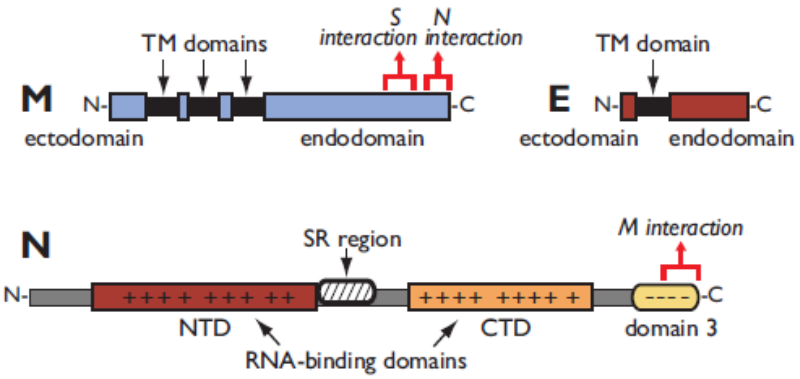


Fig. 9 Schematic illustration of Coronavirus N, M and E proteins. Reprinted by Knipe, D. M., & Howley, P. M. (2013). *Fields Virology* (6th ed.). Lippincott Williams & Wilkins.

- **Spike protein (S)** anchored in the viral membrane, is a trimeric, bulb-like glycoprotein involved in host tropism, receptor binding and membrane fusion, as well as antibody induction. Each monomer is made of two portions: the receptor-binding fragment (S1) and the fusion fragment (S2). The first contains the N-terminal domain (NTD) and the receptor-binding domain (RBD), whereas the latter contains the fusion-peptide (FP), the heptad-repeat 1 (HR1), the central helix (CH), the connector domain (CD), the heptad repeat 2 (HR2), the transmembrane motif (TM) and the cytoplasmic tail (CT) (Fig. 10).

  The activation of the S protein is a complex process which requires proteolytic cleavages at two different sites: S1/S2, that is a short polybasic sequence (RRAR) and is a unique feature of SARS-CoV-2, and a second site in S2 (S2'), located immediately upstream of the fusion peptide (FP) [1, 13, 41]. S1/S2 region can be recognized by a furin-like protease and is cleaved during biosynthesis, in order to form the pre-fusion conformation [1, 13, 40].

  On the other hand, when the RBD binds to the target receptor, at the early stage of infection, S2' undergoes a cleavage performed by a transmembrane serine protease (TMPRSS2), which triggers the

irreversible rearrangement of S protein and allows the exposure of the fusion peptide (FP) [14, 41].

In the pre-fusion form, three copies of the receptor binding domain are surrounded by three copies of the N-terminal domain.

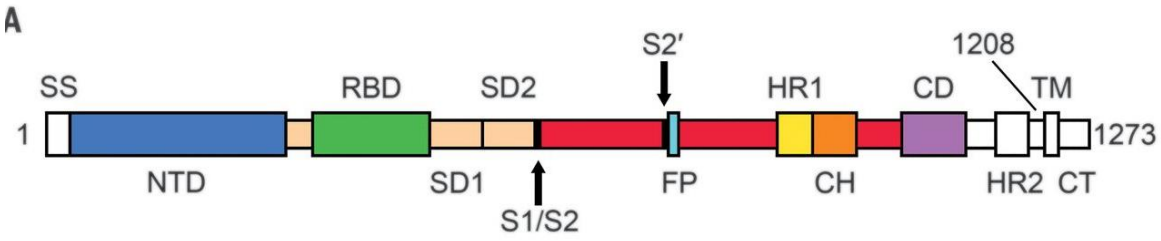


Fig. 10 SARS-CoV-2 Spike protein. (A) Schematic representation of S protein domains. Adapted from "Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation", Wrapp, D., *et al.,* 2020, *Science*, 367(6483), 1260-1263.

There are two possible conformations in the pre-fusion form (Fig. 11a, 11b): the closed conformation and the open conformation. In the closed one "all three copies of the RBD lie flat on the Spike surface", thus hiding the receptor-binding motif, whereas in the open pre-fusion conformation one or multiple RBDs lift-up and expose the receptor-binding motif [64]. After the receptor binding, a structural transition to the post-fusion conformation occurs, bringing the fusion peptide and the transmembrane domain together [64]. During infection, the S protein can stimulate the

production of neutralizing antibodies (nAbs), being therefore the most remarkable viral component for vaccine design.

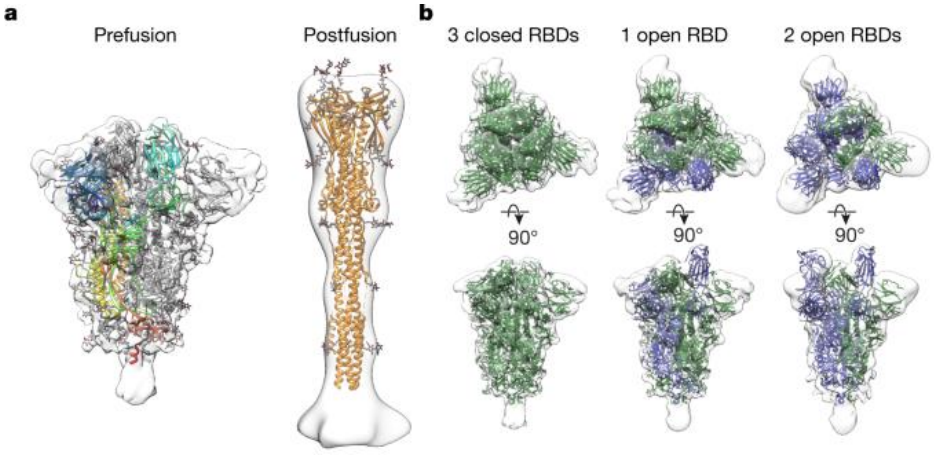


Fig. 11 (a) Structures of pre-fusion and post-fusion trimers of the S protein. (b) Possible conformations of the pre-fusion trimer: all RBDs in the closed position (left); one RBD in the open position (centre); two RBDs in the open position (right). Reprinted from "Structures and distributions of SARS-CoV-2 spike proteins on intact virions", by Ke, Z., et al., 2020, *Nature*, 588, 498–502.

2.3 Non-structural proteins in SARS-CoV-2

Non-structural proteins (nsps) are quite numerous polypeptides involved in several phases of the viral replication (Fig. 12). Their functions are not yet entirely understood. Data currently available are discussed below:

- **nsp1** is involved in IFN signalling inhibition and is a major virulence factor [38];
- **nsp2** and **nsp3** interact to act as proteases and cleave the product of

ORF1a [38];

- **nsp5** has a protease activity [36, 38];

- nsp4, nsp6 are transmembrane proteins which have been suggested to facilitate viral replication by anchoring the viral replication-transcription complex (RTC) to convoluted membranes (CMs) and to suppress host protein synthesis [37, 38];

- **nsp7** and **nsp8** are cofactors of nsp12 [37];

- **nsp9** is able to bind the RNA in complex with nsp8 [37,38],

- **nsp10** acts as a cofactor for the activity of nsp14 and nsp16 in 5' capping [38];

- **nsp11** has been shown to be essential in viral replication, although limited information on this protein is available at the moment [38];

- **nsp12** represents the RNA-dependent RNA-polymerase, which operates in association with several accessory proteins to actively replicate the viral genome [37];

- **nsp13** works as a helicase to unwind dsRNA intermediates, but it is also involved in 5' mRNA capping [38];

- **nsp14** is a 3'-5' exonuclease, ExoN is the protein with proofreading activity. It increases the fidelity of RNA synthesis by correcting nucleotide incorporation errors made by RdRp; moreover, it is involved

in the RNA 5’ Cap formation [37];

- **nsp15** is a uridylate-specific endo-nuclease which is suggested to counteract dsRNA sensing [37];

- **nsp16** is involved in the ribose 2’-O-methylation during 5’ capping [37];

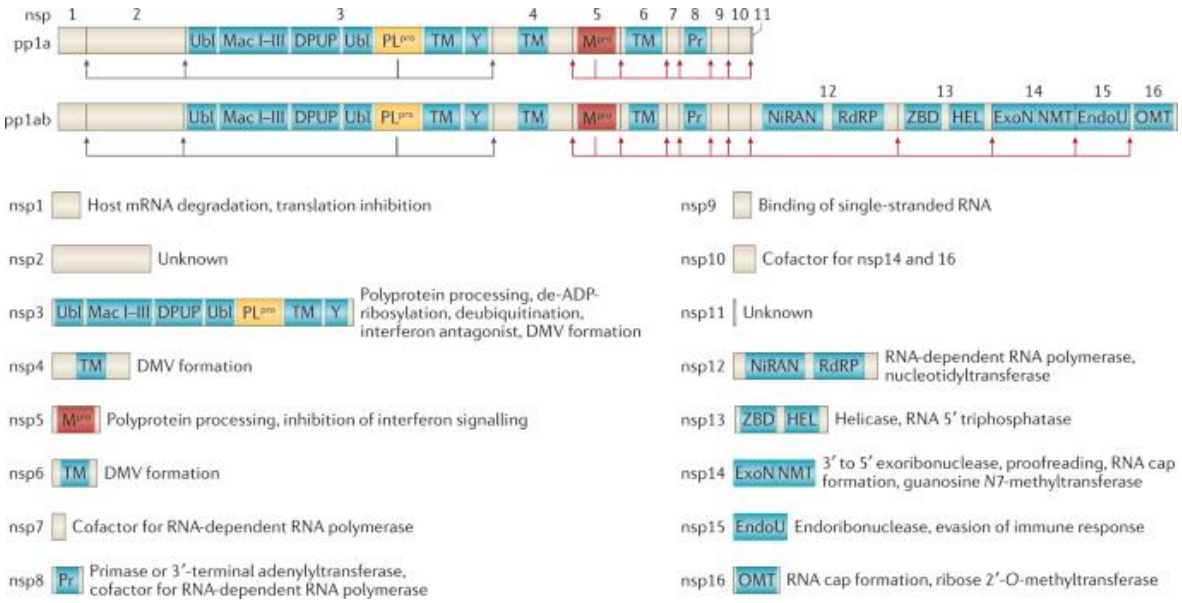


Fig. 12 Coronavirus polyprotein precursors and non-structural proteins. Reprinted from “Coronavirus biology and replication: implications for SARS-CoV-2”, 2021, V’kovski, *et al.*, *Nature Review Microbiology,* 19, 155–170.

3. The life cycle of SARS-CoV-2

SARS-CoV-2 employs the SARS-CoV cellular receptor Angiotensin converting enzyme 2 (ACE2) to entry the host cell.

3.1 Viral attachment and entry

ACE2 recognition is mediated by the RBD sequence, located in the S protein, which differs from that of SARS-CoV (Y442, L443, L472, N479, T487) by 5 crucial residues (L455, F456, F486, Q493, N501 in SARS-CoV-2) [43].

In more detail, the RBD contains five antiparallel β sheets (namely β1, β2, β3, β4 and β7) that assembly to form the core. Between the β4 and β7 strands, structural studies have revealed an extended insertion including β5 and β6 strands, as well as α4 and α5 helices, representing the receptor-binding motif (RBM), which spans from AA 438 to AA 506. The RBM is a crucial element in the overall structure of the RBD, as it comprises most of the contacting residues that bind to ACE2 (Fig. 13) [43].

Moreover, biochemical data have shown that four residues in the RBM (amino acids 482-485: G-V-E-G) seem to enhance the strength of receptor-ligand binding for SARS-CoV-2 compared with SARS-CoV [1].

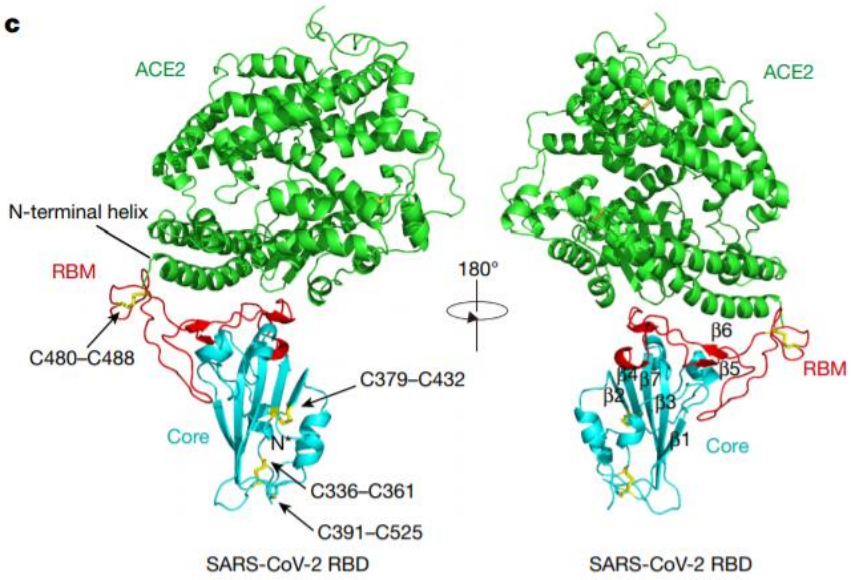


Fig. 13 Overall structure of SARS-CoV-2 RBD (red) bound to ACE2 (green). The same structure is rotated 180 degrees to show the core region (light blue). Reprinted from "Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor", by Lan, J., et al., 2020, *Nature*, 581, 215–220.

The receptor usage affects the host tropism, as proven by the heterologous expression of ACE2 into cell lines which were not permissive to SARS-CoV-2 infection [6].

The first key step to ensure viral entry in the host cell is mediated be the transmembrane serine protease (TMPRSS2), which co-localizes with ACE2 and is believed to carry out a proteolytic cleavage is S2' site, thus triggering irreversible S2 folding into conformation that provides fusion of viral envelope to cellular membrane (Fig. 14) [14].

Subsequently, virion contents are delivered into the cell to allow genome replication.

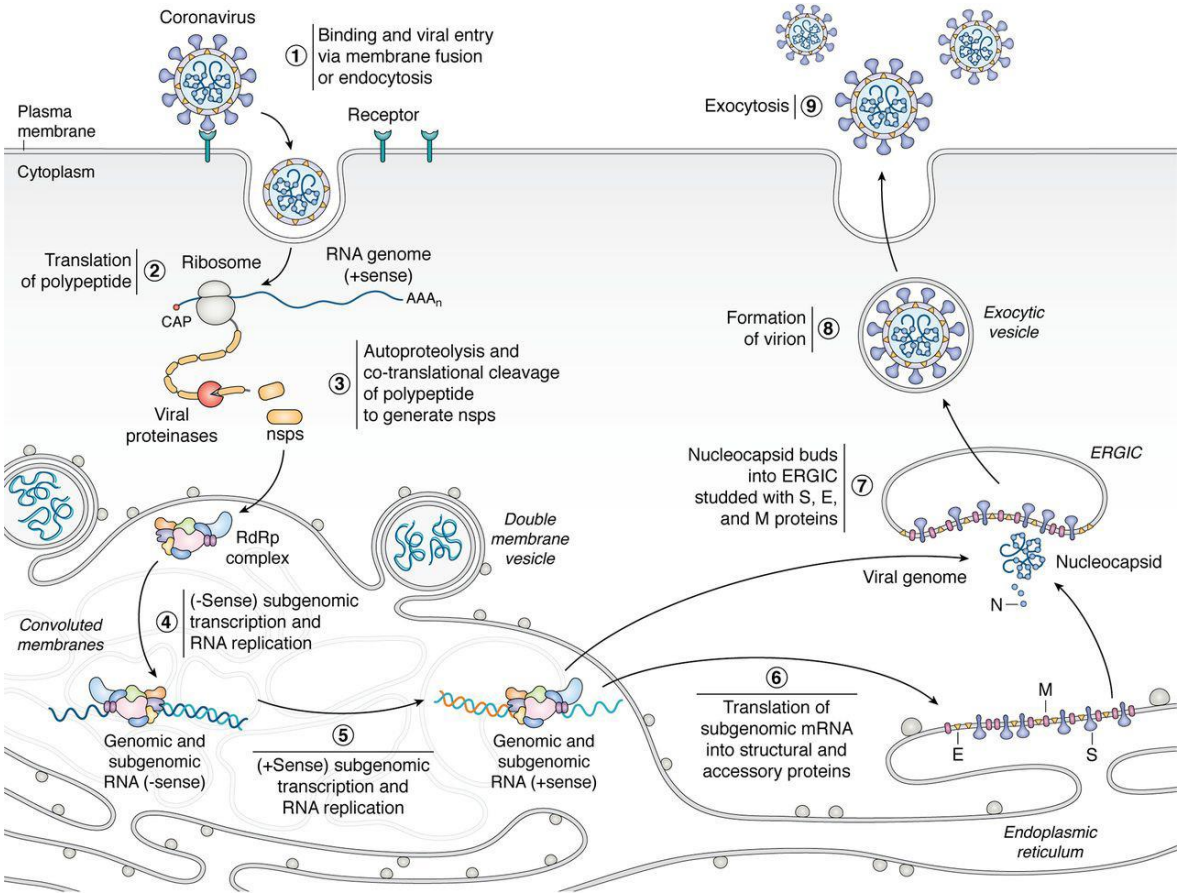


Fig. 14 Coronaviruses life cycle. Reprinted from "The molecular virology of Coronaviruses", by Hartenian, E., et al., 2020*, Journal of Biological Chemistry*, 295(37), 12910-12934.

3.2 Viral RNA replication and expression

Regardless of the infected host, Coronaviruses carry out their genome replication inside a reticulo-vesicular network that integrates convoluted membranes (CMs, depicted in bronze in Fig. 15), several interconnected double-membrane vesicles (DMVs, which diameter is approximately 200-300 nm) and "vesicle packets" (VPs), which are assembled in close connection to the ER. This mechanism represents a great chance to escape the host innate immunity, since dsRNA molecules synthetized as replicative intermediates are hidden from the surrounding environment [16].

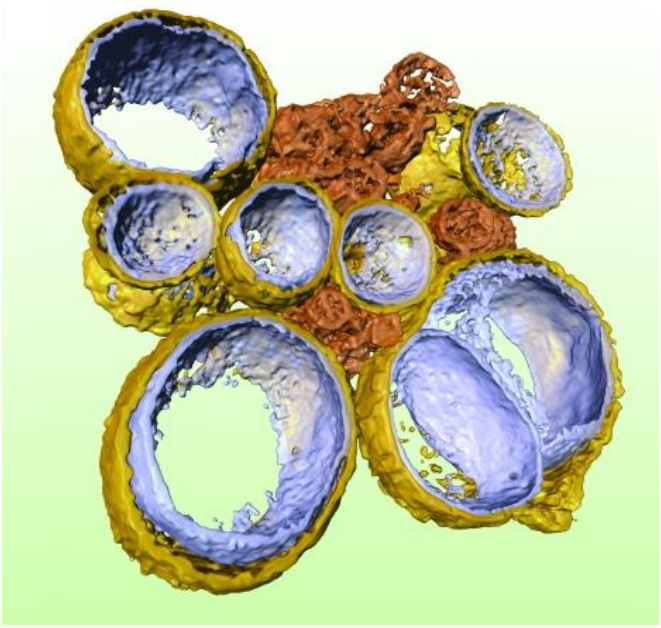


Fig. 15 3D-surface-rendered-model showing membranous compartments for RNA synthesis induced by Coronavirus infection. DMVs (silver), CMs (bronze). Reprinted from “SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum”, by Knoops, K., *et al*., 2008, *PLoS Biology*, 6(9), e226.

3.2.1 Transcription and translation

First of all, the *replicase* gene is translated by host ribosomes into two precursor polyproteins, namely pp1a and pp1b, starting at ORF1a and proceeding in ORF1b thanks to a -1 frameshift signal.

At this point, the RNA-dependent RNA-polymerase and several mature nsps are released from numerous proteolytic cleavages, provided by the main protease (Mpro), and combine to form the RTC. This complex comprises nsps, such as **nsp13** helicase and **nsp7**, to enhance the processivity of RdRp.

A remarkable proteolytic product released by Mpro is **nsp3**, which has a pivotal role in shaping the abovementioned double membrane vesicles (DMVs).

In contrast to replication, mRNA synthesis is described by a discontinuous mechanism: when RdRp crosses a TRS region, a template-switching event may occur in the (-) strand copy, implying a premature termination in the RNA synthesis (Fig. 16).

Scientists have suggested that the long-distance between TRS regions may be shortened by protein-RNA complexes that would bring two different TRS together, thus allowing the transcription of the 5'-terminal leader sequence and generating sub-genomic RNAs (sgRNA).

Subsequently, transcription of these (-) sgRNAs provides mRNAs that are finally decoded into viral proteins through the cellular translation machinery.

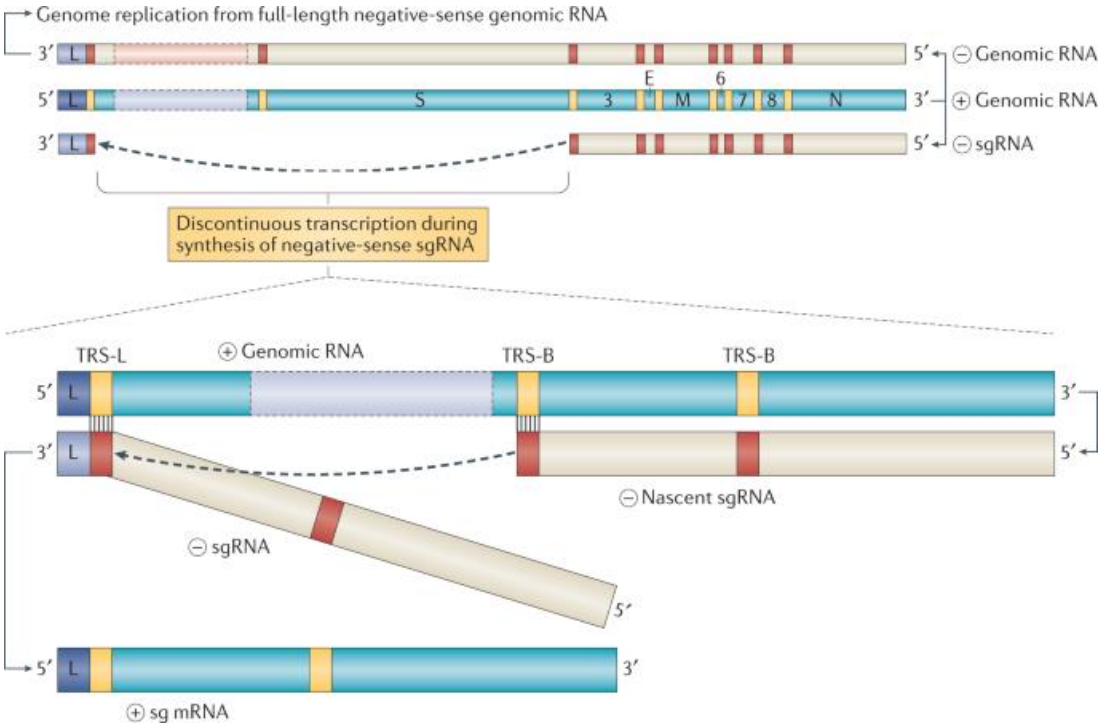


Fig. 16 Genome replication and discontinuous transcription during Coronavirus life cycle. Reprinted from "Coronavirus biology and replication: implications for SARS-CoV-2", by V'kovski, P., *et al., 2021, Nature Review Microbiology,* 19, 155–170.

3.2.2 Replication

As far as we know, Coronaviruses replication is a continuous process mediated by RdRp, which starts its activity at the 3'-end of the genome, resulting in the synthesis of a full-length (-) ssRNA that works as a complementary template to produce new genomes [18].

Moreover, genome replication is presumed to be concomitant with nucleocapsids formation; therefore, once whole-genomes are produced, they coalesce with N proteins. After transcription, all mRNAs produced are 5'-capped with the aim of being recognized by the eukaryotic translation machinery [37].

The RNA capping is carried out by several enzymes, which have been suggested to be attractive targets for novel antiviral drugs. Specifically, three main non-structural proteins work as capping machines (Fig. 17) [37]:

- **nsp13** acts as a 5'-triphosphatase, removing the 5'γ-phosphate from the 5' nucleotide; GMP is added to the 5'-diphosphate chain;
- **nsp14** catalyses the addition of a methyl group at the N7 position of the guanosine;
- **nsp16** promotes the insertion of an additional methyl group at 2'-O position on the ribose of the 5' nucleotide.

Unlike cellular mRNA 5'-capping, which takes place in the nucleus, viral capping is carried out in the cytoplasm. The addition of a 7-methyl-guanosine (m7G), linked to the 5'-phospate of the first nucleotide, prevents recognition from host antiviral mechanisms, which usually promote degradation of uncapped-RNAs (recognized as 'non self' structures) [18, 37].

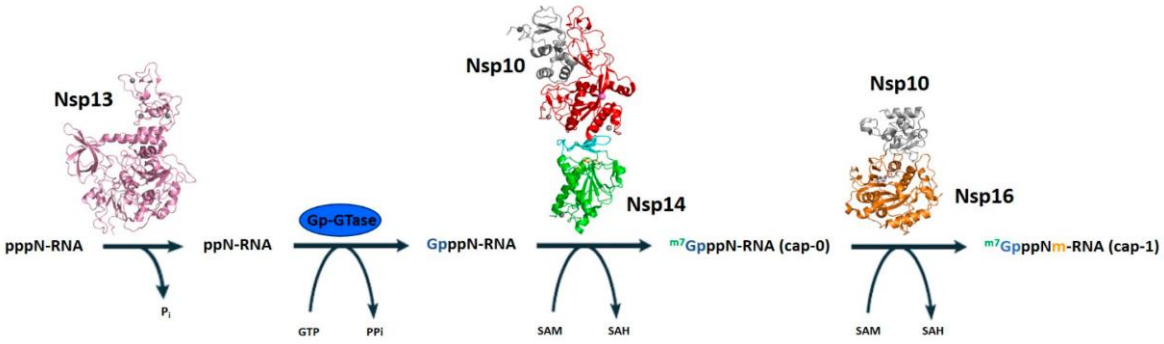


Fig. 17 The sequential enzymatic action performed during viral mRNA 5'capping. Nsp13 removes the 5'γ-phosphate from the 5' nucleotide generating the ppN-RNA; a GTase adds GMP to the 5'-terminus of ppN-RNA; Nsp10 and Nsp14 cooperate to add a methyl group to form the cap0 structure; Nsp10 and Nsp16 . Methyl donor group: S-adenosyl methionine (SAM). Reprinted from "A Structural View of SARS-CoV-2 RNA Replication Machinery: RNA Synthesis, Proofreading and Final Capping", Romano, M., *et al.*, 2020, *Cells*, *9*(5), 1267.

3.3 Assembly and Release

S, E, and M proteins are initially synthetized on and anchored to the ER exit sites (ERES), from which they migrate to reach the ERGIC or intermediate compartment (IC). In this region nucleocapsids, made of genomes and N proteins, combine with envelope components to form virions, which budding

seems to start at vacuolar domains of the IC and is likely to be based on the combination of structural proteins [6, 48]. Subsequently, virus particles are ready to move from the IC towards the plasma membrane (PM), since vesicles originating from the IC evolve to form mobile carriers (Fig. 18) [48]. Finally, budding of complete viral particles takes place and, once arrived at the plasma membrane, virions are released by exocytosis [6].

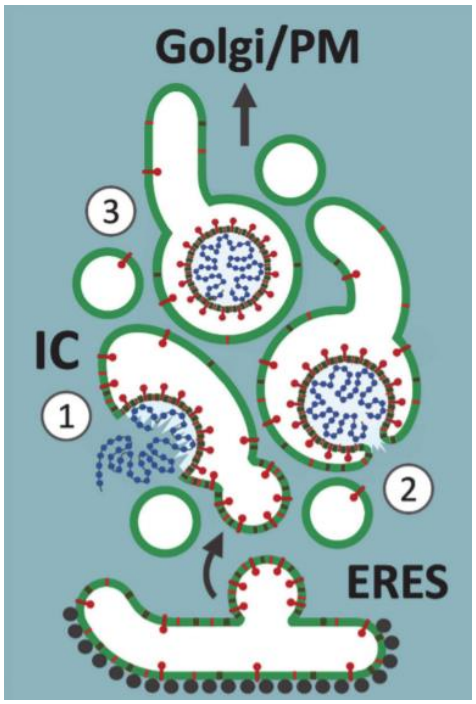


Fig. 18 Assembly process in Coronaviruses. IC: intermediate compartments. PM: plasma membrane. ERES: endoplasmic reticulum exits sites. Adapted from "Assembly and Cellular Exit of Coronaviruses: Hijacking an Unconventional Secretory Pathway from the Pre-Golgi Intermediate Compartment via the Golgi Ribbon to the Extracellular Space", by Saraste, J., *et al*., 2021, *Cells*, 10, 503.

4. Pathophysiology, immune response and immune escape strategies of SARS-CoV-2

The establishment of SARS-CoV-2 replication depends not only on the permissiveness of host cells, but also on the virus capability to inhibit and escape both innate and acquired immune response mechanisms. However, further studies are needed to fully understand all the putative evasion pathways developed by the novel Coronavirus.

4.1 Pathophysiology of SARS-CoV-2 infection

After entering the airway, SARS-CoV-2 mainly targets alveolar epithelial cells, vascular endothelial cells and alveolar macrophages, due to the high expression of ACE2 and TMPRSS2 in these cell types [19].

These entry factors are also expressed at high levels in multiple organs, including pharynx, liver, heart, kidney and gastrointestinal tract (e.g. duodenum, rectum, gallbladder, etc.) [34].

Consistently, post-mortem examinations of COVID-19 patients revealed the presence of SARS-CoV-2 in organs beyond the respiratory tract.

These information further support the hypothesis according to which SARS-CoV-2 has a broad organotropism and may lead to a multi-organ injury [33,

34].

ACE2 plays a key role in the renin-angiotensin-aldosterone system (RAAS), a pathway which dysregulation is involved in numerous diseases, such as atherosclerosis and diabetes.

In the RAAS system, the angiotensin-converting enzyme (ACE) generates angiotensin II (ANGII), an effector peptide able to stimulate vasoconstriction. Subsequently, ANGII is converted in ANG(1-7) by a proteolytic cleavage carried out by the angiotensin-converting enzyme 2 (ACE2), therefore promoting vasodilatation (Fig. 19) [19, 34].

Pathologies such as diabetes, obesity and hypertension are closely related to a high-risk severe COVID-19, since they are associated with an over-activation of the RAAS system, thus leading to an over-expression of ACE2 and increasing the overall number of receptors available for SARS-CoV-2 attachment.

Interestingly, studies carried out on mice expressing human ACE2 have shown that a local increase in the production of ANGII can result in a leakage of blood vessels in the pulmonary tract, which is a distinctive feature of Acute Respiratory Distress Syndrome (ARDS) [35].

Moreover, patients who develop severe COVID-19 undergo an over-production of pro-inflammatory cytokines, which may accumulate in lungs,

thus leading to parenchymal damage. Indeed, they show alarming bilateral alveolar damage, hyaline-membrane formation, interstitial mononuclear infiltrates, and distinctive mucus plugs in the airway [20].

Key mechanisms for a pathogenesis to be effective are discussed below [33]:

1. **Direct viral toxicity**, including the shutdown of the cellular machinery for translation, through a suppression mediated by nsp1 [19];
2. **Endothelial cell damage** in multiple vascular types, which leads to the inhibition of fibrinolysis, an increase in thrombin production and the final deposition of microthrombi [33];
3. **Dysregulation of the immune response**, associated with an over-production of pro-inflammatory cytokines (e.g. IL-6, IL-8, etc.).

   In worst cases, the excess of inflammatory mediators may lead to the "cytokine storm", observed in irremediably critical COVID-19 patients [19, 33]. Furthermore, the establishment of neutrophils extracellular traps (NETs) into small vessels contribute to the rapid and irreversible progression of multiple organs failure, through a disseminated intravascular coagulation (DIC) [19].

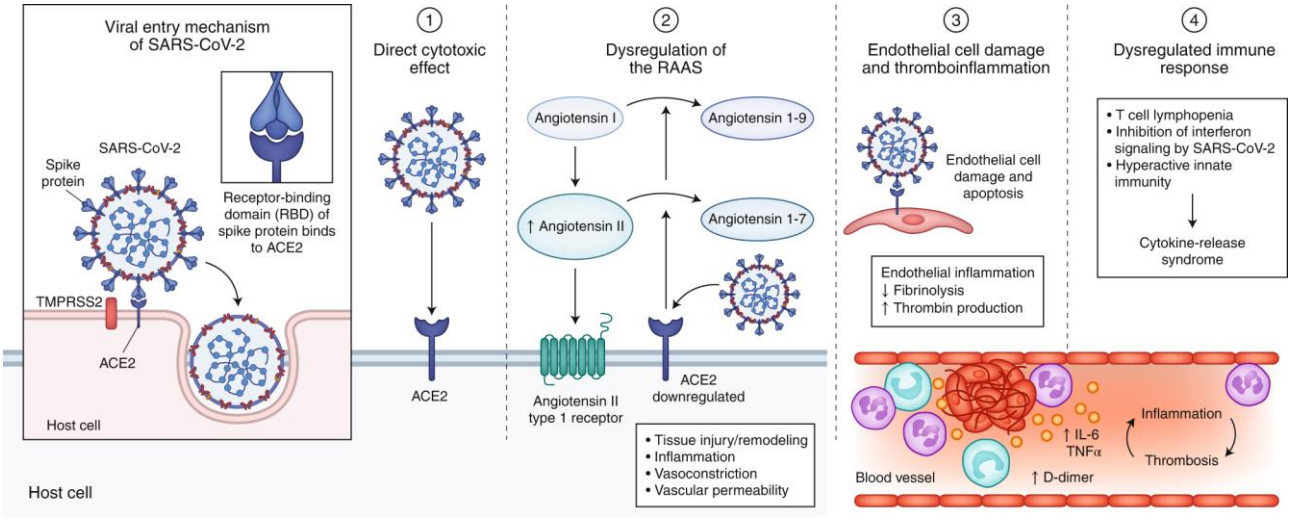


Fig. 19 Pathophysiology mechanisms of SARS-CoV-2 infection. After ACE2-RBD interaction, SARS-CoV-2 causes a direct cytotoxic effect (1). At the same time, dysregulation of RAAS occurs (2), as well as an increase in endothelial inflammation and thrombotic events (3). Finally, cytokine-storm may occur because of immune response dysregulation (4). Reprinted from "Extrapulmonary manifestations of COVID-19", by Gupta, A. et al. (2020). *Nature Medicine*, 26, 1017–1032.

4.2 Immune response to SARS-CoV-2

A key prerequisite for a pandemic is the absence of pre-existing immunity in the worldwide population. This is the case for COVID-19 pandemic.

In order to have a long-term protection against a pathogen, the adaptive immune response has to implement different strategies, such as the production of B cells that synthesize multiple classes of antibodies, as well as T cells, which play a pivotal role in eliminating virus-infected cells [51].

Regarding seroconversion, three main classes of antibodies are elicited over the course of SARS-CoV-2 infection: IgA, IgM, and IgG.

IgM dynamics seems to follow a 'rise and fall' trend, meaning that IgM levels show a peak spanning from the second to the fifth week after illness onset, and subsequently IgM decline and become undetectable [50].

On the other hand, IgA levels have been observed to increase between 16 to 22 days after symptoms onset [50].

Finally, IgG levels show a trend that consists in a peak (3-7 weeks after symptoms onset), plateau, and persistence at lower levels. As stated in numerous studies, a constant decrease in IgG levels has been suggested to take place after about eight weeks from symptoms onset [50].

Neutralizing antibodies (nAbs) are those antibodies (IgA, IgM and IgG) able to interfere with the entry of the virus into the cell, are made against several

crucial epitopes in the S protein and are generally detectable using *in vitro* biological assays, between 7 to 15 days after the disease onset, then an increase occurs during days 14-22 before reaching a plateau and, finally, decline over a period of six weeks (Fig. 19) [50].

On the other hand, current evidence on antibodies elicited against N protein suggests that they may not impair the overall infection. Nevertheless, N protein is a suitable candidate for early SARS-CoV-2 diagnosis, due to its conserved epitopes and high immunogenicity [51].

Thanks to the antibodies ability to bind different epitopes on target antigens, they take the name of *polyclonal* antibodies.

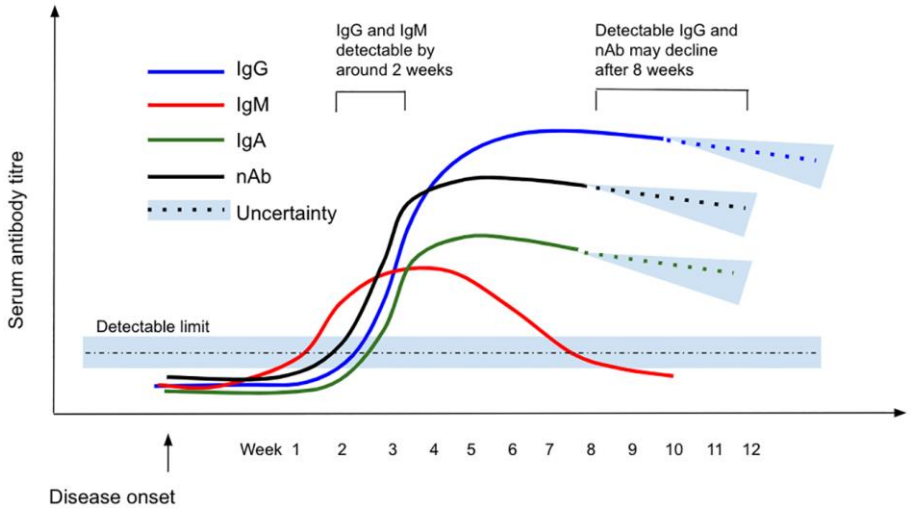


Fig. 19 IgG/IgM/IgA/Neutralising Ab response over time, starting from the disease onset. Reprinted from "Antibody response to SARS-CoV-2 infection in humans: A systematic review", by Post, N., *et al*., 2020, *PLoS ONE*, 15(12), e0244126.

4.2.1 Neutralizing antibodies (nAbs)

In general, neutralizing antibodies (nAbs) are synthetized by B cells and can block the virus attachment to the target receptor by recognizing specific epitopes on surface viral proteins. The recent isolation of SARS-CoV-2 nAbs from recovered COVID-19 patients has proven their ability to target different epitopes on the S protein, and more specifically they can recognize the RBD [55, 56].

Indeed, this domain in the S protein represents the most "immune-dominant neutralizing epitope capable of eliciting virus neutralization" [56].

nAbs can inhibit viral entry to the host cell by interacting with the S protein in multiple ways (Fig. 20) [53]:

- nAbs binding to the RBM compete for ACE2 recognition, therefore directly blocking ACE2–RBD interactions by steric hindrance (Fig. 20 A);
- nAbs binding to the N-terminal domain (NTD) or S2 subunit do not compete with ACE2 binding, but these nAbs may exhibit viral neutralization activity via unknown mechanisms (Fig. 20 B);
- nAbs binding to the RBD, but not to the RBM, may compete (Fig. 20 C) or not (Fig. 20 D) for ACE2 binding, either by inducing

conformational changes, destabilizing pre-fusion conformation, or through steric hindrance;

- Antibody cocktails that bind to multiple epitopes could also mediate virus neutralization by restricting conformational changes in the S protein (Fig. 20 E).

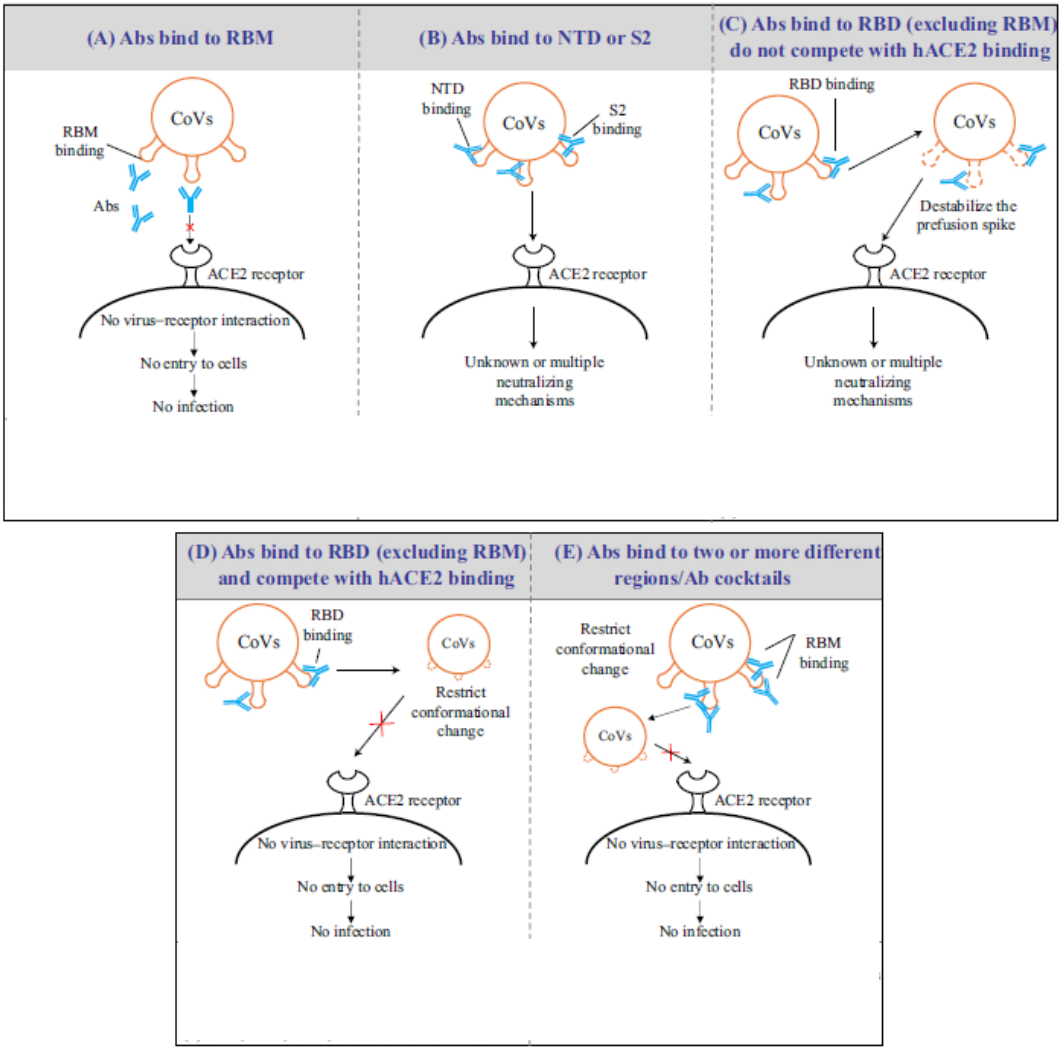


Fig. 20 Possible modes of interaction between nAbs and SARS-CoV-2 Spike protein.

Adapted from "Structural Basis of SARS-CoV-2 and SARS-CoV Antibody Interactions", by Gavor, E., et al., 2020, *Trends in Immunology*, 41(11), 1006-1022.

However, nAbs targeting the RBD of the S protein tend to have higher potency than non-RBD targeting antibodies [53]. In general, nAbs in blood mainly belong to the IgG isotype, however the IgA isotype is predominant at mucosal sites [54]. Their elicitation likely represents a fundamental key for protection against SARS-CoV-2 infection, or re-infection, although some outstanding questions are still to be answered, such as nAbs duration over time and the precise titre required to prevent reinfection [50, 52, 56].

Longitudinal analyses have demonstrated that nAbs positively correlate to disease severity: they tend to appear earlier and reach higher levels rapidly in those who develop moderate to severe pneumonia than in those with mild or asymptomatic disease [56, 57].

According to current data, high levels of nAbs provide an excellent indication in preventing infection in vaccinated individuals, and reinfection in previously infected subjects [48, 51].

A longitudinal study conducted by Lau *et al*. demonstrated a very variable nAbs titre during the 210 days after COVID-19 onset, depending on the disease severity; the variability was especially high in those who were asymptomatic (Fig. 21) [56].

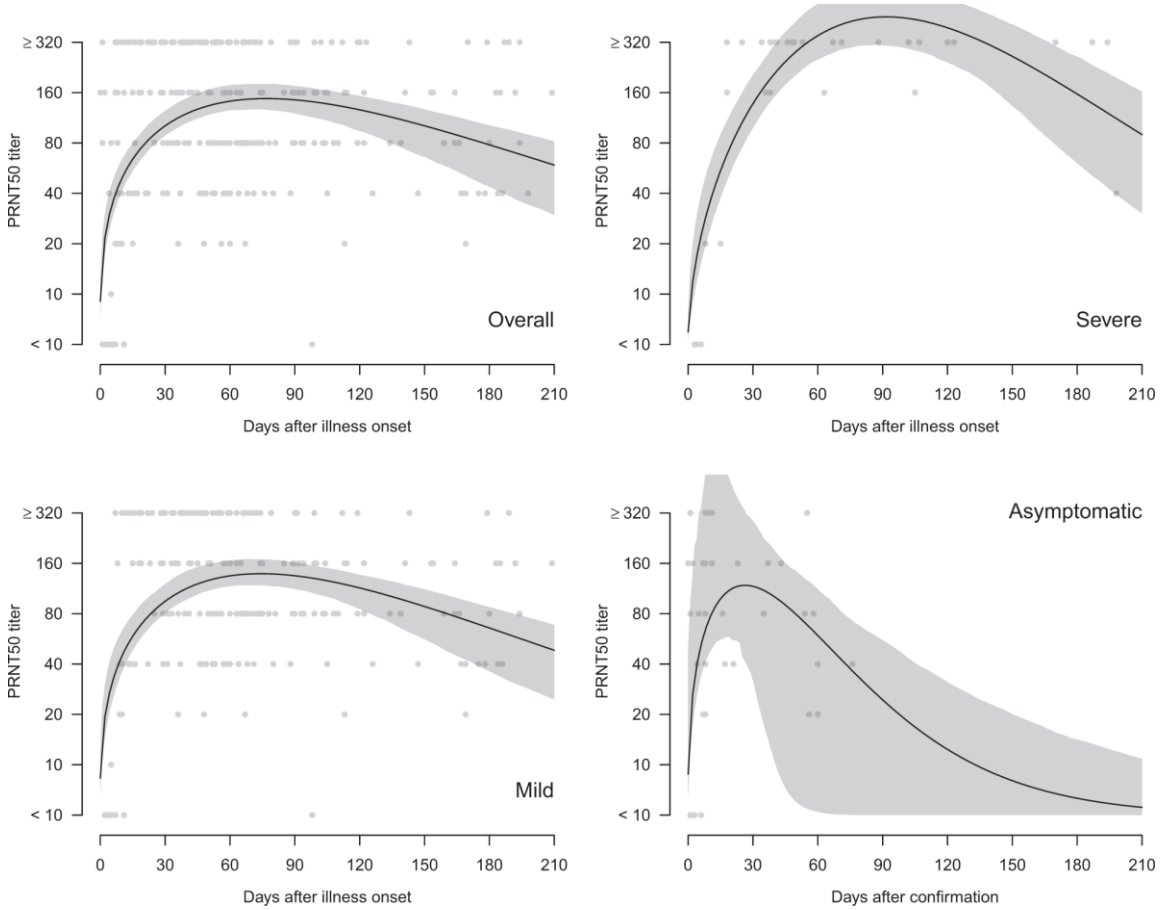


Fig. 21 Plaque-reduction neutralization test performed on serum samples from COVID-19 patients with varying degrees of disease severity over 210 days after illness onset. Reprinted from "Neutralizing antibody titres in SARS-CoV-2 infections", by Lau, E. H. Y., *et al.*, 2021, *Nature Communications*, 12, 63.

4.3 Immune escape strategies

As previously stated, the assembly of double-membrane vesicles consists in one of the most valuable strategies implemented by SARS-CoV-2 to protect its dsRNA replicative intermediates from the IFN pathways.

Moreover, several nsps have been proposed as capable of suppressing type I and type III IFN in human respiratory epithelial cells, namely nsp1, nsp3, etc. [16].

However, it is noteworthy to consider that as long as a virus circulates in a population, it is more likely to develop mutations, thus leading to alarming consequences. Indeed, one of the most remarkable mechanisms that viruses have developed to evade host immunity is based on generating point mutations in genes encoding surface antigens, which work as targets of neutralizing antibodies [54].

4.3.1 SARS-CoV-2 emerging variants

Since the pandemic began, a great number of mutations throughout the SARS-CoV-2 genome have been reported all over the world [22].

In general, RNA viruses are successful at mutating randomly because of the lack of proof-reading activity during their replicative cycle. Compared to other RNA viruses (e.g. HIV, HCV, etc.) Coronaviruses have little likelihood

of acquiring mutations, because they have evolved a proof-reading mechanism in order to maintain their long RNA genomes, which is mediated by the 3'-5' exonuclease activity in the N-terminal domain of nsp14 [25, 37].

However, the massive spreading of SARS-CoV-2 has made genetic variations more likely to occur. This phenomenon is an "interplay of natural selection and chance events that shape virus evolution within hosts, in communities and across countries" [24].

Consequently, mutations that may escape therapeutics, as well as antibodies elicited by natural infection or vaccination, pose a major threat especially in vaccines design. Indeed, they can increase virus adaptation and transmissibility, as well as worsen disease symptoms [53].

All the most alarming SARS-CoV-2 variants carry multiple deletions and substitutions on the S gene, which may compromise the efficacy of neutralizing antibodies as well as increase transmissibility (Fig. 22).

At the beginning of SARS-CoV-2 spreading out of China, an increasing number of cases were reported to bear the D614G mutation, which occurs in the S2 region of the Spike protein, and consists in the replacement of the Aspartic Acid in 614 position with a Glycine. By now, this is the most frequent substitution found in viral isolates [26].

This single variation has been the focus of several studies, such as the one

conducted by Plante, J. A., *et al*. This research group has proven this variation to significantly enhance SARS-CoV-2 fitness *in vivo* [25, 26].

An increased viral replication was shown in "primary human upper way tissues" rather than in the human lung cell line Calu-3, thus suggesting a role of the D614G mutation in viral transmissibility [26].

However, this single variation does not seem to influence neutralization provided by natural and vaccine-induced antibodies [26].

In more detail, the most concerning SARS-CoV-2 strains have acquired mutations in the RBD, that is thought to be the 'Achilles' heel' of the virus. Indeed, the RBD-ACE2 interaction, hence the whole infection, can be blocked by neutralizing antibodies (see section 4.2.1).

Greaney, A. J., *et al*. carried out an antibody depletion assay in human sera samples, consisting in the removal of RBD-binding antibodies via RBD-conjugated beads. This experiment proved RBD-binding antibodies to dominate the neutralizing activity in >90% of the tested samples [63].

Therefore, there is a reasonable probability that selective pressure for non-synonymous mutations in the RBD may help the virus to escape natural and vaccine-induced nAbs.

The RBD ranges from AA 319 to AA 541, and all the mutations emerging in the corresponding nucleotide sequence must be monitored.

Therefore, a strict surveillance must be implemented, both through sequence-based studies, epidemiological investigations and, last but not least, bioinformatic analysis over structural changes.

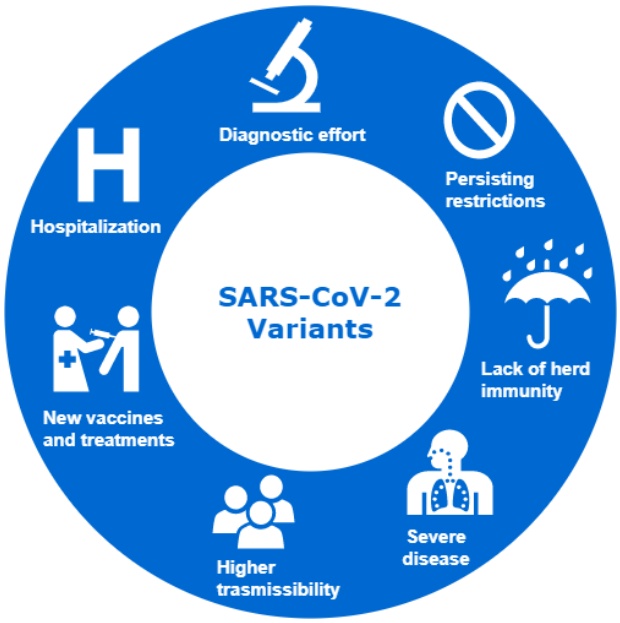


Fig. 22 Implications for SARS-CoV-2 emerging variants.

Over the last year, the naming of SARS-CoV-2 variants has been modified several times to phylogenetically organize the obtained sequences (single gene or whole genome sequences) into genetic clades as well as lineages. In such a way, a single viral variant can be described with different names, depending on the software used to perform the phylogenetic analysis. Indeed, variants identifying names vary between GISAID (Global Initiative on

Sharing Avian Influenza Data), NEXTSTRAIN (an open-source project created to constantly update publicly available pathogen genome data) and PANGO (Phylogenetic Assignment of Named Global Outbreak). However, SARS-CoV-2 lineages are usually named according to the earliest documented samples, for this reason WHO has recently decided to label the variants following the Greek alphabet. The Centre for Disease Control and Prevention (CDC) has established the following classification, which reflects the degree of attention that each viral variant deserves in terms of public-health threat (Tab. 1) [58, 59].

- **Variants of Interest (VOIs)**, associated with a globally limited prevalence.

  - B.1.526 (iota) strain, identified in New York in November 2020. This lineage bears T95I, D253G, D614G. E484K mutation was found in a small amount of B.1.526 isolates.

  - B.1.525 (eta) lineage was identified in Nigeria in late December 2020, and displays mutations such as A67V, 69/70 deletion, 144 deletion, E484K, Q677H, F888L.

- **Variants of High Consequence**, for which there is evidence that prevention medical countermeasures (MCMs) significantly reduce their effectiveness compared to other circulating variants. Therefore, they may fail diagnostics and cause a disproportionately high number of vaccine failure cases involving hospitalizations. Currently there are no SARS-CoV-2 variants included in the level of high consequence.

- **Variants of Concern (VOCs)**, associated with:

  - Evidence of escape from neutralizing antibodies, elicited not only by natural infection (meaning that a patient may be reinfected), but also by vaccination;

  - Failure of commercial nucleic acid amplification tests (NAATs);

  - More serious disease;

  - Resistance to mAbs and other antiviral therapeutics;

  - Rapid overburdening of health services;

  - Increase of infectivity, hence more rapid spreading. Indeed, the B.1.351 variant has been estimated to be 50% more transmissible than pre-existing variants in South Africa, and B.1.1.7 seems to be about 43% more transmissible than variants that appeared earlier in the UK [58].

- B.1.1.7 (alpha), was found in the South East of the United Kingdom in October 2020.

This lineage carries several aminoacidic substitutions, such as N501Y, in which an Asparagine has been replaced by a Tyrosine [25]. This single variation does show enhanced affinity to engineered wild-type ACE2 receptor [29], thus suggesting that this lineage is far more transmissible than others, as confirmed by several epidemiological evidences [28]. Among the signature changes of B.1.1.7 lineage, it is worth reporting 69/70 and 144/145 deletions , A570D, P681H, T716I, S982A, and D1118H [58, 60].

Up to now, this lineage has shown a slight decrease in nAbs titres, meaning that both a prior infection and vaccination are likely to provide an adequate protection against the B.1.1.7 variant [23].

- P.1 (gamma) was detected for the very first time in some travellers from Brazil during a routine screening in a Japanese airport. It contains K417T, N501Y and E484K mutations. Alarmingly, this lineage has been reported in several cases of reinfection [23].

- B.1.351 (beta), found in South Africa, bears three RBD variations, namely K417N, E484K, N501Y and several other variations out of the

RBD [23, 58, 59].

Experimental investigations conducted on pseudo-viruses have suggested that mutants for the RBD are for themselves capable of escaping nAbs, but they seem to be more effective when associated to additional variations out of the RBD [23]. Both P.1 and B.1.351 variants share K417N/T and E484K mutations, which suggest an increase in the binding affinity of the RBD to the ACE2 receptor [58].

- B.1.617.2 (delta), recently emerged in India and bears several mutations of interest on the S protein, such as T19R, (G142D), 156/157 deletion, R158G, L452R, T478K, P681R, D950N. This variant lacks mutations at AA positions 501 and 484, commonly associated with all the other VOCs [62], and is now dominant in the UK, indeed it is rapidly replacing the B.1.1.7 lineage.

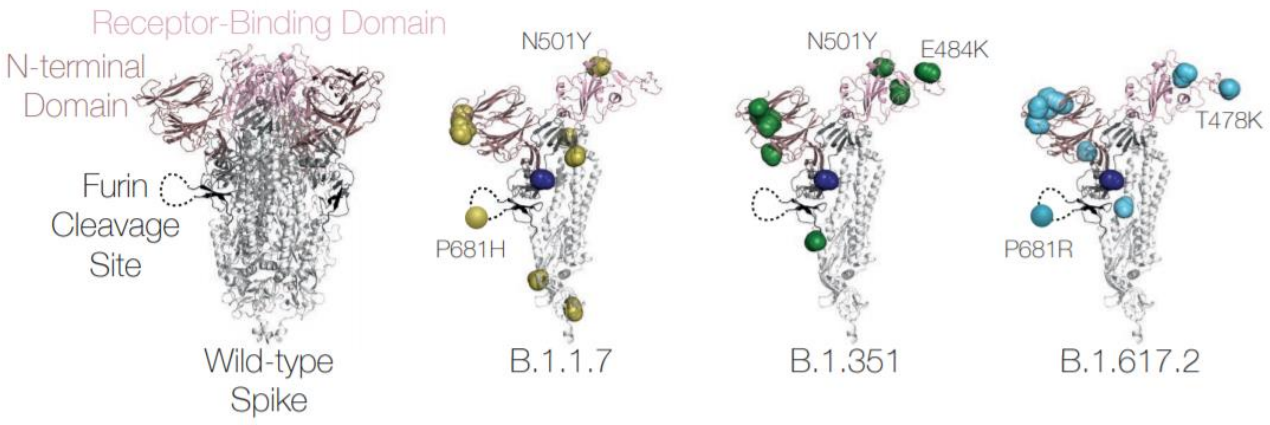


Fig. 23 SARS-CoV-2 S protein with selected AA mutations in some VOCs and B.1.617.2. Adapted from "Neutralising antibody activity against SARS-CoV-2 VOCs B.1.617.2 and B.1.351 by BNT162b2 vaccination", by Wall, E.C., *et al*., 2021, *The Lancet.*

WHO label	Name (PANGO Lineage)	Spike Protein Substitutions	First detected	VOI	VOC
Alpha	**B.1.1.7**	69/70del, 144del, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H	United Kingdom, October 2020	NO	YES
Beta	**B.1.351**	D80A, D215G, 241del, 242del, 243del, K417N, E484K, N501Y, D614G, A701V	South Africa	NO	YES
Gamma	**P.1**	L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I	Brazil	NO	YES
Delta	**B.1.617.2**	T19R, 156/157del, R158G, L452R, T478K, D614G, P681R, D950N	India, December 2020	NO	YES
Eta	**B.1.525**	A67V, 69/70del, 144del, E484K, D614G, Q677H, F888L	Nigeria, December 2020	YES	NO
Iota	**B.1.526**	T95I, D253G, D614G,	New York (USA), November 2020	YES	NO

Tab. 1 Summary of VOIs and VOCs described and their Spike protein substitutions.

5. Overview on SARS-CoV-2 vaccines

Vaccines development and prevention countermeasures (such as contact tracing and social distancing) represent crucial elements to curbing the COVID-19 pandemic.

Since SARS-CoV-2 genome sequence became available in early January 2020, vaccines development was initiated in laboratories worldwide and has moved at an unprecedented speed. It is worth pointing out that no vaccines against other coronaviruses had been licensed for use in humans before SARS-CoV-2 vaccines [76]. Furthermore, the impact of vaccination on the spread of SARS-CoV-2 in the population is not yet known, therefore further studies are needed to assess whether vaccinated people are still susceptible to infection and able to spread the virus.

As previously stated, several analyses conducted on sera from patients infected with SARS-CoV-2 indicated that nAbs primarily target the RBD of the S1 subunit, and its pre-fusion conformation represents the immunodominant antigen during natural infection [75].

Consequently, a major challenge was to induce an effective immune response to SARS-CoV-2, which would include the production of nAbs, the generation of an effective T-cell response, and as few side effects as possible.

Regarding humoral immune response, natural infection leads to the generation of a heterogeneous set of nAbs, targeting many different antigens which originate from the entire viral particle, such as the membrane protein (M), the envelope protein (E) and nucleoprotein (N). In contrast, immunization mediated by vaccines may lead to the generation of a limited range of human antibodies.

Depending on the technology, one or more antigens can be released during vaccine immunization. Regarding the types of SARS-CoV-2 vaccines, several different approaches have been proposed; however, only a few of these led to significant results. Five different types of vaccines are discussed below (Fig. 24).

5.1 Inactivated vaccines

Inactivated vaccines are generated by growing SARS-CoV-2 in cell cultures, such as Vero cells cultures. Subsequently, a chemical inactivation of the virus occurs. Although this type of vaccines is relatively easy to produce, the overall yield could be low and inconvenient in terms of time and costs (e. g. requirement for cell cultures and a BSL-3 facility). On the other hand, a remarkable advantage is that inactivated vaccines can be stored at fridge temperatures.

After the inoculation of the SARS-CoV-2 inactivated vaccine, the whole virus is presented to the immune system; therefore, the immune response is likely to target not only the S protein but also the membrane (M), envelope (E) and nucleoprotein (N) [76]. An example of inactivated vaccine is CoronaVac, commercialized by Sinovac Biotech (China) and approved for emergency use by the World Health Organization (WHO) in June 2021.

5.2 Recombinant protein vaccines

Vaccines consisting in viral recombinant proteins can be produced by using different expression systems, such as insect cells, mammalian cells or yeasts [76]. A considerable advantage of these vaccines is that no handling of live virus is required. However, depending on the expression system, yields and post-translational modifications may vary.

Novavax has manufactured the NVX-CoV2373 recombinant vaccine, designed from the full-length wild-type SARS-CoV-2 S protein and expressed in an insect cell expression system. Recombinant S protein includes 682-QQAQ-685 mutations at the S1/S2 cleavage sites, which confer resistance to human proteases. Furthermore, K986P and V987P substitutions in the S2 subunit are designed to stabilize the pre-fusion conformation [77]. This vaccine has shown 89.3 % efficacy after two doses (data from UK) [76].

5.3 Viral vector vaccines

Such vaccines are typically based on properly engineered viruses, which are designed to express the S protein, and at the same time are incapable of replication *in vivo,* because deletions of several parts of their genome have been performed.

However, a concerning disadvantage is that some of these vectors could be partially neutralized by pre-existing vector immunity. This problem can be circumvented by using vectors that are derived from animal viruses, or by using viruses that do not induce much immunity by themselves, such as adeno-associated viruses (AAVs) [75, 76].

Among the licensed vaccines, AstraZeneca has designed the AZD1222 vector, based on a chimpanzee Adenovirus. This vaccine has is 63.09% effective in preventing symptomatic SARS-CoV-2 infection [83]. Other AdV-based vectors have been commercialized by Janssen (Ad26.CoV2.S vaccine, 66.9 % efficacy [84]), and by the Gamaleya National Research Center for Epidemiology and Microbiology (Gam-COVID-Vac, also known as Sputnik V, 91.6 % efficacy [85]).

5.4 DNA vaccines

These vaccines are based on plasmid DNA, which can be propagated at large scale in bacteria (such as *E. coli*) and typically encode the S protein under the control of a mammalian expression promoter. Although the plasmid DNA has a high stability, these vaccines show low immunogenicity, and require delivery devices for administration, such as electroporators. Up to now, there are no licensed DNA vaccines against SARS-CoV-2 [76].

5.5 mRNA vaccines

Vaccines based on mRNA are quicker to produce and high yielding than those obtained by traditional approach, consisting in growing the virus in cells or in chicken eggs. Indeed, these vaccines can be produced entirely *in vitro* using a cell-free transcription from the corresponding DNA templates, a molecularly well-defined process, free of materials of animal origin [80].

SARS-CoV-2 mRNA vaccines rely on the use of a RNA oligonucleotide encoding the S protein, which is usually delivered via lipid nanoparticles (LNPs).

It is commonly accepted that exogenous mRNA has an intrinsically immunostimulatory activity [79]. Several studies focused on mRNA vaccines proved that the incorporation of chemically modified nucleosides, such as

pseudouridine or 1-methylpseudouridine, prevents the activation of several innate immune sensors (e. g. TLR7, TLR8, etc.), thus blocking type I interferon pathway. Furthermore, nucleoside modification seems to partially suppress the recognition of dsRNA and improve translation [79].

The main target of mRNA vaccines is represented by antigen presenting cells (APCs) which are highly susceptible to mRNA transfection. Once the mRNA enters these target cells, their translational machinery synthetizes the S protein, which is subsequently processed and trafficked to the cell membrane for display to CD8+ and CD4+ T cells, by exposure on major histocompatibility complexes (MHC) class I [78, 79].

As of June 2021, two mRNA vaccines are authorized in Italy, namely mRNA-1273 and BNT162b2:

- mRNA-1273, commercialized by Moderna (US), is a single-stranded, 5'-capped mRNA encoding the S protein of SARS-CoV-2 [78]. This vaccine is 94.1% effective at preventing COVID-19 [81];
- BNT162b2, commercialized by Pfizer-BioNTech (US), encodes the full-length S protein, stabilized in the pre-fusion conformation by replacing 986 and 987 residues with two prolines [82].

  This vaccine has shown 95% efficacy in preventing COVID-19.

  However, an important disadvantage of mRNA vaccines is that of long-

term storage stability, because frozen storage (-70°C / -80 °C) is required.

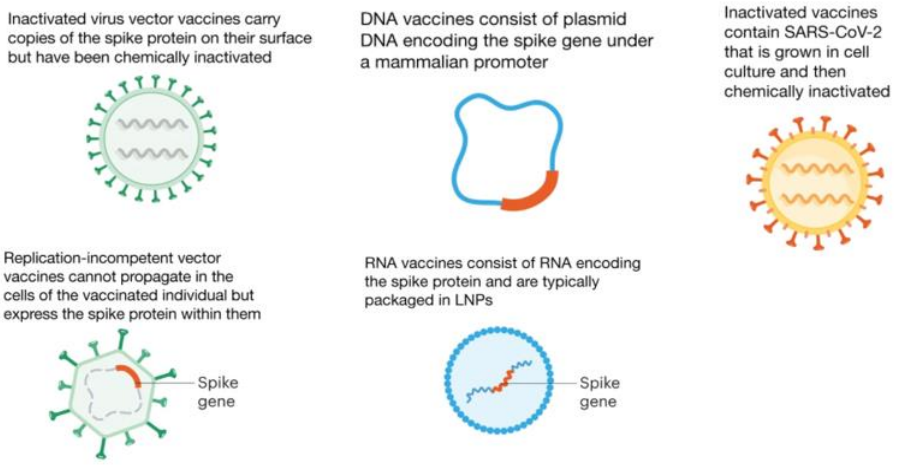


Fig. 24 Schematic illustration of different types of SARS-CoV-2 vaccines. Reprinted from "SARS-CoV-2 vaccines in development", by Krammer, F., 2020, Nature, 586, 516–527.

Name	Company	Type	Overall efficacy	Doses
AZS1222	AstraZeneca / Oxford	Viral vector	63,09%	x2 28 days apart
BNT162b2	Pfizer-BioNTech	mRNA	95%	x2 21 days apart
mRNA-1273	Moderna	mRNA	94,1%	x2 28 days apart
NVX-CoV2373	Novavax	Protein subunit	89,3 % *in the UK	x2
Ad26.CoV2.S	Janssen / Johnson & Johnson	Viral vector	66.9%	x1
CVnCoV	CureVac / GlaxoSmithKline	mRNA	Unknown	x2 28 days apart
Gam-COVID-Vac (Sputnik V)	Gamaleya National Research Center for Epidemiology and Microbiology	Viral vector	91,60%	x2 21 days apart
CoronaVac	Sinovac Biotech	Inactivated virus	51%	x2 14 days apart

Tab. 2 Summary table of the commercialized SARS-CoV-2 vaccines mentioned in the text.

6. Materials & Methods

6.1 Vero E6 cell cultures

To effectively isolate, propagate and study SARS-CoV-2, permissive cell lines for viral infection must be used. Vero E6 cells are derived from the kidney of an African green monkey, namely *Cercopithecus aethiops,* and represent one of the most common mammalian continuous cell lines used in research [74].

Since ACE2 receptor is abundantly expressed on Vero E6 cells, they have been extensively used since 2003 for SARS-CoV research by many laboratories. Moreover, they lack the ability to produce interferon, thus allowing effective viral replication [71].

Another considerable advantage of using Vero E6 cells is that they undergo cytopathic effect after viral replication, thus providing an effective tool to visually recognize an ongoing infection.

This cell line is anchorage-dependent, therefore it grows as a monolayer. Moreover, Vero E6 cells undergo contact inhibition, so that when confluency is reached, they stop growing and begin to die [74]. For that reason, it is extremely important to daily monitor cell cultures and subculture them as soon as they form confluent monolayers.

Cell cultures were grown in complete growth medium, i.e. Dulbecco's modified Eagle medium (DMEM, Euroclone, Milano, Italy), supplemented with 10% Fetal Calf Serum (FCS, Euroclone, Milano, Italy), and antibiotic-antimycotic mixture (100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B).

Vero E6 cells were maintained in T75 tissue culture flasks (CELLSTAR, Greiner Bio-One, Frickenhausen, Germany) and kept in a cell culture incubator at 37°C, in 5% CO2 and 95% humidity.

Twice a week the confluent monolayer was split at 1:10 ratio using T75 flasks following trypsinization. Briefly, confluent monolayer was washed with 2 mL of Dulbecco's Phosphate Buffered Saline (PBS, Euroclone, Milano, Italy) After washing, 2 mL of 1X Trypsin-EDTA (Gibco, Thermo Fisher Scientific) was added to the cell culture and incubated for about 5 minutes, until the complete dissociation of the monolayer occurred, 3 mL of pre-warmed complete growth medium was then added to inactivate trypsin. Finally, cells were entirely resuspended by pipetting several times and counted using a Burker's counting chamber. Finally, $4 * 10^6$ cells were plated in a new T75 flask.

6.2 Virus isolation

Five different viral stock belonging to as many lineages of SARS-CoV-2 were used: B.1 (EPI_ISL_417491), B.1.1.7 (EPI_ISL_778869), P.1 (EPI_ISL_1118260), B.1.351 (EPI_ISL_1118258) and B.1.526 (EPI_ISL_1321993). This viral stock was obtained from nasopharyngeal swabs collected from 5 patients tested positive for SARS-CoV-2 by RT- PCR test who were randomly selected as part of the surveillance program for the emergence and control of viral variants. The lineage assignment was achieved by sequencing viral isolates using Sanger and NGS sequencing approaches.

Primary isolate

SARS-CoV-2 isolation was performed as follows: a swab aliquot (0.5 mL) was filtered in a sterile tube using a 0.2 μm filter and incubated with $2 * 10^6$ Vero E6 cells suspension in 2 mL of complete growth medium for 1 h at 37 ◦C and 5% CO2. Subsequently, 4 mL of complete medium was added, and the suspension was finally transferred into a T25 tissue culture flask (CELLSTAR, Greiner Bio-One, Frickenhausen, Germany), maintained at 37 °C, 5% CO2 and 95% humidity.

The inoculated cell culture was examined using an inverted microscope (10× or 20× magnification) to assess the occurrence of the typical cytopathic effect

(CPE), consisting of rounded-shape, refractile cells undergoing a detachment [70]. Once 80% CPE was reached, the primary isolate underwent another passage (also named P1) and one aliquot was stored at -80 °C.

6.3 Virus stock preparation

In order to perform the P1 passage, $4 * 10^6$ Vero E6 cells were seeded in a T75 flask 24 hours before infection. 2 mL of the primary isolate was added to the cell monolayer and incubated for 1 hour at 37 °C, 5% CO_2. Subsequently, 8 mL of complete medium was added to the cell monolayer and incubated at 37 °C, 5% CO_2.

CPE was detectable 2-4 days after inoculation. To create viral stocks for experimental purposes, P1 supernatants were harvested three days after infection (at 80% CPE), then centrifuged at 3000 rpm for 10 minutes to remove cellular debris. The supernatant was filtered using a 0.2 μm filter, aliquoted and stored at -80 °C.

6.4 RT-qPCR assay

Viral stocks for each SARS-CoV-2 lineage was thawed and viral RNA was extracted using the Kit QIAsymphony DSP Virus/Pathogen Midi kit on the

QIAsymphony automated platform (QIAGEN, Hilden, Germany), to assess the number of genome copies from the obtained isolates.

RT-qPCR reactions were performed in a final volume of 25µl using 4.375 µL of MgSO4 (6 mM), 1.875 µL of Combined Primer/Probe Mix, 0.5 µL of H_2O, 0.75 µL of SuperScript™ III Reverse Transcriptase (ThermoFisher Scientific, Waltham, Massachusetts, USA*),* 12.5 µL of 2X Reaction Mix and 5 µL of template RNA. Subsequently, the plate was loaded on the 7500 Fast Dx Real-Time PCR Instrument (Thermo Fisher Scientific). The oligonucleotide primers and dual-labelled hydrolysis probes (TaqMan ®) used to detect and quantify SARS-CoV-2 genome copies were selected from regions of the virus nucleocapsid (N) gene. RT-qPCR assay was performed according to the directives of the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel [72].

Primers	**Sequence (5'-3')**
2019-nCoV_N2-F	5'-TTA CAA ACA TTG GCC GCA AA-3'
2019-nCoV_N2-R	5'-GCG CGA CAT TCC GAA GAA-3'
Probe	**Sequence (5'-3')**
2019-nCoV_N2-Probe	FAM-5'-ACA ATT TGC CCC CAG CGC TTC AG-3'-BHQ1

Tab. 3 Primers and probe sequences used for the RT-qPCR assay.

Thermal Profile	Time (min)	Cycles
55 °C	10:00	1
94 °C	03:00	1
94 °C	00:15	45
55 °C	00:30	

Components	Volume per reaction
MgSO4	4.375 µL
Combined Primer/Probe Mix	1.875 µL
SS III	0.75 µL
H2O	0.5 µL
2X Reaction Mix	12.5 µL
Template RNA	5 µL
Tot.	25 µL

Tab. 4 Thermal profile setup used for the RT-qPCR assay (left). RT-qPCR Master Mix (right).

The calibration curve was obtained by performing 10-fold serial dilutions (10^5 to 10^3 copies/reaction) of a standard certified plasmid (2019-nCoV Positive Control, nCoVPC, IDT), which was included in each RT-qPCR session along with a negative control.

6.5 Viral stock titration

Viral titre for each lineage was obtained by performing serial dilutions of each viral stock to obtain the TCID50 / mL value. The amount of infective virus present in the different viral stocks used in the experiments was determined by the TCID50 method which consists in computing the endpoint dilution at which 50% of the inoculated cell cultures show the CPE.

To determine the TCID50 of each viral stock, 24 hours before infection Vero E6 cells were seeded in 96-well plates at $2{,}6 * 10^4$ cells per well in 150µL of complete medium.

Eight 10-fold serial dilutions (ranging from 10^{-1} to 10^{-8}) were prepared for each viral stock by pipetting 50 µL of viral stock in 450 µL of complete medium (total volume = 500 µL).

Subsequently, cells were inoculated in sextuplicate with 50 µL of each dilution and incubated at 37 °C, 5% CO_2 for 2 hours. Following incubation, the inoculum was removed, cells were washed twice and 100 µL of complete medium was added.

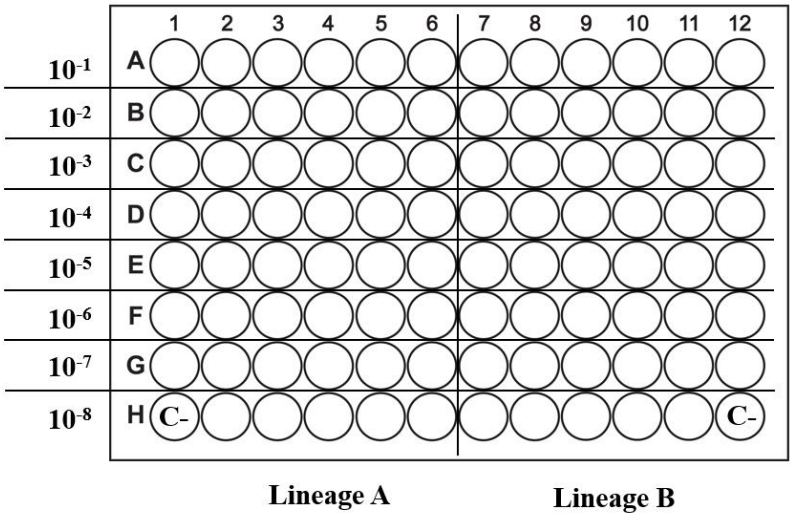


Fig. 25 Schematic illustration of the 96-well cell culture plate used for viral titration.

After 72 hours, the occurrence of CPE was evaluated in each well and the Reed-Muench method was used as follows to assess the TCID50 of each viral stock:

1. The cumulative number of infected and non-infected hosts was calculated for each dilution. After that, the following formula was applied to compute the proportional distance:

$$\frac{(\%\, positive\ value\ >\ 50\%)\ -\ 50\%}{(\%\, positive\ value\ >\ 50\%)\ -\ (\%\, positive\ value\ <\ 50\%)}$$

2. Subsequently, the following formula was used to compute the TCID50 in 50 µL:

$$10^{dilution\,>\,50\%}\ *\ 10^{proportional\ distance}$$

3. The TCID50 in 1 mL can be obtained by dividing the TCID50 in 50 µL by the corresponding volume in millilitres, that is 0,05 mL:

$$\frac{\text{TCID 50 in 50 } \mu L}{0.05\ mL}$$

Dilution		1	2	3	4	5	6	Infected	Non-infected	Total infected	Total non-infected	Percent infected (%)
-1	**A**	+	+	+	+	+	+	6	0	13	0	100
-2	**B**	+	+	+	+	+	-	5	1	7	1	87,5
-3	**C**	-	+	+	-	-	-	2	4	2	5	28,5714
-4	**D**	-	-	-	-	-	-	0	6	0	11	0
-5	**E**	-	-	-	-	-	-	0	6	0	17	0
-6	**F**	-	-	-	-	-	-	0	6	0	23	0
-7	**G**	-	-	-	-	-	-	0	6	0	29	0
-8	**H**	-	-	-	-	-	-	0	6	0	35	0

Tab. 5 Example of TCID50 plate view calculation using the Reed - Muench method.
Infected hosts are marked by a plus, non-infected hosts are marked by a minus.
The endpoint on infectivity (50%) ranges from 10^{-2} and 10^{-3} dilutions.
Proportional distance: (87,5 – 50) / (87,5 – 28,5714) = 0,63
The endpoint of infectivity (50%) is at a dilution of $10^{2} * 10^{0,63} = 10^{2,63}$
Infectious dose in 50 µL = $10^{2,63} = 4,33 * 10^{2}$ TCID50 in 50 µL
TCID50 in 1 mL = $4,33 * 10^{2}$ / 0,05 mL = $8,66 * 10^{3}$ TCID50/mL

SARS-CoV-2 lineage	Infectious titre (TCID50/mL)
B.1	2,00E+07
B.1.1.7	5,02E+05
P.1	4,62E+06
B.1.351	6,32E+05
B.1.526	8,66E+04

Fig. 26 TCID50/mL obtained after viral titration for each lineage.

Viral stocks were all normalised in the experiments at the concentration of 100 TCID50 in 50 µL.

6.6 Kinetics of SARS-CoV-2 replication in Vero E6 cells

Replication kinetics of the abovementioned five viral lineages was tested in the same cell culture plate performing a proper dilution for each lineage to reach the concentration of 100 TCID50 in 50 µL.

$2{,}6 * 10^4$ cells / well were seeded in a 96-well plate the day before infection.

After 24 hours, complete medium was removed and each well was inoculated with 50 µL of viral suspension containing 100 TCID50, and incubated for 2 hours at 5% CO_2, 37 °C. Subsequently, the inoculum was removed, cells were washed twice and 100 µL of fresh medium was added. Each viral lineage was tested in two replicates.

Supernatants were collected at regular intervals (6, 12, 24, 48 and 72 hours after inoculation) and stored at -80 °C.

Subsequently, viral RNA extraction for each supernatant was carried out using the Kit QIAsymphony DSP Virus/Pathogen Midi kit on the QIAsymphony automated platform (QIAGEN, Hilden, Germany).

Finally, RT-qPCR was performed manually (see section 6.4), to evaluate viral genome copies released in the cell supernatant.

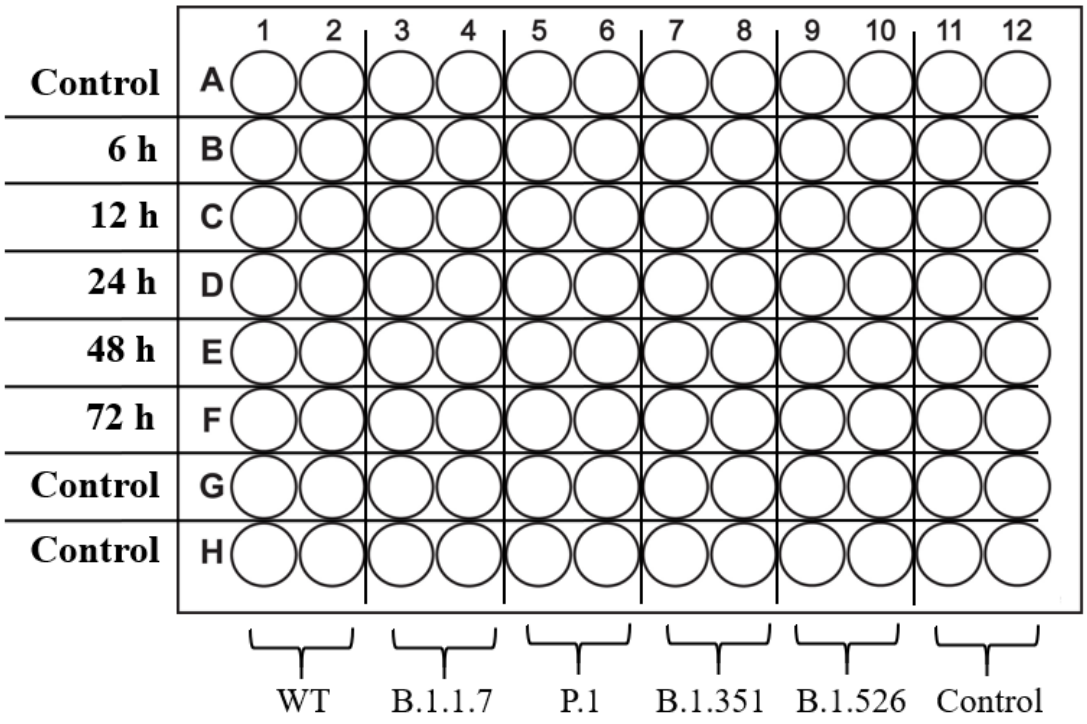


Fig. 27 Schematic representation of 96-well cell culture plate used for kinetics of SARS-CoV-2 lineage

6.7 Microneutralization assay

This technique is considered the "gold standard" for measuring levels of neutralizing antibodies in many viral diseases.

In the present study, the microneutralization test aims to evaluate the titre of neutralizing antibodies against SARS-CoV-2 in human serum, that is the highest serum dilution able to inhibit infection in 50% of cell cultures *in vitro* [73]. To assess the neutralizing antibody titre in human sera samples, $2{,}6 * 10^4$ Vero E6 cells were seeded in 96-well cell culture plates 24 hours before infection.

Human sera samples were inactivated at 56 °C for 30 minutes, then serial two-fold dilutions (ranging from 1:10 to 1:640) were carried out in three replicates in U-shaped-bottom microplates.

To perform dilutions, 10 µL of serum were added to 90 µL of complete medium (1:10 dilution) in the first line of the 96-well plate. The remaining wells were filled with 50 µL of complete medium.

After that, serial two-fold dilutions were performed along the plate, pipetting 50 µL from the first line (1:10 diluted serum) to the second line (1:20 diluted serum), then 50 µL from the second to the third line (1:40 diluted serum), and so on. The last line was serum-free, representing the control line. A proper dilution of each viral stock was prepared in a vessel to reach the standard

concentration of 100 TCID50 in 50 µL. Subsequently, 50 µL of each diluted viral stock was dispensed in each well of the U-shaped-bottom microplate containing sera dilutions, thus performing a further 1:2 serum dilution (1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280 final serum dilutions).

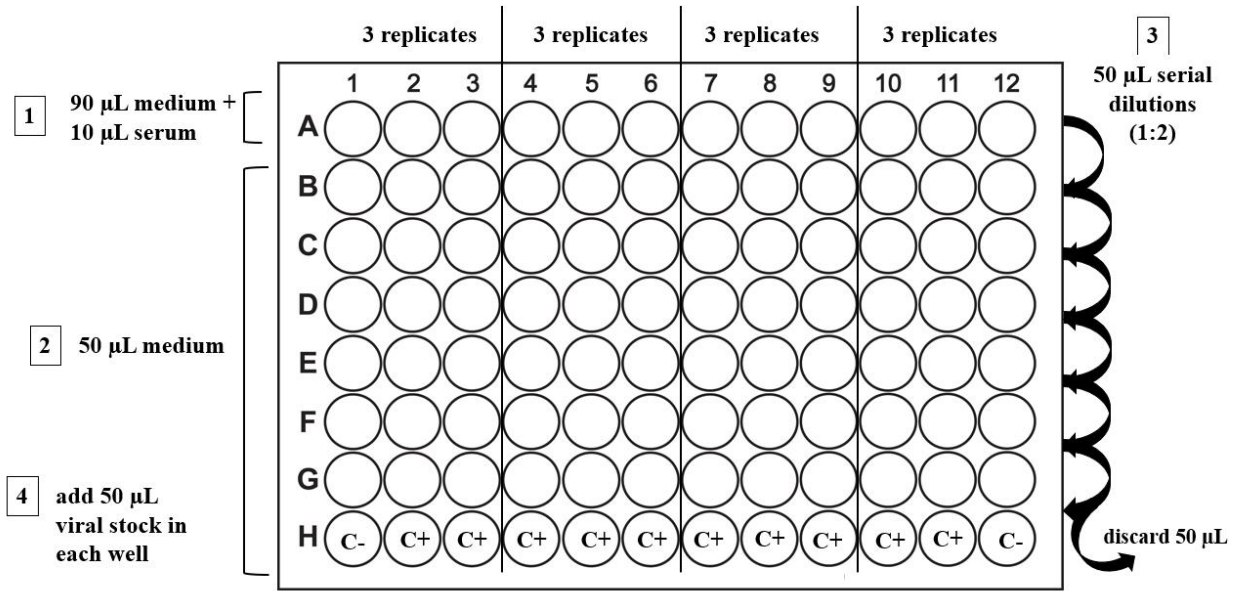


Fig. 28 Schematic representation of the plate view in microneutralization assay.
Serial dilutions for one lineage were performed in three replicates. 10 µL of serum was added to 90 µL of complete medium in the A line (1). 50 µL of medium was added in B, C, D, E, F, G lines (2). 50 µL serial dilutions were performed (1:2) from A line to G line (3). 50 µL of properly diluted viral stock was added in each well (4). H line: control.

Subsequently, the microplate containing both virus and serum was incubated at 37 °C, 5% CO_2 for 1 hour.

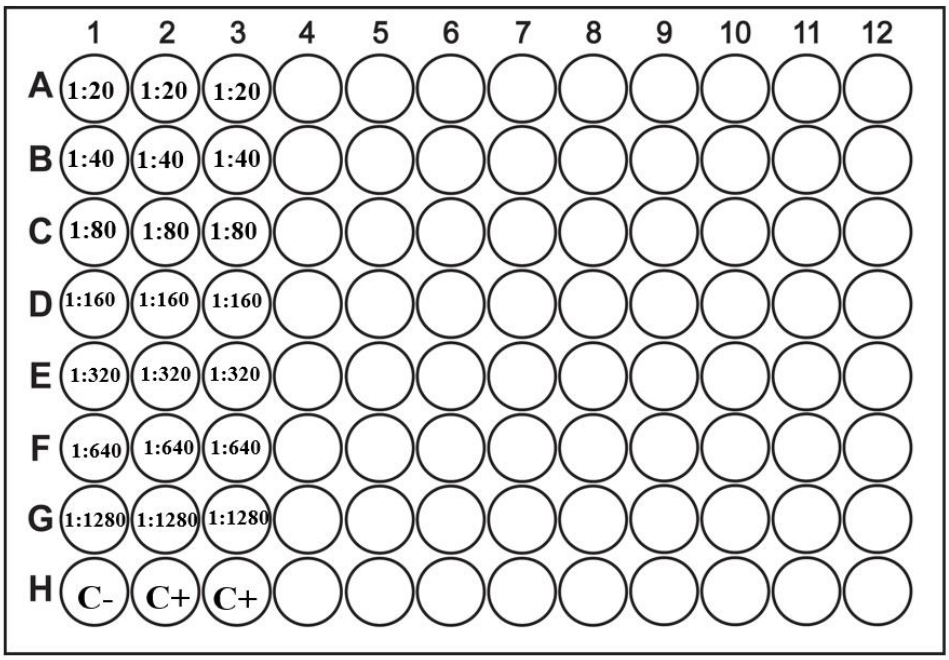


Fig. 29 Schematic representation of final serum dilutions along the 96-well plate in microneutralization assay.

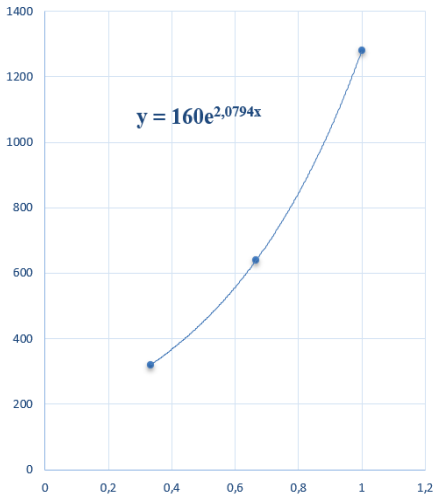
The antibody-virus suspension content of the U-shaped-bottom microplate was added to Vero E6 cells after medium removal. Positive (Vero E6 cells and virus without serum) and negative (Vero E6 cells not infected with virus) controls were established for each plate. After 72 hours of incubation, the presence of CPE was evaluated for each well by using the inverted microscope. Then, the wells displaying CPE were counted and recorded for

each serum dilution. Subsequently, to calculate the neutralizing antibody titre, the frequency of wells displaying a visible CPE for each serum dilution was interpolated in an exponential curve. The following formula was used:

$$y = k * e^{exp*x}$$

y is the virtual highest serum dilution that can be performed to reach 50% viral neutralization, that is the antibody titre; *k* is a multiplying factor and *x* is fixed at 0.5.

Dilution	Wells displaying CPE	Total wells	CPE Frequency
1:20	0	3	0
1:40	0	3	0
1:80	0	3	0
1:160	0	3	0
1:320	1	3	0,333333333
1:640	2	3	0,666666667
1:1280	3	3	1



Tab. 6 Example of antibody titre calculation for one serum sample (three replicates). *y:* serum dilution, *x:* CPE frequency, *k:* multiplying factor = 160, *e:* exponent = 2,0794. y = 160 * EXP (2.0794*0,5). Final antibody titre = 1: 544

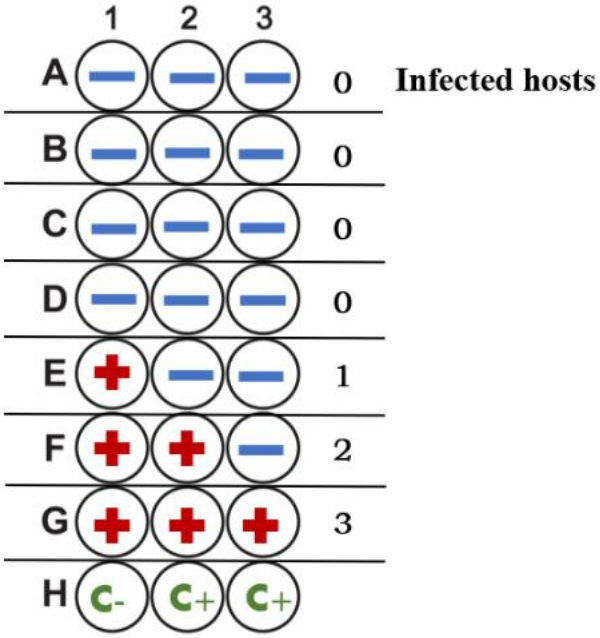


Fig. 30 Schematic illustration of CPE evaluation and recording after 72 hours of incubation.

6.8 Human sera samples

Serum samples were collected from a cohort of vaccinated healthcare workers at Ospedali Riuniti of Ancona. Sera from BNT162b2 vaccinees were collected 21 days after the first dose and 15 (± 1) days after the second dose and divided in the following groups: 50 naïve vaccinees, 15 vaccinees with previous infection, 8 vaccine failures.

Additional 20 sera from University staff were obtained after three weeks from the first dose of AZD1222 vaccine and divided in 10 naïve vaccinees and 10 vaccinees who experienced SARS-CoV-2 infection.

Sera from convalescent individuals were collected from blood donors (Marche region, Italy) between June and September 2020 with PCR-confirmed SARS-CoV-2 infection during the first wave. In addition, 15 convalescent sera were obtained from individuals infected with the B.1.1.7 lineage (n = 13), B.1.351 (n = 1) and P.1 (n = 1). The following table shows the number of samples, the collection period, sex and age of each serological group.

	Serological group	Num. Samples	Serum collection	% M	% F	Age
BNT162b2	Naïve vaccinees	50	15 (±1) days after the II dose	24%	76%	26 - 67
	Previous infection	15	3 - 11 months after infection	20%	80%	25 - 59
	Failure vaccinees	8	1 - 4 months after vaccination	37,50%	62,50%	24 - 61
AZD1222	Naïve vaccinees	10	3 weeks after the I dose	50%	50%	33 - 68
	Previous infection	10	4 - 13 months after infection	50%	50%	26 - 60
Convalescent	Infected with B.1	33	2 - 6 months after infection	90,90%	9,10%	31 - 62
	Infected with B.1.1.7	13	3 - 10 weeks after infection	76,92%	23,08%	23 - 75
	Infected with B.1.351	1	9 weeks after infection	-	100%	47
	Infected with P.1	1	5 weeks after infection	100%	-	54

Tab. 7 Serological group, number of samples, collection period, sex and age of tested samples

6.9 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9. Non-parametric *Wilcoxon matched pairs signed rank test* and *Mann-Whitney test* were applied where appropriate. Statistical significance was defined as $p < 0.05$.

7. Aim of the study

As previously stated, RNA viruses are likely to acquire mutations along their genome because of the absence of the proof-reading activity. However, thanks to nsp14, which mediates the 3'-5' exonuclease activity, Coronaviruses are able to prevent their long RNA genomes from changing [25, 37].

Therefore, as a result of ExoN activity, Coronaviruses show a lower frequency of escape from nAbs than other RNA viruses which lack such type of enzyme [61]. At the moment, a large number of studies are being undertaken to assess whether the abovementioned VOCs may escape nAbs elicited by natural infection as well as vaccination. A major concern is to identify and combat the possible patterns of antibody resistance. However, the degree at which SARS-CoV-2 might succeed in escaping nAbs is still unclear.

As soon as SARS-CoV-2 variants emerge worldwide, it is crucial to investigate their replicative as well as neutralizing properties, which may provide useful information about the biology of each lineage, as well as expand and update the current knowledge on vaccines effectiveness and make predictions about the behaviour of possible future variants.

Despite the large amount of data and studies available to date, it remains unclear whether convalescent sera derived from patients infected with the original SARS-CoV-2 strain would still be effective against new and emerging variants. At the same time, it is not clear whether authorized vaccines, which were formulated on the basis of the original SARS-CoV-2 strain, would still work at a satisfactory level against variants circulating on a global scale.

Furthermore, understanding the differences between nAbs titres elicited during natural infection and those induced by vaccination is a subject of great interest as well. Indeed, natural infection allows the generation of a highly heterogeneous set of binding and neutralizing antibodies against multiple immunogenic targets, such as several epitopes on Nucleocapsid and Spike proteins, although neutralization seems to be mediated exclusively by nAbs against the Spike protein (see section 4.2.1).

On the other hand, vaccines are designed to elicit the production of both binding and neutralizing antibodies against an unique target of interest, that is the Spike protein.

Despite the high efficacy of several licensed vaccines (see *Overview on SARS-CoV-2 vaccines*), there are no 100% effective vaccines available to date, thus a certain percentage of the vaccinated population remains

susceptible to the infection.

For that reason, it would be interesting to study nAbs titres in individuals who have tested positive for SARS-CoV-2. On the other hand, many individuals who developed COVID-19 in the months before vaccination are expected to show an unique nAbs response, compared with that of naïve vaccinees [66]. This issue in particularly important, as governments urgently need to develop policies that should maximize the number of people who get vaccinated, without sacrificing the efficacy of immunization.

Several strategies have been recently proposed to achieve this goal, for example giving a single dose of vaccine to those who previously had COVID-19, as well as delaying the second dose for everyone [66]. A high number of studies are being carried out to investigate whether these proposals represent the best solutions.

The present study aimed at investigating the replicative and neutralizing properties of five different lineages of SARS-CoV-2, namely B.1, B.1.1.7, P.1, B.1.351 and B.1.526. Therefore, both molecular and serological approaches were adopted to investigate and gain a deeper insight in the biology of five viral variants *in vitro*.

Firstly, the abovementioned five viral lineages were isolated and titrated, then the ratio between TCID50 and genome copies was calculated. This ratio is

indicative of the phenotypical properties of a viral lineage and describes the extent at which a viral variant fails in generating infectious particles after replication. Specifically, the lower the ratio TCID50 / genome copies, the worst the ability of the viral lineage to generate effective infectious particles.

Subsequently, viral replication kinetics was determined in Vero E6 cells for each viral lineage to understand whether different viral lineages may show different replication kinetics. Furthermore, to provide a comprehensive view of variant replicative properties, *in vitro* cytopathic effect caused by each viral lineage was evaluated.

In the second part of this study, the sera from different groups of individuals were evaluated for their cross-neutralizing properties: 1) convalescents who previously had an infection caused by the B.1 lineage, 2) vaccinees after either first or second dose, 3) convalescents who underwent vaccination and 4) individuals infected by viral variants different from B.1 (described in *Materials & Methods*).

This evidence, together with structural, epidemiological, phylogenetic and clinical studies carried out in laboratories worldwide, will contribute not only to virological research but also to help governments to take proper decisions that may affect the pandemic progress.

8. Results

8.1 TCID50 and viral RNA copy number

The first approach adopted to study the replicative properties of five different variants from a phenotypical point of view was the assessment of the TCID50 / mL value and the number of genome copies / mL of the respective primary isolates (Fig. 31). TCID50 / mL was obtained by performing viral titration (see section 6.5), whereas genome copies / mL were calculated using a quantitative real-time assay (see section 6.4).

cp/µL standard plasmid	Log cp/µL standard plasmid	Threshold cycle
100000	5	26,71
10000	4	30,2
1000	3	33,33

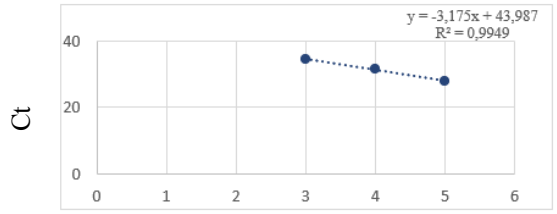


Fig. 31 Calibration curve obtained from three 10-fold dilutions of a standard plasmid.

y: PCR threshold cycles

x: Log cp/ µL standard plasmid

TCID50 / mL values for each SARS-CoV-2 lineage and the corresponding genome copy number in Vero E6 cells culture supernatants are reported below.

SARS-CoV-2 lineage	Infectious titre (TCID50/mL)	Viral RNA copy number (copies/mL)
B.1	2,00E+07	2,32E+11
B.1.1.7	5,02E+05	8,41E+10
P.1	4,62E+06	3,75E+11
B.1.351	6,32E+05	3,20E+11
B.1.526	8,66E+04	2,02E+10

Tab. 8 TCID50 and Viral RNA copy number calculated for five different SARS-CoV-2 lineages. (Viral RNA was extracted using the Kit QIAsymphony DSP Virus/Pathogen Midi kit on the QIAsymphony automated platform (QIAGEN, Hilden, Germany). RT-qPCR was performed manually as well as TCID50 assay. TCID50 was calculated using the Reed-Muench method).

Subsequently, the percentage ratio of TCID50 on the total number of genome copies / mL was calculated.

Resulting values show the first italian strain of SARS-CoV-2 as the most effective viral lineage in generating infectious particles, being the % TCID50 = 0.00863 %. Interestingly, the South-African lineage (B.1.351) represents the least productive viral variant in terms of infective doses.

	% TCID50
B.1	0,00863
B.1.1.7	0,00060
P.1	0,00123
B.1.351	0,00020
B.1.526	0,00043

Tab. 9 Percentage of TCID50 / mL on total genome copies / mL for each SARS-CoV-2 variant.

8.2 *In vitro* cytopathogenicity of five different lineages of SARS-CoV-2 on Vero E6 cell cultures

In general, a syncytium is defined as a multinucleated cellular structure generated by mononucleate cells undergoing multiple fusions [68].

Both viral replication and cellular mechanisms play an essential role in the formation of syncytia, although the regulation of this process remains largely elusive. SARS-CoV-2 infected cells express the Spike protein at their surface, therefore they can fuse with neighbouring ACE2 expressing cells to form syncytia. Furthermore, virus-induced cell fusion facilitates the transfer of viral material to the adjacent cells [68]. There is evidence that the expression of the S protein without any other viral protein triggers syncytia formation [67].

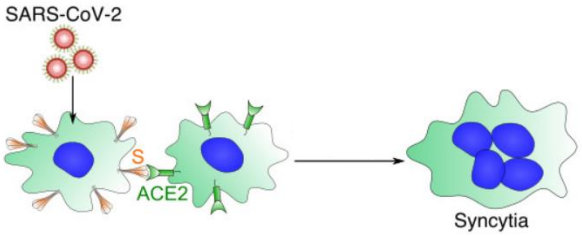


Fig. 32 Syncytia formation mechanism. Adapted from "Syncytia formation by SARS-CoV-2-infected cells", by Buchrieser, J., et al., 2020, *The EMBO Journal*, 39, e106267.

In order to investigate the replication kinetics of different SARS-CoV-2 lineages, primary viral isolates were synchronized in parallel cultures using 100 TCID50 in 50 µL (as described in *Materials & Methods*).

Microscopical visualization of infected cell cultures was carried out at 10X as well as 20X magnification, using an inverted microscope connected to a Nikon D3300 camera. The following pictures were taken after 48 and 72 hours after cell cultures infection.

Negative control: non-infected Vero E6 cells

To evaluate the level of cytopathic effect (CPE) for each viral lineage, a non-infected monolayer of Vero E6 cells was used as a negative control to compare live and infected cells morphology.

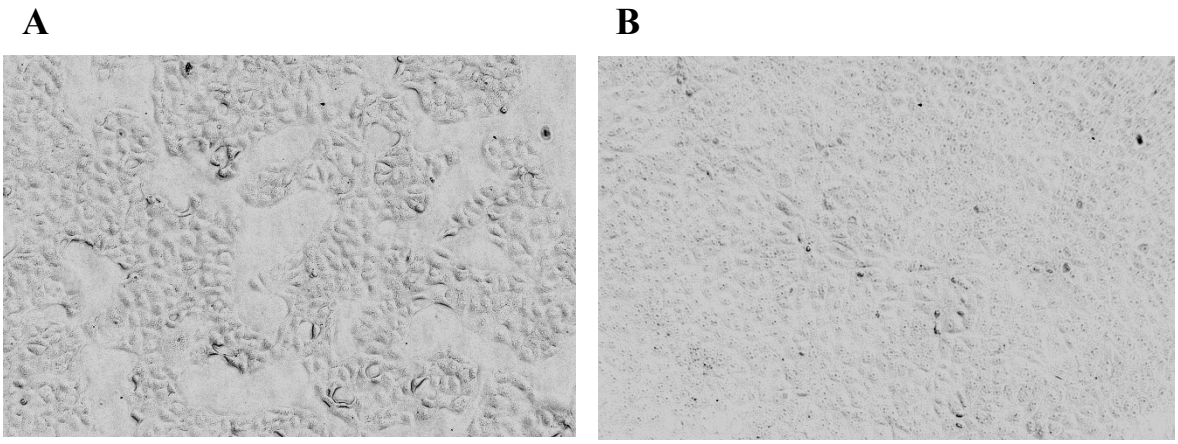


Fig. 33 Negative controls. Vero E6 cells growth leads to 70% (A) and 100% (B) confluency in complete growth medium. Vero E6 cells are non-infected.

B.1 lineage (D614G)

Vero E6 cells were examined after 48 (Fig. 34 A) and 72 hours (Fig. 34 B) to assess the occurrence of the typical CPE, consisting of rounded shape, refractile cells undergoing a detachment.

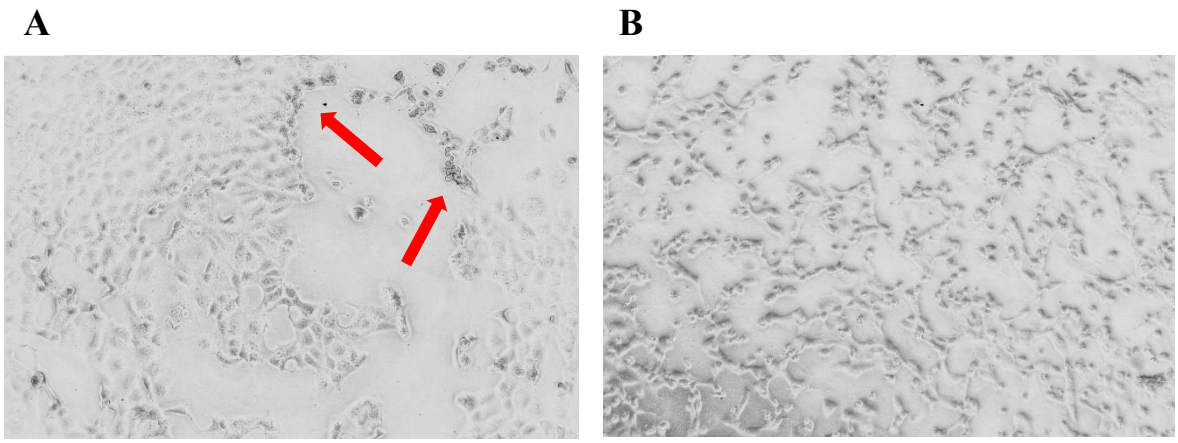


Fig. 34 B.1 lineage of SARS-CoV-2 induces cytopathic effect in Vero E6 cells after 48 (A) and 72 h (B). Rounded-shape undergoing detachment are marked with red arrows.

B.1.1.7 lineage (United Kingdom)

The B.1.1.7 lineage showed a unique CPE compared with the other examined viral lineages. Indeed, several syncytia are visually detectable 48 hours after infection (Fig. 35 A), consistent with what observed by Rajah *et al*. [69].

Furthermore, CPE induced by B.1.1.7 lineage was less conspicuous than CPE observed for B.1 lineage, suggesting that this viral variant requires slightly more time to replicate in Vero E6 cells monolayer. This evidence is confirmed by the growth replication kinetics assay (see section 8.3).

In the picture below, the low-level CPE with enlarged cells induced by B.1.1.7 lineage is visible.

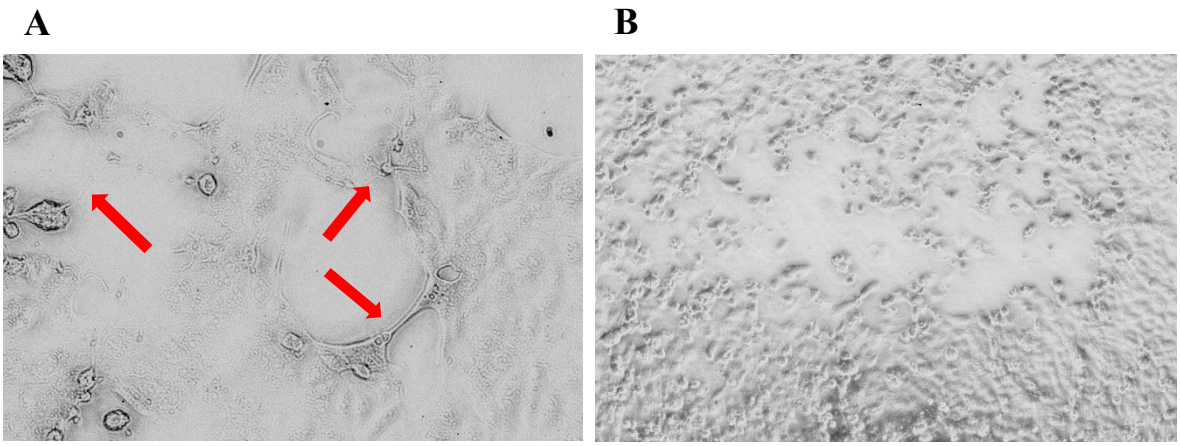


Fig. 35 B.1.1.7 lineage of SARS-CoV-2 induces syncytia formation 48 hours after infection (A). Visible syncytia are marked with red arrows. B.1.1.7-induced CPE 72 hours after infection (B).

P.1 lineage (Brazil)

CPE induced by P.1 lineage did not show significant differences with B.1 lineage, and syncytia were not frequent. CPE at 48 hours after infection seemed slightly less evident than CPE observed for B.1 lineage.

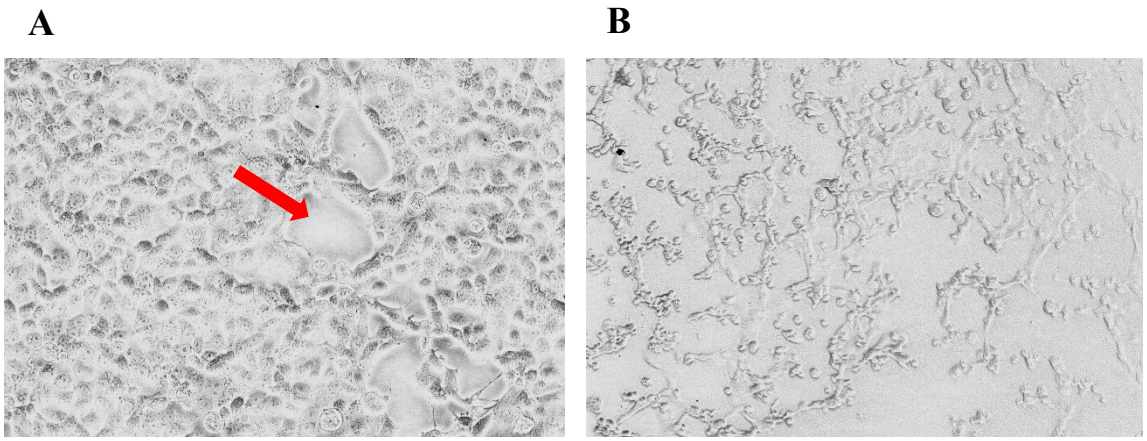


Fig. 36 P.1 lineage of SARS-CoV-2 shows a slightly less evident CPE than B.1 48 hours after infection. Death cells leave empty spaces (A). No significant differences with B.1 were observed in terms of CPE 72 hours after infection (B).

B.1.351 lineage (South Africa)

In contrast to B.1.1.7, the B.1.351 lineage bears several mutations which do not seem to increase syncytia formation, whereas only one mutation, namely D215G, has been suggested to modestly increase cell-cell fusion phenomena [69].

However, syncytia were not clearly detectable in Vero E6 cells infected by the B.1.351 variant and the overall CPE was similar to what observed for B.1 lineage.

A **B**

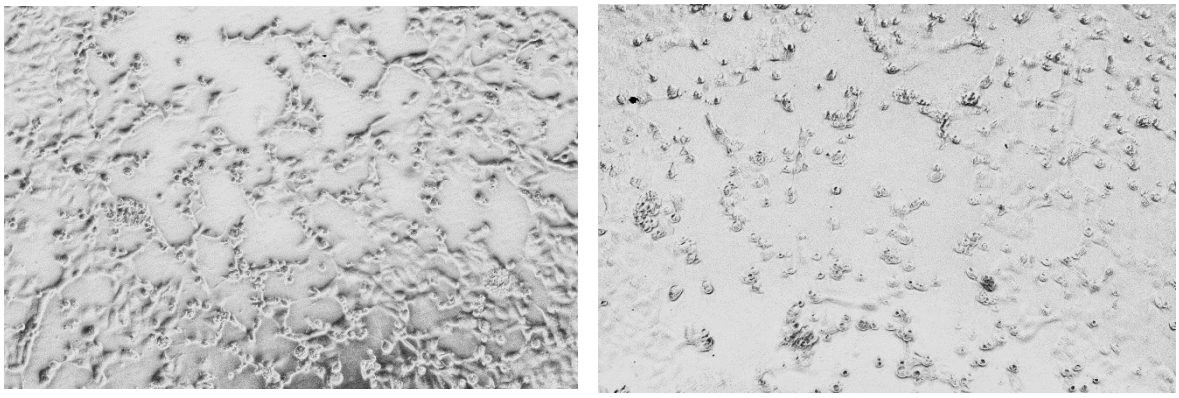


Fig. 37 Both (A) and (B) pictures show CPE inuced by B.1.351 lineage in Vero E6 cells 72 hours after infection.

B.1.526 lineage (New York)

Also B.1.526 did not show syncytia formation after 48 and 72 hours following infection.

Therefore, the overall CPE was very similar to that of the B.1 lineage, although the B.1.526 lineage seemed to grow slowly compared to the B.1 lineage, consistently with results obtained from the viral replication kinetics (see section 8.3*)*.

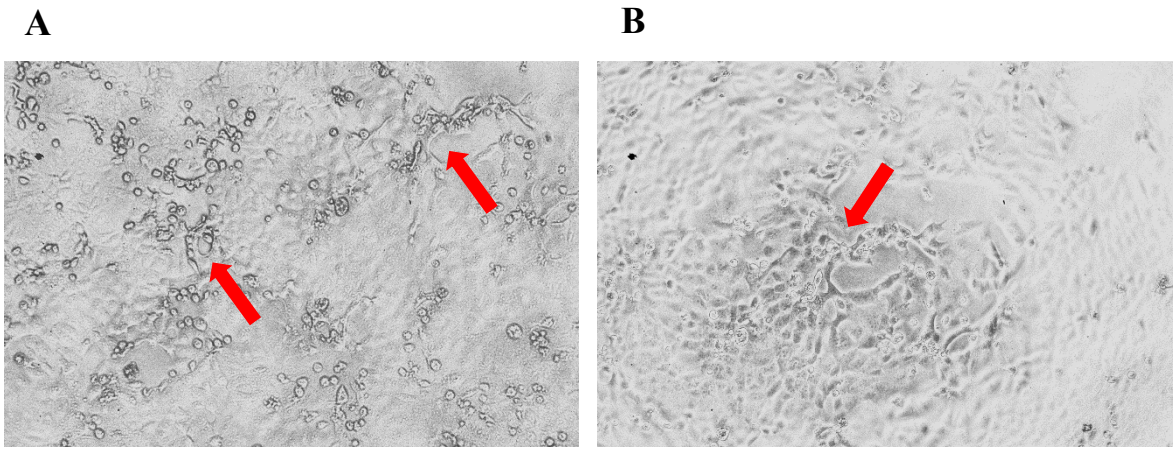


Fig. 38 Both (A) and (B) pictures show CPE inuced by B.1.526 lineage in Vero E6 cells 48 hours after infection. Cells undergoing detachment are marked by red arrows.

8.3 Replication kinetics of different lineages of SARS-CoV-2

Interestingly, the five lineages showed differences also in their replication kinetics. The B.1.1.7 lineage (the most syncythiogenic) showed a slower growth in Vero E6 cell cultures than other lineages, as well as B.1.526, which did not display syncytia formation.

In contrast, both P.1 and B.1.351 lineages seemed to grow faster than B.1.1.7 and B.1.526 lineages. However, all lineages showed a considerably slower replication kinetics compared to that of the B.1 strain.

	B.1	**B.1.1.7**	**P.1**	**B.1.351**	**B.1.526**
6 h	3,94E+04	2,19E+06	5,75E+05	3,18E+06	1,08E+07
12 h	7,49E+06	2,54E+06	3,24E+06	2,18E+07	1,07E+07
24 h	8,74E+09	1,33E+08	2,31E+08	3,28E+09	2,52E+08
48 h	3,92E+11	3,26E+10	1,62E+11	4,54E+11	1,06E+11
72 h	7,74E+11	5,03E+11	1,36E+12	1,11E+12	6,11E+11

Tab. 10 Kinetic replication values for each viral lineage (genome copies / mL). Samples were collected at 6, 12, 24, 48, 72 hours after infection.

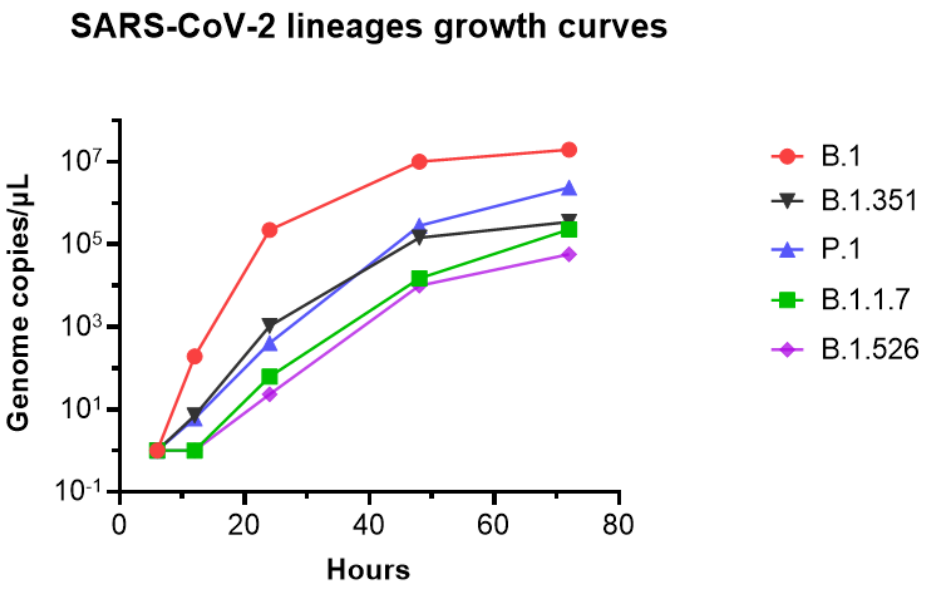


Fig. 39 Growth curve of five different lineages of SARS-CoV-2. Kinetic replication values were normalized for each lineage by dividing each value by the 6 h value before plotting.

8.4 Microneutralization assay

In order to establish the highest possible standardization across sera and lineages, each serum was tested in the same experiment and in parallel against each lineage.

8.4.1 nAbs elicited by BNT162b2 vaccine and natural infection

50 sera from subjects vaccinated with the BNT162b2 vaccine were obtained 15 (±1) days after the second dose and were tested against the B.1, B.1.1.7, P.1, B.1.351 and B.1.526 lineages. 33 sera (median 110 days, iqr 96-143 days from infection) were collected from naturally infected individuals before the arrival of the B.1.1.7 lineage in Italy (specifically, before December 2020), meaning that convalescent patients were most probably infected with the B.1 lineage. The unpaired, non-parametric Mann-Whitney test was performed to compare, for each viral variant, the nAbs titres obtained from vaccinees and convalescent individuals; whereas the paired, non-parametric Wilcoxon test was carried out to compare the nAbs titres obtained from vaccine-derived (as well as convalescent) sera against all the five viral lineages.

Resulting data are listed below:

- **B.1 lineage**: both vaccine-derived and convalescent sera showed a good level of neutralization against the B.1 (D614G) lineage, with a median titre of 367,5 for vaccinees and 192,0 for convalescent individuals. The difference between vaccine-induced and convalescent sera was statistically significant ($p < 0.0001$), thus meaning that vaccine-induced nAbs seem to be more powerful than those elicited during natural infection.

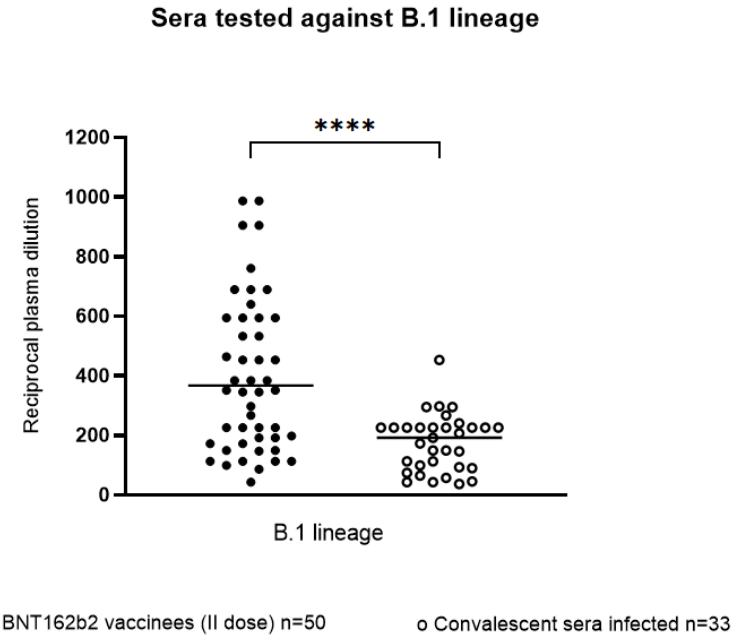


Fig. 40 BNT162b2 vaccinees versus convalescent sera neutralizing capacity tested against B.1 lineage ($p < 0.0001$).

- **B.1.1.7 lineage**: consistently with what observed for the B.1 lineage, the B.1.1.7 lineage was best neutralized by nAbs from vaccine-induced rather than convalescent sera ($p < 0.0001$). The median nAbs titre values for vaccinees and convalescent patients were respectively 113,0 and 37,0.

  Despite this similar trend, a strong decrease in the neutralizing capacity of both populations was observed by comparing the nAbs titre obtained against B.1.1.7 and B.1 lineages (Fig. 45), following a paired, non-parametric Wilcoxon test; the obtained difference was statistically significant ($p < 0.0001$).

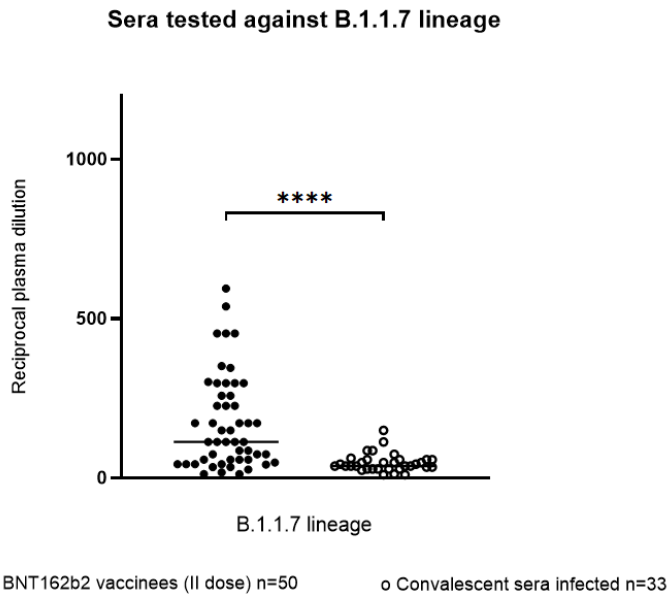


Fig. 41 BNT162b2 vaccinees versus convalescent sera neutralizing capacity tested against B.1.1.7 lineage ($p < 0.0001$).

- **P.1 lineage**: also in the case of the P.1 lineage, nAbs elicited following the second dose of the BNT162b2 vaccine showed a higher titre than those observed in convalescent individuals (previously infected with the B.1 lineage).

  Median titres were 104,5 and 37,0 respectively for vaccinees and convalescent patients. The latter median titre was the same as the one obtained for convalescent sera tested against the B.1.1.7 lineage, meaning that naturally infected individuals develop nAbs with a very similar neutralizing power against B.1.1.7 and P.1 variants.

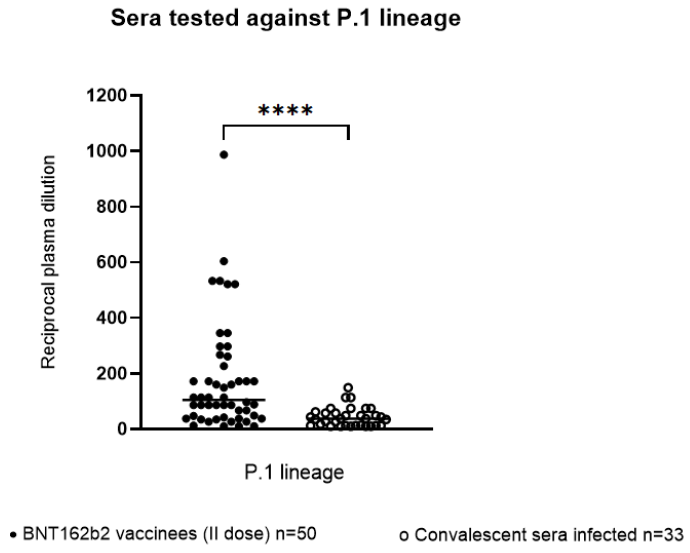


Fig. 42 BNT162b2 vaccinees versus convalescent sera neutralizing capacity tested against P.1 lineage ($p < 0.0001$).

- **B.1.351 lineage**: unlike all the other tested lineages, nAbs titre obtained from vaccine-derived and convalescent sera showed a statistically non-significant difference when tested against the B.1.351 lineage (median = 10,0 for both vaccinees and convalescent individuals). Indeed, the B.1.351 lineage seemed to be remarkably capable of escaping nAbs elicited not only from the BNT162b2 vaccine, but also from natural infection, as both populations showed a drastic loss of their neutralizing power (Fig. 45).

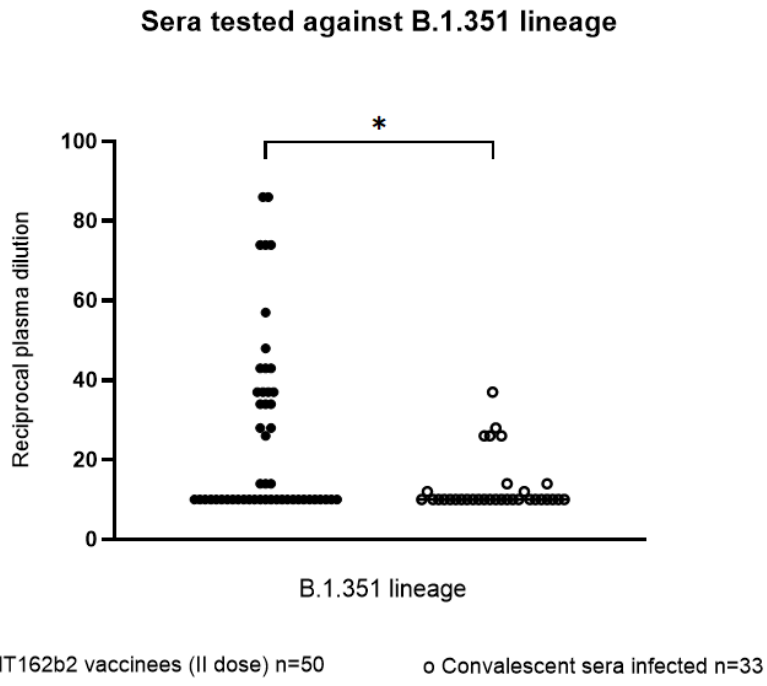


Fig. 43 BNT162b2 vaccinees versus convalescent sera neutralizing capacity tested against B.1.351 lineage (non significant difference, p = 0.0148).

Finally, BNT162b2 sera were tested against the B.1.526 lineage, unfortunately it was not possible to test convalescent sera against this viral variant. Consistently with what observed for all other lineages, resulting nAbs titres computed for the B.1.526 lineage showed a statistically significant difference compared to the B.1 lineage nAbs titre, being the median neutralizing titre similar to that of P.1 and B.1.1.7 (respectively 104,5 and 113,0).

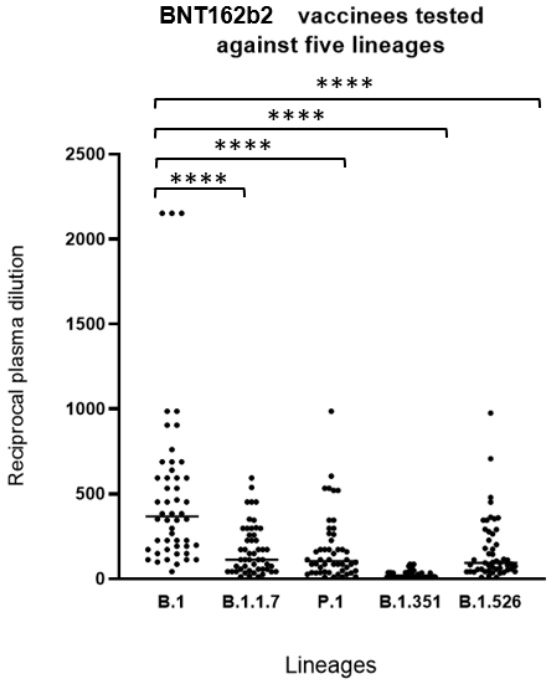


Fig. 44 BNT162b2 vaccinees nAbs titres obtaines against five different lineages ($p < 0.0001$).

The figure below shows the comprehensive data resulting from paired, non-parametric Wilcoxon tests carried out for vaccinees and convalescent sera. Statistically significant ($p < 0.0001$) differences were noticed in each serological group, meaning that each viral lineage is neutralized differently in vaccinees as well as convalescent individuals.

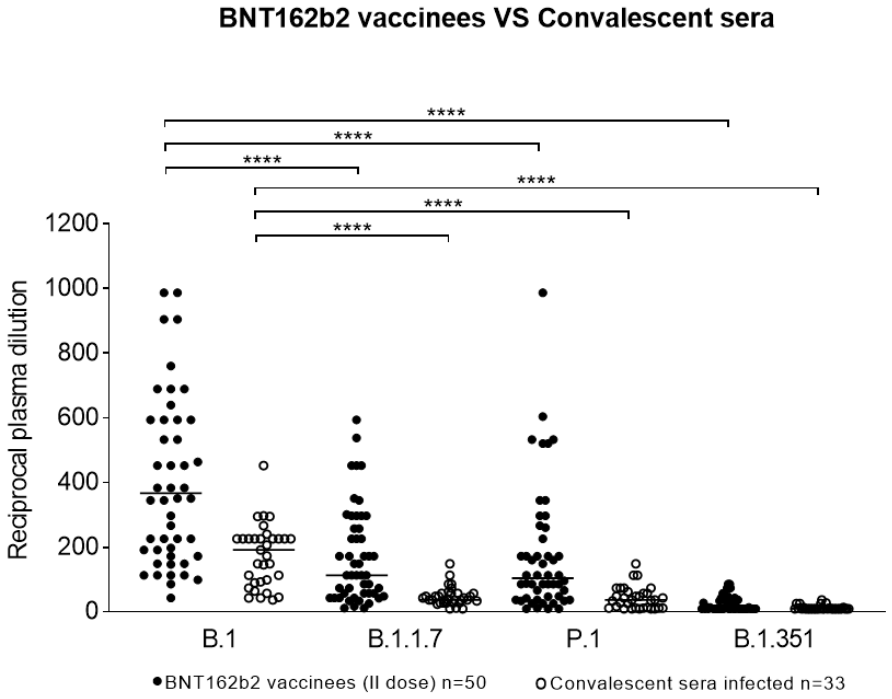


Fig. 45 Comprehensive plot showing nAbs titres resulting from sera, derived from BNT162b2 vaccinees and convalescent patients. Each comparison was statistically significant ($p < 0.0001$).

8.4.2 Convalescent sera from invidivuals infected with lineages other than B.1

In order to assess whether the experimental design was adequate for evaluating lineage-specific antibody response, 13 sera (median 31 day, iqr 19-44 days after the first positive test for SARS-CoV-2) derived from B.1.1.7 infection, as well as two sera derived from P.1 and B.1.351 infected individuals respectively, were tested against all viral lineages.

Remarkably, 13 sera from convalescent individuals infected with the B.1.1.7 lineage displayed a significantly higher neutralizing capacity against this specific lineage ($p = 0.0171$). Similarly, sera derived from P.1 and B.1.351 infections were also primarily capable of neutralizing P.1 ($p = 0.0001$) and B.1.351 ($p = 0.0002$) lineages, respectively.

Moreover, the nAbs titre obtained from the serum belonging to the patient infected with the B.1.351 lineage was the most cross-reactive when tested against all other viral lineages. Indeed, nAbs titres were very similar for each variant.

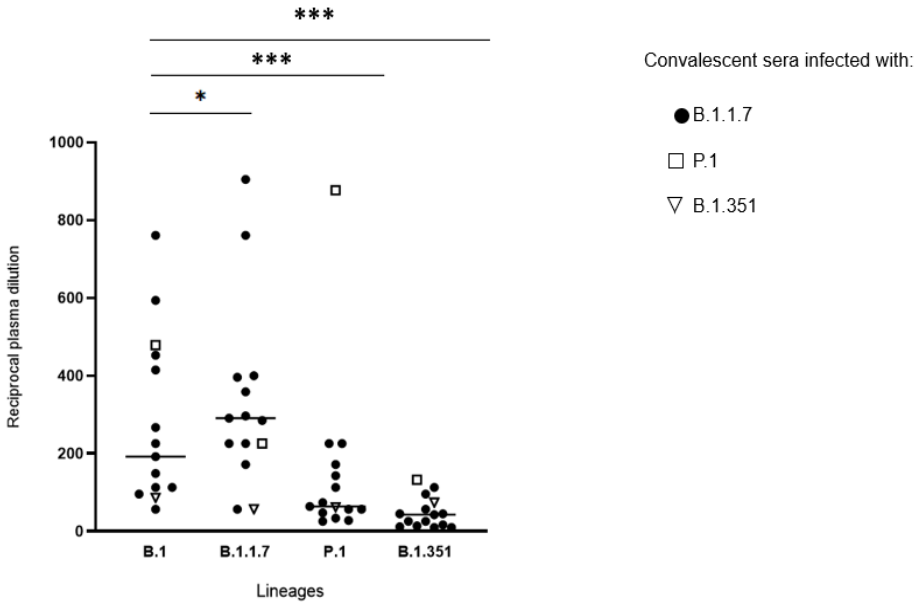


Fig. 46 Convalescent sera derived from patients infected with B.1.1.7, P.1 and B.1.351 lineages tested against four viral variants.

8.4.3 Vaccine failures

Another aspect that deserves attention is understanding whether the *in vitro* neutralizing activity of sera derived from vaccinees could represent a valuable predictive marker for vaccine failure that might occur in these individuals. In order to investigate this, a small cohort of 8 sera derived from individuals who experienced COVID-19 after the second dose of BNT162b2 vaccine (median 39 days, iqr 32-45 days) were tested against each viral lineage. Subsequently, the resulting nAbs titres were compared to sera of the previously analyzed vaccinated individuals.

The neutralizing power against the B.1 lineage of sera from vaccine failures, compared to that of successful vaccinees against the B.1 lineage, was slightly and non-significantly inferior. However, all individuals who experienced vaccine failure were infected with the B.1.1.7 lineage.

Therefore, the nAbs titres against the B.1.1.7 lineage from vaccine failures were assessed, which resulted comparable to that observed in the group of vaccinees previously tested. A significant decrease in neutralizing power was observed in vaccine failures when comparing nAbs titre obtained in the case of all other lineages, consistently with what observed in the general population of vaccinees.

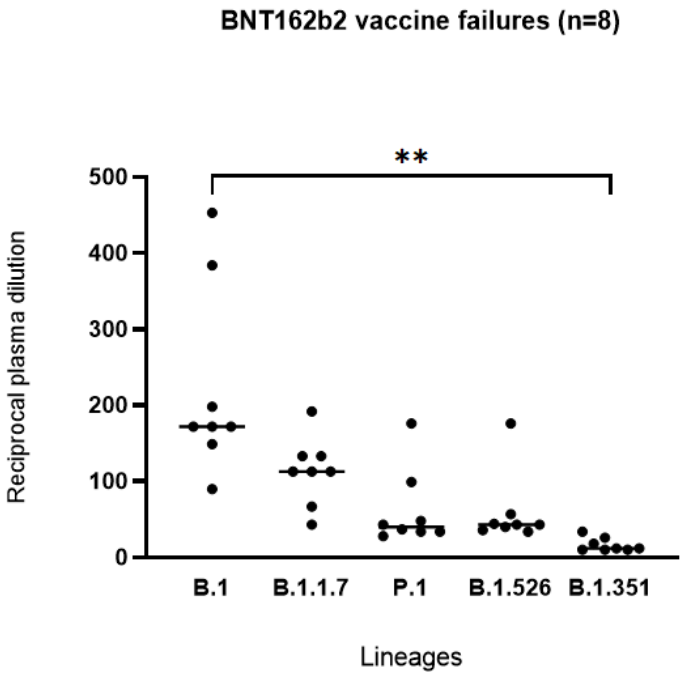


Fig. 47 Sera derived from patients who experienced vaccine failure, tested against B.1, B.1.1.7, P.1, B.1.526 and B.1.351 lineages (p = 0.0078).

8.4.4 nAbs titre in BNT162b2 vaccinees with previous SARS-CoV-2 infection

Neutralization test was performed on sera derived from individuals who experienced natural infection before BNT162b2 vaccination, in order to assess the effect of this vaccine as an anamnestic response, also in terms of cross-reactivity against multiple lineages. The 15 sera analyzed for this purpose derived from individuals who showed a variable time between the previous infection and vaccination (median 96 days, iqr 81-319).

Firstly, neutralizing activity of these 15 sera was tested against each viral lineage, showing similar trends of those observed in the group of non-infected vaccinees (n = 50): a significant decrease in nAbs titre was proven by comparing nAbs titres against the B.1 lineage to those against all other lineages ($p < 0.0001$).

However, vaccinees with previous SARS-CoV-2 infection showed a significantly higher nAbs titre compared to the group of non-infected vaccinees. Moreover, the most significant finding that emerges from this analysis is that the neutralizing activity was notably higher when tested against the B.1.351 lineage: a notable decrease ($p < 0.0001$) was calculated; in more detail the median titre for previously infected vaccinees (n = 15) tested

against the B.1.351 lineage was 149, whereas the median titre for non-infected vaccinees (n = 50) tested against the same viral variant was 10.

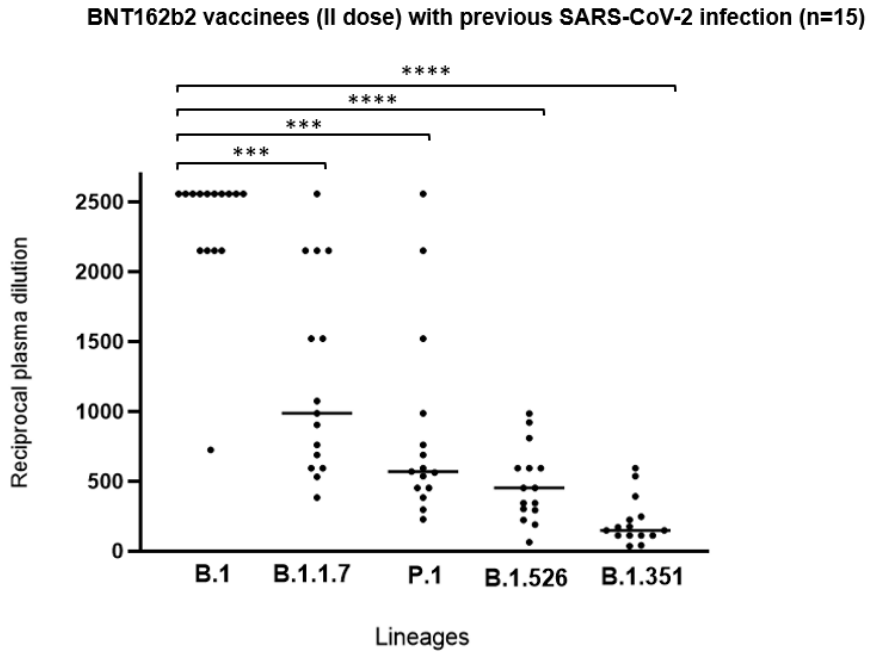


Fig. 48 Sera derived from patients who experienced natural infection, tested against B.1, B.1.1.7, P.1, B.1.526 and B.1.351 lineages.

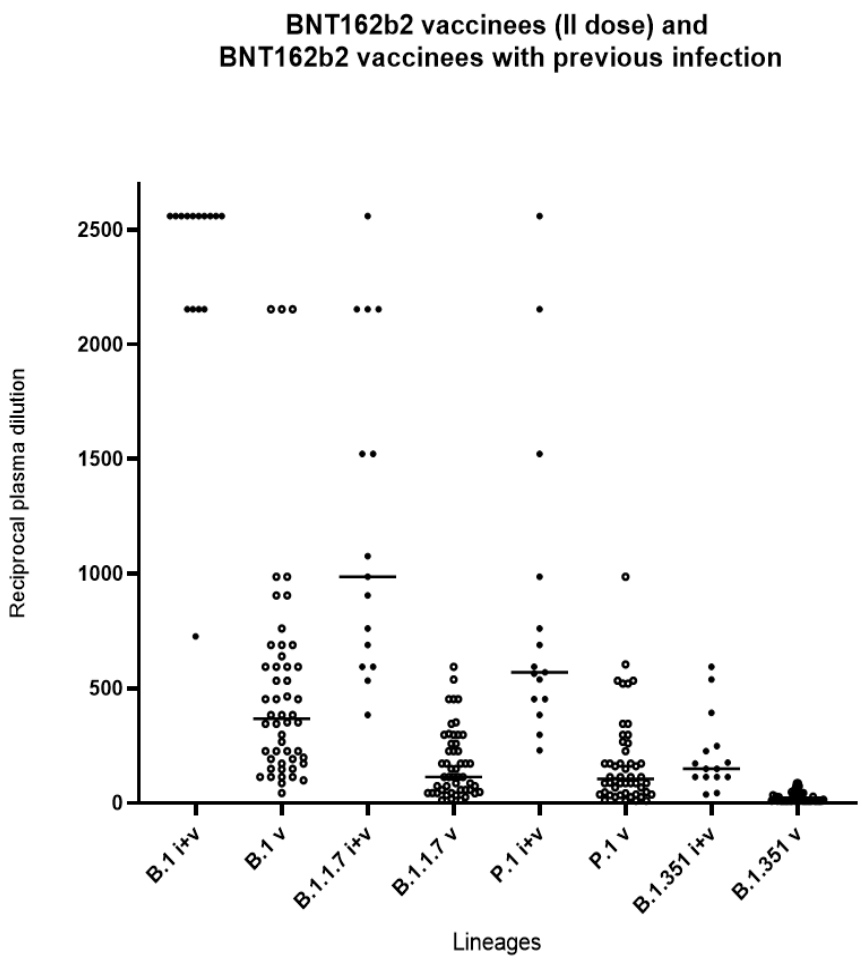


Fig. 49 Sera derived from patients infected with SARS-CoV-2 before the BNT162b2 vaccine (n = 15) compared to non-infected vaccinees (n = 50), tested against B.1, B.1.1.7, P.1, B.1.526 and B.1.351 lineages.

To gain more insight in anamnestic response, sera derived from previously infected subjects (median 13 weeks) were collected after the first dose and after the second dose of the BNT162b2 vaccine (n = 3), and were tested against each viral lineage, in order to investigate whether a single dose of the BNT162b2 vaccine was sufficient to stimulate an adequate immune response in individuals who had already experienced COVID-19 (Fig. 50).

Interestingly, this small cohort of individuals showed a non-significant variation ($p > 0.05$) in nAbs titre between the first and the second dose of vaccine; moreover, the neutralizing power evaluated in the sample collected after the first dose was similar to that observed in the non-infected vaccinees group who received a double dose of the BNT162b2 vaccine.

This result suggests that a single dose of the BNT162b2 vaccine would be sufficient to stimulate a protective immune response against re-infection even by different lineages.

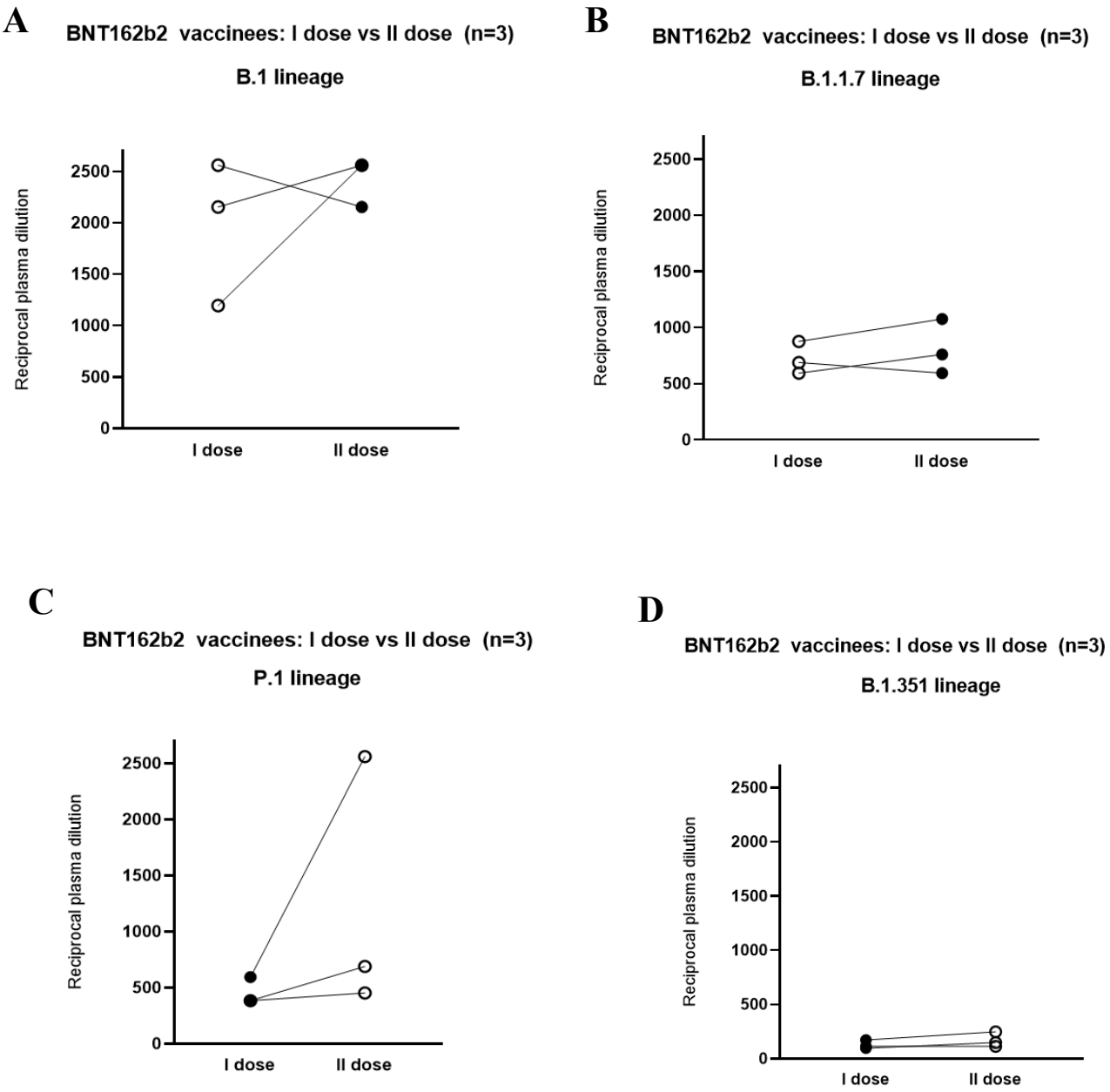


Fig. 50 Three serum samples (I and II dose) derived from previously infected BNT162b2 vaccinees. nAbs titre was evaluated after the I and the II dose (p value: non significant).

8.4.5 nAbs titre in AZD1222 non-infected vaccinees and AZD1222 vaccinees with previous SARS-CoV-2 infection

Sera obtained from two groups of individuals vaccinated with the AZD1222 vaccine (n = 20) were collected 3 weeks after the first dose and tested against B.1, B.1.1.7, P.1, B.1.351 and B.1.526 lineages. In more detail, 10 of these 20 AZD1222 vaccinees experienced SARS-CoV-2 infection in the period February-December 2020. This group showed a significantly higher neutralizing power ($p < 0.01$) compared to the 10 naïve vaccinees, who showed low or absent nAbs titres against all viral lineages, as reported in Fig. 51.

Furthermore, AZD1222 vaccinees who experienced COVID-19 (n = 10) showed a lower neutralizing activity when tested against lineages other than B.1, consistently with what observed in BNT162b2 infected vaccinees.

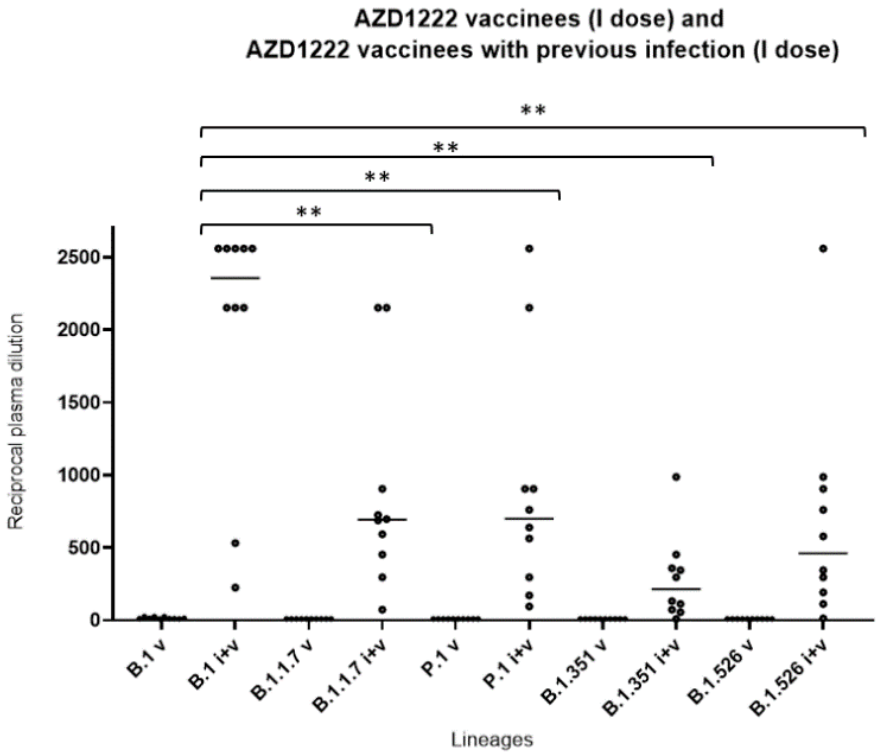


Fig. 51 AZD1222 vaccinees (n = 20). Sera were collected 3 weeks after the first dose of the AZD1222 vaccine. Vaccinees with previous infection showed a significantly decreasing neutralizing power against lineages other than B.1 ($p < 0.01$).

8.4.6 Comparison between I wave infected vaccinees (March – September 2020) and II wave infected vaccinees (October – December 2020)

In order to investigate whether nAbs titres obtained from vaccinees who experienced COVID-19 could change depending on the time elapsed between infection and vaccination, two groups of infected vaccinees (either with the BNT162b2 or AZD1222 vaccine) were further divided into two categories (COVID-19 I wave and II wave) depending on the period in which they were tested positive for SARS-CoV-2.

AZD1222 vaccinees: I wave and II wave

AZD1222 sera were collected three weeks after the first dose of vaccine. No significant variations in nAbs titre against multiple lineages ($p > 0.05$) were observed in AZD1222 vaccinees who experienced COVID-19 either in the I wave ($n = 5$) or in the II wave ($n = 5$), with the exception of the B.1.1.7 lineage. Indeed, in vaccinees who recently experienced SARS-CoV-2 infection (II wave) the neutralizing power was slightly lower than in those who were infected during the I wave ($p < 0.05$).

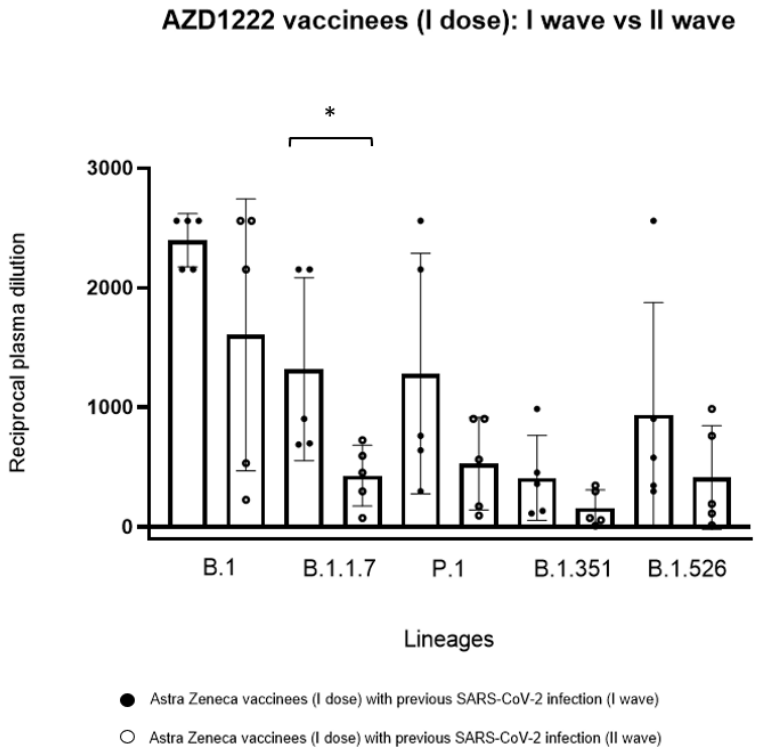


Fig. 52 AZD1222 vaccinees (I dose) tested against five different lineages of SARS-COV-2. Mean and standard deviation are reported for each group of sera, respectively belonging to the I wave and the II wave.

BNT162b2 vaccinees: I wave and II wave

Unlike the previous group, sera from vaccinees with the BNT162b2 vaccine were collected after the second dose. Even in this case, no significant variations in nAbs titre against multiple lineages were observed in BNT162b2 vaccinees who tested positive for SARS-CoV-2 infection either in the I wave (n = 7) or in the II wave (n = 8).

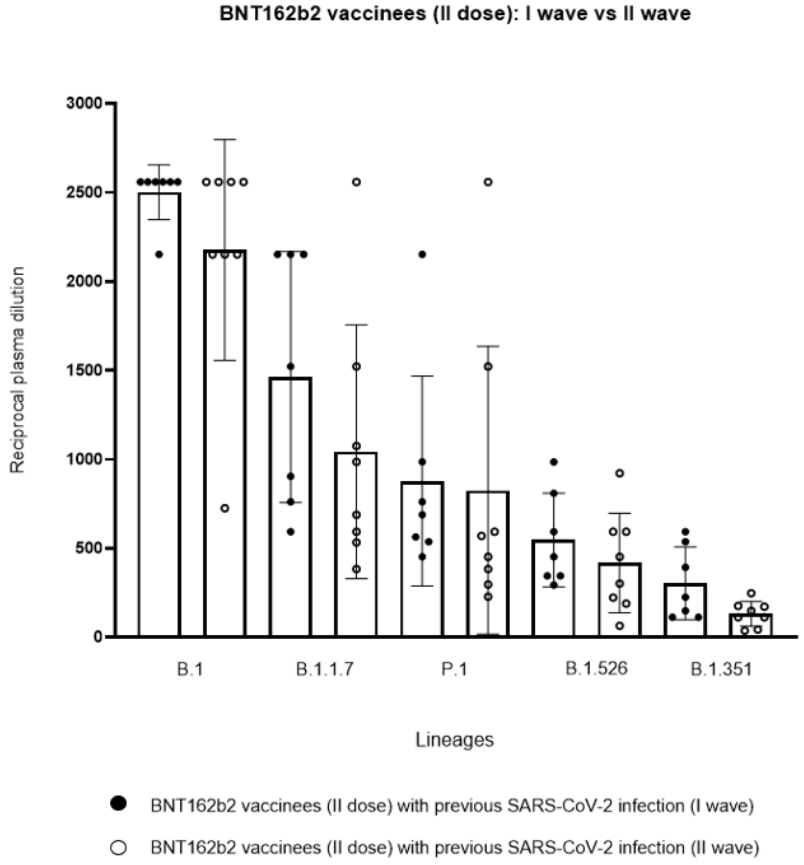


Fig. 53 BNT162b2 vaccinees (II dose) tested against five different lineages of SARS-COV-2. Mean and standard deviation are reported for each group of sera, respectively belonging to the I wave and the II wave.

9. Discussion

A better knowledge of the replication and immune response dynamics of SARS-CoV-2 is crucial for public health management, as well as for the development of effective antiviral therapies, vaccines and for the implementation of functional strategies of epidemiological control.

Over the last year, SARS-CoV-2 has significantly evolved in new lineages, which display a concerning degree of divergence from the wild-type strain firstly detected in Wuhan.

Scientists have focused most of their efforts on developing safe vaccines in record time. Up to now, more than 1.7 billion doses have been administered worldwide, and an enormous effort in terms of epidemiological data monitoring is being made by public health organizations, in order to understand whether vaccines might shape the course of the COVID-19 pandemic, which has already caused more than 3.5 million deaths [90].

Moreover, there is an ongoing discussion over which aspects of the immune response, elicited during SARS-CoV-2 infection as well as vaccination, provide hallmarks of immune protection. It is widely accepted that vaccines efficacy should not be expected to be associated with high antibody concentrations, which are mainly typical of the acute immune reaction. It

seems that protection is well maintained during the memory phase. In most of the patients who recovered from COVID-19, an induction of long-term immunity has been noticed [86].

Concerning neutralization, many of the studies available as of June 2021 were conducted by generating a panel of recombinant pseudo-viruses expressing the Spike protein on their surface (provided with specific mutations on the RBD) and by incubating them with human sera samples to evaluate the neutralization level; indeed these recombinant systems were proven to be highly comparable to live viruses, but easier to handle (BSL-2 environment).

Greaney *et al*. showed that residue in position 484 of the Spike protein is one of the most concerning variation sites. Indeed, the effect of three mutations at 484 amino acid position (namely E484K, E484Q, E484P displayed on the surface of pseudo-viruses) was striking: several of the tested sera samples showed a drop in the neutralization titre by over one order of magnitude [63].

Moreover, in Chen *et al*., convalescent serum neutralization titres were 5-fold lower against the VOCs carrying the E484K and N501Y mutations (namely P.1 and B.1.351) [60]. In the same study, sera derived from individuals who received the BNT162b2 vaccine were tested against different viral lineages, resulting in a moderate reduction of the neutralizing activity against the B.1.1.7 lineage (2-fold) and a larger decrease against strains bearing the

E484K/N501Y mutations (4-fold), whereas the most relevant decrease was observed in the B.1.351 lineage (10-fold), similarly to what previously observed in convalescent sera [60].

Considering this evidence, the combination of E484K, N501Y and K417N point mutations seems to result in a higher infection rate as well as reduced neutralizing capacity of nAbs elicited during infection with lineages free of these RBD mutations of concern. All these data are consistent with what reported in the present study: a sizeable decrease in neutralizing power was observed in sera from both vaccinated and convalescent individuals tested against the B.1.351 lineage, which carries all the three abovementioned mutations.

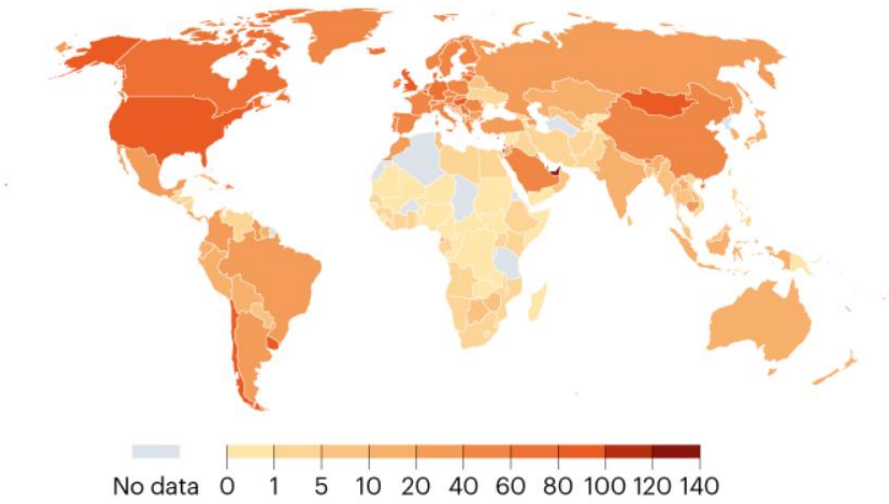


Fig. 54 Global map regarding on mass vaccination in worldwide countries as of June 2021. Reprinted from "Six months of COVID vaccines: what 1.7 billion doses have taught scientists", by Ledford, H., 2021, *Nature*.

This study has been carried out by using live viral lineages, for which a BSL-3 laboratory was required, in order to create *in vitro* conditions that could effectively display *in vivo* properties of SARS-CoV-2.

9.1 Replicative properties

Increasing transmission rates of SARS-CoV-2 VOCs have been reported worldwide but, despite this, still very limited information concerning other biological aspects (e.g. replication kinetics and cytopathological phenotypes) is currently available.

This study aimed at investigating the replicative properties of the emerging SARS-CoV-2 lineages, and is a part of the large number of studies which are being undertaken to understand whether the replication dynamics of each VOC does correlate with their *in vivo* properties, such as transmissibility, viral load, etc. [89]. In the present study, the virus–host interaction was simulated in a Vero E6 cell culture system, showing that different lineages replicate at different speeds. In more detail, B.1 (or D614G) turned out to be the most efficient lineage in terms of replication speed from 6 to 12 hours from inoculation (1.5-fold). On the other hand, B.1.1.7 and B.1.526 lineages replicated at a similar pace, resulting in a slower replication than B.1 lineage. Vero E6 cells infected with the B.1.1.7 lineage displayed remarkable syncytia

formation, consistently with what observed in Rajah *et al*. [69]. This evidence suggests that the B.1.1.7 Spike protein, which is transferred to the cell membrane during viral assembly, has some special structural properties which may cause the rapid formation of syncytia. Consistently, site-directed mutagenesis experiments showed that B.1.1.7 carries two mutations that sensibly increase cell-cell fusion: P681H and D1118H [69].

On the other hand, 12 hours after inoculation, B.1.351 and P.1 lineages showed a faster replication dynamics than B.1.1.7 and B.1.526 lineages, following a similar trend.

Furthermore, the ratio between genome copies and TCID50 provided useful information about the ability of each lineage to generate complete particles during viral replication. B.1 turned out to be the most effective viral lineage in generating complete virions during cell culture infection, whereas B.1.526 and B.1.351 showed a slightly inferior capability to generate complete viral particles.

However, it remains unclear whether *in vitro* cytopathology and replication kinetics could be related to *in vivo* viral replication of SARS-CoV-2, as well as the transmissibility of each VOC.

9.2 Neutralizing assays

Up to now, many of the published studies concerning immunity to SARS-CoV-2 were limited to short-time analyses. Indeed, the longest follow-up reported so far is the one conducted in Israel by Haas *et al*., who studied BNT162b2 vaccine efficacy after 7 weeks from the second dose. Therefore, although current data suggest optimism, longer-term analyses are needed [87].

Convalescent and vaccine-derived sera

nAbs titres resulting from vaccine-derived and convalescent sera turned out to be significantly different between the two groups: individuals who received two doses of the BNT162b2 vaccine neutralized B.1, B.1.1.7 and P.1 lineages more efficiently than convalescent patients. In contrast, an alarming result was derived from microneutralization tests carried out against the B.1.351 lineage: this variant was weakly neutralized by either vaccine-derived or convalescent sera, consistently with what observed by Liu *et al*., who noticed that B.1.1.7 and P.1 pseudo-viruses showed an approximately equivalent nAbs titre, whereas pseudo-viruses displaying the full set of mutations of the B.1.351 lineage showed a lower neutralization titre [60, 92]. Therefore, even a limited number of mutations in lineages such as B.1.351 can erase most of the neutralizing power in the immune response.

Apart from the B.1.351 lineage, this data represents a noticeable indication: periodical immunological stimulations seem to boost cross-lineage nAbs generation, which seem to be weaker in naturally infected individuals who were not further stimulated against SARS-CoV-2. These considerations are further confirmed by microneutralization tests carried out for vaccinated individuals who previously experienced SARS-CoV-2 infection. Interestingly, in this case the B.1.351 lineage was neutralized more efficiently, whereas all other lineages were neutralized similarly compared to the naïve vaccinees group. Furthermore, three individuals who were tested against all lineages after the first dose of BNT162b2 vaccine showed a neutralizing power which turned out to be very similar to that of sera collected after the second dose in naïve vaccinees, thus suggesting that one only dose of vaccine may be sufficient for those with prior SARS-CoV-2 infection to boost the immune system and achieve a satisfactory level of protection, even if they are vaccinated after a year with a single dose of vaccine. This result is consistent with the induction of SARS-COV-2 long-term immunity demonstrated by Radbruch *et al*. [86].

Furthermore, data reported in the present study are in accordance with what stated by several independent research groups, which reported high nAbs titres after the first dose of the BNT162b2 vaccine, as well as the mRNA-

1273 vaccine, in individuals who experienced natural infection [66]. Despite the major limitation of these studies, such as the small number of participants, they all agree that pre-existing immunity to SARS-CoV-2 can elicit a high level of nAbs, thus supporting the hypothesis that a single vaccine dose acts as a sufficient boost for a seropositive patient, who would be protected [92].

In addition to this data, the present study showed that reaching a satisfactory level of cross-lineage neutralization is possible in previously infected individuals who are given a single dose of vaccine. Two major advantages derive from this evidence: on one hand, the administration of one only dose of vaccine may limit side effects, on the other hand there would be a greater vaccine supply to protect more individuals now at risk. Finally, follow-up studies that will be carried out in the next future may prove whether these differences in different serological groups are maintained over a prolonged time.

In addition, regarding individuals who were infected with B.1.1.7, P.1 and B.1.351 lineages, each serum displayed a stronger neutralizing activity against the lineage by which each patient had been infected, thus proving that the microneutralization experimental design was adequate to test multiple types of sera. Notably, the serum sample derived from the B.1.351 infection showed a strong cross-lineage neutralizing power against all lineages, thus suggesting

that the mutations in the South African lineage Spike protein may increase the elicitation of broadly neutralizing antibodies.

Vaccine failure prediction

The molecular epidemiology of SARS-CoV-2 infection in Italy at the time is dominated by the B.1.1.7 lineage. As stated in 8.4.3 section, all individuals who experienced vaccine failure were infected with the B.1.1.7 lineage. However, the small amount of tested samples (n = 8) does not allow a discussion on whether this viral lineage could be the most likely to cause vaccine failure.

In these subjects, a slight and non-significant decrease in the neutralizing power was demonstrated against the B.1 lineage compared to other vaccinees. Therefore, the resulting data showed that the neutralizing activity of antibodies tested against a heterogeneous set of viral variants did not seem to be a reliable serological marker to predict vaccine failure in single individuals.

In section 8.4.6, nAbs elicited after vaccination (II dose) show a similar neutralizing power, regardless the time elapsed since the natural infection. This interesting feature of the anamnestic response was observed for BNT162b2 as well as AZD1222 vaccinees.

Furthermore, although the AZD1222 vaccine does not seem as efficient as the BNT162b2 vaccine at eliciting neutralizing antibodies, it results in an overall good efficacy when used as a single-dose booster after natural infection, comparable with what observed for infected vaccinees after the first dose of the BNT162b2 vaccine.

In conclusion, the enormous amount of published and ongoing studies concerning SARS-CoV-2 immunity, carried out in laboratories worldwide, is helping to understand how long the immune response could last and how effective it could be against new and emerging viral variants, both in previously infected and vaccinated individuals.

However, one of the most important questions to be answered is whether the currently licensed vaccines could prevent viral transmission or not. This issue represents a crucial aspect for bringing the pandemic under control, but demonstrating vaccine efficacy in blocking transmission in a general population is difficult because countermeasures, such as local lockdowns, may obscure this effect [91].

Another aspect that deserves consideration is to understand the durability of vaccine-induced immunity, and the rational planning of booster shots in the next few months. Another possibility is that vaccines might make infected people less liable to pass the virus on to others. For example, research groups

in Israel measured the viral load in individuals who tested positive for SARS-CoV-2 after vaccination, which turned out to be a good marker for infectiousness [91].

However, as the pandemic is still ongoing, further studies in the field of epidemiology, structural biology, genomics, immunology and proteomics are required to make accurate predictions of the outcome of the current COVID-19 pandemic.

10. Acronyms

ACE2: angiotensin converting enzyme 2

ANG-I: angiotensin-I

ANG-II: angiotensin-II

APC: antigen presenting cell

BAL: bronchoalveolar lavage

CDC: centre for disease control and prevention

CM: convoluted membranes

CPE: cytopathic effect

CTD: C-terminal domain

DIC: disseminated intravascular coagulation

ERES: endoplasmic reticulum exit sites

ERGIC: endoplasmic reticulum Golgi intermediate compartment

GISAID: global initiative on sharing avian influenza data

IC: intermediate compartment

LNP: lipid nanoparticle

MHC: major histocompatibility complexes

MCM: medical countermeasures

MERS: middle east respiratory syndrome

Mpro: main protease

nAbs: neutralizing antibodies

NET: neutrophils extracellular trap

Nsp: non-structural protein

NTD: N-terminal domain

PANGO: phylogenetic assignment of named global outbreak

PM: plasma membrane

RBD: receptor binding domain

RBM: receptor binding motif

RTC: replication transcription complex

SARS: severe acute respiratory syndrome

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