UNIVERSITAT DE BARCELONA

Naturally Acquired and Vaccine-Induced Neutralizing Humoral Responses to SARS-CoV-2

Edwards Pradenas Saavedra

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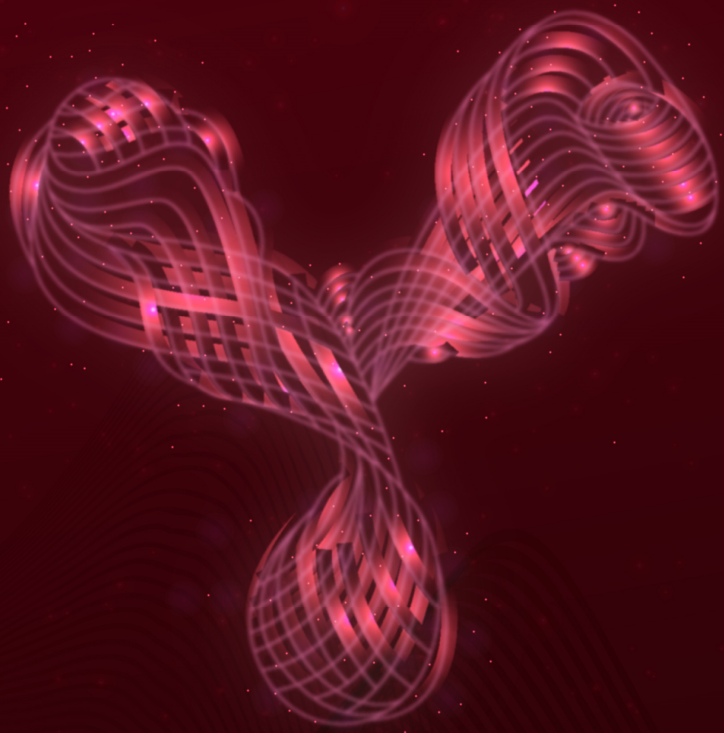

UNIVERSITAT DE BARCELONA

Faculty of Medicine and Health Sciences
Doctoral Programme in Biomedicine

Naturally Acquired and Vaccine-Induced Neutralizing Humoral Responses to SARS-CoV-2

Thesis submitted by

Edwards Pradenas

Edwards Pradenas Saavedra

to qualify for the degree of doctor by the University of Barcelona.

The presented work has been performed in the Cell Virology and Immunology group, at the IrsiCaixa AIDS Research Institute, supervised and tutored by

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Dr. Julià Blanco Arbués	**Dr. Jorge Carrillo Molina**	**Dr. Manel Juan Otero**
Director	Director	Tutor

Barcelona, June 2023

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certify

that the experimental work and the writing of the present Doctoral Thesis entitled

"Naturally Acquired and Vaccine-Induced Neutralizing Humoral Responses to SARS-CoV-2"

have been carried out by Edwards Pradenas Saavedra under their supervision, and

consider

that it is suitable to be presented and to qualify for the degree of Doctor in Biomedicine at the University of Barcelona.

For the record, they sign this document at

Badalona, June 10th, 2023

Julià Blanco

Dr. Julià Blanco Arbués

Jorge Carrillo

Dr. Jorge Carrillo Molina

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Antibodies represent the key molecules mediating protection from viral infections. Viruses illustrate SARS-CoV-2 infection. COVID-19 vaccination is represented by spikes. The waves symbolize different epidemiological periods of COVID-19 in Spain. Background stippling represents droplet and airborne transmission of SARS-CoV-2.

Naturally Acquired and Vaccine-Induced Neutralizing Humoral Responses to SARS-CoV-2

Thesis for doctoral degree (Ph.D.)

by

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In memory of my parents, María Saavedra and Florencio Pradenas
For their love, education, support and sacrifice

To my family and friends

To all victims of the COVID-19 pandemic

Some lessons from Ebola outbreaks:

"Be fast. Have no regrets. You must be the first mover. The virus will always get you if you don't move quickly. If you need to be right before you move, you will never win. Perfection is the enemy of the good when it comes to emergency management. Speed trumps perfection; and the problem in society we have at the moment is everyone is afraid of making a mistake, everyone is afraid of the consequence of error. But the greatest error is not to move. The greatest error is to be paralyzed by the fear of failure"

Michael J. Ryan, 2020
Executive Director of WHO's Health Emergencies Programme

Pandemic Influenza Leadership Forum:

"We don't know when a pandemic might strike. But we can be sure of two things. Everything we do before a pandemic will seem alarmist. Everything we do after a pandemic will seem inadequate. This is the dilemma we face, but it should not stop us from doing what we can to prepare. We need to reach out to everyone with words that inform, but not inflame. We need to encourage everyone to prepare, but not panic"

Michael O. Leavitt, 2007
Former US Department of Health and Human Services Secretary

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List of Scientific Articles

This thesis is based on the following publications:

1. Trinité B, Tarrés-Freixas F, Rodon J, **Pradenas E**, Urrea V, Marfil S, Rodríguez de la Concepción ML, Ávila-Nieto C, Aguilar-Gurrieri C, Barajas A, et al. SARS-CoV-2 infection elicits a rapid neutralizing antibody response that correlates with disease severity. *Sci Rep* (2021) **11**:1–10. doi:10.1038/s41598-021-81862-9

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Foreword

It is a real pleasure to write this foreword to Edwards Pradenas Saavedra's thesis. A doctoral thesis is the summit of the academic training and, therefore, a unique moment in the life of a researcher. In this particular case, different unique aspects make this moment more special.

First of all, we would highlight the personal aspect. It has been our privilege to be able to supervise the doctoral work of Edwards Pradenas Saavedra. He has demonstrated two valuable qualities, a tireless desire to grow professionally fighting against all the elements (visas included) and a high care and rigor in the work that is demonstrated on each page of his doctoral thesis.

Secondly, it is worth putting this doctoral thesis in context. The irruption of the COVID-19 pandemic in the midst of a thesis initially conceived to analyze neutralizing humoral responses against HIV. IrsiCaixa's commitment to SARS-CoV-2 research forced a change of objectives that implied a certain risk for the thesis. However, Edwards' ability to work with the new virus was instrumental in his ultimate success. The quantity and quality of the results indicate that we made the right decision.

Third, the research on SARS-CoV-2 has meant an avalanche of information that needed to be managed. For a doctoral student, this was an additional challenge that Edwards has overcome by far.

The final result is an excellent thesis, that contains only a part of the work done by Edwards in the last years. We hope the readers will enjoy this document that represents a timely and valuable contribution to our understanding of neutralizing humoral responses against SARS-CoV-2 and its intricate relationship with the continuous viral evolution.

Dr. Julià Blanco and Dr. Jorge Carrillo

Summary

Neutralizing antibodies represent one of the major correlates of protection against viral infections. Their elicitation is one of the main goals of vaccine development. The longitudinal kinetics, the factors that determine the neutralizing activity generated by vaccination, natural infection, or both (hybrid immunity), and their ability to neutralize different variants are key pieces of information to evaluate the immune status against SARS-CoV-2. These data would inform us to take key decision on immunization schedules and/or vaccination strategies. Despite the plethora of published studies in this matter, at the beginning of the COVID-19 pandemic the evidence was scarce and uncertain, and today there are still gaps in our knowledge about the neutralizing response.

In this thesis we evaluate in detail the anti-SARS-CoV-2 neutralizing humoral response elicited by natural infection, vaccination, or both. We report that the neutralizing response to natural SARS-CoV-2 infection is elicited early after infection and is long-lasting (beyond one year). However, it induces limited long-term variant cross-neutralization. The level and quality of neutralization is determined by disease severity, and specifically, the magnitude of the humoral response in asymptomatic or mildly symptomatic individuals is associated with duration of symptoms and age, with no apparent contribution from gender and viral load. Early vaccination schedules generated neutralizing antibodies that decay over time and poorly neutralize emerging variants. However, vaccination of convalescent individuals boosts pre-existing natural immunity and provides better variant coverage. The third dose of vaccine broadens the cross-neutralization by generating a neutralizing response that mimics hybrid immunity. Omicron breakthrough infection have a positive impact on the neutralizing response to Omicron subvariants.

All these results demonstrate the complexity of the neutralizing immune response to SARS-CoV-2, and how the magnitude and quality of this response are influenced by several factors, such as the clinical course of the disease, demographic factors, and the nature and number of antigenic exposures. This information can be useful to evaluate and predict the population protective status and to guide future health strategies.

Resumen

En el contexto de las infecciones virales, los anticuerpos neutralizantes representan uno de los principales correlatos de protección. Su generación es uno de los objetivos primordiales a conseguir mediante la vacunación. Comprender la cinética de los anticuerpos neutralizantes a lo largo del tiempo, sus determinantes tras la vacunación, la infección natural, o ambos eventos (inmunidad híbrida), y su capacidad para neutralizar diferentes variantes, representan uno de los pilares fundamentales sobre el cual evaluar la respuesta inmunológica contra el SARS-CoV-2. Esta información proporciona una base sólida sobre la cual establecer esquemas de inmunización y/o estrategias vacunales. A pesar del considerable número de publicaciones que abordan este tema, todavía existen vacíos en nuestra comprensión sobre la respuesta neutralizante.

En nuestros estudios evaluamos detalladamente la respuesta humoral neutralizante contra el SARS-CoV-2 obtenida por la infección natural, la vacunación, o la combinación de ambos eventos. Observamos que la respuesta neutralizante a la infección natural por SARS-CoV-2 se genera rápidamente tras la infección y es duradera (más allá de un año), pero presenta una limitada neutralización cruzada contra diferentes variantes virales. La gravedad de la enfermedad determina los niveles y la calidad de la neutralización, mientras que la magnitud de la respuesta humoral en individuos asintomáticos o con enfermedad leve está asociada con la duración de los síntomas y la edad, sin contribución aparente del sexo y la carga viral. La vacunación genera anticuerpos neutralizantes que disminuyen con el tiempo y muestran una reducida actividad contra las variantes emergentes. Sin embargo, la vacunación en convalecientes refuerza la inmunidad natural preexistente y confiere una mayor amplitud de neutralización. La tercera dosis de la vacuna amplía la neutralización cruzada, generando una respuesta similar a la inmunidad híbrida. Las infecciones tras la vacunación causadas por Omicron, tienen un impacto positivo en la respuesta neutralizante a las subvariantes Omicron.

Estos resultados evidencian la complejidad de la respuesta humoral neutralizante y, tanto la magnitud como la calidad de la misma, está influenciada por varios factores, tales como el curso clínico de la enfermedad, características demográficas, y la naturaleza y número de exposiciones antigénicas. Esta información resulta útil para conocer y predecir el estado de protección de la población y guiar futuras estrategias sanitarias.

Resum

En el context de les infeccions virals, els anticossos neutralitzants representen un dels principals correlats de protecció. La seva generació és un dels objectius primordials a aconseguir mitjançant la vacunació. Comprendre la cinètica dels anticossos neutralitzants al llarg del temps, els seus determinants després de la vacunació, la infecció, o ambdós esdeveniments (immunitat híbrida), i la seva capacitat per neutralitzar diferents variants, representa un dels pilars fonamentals sobre els quals avaluar la resposta immunològica contra el SARS-CoV-2. Aquesta informació proporciona una base sòlida per establir esquemes d'immunització i/o estratègies de vacunació. Malgrat el considerable nombre de publicacions que aborden aquest tema, encara hi ha buits a la nostra comprensió de la resposta neutralitzant.

Als nostres estudis, vam avaluar detalladament la resposta humoral neutralitzant contra el SARS-CoV-2 obtinguda per la infecció natural, la vacunació o ambdós. Vam observar que la resposta neutralitzant a la infecció natural per SARS-CoV-2 es genera ràpidament després de la infecció i és duradora (més enllà d'un any), però amb una limitada neutralització creuada contra variants virals a llarg termini. La gravetat de la malaltia determina els nivells i la qualitat de la neutralització, mentre que la magnitud de la resposta humoral en individus asimptomàtics o amb malaltia lleu està associada amb la durada dels símptomes i l'edat, sense contribució aparent del sexe i la càrrega viral. La vacunació genera anticossos neutralitzants que disminueixen amb el temps i mostren una activitat reduïda contra les variants emergents. Tanmateix, la vacunació en persones convalescents reforça la immunitat natural preexistent i confereix una major amplitud de neutralització. La tercera dosi de la vacuna amplia la neutralització creuada, generant una resposta similar a la immunitat híbrida. Les infeccions després de la vacunació causades per Omicron tenen un impacte positiu en la resposta neutralitzant a les subvariants Omicron.

Aquests resultats evidencien la complexitat de la resposta humoral neutralitzant, i què tant la magnitud com la qualitat d'aquesta està influïda per diversos factors, com el desenvolupament clínic de la malaltia, les característiques demogràfiques i la naturalesa i nombre d'exposicions antigèniques. Aquesta informació és útil per a conèixer i predir l'estat de protecció de la població i guiar futures estratègies sanitàries.

1

INTRODUCTION

Every day the world faces new challenges, and pandemics have been a part of our lives since ancient times; therefore, we must learn from past experiences and apply those lessons to deal with current and future outbreaks effectively.

Throughout humankind's history, many pandemics have caused irreparable devastation to entire continents. The tragic and overwhelming loss of millions of lives has been a common outcome of pandemics; starting from the earliest recorded plagues (Athenian, Antonine, and Justinian), going through the Black Death, smallpox, and the Spanish influenza H1N1, to the present day with Acquired Immunodeficiency Syndrome (AIDS) and Coronavirus Disease 2019 (COVID-19). Regrettably, some of these pandemics continue to claim thousands of lives[1,2].

Emerging and reemerging infectious diseases are one of the major threats to public health[3–5], especially those that are deadly or cause serious and irreparable damage to people's health, and have a strong impact on our socio-economic structure. Many epidemics and pandemics are zoonotic[6], meaning that they are transmitted between vertebrate animals and humans[7]. It is calculated that ≈1.7 million viruses are yet to be discovered in mammals and birds, and nearly half of them have zoonotic potential[8]. In fact, it is estimated that more than 6 out of 10 human pathogens can be transmitted from animals, and that more than 70% of emerging infectious diseases originate from wildlife species[4,6,9].

In the last two decades, the world has experienced a surge of emerging and reemerging infectious diseases. Among them, it is possible to mention the 2003 Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV or SARS-CoV-1) outbreak, the 2009 influenza A virus (H1N1, swine influenza) pandemic, the 2012 Middle East Respiratory Syndrome Coronavirus (MERS-CoV) outbreak, the 2013-2016 Ebola virus disease epidemic, the 2015 Zika virus epidemic, the 2019 Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pandemic, and more recently, the 2022 Monkeypox outbreak. Importantly, the fact that numerous outbreaks, epidemics, and pandemics are caused by respiratory viruses is probably due to their transmissibility and route of transmission[10–15].

The host immune response plays a critical role in the outcome of a pandemic, as it determines the susceptibility of individuals to the pathogen, the severity of the disease, and the ability to develop immunologic memory and herd immunity[16–18]. Furthermore, the immune response can shape the course of the pandemic by influencing the transmission dynamics of the infectious agent[19,20]. The pathogen needs to evade or subvert the host immune response to survive and propagate, while the host immune response needs to effectively recognize and eliminate the pathogen. Therefore, in the context of the current pandemic, a thorough understanding of the interaction between SARS-CoV-2 and the host immune response is essential to develop effective strategies to control the spread of the virus and reduce the disease burden.

Overview of COVID-19 pandemic

COVID-19 inception

SARS-CoV-2 is the etiologic agent of COVID-19, one of the best-documented human pandemics, thanks to the remarkable international cooperation that has led to a plethora of scientific publications in a short lapse of time[21].

In December 2019 (**Figure 1**), a cluster of patients exhibiting atypical pneumonia and not responding adequately to conventional treatments was detected in Wuhan city (Hubei, China)[22–24]. On 31 December 2019, Wuhan Municipal Health Commission reported an outbreak of pneumonia of unknown cause, and several cases seemed to be connected to the Huanan Seafood Wholesale Market (Wuhan)[25,26].

Retrospective studies suggest a likelihood that SARS-CoV-2 had spilled over into humans between early October and mid-November 2019[27–30]. Evidence collected to date indicates that the origin of SARS-CoV-2 may be wildlife. Although exactly how, when, and where it emerged is still a mystery. The strongest theory is that it jumped from bats (or other animal species, such as pangolins)[22,31–34] to an intermediate host animal (e.g., raccoon dogs, foxes, minks, etc.), and from there to humans; and that the Huanan Seafood Wholesale Market was the initial epicenter of the pandemic[35–39].

During the first days of January 2020, WHO reported that the outbreak was caused by a new coronavirus, and on January 10-12, China shared the genetic sequence of SARS-CoV-2 (at that time named 2019 Novel Coronavirus, 2019-nCoV)[40–42]. On January 13, the first detection protocol using reverse-transcription polymerase chain reaction (RT-PCR) was published[43]. Since then, cases began to be detected outside China and the first deaths were registered.

On January 19-22, the WHO announced that there was evidence of human-to-human transmission; and 1 day later, in an attempt to contain the spread of the virus, Wuhan—and other cities in Hubei province—was put under lockdown, which had remarkable success in decreasing the growth rate of cases[44–46]. On January 30, WHO considered the outbreak to constitute

a Public Health Emergency of International Concern (PHEIC). On February 11, it was announced that the name of the new coronavirus would be SARS-CoV-2, and the disease it produced would be called COVID-19. One month later, the WHO characterized it as a pandemic, Europe became the epicenter, and the world began an unprecedented shutdown, leading to the confinement of more than half of humanity to their homes[47,48]. The number of infections and deaths has increased steadily ever since.

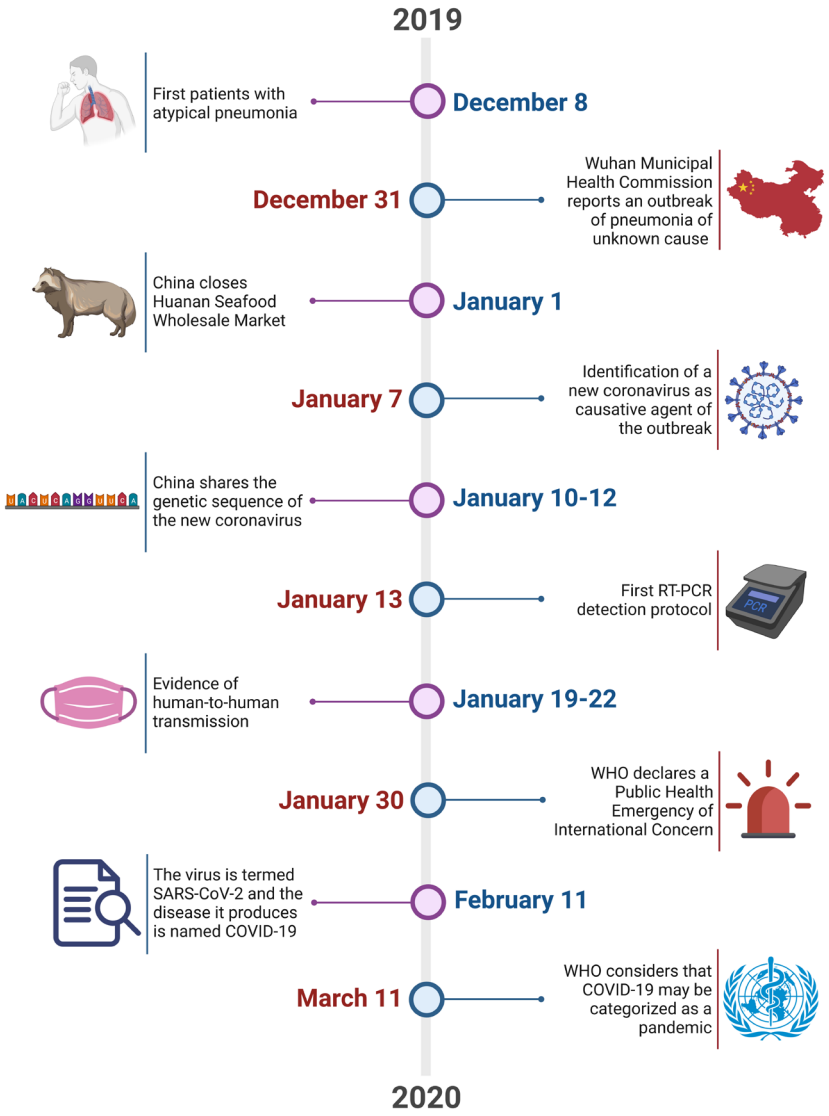


Figure 1 | **COVID-19 timeline**. Key events from the first SARS-CoV-2 cases to the COVID-19 pandemic categorization. *Created with BioRender.com*

More than three years later (May 2023), after several COVID-19 waves around the world, WHO estimates that there have been over 760 million confirmed SARS-CoV-2 infections that

have led to nearly 7 million deaths worldwide[49] and considerable morbidity in the population[50,51]. However, the epidemiological data is most likely underestimated[52–55]. Analyses have projected a cumulative excess of more than 20 million deaths associated with the COVID-19 pandemic[56], making it one of the leading causes of death in the world[57–61]. In Spain, there are 13.8 million confirmed cases registered and 121,000 deaths since the beginning of the pandemic[49,62]. Today, approximately more than a thousand new cases and 10-30 deaths are reported daily in this country[49,62]. The decreasing trend in deaths and the decline in hospitalizations and intensive care unit (ICU) admissions led WHO to indicate in January 2023 that the COVID-19 pandemic was probably at a transition point[63,64]. Recently (5 May 2023), WHO has declared that COVID-19 is now an well-established and ongoing health issue which no longer constitutes a PHEIC[65].

Coronaviruses

SARS-CoV-2 is a nidovirus, member of the genus *Betacoronavirus* in the *Coronaviridae* family[66,67]. Coronaviruses are so named because they have spike-like proteins on their surface, which under electron microscopy resemble a crown (coronavirus derives from Latin *corona*, itself a borrowing from Greek *κορώνη*, meaning "crown"), reminiscent of the solar corona[68].

Coronaviruses were first identified in the 1930s[69–71] and constitute a large and heterogeneous group of enveloped positive-sense single-stranded RNA viruses. They infect and cause diseases in mammals (including humans, livestock, and pets) and avian species[72,73]. Human coronaviruses were first discovered in the mid-1960s[74–79]. Currently, seven coronaviruses capable of infecting humans have been identified, four of them (HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1) are endemic, account for 10% to 30% of upper respiratory tract infections in adults, and generally cause "common cold" symptoms[80,81]; while SARS-CoV, MERS-CoV, and SARS-CoV-2 are highly pathogenic and have the potential to cause severe respiratory disease and affect multiple tissues.

More than 50% of the known human coronaviruses have been discovered in the 21st century[81–85]. SARS-CoV was identified in 2002-2003 and had a case fatality rate (CFR) of about 10%; chronologically no more SARS-CoV cases have been reported since 2004[86,87]. MERS-CoV was identified in 2012 and had a CFR of 30-36%; to date there are still reports of sporadic new cases, most of them from Saudi Arabia[87–89]. In contrast, the estimated CFR for SARS-CoV-2 was relatively low (1-3%), although it varies across the different viral variants[87,88,90,91].

SARS-CoV-2 Virology

The virion

SARS-CoV-2 viral particles are spherical to pleomorphic[92] with a diameter around 100 nm (ranging from 60 to 140 nm)[29,93]. The SARS-CoV-2 genome encodes sixteen non-structural proteins (NSP-1 to NSP-16), nine putative accessory proteins, and four structural proteins (**Figure 2**) in varying abundance: spike (S)-glycoprotein with around 20-40 copies per particle (irregularly arranged on the surface of the virion)[93,94], envelope protein (E) with ≈20 copies/virion, membrane protein (M, ≈2,000 copies), and nucleocapsid protein (N, ≈1,000 copies)[95].

SARS-CoV-2 contains a large genome (almost 30 kb in length) encapsidated within a helical nucleocapsid. The genome is constituted by several open reading frames (ORFs, **Figure 2**) that play an essential role in viral pathogenicity and infection. The 5'-terminal region contains two overlapping ORFs, ORF1a and ORF1b, which represent more than two-thirds of the genome and are responsible for encoding two precursor polyproteins (pp1a and pp1ab) that are co-translationally and post-translationally processed into NSPs that form the replicase–transcriptase complex (RTC) and are essential for viral replication. These proteins include an RNA-dependent RNA polymerase, RNA binding proteins, co-factors involved in replication, an exonuclease for proofreading, a 3-chymotrypsin-like protease, a papain-like protease, a helicase, a 3'-5' endonuclease, N7 and 2'-O-ribose methyltransferase, and others[96]. In the 3'-terminal region, there are four genes encoding four structural proteins (S, E, M, N), and several ORFs encoding accessory proteins (ORF3s, ORF6, ORF7s, ORF8, ORF9s, ORF10)[97,98].

Structural proteins are crucial for the viral life cycle and are important targets for immune responses and diagnostic tests. The S-glycoprotein, situated on the surface of the virus, mediates viral entry into host cells, determines the viral host range, tissue tropism, serves as a crucial target for neutralizing antibodies, and is vital for the development of vaccines and therapeutics[99,100]. The E protein is the smallest (75 amino acids [aa]) of all the structural proteins, participates in the assembly and release of viral particles. It contributes to viral morphogenesis and plays a role in maintaining the overall structure of the virus[99,100]. The M protein, an essential component of the viral envelope, interacts with the S-glycoprotein and aids in the assembly of the virus. It also contributes to viral budding and assembly processes[99,100]. The N protein encapsulates the viral RNA genome, forming the nucleocapsid structure. It is involved in viral replication and packaging of the viral RNA. The N protein elicits a strong immune response and is frequently employed in diagnostic tests to detect SARS-CoV-2 infections[99,100].

Accessory proteins are defined as non-essential proteins for virus growth in cell culture. However, many of these proteins play crucial roles in viral pathogenicity and can modulate host immune responses. For example, they are involved in inhibiting cytokine secretion (ORF9c) and counteracting the antiviral effects of type I interferon (ORF3b, ORF6, ORF7a, ORF8,

ORF9b). Moreover, these proteins also influence significant cellular processes such as autophagy and apoptosis (ORF3a), mitochondrial function (ORF3d), and activation of the inflammasome (ORF9b)[97,100].

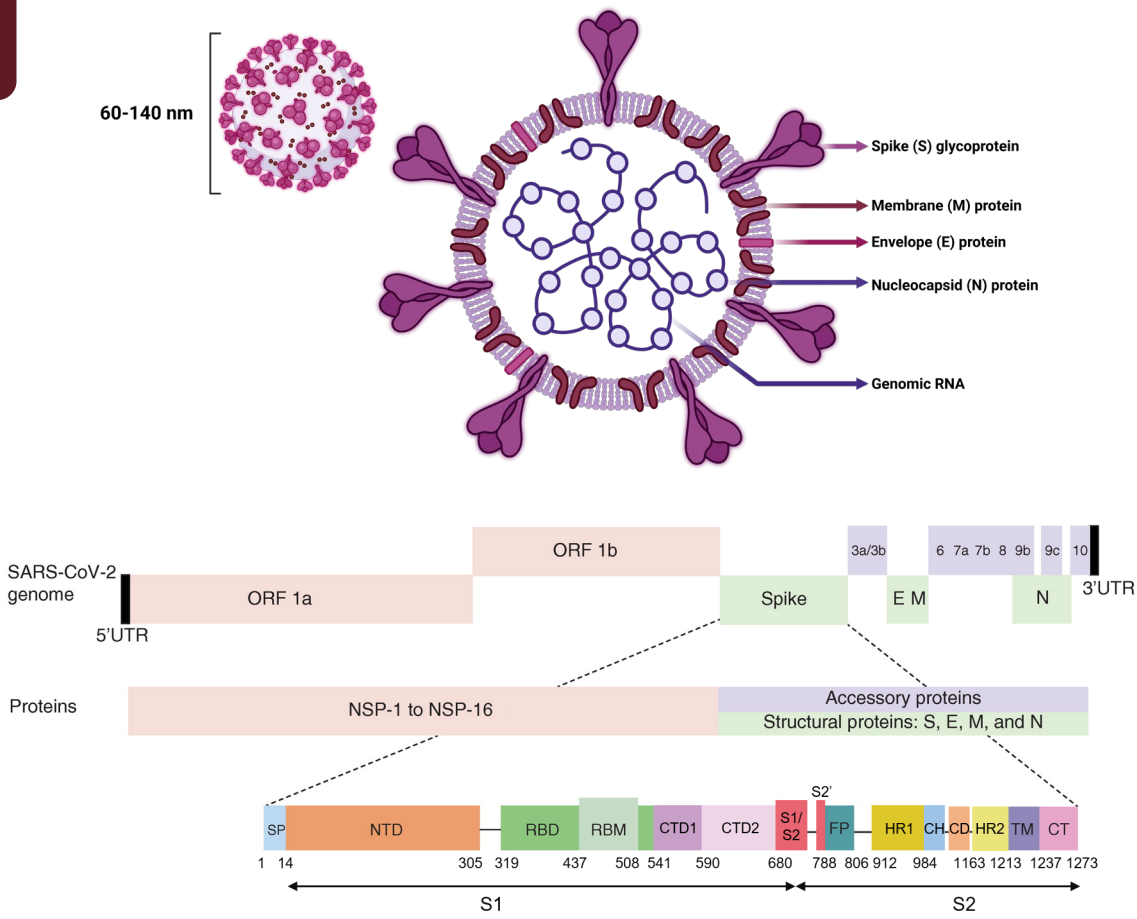


Figure 2 | **SARS-CoV-2 structure and genome**. The upper panel depicts a schematic representation of SARS-CoV-2, indicating its size, structural proteins, and genetic material. *Created with BioRender.com*. The lower panel illustrates the genomic organization of SARS-CoV-2 with a specific emphasis on the S-glycoprotein. *Adapted with permission from Rani Rajpal et al.*[101]

SARS-CoV-2 is genetically related to other coronaviruses isolated from bats, such as BatCoV RaTG13 and BANAL-20-52, with which it shares 96% and 97% of the genetic sequence, respectively[102,103]. In addition, SARS-CoV-2 shows genomic homology with other human coronaviruses associated with severe acute respiratory disease, i.e. SARS-CoV and MERS-CoV (79% and 50% of homology, respectively[87,104]).

Spike glycoprotein

The S-glycoprotein of SARS-CoV-2, a class I homotrimeric fusion protein, like the hemagglutinin of influenza virus and the gp160 envelope glycoprotein of HIV, is distributed on the surface of the virion and is relatively conserved among some human coronaviruses (e.g. the SARS-CoV-2 spike has been found to have about 76% homology with the SARS-CoV spike, while it only shares about 35% homology with the MERS-CoV spike)[105–109]. Notably, although SARS-CoV and SARS-CoV-2 are relatively homologous and both bind to the same cellular receptor, some data suggest that the latter virus is much more contagious because it has a higher binding affinity (2 to 20-fold) to angiotensin-converting enzyme 2 (ACE2) compared to SARS-CoV[110–113]. The receptor binding domain (RBD) of SARS-CoV-2 has a more compact conformation (compared to the SARS-CoV RBD), which, in addition to several residue changes, may contribute to stabilizing the hotspots of the ACE2-RBD binding interface, potentially explaining the observed phenomenon[114]. Moreover, another important difference between the two viruses is that the cleavage of the SARS-CoV spike does not involve furin-like enzymes as it does for the SARS-CoV-2 spike (as well as other proteases)[115,116]. Together, these data could explain the transmission qualities of SARS-CoV-2.

The S-glycoprotein exists mostly in a metastable prefusion conformation, and is densely glycosylated (about 66 putative glycosylation sites, ≈40% of the protein surface and ≈17% of the total molecular weight of the trimer)[117–119] mainly by N- and O-glycans that play a crucial role in viral pathobiology and act as shields to evade the immune response[93,120–124]. The host endoplasmic reticulum (ER) modifies the S-glycoprotein through co-translational and post-translational modifications, which involve extensive glycosylation, signal peptide removal, trimerization, and subunit cleavage[125].

As mentioned above, each virion contains around 20-40 spikes, a higher density compared to HIV (7-14 per virion) which is similar in size (≈100 nm)[126–129], which could imply a higher immunogenic potential. A greater contact surface area could provide more targets for immune system recognition.

The S-glycoprotein (**Figure 3**) is 180-200 kDa in size and has a total length of 1273 aa (**Figure 2**). Each monomer consists of a signal peptide (SP, aa 1-13), and two functionally distinct non-covalently associated subunits (S1 and S2). These subunits contribute to the formation of distinct regions in the protein structure, with the S1 subunit comprising the bulbous head and the S2 subunit forming the stalk region[130]. The surface subunit S1 (residues 14-685) contains the N-terminal domain (NTD, residues 14-305), the receptor-binding domain (RBD, residues 319-541), which is responsible for the interaction with the host cell receptor and includes the receptor binding motif (RBM, residues 437-508), a portion that directly contacts ACE2, and two carboxy-terminal domains (CTD1 and CTD2)[113,131,132]. Importantly, unlike SARS-CoV and other related coronaviruses, the S-glycoprotein of SARS-CoV-2 contains a polybasic cleavage site (PRRAR) between the S1/S2 boundary, which permits efficient cleavage by the prototype proprotein convertase furin and appears to play an important role in viral pathogenesis[133,134]. The S2 transmembrane domain (residues 686-1273) mediates the fusion of viral and cell membranes, and contains a N-terminal region (residues 686-787) that is cleaved (S2')

by serin proteases or cathepsins (cell surface expressed or endosomal) to release the fusion peptide (FP, residues 788-806), the central helix with heptad repeated 1 (CH and HR1, residues 912-1035), the connecting domain (CD, residues 1080-1135), the heptad repeated 2 (HR2, residues 1163-1213), the transmembrane domain (TM, residues 1214-1237), and the cytoplasmic tail (CT, residues 1238-1273)[113,131,132].

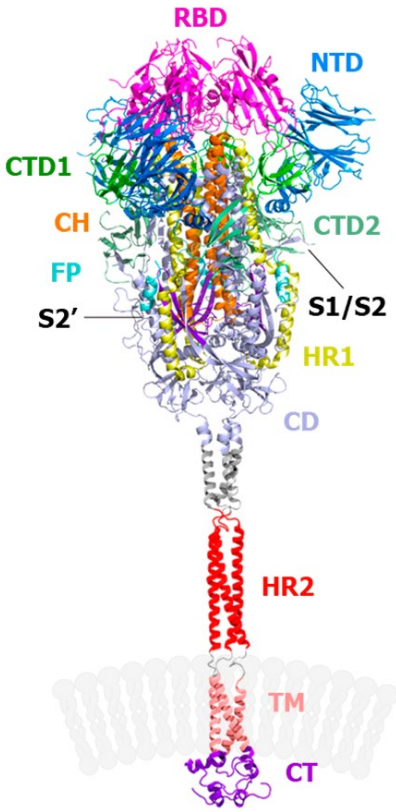


Figure 3 | SARS-CoV-2 S-glycoprotein. Structure of the SARS-CoV-2 spike in the prefusion state, with all three RBDs in the down conformation. The colors represent the different regions. S1/S2 cleavage site, and S2' cleavage site are indicated. *Adapted with permission from Cai et al.*[135] *and Casalino et al.*[122]

Importantly, S-glycoprotein plays a key role in the infectivity and transmissibility of SARS-CoV-2, but it is also the main target for humoral immune surveillance, and the development de human interventions strategies (vaccines, monoclonal antibodies and some antivirals)[136–138] since it is highly immunogenic and is exposed on the surface of the virus.

Replication cycle

Viruses depend on the molecular machinery of the host cell to generate progeny and perpetuate themselves; this is the purpose of the viral replication cycle. Each SARS-CoV-2 replication cycle is estimated to take about 10 hours[95].

Briefly, the SARS-CoV-2 life cycle (**Figure 4**) begins with the recognition and binding of the S-glycoprotein, specifically the RBD, to receptors present on target cells, mainly the cell surface expressed ACE2 (the same receptor of SARS-CoV and HCoV-NL63)[139,140]. Next, cellular proteases, such as transmembrane serine protease 2 (TMPRSS2), act as entry activators and cleave the S-glycoprotein, and the S2 domain mediates fusion of the virus with the cell membrane after extensive conformational rearrangements[138,141–143].

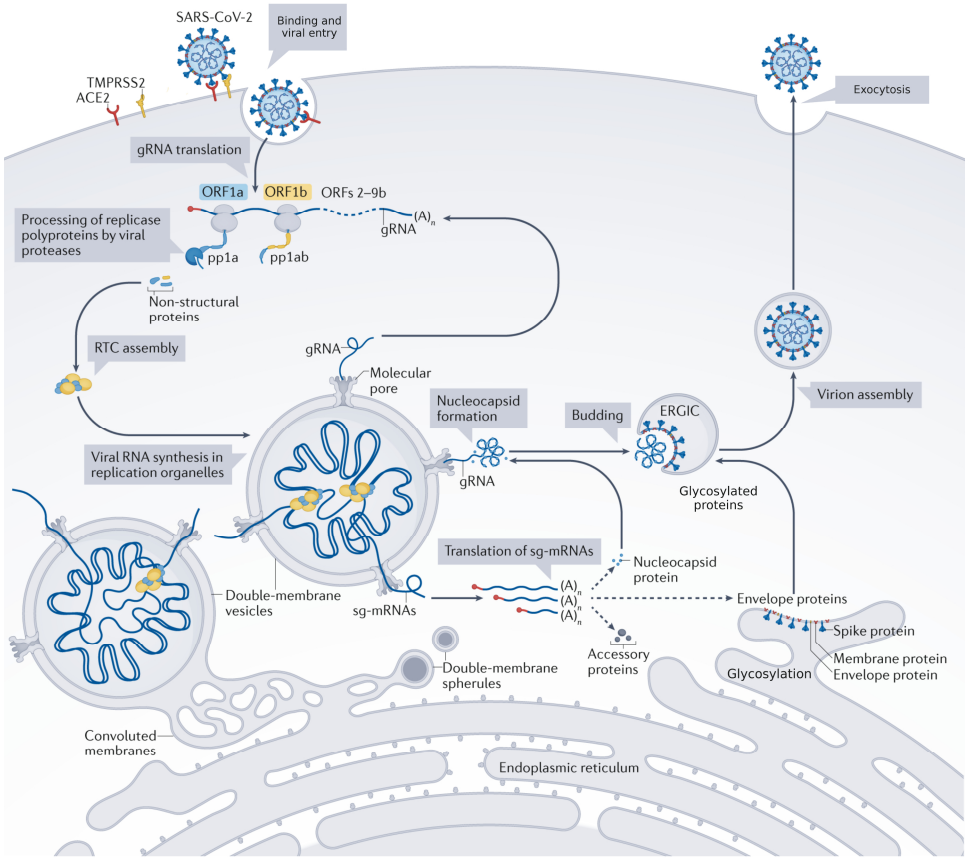


Figure 4 | SARS-CoV-2 infection cycle. The diagram indicates the different stages of the SARS-CoV-2 viral replication cycle, from binding to cell receptors to budding. See details in the main text. *Modified with permission from Malone et al.*[144] *with BioRender.com*

SARS-CoV-2 entry can also occur via the endocytic pathway (**Figure 5**), in which cathepsins can cleave the S-glycoprotein, although this pathway was not efficiently used by previous

viral variants until the emergence of the Omicron subvariants. Omicron changed this paradigm, preferring endocytic fusion, with relative alteration of cell tropism and implications for viral pathogenesis[145–148]. In any case, it always requires a prior recognition step involving the ACE2 receptor[143,149]. Therefore, it is worth noting that cell tropism for SARS-CoV-2 is complex and is generally determined by the co-expression of ACE2 (or other potential receptors)[150–153] and host proteases.

Other co-receptors involved in SARS-CoV-2 entry into host cells have been proposed, but their contribution to SARS-CoV-2 pathogenesis remains unclear[154–157]. Subsequently, the nucleocapsid is released into the cytoplasm and the genomic RNA (gRNA) is translated on cellular ribosomes to produce several NSPs (**Figure 4**). The subgenomic mRNAs (sg-mRNAs) encode structural and accessory proteins. The structural proteins S, M, and E insert into the membrane of the ER, and translocate to the compartment between the endoplasmic reticulum and the Golgi complex (ERGIC) to find the N protein, which encapsulates the viral RNA. The host cells' glycosylation machinery facilitates N- and O-glycosylation of the virus proteins during their transit through the ER, ERGIC, and Golgi[124]. The assembled viral particles are transported to the cell membrane by vesicles and released by exocytosis[93]. For a more detailed explanation, see reviews by V'kovski et al.[73] and Malone et al.[144].

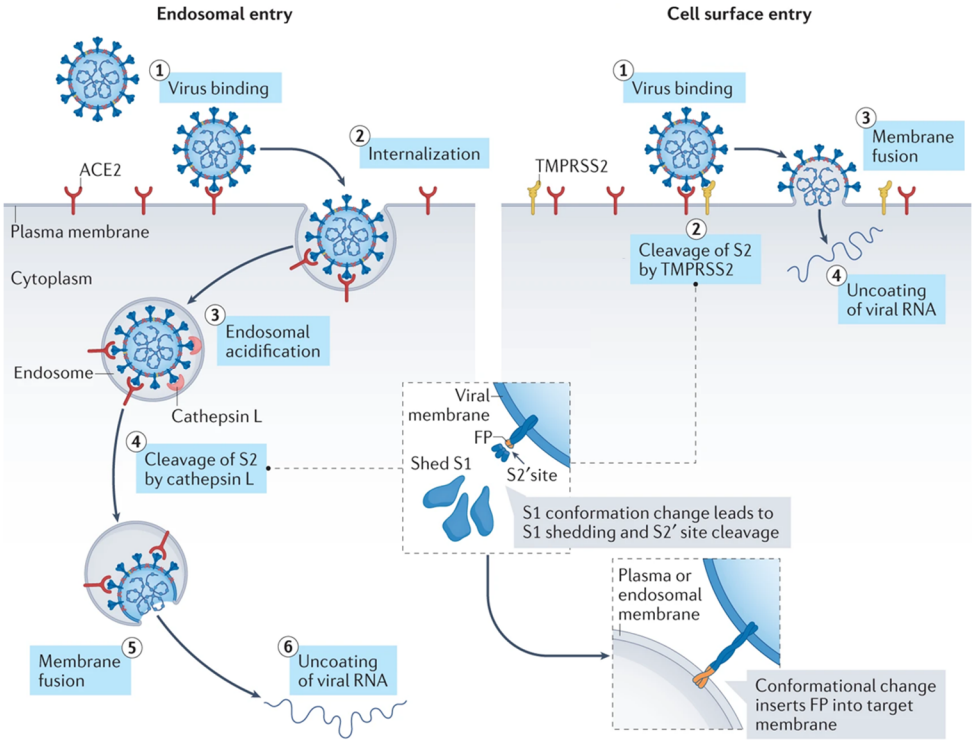


Figure 5 | SARS-CoV-2 entry pathways. The scheme shows the two different entry pathways of SARS-CoV-2 into cells. The S2' site is cleaved by different proteases depending on the endosomal or cell surface entry. The endosomal route is preferentially exploited by the Omicron subvariants. *Modified with permission from Jackson et al.*[143]

Transmission and pathophysiology

Respiratory viruses are characterized by a high incidence in the human population[158–163], in fact, lower respiratory tract infections represent the fourth most common cause of death globally, and the second leading cause of death in low-income countries (WHO Global Health Estimates)[164].

Since the SARS-CoV-2 outbreak, the prevalence of respiratory infectious diseases has decreased notably, probably due—at least in part, to the prevention measures adopted during the COVID-19 pandemic[165–170]. However, a reemergence and even an increase in the incidence of some of the respiratory pathogens is being observed[165,171–173].

Transmission

SARS-CoV-2 is transmitted mainly via airborne droplets and aerosols[15,174–177], and less frequently by direct or indirect (via fomites) contact[178–180] (**Figure 6**). There is also the potential for fecal-oral[181,182] and vertical transmission (from mother to fetus)[183,184]; however, evidence is still scarce and unclear. Importantly, although the stability of different SARS-CoV-2 variants on surfaces or aerosols varies[179,185] and might influence their transmissibility, small differences in half-lives are unlikely to influence epidemiology, as infectivity decreases rapidly in the first 5 minutes after aerosolization[186]. Remarkably, despite its putative zoonotic origin, and that many animals species are susceptible to SARS-CoV-2 infection, whether they may represent a viral reservoir is not yet known[187–192].

The basic reproduction number (R_0, expected number of new cases that can be caused by one infected person during the infectious period in a naïve, uninfected population) of the ancestral SARS-CoV-2 was about 2-3[184,193–195], similar to the Spanish influenza of 1918[196–198], and about five times less than measles[199], one of the most contagious human viruses known today. Additionally, the serial interval, which measures the time between illness onset in the primary case and illness onset in secondary cases, is estimated to range from 4 to 8 days[200–203], indicating the potential for relatively rapid transmission of SARS-CoV-2 within communities.

The effective reproduction number (R_e, also known as R_t, captures the total number of secondary infections each case generates in a population where some individuals may no longer be susceptible) changes constantly as the population becomes immunized and is affected by the variants, people's behavior, recovery time and death[204]. In the first months of the pandemic, a R_e of approximately 0.66-2.04 was estimated. Before vaccination, the R_e was estimated to be between 0.82 and 1.77. As of March 2023, the R_e offers a highly heterogeneous scenario, with some countries, such as Italy, Spain and others, having a $R_e<0.5$, while some countries, such as Australia, Portugal, Ukraine, etc., have a $R_e>3.0$[205].

Although R_0 and R_e can be used as initial benchmarks to understand the propagation speed of an infectious disease in different scenarios[206,207], they are not sufficient, since the dynamics in

the transmission of infections are complex and heterogeneous, and do not exhibit a single pattern. Superspreading events (as seen with influenza virus, SARS-CoV, MERS-CoV, Ebola virus, etc.) were frequent at the beginning of the pandemic and a dispersion factor *k* of 0.1-0.2 was estimated[208–213], meaning that a small fraction of individuals infected a large number of people[214]. By contrast, the dispersion factor for the Spanish influenza (1918) was calculated to be close to 1[215]. Epidemiological analyses suggest that 10% to 20% of cases were responsible for 80% to 90% of secondary infections[210,212,216–220]. Importantly, transmissibility is the primary driver of SARS-CoV-2 evolution[221] (*see viral evolution and immune escape section*).

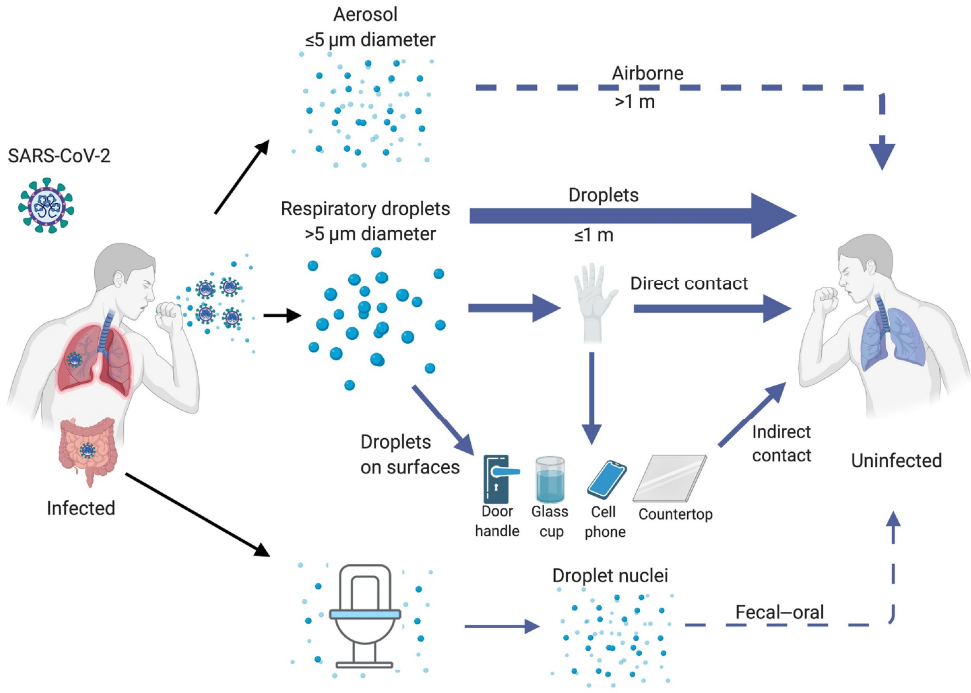


Figure 6 | SARS-CoV-2 transmission. Schematic representation of the main routes of SARS-CoV-2 transmission. *Reproduced with permission from Harrison et al.*[222]

Pathogenesis

The nasal cavity is the gateway to the respiratory system and is an anatomical site that is naturally highly exposed to the environment. In the upper respiratory tract, nasal ciliated cells express the highest levels of ACE2[223–225] and seem to be the first targets for SARS-CoV-2 replication in the early stages of infection[226,227]. However, ACE2 expression decreases in the lower respiratory tract in parallel with SARS-CoV-2 replication[228,229].

Following SARS-CoV-2 infection, there is an incubation period (time from infection to the onset of clinical signs and symptoms) characterized by low viral titers lasting approximately 4-7 days[90,203,230–233]. After that, the virus undergoes exponential growth and nasopharyngeal

viral load peak is detected by day ≈7 from symptom onset[234–236]. It is estimated that during this peak, a person carries between 1 and 100 billion of SARS-CoV-2 virions, which represents a total mass ranging from 1 μg to 100 μg[237].

SARS-CoV-2 infection is heterogeneous in symptoms, disease severity, and outcomes after resolution. Thus, the infection can be asymptomatic, or develop mild, moderate, severe, or critical disease, that eventually can provoke the death. After resolution, patients may be fully recovered, but may also show secondary damage or post-COVID conditions (e.g. long-COVID, *see immunopathology section*)[238].

Systematic reviews and meta-analyses indicate that between 20% to 40% of SARS-CoV-2 infections are asymptomatic[239–243]. Among patients presenting clinical symptoms, the majority typically experience mild to moderate disease (around 80%), while approximately 15% develop severe COVID-19 requiring oxygen support[244,245]. A smaller proportion, about 5%, experience critical illness with complications such as respiratory failure, acute respiratory distress syndrome (ARDS), sepsis and septic shock, thromboembolism, and/or multi-organ failure, including acute kidney injury and cardiac injury[244,245]. Importantly, these numbers varies among studies, probably due to differences in the methodology, regional variance in demographics, the involved SARS-CoV-2 variants, vaccination status, or other factors[246–250].

COVID-19 presents with a wide range of clinical manifestations, ranging from asymptomatic to critical illness, although the frequency and spectrum of symptomatology also varies across variants. In general, signs and symptoms may include cough, sore throat, fever, dyspnea, fatigue, myalgias, headache, anosmia, dysgeusia, gastrointestinal symptoms, chest pain, and may progress to ARDS[90,251–255]. The mechanisms underlying the various manifestations caused by SARS-CoV-2 infection are still not fully understood.

As mentioned above, the cell tropism of SARS-CoV-2 is complex, and it is closely related to viral pathogenicity. SARS-CoV-2 has the ability to infect many tissues, which can result in multi-organ dysfunction. The main target of SARS-CoV-2 are cells from the respiratory tract, but it has also been found in other organs that show a high expression of ACE2 and/or proteases, such as heart, kidneys, liver, small intestine, colon, pancreas and brain[153,256–265]. Additionally, multiple publications suggest that the renin-angiotensin system is involved in the pathogenesis of SARS-CoV-2[266–270]. Likewise, SARS-CoV-2 seems to disrupt immune functions[271–273].

The natural course of SARS-CoV-2 infection comprises essentially 3 stages (**Figure 7**): 1) the early infection, 2) the pulmonary phase and, 3) the hyperinflammatory phase. Phase 1 is characterized by viral replication with mild to moderate symptoms (or asymptomatic) and a strong antiviral innate immune response. In the pulmonary phase, viral replication continues and more severe symptoms such as pneumonia and dyspnea may occur. In the absence of effective immune control of viral replication, infection will evolve to the hyperinflammatory phase, in which there is an elevated proinflammatory host immune response, characterized by a cytokine storm, potentially leading to sepsis, respiratory failure, ARDS, and ultimately death[274].

Comorbidities are a growing concern for their impact on the progression and outcome of COVID-19. It is estimated that 1.7 billion people, comprising nearly a quarter of the world's population, have at least one comorbidity that is associated with an increased risk of developing severe COVID-19[275]. The main risk factors (in no particular order of relevance) for COVID-19 are: age, sex, genetic factors, vaccination status, hypertension, hyperlipidemia, diabetes, chronic lung disease and smoking[276,277]. In addition to clusters of conditions that indicate potential biological mechanisms, the likelihood of infection and subsequent outcomes is also influenced by interactions among demographic and socioeconomic factors, ethnicity, and the environment[276]. The presence of multimorbidities further increases the risk of mortality and the impaired quality of life of the survivors[276,277]. Finally, severe disease and worse prognosis are associated with lymphopenia, thrombocytopenia, and elevated levels of D-dimer, lactate dehydrogenase, C-reactive protein, ferritin, liver enzymes, interleukin 6, tumor necrosis factor alpha, troponin, creatine phosphokinase, and prolonged prothrombin time[278].

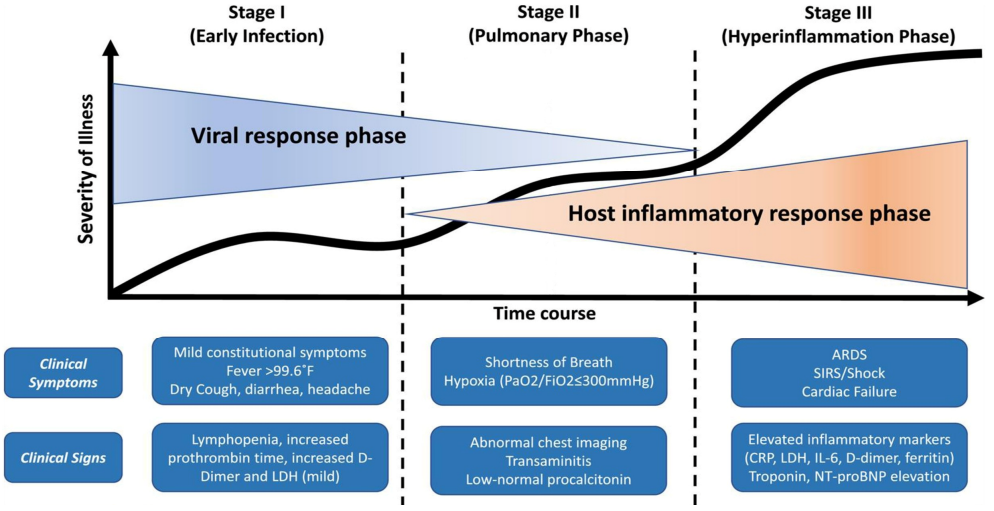


Figure 7 | COVID-19 pathogenic phases. Schematic representation of the natural history of SARS-CoV-2 infection (depending on disease severity), indicating viral and inflammatory response and clinical signs and symptoms over time. *Adapted with permission from Hariram Nile et al.*[279]

The host immune response to SARS-CoV-2 infection and vaccination

The immune system is essential for life, and encompasses a complex integrated network of cells and molecules that collectively communicate with each other to fight invading pathogens[280,281].

Most respiratory viruses generally produce acute infections located in the upper respiratory tract and are successfully controlled and eliminated by the immune system[282,283]. The mechanisms by which the immune system recognizes, fights and eliminates viruses in our body have not been fully elucidated, and this lack of knowledge is a hindrance to the development of effective vaccines or treatments against many respiratory viruses. Both mucosal and systemic immunity against SARS-CoV-2 are relevant; however, in the following subsections we will focus on developing the knowledge available on the immune response at the systemic level (which is the most widely studied), without neglecting an area of enormous relevance in SARS-CoV-2 infection, such as mucosal immunity[284–287].

In general terms, the immune system can cope with these threats by means of two major arms, innate and adaptive immunity. However, the relative contributions of innate and adaptive immunity in modulating the severity of SARS-CoV-2 infection are difficult to deconvolute and remain relatively poorly understood at present.

Innate immunity

Innate immunity is the first line of defense against pathogens and is composed of anatomical (e.g., skin and mucous membranes) and physical-chemical (e.g., temperature, hydrochloric acid, chemical mediators) barriers[280]. Importantly, it is also integrated by a variety of cells of different lineages and multiple functions, such as neutrophils, monocytes, macrophages, basophils, eosinophils, mast cells, dendritic cells (DCs), natural killer (NK) cells, and gamma-delta T cells[288,289]. The innate immune response is a rapid, potent, and non-antigen-specific defense mechanism that precede and play a major role in the activation of the adaptive immune response. The innate immune response limits the entry, translation, replication and assembly of viral particles, and helps to identify and eliminate infected cells[290].

As mentioned above, SARS-CoV-2 primarily infect nasal epithelial cells. Once the virus has established infection, infected cells and some immune cells, such as macrophages and DCs, detect SARS-CoV-2 molecules using pathogen recognition receptors (PRRs), such as the toll-like receptors (TLRs), RIG-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs)[291,292]. Epithelial, endothelial, and tissue-resident immune cells initiate a signaling cascade with the subsequent production of various cytokines and chemokines, which alert and signal the site of infection for immune cell recruitment. The main antiviral

cytokines are type I (IFN-α and IFN-β) and type III interferons (IFN- λ), TNF-α, IL-1 and IL-6[283,293].

Current evidence suggests that type I interferon-mediated immunity appears to be fundamental over the clinical course of the disease and the subsequent adaptive immune response[294–296]. Interestingly, *ACE2* is a human interferon-stimulated gene (ISG), suggesting that SARS-CoV-2 could exploit IFN-driven *ACE2* upregulation to enhance infection[297]. Furthermore, in patients with SARS-CoV-2, autoantibodies against type I IFNs have an increased risk of developing severe COVID-19[298–300]. Some studies found that patients with low IFN-α levels had a poorer prognosis and a higher viral load[294,301]. In contrast, elevated blood levels of IL-1β and IL-18, correlate with disease severity[302,303]. In addition to this, synergism of TNF-α and IFN-γ (type II interferon) contributes to the pathogenesis of COVID-19 by inducing inflammatory cell death (PANoptosis), perpetuating the cytokine storm[304], and may have a possible link to multisystem inflammatory syndrome in children (MIS-C) (*see immunopathology section*). In response to innate defense mechanisms, coronaviruses have evolved evasion strategies to limit host control and enhance viral replication and transmission[305–307].

During the initial stages of an acute respiratory infection, NK cells and neutrophils are the predominant cells in the lung[308–310]. NK cells directly recognize infected cells leading to increased cytolysis and production of IFN-γ (a cytokine involved in the regulation of immune and inflammatory responses)[311,312]. However, the precise role of neutrophils in respiratory virus clearance has not yet been well elucidated. Some studies suggest that they are not major players in the control of viral replication and may even contribute to the severity of the disease, while other reports attribute to them a protective role during acute viral infection[313–317].

A few hours after the establishment of infection, there is increased trafficking of DCs from the lung to the lymph nodes (mainly CCR7-dependent[318–320]), a process of vital importance for the generation of adaptive immunity[321,322]. However, in some cases of acute COVID-19, depletion of germinal centers in the spleen and lymph nodes has been evidenced[323], which could interfere with a proper humoral response.

Finally, it is noteworthy that some lymphoid structures located in the airways, such as nasopharynx-associated lymphoid tissue (NALT) and inducible bronchus-associated lymphoid tissue (BALT), may have an important role in the antiviral response by generating potential adaptive immune responses[324–327], as they exhibit lymph node-like organization, high endothelial venules, and expression of chemokines important for DC and naïve T-cell migration[283,328].

Innate immunity is also triggered by COVID-19 vaccines[329,330]. It has been observed that in mice, the BNT162b2 vaccine substantially stimulated the innate response following secondary immunization reflected in increased levels of serum IFN-γ[331]. NKs and $CD8^+$ T cells present in the draining lymph nodes were mainly responsible for this circulating IFN-γ[331]. In this study, an increase in the frequencies of monocytes, plasmacytoid, migratory and resident DCs in the draining lymph nodes was observed, although with different behavior. In conjunction, monocytes, macrophages and DC subsets remained highly activated until day 3 post-immunization[331]. A release of multiple cytokines (MCP1, MIP1b, IL-6 and CXCL10) was also found,

which returned to normal levels at day 3 after immunization[331]. Arunachalam et al. described some similar results in humans with the BNT162b2 vaccine. The second dose showed an increased frequency of $CD14^{+}CD16^{+}$ inflammatory monocytes, a higher concentration of plasma IFN-γ, and a transcriptional signature of innate antiviral immunity[332]. The third dose of BNT162b2 vaccine increased the levels of classical and intermediate inflammatory monocytes, different subsets of NK cells, and IFN-γ in humans[333].

A more complete overview of innate immunity against SARS-CoV-2 can be consulted in the review by Diamond and Kanneganti[290] and the innate mechanisms of mRNA vaccines in the relatively recent publication by Verbeke et al[329].

Adaptive immunity

Adaptive immunity is considered the second line of defense against viruses. Unlike innate immunity, it is antigen-specific, and is capable of generating long-lasting immunological memory, which is crucial to respond faster and more effectively in case of a new exposure to the pathogen. For practical purposes, it is generally divided into two complementary branches: cell-mediated immunity and humoral immunity.

Cell-mediated immunity is governed by $CD4^{+}$ and $CD8^{+}$ T lymphocytes, is more related to protection against severe disease (although antibodies also play a potential role) and has among its main objectives the killing of infected cells. This is because T cells cannot detect a virus until the cells are infected. Whereas humoral immunity is composed of B cells, plasma cells and antibodies, and is more related to protecting against infection (with a contribution of $CD4^{+}$ and $CD8^{+}$ T cells) (**Figure 8**). Antibodies exhibit higher efficacy against extracellular virus than infected cells (although they also trigger important F_c-dependent effector functions that help eliminate infected cells), and therefore, they tend to be most effective when present before the start or in the early stage of an infection[334,335].

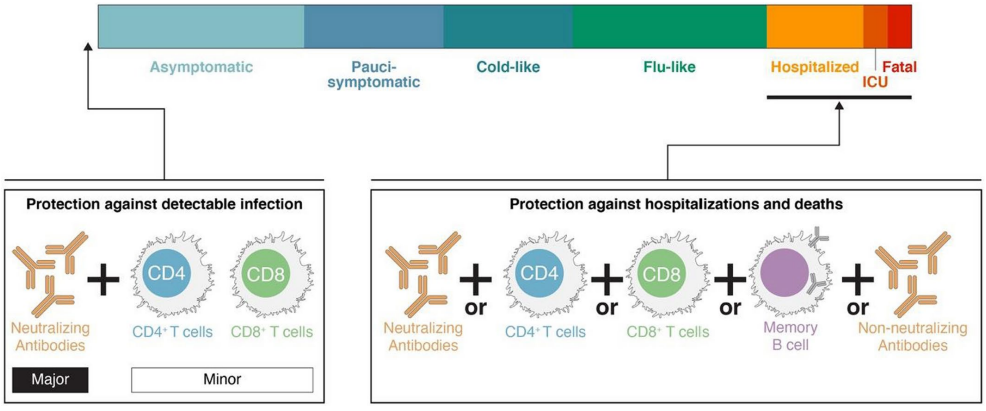


Figure 8 | Immune responses for protection against SARS-CoV-2. The importance of the protective role of different players of adaptive immunity in the course of SARS-CoV-2 infection. *Reproduced with permission from Goldblatt et al.*[335]

Cell-mediated immunity

$CD4^+$ and $CD8^+$ T lymphocytes continuously scan antigen-presenting cells (APCs) for the presence of cognate antigen/MHC complexes[336,337]. Thus, the response of naïve T cells depends strongly on how APCs process antigenic peptides and induce co-stimulatory ligands and cytokines[338,339]. Additionally, to generate effective immune responses to infections and to respond adequately to vaccination, the maintenance of a vast T-cell repertoire through genetic recombination is crucial[340,341].

Cellular immunity plays a central role in the control of SARS-CoV-2 and protection from disease severity[342,343]. The favorable contribution of T-cells on the course of the disease has been widely demonstrated[344–347]. During the infection, dendritic cells from the lung interact with naïve T-cells in the lymph nodes and initiate a sustained SARS-CoV-2 specific effector T-cells proliferation. $CD8^+$ T-cells are observed at approximately 7 days post-infection and have an immediate effect in decreasing viral load and correlate with mild disease[342,346]. Grifoni et al. identified SARS-CoV-2-specific $CD8^+$ and $CD4^+$ T-cells in ≈70% and ≈100% of COVID-19 convalescent patients, respectively. $CD4^+$ T-cell responses were predominantly T_H1, generally with undetectable T_H2 cells[348]. $CD4^+$ and $CD8^+$ T cell responses can be detected early in the upper respiratory tract post-SARS-CoV-2 infection[349,350]. Furthermore, the existence of T cell responses has been noted in the lungs following SARS-CoV-2 infection, despite the absence of virus-specific T cells in the periphery in some individuals[351,352].

Early SARS-CoV-2-specific $CD4^+$ T-cell responses have been found to be associated with reduced disease severity[353,354]. In addition, it has been observed that $CD8^+$ T-cells contribute to protection in the context of low antibody titers[355]. Indeed, a cellular response to SARS-CoV-2 has been described in many individuals who have had high exposure to SARS-CoV-2 (but have not been infected), in the absence of specific antibodies[356–358]. Moreover, even patients with X-linked agammaglobulinemia have been reported to recover and have mild disease, suggesting that cell-mediated immunity is vital in the absence of a proper humoral response[359–361]. Although the clinical benefit of early treatment with monoclonal antibodies (mAbs) is significant in preventing infection, the impact of mAbs in hospitalized patients has not been shown to be as beneficial and has a modest effect on viral load[362–364]. This suggests that although antibodies can contribute to viral clearance, T cell are the main actors controlling viral replication after infection[335]. This becomes more relevant because some severe or fatal COVID-19 cases showed a loss of T_{FH} cells in germinal centers expressing BCL6[323,365], a transcriptional repressor required during B-cell development.

T-cell responses recognize multiple SARS-CoV-2 epitopes[366], with a strong dominant $CD4^+$ and $CD8^+$ T cell responses to spike protein[348,367]. Followed by M, N, and other non-structural proteins (**Figure 9**)[368]. All these antigens are of great interest for the design of pan-coronavirus vaccines[369]. It is unclear why the spike protein is the primary target in SARS-CoV-2 infection, but its large size (containing many epitopes) and high level of expression are likely contributing factors[370].

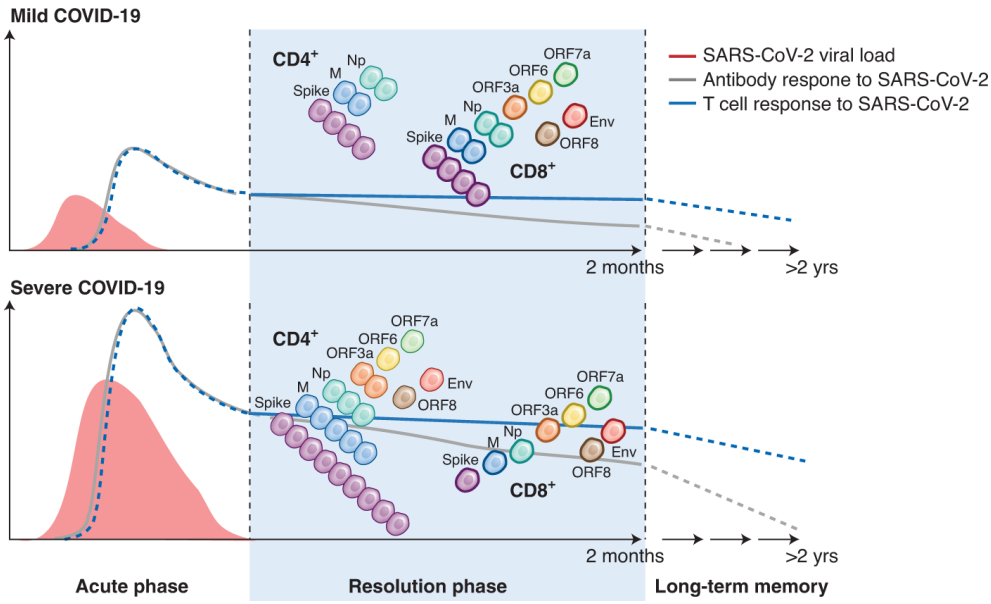


Figure 9 | T cell response against SARS-CoV-2. Figure based on the study by Peng et al.[346]. $CD4^+$ and $CD8^+$ T cell response (cell colors and numbers represent relative frequencies of indicated protein specificities) in the resolution phase of mild or severe COVID-19. The total T cell response (solid blue line) is stronger and broader in severe cases (assumed to have had higher viral burden, red curve), correlating with stronger antibody responses (solid gray line). However, there are, proportionally, more $CD8^+$ T cells in mild disease. *Reproduced with permission from Swadling and Maini*[371]

The Immune Epitope Database records more than 3,000 T-cell epitopes from SARS-CoV-2[372]; however, the breadth of response is estimated to be approximately 30-40 epitope-specific, by $CD4^+$ or $CD8^+$ T cells after natural infection, in most individuals[373]. In both natural infection and vaccination, a median of 10-11 epitopes specific to the S-glycoprotein are recognized[368]. One study suggests that mRNA vaccination may lead to a stronger T-cell response against the S-glycoprotein compared to natural infection[374]. Additionally highlights the potential benefits of booster vaccination for convalescent individuals by increasing the breadth of spike-specific T-cell response[374]. However, this may also be influenced by many other factors, although some of them, such as the contribution of age and gender have not yet been fully elucidated[370].

Vaccination also provides a potent (of greater magnitude in adenovirus-based vaccines compared to mRNA vaccines[375,376]) and durable cellular response[377–379], although inactivated virus vaccines apparently do not induce a proper $CD8^+$ T-cell generation[380–382]. Additionally, protection following a single dose of mRNA vaccine was associated with the appearance of T-cell responses[377,383,384]. Circulating T follicular helper (T_{FH}) cells have also been detected after mRNA vaccination[385–388], which strongly correlated with neutralizing antibody development[388]. However, the data indicate that cellular responses decrease more gradually than an-

tibodies after two doses of mRNA vaccination[389–391]. Importantly, immunocompromised individuals represent a high-risk group for SARS-CoV-2 infection, complications and death[392,393]. However, although alterations in the B-cell compartment may lead to a deficient humoral response, T-cell-mediated immune responses are generally conserved after vaccination and are associated with significant protection from SARS-CoV-2[394–396]. In general, heterologous immunization improves the cellular immune response in comparison with homologous vaccination, but this is highly dependent on the types of vaccines used in the different regimens[370].

Another aspect to consider is the cross-reactivity offered by cell-mediated immunity. Vaccination generates a good cellular cross-reactive response against SARS-CoV-2 variants, that is boosted by previous infection (hybrid immunity)[385,397,398]. Several studies have shown some T-cell cross-reactivity between seasonal coronaviruses and SARS-CoV-2 proteins[348,399–401]; in fact, one study demonstrated that SARS-CoV-specific memory T cells are reactive even 17 years after infection, and show robust cross-reactivity to the SARS-CoV-2 N protein[399]. Bearing in mind that more than 90% of the population has serum antibodies to endemic coronaviruses[402,403], suggesting that most people have experienced a coronavirus infection, this is a relevant point, as preexisting immunity could induce a faster and stronger immune response to SARS-CoV-2, limiting the severity of the disease[404]. Importantly, $CD4^+$ T cells also differentiate into T_{FH}, which are crucial for the development of antibody-producing cells, and correlate positively with the humoral response[405,406]. A higher proportion of SARS-CoV-2 specific circulating T_{FH} and T_H1 cells has been linked to mild COVID-19, and this has been found to be associated with sustained anti-spike antibody responses following viral clearance[366]. Additionally, $CD4^+$ T cell responses to SARS-CoV-2 have been shown to predict the magnitude, breadth, and duration of subsequent neutralizing antibody responses[407].

In terms of viral variants, it has been shown that mutations in the S-glycoprotein could result in decreased T-cell recognition[408]. However, previously infected or vaccinated individuals have been found to elicit a robust cross-reactive cellular response against SARS-CoV-2 variants, including the highly transmissible and immuno-resistant Omicron variant[368,409–411]. This is probably due to the high conservation of T cells epitopes, where approximately only 3-7% of these epitopes are affected by the mutations[410]. At the population level, a conservation of T-cell reactivity of ≈80% was detected and, in most cases, no reduction was observed. However, significant decreases were observed in certain specific combinations of individual subjects and variants[410,412–416]. These findings collectively suggest that T-cell escape is not the driving force behind variant evolution at the population level[417].

Finally, it is worth mentioning that after the resolution of viral infections, effector T cells enter a process of programmed cell death, termed apoptosis, in which 90-95% of virus-specific T cells are destroyed, leaving a stable effector memory cell pool needed to respond to reinfections[418–420]. Although this subset of cells gradually decreases over time after infection, a very low level is maintained for years by continual recruitment from the circulation[421–423], which could explain the long-term persistence of memory T cells responding to coronaviruses[424,425]. Based on several studies, it is estimated that the duration of memory T cells followed by infection could persist for many years and that this duration could be independent of disease

severity, although a percentage of individuals (≈30%) may not have a robust memory CD8 T-cell response[426–432].

Relying on the potential beneficial effect of preexisting cellular immunity against respiratory viruses, as seen against influenza[433–435], and given the precedent that memory T cells can be obtained by natural infection and/or vaccination and persist over time, in addition to showing good cross-reactivity against the different SARS-CoV-2 variants, we are facing a promising scenario to deal with reinfections and the emergence of new viral lineages or variants.

Humoral immunity

The humoral response plays a relevant role in the protection and resolution of many infections, such as influenza and highly pathogenic viruses[436,437]. Antibody-mediated protection is primarily attributed to the capacity of antibodies to bind and neutralize pathogen infectivity in association with diverse F_c-effector functions.

Plasma and B cells

Humoral immunity refers to soluble antibodies, however, there are specialized lymphoid structures and a palette of vital cells that work in orchestration for the generation and maturation of these molecules. Humoral memory involves long-lived plasma cells (secrete antibodies) and memory B cells (remain in a quiescent state and can respond to reinfection and viral variants).

In general, during a primary immune response (**Figure 10**), DCs present the antigen to naïve $CD4^+$ T cells, and these in turn to naïve B cells. Already in the B-cell follicles, with the collaboration of T_{FH} cells, the activated B cells proliferate and differentiate into short-lived plasma cells (low-affinity antibody-producing and their lifespans are generally limited to the course of infection), become memory B cells and generate germinal center-independent immunological memory, or develop germinal centers to undergo maturation processes[438,439]. Importantly, short-lived cells can also be produced as a result of an extrafollicular response, which does not necessarily imply immunoglobulin evolution through somatic hypermutation or the selection of high-affinity B cells[440–442]. In fact, there has been observed a strong correlation between extrafollicular activation and a significant expansion of antibody-secreting cells, leading to early production of high concentrations of anti-SARS-CoV-2 neutralizing antibodies[443].

Once in the germinal centers, in the dark zone, B cells undergo proliferation and somatic hypermutation, then mobilize to the light zone, where activated B cells recognize antigens presented by follicular dendritic cells (FDCs) and undergo affinity selection with the help of T_{FH} cells[438,439]. Interestingly, while somatic hypermutation takes place in germinal centers, it has been observed that class-switching recombination occurs prior to the formation of these

specialized structures[444]. Germinal center B cells either become long-lived memory B cells (quiescent cells residing in niches within secondary lymphoid organs and other tissues), differentiate into long-lived plasma cells (reside in the bone marrow and persist for years), or re-enter the dark zone and begin a feedback process (between light and dark zone) of clonal expansion, BCR diversification and affinity maturation[438,439].

During a secondary immune response, pre-existing circulating antibodies function as the first line of defense (constitutive humoral memory). If this is not sufficient, memory B cells are activated and differentiate into long-lived plasma cells or rapidly enter germinal centers to undergo rounds of expansion, somatic hypermutation and selection to produce antibodies (reactive humoral memory)[283,439]. For further details, see the reviews by Akkaya et al.[438] and Kurosaki et al.[439].

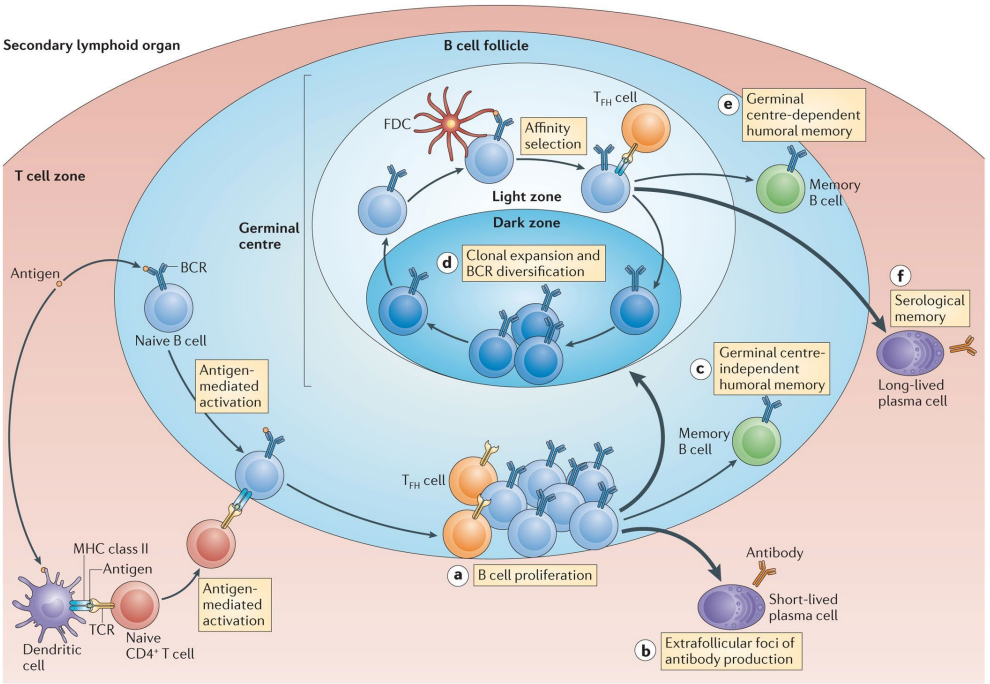


Figure 10 | T cell-dependent B cell responses. Activation, proliferation, differentiation, expansion, and maturation of B cells. *Reproduced with permission from Kurosaki et al.*[439]

As mentioned above, clonally expanded B cells differentiate into plasma cells, which are authentic antibody factories. Antibody-producing cells generate antibody molecules at a rate of approximately 2,000 and up to 5,000-10,000 antibody molecules per cell per second[440,445]. Recently, using a murine model, it has been described that plasma cells accumulate in the bone marrow at a constant rate of approximately one cell per hour from early on and for several weeks after a single immunization[446]. In addition, it is important to note that a singular long-lived plasma cell is specific for a single epitope, so the singular response may be poor against viral variants. However, it must be kept in mind that the immune responses are polyclonal in nature. If upon a second exposure, the pathogen is not completely neutralized,

memory B cells will engage by responding positively to variants that escape to antibody-mediated neutralization governed by long-lived plasma cells[447,448]. In short, memory B cells have two main functions. The first is to act as a cellular source for the anamnestic antibody response, which means they can potentially reactivate and produce antibodies within a relatively short period of days[449]. The second function of memory B cells is to serve as a repository by the immune system for epitopes conserved in future viral variants[426,447].

Historically, several non-exclusive models have been proposed to explain the presence of long-term circulating antibodies; however, the generation of long-lived plasma cells seems to best explain sustained antibody responses[440,450–454]. As shown in **Figure 11**, after antigenic exposure and stimulation of naïve B cells, the number of antigen-specific B lymphocytes and antibodies increases exponentially to a peak (expansion phase), during this process, proliferation and differentiation of B lymphocytes into short-lived plasma cells occurs. When short-lived plasma cells die, the antibodies they produce decay; this phenomenon is known as contraction of the immune response. B cells mature in germinal centers, increasing antigen recognition to give rise to long-lived plasma cells and memory B cells. Long-lived plasma cells are responsible for continuing to produce antibodies and, although for some pathogens antibody levels decrease over time, the quality could increase by an evolutionary process of the immune response (memory and evolution phase). Upon antigenic re-exposure, memory B cells are activated and differentiate into antibody-producing plasma cells. Compared to the primary immune response, recall response is much faster, stronger and of higher affinity[439,455,456].

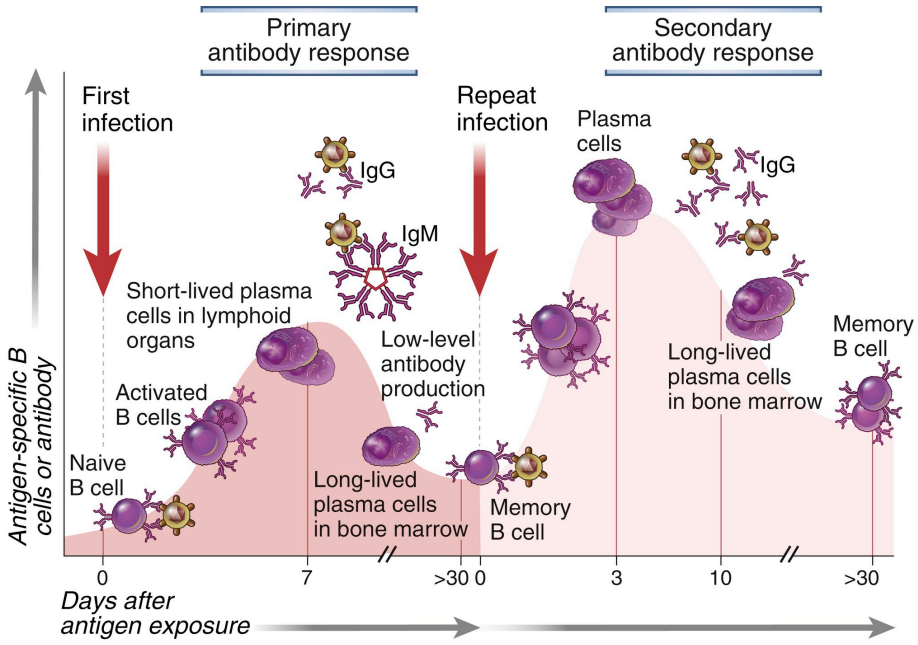


Figure 11 | B-cell and antibody immune responses. Kinetics of primary and secondary humoral responses to primoinfection and reinfection, respectively. *Reproduced with permission from Abbas et al. ©Elsevier (2022)*[455]

Memory B cells can persist for many years, for example, the smallpox vaccine induces virus-specific memory B cells, which, although initially decreased after immunization, reached a plateau and remained stable for more than 50 years at a frequency of ≈0.1% of total circulating IgG^+ B cells[457]. Along the same lines, circulating memory B cells against 1918 H1N1 influenza virus were detectable about 90 years after the pandemic[458]. Importantly, it has been demonstrated that SARS-CoV-2 infection generates circulating memory B cells, tissue-resident B cells, and long-lived bone marrow plasma cells[351,459–463]. SARS-CoV-2 specific memory B cells are detected within two weeks post-symptom onset (PSO) and their frequency increases continuously over the course of 3 to 6 months after infection[427,428], remaining detectable for more than 15 months[464,465]. However, most of these memory B cells are IgG^+, with low frequency of IgM^+ and IgA^+ spike-specific B cells, but with a longer detection time of the later ones[427,428]. Importantly, SARS-CoV-2 infection induces spike-specific germinal center B cells and T_{FH} cells in human tonsils, whose responses were detectable in a percentage of individuals up to seven months following infection[466].

It has also been observed that mRNA-based COVID-19 vaccines induce memory B cells and persistent germinal center responses in humans[385,387,467–473]. A fraction of RBD-binding IgG^+ memory B cells from individuals immunized with 2 doses of mRNA vaccines also bind to the RBDs of SARS-CoV-2 variants[385,417], although affinity maturation after two doses of mRNA vaccine is qualitatively poor compared to SARS-CoV-2 infection[426,470]. This difference may be due, at least in part, to the time elapsed between the two doses of vaccine, since a longer time than the usual schedule results in a greater magnitude and quality of the neutralizing response[474,475]. Importantly, germinal centers appear to be crucial for generating an adequate immune response to COVID-19 vaccines. After mRNA vaccination, germinal centers in kidney transplant recipients, in comparison to healthy individuals, exhibited severely attenuated SARS-CoV-2-specific germinal center B cell responses, along with severely impaired T_{FH} cell responses, SARS-CoV-2 RBD-specific memory B cells and neutralizing antibodies[388]. Other types of vaccines, such as Ad26.COV2.S (non-replicating viral vector), NVX-CoV2373 (protein subunit) and AZD1222 (non-replicating viral vector), also generate specific circulating memory B cells[391,476]. Additionally, one study detected, more than six months after immunization with the BNT162b2 vaccine, spike-specific IgG-secreting long-lived bone marrow plasma cells, which exhibited high frequencies of somatic hypermutation relative to other B-cell compartments[473]. Although strong evidence on the elicitation of long-lived plasma cells by COVID-19 vaccination is still scarce and has not been studied in all types of vaccines, the persistence of B cells in germinal centers and plasmablasts in lymph nodes is a positive indicator for the induction of long-lived plasma cells[477]. However, the fact that antibodies drop rapidly after vaccination suggests that the long-lived plasma cells apparently represent a small fraction and may not be well produced by current vaccination[335,426].

Finally, vaccination boosts RBD-specific memory B cells in previously SARS-CoV-2 infected individuals. These cells exhibit greater somatic hypermutation and affinity maturation compared to vaccination alone, which is also reflected in antibodies with greater potency and breadth of neutralization[385,464,471].

Antibodies

Probably, the first reference to antibodies dates back to 1890 by Emil von Behring and Shibasabura Kitasato, in their experiments on serum transfer from animals that had survived the disease to infected animals[478]. During the early years of the 20th century, Paul Ehrlich proposed his famous side-chain theory of immunity, where side-chain receptors on cells bind to a given pathogen, which explained the interaction of antibodies and antigens, and how antibodies are produced[479]. Since then, the importance of antibodies as anti-pathogen and immunomodulatory agents, and their versatility as therapeutics has been widely demonstrated[480].

Immunoglobulins (or antibodies) are glycoproteins composed of heavy chains and light chains, which together form a Y-shaped structure. The Y-shaped arms, known as the F_{ab} (antigen-binding fragment) regions, are responsible for recognizing and binding to specific antigens. The stem of the Y, known as the F_c (crystallizable fragment) region, mediates various immune functions by interacting with immune cells and molecules[481]. Immunoglobulins exhibit different isotypes, including IgA, IgD, IgE, IgM, and IgG, each with distinct structural and functional characteristics. Among the isotypes, IgG and IgA are divided in subclasses with slight structural and functional differences[482–484]. For IgG, subclasses include IgG1, IgG2, IgG3, and IgG4, and for IgA, subclasses include IgA1 and IgA2. Immunoglobulins can also exhibit allotypic variation, representing genetic polymorphisms within a species[482,485], and idiotypes, which are unique antigen-binding sites determined by specific amino acid sequences[486].

In the realm of infectious viral diseases, IgG, IgA and IgM are of utmost importance[487–489]. IgG is the most extensively studied immunoglobulin class due to its important role in immunotherapy and viral immunity. It is the most abundant immunoglobulin in human serum, and although there are several subclasses, IgG1 represents the majority of serum antibodies and is primarily responsible for protection against infection[482,487]; although IgG3 has some interesting and unique features that may play an important role in infectious diseases. For instance, certain IgG3s may exhibit enhanced cellular cytotoxic activity, opsonophagocytosis, complement activation, and neutralization compared with other IgG subclasses[490,491].

Contrary to what might be thought, IgA is the most common antibody class produced in the human body[492]. It is an immunoglobulin isotype mostly concentrated in the mucosa, but it is also found in serum and other external secretions, including tears, mucus, breast milk and saliva[492,493]. Although IgA has typically been associated with passive immunity, it has recently been discovered that it can induce active immunity by contributing to the initiation of inflammation, both in mucosal and non-mucosal sites, by modulating the production of several cytokines[494,495]. Secretory IgA found in mucus, such as nasal secretions, is essential to achieve sterilizing immunity against SARS-CoV-2 by preventing its adhesion to epithelial cells[496].

B cells are primarily responsible for the production and release of virus-specific IgM antibodies; then, $CD4^+$ T cells promote class switching from early IgM to higher-affinity IgG or IgA isotypes[497,498]. SARS-CoV-2 infected individuals show a high percentage of seroconversion (≈90% 10 days) within 5-15 days PSO[499]. Seroconversion to the SARS-CoV-2 S-glycoprotein

ranges from 91-99%[500–502]. The Canadian COVID-19 Antibody and Health Survey found that, between April and August 2022, 98% of Canadians adults had antibodies to SARS-CoV-2 either from vaccination, previous infection, or both[503]. Likewise in February 2023, in the United Kingdom, it was estimated that more than 90% of the population (≥16 years old) had antibodies against SARS-CoV-2, reaching values close to or above 98% in people over 60 years of age[504]. Similarly, in Switzerland, 94% of the population was estimated to have anti-SARS-CoV-2 antibodies[505]. Antibodies directed against the N protein are also commonly detected. In the United States, as of February 2022, the prevalence of anti-N antibodies was 57.7%, and approximately 75% of children and adolescents had serologic evidence of previous infection with SARS-CoV-2[506]. In June 2022, a 72.4% prevalence of infection-induced antibodies was estimated in Switzerland[505]. In Navarre (Spain), in May 2022, the seroprevalence of anti-N and anti-S antibodies was 58.9% and 92.7%, respectively[507]. Importantly, anti-SARS-CoV-2 antibodies can also be detected in saliva and breast milk[508–510]. Furthermore, SARS-CoV-2 has the capability to infect some animals, a study conducted in Spain reported a seroprevalence of 3.6% in companion animals (dogs and cats) and showed a correlation between the incidence of COVID-19 in owner and positivity to antibody detection in pets[511].

The antibody response peaks between the third and fifth week after infection and is characterized by the presence of IgA, IgM and IgG isotypes in plasma and saliva[512–515]. Circulating IgA and IgG antibody titers correlate with their corresponding immunoglobulin class in saliva, with a steeper decline in antibody titer in saliva compared to blood antibodies[508,516]. Interestingly, there is a slightly weaker correlation between serum and saliva IgA, suggesting a potential compartmentalization of the mucosal immune response[508,517]. In a study conducted in children attending summer schools in Barcelona (Spain), asymptomatic individuals had higher levels of IgA, IgM and IgG in saliva compared to symptomatic cases, suggesting a strong neutralizing response at the mucosal level that could prevent symptomatic infection[518]. Supporting this, mucosal neutralization has been associated with nasal SARS-CoV-2-specific IgA[519]. Airway IgG and IgA antibodies decline significantly within 3 months[520], although salivary IgG antibodies may remain detectable for up to 9 months post-infection[521].

Early publications pointed to a rapid and sustained decrease in systemic antibody levels[522–524], probably because they analyzed the acute and early convalescent phase of COVID-19. However, subsequent studies showed sustained seropositivity for more than 1 year[465,525–530]. In fact, a couple of studies found that anti-S IgG antibodies, and to a lesser extent anti-S IgAs, can be detected for more than 20 months, showing a slower decay than anti-N IgGs[531,532]. In contrast, IgM antibodies had a considerably shorter duration[528,531]. At month 20, the seropositivity for anti-S IgM was found to be less than 10%, whereas IgG and IgA showed seropositivity rates of over 95%[531].

The generation of antibodies induced by vaccination is broad and heterogeneous, for example: the measles vaccine provides life-long protection; indeed, the half-life of measles antibodies has been estimated to be more than 3,000 years[456]. Other vaccines, such as tetanus and diphtheria vaccines, induce antibodies that last approximately 1 to 2 decades[456]. The immune response induced by influenza vaccine is short-lived; in contrast, the response to natural infection appears to be long-lasting[436]. Due to the relatively recent emergence of SARS-CoV-2 and

the widespread coverage of vaccines (in developed and developing countries), the accurate duration of SARS-CoV-2 antibodies produced by natural infection is still unknown, but based on data and different analyses, a half-life of more than two years is estimated[533–538]. On the contrary, numerous studies have determined a relatively rapid decay of antibodies induced by the different COVID-19 vaccines. Antibodies elicited by two doses of mRNA vaccines wane significantly, by a factor of 8 to 10 over 6-8 months[389,390,539,540]. On average, two doses of the mRNA-1273 vaccine elicits higher levels of anti-SARS-CoV-2 antibodies at the peak of the humoral response compared with the BNT162b2 vaccine[541], probably due to the higher amount of mRNA in the former one[542]. AZD1222 (two doses) and Ad26.COV2.S (one dose) vaccines generate a lower antibody titer and faster decay than mRNA vaccines[543–548]. The NVX-CoV2373 protein subunit-based vaccine induces lower levels of anti-spike IgG antibodies (and a poor $CD8^+$ T cell response) than the mRNA vaccines, but a comparable decay rate to the mRNA-1273 and BNT162b2 vaccines[391,549]. The inactivated CoronaVac vaccine induced virtually no anti-N antibody response, and a lower level of anti-spike antibodies that decayed more rapidly over time compared to the BNT162b2 vaccine[550,551]. The PHH-1V vaccine showed significantly higher and more slowly decaying anti-RBD antibody titers than those induced by the BNT162b2 vaccine[552].

In general, the third vaccine dose increases the magnitude of the humoral response and generates longer lasting antibodies than two doses[553–557]. Heterologous vaccination schedules resulted in more robust immune responses than the homologous booster dose[558–561]. The fourth dose of mRNA vaccine boosts immunity and achieves similar, or potentially higher, peak responses than those obtained with the third dose[562–564]. However, the fourth dose is mostly recommended for the elderly, immunocompromised and other individuals with a high risk of developing severe COVID-19 or who are highly exposed, such as healthcare workers[565–567]. Although booster doses elicit a strong systemic response, they do not appear to generate a highly effective mucosal response[532–535].

Hybrid humoral immunity is more complex, and is apparently influenced by several factors, such as the time and course of infection, the number and nature of exposures, the infecting variant, the duration of disease, the type and number of vaccine doses, breakthrough infections, etc.[572–575]. However, studies indicate that anti-S antibody titers are higher than those generated by infection and far superior to the ones elicited by vaccination alone, in both immunocompetent and immunocompromised populations[576–582].

Usually, antibodies elicited by infection and/or vaccination are characterized by neutralizing properties, linked to F_{ab}, and effector functions mediated by F_c[583–587]. However, some antibodies can exhibit noncanonical functions that may contribute to immune defense and regulation; for instance, direct pathogen inactivation, catalytic activity, control of bacterial metabolite penetration, acting as agonist molecules, counterbalance of immune pathology, etc.[588]. Intriguingly, at the opposite pole, some antibodies may play a pathogenic role, e.g. antibody-dependent enhancement (ADE). In this section, we will discuss anti-SARS-CoV-2 neutralizing (the main focus of this thesis) and non-neutralizing antibodies (specifically F_c-mediated effector functions). Next, in the immunopathology section, we will take a brief look at the potential pathogenic contribution of antibodies on COVID-19.

Neutralizing antibodies

Neutralizing antibodies (NAbs) have been shown to be one of the best mechanistic correlates of protection against multiple viral infections[589–591], and their elicitation has become a prime goal for many vaccines[592–597]. Passive transfer of NAbs protects animal models from SARS-CoV-2 challenge[598,599]. In addition, they are highly predictive of immune protection against SARS-CoV-2 infection in humans[335,600–605]. Besides the preventive effect of NAbs, they have also proven to be excellent therapeutic agents for COVID-19[585,606–608], although its usefulness has now been hampered by the emergence of neutralization-resistant SARS-CoV-2 variants.

NAbs bind to specific epitopes to block the infection process. Therefore, we could say that neutralization is a functional definition. Importantly, not all antibodies are neutralizing; in fact, only a subset of all antibodies produced have neutralizing capacity[609–611].

In this thesis, the neutralization of viruses is defined as the abrogation of virus infectivity *in vitro* by the binding of antibodies to SARS-CoV-2 virions[612]. Although the blockade of binding of the virus to the target receptor is one of the most common mechanisms of neutralization, NAbs can also block virus entry into cells by inhibiting spike conformational changes, binding to other spike key sites during membrane fusion or causing aggregation of viral particles[487,613–616]. Therefore, the S-glycoprotein of SARS-CoV-2 is the main target of NAbs. It is important to understand that neutralization is a multifactorial phenomenon that depend on several factors, such as the nature of the virus, the properties of the epitope targeted by NAbs and the binding affinity, the antibody isotype and subclass, and the virus:immunoglobulin ratio[617].

Measurement of neutralizing activity is not common in clinical laboratories, but is performed in research institutions due to equipment, infrastructure, time and costs. There are different methods to measure neutralizing activity: plaque reduction neutralization test (PRNT), focus-reduction neutralization test (FRNT), virus neutralization assay (VNA), pseudovirus-based neutralization assay (PBNA), and most recently, surrogate virus neutralization test (sVNT). Each has its advantages and disadvantages, and their sensitivities are different. In the studies described in this thesis, we chose to use PBNA because it is a highly reproducible technique with low operator dependence, it is highly sensitive, specific, reliable and versatile, and it is safe for the analyst[618,619].

In sync with seroconversion, the presence of NAbs is induced early (6-15 days post symptom onset) and mainly targets the RBD, which is the immunodominant region and harbors about 90% of the neutralizing activity of sera from individuals previously infected with SARS-CoV-2[620–623]. However, NAbs targeting other regions of the S-glycoprotein (**Figure 12**) have also been identified (e.g., NTD in S1, and stem helix [SH] and FP in S2)[584,624,625]. Interestingly, the combination of these NAbs can offer valuable therapeutic strategies[626]. Importantly, anti-SARS-CoV-2 mAbs isolated from memory B cells have shown increased somatic hypermutation, binding affinity and neutralization potency over time, evidencing prolonged antibody affinity maturation[464,627].

NAbs are also induced after vaccination. Levin et al. observed that the BNT162b2 vaccine generates NAbs that peak between day 4-30 after the second immunization; however, these

antibodies evidenced a steep decline during the first three months with a relatively slow decline thereafter[539]. Importantly, booster doses restore the magnitude and quality of the neutralizing response, even improving it[628–630]. However, the fourth dose of the new bivalent mRNA vaccines do not seem to substantially improve NAbs levels (neither in the short nor mid-term) against the new Omicron subvariants (although there is a slight upward trend), compared to a fourth dose of monovalent vaccine based on the spike of the ancestral virus (there also appears to be no difference in cell-mediated immunity between the two vaccines)[631–634]. The results suggested that the marginal difference observed is unlikely to be of clinical significance[635]; however a large cohort study demonstrated a higher effectiveness of bivalent booster compared to monovalent booster against hospitalizations and deaths from the new Omicron subvariants[636]. This effectiveness decreases by almost 30 percentage points at month five[637]. Regardless, the findings on the neutralizing activity bring to the forefront the potential role of immunological imprinting by previous antigenic exposure (*see immunopathology section*). In comparison, the BA.5 breakthrough infection exhibited significantly higher NAbs titers against the Omicron subvariants, specifically after 3 months. NAbs levels declined approximately 2-fold over a 2-month period[633,638]. By month three, NAb titers to XBB.1 and XBB.1.5 declined to baseline levels prior to boosting[638].

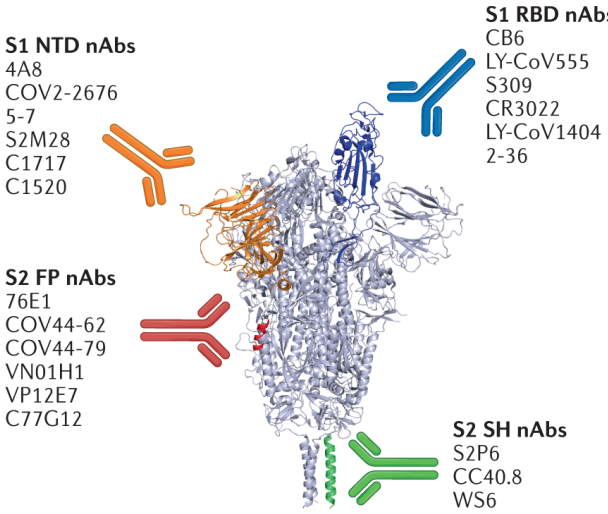


Figure 12 | Anti-SARS-CoV-2 neutralizing antibodies. Neutralizing antibodies targeting different regions in the spike of SARS-CoV-2. As an example, some NAbs were listed. *Adapted with permission from Chen et al.*[584]

The incidence of reinfections has increased in recent times[639–643]. In UK, the majority of reinfections occurred in the period when the Omicron subvariants were dominant, with BA.4 and BA.5 accounting for almost 50% of all secondary infections identified[504]. A meta-analysis concluded that protection from past infection was substantially lower for the BA.1 subvariant compared to the previous circulating variants[644]. Importantly, although reinfection with SARS-CoV-2 occurs, even in the presence of neutralizing antibodies, prior infection is associated with an 84% lower risk of reinfection at 7 months[645]. Additionally, a lower risk of

reinfection was observed in infected and previously vaccinated individuals compared to infected but unvaccinated individuals[646]. In this context, NAbs may play a critical role in reducing reinfection and potential transmission[601,602,647].

Further details on systemic anti-SARS-CoV-2 NAbs, including their duration, kinetics, factors associated with their generation, and cross-reactivity against several SARS-CoV-2 variants in different antigenic exposure scenarios (infection, vaccination, pre- and post-vaccination infection) are described, discussed and highlighted in chapters 3 (Results), 4 (Discussion) and 5 (Concluding Remarks) of this thesis.

Within the S-glycoprotein, the RBD exhibits remarkable motility. Various structures of viral particles and spike protein ectodomains have shown that there are three distinct conformations. One conformation has all RBDs in a "down" (horizontal) state (3-RBD-down), while the other two have either one (1-RBD-up) or two (2-RBD-up) RBDs in an "up" (vertical) position[135,585]. RBD-directed NAbs are generally classified according to their structure and recognition mode of the RBD in four main classes (**Figure 13**)[648]. Class 1: NAbs mostly encoded by VH3-53 and VH3-66 germ lines with short CDRH3 loops that block ACE2 and bind to “up” RBDs. Class 2: NAbs recognize both “up” and “down” RBDs. Class 3: NAbs bind outside the ACE2-binding region, and they can also bind to both “up” and “down” RBDs. Class 4: antibodies that do not block ACE2 and bind only to “up” RBDs[648]. Classes 1 and 2 anti-RBD NAbs target key RBD residues that are mutated in SARS-CoV-2 variants. While NAbs of classes 3 and 4 bind to more conserved epitopes, making them promising candidates for neutralizing variants of SARS-CoV-2 and other SARS-like coronaviruses[584,649].

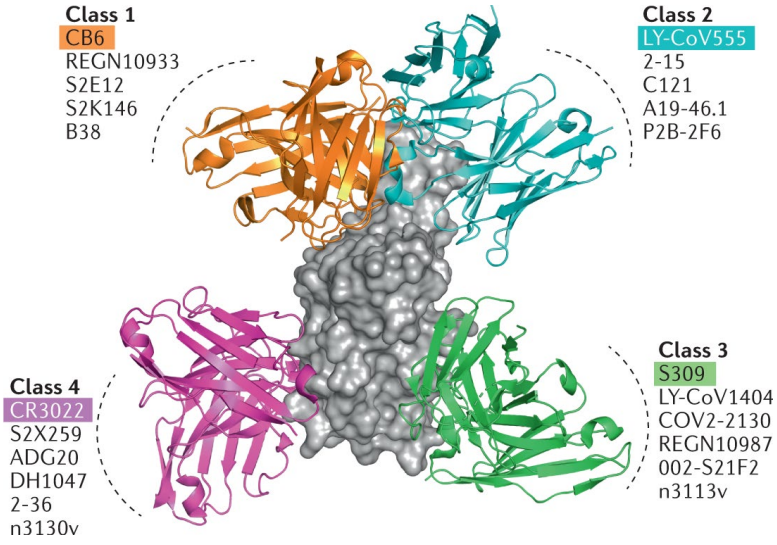


Figure 13 | Classes of anti-RBD neutralizing antibodies. Representation of the four classes of RBD-targeted neutralizing antibodies within the S-glycoprotein of SARS-CoV-2. *Adapted with permission from Chen et al.*[584]

Importantly, SARS-CoV-2-specific $CD4^+$ T cells are associated with long-term persistence of NAbs, highlighting the relevance of coordinating cellular and humoral immunity to achieve long-term protective immunity[650].

Many mutations in the S-glycoprotein of SARS-CoV-2 can affect neutralizing activity. So far, several key mutations have been described in the viral variants that exhibit some degree of neutralizing evasive capacity, such as: E484K, N501Y, K417N/T, L452R/M, R346S, N439K, A475V, Q493R, V483A, S477N, F486P, etc. (*see viral evolution and immune escape section*). However, although some single mutations seem to confer immuno-evasive properties to SARS-CoV-2, it is important to understand that mutations occur in favor of virus survival, and generally— but not always[651]—exhibit a high degree of synergism. This implies that we must evaluate mutations in combination and not independently.

Some antibodies may be abolished their neutralizing capacity for new mutations; however, there is a subset of antibodies that are able to neutralize a large number of viral variants, and they are known as broadly neutralizing antibodies (bNAbs). bNAbs are present in many infections, such as influenza, hepatitis C, dengue, Ebola, etc., and have been extensively studied in HIV infection (one of the most genetically diverse viruses known)[652–654]. Fortunately, bNAbs have also been detected in SARS-like virus infections; two clear examples include S309 and 17T2. S309 is an antibody isolated from memory B cells from an individual infected with SARS-CoV in 2003[655]. While 17T2 was isolated from an individual infected during the first COVID-19 wave in Spain. This antibody is derived from a RBD-specific IgA memory B cell and is capable of neutralizing a large number of variants, including some of the most resistant ones, such as BA.5 and BQ.1.1, with higher potency than S309[656]. Importantly, pan-betacoronaviruses bNAbs have also been described[657].

Non-neutralizing antibodies

Antibodies are "generally" associated with neutralization-mediated protection that prevents the entry of pathogens into cells and inhibits bacterial toxins. However, most antibodies are not neutralizing. These antibodies can mediate other protective mechanisms by promoting the control and elimination of infectious agents through their interaction with the innate and adaptive immune system. Specifically, antibodies can bind to pathogens forming immune complexes that stimulate innate immune cells favoring the uptake of pathogens, clearance of toxins and infected cells, enhance antigen presentation and regulate inflammation[586].

Non-neutralizing antibodies encompass a wide umbrella of functions. In this section we will focus on F_c-dependent effector functions, which are the most studied and for which there is evidence of their role in SARS-CoV-2 infection. It is important to emphasize that neutralizing antibodies can also induce F_c-dependent functions. F_c-mediated effector functions (**Figure 14**) mainly include Antibody-Dependent Cellular Cytotoxicity (ADCC), Antibody-Dependent Cellular Phagocytosis (ADCP) and Antibody-Dependent Complement Deposition (ADCD). Additionally, the F_c domain can increase or inhibit the affinity and avidity of the F_{ab}

domain for the antigen[658–660]. In turn, the stoichiometry of the F_{ab} domain binding to the antigen determines the quality of immuno-complex formation, which influences F_c-dependent effector functions[661,662].

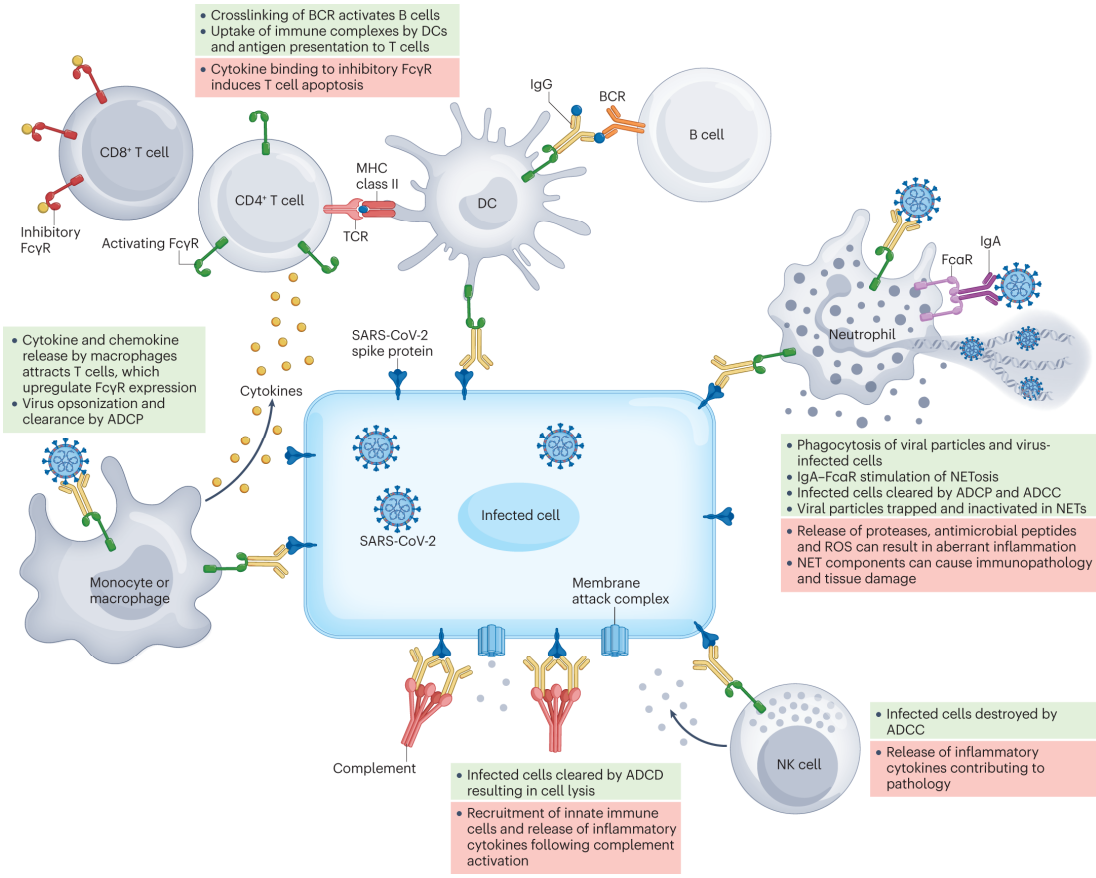


Figure 14 | F_c-dependent antibody effector functions. Non-neutralizing antibodies can bind to crystallizable fragment (F_c) receptors (F_cRs) on various immune cells to initiate effector functions that can impact the outcome of the infection. Additionally, antibodies that attach to the spike protein of SARS-CoV-2 can lead to complement fixation. F_c-dependent antibody responses tend to have antiviral functions that mediate disease resolution when properly regulated (green boxes) but may contribute to immunopathology and exacerbate disease when dysregulated (red boxes). ADCC, antibody-dependent cellular cytotoxicity; ADCD, antibody-dependent complement deposition; ADCP, antibody-dependent cellular phagocytosis; BCR, B cell receptor; NET, neutrophil extracellular trap; ROS, reactive oxygen species; TCR, T cell receptor. *Reproduced with permission from Zhang et al.*[587]

The importance of F_c-mediated effector functions of antibodies has been extensively demonstrated; a clear example comes from the RV144 vaccine clinical trial in 2009, which showed partial, although insufficient, efficacy[663,664]. Prevention of HIV infection was not associated with total anti-HIV-envelope antibody levels, but immunological correlates included $CD4^+$ T cell response, epitope-specific IgG antibodies, IgA and IgG3 levels, and ADCC[663,664].

Non-neutralizing antibodies seem to play a role in the mitigation of severe COVID-19 and resolving SARS-CoV-2 infection[665,666]. In this context, some studies in animal models showed that the neutralizing potency of antibodies did not always correlate with *in vivo* protection, and demonstrated that F_c-dependent effector functions explained this observation[667,668]. However, this does not mean that the F_c-mediated effector activities overlap with the neutralization, but rather that act synergistically.

In COVID-19 patients, the development of effective F_c-mediated responses has been demonstrated. One study observed that ADCC was elicited by 10 days post-infection, peaked by 11-20 days, and remained detectable until 400 days post-infection[669]. Additionally, patients who experienced severe disease and recovered had higher ADCC activities compared to patients who had severe disease and deceased[669]. Another study observed that both SARS-CoV-2 infection and COVID-19 vaccination induced ADCC activity[670].

Adeniji and colleagues isolated anti-S1 and anti-RBD IgG antibodies, from 60 individuals (hospitalized and non-hospitalized), and analyzed their F_c-dependent effector functions[671]. They found that antibodies from patients with more severe disease had higher ADCD activity, but lower ADCP compared to those with mild disease. ADCD activity was associated with greater systemic inflammation, raising the potential contributions of specific antibodies to the severity of COVID-19[671]. Consistently, another early study suggested that F_c-dependent antibody profiles predict COVID-19 trajectory[672]. Zohar et al. found that spike-specific antibodies mediating ADCP are associated with survival[673]. It is worth adding that association does not necessarily imply causation. The F_c-mediated effector functions of antibodies depend on many factors, such as the specific F_cRs that the antibodies engage, the cell type expressing those F_cRs, the antigen or epitopes to which they bind, and the kinetics with which they act during infection[587].

In conclusion, the F_c-dependent effector functions of anti-SARS-CoV-2 antibodies should be further studied to understand the balance between their protective and pathogenic role in COVID-19. Further details on these antibody functions in SARS-CoV-2 infection were excellently reviewed by Zhang et al.[587]

Viral evolution and immune escape

Viruses are considered the most abundant and genetically diverse biological entities on Earth and are one of the best examples of the theory of evolution by natural selection conceived by Charles Darwin (predominate those viral variants with mutations advantageous for their survival)[674–678]. Viruses are characterized by high rates of genetic mutation, especially RNA viruses. Although nidoviruses, such as coronaviruses, generally have a lower mutational rate than other RNA viruses, probably because they have a corrective exoribonuclease activity[679,680].

Early sequencing data revealed that SARS-CoV-2 acquires approximately two mutations per month in the global population[681]. By comparison, the influenza virus accumulates twice as many mutations per month, and the human immunodeficiency virus (HIV) accumulates four times as many mutations as SARS-CoV-2[682]. Interestingly, most of the mutations are deleterious and cause the virions to be unable to replicate successfully[679,683,684]. The estimated mutation rate of SARS-CoV-2 is around $1.3\times10^{-6} \pm 0.2\times10^{-6}$ mutations per nucleotide per replication cycle[685]. This rate is lower than the rates typically observed in other RNA viruses like hepatitis C virus ($\approx2.5\times10^{-5}$ mutations per nucleotide per replication cycle) and HIV ($\approx1.4\times10^{-5}$ mutations per nucleotide per replication cycle)[686,687].

Although SARS-CoV-2, has this proofreading enzymatic capacity and a relatively low degree of genetic diversity (compared to the above viruses exemplified)[688,689], we have witnessed its evolution, mutating towards more transmissible and/or immune-escaping variants, leading to successive COVID-19 waves[681,690].

Over the course of the COVID-19 pandemic, the scientific community has thoroughly monitored of the evolution of SARS-CoV-2 and its impact on public health (transmissibility, pathogenesis, immunity, treatments and diagnosis). As of April 2023, more than 15 million genomic sequences of SARS-CoV-2 are recorded in the GISAID database[691], making it one of the most massively sequenced human virus. Given the complexity of the multiple variants, in May 2021, the WHO announced a simple way to name the key variants of SARS-CoV-2 using letters of the Greek alphabet[692,693]. Currently, there is a categorization of variants, depending on the impact they have on global public health: variant of concern (VOC), variant of interest (VOI), and variant under monitoring (VUM). In 2022, the WHO added a new category labeled "Omicron subvariants under monitoring" due to the complex evolution of the Omicron variant that has originated multiple sublineages; but recently (15 March 2023) WHO updated the definitions where the Greek letter only will be assigned to VOCs, while VOIs will be referred to using scientific nomenclature (Nextrain and Pango)[694]. Additionally the category Omicron subvariants under monitoring was removed, and WHO will consider the classification of each Omicron sublineage independently, and will not automatically classify it as a VOC merely because it is a sublineage of the established Omicron VOC[694].

Categorization is performed by evaluating several parameters that affect the properties of SARS-CoV-2, such as changes (evident, predictable, or suspected) in transmissibility, patho-

genesis, and impact on public health and social measures, immune response, diagnosis, therapies and/or vaccines. For a more detailed definition, *see glossary*. Currently (17 May 2023), according to WHO, there are not circulating VOCs, XBB.1.5 and XBB.1.16 are the only two VOIs, and there are seven VUMs (BA.2.75, CH.1.1, BQ.1, XBB, XBB.1.9.1, XBB.1.9.2 and XBB.2.3)[695]. The European Centre for Disease Control and Prevention (ECDC) considers four and two Omicron subvariants as the only VOIs (BA.2.75, BQ.1, XBB and XBB.1.5) and VUMs (CH.1.1 and XBB.1.16), respectively[696].

Some variants have accumulated many mutations (especially the Omicron subvariants) that have deeply diverged them from the ancestral virus (**Figure 15**). Importantly, genetic distance predicts vaccine effectiveness from symptomatic SARS-CoV-2 infection[697].

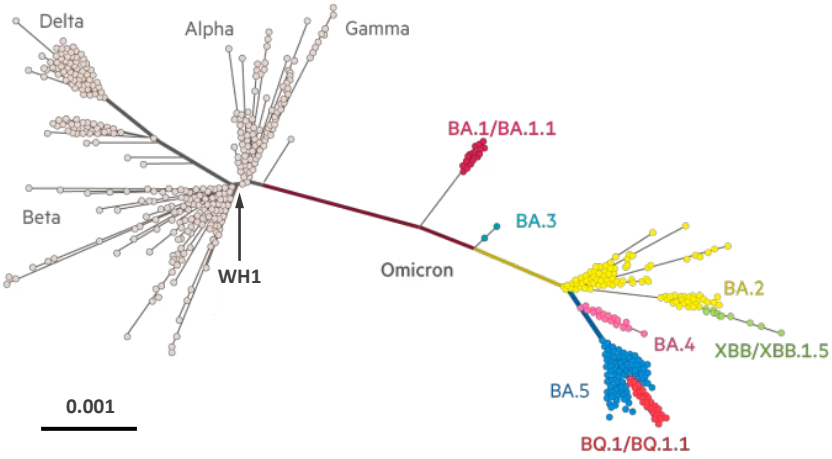


Figure 15 | **Phylogenetic tree of SARS-CoV-2**. Representation of the genetic distance from the sequences between the most relevant SARS-CoV-2 variants. The scale bar indicates the genetic distance. *Adapted under the terms of CC BY license from Wang et al.*[698]

SARS-CoV-2 variants

The D614G variant

D614G (substitution of the aspartic acid at position 614 by a glycine in the S-glycoprotein) emerged during the first months after the outbreak (January-February 2020), and several months later became the prevalent variant around the world, replacing the ancestral Wuhan-Hu-1 (WH1) virus identified in China[699]. D614G SARS-CoV-2 showed increased infectivity and transmission compared to WH1[699–705]. It has also been associated with higher viral load at the nasopharyngeal level and enhanced viral replication in pulmonary epithelial cells, however, it does not cause more severe disease[699–705].

In addition, this mutation promotes a more open conformational state of the S-glycoprotein, increasing the exposure of the RBD epitopes and making it more accessible to NAbs, resulting in a moderately higher sensitivity to neutralization compared to WH1 virus[706–708]. In spite of that, D614G mutation seems to be essential for SARS-CoV-2 survival and it is conserved in all major subsequent variants.

The Alpha variant

In September 2020, the B.1.1.7 lineage (Alpha variant) containing 17 mutations (9 of them in the S-glycoprotein compared to WH1) was detected in the United Kingdom. This variant had increased transmissibility, a higher reproduction number, and a higher secondary attack rate than the pre-existing variants[709–716]. Although with some controversies, B.1.1.7 was associated with an increased risk of hospitalization and mortality[717–724]. In December 2020, it was designated as the first VOC and was detected in 184 countries[725].

The key mutations (in addition to D614G, **Figure 18**) that significantly affect its behavior are: 1) N501Y, one of the key contact residues within the RBM[726], that enhances virus infection and transmission by increasing binding to ACE2 receptor[727,728]; 2) ΔH69/V70, this deletion increased infectivity and was associated with better incorporation of the spike into the virions[729]; and 3) P681H/R that facilitates access to the furin cleavage site, improving S processing by host proteases and viral entry into cells. In addition, this mutation confers resistance to IFN-β[730]. Remarkably, ΔH69/V70 causes S-gene target failure (SGTF) in at least one RT-PCR diagnostic assay[731].

N501Y is conserved in most major SARS-CoV-2 variants (except the Delta variant), while P681H/R is found in the Delta and Omicron variants (and their diverse subvariants).

The Beta variant

In December 2020, a new variant (detected early in May 2020) was reported and spread rapidly in South Africa[732], but did not become globally prevalent. The variant was named Beta (B.1.351) and became the second VOC. The Beta variant is characterized by at least eight mutations in the S-glycoprotein (**Figure 18**), including three relevant substitutions (K417N, E484K, and N501Y)[732]. Like the Alpha variant, it contained the N501Y mutation, although they came from different viral ancestors.

K417N increases affinity to hACE2 (compared to WH1), enhancing viral infectivity[733], although this affinity is drastically reduced compared to the Alpha variant[734,735], making it less transmissible than the latter. The K417N mutation is also found in the Omicron subvariants.

E484 is a hotspot for the binding of neutralizing antibodies[648,735], which explains why the E484K mutation is associated with reduced neutralization activity[736–739]. The E484K modification is also shared with the Gamma variant, while in the Omicron subvariants, the glutamic acid is substituted by an alanine (E484A).

Beta is characterized by a powerful ability to resist neutralization[740]. Both K417N and E484K have been shown to contribute to increased resistance to neutralization, including class 1 and 2 NAbs[741–743].

The Gamma variant

In early 2021 (in Japan and Brazil) the third VOC, the Gamma variant, was described (P.1, B.1.1.28.1)[744]. First detected in Brazil (November 2020), but like Beta, it did not become a dominant variant in the world but was predominantly in South America. According to one study, P.1 had higher transmissibility (1.7-2.4-fold compared to non-P.1 variants) and mortality, and was able to evade 21-46% of the immunity generated by previous SARS-CoV-2 infections[745]. In addition, it generated an approximately 10-fold higher viral load compared to non-P.1 variants[746].

It contains 17 amino acid changes, and 12 of them are found in the S-glycoprotein (**Figure 18**), including the key mutations K417T, E484K and N501Y. Unlike the Beta variant, where a lysine was replaced at position 417 by an asparagine (K417N), in this new variant the lysine was replaced by a threonine at the same position (K417T). Both K417T and N501Y directly interact with ACE2[111], but K417T reduces binding affinity (as well as affects neutralization)[734], whereas the N501Y mutation increases molecular interactions[747]. The combination of these three mutations resulted in a 5.3-fold improvement in affinity compared to WH1[748].

The Gamma variant shows some resistance to monoclonal neutralizing antibodies, convalescent and vaccinated plasma samples compared to the ancestral virus[749,750]. The sensitivity to neutralization is higher than the Beta variant, despite having a virtually identical triad of mutations in the RBD (K417N/T, E484K, and N501Y), suggesting that the overall constellation and combination of mutations—including those outside the RBD—may impact neutralizing activity[749,750].

The Delta variant

Shortly thereafter, the B.1.617.2 lineage (Delta variant) emerged in India (October 2020)—becoming the fourth VOC (May 2021), which replaced all previous VOCs rapidly and became the dominant variant worldwide. Probably due to the combination of its high transmissibility and the relaxation of COVID-19 pandemic prevention and control measures.

The Delta variant had ten mutations in the S-glycoprotein (**Figure 18**) compared to WH1. It exhibited a median 1.5-fold increased transmissibility and 20% reduced sensitivity to host immune response compared to non-Delta variants[751,752]. It has an increased risk of severe disease and hospitalization compared to the Alpha variant[753–755]. Estimated increase in effective reproduction number compared to Alpha, Beta and Gamma variants of 55%, 60% and 34% respectively[756]. It was determined *in vitro* that the Delta variant presented a resistance between 6 and 8 times to the neutralizing response developed by infection or vaccination, compared to D614G variant[757].

Key mutations are L452R, T478K and P681R. The first two mutations were not reported in any previous VOC. In addition, the P681R mutation was also exclusive of the Delta variant, since a P681H mutation occurred in Alpha and subsequent Omicron subvariants.

L452R increased spike stability and enhanced binding capacity to the ACE2 receptor, which may have provided a spreading advantage over other variants[758]. Additionally, this mutation contributes to the escape from HLA-restricted T-cell immunity[758] and decreases the neutralizing capacity[759–761].

T478K is located within the epitope region of class 1 NAbs, affecting antibodies directed to this site[762,763]. In addition, it contributes to increased affinity for ACE2 and appears to be associated with increased transmissibility[764].

P681R enhances the S1/S2 cleavage in the S-glycoprotein and increases transmissibility compared to the Alpha variant, resulting in a shift in its prevalence worldwide[765].

The Omicron subvariants

In November 2021, the world saw the rise of a new lineage (B.1.1.529) that quickly displaced the Delta variant. The variant was named Omicron, was detected in multiple countries, and was quickly designated by the WHO as the fifth VOC, due in part to the high percentage of mutations in the spike[766]. It is currently the dominant variant circulating globally, accounting for >98% of the viral sequences (GISAID)[691]. The Omicron variant accumulates more than 30 mutations in the S-glycoprotein (**Figure 18**) and most of them are shared in all the subsequent Omicron subvariants (BA.1, BA.2, BA.4, BA.5, BA.2.12.1, BA.2.75, BQ.1, XBB, XBB.1.5, etc.). 15 mutations were located in the RBD, of which, 11 are located in the RBM, and 7 of them are ACE2 contact residues[110,767]. This constellation of mutations (losing important residues for ACE2 binding, but compensating them with other interactions and new biochemical bridges[768]) conferred to all Omicron subvariants a remarkable immuno-evasion ability[741,769,770], high transmissibility, and a favorable endosomal entry pathway[145,146,148,771], although it causes a less severe disease compared to some earlier variants[772]. Omicron had a fivefold increased relative risk of reinfection compared to the Delta variant, increasing to more than 6 times when unvaccinated individuals are considered[773].

There are several theories that explain Omicron evolution[774]. Antigenic cartography (**Figure 16**) analyses reveal that the Omicron subvariants are largely antigenically distinct from the

other variants[775–778]. In short, the family of Omicron subvariants exhibits major structural and functional differences, such as immune-escape, fitness and tropism, compared to previous variants, that open the gateway to consider it as a separate serotype[779,780].

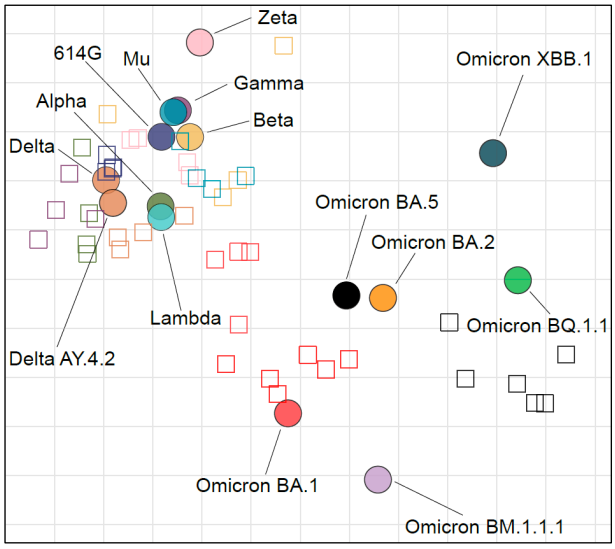


Figure 16 | **Antigenic cartography of SARS-CoV-2 variants**. Multidimensional scaling was used to generate an antigenic map from PRNT50 titers generated against multiple SARS-CoV-2 variants. Viruses are shown as circles and anti-sera as squares of matching color. Distances between viruses and antisera in the map are inversely related to PRNT50 titers with minimized error. The grid represents two-fold dilutions in titrations. PRNT50: plaque reduction neutralization titer resulting in 50% plaque reduction. *Reproduced under the terms of CC BY-NC-ND license from Mykytyn et al.*[778]

Of greater concern today is the emergence of recombinant variants, such as XBB (**Figure 17**), which is a result of the recombination of BJ.1 and BM.1.1.1 (BA.2 lineage). As of April 2023, the predominant variant worldwide is XBB.1.5[781], which contains a key—and unusual—mutation in the RBD, F486P. This mutation requires a change of two nucleotides and is associated with superior binding affinity, transmissibility and immune escape than previous circulating Omicron subvariants[782]. Recently, the WHO elevated XBB.1.16 to a variant of interest because in the last weeks it is being observed that this variant represents the majority of cases in India[783], almost 15% in the USA (in addition to XBB.1.9.1)[784], and has a relative growth advantage of ≈35%[785]. In the UK, XBB.1.16 is estimated to have a growth rate of about 50%, making it the fastest growing variant. It is followed relatively closely by XBB.1.5.13, which has a growth rate of around 30%[786].

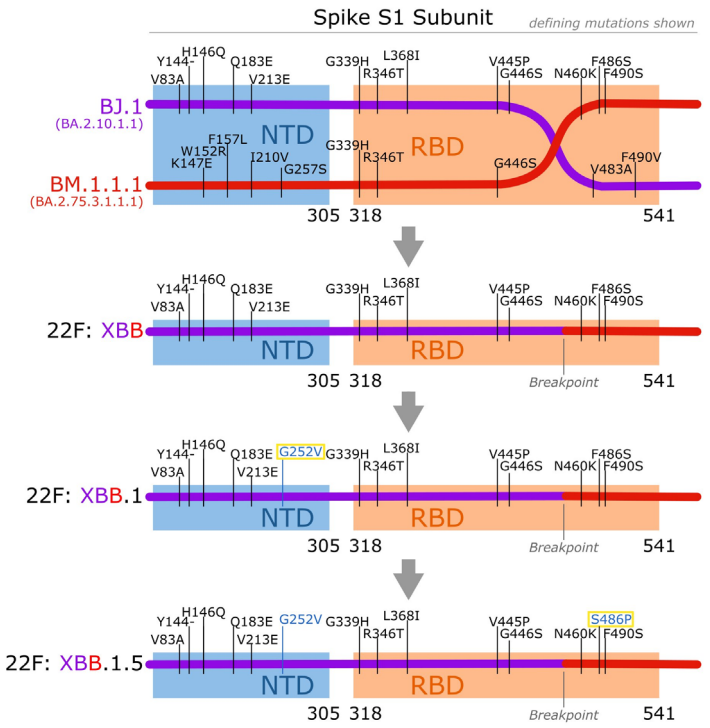


Figure 17 | SARS-CoV-2 genetic recombination variants. The diagram shows mutations in the RBD and NTD regions of the S-glycoprotein. The yellow boxes highlight the additional mutations of each subvariant with respect to the previous one. *Reproduced with permission. Courtesy of Emma B. Hodcroft*

The functional properties of many of the mutations described for the Omicron variant still need to be studied—individually and in combination—in greater detail to understand their behavior. The body of evidence suggests that although we are dealing with a large genetic diversity of Omicron sublineages, the reports indicate mild clinical course with differences in immune escape potential[787]. Epidemiological, virological and immunological information on the new Omicron subvariants is limited and continues to emerge. For more details about Omicron subvariants, consult the review by Chatterjee et al.[788]

Other variants, such as Kappa (B.1.617.1), Delta plus (AY.4.2), Epsilon (B.1.427/B.1.429), Zeta (P.2), Eta (B.1.525), Theta (P.3), Iota (B.1.526), Lambda (C.37), and Mu (B.1.621) did not have a remarkable impact, and they never came to be considered as VOCs.

Figure 18 | Representation of mutations in the S-glycoprotein between different SARS-CoV-2 variants. Former VOCs and the most globally relevant Omicron subvariants are represented. The color intensity indicates the prevalence of the mutation. Red and yellow highlight the mutation of concern or interest, respectively. Boxes filled with a diagonal striped pattern indicate that the mutation has not been detected. *Data extracted from Outbreak.info*

Impact of SARS-CoV-2 variants on the dynamics of the COVID-19 pandemic

The dynamics of a pandemic is complex and can be influenced by several factors, including the infectiousness and transmissibility of the pathogen[789–791], the susceptibility of the population[17,792], the effectiveness and availability of interventions such as vaccines and treatments[793,794], the speed and extent of public health measures such as testing, contact tracing and confinament[791,795–797], the level of compliance and adherence to these measures, as well as socio-economic and environmental factors such as population density, mobility, and access to healthcare[798–800]. Other factors that may play a role include the emergence and spread of new variants of the pathogen, the level of cross-immunity from previous infections or vaccinations, and the level of public awareness and perception of the threat posed by the pandemic[801,802]. In this subsection we will focus on the impact of viral variants (and their mutations) over the course of the pandemic.

The COVID-19 pandemic has been characterized by several waves around the world associated with the emergence of new SARS-CoV-2 variants better adapted to novel population scenarios (**Figure 19** and **20**).

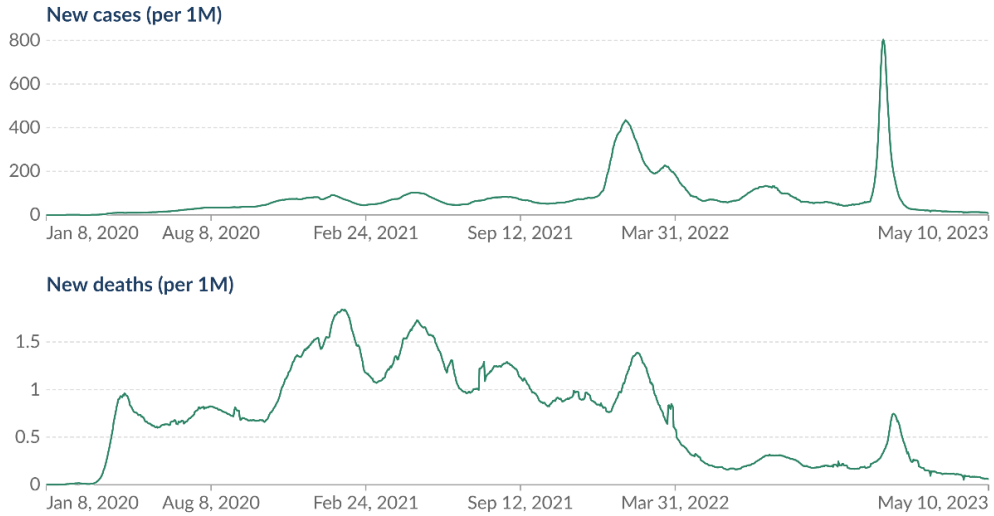


Figure 19 | COVID-19 cases and deaths worldwide. Confirmed COVID-19 cases and deaths per million people from January 2020 to April 2023. *Retrieved from OurWorldinData.org*[62]

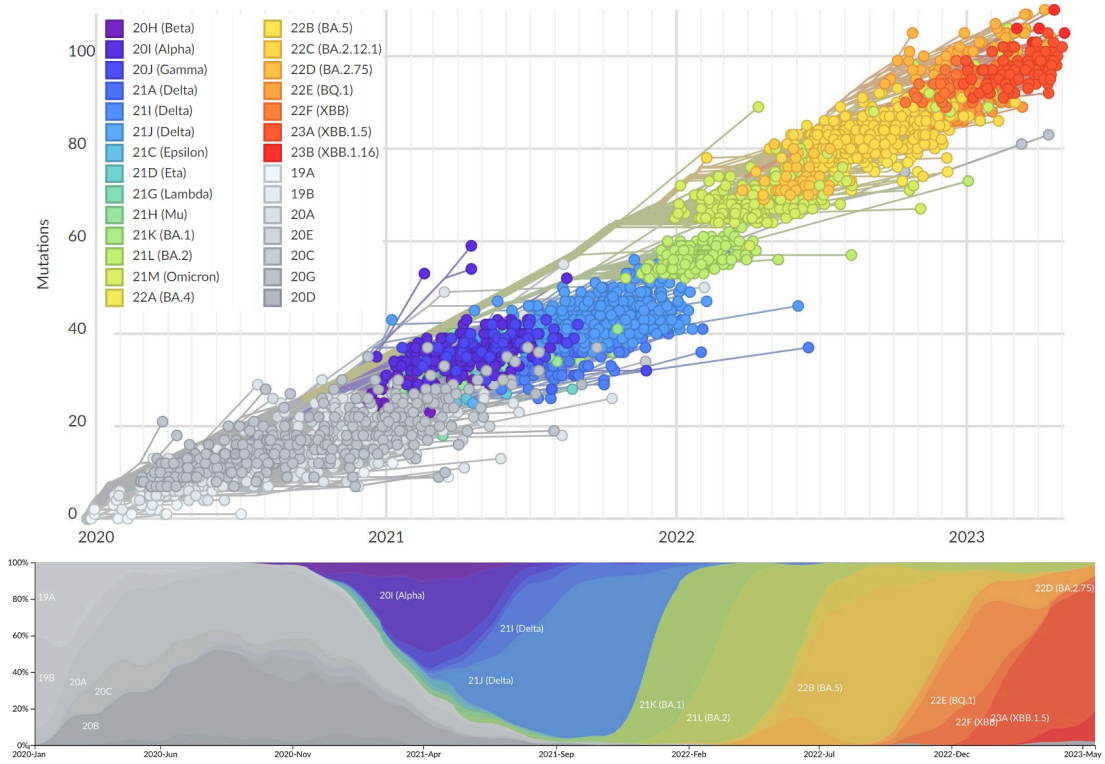


Figure 20 | **SARS-CoV-2 variants over time**. The upper panel illustrates a phylogenetic tree of SARS-CoV-2 embedded as a root-to-tip plot. The x-axis represents the sampling date of each virus. The y-axis represents the number of genomic mutations that have occurred since the phylogeny root (WH1 virus). The lower panel indicates the relative worldwide frequency of the different variants and subvariants from January 2020 to May 2023. *Retrieved from nextstrain.org with data provided by GISAID.*

It is widely recognized that viruses possess the ability to undergo genetic variation as a means of ensuring their survival. In particular, RNA viruses are known for their high mutation rates, high yields, and short replication times[803]. Viral evolution may influence several aspects of transmissibility, which may have implications for epidemiological outcomes (**Table 1**)[221]. In this context, variant fitness is a crucial characteristic that impacts the transmission dynamics[804,805]. This property encompasses lineage growth, basic reproduction number, generation time and degree of immune evasion[806]. While repeated bottleneck events lead to fitness loss, large population passages result in fitness gain[803]. These fitness gains in specific environments may not be advantageous in alternative scenarios, forcing viruses to evolve to favor this phenotypic feature[803]. Viral fitness studies have revealed different transmission patterns among SARS-CoV-2 variants[765,806–808]. **Figure 21** shows a modeling of the relative fitness of different SARS-CoV-2 lineages based on viral growth as a linear combination of the effects of individual mutations. This study demonstrated a relative upward trend in fitness among different lineages over time, with the Omicron subvariants having dramatically higher viral fitness (BA.2 had 8.9 times higher fitness than the original Wuhan lineage)[806].

Table 1 | Transmission-enhancing properties of SARS-CoV-2 influenced by viral evolution, and their effect on epidemiological parameters and population outcomes

Elements of transmissibility affected by viral evolution	Effects on SARS-CoV-2 epidemiological outcomes							
	R_0	R_e	Generation interval	Incidence of infection	Prevalence of infection	Reinfection rates	Epidemic growth rate	Burden of disease
Intrinsic transmissibility	Y	Y	N	Y	Y	Y#/N	Y	Y
Duration of infectiousness	Y	Y	N	Y	Y	Y#/N	Y	Y
Early onset of infectiousness	N	N	Y	Y	Y	Y#/N	Y	Y
Immune escape	N	Y	N	Y	Y	Y	Y	Y

Y = Effect N = No effect #Only in combination with immune escape
R_0: basic reproduction number; R_e: effective reproduction number
Adapted with permission from Markov et al.[221]

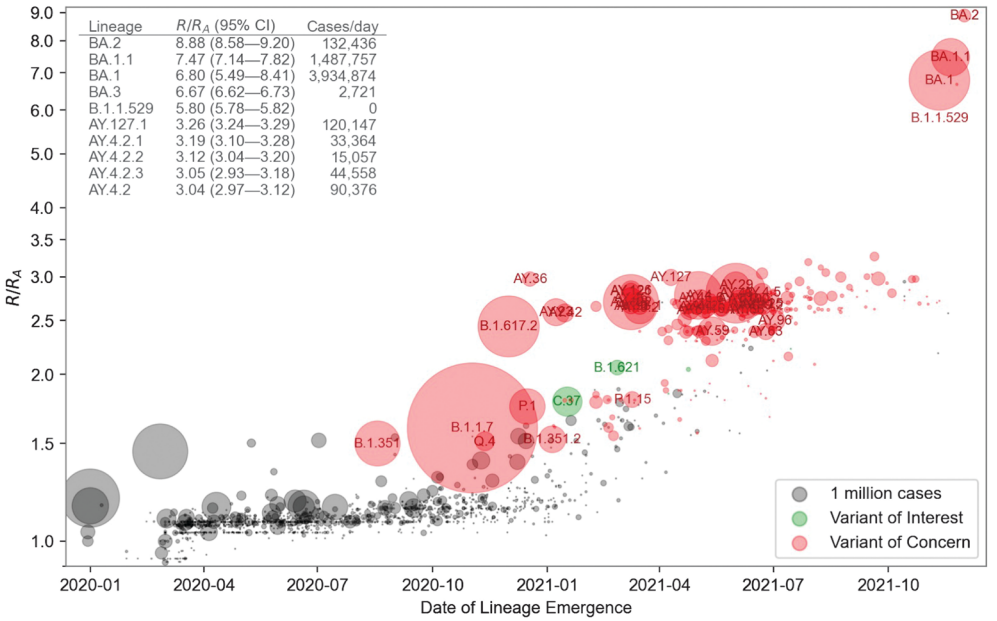


Figure 21 | Relative fitness versus date of lineage emergence. Circle size is proportional to cumulative case count inferred from lineage proportion estimates and confirmed case counts. The inset table lists the 10 fittest lineages inferred by the model. R/R_A is the fold increase in relative fitness over the Wuhan (A) lineage, assuming a fixed generation time of 5.5 days. *Reproduced with permission from Obermeyer et al.*[806]

Historically, the first notable selective advantage was the D614G mutation, whose variant had a relative growth of approximately 20% compared to the WH1 virus[705]. This mutation alters SARS-CoV-2 fitness by enhancing virion infectivity and stability[700]. An analysis of population dynamics indicated a selective sweep of G614 over D614. The higher transmissibility

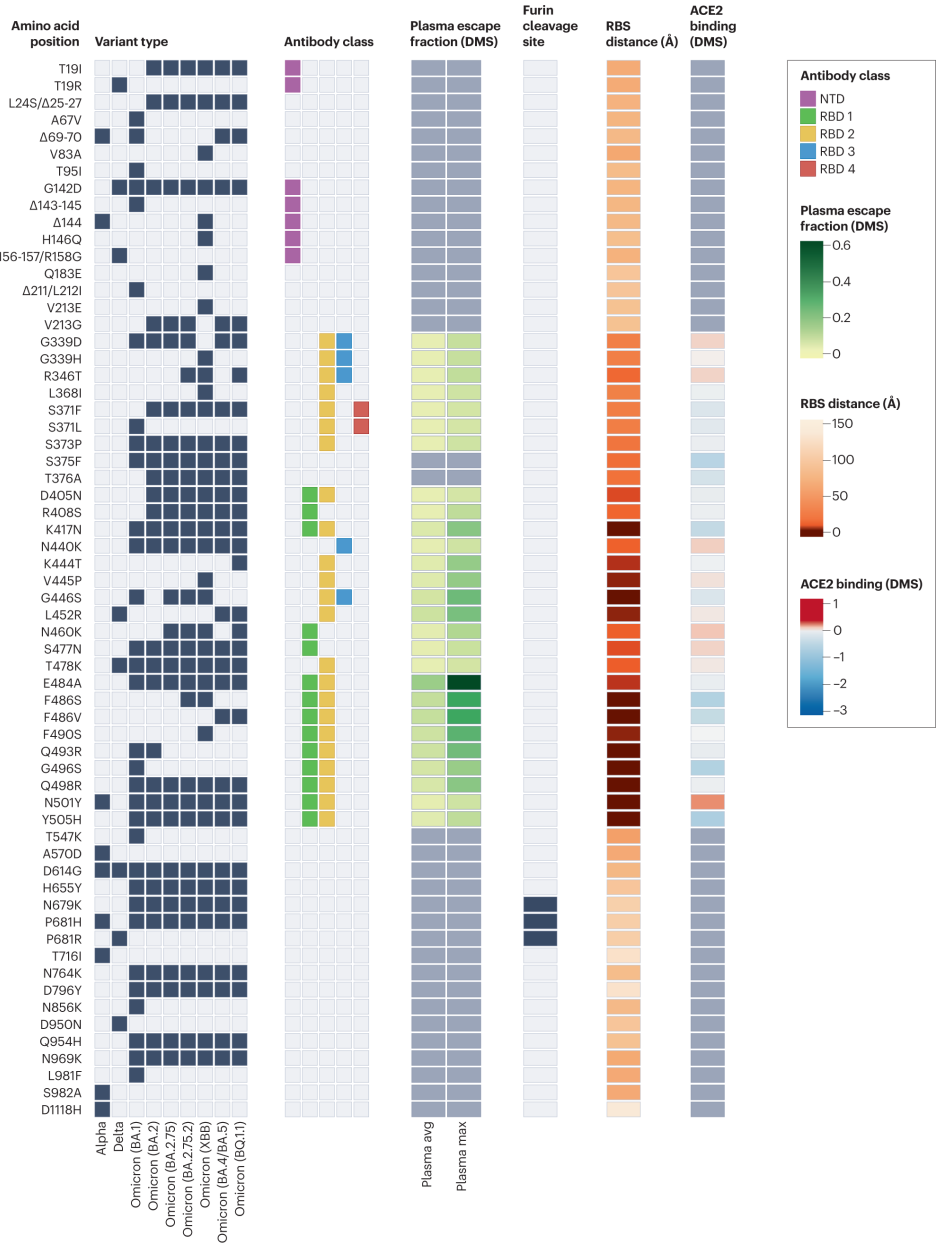
may partly explain the increased propagation of this variant[699,809]. Since October 2020, variants with an additional number of mutations have started to emerge[810]. These variants present alterations in transmissibility, antigenicity, and virulence. Throughout the pandemic, WHO has declared five VOCs, but all of them have been de-escalated, and today there are only VOIs and VUMs. The Alpha variant affected mostly Europe, the Beta variant was prevalent in South Africa and the Gamma variant in South America, while the Delta variant and the different Omicron subvariants had a global impact[811].

The evolution of SARS-CoV-2 initially progressed at a slow pace but gained momentum over time. Early variants of the virus exhibited limited immune evasion capabilities and focused more on reproductive success through adaptation of intrinsic biological features, such as through increased exposure of the RBD (D614G)[704,706,708] or improved furin cleavage and ACE2 binding (Alpha variant)[712,727]. This may have been facilitated by the relatively low population immunity at that stage. However, as the population started to develop immunity through infection and vaccination, new variants emerged that displayed greater resistance to immune responses. The Beta variant, for instance, showed reduced susceptibility to neutralization compared to previous circulating variants, but its lower transmissibility limited its global diversification[812]. A similar pattern was observed with the Gamma variant[813]. In contrast, the Delta variant acquired additional mutations that significantly increased its transmissibility[814,815]. Eventually, the Omicron subvariants (dominant in the current population scenario) emerged, which exhibit even higher transmissibility and a heightened ability to evade neutralizing responses (critical components that influenced its transmission dynamics)[816–820]. On the contrary, the 20E (EU1) variant, which had an impact on the number of cases in Western Europe, did not show great selective advantages and was considered an opportunistic variant (see below)[821].

As mentioned above, mutations in the emerging variants of SARS-CoV-2 are of great relevance in explaining its virological and immune-evasive properties. Bloom and Neher have described a tool to visualize the fitness effects of mutations in SARS-CoV-2 proteins[822]. For most genes, synonymous mutations have minimal impact, stop-codon mutations are deleterious, and amino acid mutations have a range of effects, while certain viral accessory proteins may undergo negligible or limited selection pressure[822]. Obermeyer and colleagues determined that mutations in the S, N, M and ORF1 polyprotein genes were most associated with SARS-CoV-2 fitness. Within the S gene, three hotspots of fitness-enhancing mutations were found: NTD, RBD, and the furin-cleavage site[806]. Mutations in the S-glycoprotein are considered to be the most significant in terms of their potential to promote infectivity and escape from neutralization[681,823–826]. Carabelli et al. showed schematically the effects on neutralizing response and other antigenic properties of spike mutations in different SARS-CoV-2 variants (**Figure 22**)[808]. Some mutations such as L452R, N439R, N501Y and E484K have been associated with improved infectivity, increased transmissibility, enhanced binding to ACE2, and antibody escape, respectively[739,760,827,828].

The functional furin-cleavage site has been shown to be a critical factor contributing to the high transmission rates of SARS-CoV-2[115,829]. Over time, there have been continuous adaptations of this site, resulting in improved furin cleavage of the spike proteins in the Alpha and Delta variants[830–832]. These mutations, along with other changes that improve ACE2 binding,

are believed to have played a role in the increased transmissibility and fitness of the Alpha and Delta variants, which showed 65% and 55% higher relative transmissibility, respectively, compared with the variants they replaced[712,756,765]. Meanwhile, the Omicron subvariants are mostly characterized by a modified entry phenotype and a significant immune evasion capacity[145]. This alternative mechanism exploited by Omicron could explain its diminished pathogenicity, due to lower fusogenicity and potentially altered cell tropism, with a bias towards the upper respiratory tract[808,833,834], which in turn could influence its transmissibility[834–836].



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Additionally, other mutations in both structural proteins outside the spike (i.e., M, E and N) and in other nonstructural proteins may contribute to changes in the intrinsic properties of the variants[808]. For example, the R203K and G204R (found in Alpha, Gamma and Omicron variants) mutation in the N protein increases infectivity, fitness and virulence[837,838]. The BA.1 variant contains mutations in the M and E proteins that result in reduced entry of virus-like particles into cells. Nevertheless, these mutations are compensated for by additional substitutions in the S and N proteins[839]. A study has shown that the T9I mutation found in the E protein of Omicron subvariants results in the formation of a non-selective ion channel that could be responsible for the less efficient release and lower level of cell damage induced by these subvariants[840]. As well, a notable trend observed in the latest Omicron subvariants is the downregulation of ORF8 expression[841]. Until now, it was considered to play a key role in modulating the host immune response[842–845].

Another significant factor that contributes to the dominance of specific variants is their ability to evade innate immunity[306,846]. There have been also identified mutations that evade cell-mediated immunity, such as the P272L mutation in the spike protein, which has been demonstrated to lead to immune evasion of a dominant T cell epitope[847]. Furthermore, other mutations in T cell epitopes have been found to decrease or completely inhibit MHC class I presentation[848]. SARS-CoV-2 infection is known to cause dysregulation of the production of different types of IFN[849–851] and other cytokines[852–854]. This virus may also lead to functionally impaired dendritic and T cells[855], affect macrophages[856–858] and NK cells[859].

Figure 22 | Properties of amino acid substitutions or deletions in SARS-CoV-2 variants. The dark blue boxes in the "variant type" panel identify the mutations present in the viral variants. In the "antibody class" panel, residues that affect the binding and/or neutralizing activity of NTD antibodies and/or the different classes of RBD antibodies are colored. For RBD residues, the "plasma escape fraction" panel indicates, by plotting a heat map, the escape fraction of the polyclonal antibody binding for each mutant averaged across plasma ("plasma avg") and for the most sensitive plasma ("plasma max"), illustrating consistency or variation in the effect of a mutation depending on differences in the antibody repertoire of individuals. Mutations in the furin cleavage site are highlighted. Orange shading indicates the distance to ACE2-contacting residues that form the receptor-binding motif (RBM/RBS). Finally, ACE2-binding scores representing the binding constant ($\Delta\log_{10} K_D$) relative to the wild-type reference amino acid are shown in shades of red or blue. NTD: N-terminal domain; RBD: receptor binding domain; DMS: deep mutational scanning. *Reproduced with permission from Carabelli et al.*[808]

Along the same lines, a crucial factor to consider is virulence, as it plays a significant role in determining the morbidity and mortality of a population. Virulence does not necessarily decrease over time, as there is a trade-off between transmission rate and virulence[860]. During the COVID-19 pandemic, different SARS-CoV-2 variants have shown varying levels of disease severity. Alpha and Delta variants showed higher clinical severity compared to previous variants, while Omicron showed reduced pathogenicity[861–863]. Some hypotheses suggest that the lower virulence of Omicron is due to reduced fusogenicity of the spike protein, resulting in less damage to tissues, as well as a change in its preference for infecting cells in the upper respiratory tract[833,834]. Importantly, the pathogenicity of a variant can be influenced by several factors (e.g., the immune status of an individual, age, comorbidities, genetics, etc.).[861,864,865].

In summary, both virological (e.g., transmissibility and virulence) and immunological (e.g., escape to neutralizing antibodies and/or cell-mediated immunity) properties seem to be key factors to determine the increased fitness of viral variants, impacting pandemic dynamics. However, the dependence of transmissibility, pathogenicity and immune escape is complex. While the Alpha and Delta variants were characterized by higher transmissibility and pathogenicity, the Beta and Gamma variants gained higher immuno-evasive capacities[866]. The Omicron subvariants are characterized by high transmissibility, high capacity for reinfection, lower pathogenicity, and an impressive immune escape to both monoclonal neutralizing antibodies and polyclonal responses in previously infected and/or vaccinated individuals[867]. The results suggest that the future fitness and successful evolution of SARS-CoV-2 variants will depend more on their ability to present antigenic novelties and evade the immune system than on further optimization for human-to-human transmission, although this remains a potential strategy[808]. Nevertheless, understanding the evolution of SARS-CoV-2 variants in response to changing selection pressures is crucial and epidemiological surveillance should be maintained to assess the impact of new variants and the risk they pose to the population, especially for the most vulnerable groups (immunocompromised and elderly).

COVID-19 waves in Spain

SARS-CoV-2 has caused nearly 14 million confirmed cases and more than 120,000 deaths in Spain since the first reported diagnosis on January 31, 2020[868]. During the first months of the pandemic (February-March 2020), Spain (jointly with Italy) was one of the most affected countries in the world, reporting a high mortality rate (more than 750 daily deaths from SARS-CoV-2). Between September 2022 and April 2023, the pandemic in Spain was at an apparent control point (about 10-30 deaths per day), representing a relative change of more than 98%[49,62]. As of April 2023, seven COVID-19 epidemic periods have been identified in Spain (**Figure 23**).

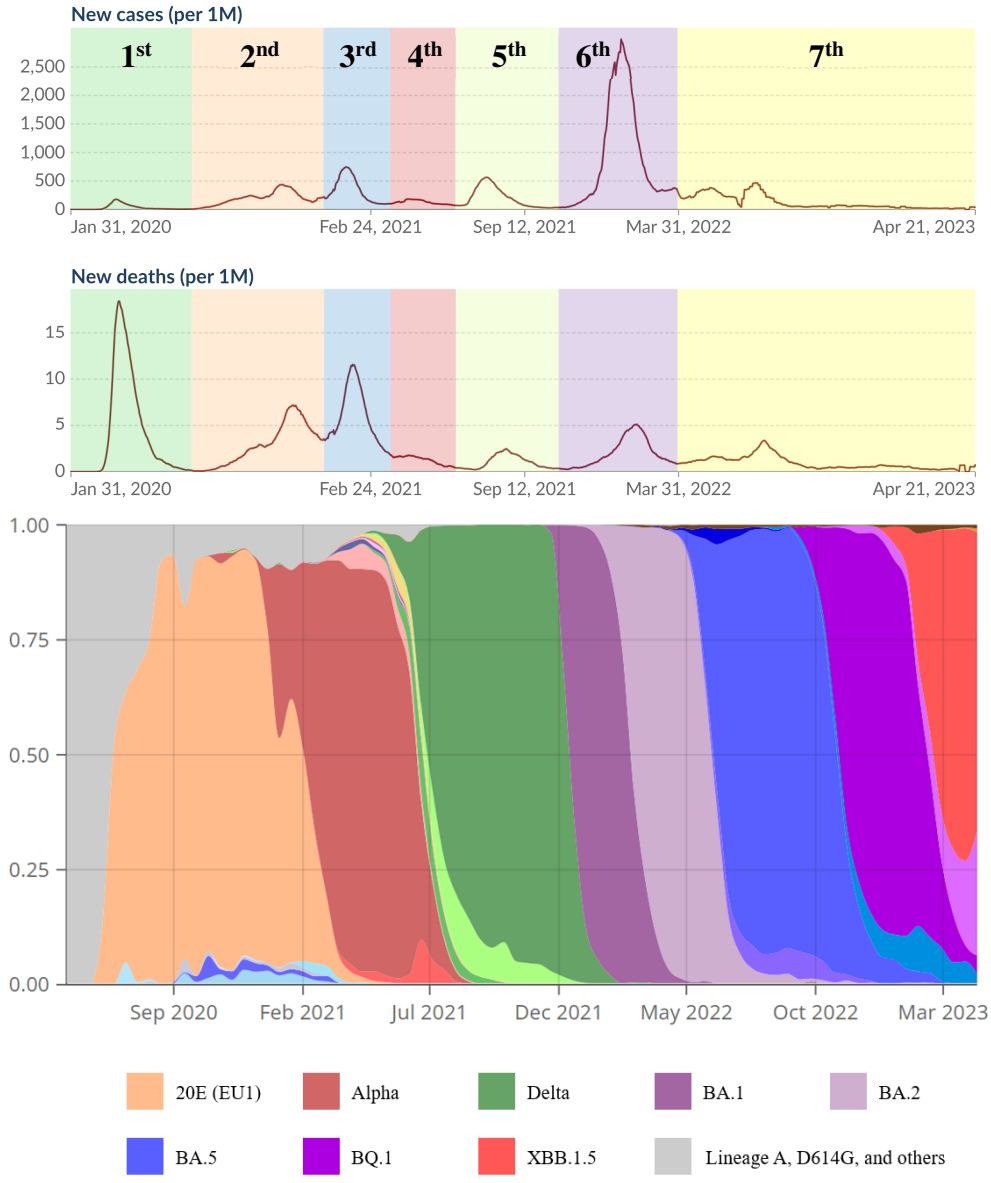

Figure 23 | COVID-19 waves in Spain. The two upper graphs represent the number of cases and deaths per million inhabitants in Spain. The seven epidemic periods in Spain (from March 2020 to April 2023) are highlighted in different colors. The lower graph shows the relative frequency of the different SARS-CoV-2 variants and subvariants over this time period. The colors corresponding to each variant are indicated at the bottom (these colors are independent of those used to differentiate the epidemic periods). *Retrieved from OurWorldinData.org*[62] *and CoVariants.org*[811]

First period (March 2020 - June 2020): Spain had one of the highest incidences of SARS-CoV-2 in Europe[869,870] with a seroprevalence ≈10% in Madrid and ≈7% in Barcelona[869]. The first weeks of the pandemic were dominated by variants of lineage A, contrary to what was observed in the rest of European countries, where lineage B prevailed[871]. The B.1.5 lineage (carrying the D614G mutation) was introduced later in Spain, approximately in March 2020, when a strict lockdown on non-essential services and movements was established in the country[871]. Next, the B.1.1 lineage emerged as the dominant[872]. All of these lineages collectively predominated during the first COVID-19 wave in Spain. A peak of ≈180 cases and ≈18 deaths per million population per day was reached, with over 250,000 accumulated cases and nearly 30,000 deaths[873]. Although during this pandemic phase there was poor data collection[869]. The "state of alarm" in Spain comprised this entire period of time (14 March 2020 to 21 June 2020)[870].

Second period (June 2020 - December 2020): From June 2020 Spain began the lifting of the confinement measures; however, during this period 20E (EU1) or B.1.177 lineage emerged. This variant was identified in Spain and became prevalent in Western Europe by the end of 2020, although interestingly it did not show a high transmissibility pattern[821]. It was characterized by an additional mutation in the S-glycoprotein (A222V), although this mutation did not affect the antigenicity of the spike, nor an increase in infectivity as seen for D614G[702,704]. Thus, all the variables evaluated did not result in this variant being of concern, despite its prevalence. Therefore, it was concluded that it was an opportunistic variant that prevailed due to favorable epidemiological circumstances[821]. During this period, 435 cases and 7 deaths per million inhabitants per day were reached, with more than 1.7 million cases and 50,000 cumulative deaths. There were more than 300 hospital admissions and 60 patients requiring intensive care (ICU) per million inhabitants per day[873].

Third period (December 2020 - March 2021): The first COVID-19 Christmas. The vaccination campaign in Spain started on December 27th[874]. In this period, 745 cases and 12 deaths per day per million population were reached. The number of cases exceeded 3 million and deaths increased by 50% compared to the first two waves. The number of hospital admissions exceeded 470 and 100 ICU patients per day per million inhabitants[873]. During this wave there was a codominance of 20E (EU1) and the Alpha variant.

Fourth period (March 2021 - June 2021): Due to the availability of vaccines, statistics were much lower than in previous waves. In this period, 180 cases and 2 deaths per day per million population were reached. The number of cases increased by 570,000 compared to the third wave and the number of deaths by 5,000. 150 hospital admissions and 50 ICU patients per day per million population were reached[873]. Alpha was the predominant variant.

Fifth period (June 2021 - October 2021): The “EU Digital COVID Certificate” entered into application on 1 July 2021[875]. In this period, 560 cases and 2 deaths per day per million population were reached. Cumulative cases exceeded 5 million since the beginning of the outbreak and about 6,000 deaths were confirmed in this fifth period. The number of hospital admissions exceeded 170 and 40 ICU patients per day per million population[873]. In this wave, the Delta variant prevailed.

Sixth period (October 2021 - March 2022): In this period, 2,900 cases and 5 deaths per day per million population were reached. It has been the worst COVID-19 wave (in number of cases), with more than 6 million confirmed cases in this 5-month period, and cumulative deaths exceeded 100,000. More than 300 hospital admissions and 40 ICU patients per day per million inhabitants were exceeded[873]. This epidemic period was initially characterized by the Delta variant, which was later replaced by the first Omicron variant (BA.1).

Seventh period (March 2022 - present): On 28 March 2022, Spain implements a new surveillance strategy that only records diagnoses in people aged 60 years or older and hospitalized cases of any age[876]. The control strategy was also updated, establishing that mild and asymptomatic confirmed cases will not be isolated and close contacts will not be quarantined[876]. In this period, 467 cases and 3 deaths per day per million inhabitants were reached. Less than 20,000 COVID-19 deaths were recorded in one year. There were more than 220 hospital admissions and 10 ICU patients per day per million population[873]. However, the statistics decreased markedly from September 2022 and remained relatively low until most of April 2023, with a small increase in the last days of this month[873]. This last epidemic period has been characterized by the successive emergence and dominance of several Omicron subvariants (BA.2, BA.5, BQ.1 and XBB.1.5).

The prevalence of past, current, and future variants invites us to be alert to possible evolutionary changes in SARS-CoV-2, whether due to chance events[690,877,878], prolonged infection in immunosuppressed individuals[879–881], and changes in the host or potential animal reservoirs[192,882,883]. Understanding the behavior of the different SARS-CoV-2 variants and how mutations contribute to changes in important properties of the virus, such as immuno-escape, transmissibility, virulence, etc., are key for evaluating the efficacy of preventive strategies and therapeutic treatments, the immunity conferred by both infection and/or vaccination, and health prevention and control measures.

Immunopathology

Significant global research endeavors have been dedicated to unraveling the immune response elicited by SARS-CoV-2 infection. It is known that SARS-CoV-2 infection can trigger an unbalanced immune response that can result in severe disease, persistence of symptomatology, or death, but the underlying mechanisms are not fully understood. For example, severe COVID-19 can lead to lymphopenia, with total T-cell count being a potential predictor of a more favorable clinical outcome[884].

In this section we will vaguely discuss some immunopathological processes resulting from the interaction of the immune system and SARS-CoV-2; all of them with many unanswered questions, such as cytokine storm, antibody-dependent enhancement, and immunological imprinting, and two important and still poorly understood pathological outcomes following SARS-CoV-2 infection, long COVID and multisystem inflammatory syndrome in children.

Cytokine storm

As mentioned above, cytokines are immune mediators necessary for a proper antiviral response[885,886]. However, like almost everything else, the response must be controlled, to avoid major side effects that can be life-threatening.

In 1993, the first report on a new concept in medicine, cytokine storm, was published[887]. This phenomenon is characterized by an uncontrolled overproduction, both at local and systemic levels, of cytokines. Although it was originally used to describe the engraftment syndrome of acute graft-versus-host disease[888], it is now known that it can be triggered by a multitude of factors, such as infections, therapies, cancers, autoimmune conditions and monogenic disorders[889].

It has been widely reported that this phenomenon also occurs in patients affected from severe COVID-19, and can lead to tissue damage, disseminated intravascular coagulation, ARDS, multiorgan failure and death[890,891]. The underlying mechanisms responsible for the unbridled release of inflammatory factors are still obscure[892]. Some studies suggest that this phenomenon is related to pyroptosis, which triggers the release of proinflammatory cytokines and affects the functions of macrophages and lymphocytes[893–897]. Several cytokines and chemokines, including IL-1β, IL-6, IP-10, TNF, IFN-γ, MIP-1α and MIP-1β, and VEGF, are elevated in patients with inflammation and lung damage, and some of them are independent predictors of COVID-19 survival[25,898,899]. For this reason, anti-inflammatory agents, such as corticosteroids, and drugs that inhibit the action of cytokines involved in this phenomenon, such as anakinra (IL-1Ra) and tocilizumab (anti-IL-6R), have been authorized (*see treatments and therapies section*).

Long COVID

Most people infected with SARS-CoV-2 recover completely, however, between 10% and 20% of the population experience medium- to long-term sequelae following infection, a condition known as long COVID (formally "post-acute sequelae of COVID-19, PASC")[900]. WHO defines this multisystemic condition as the continuation or development of new symptoms by persons with a history of probable or confirmed SARS-CoV-2 infection, usually within three months of the onset of COVID-19, with symptoms and effects lasting at least two months that cannot be explained by an alternative diagnosis[901,902].

It is estimated that at least 65 million people in the world have long COVID[903], with an incidence ranging from 10-30% of non-hospitalized cases, 50-70% of hospitalized cases and 10-12% of vaccinated cases[904–907]. Although long COVID affects all ages, most cases are diagnosed in middle age adults and in individuals who have experienced mild disease[908]. Patients reveal variably symptoms across multiple organs, including heart, lungs, gastrointestinal tract, neurological system, reproductive system, among others[903]. The most frequent signs and symptoms are fatigue (58%), headache (44%), attention disorder (27%), hair loss (25%),

dyspnea (24%), ageusia (23%), anosmia (21%), polypnea (21%), joint pain (19%) and cough (19%)[909]. These symptoms can last for years and significantly affect patients' quality of life[910].

The underlying causes of long COVID are still unknown, however, it is likely to be a multifactorial process[911]. The comprehensive review by Davis et al. has gathered several hypotheses for the pathogenesis of the disease: persistent reservoirs of SARS-CoV-2 in tissues, immune dysregulation with or without reactivation of underlying pathogens, impacts of SARS-CoV-2 on the microbiota including the virome, autoimmunity and priming of the immune system from molecular mimicry, microvascular blood clotting with endothelial dysfunction, and dysfunctional signaling in the brainstem and/or vagus nerve[903].

Studies have found that the disease is associated with various immune alterations, such as diminished numbers of $CD4^+$ and $CD8^+$ effector memory cells, exhausted T cells, and elevated PD1 expression on central memory cells[912,913]. Additionally, this condition seems to be associated with highly activated innate immune cells, a lack of naive T and B cells, and increased expression of IFN-β and IFN-λ1[914]. One study observed that patients with long COVID had elevated the IL-1β, IL-6, and TNF cytokine triad[915].

Additionally, long COVID has been found to have increased levels of autoantibodies, including autoantibodies to ACE2[916,917]. Levels of certain autoantibodies correlate negatively with the titer of protective antibodies, which may suggest that patients with elevated autoantibodies levels are more susceptible to breakthrough infections[917]. A weak anti-SARS-CoV-2 antibody response has been associated with an increased risk of developing long-term symptoms[918,919]. Interestingly, a recent preprint suggests a potential relationship between the breadth and decay rate of the neutralizing response with different long COVID phenotypes[920]. Another preprint reports that cross-immunity to endemic human coronavirus OC43 may be associated with long COVID[921]. The study conducted by Hu et al. found that viral persistence and periodic shedding in the gastrointestinal tract, which may further perpetuate chronic immune activation in long COVID, was related with lower levels of and slower generation of anti-RBD-specific IgA and IgG antibodies[922]. These results could be useful not only to characterize the long COVID but also to predict it.

One study found a strong association between innate immune cell count, kynurenine, and lipid metabolites, revealing strong interpatient and intrapatient temporal covariation. These biomarkers appeared to be indicative of the likelihood of resolution, mortality, and long COVID[923]. The predictive tool that the authors developed could be valuable for early treatment and for preventing or reducing long COVID.

Multisystem inflammatory syndrome in children

Most children experience mild or asymptomatic COVID-19, but a small percentage of them are at risk of developing severe disease and death[924–926]. Early in the pandemic, cases of hyperinflammatory shock in children, similar to Kawasaki's disease, began to be reported[927–930]. Today, this condition is known as inflammatory syndrome in children (MIS-C).

MIS-C is a hyperinflammatory complication of COVID-19 and generally occurs 2-6 weeks following SARS-CoV-2 infection. The children with MIS had a more inflammatory profile and severe clinical phenotype[924], characterized by cardiovascular, respiratory, neurologic, gastrointestinal, mucocutaneous manifestations and multiorgan dysfunction, and more than 50% of patients can require ICU admission[931–933]. This syndrome leads to serious and life-threatening illness in previously healthy children and adolescents[934].

The pathophysiology is not yet well understood, but some papers suggest that it is the result of immune dysregulation[935,936]. Thus, this phenomenon might be related to an IgG-mediated disease enhancement (ADE), or to a delayed cytokine storm[937–940] (because coronaviruses have the potential to block type I and type III interferon responses[941]). However, there are other potential hypotheses[936]. In addition, patients with MIS-C, unlike children with acute SARS-CoV-2 disease, maintained high levels of inflammatory monocyte-activating SARS-CoV-2 IgG antibodies, high levels of pro-inflammatory cytokines, lymphopenia and increased $CD8^+$ T-cell activation, among other laboratory findings that are common but not ubiquitous[942–945]. Although the exact mechanisms by which an abnormal immune response to SARS-CoV-2 is triggered in children are unknown, an early diagnosis and treatment lead to a favorable outcome. In addition, there is evidence that vaccines protect from MIS-C[946–951]. For more details on this syndrome, see the excellent publication by Feleszko and colleagues[936].

Antibody-dependent enhancement

Beyond the beneficial effects of antibodies and the fact that they are a good correlate of protection from viral diseases, they can also be deleterious or interfere with the functions of other antibodies[952,953]. Antibody-Dependent Enhancement (ADE), a phenomenon frequently associated with pre-existing non-neutralizing or subneutralizing antibodies, has been documented for several pathogens, such as dengue and respiratory syncytial virus (RSV)[954]. ADE use anti-pathogen antibodies to promotes infection of cells bearing F_c or complement receptors[954].

Kam et al. demonstrated, *in vitro*, that anti-S antibodies mediated ADE for SARS-CoV and can affect B cells[955]. Other studies showed that the antibodies were able to enhance viral infection in monocytes and macrophages, broadening the cell tropism of SARS-CoV[956–958], but it does not sustain productive viral replication and shedding in the infected cells[959].

Houser et al. inoculated rabbits intranasally with MERS-CoV and observed that the antibodies developed lacked neutralizing activity and did not protect the animals from reinfection; in fact, reinfection resulted in enhanced lung inflammation, with the contribution of complement proteins[960]. Another study described that a neutralizing anti-MERS-CoV mAb (Mersmab1) produces conformational changes in the spike, makes it prone to proteolytic activation, and mediates viral entry into $F_c\gamma$ receptor-expressing cells through canonical viral-receptor-dependent pathways, revealing a novel mechanism for ADE[961]. With this background in SARS-like viruses, an early publication put this phenomenon into perspective for SARS-CoV-2 infection and COVID-19 vaccines[962].

At least two potential mechanisms by which SARS-CoV-2 antibodies may mediate ADE have been described. Anti-RBD antibodies may enhance infection via F_c receptors, whereas anti-NTD antibodies may promote virus binding to ACE2-expressing cells via conformational changes in S-glycoprotein[963]. Wang et al. described two neutralizing mAbs (MW01 and MW05) that could enhance the infection of SARS-CoV-2 pseudovirus on $F_c\gamma$RIIB-expressing B cells[964]. Additionally, Maemura et al. evaluated ADE of infection by using COVID-19 convalescent plasma, and found that $F_c\gamma$RIIA and $F_c\gamma$RIIIA mediated modest ADE of SARS-CoV-2 infection[965]. Furthermore, Liu and colleagues demonstrated that some NTD site-specific antibodies lead to an open state of the RBD, which enhances the infectivity of SARS-CoV-2[966].

Whether this occurs in humans is still less clear, and ADE of infection observed *in vitro* and animal models do not predict risk of ADE of disease. In part, protective and potentially detrimental antibody-mediated mechanisms are the same[967], and antibodies with effector functions, in an inflammatory environment, could shift the balance more towards a protective rather than a harmful mechanism[967,968].

In conclusion, while theory suggests that ADE events of infection can occur, cases in practice are extremely sporadic[969]. Anyway, further research is needed to clarify the doubts regarding this phenomenon of enhancement of SARS-CoV-2 infection.

Antigenic original sin

The concept of original antigenic sin arose in 1960[970] to describe patterns of antibody response to influenza vaccination. This immunological concept attempts to explain the ineffective or null response to an antigen related (slightly variable) to previous exposure, due to the fact that the immune system prioritizes immunological memory. This phenomenon has been documented for some viruses, such as influenza[971,972] and dengue[973,974].

For COVID-19, the hypothesis is on the table but has not been studied further. The close relationship between different human coronaviruses and between SARS-CoV-2 variants could lead to the development of this phenomenon. $CD4^+$ T cells with cross-reactivity between SARS-CoV-2 and other endemic coronaviruses have been detected in individuals not exposed to SARS-CoV-2[975]. Evidence of immunological imprinting by earlier seasonal coronaviruses has also been found, with pre-existing cross-reactive memory B cells and the presence of anti-SARS-CoV-2 antibodies linked to back-boosting of antibodies against conserve regions of betacoronaviruses[976,977]. In addition, it has been shown that mRNA vaccination substantially increased the antibody responses against the S-glycoprotein of betacoronaviruses[610]. However, although encouraging, whether this boost effect might protect from SARS-CoV-2 infection or disease remain unknown. Interestingly, according to available precedents on influenza virus, the mortality rate associated to the infection by the swine-origin H1N1 influenza virus (2009) was lower at older ages, attributing this fact to a possible relationship with previous exposition to the 1918 H1N1 Spanish influenza[458,978,979]. On the other side and along the same lines as other studies published[631–633], a recent non-peer-reviewed

publication showed similar neutralizing antibody titers against the Omicron subvariants between the monovalent and bivalent vaccine following the fourth dose[980]. Analyses indicated that inclusion of the ancestral spike in the bivalent vaccine could prevent broadening of the antibody response to BA.5[980], since the response was probably directed to epitopes shared between the ancestral virus and the BA.5 subvariant, rather than mounting a neutralizing humoral response against novel epitopes. The authors suggest that the ancestral spike in the current bivalent COVID-19 vaccine is the cause of deep immunological imprinting; however, these results should be interpreted with caution and should be studied further.

The overall findings could be interpreted in a positive or negative way. Immune cross-reactivity between different coronaviruses could support the immune memory and generate more successful responses (back-boosting); or, the immune system, encountering previously seen conserved epitopes, could draw on immunological memory and generate a delayed or non-appropriate response to viruses with minor antigenic variations (original antigenic sin). Thus, the hypothesis of antigenic distance[981] becomes an important question still unresolved. The impact of antigenic evolution and original antigenic sin on SARS-CoV-2 immunity was more extensively reviewed by Aguilar-Bretones et al.[982]. More detail about immunological imprinting and COVID-19 was recently discussed by Koutsakos and Ellebedy[983].

Diagnostic tools

The symptoms caused by SARS-CoV-2 are very similar to those produced by other respiratory viruses so clinically it is very difficult to distinguish among them[984]. The diagnosis of COVID-19 is made on the basis of laboratory testing, commonly by detecting SARS-CoV-2 RNA by RT-PCR through nasopharyngeal swab, saliva[985], or other respiratory tract specimens (oropharyngeal swab, middle turbinate and anterior nares, sputum, tracheal aspirate or bronchoalveolar lavage)[986,987].

RT-PCR assays for SARS-CoV-2 begin to test positive a few days after infection and before symptoms onset[988,989], and may continue to give positive results for several weeks or even months[990–993]. However, SARS-CoV-2 genomic and subgenomic RNAs in diagnostic samples are not an indicator of active replication[235,994]. The presence of replication-competent viral particles is around 1-2 weeks post symptom onset, except in specific cases, such as in immunocompromised patients, where the duration may be longer[236,988,995–999]. Therefore, it is recommended that specimens be collected early during infection, since viral loads peak in most cases around the time of symptom onset and then gradually decrease[234,1000].

The SARS-CoV-2 antigen-detecting rapid diagnostic test has been widely used and has become the primary method of screening for SARS-CoV-2 infection in the non-hospitalized population, due to its wide market availability, ease of use, rapid results and cost[1001]. It should be noted that antigen tests may begin to be positive days after symptomatology[1002] and have lower sensitivity compared to molecular tests[1002–1005]. In addition, such sensitivity may vary

across SARS-CoV-2 variants[1006,1007]. For this reason, in case of symptomatology and a negative result for SARS-CoV-2 by rapid antigenic testing, it is recommended to repeat the rapid test 1 or 2 days later[1001,1002]. Antigen tests can detect several virus proteins, however, most of them are designed to detect the N protein[1008] (highly immunogenic, elevated expression during infection, and less variable than S-glycoprotein, although some antibodies [antigen tests are based on antibodies] may have a certain level of cross-reactivity with the human common cold coronaviruses[1009,1010]) in nasal, nasopharyngeal and oropharyngeal swabs[234]. The technical working group on COVID-19 diagnostic tests provides a common list of rapid antigen detection tests in the European Union[1011].

Antibody tests were used as a method of indirect detection of SARS-CoV-2[1012–1014], although their use has been limited, since most of the population is vaccinated with vaccines based on the SARS-CoV-2 spike glycoprotein, so they already have antibodies against this antigen, and would lead to false-positive results[1015–1017]. Rapid antibody tests used to trace past infections are usually designed to detect antibodies against the N protein[1008]. Importantly, anti-N antibodies may lose validity as indicators of past infections over time, because they decline more rapidly than antibodies directed against other SARS-CoV-2 epitopes and/or their higher background signal from cross-reactive anti-nucleocapsid antibodies against other human coronaviruses[529,1009,1018–1020]. Given that a significant percentage of the population remains asymptomatic and/or has never had a COVID-19 test, and because such tests do not detect infection at its earliest stage (antibodies take days to be detectable, *see adaptive immunity section*), they are not recommended in practice as a diagnostic method, nor to assess the immune status or protection from COVID-19[1013,1021,1022]. Although serological antibody tests were useful at the beginning of the pandemic, they have lost prominence and are nowadays more valuable in epidemiological studies and for confirming past infections[1023–1025].

In addition to the above and using a positive nucleic acid test as the primary diagnostic criterion, China considers other serological findings as diagnostic criteria. For example, if the IgG antibody level shows an increased by four times or more in the convalescent phase compared to the acute phase[1026].

Finally, the genetic (by sequencing) and antigenic (by neutralization assays) characterization of SARS-CoV-2 variants is also of great importance in the context of diagnostics and immune protection achieved by infection, vaccination, or the combination of both[1027].

Treatments and Therapies

Antivirals

Throughout the pandemic, multiple potential treatments have been tested against SARS-CoV-2, but few of them have shown good efficacy, while others have fallen by the wayside as SARS-CoV-2 evolved.

Paxlovid (nirmatrelvir plus ritonavir) is a SARS-CoV-2 3CL protease inhibitor. Several studies have demonstrated a efficacy greater than ≈85% in preventing severe disease, hospitalization, and death[1028–1031]. Paxlovid is administered orally and it is recommended by the FDA, EMA, NMPA (China) and WHO for people who are at high risk of hospitalization[1026,1032–1034]. In addition, Paxlovid reduces the risk of long COVID by 26% according to a recent study[1035]. However, SARS-CoV-2 $3CL^{pro}$ mutations confer resistance to nirmatrelvir and other compounds[1036,1037].

Remdesivir, originally developed to treat hepatitis C, was subsequently used as a therapeutic agent against Ebola and Marburg virus infections and showed *in vivo* activity against several other viruses (including SARS-CoV and MERS-CoV)[1038,1039]. Remdesivir, is a nucleotide analogue that inhibits viral RNA polymerases, and has been used since the beginning of the COVID-19 pandemic[1040,1041]. It was the first drug approved by the FDA and over time has shown good efficacy in reducing severe COVID-19, although its beneficial effect on mortality in certain cases is still unclear[1042–1046]. Remdesivir is administered intravenously and is recommended by the FDA, EMA and WHO for hospitalized and non-hospitalized patients who are at high risk of developing severe COVID-19[1032–1034]. A recent study found, in adults with mild to moderate COVID-19, that an oral analogue of remdesivir (VV116) is as effective as nirmatrelvir plus ritonavir with respect to clinical recovery time and shows fewer adverse effects[1047]. This year (2023), China has authorized its use for the treatment of adult patients with mild to moderate COVID-19[1048].

Molnupiravir, another oral drug against COVID-19, is an RNA-dependent RNA polymerase inhibitor. Studies have shown that it is less effective than the previous treatments in terms of reducing the risk of hospitalization[1049–1052]. The PANORAMIC multicenter trial found that molnupiravir does not reduce COVID-19 hospitalization or deaths in vaccinated people at high risk[1053]. Molnupiravir acts by inducing mutations in the virus genome during replication, most of which appear to be deleterious and result in non-viable viral progeny[1054,1055]. Importantly, a preprint published earlier this year suggests that molnupiravir might, in some cases, drive the evolution of viral lineages[1056]. NMPA has approved it for adults with mild to moderate SARS-CoV-2 infection at high risk of progression to severe disease[1057]. The FDA and WHO recommend it only if other options are not available, feasible to use, or clinically appropriate, and advise that it is contraindicated in children and pregnant or breastfeeding women[1032,1033]. The EMA recommended the refusal of marketing authorization, but the company (MSD) requested a re-examination[1034].

A new oral antiviral known as Ensitrelvir, a potent protease inhibitor, has been shown to be effective and safe from the phase III clinical trial. The results were presented at Conference on Retroviruses and Opportunistic Infections (CROI) 2023 (abstract #166). It is the first drug to statistically significantly reduce the number of days that people test positive for SARS-CoV-2[1058]. As a result, it has received emergency approval in Japan, but not yet in other parts of the world (FDA has granted Fast Track designation[1059]). Importantly, Ensitrelvir showed a reduction in the relative risk of long COVID (CROI 2023).

Metformin, a first-line drug indicated for the treatment of type 2 diabetes mellitus, is a potential therapeutic agent for COVID-19 because of its putative action against proteins involved in translation. In addition, it shows *in vitro* antiviral effect, and anti-inflammatory and antithrombotic activities[1060–1065]. However, some studies indicate that metformin does not reduce the risk of hospitalization or death from COVID-19[1066,1067]. While a phase IIb clinical trial revealed that patients treated with metformin glycinate reduces SARS-CoV-2 viral load in 3.3 days, while the control group required 5.6 days[1068]. Results presented at CROI 2023 (abstract #170), from a phase III clinical trial, are consistent in concluding that metformin decreases SARS-CoV-2 viral load and, furthermore, the authors indicate that the magnitude of antiviral effect was similar to nirmatrelvir at day 5 and greater than nirmatrelvir at day 10. Additionally, in a recent study not yet peer-reviewed, metformin was found to reduce the relative risk of long COVID by 42%[1069]. Due to the lack of evidence, metformin is not recommended as a COVID-19 treatment, except in clinical trials.

Sabizabulin is a novel microtubule disruptor with potential antineoplastic, antiviral, and anti-inflammatory activities. One study found a 55% relative reduction (25% absolute reduction) in inpatient deaths from COVID-19 compared to placebo[1070]. This drug is under review by the EMA (article 18). The FDA declined to grant an emergency use authorization.

China has approved several antivirals for the treatment of COVID-19 that have not yet been approved by either the FDA or the EMA. These antivirals are: Azvudine, Xiannuoxin, Simnotrelvir plus Ritonavir, VV116 and RAY1216[1057].

Some drugs with antiviral potential that have been evaluated and have not shown benefit—at least clearly and conclusively—against COVID-19, include: favipiravir[1071–1073], ribavirin[1074,1075], fluvoxamine[1076–1079], lopinavir plus ritonavir[1080,1081], arbidol[1082], nitazoxanide[1083] and camostat mesylate[1084–1086].

Ivermectin, an anti-parasitic drug, was a controversial candidate at the beginning of the pandemic and even transcended far beyond science[1087]. It was known as the "miracle drug" because it inhibited SARS-CoV-2 replication *in vitro*[1088] and preliminary reports (later retracted due to serious errors and potential fraud in the data) showed a surprisingly effect reducing SARS-CoV-2 mortality[1089–1092]. However, the vast body of accepted evidence indicates that it has no effect on the course of COVID-19[1093–1101].

Similarly, although there was a great expectation at the beginning of the pandemic with chloroquine and hydroxychloroquine, different studies showed that they have a limited effect[1102–1106]. A possible explanation could be because the endocytic pathway is not the main mechanism of SARS-CoV-2 entry (except for Omicron subvariants)[143,149].

Some spike-targeted antivirals, such as cyanovirin-N, have shown promising results *in vitro* and/or in animal models[136,137,1107], but have not been tested in clinical trials to evaluate their efficacy.

In summary, to date, antiviral alternatives are limited; however, remdesivir, nirmatrelvir, molnupiravir and ensitrelvir remain active against the different Omicron subvariants, including BQ.1.1 and XBB.1.5[1108–1110].

Potential drugs that show promising results in clinical trials in reducing the risk of long COVID, raise the question of whether all SARS-CoV-2 infected individuals should take them to reduce the risk of post-COVID sequelae.

SARS-CoV-2 monoclonal antibodies

NAbs are the basis of many approaches with human interventions, either by active induction (vaccines) or passive infusion (mAbs, convalescent plasma, hyperimmune immunoglobulin). These approaches have been extensively studied in SARS-CoV-2 infection. As mentioned above, and roughly speaking, antibodies can mediate several anti-viral functions. Antibodies can neutralize SARS-CoV-2 by targeting the S-glycoprotein; however, they can also be used to reduce the severity of COVID-19 by targeting pro-inflammatory cytokines and chemokines involved in SARS-CoV-2 infection[1111].

Monoclonal antibodies (mAbs) have been shown to decrease the risk of symptomatic SARS-CoV-2 infection in the context of pre- or post-exposure prophylaxis, and to reduce the risk of progression to severe disease, hospitalization and death if used early in the course of infection[1112–1114]. To date, a total of six anti-SARS-CoV-2 monoclonal antibody products have received FDA (tixagevimab plus cilgavimab [Evusheld], bamlanivimab plus etesevimab, casirivimab plus imdevimab, sotrovimab, and bebtelovimab)[1115] and EMA (tixagevimab plus cilgavimab, regdanvimab, casirivimab plus imdevimab, and sotrovimab)[1034] authorization for use as COVID-19 therapy and/or prophylaxis. However, both agencies agree in no longer recommending or restricting the use of anti-spike mAbs due to reduced or no efficacy against dominant Omicron subvariants[1116,1117]. For example, BQ.1.1 and XBB.1.5 show incredible resistance to most of the antibodies used individually or as a cocktail[1118,1119]. China recommends the use of ambavirumab plus romisevirumab (also known as amubarvimab plus romlusevimab or BRII-196 plus BRII-198) for adults and adolescents with mild to moderate COVID-19 and who have high-risk factors for severe disease[1026]. One study showed high resistance of BA.2.75.2 and BQ.1.1 to these two antibodies both individually and in combination (**Figure 24**)[1118]. Recently, a handful of publications have characterized mAbs with broad neutralizing activity across different variants (bNAbs) and have been shown to be effective even against Omicron subvariants[656,1120–1123].

		B.1	BA.1	BA.4–5	BA.4.6	BA.2.75.2	BJ.1	BQ.1.1
Single mAbs	Casirivimab	21	1890	>50000	>50000	>50000	880	>50000
	Imdevimab	19	>50000	994	2109	>50000	>50000	>50000
	Bamlanivimab	16	>50000	>50000	>50000	>50000	>50000	>50000
	Etesevimab	53	>50000	>50000	>50000	>50000	>50000	>50000
	Cilgavimab	37	2658	88	24200	>50000	>50000	>50000
	Tixagevimab	7	173	10090	27740	>50000	304	>50000
	Amubarvimab	53	5641	1234	1290	>50000	4762	>50000
	Romlusevimab	852	866	8279	>50000	>50000	>50000	>50000
	Adintrevimab	14	23	>50000	>50000	>50000	>50000	>50000
	Regdanvimab	7	>50000	>50000	>50000	6336	>50000	>50000
	Bebtelovimab	5	7	6	7	14	>50000	>50000
	Sotrovimab	157	833	5554	13000	3239	825	>50000
Cocktails of mAbs	Casirivimab–imdevimab	9	3642	2611	5395	>50000	2456	>50000
	Bamlanivimab–etesevimab	18	>50000	>50000	>50000	>50000	>50000	>50000
	Cilgavimab–tixagevimab	7	97	155	7131	>50000	482	>50000
	Amubarvimab–romlusevimab	64	657	1819	1015	>50000	5359	>50000

EC50 (ng/ml)

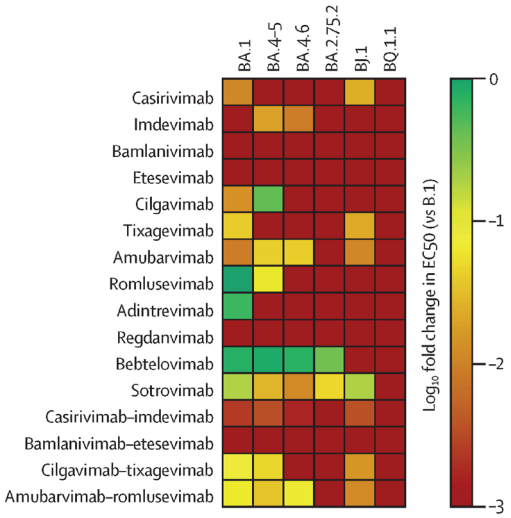

Figure 24 | **Neutralization profile of anti-SARS-CoV-2 monoclonal antibodies**. The upper panel (table) shows the IC50 (ng/ml) of mAbs evaluated individually and combined against different Omicron subvariants and the B.1 lineage. The lower panel represents, by heatmap, the fold change in IC50 compared to B.1 pseudovirus. *Reproduced with permission from Arora et al.*[1118]

The evolution of SARS-CoV-2 and the remarkable resistance to spike-targeted neutralizing mAbs have paved the way for additional antibody approaches. Mutations have driven the avoidance of antibody recognition, but still, new emerging variants require binding to ACE2 through the S-glycoprotein; although this binding affinity changes due to mutations. Employing ACE2 derivatives as a viral antagonist is a viable strategy[1124]. hACE2.16 (anti-ACE2 antibody) blocks infection and does not affect ACE2 expression or enzymatic activity[1125]. At CROI 2023 (abstract #107) results were presented on anti-hACE2 antibodies as prophylactic

agents effective against, in theory, any current and future SARS-CoV-2 variants, as well as other hACE2-binding sarbecoviruses[1126]. P2C-1F11 is an ACE2-mimicking antibody, which in practice acts as a "neutralizing antibody", has a high binding affinity for RBD and confers protection against SARS-CoV-2 infection in mice[1127]. S2K146 is another antibody that acts by ACE2 molecular mimicry, protects hamsters challenged with the Beta variant and none of the single mutations in the Omicron variant affected antibody binding[1128]. In fact, one study revealed that S2K146 had greater neutralizing potency against Omicron compared to the ancestral virus[1129], probably because Omicron has a higher binding affinity for ACE2[1130]. This set of results encourages clinical studies of these types of mAbs. These approaches could lead to the development of novel potential therapeutic and/or prophylactic agents capable of circumvent the mutations found in the emerging variants.

Finally, the joint EMA-FDA workshop (December 2022) discussed the efficacy of mAbs in the context of rapidly evolving SARS-CoV-2 variants. It highlighted the importance of using an immunobridging-based biomarker approach (geometric mean titer [GMT] of neutralizing antibodies at specific time points) and concluded that there is a need to accelerate the development of new mAb products against emerging variants (especially in the context of pre-exposure prophylaxis for immunocompromised patients)[1116].

Other therapies

Interferons

Many potential interferon treatments have not been successful; for example, a phase 2 study found interferon beta-1a SNG001 (SPRINTER) reduced the risk of COVID-19 severity[1131], but the phase 3 clinical trial did not support those findings[1132]. Results presented at CROI 2023 (abstract #169) showed that inhaled nebulized SNG001 did not reduce SARS-CoV-2 RNA levels in the nasopharynx nor decrease the time to improvement of COVID-19 symptoms in outpatients. Along the same lines, interferon beta-1a plus remdesivir was not superior to remdesivir alone in hospitalized patients, and even proved to be worse than placebo in the group of patients requiring high-flow oxygen[1133]. However, interferon λ may accelerate the decline in viral load and viral clearance in outpatients with COVID-19[1134]. Additionally, a recent study indicates that interferon λ reduces COVID-19 hospitalizations by 50%[1135]. Nevertheless, for the time being, the FDA does not recommend the use of interferons for the management of COVID-19 (except in clinical trials)[1032].

Convalescent plasma

Convalescent plasma has been used for many years and has proven to be effective in several cases of acute infections[1136–1139]. However, the benefits of convalescent plasma on COVID-19 remain controversial[1140–1143]; most clinical studies suggest that infusion of convalescent plasma does not have an effect on the course of COVID-19 disease, nor does it reduce viral

load levels[1144–1149]. A group of researchers in the UK stopped a trial involving 10,000 people when they observed that the convalescent plasma had no beneficial effect[1146,1150], so did the NIH[1151], and therefore health authorities (FDA, EMA, WHO) have restricted or not recommended it in their guidelines as a therapy for COVID-19, except in the case of immunocompromised patients or those receiving immunosuppressive treatment, where it appears to be beneficial[1152–1154]. China indicates that it can be used in patients with high-risk factors, high viral load and rapid disease progression in the early stage of the disease[1026].

Hyperimmune immunoglobulin

Partly because convalescent plasma did not show promising results, hyperimmune immunoglobulin (hIG) was evaluated as a possible therapeutic option. This product contains a concentrate of purified antibodies obtained from a pool of convalescent COVID-19 plasmas or prepared from animal sources through immunization[1155]. hIG has been used for the treatment and prophylaxis of several viral infections, including cytomegalovirus, varicella, rubella, and hepatitis A and B[1156–1159]. Despite the fact that hyperimmune immunoglobulins may have several effector functions *in vitro* against SARS-CoV-2, including neutralizing activity[1160,1161], and that a few studies have shown some positive effect in reducing the risk of disease progression[1162,1163], the body of evidence suggests that hIG products do not demonstrate clear clinical efficacy[1155,1164,1165] and therefore are not recommended in COVID-19 treatment guidelines. However, like convalescent plasma, it may be beneficial in immunocompromised patients[1163].

Immunomodulators, other drugs and supplements

Because the hyper-inflammatory response to SARS-CoV-2 infection plays a central role in COVID-19 pathogenesis[1166,1167], the use of immunomodulators is recommended in some cases.

The FDA's treatment guidelines include the use of the following immunomodulators: corticosteroids (dexamethasone), interleukin-6 (tocilizumab or sarilumab), interleukin-1 (anakinra) and Janus kinase (baricitinib or tofacitinib) inhibitors[1032]. At present, there is not sufficient evidence to include inhaled corticosteroids as immunomodulatory therapy, and it does not recommend the use of canakinumab (IL-1 inhibitor) for the treatment of COVID-19[1032]. Additionally, in patients with a high suspicion of thromboembolic disease, those requiring extracorporeal membrane oxygenation, continuous renal replacement therapy, or have thrombosis related to extracorporeal catheters or filters, the FDA recommends anticoagulant (heparin) and antiplatelet therapy (not recommended in non-hospitalized patients, nor as prophylaxis after hospital discharge)[1032].

WHO suggests not to use tofacitinib or ruxolitinib (Janus kinase inhibitors), and consider them only if neither baricitinib nor IL-6 receptor blockers are available[1168]. In December 2022, the company (Lilly Netherlands) withdrew its application to the EMA for the use of baricitinib as a COVID-19 treatment[1169].

Tocilizumab is an anti-IL-6R (interleukin-6 receptor) mAb used for the treatment of rheumatoid arthritis and other inflammatory pathologies. Different clinical trials provide evidence that tocilizumab, when administered together with corticosteroids, shows a moderate survival benefit in patients with severe or critical COVID-19[659,1170,1171]. However, other research groups did not observe a significant benefit[1172–1174]. FDA recommends its use in COVID-19 patients requiring oxygen in combination with dexamethasone. The EMA and WHO also recommend it in their therapeutic guidelines[1032–1034]. The NMPA (China) approved its marketing[1026,1057]. The FDA does not recommend the use of siltuximab, as the evidence is currently limited, but does recommend sarilumab when tocilizumab is not available or not feasible to use[1032].

Anakinra is another drug used for rheumatoid arthritis. It is a recombinant protein that emulates the human IL-1 receptor antagonist. Some studies suggest that anakinra is beneficial for severe COVID-19 patients by reducing both the need for invasive mechanical ventilation and mortality risk of hospitalized non-intubated patients[1175–1177], but another publications concludes that anakinra has no effect on hospitalized adults with SARS-CoV-2 infection[1178–1180]. The FDA indicates that the evidence is insufficient to include anakinra in its treatment guidelines[1032]. The EMA has authorized its use for COVID-19 patients[1034].

Regarding to corticosteroids, China recommends the use of dexamethasone or methylprednisolone in very specific cases and warns about possible side effects due to high doses and long-term use[1026]. The WHO (and the FDA) recommends the use of dexamethasone in patients with severe or critical COVID-19; alternative acceptable regimens include hydrocortisone, methylprednisolone and prednisone[1032,1168].

At CROI 2023 (abstract #542), a Spanish study indicated that daily zinc supplementation during the acute phase of SARS-CoV-2 infection resulted in lower rates of severity (less deaths and ICU admissions) and faster clinical recovery along with shorter hospital stay. However, scientific evidence is still insufficient to recommend either for or against the use of supplements such as Vitamin C, D and Zinc[1032].

The use of antipyretics, analgesics and antitussives is adequate to reduce symptoms such as fever, headache, myalgia and cough[1181]. Importantly, contraindications and interactions with other drugs should be considered in all the treatments mentioned above. In addition to efficacy, tolerability, allergic reactions and other side effects must be closely monitored.

The prone position has been shown to improve oxygenation in patients with hypoxic respiratory failure and, in some cases, reduces mortality in patients with moderate to severe ARDS when used early and for prolonged periods[1182–1186]. In certain cases COVID-19 may be beneficial[1187–1189]; however, because the evidence is not clear, it is not mentioned as a COVID-19 therapy, except in Chinese treatment guidelines[1026]. China also mentions traditional Chinese medicine therapy in its guidelines[1026].

Other supportive treatments and psychological interventions according to the clinical status and condition of the patient should be considered.

Preventive measures and vaccines

Prevention

"Prevention is better than cure" and *"primum non nocere"* are two maxims of the precautionary principle, which have deeply touched society after suffering one of the most devastating pandemics (from different perspectives) of recent times.

In the beginning, long before the first vaccines, some countries, such as Sweden, had opted—or so it is presumed—for rapidly achieving herd immunity through natural infection, and did not recommend the use of face masks or the closing of public spaces, which led Sweden to have the highest mortality rate in all of Europe, even ten times higher than its neighboring country, Norway[1190]. However, at the other extreme, the zero COVID policy applied in China for almost 3 years (from January 2020 to December 2022) was also strongly criticized due to the aggressive quarantine, blockade and control measures[1191,1192]. "Living with COVID-19" has been one of the most balanced strategies[1193,1194].

Different prevention measures were read and heard daily in the mass media (**Figure 25**). All these measures were tremendously effective in reducing the number of infections[1195–1197]: the use of masks, keeping a safe distance, and some human practices that we should internalize, such as proper hand washing, coughing or sneezing by covering your mouth and nose with your elbow, ventilation of enclosed spaces and disinfecting surfaces. In addition, vaccines represent the best weapon available against coronavirus and do not cause a disruption of our social structure. The use of anti-SARS-CoV-2 monoclonal antibodies as pre- or post-exposure prophylaxis is not recommended due to the substantial loss of efficacy against the newly prevalent Omicron subvariants[1032]. Currently, there is insufficient scientific evidence to either support or refute the use of supplements, such as vitamin C, D, and Zinc, as a preventive measure against COVID-19[1032].

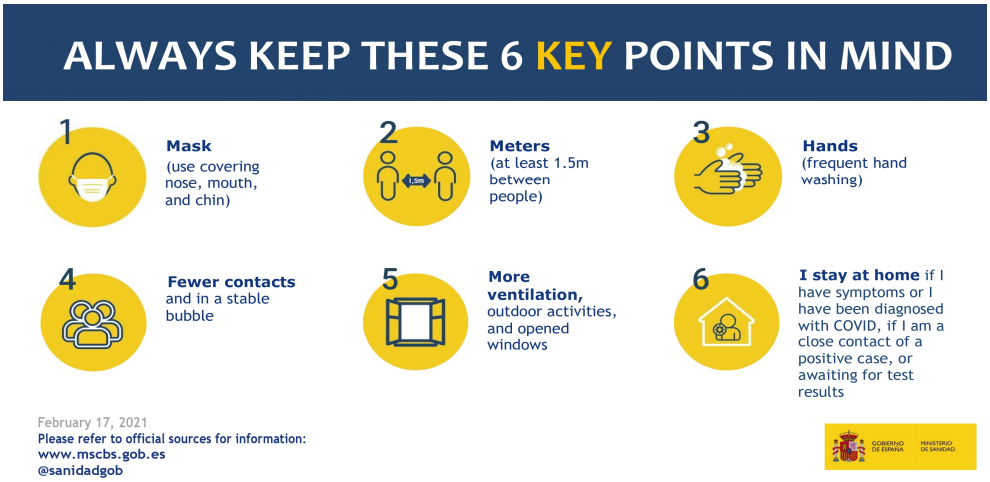

Figure 25 | COVID-19 prevention measures. 6 key points to prevent SARS-CoV-2 infection recommended by the Spanish government. *Adapted from the Spanish Ministry of Health, 2021.*

Vaccines

We moved from the miasma theory and the Hippocratic humoral theory during the first pandemics, to one of the most successful medical interventions against infectious diseases and one that has saved more lives in all of human history: vaccines[1198]. In addition, vaccines are one of the most cost-effective measures that have benefited society as a whole[1199–1203]. Thanks to vaccination campaigns, smallpox (in 1980) and rinderpest (in 2011) were fully wiped out from the face of the earth, and there are many other infectious diseases on the path towards eradication (poliomyelitis, measles, mumps, etc.)[1204].

Vaccines have transformed public health by protecting against more than 20 life-threatening diseases[1205]; however, more successful vaccines have yet to be developed to fight numerous human and animal pathogens; for instance, against another major pandemic that claims millions of lives annually, AIDS, for which after 40 years (since 1981), there is still no effective vaccine to combat HIV, making it one of the greatest challenges of modern medicine.

As a result of the COVID-19 pandemic, we have witnessed the largest vaccination campaign in history, in just over 2 years more than 13 billion doses have been administered globally[49]. This global rollout, in terms of speed, scale, and the diverse populations reached, is an unprecedented historic achievement. Humanity is getting closer and closer to reaching 70% immunization coverage against COVID-19, which was the original target for achieving herd immunity[1206]. However, as a society, we cannot ignore the colossal differences in the distribution of vaccines (**Figure 26**); while some countries have surpassed the 90% rate of their population with the initial full vaccination schedule (all of them high-income economies, e.g. United Arab Emirates, Qatar, Chile), others do not even reach 5% (all of them low or lower-middle income economies, e.g. Burundi, Haiti, Yemen, Papua New Guinea)[62,1207].

Both prior knowledge about SARS-CoV and MERS-CoV, and decades of R&D on mRNA-based vaccines led to the rapid development (in less than a year) of highly effective vaccines against SARS-CoV-2[867,1208–1210]. To date, 50 COVID-19 vaccines, based on different technologies, have been authorized by at least one country, and more than 350 vaccines continue to be developed[1211,1212]. The WHO includes 11 vaccines in its emergency use list, the EMA has authorized 8 vaccines for use in the European Union, while the FDA has only authorized 4 vaccines for use in the United States (see **Table 2** for more details), without considering Omicron-adapted vaccines. It is worth noting that the EMA recently (30 March 2023) recommended the authorization of the first Spanish COVID-19 vaccine developed by HIPRA in collaboration with multiple hospitals and scientific institutions, including IrsiCaixa AIDS Research Institute[1213].

All FDA and/or EMA approved COVID-19 vaccines are for intramuscular injection, however, there are studies in development for intranasal vaccines[1214,1215]. Intranasal vaccines target the nasal mucosa, which is the gateway for SARS-CoV-2, hence successful stimulation of mucosal immunity at this level could further reduce transmission and provide sterilizing immunity[1216,1217]. In September 2022, China approved an aerosolized version of the Convidecia vaccine, named “Convidecia Air”, for use as a booster dose[1218–1220]. iNCOVACC (BBV154) is

an intranasal vaccine that has been shown to be effective in preclinical and clinical trials and has been approved in December 2022 by India[1221–1225].

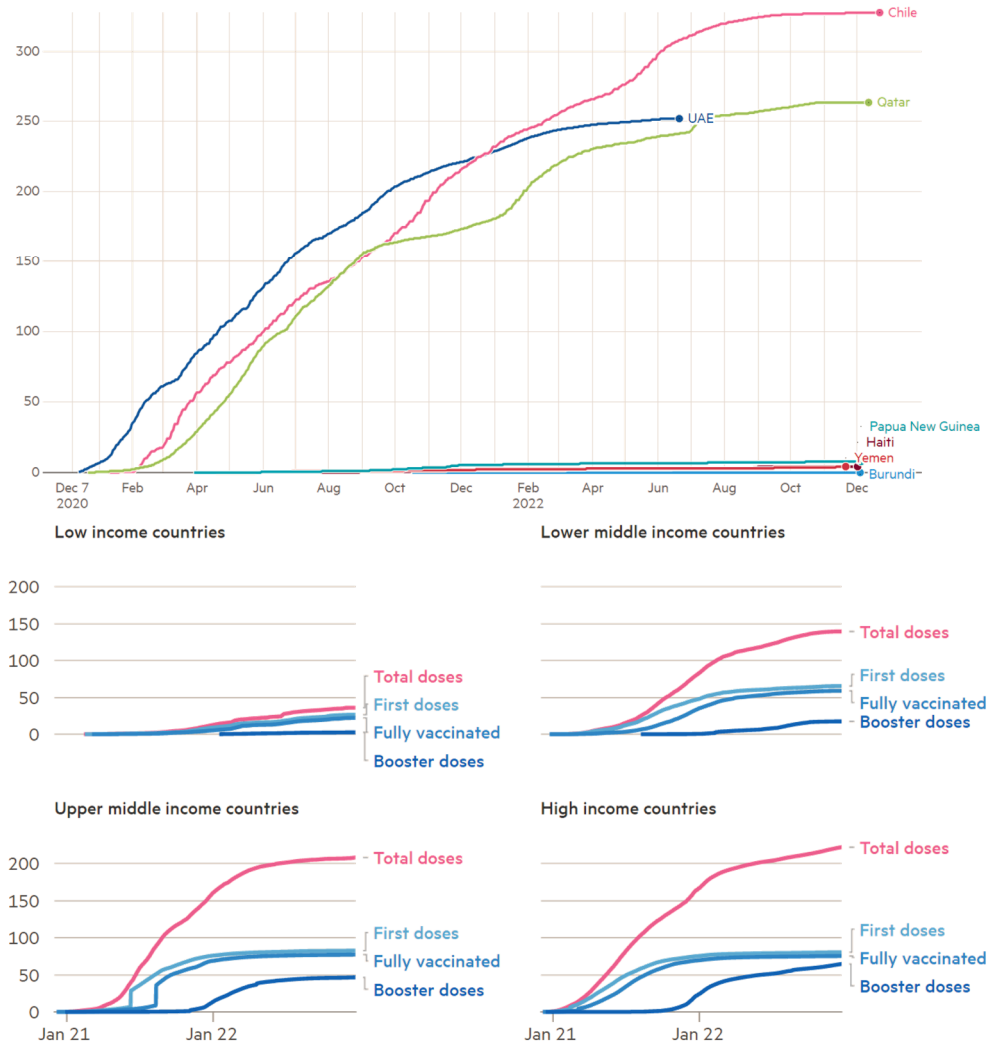

Figure 26 | Cumulative COVID-19 vaccination doses. The upper panel shows the total doses administered per 100 residents for the countries with the highest and lowest vaccination rates. The lower panel shows total, first, full and booster doses administered per 100 residents by income classification according to the World Bank. *Retrieved with permission from FinancialTimes.com (Covid-19 vaccine tracker: the global race to vaccinate, by FT Visual & Data Journalism team, 23 December 2022). © The Financial Times Limited 2023. All Rights Reserved.*

There are at least four main goals of COVID-19 vaccination: protection against the acquisition of SARS-CoV-2, prevention of transmission, protection from severe disease and death, and prevention of long COVID[1226]. In this sense, and achieving the goals, it is estimated that the vaccines prevented nearly 20 million potential COVID-19 deaths worldwide in the course of

one year[794]. Vaccination reduces the probability of SARS-CoV-2 transmission[1227–1230], is effective against infection and reinfections[1231–1235], and reduces the likelihood, severity and duration of long COVID[1236,1237]. Additionally, COVID-19 vaccines have been demonstrated to be safe—for the most part, in children, and pregnant women, causing mild and transient adverse events[1238–1242]. Importantly, some COVID-19 vaccines may present a higher risk (albeit low incidence) of serious adverse events than other COVID-19 vaccines[1243–1252].

Table 2 | Vaccines currently included in WHO, EMA and/or FDA authorization lists

Name and Company	Type	Doses	Virus/Variant	WHO	EMA	FDA	Countries
Comirnaty (BNT162b2) [Pfizer/BioNTech]	mRNA	2	Ancestral Ancestral+BA.1 Ancestral+BA.4/5	●	●	●	149
Spikevax (mRNA-1273) [Moderna]	mRNA	2	Ancestral Ancestral+BA.1 Ancestral+BA.4/5	●	●	●	88
Jcovden (Ad26.COV2.S) [Janssen]	Non-Replicated Viral Vector	1	Ancestral	●	●	●	113
Vaxzevria (AZD1222) [Oxford/AstraZeneca]	Non-Replicated Viral Vector	2	Ancestral	●	●		149
Convidecia (Ad5-nCoV) [CanSino]	Non-Replicated Viral Vector	1	Ancestral	●			10
Covishield [Serum Institute of India]	Non-Replicated Viral Vector	2	Ancestral	●			49
Covovax [Serum Institute of India]	Protein Subunit	2	Ancestral	●			6
Nuvaxovid (NVX-CoV2373) [Novavax]	Protein Subunit	2	Ancestral	●	●	●	40
Covaxin (BBV152) [Bharat Biotech]	Inactivated	2	D614G	●			14
Covilo (BBIBP-CorV) [Sinopharm]	Inactivated	2	Ancestral	●			93
CoronaVac [Sinovac]	Inactivated	2	Ancestral	●			56
COVID-19 Vaccine (VLA2001) [Valneva]	Inactivated	2	Ancestral		●		33
VidPrevtyn Beta [Sanofi Pasteur/GSK]	Protein Subunit	Booster	Beta		●		30
Bimervax (PHH-1V) [HIPRA]	Protein Subunit	Booster	Alpha+Beta		●		30

The number of doses indicated refers to the first series of vaccines, without considering the doses of vaccines adapted to the new variants or the booster doses. The last column indicates the number of countries where the original vaccine has been approved or licensed for emergency use. WHO: World Health Organization; EMA: European Medicines Agency; FDA: U.S. Food and Drug Administration.

Initial reports showed 94-95% protection from symptomatic COVID-19 for mRNA vaccines[1253,1254], although its effectiveness decreases over time and with the emergence of more immuno-resistant viral lineages[1255–1258]. This led to the promotion of booster doses administration and the development of vaccines adapted to new variants. Both strategies have shown evidence of restoring the effectiveness of primary vaccination and improving immunity against the new SARS-CoV-2 variants[636,1258–1261].

Heterologous vaccination, booster doses (with monovalent or bivalent vaccine) and "hybrid immunity" (conceived as immunity conferred by vaccination in previously infected individuals and/or with breakthrough infections) have been shown to positively impact on the immune response and provide additional protection against severe SARS-CoV-2 infection[574–578,636,1262–1265], with comparable levels (breakthrough infection and severe outcomes) between patients with and without immune dysfunction (abstract #214, CROI 2023).

In addition to the decline in vaccine efficacy due to the antibody decay over time and the emergence of new variants, other major concerns, such as global access and vaccine hesitancy, have had a significant impact on the course of the pandemic[1266–1268]. Vaccine hesitancy has led to enormous differences in vaccination rates, especially in children[1269–1272]. For example, in Chile, one of the countries with the highest COVID-19 vaccination rates in the world, about 90% of children aged 3 to 17 years are fully immunized; in Spain, the vaccination rate in children barely exceeds 50%, while in other European countries, such as France, Switzerland and England, less than 5% of children are vaccinated against SARS-CoV-2[1273]. This issue raises concerns considering the widespread availability of authorized vaccines for that specific age range and the non-negligible, albeit relatively low, risk of hospitalization, death, and MIS-C in this pivotal segment of the population[1274–1278].

Vaccines should safely and successfully stimulate an immune response that confers protection against infection and/or disease upon subsequent exposure to a pathogen[1279,1280]. In general, COVID-19 vaccines induce both cellular and humoral responses, although to a lesser magnitude in elderly and immunosuppressed patients[1281–1285]. In addition, many factors can impact on immune responses. For example, intrinsic, extrinsic, environmental, behavioral or nutritional factors, or even circadian rhythm, could influence the immune response acquired by vaccination and/or infection[1286–1292]. This highlights the multifactorial nature of the immune response elicited by vaccination (and infection), and it is worth emphasizing that the combined response of all immune arms is important to provide a successful local and systemic response. However, at present, the major correlate—but not exclusive—of protection (common to all COVID-19 vaccines) is attributed to the induction of NAbs[601–604,1293].

Several COVID-19 vaccines induces IgG and IgA in serum, saliva and breast milk (at different levels)[1294–1298] and higher antibody titers are associated with higher estimates of efficacy[1299]. However, Tang and colleagues found that COVID-19 mRNA vaccination induced significantly lower levels of neutralizing antibodies (against multiple variants) in bronchoalveolar fluid when compared to convalescent individuals[569]. The same paper showed that vaccination induced significant circulating S-specific B and T cell immunity that were absent in the bronchoalveolar lavage fluid[569]. However, vaccine-elicited memory B cells are highly

durable and may contribute to protection from disease[385,469]. In addition, SARS-CoV-2–specific memory T cells durably persist after vaccination or infection[385,1300,1301]. Because T cells recognize short linear peptides of 8 to 15 amino acids (beyond the RBD and NTD) that are highly conserved among the different viral variants, T cell responses remain practically intact against Omicron subvariants[417,1300]. In contrast, many vaccines are designed on a stabilized S-glycoprotein, and most of the humoral response is targeted to the highly variable RBD, so the new variants challenge the neutralizing response, and seem to decline rapidly against the Omicron subvariants[1302–1305]. In addition, the vaccines also elicit antibodies with F_c-dependent effector functions[1306–1308], which contribute to the protection from SARS-CoV-2 in combination with neutralization. However, F_c-mediated effector functions seem to be better maintained than neutralization across the different variants[1309,1310].

In summary, COVID-19 vaccines induce—at least—binding antibodies, with neutralizing activity and F_c-dependent effector functions, generation of memory B cells, germinal center responses, and specific $CD4^+$ and $CD8^+$ T cells; however, it does not seem to stimulate adequate mucosal immunity and the antibody response declines rapidly[568,571,1311]. In conclusion, vaccines work; however, the complexity of the immune system limits our comprehensive understanding of the mechanisms underlying the immune response elicited by infection and/or vaccination; nevertheless, the message is clear, vaccination is safe and effective, and its benefits far outweigh the potential risks.

2

HYPOTHESES AND OBJECTIVES

Hypotheses

Considering the current knowledge on the humoral immune response against human coronaviruses[1020,1312–1318], the apparent impact of clinical and/or demographic parameters on the levels of antibodies developed against some of them[1319–1322], the potential for cross-reactivity among related coronaviruses[1323–1325], and that NAbs represent a robust surrogate for protection against viral infection in several vaccines[590–597], our hypotheses are as follows:

1. The knowledge of the durability of neutralizing immune responses against SARS-CoV-2 will help to define the vaccination strategies.
2. The interindividual heterogeneity in the magnitude of the neutralizing response to SARS-CoV-2 could be explained by clinical-demographic factors.
3. Previous SARS-CoV-2 infection may modulate the magnitude and quality of neutralizing responses to COVID-19 vaccines.
4. Repeated exposure to antigens may modulate the magnitude and quality of neutralizing response in terms of cross-neutralization of viral variants.

Aim

To characterize the neutralizing humoral immune responses elicited by natural infection, vaccination, and combination thereof, against the original SARS-CoV-2 virus (WH1) and its major variants at short, mid and long-term.

Objectives

1. To develop a SARS-CoV-2 pseudovirus-based neutralization assay.
2. To longitudinally evaluate the SARS-CoV-2 neutralizing humoral response induced by vaccination, infection, and the combination of both events.
3. To identify the factors associated to the magnitude of the neutralizing response after infection.
4. To evaluate the cross-neutralizing responses against different SARS-CoV-2 variants.

3

RESULTS

Part I: SARS-CoV-2 infection elicits a rapid neutralizing antibody response that correlates with disease severity

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SARS-CoV-2 infection elicits a rapid neutralizing antibody response that correlates with disease severity

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The protective effect of neutralizing antibodies in SARS-CoV-2 infected individuals is not yet well defined. To address this issue, we have analyzed the kinetics of neutralizing antibody responses and their association with disease severity. Between March and May 2020, the prospective KING study enrolled 72 COVID-19+ participants grouped according to disease severity. SARS-CoV-2 infection was diagnosed by serological and virological tests. Plasma neutralizing responses were assessed against replicative virus and pseudoviral particles. Multiple regression and non-parametric tests were used to analyze dependence of parameters. The magnitude of neutralizing titers significantly increased with disease severity. Hospitalized individuals developed higher titers compared to mild-symptomatic and asymptomatic individuals, which together showed titers below the detection limit in 50% of cases. Longitudinal analysis confirmed the strong differences in neutralizing titers between non-hospitalized and hospitalized participants and showed rapid kinetics of appearance of neutralizing antibodies (50% and 80% of maximal activity reached after 11 and 17 days after symptoms onset, respectively) in hospitalized patients. No significant impact of age, gender or treatment on the neutralizing titers was observed in this limited cohort. These data identify a clear association of humoral immunity with disease severity and point to immune mechanisms other than antibodies as relevant players in COVID-19 protection.

In December 2019, a novel severe acute respiratory disease was reported in China[1]. Following the early identification, in January 2020[2], of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as the etiologic agent of the Coronavirus disease-19 (COVID-19), the new virus rapidly spread to generate a pandemic with a deep impact in global human health. The virus has caused more than 32,800,000 infections and more than 990,000 deaths (as of September 27th, 2020) despite worldwide restrictions in economic activities and mobility.

This massive impact has prompted an unprecedented research taskforce to define the epidemiological features of SARS-CoV-2 transmission, to identify new antivirals and to develop new vaccines able to generate protective immunity against the virus[3,4]. To guide vaccine development, the understanding of the interplay between the virus and the immune system as well as the definition of protective mechanisms have also been established as research priorities[5]. The current knowledge indicates that COVID-19 patients elicit a rapid humoral response

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	Uninfected n = 6	SARS-CoV-2 infected		p-value
		Non-Hospitalized n = 32	Hospitalized n = 40	
Gender. Female, N (%)	3 (50)	19 (59)	13 (33)	**0.066**[a]
Age (years), Median [IQR]	50 [43–62]	51 [42–55]	63 [56–70]	**< 0.0001**[b]
Days from symptoms Median [IQR]	–	27 [18–30]	28 [13–35]	ns[b]
Hospitalization days Median [IQR]	–	–	22 [16–28]	**< 0.001**[c]
Severity n (%)				
Asymptomatic	NA	7 (22)	0 (0)	
Mild/asymptomatic	NA	25 (78)	0 (0)	
Hospital Non-severe	NA	0 (0)	13 (33)	
Hospital Severe	NA	0 (0)	22 (55)	
Hospital ICU	NA	0 (0)	5 (13)	
Treatment, N (%)				
Corticosteroids	0 (0)	0 (0)	20 (50)	
Tocilizumab or equivalent	0 (0)	0 (0)	11 (28)	
OHCQ or CQ	0 (0)	1 (4)	39 (98)	
Type I IFN	0 (0)	0 (0)	8 (20)	
PI	0 (0)	0 (0)	17 (43)	
Exitus, N (%)	0 (0)	0 (0)	4 (10)	

Table 1. Description of participants. Bold values indicate statistically significant differences. *NA* Not applicable. [a]Fisher exact test. [b]Kruskal–Wallis rank sum test. [c]Mann Whitney test.

against the virus, all of them seroconverting 19 days after symptom onset, with heterogeneous kinetics of IgM and IgG subclasses[6]. Elicited antibodies show reactivity against multiple viral proteins including the outer Spike (S) protein, which is the target of neutralizing antibodies. These include mainly, but not exclusively, antibodies blocking the binding of the S protein to the ACE-2 receptor through interaction with different epitopes of the receptor binding domain (RBD)[7–13]. These antibodies, which are elicited in most infected individuals, are able to protect golden Syrian hamsters from acquisition of SARS-CoV-2 infection[12,14], and are thought to play a relevant role in viral clearance after infection[15]. Consistently, different S protein-based vaccines are able to induce neutralizing responses and mediate protection in different animal models[16]. In contrast, the implication of antibodies in exacerbated inflammatory responses and in antibody-dependent enhancement of infection (ADE) phenomena are among the potential drawbacks of the humoral response in COVID-19 patients[15].

Most of the knowledge generated on humoral responses against SARS-CoV-2 is based on severe/hospitalized patients. However, epidemiological data indicate that up to 80% of infected individuals undergo mild symptoms[17]. Importantly there is an undetermined number of infected individuals (reaching 40% in some studies) that do not develop symptoms[18]. Given the high percentage of mild and subclinical cases, the analysis of these individuals may be valuable to understand the global kinetics of herd immunity against the virus.

Here, we longitudinally assessed 72 patients from North Barcelona area displaying a wide range of clinical manifestations (from critical to asymptomatic infection) and we have systematically evaluated their ability to generate neutralizing antibodies. Our data show a rapid elicitation of neutralizing antibodies in hospitalized patients reaching 80% maximal levels 17 days after symptoms onset. In contrast, mild-symptomatic and asymptomatic patients developed lower and sometimes undetectable neutralizing antibodies. These data associate humoral immunity with disease severity and point to immune mechanisms other than neutralizing antibodies as relevant players in COVID-19 protection.

Results

Description of participants. The KING study recruited 78 individuals suspected from COVID-19 symptoms. As shown in Table 1 (and supplementary Fig. 1), six individuals gave negative results in both serologic and molecular diagnostic tests and were included in the control uninfected group, while 72 individuals were found positive for SARS-CoV-2 infection by either serological or nucleic acid detection tests and were monitored longitudinally, when possible. From positive individuals, 32 (44%) did not require hospital admission, most of them were identified by mild symptoms (25 individuals), while seven individuals with no symptoms were identified in routine serologic tests. The hospitalized participants (n = 40) were classified according to severity (cutoff pO_2 saturation 94%) and need of intensive care (Supplementary Fig. 1). One third showed non-severe infection, while 22 patients (55% of hospitalized individuals) were severely affected and 5 required intensive care. The main characteristics of enrolled individuals are shown in Table 1. Significant differences were observed in gender and age ($p < 0.05$) between infected subgroups, with women and young participants being more represented in the non-hospitalized group. The main comorbidities in hospitalized patients were high blood pressure (19 out 40 patients, 47.5%) and respiratory diseases (10 out of 40 patients, 25%), while the main treatments were hydroxychloroquine, corticosteroids and available antivirals other than remdesivir (mainly lopinavir). Most patients received combined treatments that also included anti-IL-6 biologics (mainly tocilizumab) and Interferon-ß (Table 1).

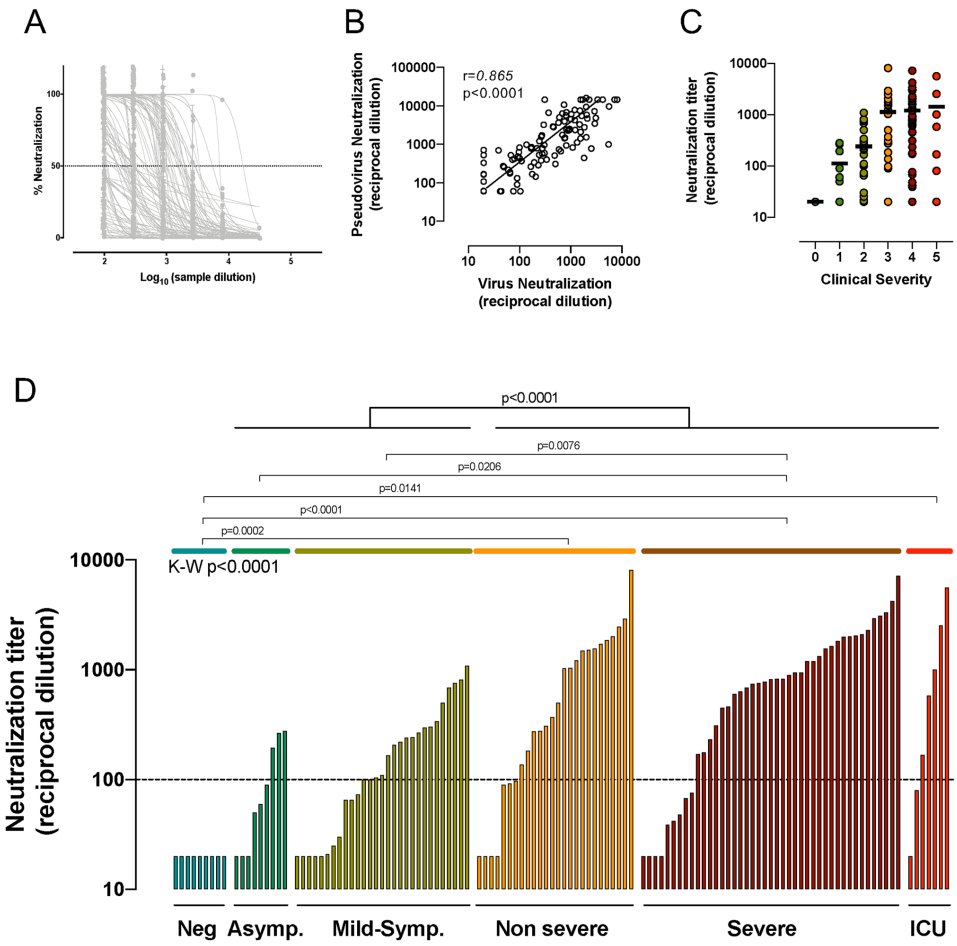

Figure 1. Neutralization activity. (**A**) Dose response of normalized neutralization data for all samples tested against replicative virus in Vero E6 cells (n = 130). (**B**) Correlation between IC_{50} values of plasma samples in replicative virus and pseudovirus neutralization assays (n = 122). Line indicates linear regression for illustrative purposes. Correlation coefficient and p-value (Spearman correlation test) are shown. (**C**) Analysis of the impact of disease severity on neutralization titers (replicative virus assay) for the whole sample set. Individual values, mean values (solid lines) are shown for each group (0 = seronegative, 1 = asymptomatic, 2 = mid-symptomatic, 3 = hospitalized non severe, 4 = severe, 5 = ICU). (**D**) Calculated IC_{50} (reciprocal dilution) in the replicative virus assay for all plasma samples tested grouped by SARS-CoV-2 positivity and clinical grade of symptoms. Comparison between groups was performed by Kruskal–Wallis test (p-value indicated in the Figure) with Dunn's correction for multiple comparisons (indicated in intergroup comparisons). Top p-value indicates the comparison of the whole hospitalized and outpatient groups.

Neutralization assays. A total of 128 plasma samples were assayed for neutralization capacity against the replication of an infectious isolate of SARS-CoV-2 in Vero E6 cells (Fig. 1A)[19] and neutralization titers were determined.

To confirm that neutralization was directly associated with the blockade of S-protein mediated viral entry, a pseudoviral neutralization assay, that uses HIV-based pseudoviruses bearing the SARS-CoV-2 S or the VSV-G proteins, was also developed (see methods). 122 plasma samples were analyzed for pseudovirus neutralization and IC_{50}s were compared with the results obtained with the replicative virus neutralization assay. Figure 1B shows

the strong correlation between the neutralization titers calculated using each method ($r = 0.865$, $p = 0.00001$, Spearman test). This result confirms that plasma-mediated inhibition of fully replicative virus is primarily associated with the presence of neutralizing anti-S antibodies.

Plasma neutralization titers from all infected participants, showed a wide range of activity with a gradual increase in median neutralization activity following disease severity (Fig. 1C). A detailed analysis showed significant differences among disease severity groups ($p < 0.0001$, Kruskal–Wallis test), that was driven by differences between seronegative individuals and hospitalized subgroups and by significant differences between asymptomatic or mild-symptomatic subgroups with severe patients (Fig. 1D, Dunn's multiple comparison test). However, no statistical differences were observed between asymptomatic and mild asymptomatic participants or among hospitalized subgroups. When subgroups were combined in non-hospitalized and hospitalized, the former group showed significant lower levels of neutralizing antibodies compared to individuals requiring hospitalization ($p < 0.0001$, K–W test). Among plasma from infected individuals, 12% of samples reached titers above 2000, with 3 samples, corresponding to three different hospitalized individuals, above 5000. At the other end, 33% of plasma samples showed neutralization titers below 100, mostly corresponding to individuals with mild/asymptomatic infection and early sampled hospitalized individuals (Fig. 1D). All control uninfected individuals showed undetectable neutralizing activity (< 50, reported as 20, Fig. 1D).

Kinetics of neutralizing antibodies. Taking advantage of the wide range of sampling times after symptoms onset, we determined the kinetics of emergence of neutralizing antibodies using nonlinear mixed-effects models. Data from hospitalized patients (who had sampling timepoints closer to symptom onset and longer follow-up periods), allowed for proper fitting of data. Kinetics were similar for severe and non-severe individuals, while ICU participants showed a trend towards faster and higher development of neutralizing activity; however, differences were not statistically significant. Fitting all pooled data showed that half maximal neutralization activity was achieved at day 10.7 (confidence interval, CI 8.3–12.9), while 17.3 days (CI 14–21.1) were required to develop the 80% maximal response, which achieved 3.12 logs (CI 2.9–3.3), i.e. 1584 (CI: 794–1995) reciprocal dilution (Fig. 2A). Interestingly, one individual from the hospitalized group failed to generate detectable neutralizing activity even after 55 days of symptoms. This individual was not included in this analysis.

Data from mild-symptomatic individuals could not be analyzed in the same way owing to different temporal distributions of data (as a consequence of difficulties in obtaining samples short term after infection) and low level of neutralizing titers observed in some individuals with late sampling. Therefore, after discarding the late samples, we analyzed the mean neutralization level overtime yielding a value of 2.4 logs (CI 2.2–2.6), i.e. 234 (CI 158–354) reciprocal dilution (Fig. 2B). The difference of this value with the plateau of neutralizing activity of hospitalized individuals was highly significant ($p < 10^{-7}$ by Z-test, and $p < 10^{-4}$ by Wilcoxon test as described in methods; Fig. 2B), and reflects the different distribution ($p = 0.0003$, Chi-square test) of individuals with undetectable (< 20), low (20–100), medium (100–1000) or high (> 1000) neutralization titers in the non-hospitalized and the hospitalized groups (Fig. 2C). Of note, almost 50% of outpatient (asymptomatic and mild-symptomatic) participants showed low neutralization titers (< 100).

Association of neutralizing antibodies with age and gender. Since hospitalized and non-hospitalized individuals showed differences in age and gender distribution, we analyzed the impact of these parameters on neutralization titers. A positive correlation was observed between maximal individual neutralization titers and age when all individuals were analyzed ($p = 0.03$, Spearman test, Fig. 3A). However, significance was lost when each group (hospitalized and non-hospitalized) was analyzed separately (Fig. 3A, dotted lines), suggesting that the main driver of the correlation is the increased age in hospitalized patients. A two-factor regression model, including age and hospitalization status, showed a strong correlation of neutralizing titers with hospitalization ($p = 0.0001$, Wald test) and a non-significant contribution of age (Table 2). Although we cannot rule out an effect of age due to the limited size of our dataset, these data suggest that severity is the major correlate of neutralizing antibody titer.

For hospitalized patients no correlation was observed between the neutralization capacity and the duration of hospital stay (Fig. 3B). Similarly, unbalanced gender distribution among groups seems to be unrelated to neutralization titer, although barely significant differences were observed when maximal titers of neutralization were compared (Fig. 3C), the kinetics and plateau of female and male participants were similar when a longitudinal analysis was performed (Fig. 3D).

Impact of treatment on neutralizing titers. We analyzed the potential impact of immunomodulatory or antiviral treatments on neutralizing titers. All participants, but one, were on hydroxychloroquine or chloroquine treatment, hampering the analysis of the effect of this drug. For other drugs, analysis was also perturbed by the different combinations administered. When drugs were analyzed individually, no differences were observed between maximal neutralization titers among participants treated with corticosteroids, tocilizumab (or other anti-IL-6 drugs), type-I IFN (mainly IFN-ß) or protease inhibitors (mainly Lopinavir, Fig. 4). Although type-I IFN seemed to negatively impact neutralization titers, this observation is caused by the high incidence of death (4 out of 8 patients) and the shorter sampling time in the IFN-treated group. We approached the analysis of drug combinations by a more general clustering analysis. However, the large amount of combinations and the limited number of participants prevented the identification of any significant relationships between severity, neutralization titer and treatment regimen (Supplementary Fig. 2).

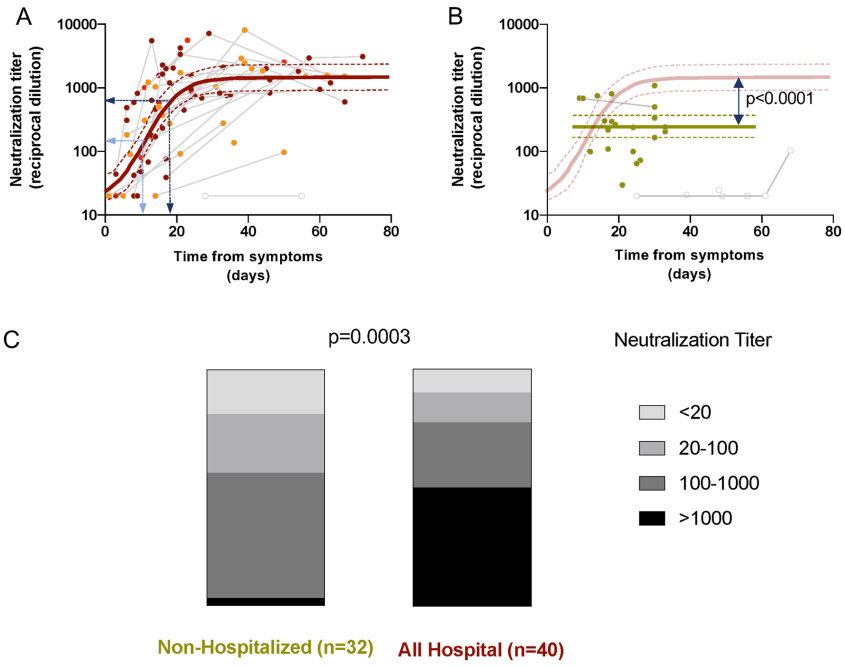

Figure 2. Longitudinal analysis and distribution of neutralization activity. (**A**) Neutralization titers from hospitalized patients were plotted against time from symptoms onset and fitted (solid line). Empty symbols indicate outliers. Light and dark blue arrows indicate the calculated time required to achieve the 50% and the 80% maximal neutralization titer, respectively. Non severe, severe and ICU groups are indicated by orange, maroon and red symbols, respectively. Analysis was performed with all the dataset. (**B**) Neutralization titers from mild-symptomatic individuals were fitted (solid line) after identification of outliers (empty symbols). The comparison of the plateau values for neutralization titers in hospitalized (light maroon line) and mild-symptomatic individuals is shown (Z test). (**C**) Representation of the frequency of undetectable, low, medium and high neutralizing individuals in non-hospitalized and hospitalized (All hospital) patients (p-value of Chi-square test).

Discussion

In this study, we analyzed the development of antibody-mediated neutralizing activity in SARS-CoV-2 infected individuals. We used, either a fully replicative SARS-CoV-2 isolate or a HIV-based pseudovirus exposing the SARS-CoV-2 S protein, similar to other recently reported assays[20–22]. The comparison of both methods yielded a high degree of identity, suggesting that the antiviral activity of plasmas samples is mostly mediated by anti-S protein antibodies (the only SARS-CoV-2 derived protein expressed on the pseudovirus). This comparison also validates the pseudoviral assay as a faster, safer and specific (compared to VSV-G pseudoviruses as control) neutralization screening method.

Our analysis of SARS-CoV-2 infected individuals highlights the association between the development of the neutralizing activity and the clinical course of the infection. First, disease severity appears to be linked to age and gender, with hospitalization rates being higher in both older and male individuals. However, the sub-analyses of hospitalized patients showed no significant differences in neutralization titers according to gender and age. We consider that we do not have enough sample size to be able to correctly assess these questions; therefore, larger studies with longer follow-up will be needed to properly address this issue. Second, hospitalized patients showed a relatively homogeneous development of neutralizing antibodies reaching titers of 3.12 logs. Only ICU cases showed a trend to elicit faster and higher titers, although no clear causality can be established from our data. The global longitudinal analysis showed 50% of response by day 11 and maximal responses (> 80%) attained by day 17 after symptoms onset. These values are similar to those reported for total antibody titers, with 11 and 16 days, respectively[15], suggesting that the early humoral response already contains neutralizing antibodies. This is consistent with the identification of neutralizing antibodies with a low somatic hypermutation that can probably arise during the first germinal center reactions[23]. No clear effect of treatment on the short-term neutralizing activity was observed as none of the treatments analyzed (tocilizumab, corticosteroids, type-I IFN or protease

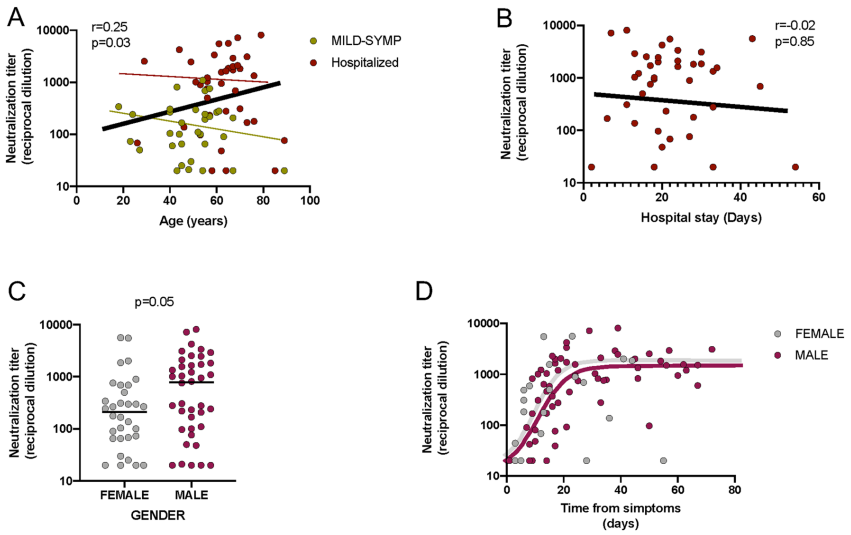

Figure 3. Factors associated with neutralizing responses. (**A**) Correlation between maximal individual neutralizing titers and age. P-value for Spearman's test correlation of all data is shown (solid line), red and green dotted lines indicate correlations for hospitalized and mild-symptomatic individuals, respectively. *P*-value for Spearman's test correlation is shown. (**B**) For hospitalized patients, correlation between neutralizing activity and duration of hospital stay. *P*-value for Spearman's test correlation of data is shown (solid line). (**C**,**D**) Analysis of gender differences in the maximal neutralization titer value of COVID-19 participants (n = 73, **C**) and in hospitalized participants (n = 40, panel D). *P*-values for Mann–Whitney tests is shown.

	Regression coefficient	Standard error	*p*-value (Wald test)
Age	− 0.004	0.006	0.523
Hospitalization	0.752	0.182	0.0001

Table 2. Two-factor regression model to assess the impact of age and hospitalization on neutralizing activity.

inhibitors) were associated with higher or lower magnitude of neutralizing responses. This fact contrasts with reported impact of corticosteroid treatment in long-lasting immunity against SARS-CoV[24]; however, the lack of long-term follow up in our samples impedes a direct comparison. Therefore, we cannot rule out a long-term impact, since immunomodulatory interventions might affect the inflammatory balance and the activation and migration of immune cells to secondary lymphoid organs. Again, the reduced sample size and the large number of treatment combinations limited our ability to assess this issue, larger cohorts with longer follow-up are required.

Importantly, our data show that mild-symptomatic participants exhibited significant lower titers of neutralizing antibodies either analyzed longitudinally or by comparing maximal individual values. Consistently, a relevant fraction, roughly 50%, of mild-symptomatic/asymptomatic patients showed neutralization titers below 100, and among them, a significant fraction of individuals with undetectable activity were also identified. This fact has been also observed by others[23,25–27]; and despite that some neutralizing antibodies have been isolated from those individuals[23], the reasons and the consequences of such a low neutralizing response remain unclear. Exceptionally, we also identified one hospitalized patient with persistent undetectable neutralization titers, despite undergoing severe infection and 33 days hospital stay before recovering.

An obvious risk for patients with low neutralizing capacity is the possibility of reinfection. Although animal models point against this possibility[28], several cases have been reported in humans[29,30], and at least one of them was associated with a poor seroconversion after the initial infection[30]. Dangers of low neutralization titer could be also associated with incomplete antibody mediated protection and ADE, a situation of antibody mediated exacerbation of the infection reported for other coronaviruses[31]. However, this is not the case for individuals with low/undetectable neutralizing activity identified in our study, since they have experienced mild-symptomatic or fully asymptomatic infection. Additionally, the absence of correlation between neutralization capacity and length of hospital stay (in the hospitalized group) could suggests that the presence of neutralizing antibodies

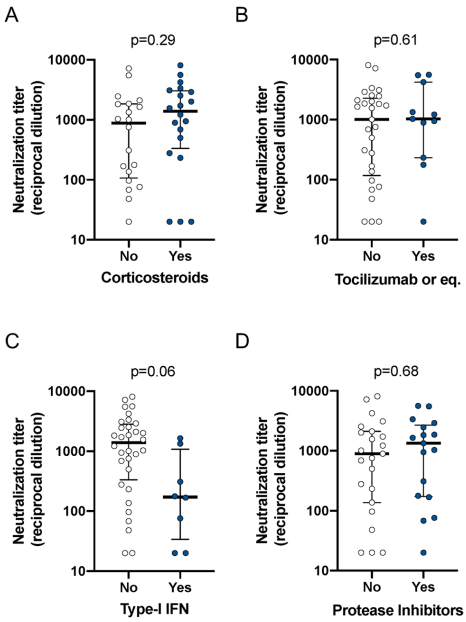

Figure 4. Effect of treatment. Maximal neutralization titers from hospitalized participants (n = 40) were analyzed according to the indicated treatments. Individual values, median and interquartile boxes (25–75) are indicated. *P*-values for Mann–Whitney tests are shown.

is not determinant for the resolution of the disease. This is consistent with published data on SARS-CoV-2[32], but contrasts with a previous study on SARS-CoV patient linking neutralization capacity with shorter illness[33]. Therefore, our data point to a contradictory situation in which neutralization titers do not associate with clinical benefit. In addition, individuals with low antibody responses, far from the doses reported to be protective in animal models[14], seem to have been protected against severe infection. This apparent contradiction should be explained by further exploration of other immunological mechanisms of viral control. Specifically, innate and/or T-cell mediated responses might play a key role promoting sufficient protection in the absence of a wide and potent B cell mobilization. While few data exist on the protective role of innate immunity against SARS-CoV-2[34], a relevant role for T-cell responses has been described[35,36]. The hypothesis of a major role of preexisting SARS-CoV-2 cross reactive T cells is of particular interest in this context. These cells could have arisen in a large fraction (roughly 50%) of SARS-CoV-2 unexposed individuals by previous infections with other human coronaviruses causing common cold[37] and could mediate cross protection as reported in animal models of SARS-CoV and Middle East respiratory syndrome coronavirus infections[38]. Alternatively, the failure to detect neutralizing activity does not rule out the presence of transient albeit low neutralizing responses, which could be sufficient to control early viral replication. Consistent with this hypothesis, low frequencies of RBD-specific B cells have been identified in low neutralizing individuals[23].

Given the seemingly relevance of asymptomatic or mild-symptomatic infection in the global COVID-19 pandemic[18], understanding the mechanisms that control viral pathogenesis will be key to assess the herd immunity (antibody-mediated or not) against SARS-CoV-2.

Materials and methods

Participants. We designed the KING observational study at the Hospital Universitari Germans Trias i Pujol (Badalona, Spain) aimed to characterize virological and immunological features of SARS-CoV-2 infection. The study was approved by the Hospital Universitari Germans Trias i Pujol Ethics Committee Board (reference PI-20-122). Participants were enrolled after a positive test of SARS-CoV-2 infection (either virological test performed by RT-qPCR analysis of nasopharyngeal swabs in routine clinical screenings or serological test performed by in-house ELISA of plasma samples). All methods were carried out in accordance with the principles of the Declaration of Helsinki. All participants provided written informed consent before inclusion. Some of the individuals recruited in the KING cohort have been included in a sub-analysis of humoral responses recently submitted (Rodriguez de la Concepción et al., submitted).

Severity of symptoms was defined by the following criteria. Asymptomatic infection (severity level 1), mild-symptomatic infection requiring medical visit but no hospitalization (severity level 2), symptomatic non-severe infection requiring hospitalization with pO2 saturation above 94% (severity level 3), severe infection requiring hospitalization and reaching pO2 saturation values below 94% (severity level 4) and very severe infection requiring hospitalization and further intensive care unit (ICU) admission (severity level 5).

Samples and COVID-19 tests. When available, nasopharyngeal swabs were obtained at time of inclusion in the study and processed by the routine clinical services. Results were categorized as positive or undetectable (considered negative). No quantitative data on viral load was available from these specimens.

Blood was collected by venipuncture in EDTA vacutainer tubes (BD Bioscience). Plasma was obtained by centrifugation of blood at 1200xg for 10 min and stored at – 80 °C until use. The presence of anti-SARS-CoV-2 antibodies in plasma samples was assayed by ELISA (Rodriguez de la Concepción et al., submitted). Briefly, the anti-6xHis antibody HIS.H8 (2 µg/mL in PBS) was coated overnight at 4 °C in MaxiSorp plates (Nunc). Then, plates were blocked using blocking buffer (BB): PBS/1% of bovine serum albumin (BSA, Miltenyi Biotec) for two hours at room temperature. After that, 50 µL of SARS-CoV-2 S2 subunit at 0.9 µg/mL and recombinant RBD at 0.3 µg/mL (both from SinoBiologicals and prepared in BB), were added and incubated overnight at 4 °C. Plasma samples were incubated at 1/100 dilution in BB for one hour at room temperature. The HRP conjugated- $(Fab)_2$ Goat anti-human IgG (Fc specific) (1/20,000), Goat anti-human IgM (1/10,000), and Goat anti-human IgA (alpha chain specific) (1/20,000) (Jackson ImmunoResearch) were used as detection antibodies. The specific signal for each sample was calculated after subtracting the background signal obtained for antigen-free wells. Negative cutoffs were defined by COVID-19 negative samples run in parallel.

Virus neutralization assay. Plasma samples were inactivated (56 °C, 30 min) before mixing at increasing dilutions (ranging from 1/100 to 1/8100) with 60 $TCID_{50}$/mL of the SARS-CoV-2 isolate Cat01 (accession ID EPI_ISL_418268 at GISAID repository: http://gisaid.org), a concentration that achieves a 50% of cytopathic effect as described previously[19]. Uninfected cells and untreated virus-infected cells were used as negative and positive control of infection respectively. In order to detect any plasma-associated cytotoxicity, Vero E6 cells (ATCC CRL-1586) were equally cultured in the presence of increasing plasma dilutions, but in the absence of virus. Cytopathic or cytotoxic effects of the virus or plasma samples were measured at 3 days post infection, using the CellTiter-Glo luminescent cell viability assay (Promega). Luminescence was measured as relative luminescence units (RLU) in a Fluoroskan Ascent FL luminometer (ThermoFisher Scientific).

Dose response neutralization curves were normalized according to positive and negative controls (% Neutralization = (RLUmax – RLUexperimental)/(RLUmax – RLUmin)*100) and fitted to a four-parameter logistic curve with variable slope using Graph Pad Prism software (v8.3.0). All IC_{50} values are expressed as reciprocal dilution.

Pseudovirus neutralization assay. HIV reporter pseudoviruses expressing SARS-CoV-2 S protein, and Luciferase were generated. pNL4-3.Luc.R-.E- was obtained from the NIH AIDs repository[39]. SARS-CoV-2. SctΔ19 was generated (Geneart) from the full protein sequence of SARS-CoV-2 spike with a deletion of the last 19 amino acids in C-terminal[40], human-codon optimized and inserted into pcDNA3.4-TOPO.

Expi293F cells were transfected using Expifectamine Reagent (Thermo Fisher Scientific, Waltham, MA, USA) with pNL4-3.Luc.R-.E- and SARS-CoV-2.SctΔ19 at a 24:1 ratio, respectively. Control pseudoviruses were obtained by replacing the S protein expression plasmid by a VSV-G protein expression plasmid as reported previously[41]. Supernatants were harvested 48 h after transfection, filtered at 0.45 µm, frozen and titrated on HEK293T cells overexpressing WT human ACE-2 (Integral Molecular, USA). For neutralization assay, 200 $TCID_{50}$ of pseudovirus supernatant was preincubated with serial dilutions of the heat-inactivated plasma samples (see above) for 1 h at 37 °C and then added onto ACE2 overexpressing HEK293T cells. After 48 h, cells were lysed with Britelite Plus Luciferase reagent (Perkin Elmer, Waltham, MA, USA). Luminescence was measured for 0·2 s with an EnSight Multimode Plate Reader (Perkin Elmer).

Neutralization capacity of the plasma samples was calculated by comparing the experimental RLU calculated from infected cells treated with each plasma to the max RLUs (maximal infectivity calculated from untreated infected cells) and min RLUs (minimal infectivity calculated from uninfected cells), and expressed as percent neutralization: $\%Neutralization = (RLU_{max} - RLU_{experimental})/(RLU_{max} - RLU_{min})*100$. IC_{50} values were calculated as described above.

Statistical analysis. Continuous variables were descriptively summarized using medians with 25^{th} and 75^{th} percentiles, and categorical factors were reported using percentages. T-test and chi-square test were used to analyze association of age and gender with the clinical severity of the infection. Association of age with neutralizing titers was analyzed fitting a multivariate linear regression adjusted by clinical severity. We used nonlinear mixed-effects models with an individual based single-level of grouping to model the levels of neutralizing antibodies overtime, estimated since the apparition of symptoms. Models were fitted to a four-parameter logistic function with a constrained lower asymptote set to the limit of detection and three parameters, the inflection point, a scale parameter and the upper asymptote. Individual-specific random effect for upper asymptote was introduced in the model and a first order autocorrelation structure was used to model the within-individuals error variance–covariance structure. In order to analyze differences in antibody concentration between genders and patients with different severity levels, models with covariate-dependent fixed effects were also fitted. Due to the lack of early timepoints in the mid-symptomatic individuals, this group was analyzed separately, estimating the mean level and its standard error of neutralizing antibodies. Comparison of neutralizing antibodies levels between mid-symptomatic and hospitalized groups was assessed in to ways, performing a Z test from estima-

tions and their standard errors (mean level for the former and upper asymptote estimation for the latter) and using Wilcoxon rank sum test to compare antibody levels between mild-symptomatic and hospitalized individuals after 14 days (estimated lower bound to reach the 80% of neutralization level). One individual from the hospitalized group and three from the mild-symptomatic group who failed to generate detectable neutralizing activity were not included in the longitudinal analyses. All analyses were performed with GraphPad Prism 8.4.3 (GraphPad Software, Inc., San Diego, CA) and R version 4.0 (R Foundation for Statistical Computing)[42]. Mixed-effects models was fitted using "nlme" R package.

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Author contributions
B.T., F.T.-F., J.R., E.P., S.M., M.L.R., C.A.-N., C.A.-G., A.B. and R.O. generated experimental data on virus neutralization, pseudovirus neutralization and ELISA. V.U. was in charge of statistical analysis. R.P., L.M., L.R., E.G., M.M., J.P., A.C. collected and analyzed clinical data. A.V., V.G., N.I.-U., J.S., J.C. interpreted and designed specific analysis. J.V.-A., J.C., B.C. and J.B. designed the study. B.T. and J.B. drafted the manuscript. All authors edited and approved the final manuscript.

Competing interests
Outside the submitted work JB and JC are founders and shareholders of AlbaJuna Therapeutics, S.L. BC is founder and shareholder of AlbaJuna Therapeutics, S.L and AELIX Therapeutics, S.L. The other authors declare no competing interests.

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SARS-CoV-2 infection elicits a rapid neutralizing antibody response that correlates with disease severity

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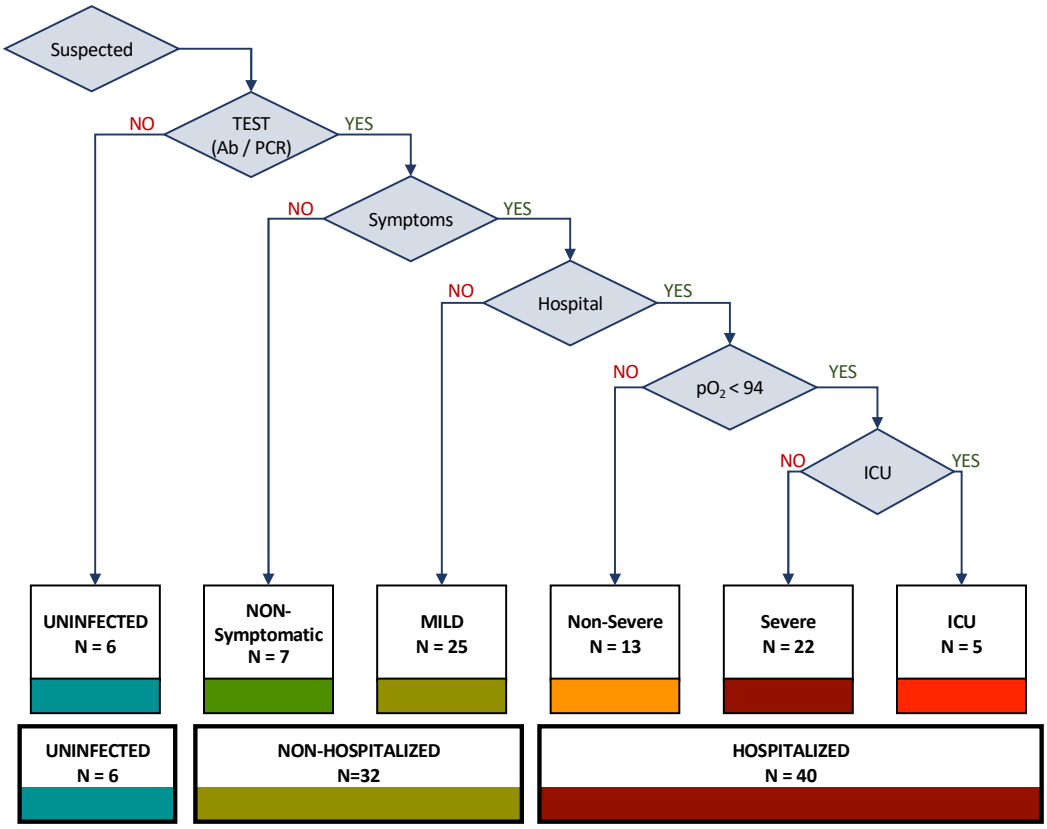
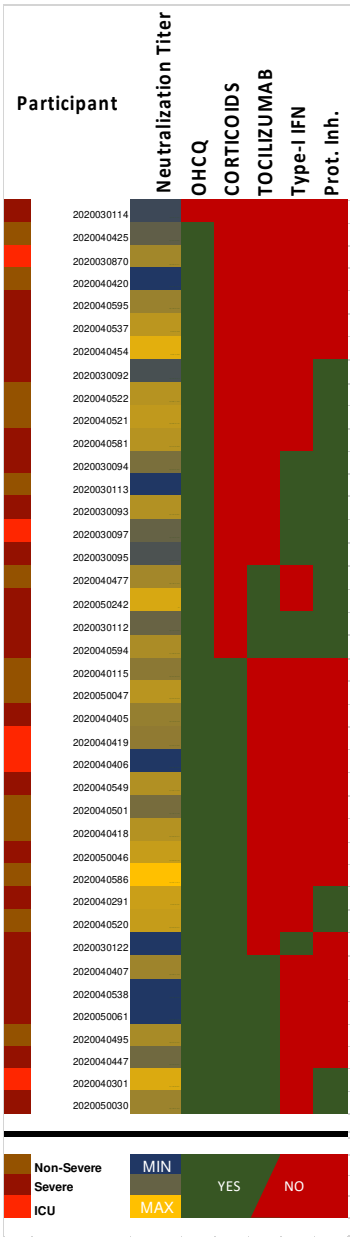
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Patients classification according to symptoms



Supplementary Figure 2. Analysis of neutralizing activity according to drug combinations.

Part II: Stable neutralizing antibody levels 6 months after mild and severe COVID-19 episodes

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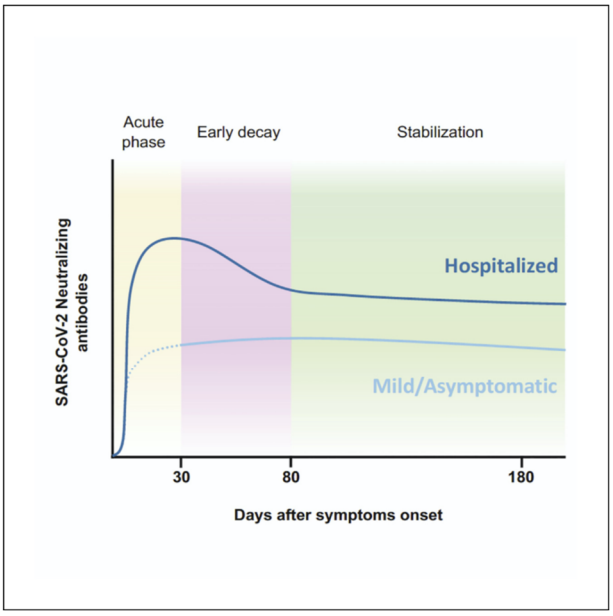
Stable neutralizing antibody levels 6 months after mild and severe COVID-19 episodes



Pradenas et al. describe the kinetics of neutralizing antibodies against SARS-CoV-2 and demonstrate their association with clinical severity and their stability for at least 6 months, despite constant decay of IgG titers. These findings help us to understand the mid-term immune response and the impact on herd immunity.

Edwards Pradenas, Benjamin Trinité, Víctor Urrea, ..., Jorge Carrillo, Bonaventura Clotet, Julià Blanco

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HIGHLIGHTS

Neutralizing activity against SARS-CoV-2 is maintained for at least 6 months

Anti-RBD and anti-S2 IgG titers show a constant decay

Maintenance of neutralizing activity suggests a potential evolution of the immunity

Hospitalized patients maintain higher neutralizing capacity than non-hospitalized

Translation to Patients

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Stable neutralizing antibody levels 6 months after mild and severe COVID-19 episodes

Edwards Pradenas,[1,10] Benjamin Trinité,[1,10] Víctor Urrea,[1] Silvia Marfil,[1] Carlos Ávila-Nieto,[1] María Luisa Rodríguez de la Concepción,[1] Ferran Tarrés-Freixas,[1] Silvia Pérez-Yanes,[1,2] Carla Rovirosa,[1] Erola Ainsua-Enrich,[1] Jordi Rodon,[3] Júlia Vergara-Alert,[3] Joaquim Segalés,[4,5] Victor Guallar,[6,7] Alfonso Valencia,[6,7] Nuria Izquierdo-Useros,[1] Roger Paredes,[1,8] Lourdes Mateu,[8] Anna Chamorro,[8] Marta Massanella,[1] Jorge Carrillo,[1] Bonaventura Clotet,[1,8,9] and Julià Blanco[1,9,11,*]

SUMMARY

Background: Understanding mid-term kinetics of immunity to SARS-CoV-2 is the cornerstone for public health control of the pandemic and vaccine development. However, current evidence is rather based on limited measurements, losing sight of the temporal pattern of these changes.

Methods: We conducted a longitudinal analysis on a prospective cohort of COVID-19 patients followed up for >6 months. Neutralizing activity was evaluated using HIV reporter pseudoviruses expressing SARS-CoV-2 S protein. IgG antibody titer was evaluated by ELISA against the S2 subunit, the receptor binding domain (RBD), and the nucleoprotein (NP). Statistical analyses were carried out using mixed-effects models.

Findings: We found that individuals with mild or asymptomatic infection experienced an insignificant decay in neutralizing activity, which persisted 6 months after symptom onset or diagnosis. Hospitalized individuals showed higher neutralizing titers, which decreased following a 2-phase pattern, with an initial rapid decline that significantly slowed after day 80. Despite this initial decay, neutralizing activity at 6 months remained higher among hospitalized individuals compared to mild symptomatic. The slow decline in neutralizing activity at mid-term contrasted with the steep slope of anti-RBD, S2, or NP antibody titers, all of them showing a constant decline over the follow-up period.

Conclusions: Our results reinforce the hypothesis that the quality of the neutralizing immune response against SARS-CoV-2 evolves over the post-convalescent stage.

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Context and significance

Assessing the durability of neutralizing responses against SARS-CoV-2 is crucial to predict the level of protection in post-convalescent COVID-19 patients. We monitored for >6 months a cohort of 210 SARS-CoV-2-infected individuals with a wide range of symptoms (from asymptomatic infection to severe disease). Our results indicate that neutralizing antibodies are stable for at least 6 months after infection. However, individuals with mild or asymptomatic infection developed lower titers of neutralizing antibodies and could be at higher risk of reinfection. Despite the maintenance of neutralizing antibodies, total antibody titers slowly but gradually declined over time without apparent stabilization. This observation requires further analysis to evaluate the potential role of viral persistence or viral re-exposure in maintaining neutralization titers.

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Fundación Canaria Doctor Manuel Morales and Universidad de La Laguna.

INTRODUCTION

While the early humoral response after severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) infection has been thoroughly described,[1–5] current data on the decay of antibody levels beyond the convalescent stage depict a heterogeneous scenario with limited information on the neutralizing activity throughout the follow-up period.[6–8] Various authors have recently suggested more complex kinetics of neutralizing activity decay as compared to total antibody titers, with clonotype-, epitope-, or subject-specific patterns that evolve in terms of potency and resistance to epitope mutations.[9–11] In this study, we longitudinally evaluated the neutralizing humoral response, in mild/asymptomatic and hospitalized individuals infected by SARS-CoV-2, over a 6-month period. These mid-term kinetics showed stable behavior of the neutralizing response in both groups, despite a clear decrease in the total viral-specific humoral response.

RESULTS

Patient selection and early neutralizing responses

Our analysis included 210 patients with RT-PCR-confirmed SARS-CoV-2 infection, recruited during the first and second waves of the coronavirus disease 2019 (COVID-19) epidemic in Catalonia (northeast Spain). Of these, 106 (50.5%) had a mild or an asymptomatic infection, and 104 (49.5%) required hospitalization because of respiratory compromise (Table 1). As reported in our country,[12] the hospitalization group showed significantly older age and lower frequency of females (Table 1). We collected samples periodically throughout a maximum follow-up period of 242 days (mean follow-up time point of patients from the first COVID-19 wave was 201 days; Figure S1). Most of the study participants developed a neutralizing humoral response against SARS-CoV-2 HIV-based pseudoviruses that was confirmed using infectious viruses.[13] However, in line with trends reported elsewhere,[6,8] mildly affected or asymptomatic individuals developed a 10-fold lower maximal neutralization titer than those who required hospitalization when the full dataset was analyzed ($p < 0.0001$, Mann-Whitney test; Figure 1A). The higher number of determinations obtained from hospitalized individuals during the acute phase permitted the clear observation of a sharp initial response (Figures 1B and 1C), also reported in previous analyses of the early response.[1–5] This was visible for individuals recruited during both the first (March–June 2020) and the second (July–October 2020) waves of the COVID-19 pandemic in Catalonia. A longitudinal analysis fitted to a 4-parameter logistic model of increase defined a 30-day sharpening phase after symptom onset, irrespective of the wave in which hospital admission occurred. Half-maximal neutralization activity was achieved on day 10 (95% confidence interval [CI] 8–11); 80% maximal response, which corresponded to 3.97 logs (i.e., 9,333 reciprocal dilution), was achieved on day 14 (Figure 1D). Moreover, as reported previously using an infectious virus neutralization assay,[13] we could not find a gender impact on the elicitation of neutralizing antibodies in hospitalized individuals. Based on these findings, irrespective of gender and wave, we decided to set day 30 after symptom onset as a starting point for the longitudinal analysis of immune response at the mid-term.

Assessment of mid-term neutralizing responses

The longitudinal modeling of the neutralizing activity at mid-term in our cohort revealed a nearly flat slope (i.e., not significantly different from 0, with a half-life of

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Table 1. Characteristics of individuals included in analysis

	Mild/asymptomatic (n = 106)	Hospitalized (n = 104)	p
Gender, female, n (%)	72 (68)	46 (44)	0.0006[a]
Age, y, median (IQR)	46.5 (38–54)	57.5 (46–66)	<0.0001[b]
Individuals with ≥2 samples, n (%)	52 (49)	59 (57)	0.278[a]
Wave of COVID-19 outbreak (first), n (%)	96 (91)	73 (70)	–
Severity, n (%)			
Asymptomatic	8 (8)	–	–
Mild	98 (92)	–	–
Hospitalized non-severe	–	59 (56.7)	–
Hospitalized severe	–	37 (35.6)	–
Hospitalized (intensive care unit)	–	8 (7.7)	–

IQR, interquartile range (25th and 75th percentiles).
[a]Chi-square test.
[b]Mann-Whitney test.

2,134 days) in individuals with asymptomatic infection or mild disease (Figure 2A). Conversely, the decrease in neutralizing activity in hospitalized individuals showed a 2-phase pattern, with a rapid decay (half-life 31 days) until day 80, which slowed down to a flat slope (half-life 753 days) from that time point on (Figure 2B). In agreement with previous data, suggesting a faster decay of neutralizing antibodies in male compared to female infected individuals,[9,14] we found significant gender differences in early decay; however, upon stabilization of neutralization titers after day 80, no gender impact was observed in our cohort (Figure S2).

The characterization of the neutralizing activity behavior at mid-term should ultimately project the proportion of post-convalescent individuals protected against new infections in the mid- and long-terms. The limited number of measures and lack of a clear threshold of neutralizing activity for preventing SARS-CoV-2 infection precluded assessing this outcome using survival analysis. Alternatively, we explored the neutralizing activity at the end of our 6-month follow-up period. Based on the mixed-effects model obtained from the longitudinal analysis, we estimated a stable mid-term neutralizing activity of 2.72 and 3.16 log for the mild/asymptomatic and hospitalized subgroups, respectively ($p < 0.0001$; likelihood ratio test; Figure 2C, dotted lines). This estimate was consistent with the observed values for the last measurement taken between days 135 and 242, a time frame centered on day 180 (Figure 2C, boxplots). Likewise, the value distribution at this time frame showed significant differences between mild/asymptomatic (median 2.5; interquartile range [IQR] 2.0–3.0) and hospitalized (3.0; 2.7–3.3) individuals ($p = 0.0012$, Mann-Whitney test). To date, no clear cutoff for a neutralizing activity that protects against new reinfection has been established. Nevertheless, data gathered from high attack rate events suggest that neutralizing activities between 1:161 and 1:3,082 are strong enough to prevent infection.[15] Hence, we assumed that reinfections would be unlikely among individuals above the 1:250 cutoff. Of the 23 hospitalized individuals with measurement beyond day 135, 21 (91%) had a mean neutralizing activity value above 1:250 and were thus considered long-term neutralizers. The corresponding proportion in the mild/asymptomatic group (58%; 26/45) was significantly lower ($p = 0.0052$, chi-square test; Figure 2D). Although this number must be considered cautiously due to the cutoff assumption, our finding suggests that hospitalized patients have a higher capacity for long-term neutralization, despite the faster initial decay in neutralization activity.

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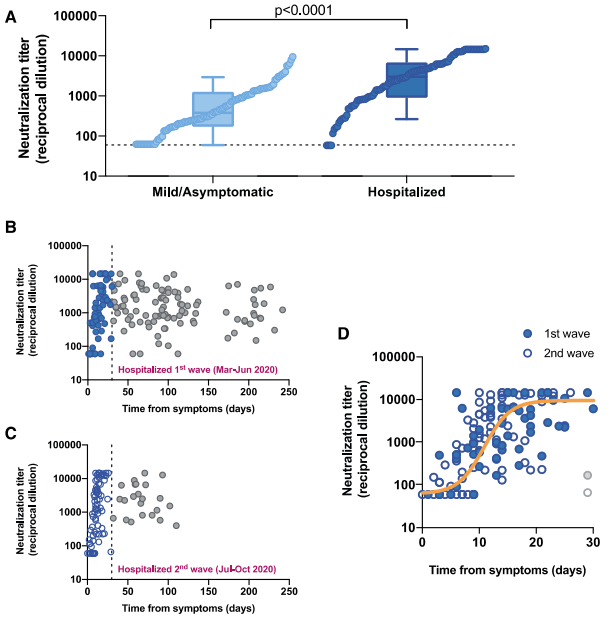

Figure 1. Neutralizing activity among study participants

(A) Maximal neutralization titer of 210 individuals recruited according to disease severity (light and dark blue for mild/asymptomatic and hospitalized individuals, respectively). Boxes show the median and the interquartile range and bars the 10th and 90th percentiles. Distributions were compared using the Mann–Whitney test. Individual values are ranked for comparative purposes.

(B and C) Longitudinal dot plot of neutralizing activity among hospitalized individuals admitted during the first (B) and second (C) waves of the COVID-19 epidemic in our area; filled (B) and empty (C) blue dots show the early (i.e., 30 days after diagnosis) increasing phase.

(D) Magnification of the early phase for individuals admitted during the first (filled symbols) and second (empty symbols) waves. No differences between waves were observed. The solid orange line shows the non-linear fit (mixed-model estimate) for the whole dataset (125 samples, 55 individuals analyzed). Two samples from late seroconverters (1 from each wave, gray dots) were excluded from the analysis.

Comparative analysis of neutralizing responses and immunoglobulin G (IgG) titers

It has recently been proposed that the kinetics of neutralizing activity may not mirror those of antibody titers.[11] Hence, we investigated the change in IgG titers in a subset of 28 individuals (14 in each severity group) with the most extended follow-up period. The analysis included antibodies against the S protein receptor-binding domain (RBD), the main target of SARS-CoV-2-specific neutralizing antibodies[16]; the S2 subunit of the S protein, which may also contribute to neutralizing activity and is more cross-reactive with other coronaviruses[17]; and the nucleoprotein (NP), which is very abundant, albeit unable to neutralize the SARS-CoV-2. The longitudinal analysis revealed a 1-phase significant ($p < 0.0001$) steady decay pattern of all tested antibodies, which was notably faster in anti-NP IgG (Figures 3A–3C). The half-lives of anti-RBD, anti-S2, and anti-NP antibodies for the period beyond day 30 were

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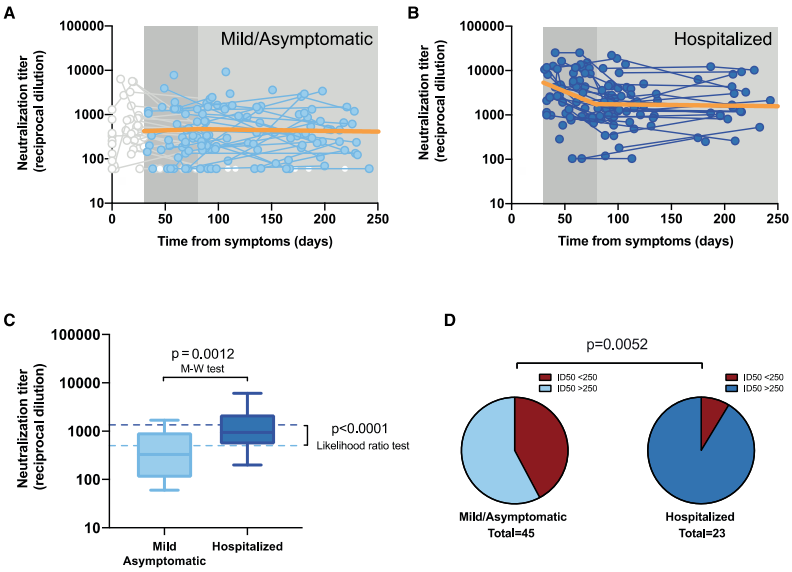

Figure 2. Longitudinal analysis of neutralizing activity

(A) Individual measurements (dots) and linear mixed model (solid orange line) of the longitudinal analysis for mild or asymptomatic individuals beyond day 30 (single-phase slope −0.00014; p = 0.75, likelihood ratio test; estimated half-life 2,134 days). Time points preceding day 30 as well as participants only showing undetectable titers were excluded from the analysis; values are shown but grayed out.

(B) The corresponding analysis for hospitalized individuals (the slopes of the linear fit for the first and second phase were −0.0096 [p = 0.0002] [half-life 31 days] and −00004 [half-life 753 days] [p = 0.78], respectively).

(C) Distribution of neutralizing activity 6 months after infection in both disease severity groups. Experimental values of mean neutralizing activities in the period 135–242 days as summarized in boxplots (as in Figure 1A; Mann-Whitney test for comparative analysis) and modeled data as dotted lines (likelihood ratio test for comparative analysis).

(D) Frequency of long-term neutralizers (i.e., individuals with mean neutralizing activity >250 in the 135–242 days period) in each severity subgroup (chi-square test p value is shown).

86, 108, and 59 days, respectively. These values were consistent with those reported by Wheatley et al.,[11] estimated on a 160-day time frame. Although the limited sample size of this sub-analysis precluded independent modeling of the decay in mild/asymptomatic and hospitalized patients, the latter showed significantly higher titers of anti-S2 at the end of the follow-up period (Figure S3), whereas no significant differences were found in other antibodies regarding disease status. Interestingly, in this subset of individuals, the decay in antibody titers contrasted with the behavior of neutralizing activity, which fitted to a 2-phase model—as in the whole dataset—with a rapid decay until day 80 (slope 0.014, half-life 22 days) and a flat slope (i.e., not significantly different from 0) afterward (Figure 3D).

DISCUSSION

Complementary data on the binding affinity and B cell clone abundance at the same time points would provide a more comprehensive picture to explain this divergent trend. However, our findings support the hypothesis of Gaebler et al.,[10] who suggested that the accumulation of IgG somatic mutations—and subsequent production of antibodies with increased neutralizing potency—allow the maintenance of

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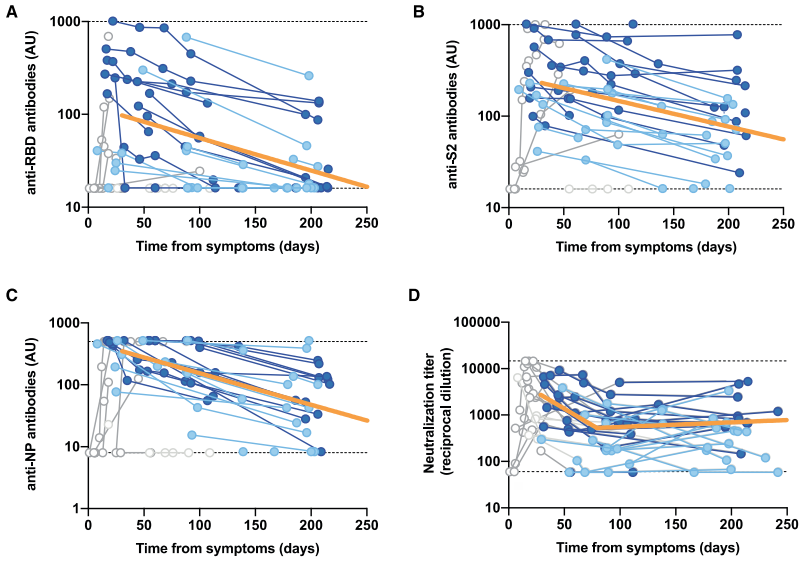

Figure 3. Longitudinal analysis of IgG titers

(A) Anti-receptor binding domain (RBD).

(B) Anti-S2.

(C) Anti-nucleoprotein.

(D) Overall neutralizing activity in the same set of samples. All of the analyses were performed on a subset of individuals with the largest follow-up (n = 14 for mild/asymptomatic in light blue and n = 14 for hospitalized in dark blue; total no. samples 94). Solid orange lines show the linear mixed model estimate for the period beyond day 30.

Kinetics of antibody decay (A–C) were calculated excluding time points preceding the maximal values for each patient. Kinetics of neutralizing antibodies excluded samples preceding day 30 (as in Figures 2A and 2B). All of the excluded values are shown but grayed out.

neutralizing activity levels, despite the decline in specific antibody titers. Of note, our follow-up period encompassed 2 waves of the COVID-19 outbreak in our country. Individuals infected during the first wave were likely to be exposed to high viral pressure in their environment, potentially favoring further virus exposure that may also contribute to maintaining humoral responses, adding to the mechanism proposed by Gaebler et al.[10]

Our longitudinal analysis supplements current evidence regarding mid-term immunity against SARS-CoV-2[8,10,11] and confirms the slow decay and mid-term maintenance of neutralizing activity observed in other cohorts, with a 5%-to-11% prevalence of hospitalized patients.[8,10] In this regard, the 2-phase behavioral pattern of neutralizing activity observed in hospitalized individuals suggests that the rapid decay reported in previous characterizations[7] may be due to the abundance of individuals in this early phase. Furthermore, apparent inconsistencies found between the declines of neutralizing activity and IgG titers reinforce the idea proposed by other authors that the behavior of antibody titers may not mirror the neutralizing activity. Interestingly, differences in decline were observed not only between neutralizing activity and anti-N antibodies, which do not contribute to neutralization, but also for anti-S2 and anti-RBD antibodies, which are major determinants of

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neutralization.[16,17] The current evidence on immunity to SARS-CoV-2 infection suggests stability of neutralizing activity, pointing toward an optimistic scenario for the establishment of infection- or vaccine-mediated herd immunity. Still, long-term data available on other human coronaviruses show waning of antibodies 1–2 years after infection,[18,19] with uncertainty regarding the immune response behavior in the context of vaccine-mediated immunity.[20] The continuity of our prospective cohort of individuals recovered from SARS-CoV-2 infection will provide novel insights into the long-term kinetics of the immune response.

Limitations of Study

Our analysis is limited by the reduced sample size, particularly in the acute phase for mild/asymptomatic subgroup, for which we failed to define the kinetics of neutralizing response development and to identify a 2-phase pattern decay. Despite the limited sample size, the availability of multiple measures along the follow-up period allowed us to provide a longitudinal perspective on neutralizing activity and antibody titer behavior.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- METHOD DETAILS
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- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.medj.2021.01.005.

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AUTHOR CONTRIBUTIONS

J.B. and B.C. designed and coordinated the study. E.P., B.T., S.M., C.A.-N., M.L.R., F.T.-F., S.P.-Y., C.R., E.A.-E., J.R., J.V.-A., J.S., and N.I.-U. performed and analyzed the neutralization and ELISA assays. V.U. performed the statistical analysis. R.P., L.M., A.C., M.M., V.G., A.V., and J.C. selected the patients and coordinated the data. J.B. and Gerard Carot-Sans drafted the manuscript, and all of the authors made substantial contributions to the revision of the subsequent versions. All of the authors approved the submitted version of the manuscript and agreed both to be personally accountable for their own contributions and to ensure answer to questions related to the accuracy or integrity of any part of the work.

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DECLARATION OF INTERESTS

J.B. and J.C. are founders of and shareholders in AlbaJuna Therapeutics; B.C. is a founder of and shareholder in AlbaJuna Therapeutics and AELIX Therapeutics (all unrelated to the present work). The other authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-6x-His clone HIS.H8	Thermo Fisher Scientific	Cat#MA1-21315; RRID: AB_557403
HRP-conjugated, F(ab')$_2$ goat anti-human IgG	Jackson ImmunoResearch	Cat#109-035-006; RRID: AB_2337578
Bacterial and virus strains		
pNL4-3.Luc.R-.E-	NIH ARP	Cat#3418
SARS-CoV-2.SctΔ19	This paper	N/A
pcDNA3.4-TOPO	GeneArt/Thermo Fisher Scientific	Cat#810330DE
pVSV-G	Clontech	[21]
Biological samples		
ELISA standard, positive plasma sample	This paper	N/A
Chemicals, peptides, and recombinant proteins		
S2 (Ser686-Pro1213)	Sino Biological	Cat#40590-V08B
RBD (Arg319-Phe541)	Sino Biological	Cat#40592-V08H
Nucleocapsid protein (NP)	Sino Biological	Cat#40588- V08B
MACS BSA solution	Miltenyi Biotec	Cat#130-091-376
Phosphate Buffered Saline	Thermo Fisher Scientific	Cat#10010015
o-Phenylenediamine dihydrochloride	Sigma-Aldrich	Cat#P8787-100TAB
H_2SO_4	Sigma-Aldrich	Cat#258105-1L-PC-M
Fetal Bovine Serum	Thermo Fisher Scientific	Cat#10270106
Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific	Cat#41966052
Expi293 Expression Medium	Thermo Fisher Scientific	Cat#A1435102
Opti-MEM I Reduced Serum Medium	Thermo Fisher Scientific	Cat#31985070
ExpiFectamine 293 Transfection Kit	Thermo Fisher Scientific	Cat#A14524
Versene	Thermo Fisher Scientific	Cat#15040033
Puromycin	Thermo Fisher Scientific	Cat#A1113803
DEAE-Dextran	Sigma-Aldrich	Cat#D9885-100G
BriteLite Plus Luciferase	PerkinElmer	Cat#6066769
Experimental models: cell lines		
Expi293F GnTI- cells	Thermo Fisher Scientific	Cat#A39240
HEK293T/hACE2 cells	Integral Molecular	Cat#C-HA101
Software and algorithms		
GraphPad Prism v8.4.3	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
R v4.0	R Foundation for Statistical Computing	https://www.r-project.org/
"nlme" R Package	R Foundation for Statistical Computing	https://cran.r-project.org/web/packages/nlme/index.html
Other		
GeneArt Gene Synthesis	Thermo Fisher Scientific	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Julià Blanco (jblanco@irsicaixa.es).

Materials availability

The plasmid pcDNA3.4 SARS-CoV-2.SctΔ19 is available upon request to the lead contact.

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Data and code availability

This study did not generate any unique datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study overview and subjects

The study was approved by the Hospital Ethics Committee Board from Hospital Universitari Germans Trias i Pujol (PI-20-122 and PI-20-217) and all participants provided written informed consent before inclusion.

Plasma samples were obtained from individuals of the prospective KING cohort of the HUGTiP (Badalona, Spain). This is an observational cohort, no blinding or randomization was applied. The recruitment period lasted from March to October 2020, thus covering the first and second waves of COVID-19 outbreak in Catalonia (dadescovid.cat). The KING cohort included individuals with a documented positive RT-qPCR result from nasopharyngeal swab and/or a positive serological diagnostic test. In addition, we performed in all individuals a confirmatory ELISA test, analyzing IgG, IgM and IgA anti-RDB and anti-S2 responses, that has been developed in our center (https://www.irsicaixa.es/sites/default/files/detection_of_sars-cov-2_antibodies_by_elisa_-_protocol_by_irsicaixa_protected.pdf). Participants were recruited irrespective of age and disease severity—including asymptomatic status- in various settings, including primary care, hospital, and epidemiological surveillance based on contact tracing. Age under 18 was the sole exclusion criterion. Stratification of participants was performed according to the WHO progression scale:[22] asymptomatic or mild (levels 1-3), and hospitalized (levels 4-10). We collected plasma samples at the time of COVID-19 diagnosis and at 3 and 6 months. Additionally, hospitalized individuals were sampled twice a week during the acute phase.

Cell lines

HEK293T cells (presumably of female origin) overexpressing WT human ACE-2 (Integral Molecular, USA) were used as target for SARS-CoV-2 spike expressing pseudovirus infection. Cells were maintained in T75 flasks with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1μg/mL of Puromycin (Thermo Fisher Scientific, USA).

METHOD DETAILS

Humoral response determination

The humoral response against SARS-CoV-2 was evaluated with an in-house sandwich- ELISA using the following antigens (Sino Biological, Germany): S2 (Ser686-Pro1213), RBD (Arg319-Phe541), both potentially contributing to neutralizing activity; and whole nucleocapsid protein (NP), which is unrelated to neutralizing capacity. Nunc MaxiSorp plates were coated with 50 μL of anti-6x-His antibody clone HIS.H8 (2 μg/mL, Thermo Fisher Scientific) in PBS overnight at 4°C. After washing, plates were blocked with 1% BSA in PBS (Miltenyi Biotec, Germany) for two hours at room temperature. Antigens were added at 1 μg/mL concentration (50 μL/well) and incubated overnight at 4°C. Plasma samples were heat-inactivated before use (56°C for 30 minutes) and analyzed in duplicate in antigen-coated and antigen-free wells in the same plate. Serial dilutions of a positive plasma sample were used as standard. A pool of pre-pandemic plasmas from healthy controls was used as a negative control. Standards, negative control, and plasma samples were diluted in blocking buffer and were incubated (50 μL/well) for one hour at room temperature. The HRP-conjugated (Fab)2 goat anti-human IgG (Fc specific, Jackson

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ImmunoResearch, UK) was then incubated for 30 minutes at room temperature. Plates were revealed with o-Phenylenediamine dihydrochloride (Sigma-Aldrich, USA) and reaction was stopped using 4N of H_2SO_4 (Sigma-Aldrich). Optical density (OD) at 492 nm with noise correction at 620 nm were used to calculate specific signal for each antigen after subtracting the antigen-free well signal for each sample. Standard curves were fitted to a 5-parameter logistic curve and data was expressed as arbitrary units (AU) according to the standard.

Pseudovirus generation and neutralization assay

HIV reporter pseudoviruses expressing SARS-CoV-2 S protein and Luciferase were generated. pNL4-3.Luc.R-.E- was obtained from the NIH AIDS Reagent Program.[23] SARS-CoV-2.SctΔ19 was generated (GeneArt) from the full protein sequence of SARS-CoV-2 spike with a deletion of the last 19 amino acids in C-terminal,[24] human-codon optimized and inserted into pcDNA3.4-TOPO. Expi293F cells were transfected using ExpiFectamine293 Reagent (Thermo Fisher Scientific) with pNL4-3.Luc.R-.E- and SARS-CoV-2.SctΔ19 at a 24:1 ratio, respectively. Control pseudoviruses were obtained by replacing the S protein expression plasmid with a VSV-G protein expression plasmid as reported.[21] Supernatants were harvested 48 hours after transfection, filtered at 0.45 μm, frozen, and titrated on HEK293T cells overexpressing WT human ACE-2 (Integral Molecular, USA). This neutralization assay has been previously validated in a large subset of samples.[13]

Neutralization assays were performed in duplicate. Briefly, in Nunc 96-well cell culture plates (Thermo Fisher Scientific), 200 $TCID_{50}$ of pseudovirus were preincubated with three-fold serial dilutions (1/60–1/14,580) of heat-inactivated plasma samples for 1 hour at 37°C. Then, $2x10^4$ HEK293T/hACE2 cells treated with DEAE-Dextran (Sigma-Aldrich) were added. Results were read after 48 hours using the EnSight Multimode Plate Reader and BriteLite Plus Luciferase reagent (PerkinElmer, USA). The values were normalized, and the ID_{50} (the reciprocal dilution inhibiting 50% of the infection) was calculated by plotting and fitting the log of plasma dilution versus response to a 4-parameters equation in Prism 8.4.3 (GraphPad Software, USA).

QUANTIFICATION AND STATISTICAL ANALYSIS

Continuous variables were described using medians and the interquartile range (IQR, defined by the 25^{th} and 75^{th} percentiles), whereas categorical factors were reported as percentages over available data. Quantitative variables were compared using the Mann-Whitney test, and percentages using the chi-square test. All experimental data were generated in duplicates. Kinetics of neutralizing activity and antibody titers (Log_{10} transformed to approximate to a normal distribution) were estimated from symptom onset—or serological diagnosis in asymptomatic individuals—and modeled using mixed-effects models in two steps. First, a 4-parameter logistic function was adjusted for the first 30 days after diagnosis using non-linear mixed models. Mid-term decay was analyzed using a piecewise regression with two decline slopes for data beyond 30 days, with a breakpoint at 80 days. For the latter analysis, linear mixed-effect models with random intercepts and slopes were used, and different breakpoints were tested; the best fit was chosen. For the longitudinal analysis of neutralizing activity, patients were grouped into two severity groups according to the WHO progression scale:[22] asymptomatic or mild (levels 1-3), and hospitalized (levels 4-10). Differences between the two severity groups were assessed using the likelihood ratio test. Association of neutralizing titers with gender was analyzed adjusting fitted models by gender and computing the corresponding likelihood ratio test. The longitudinal analysis of antibody titers was

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performed on a subset of 28 individuals (14 in each severity group) with the highest number of measures during the follow-up; owing to the limited sample size, all individuals were analyzed as a single group. Analyses were performed with Prism 8.4.3 (GraphPad Software) and R version 4.0 (R Foundation for Statistical Computing). Mixed-effects models were fitted using "nlme" R package.

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Supplemental information

Stable neutralizing antibody levels 6 months after mild and severe COVID-19 episodes

Edwards Pradenas, Benjamin Trinité, Víctor Urrea, Silvia Marfil, Carlos Ávila-Nieto, María Luisa Rodríguez de la Concepción, Ferran Tarrés-Freixas, Silvia Pérez-Yanes, Carla Rovirosa, Erola Ainsua-Enrich, Jordi Rodon, Júlia Vergara-Alert, Joaquim Segalés, Victor Guallar, Alfonso Valencia, Nuria Izquierdo-Useros, Roger Paredes, Lourdes Mateu, Anna Chamorro, Marta Massanella, Jorge Carrillo, Bonaventura Clotet, and Julià Blanco

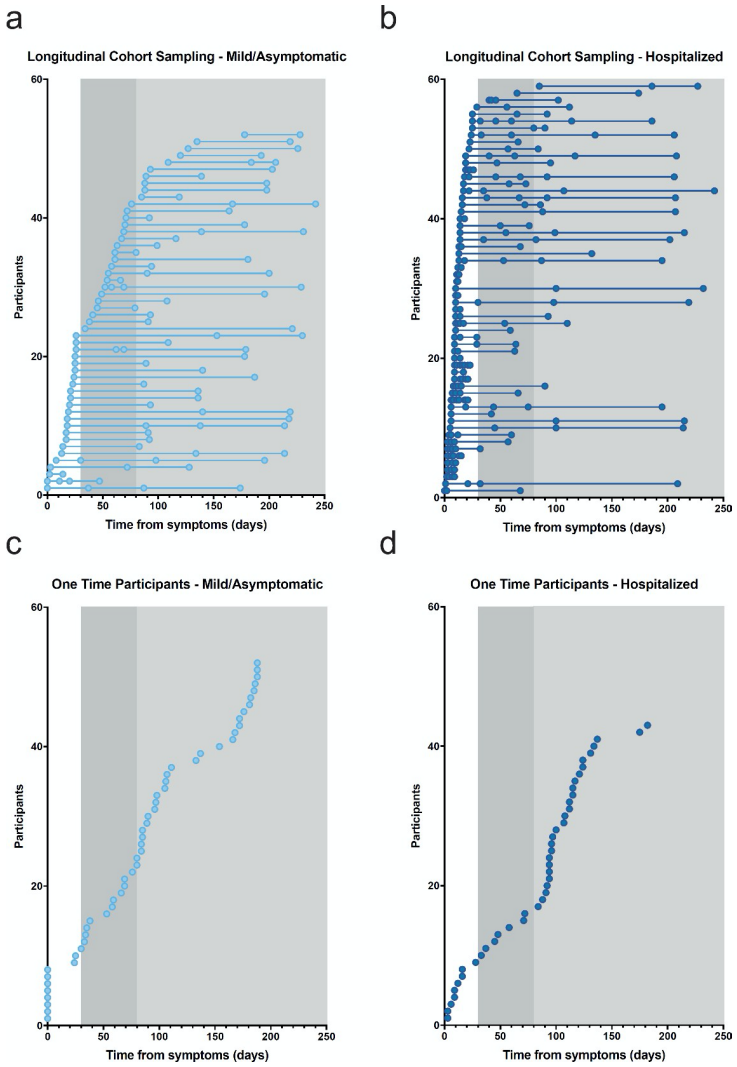

Figure S1. Patient and sampling distribution across the follow-up period, related to Figure 1. Top panels show the time points for sample collection among mild/asymptomatic (**a**) and hospitalized (**b**) individuals. Bottom panels show the time points for samples of individuals with a single measurement: **c**, mild/asymptomatic; **d**, hospitalized. Time count starts on the day of symptom onset, except for asymptomatic individuals, for whom the serological diagnosis was considered. The areas define the periods considered for the longitudinal analysis: days 0-30 (white), 30-80 (dark grey) and after 80 days (light grey).

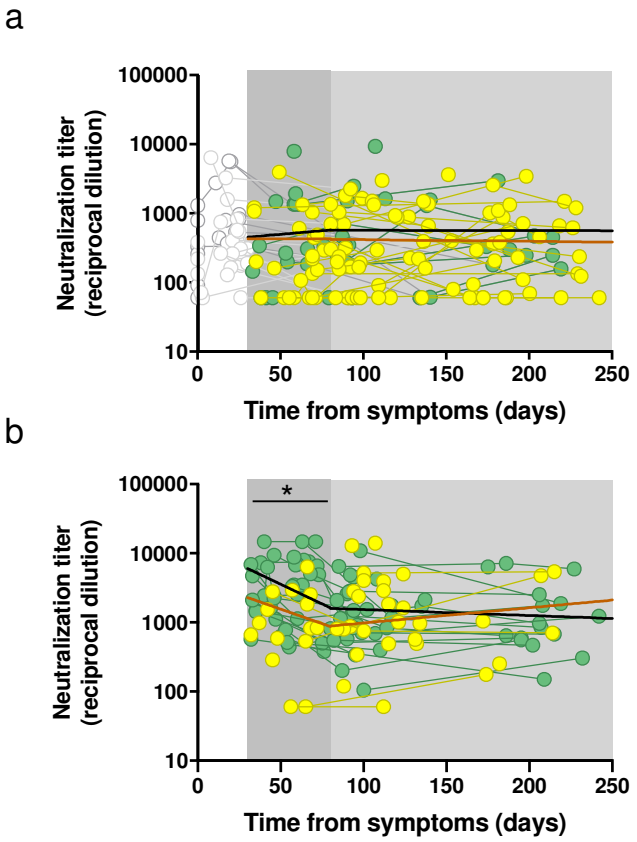

Figure S2. Longitudinal analysis of neutralizing activity with gender comparison, related to Figure 2. a, Analysis of figure 2a (mild/asymptomatic participants) comparing males (green dots) and females (yellow dots). Linear mixed model is shown for males (black lines) and female (brown line). No effect of gender was detected (p=0.75, likelihood ratio test). Time points preceding day 30 as well as participants only showing undetectable titers were excluded from the analysis, values are shown but grayed out. **b,** Equivalent reanalysis for figure 2b. Statistical difference between the male and female during the initial slope (day 30-80; p=0.014, likelihood ratio test initial slope, asterisk) but not the second slope (day >80; p=0.16, likelihood ratio test).

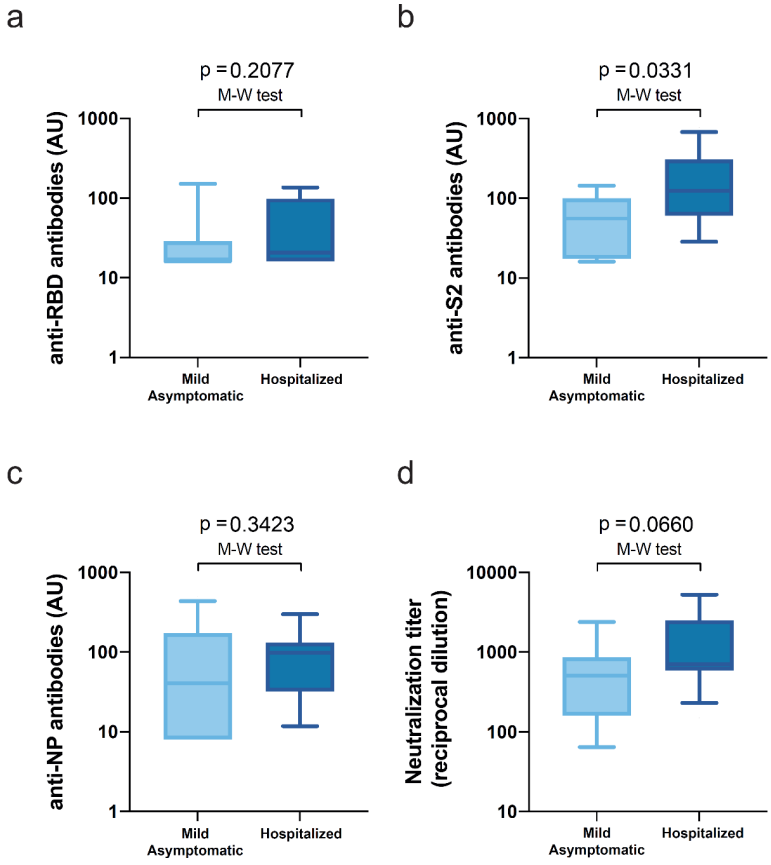

Figure S3. Antibody titers at the end of the follow-up period, related to Figure 3. Antibody titers of the last measure for IgG against the receptor binding domain (RBD) (**a**), S2 (**b**), and nucleoprotein (NP) (**c**) on a subset of individuals with largest follow-up (n=14 for mild/asymptomatic and n=14 for hospitalized). Panel **d** shows the neutralizing activity of the same subset of individuals at the end of the follow-up period. Boxes show the median and the interquartile range, and bars the 10th and 90th percentiles. Severity groups (i.e., mild/asymptomatic and hospitalized) were compared using the Mann-Whitney test.

Part III: Clinical course impacts early kinetics, magnitude, and amplitude of SARS-CoV-2 neutralizing antibodies beyond 1 year after infection

Edwards Pradenas[•], Benjamin Trinité[•], Víctor Urrea, Silvia Marfil, Ferran Tarrés-Freixas, Raquel Ortiz, Carla Rovirosa, Jordi Rodon, Júlia Vergara-Alert, Joaquim Segalés, Victor Guallar, Alfonso Valencia, Nuria Izquierdo-Useros, Marc Noguera-Julian, Jorge Carrillo, Roger Paredes, Lourdes Mateu, Anna Chamorro, Ruth Toledo, Marta Massanella, Bonaventura Clotet, Julià Blanco✉

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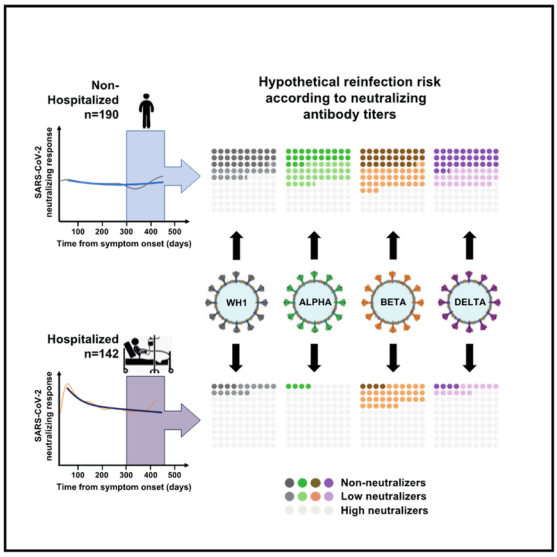
Clinical course impacts early kinetics, magnitude, and amplitude of SARS-CoV-2 neutralizing antibodies beyond 1 year after infection

Graphical abstract



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In brief

Pradenas and Trinité et al. analyze the kinetics of neutralizing antibodies in response to natural SARS-CoV-2 infection and evaluate the long-term (beyond 1 year) stability and breadth of the neutralizing response before subsequent vaccination.

Highlights

- Long-term persistence (>12 months) of neutralizing antibodies against SARS-CoV-2
- Severity of infection determines the magnitude and quality of neutralizing response
- Vaccination boosts neutralizing response to natural infection

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Clinical course impacts early kinetics, magnitude, and amplitude of SARS-CoV-2 neutralizing antibodies beyond 1 year after infection

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SUMMARY

To understand the determinants of long-term immune responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the concurrent impact of vaccination and emerging variants, we follow a prospective cohort of 332 patients with coronavirus disease 2019 (COVID-19) over more than a year after symptom onset. We evaluate plasma-neutralizing activity using HIV-based pseudoviruses expressing the spike of different SARS-CoV-2 variants and analyze them longitudinally using mixed-effects models. Long-term neutralizing activity is stable beyond 1 year after infection in mild/asymptomatic and hospitalized participants. However, longitudinal models suggest that hospitalized individuals generate both short- and long-lived memory B cells, while the responses of non-hospitalized individuals are dominated by long-lived B cells. In both groups, vaccination boosts responses to natural infection. Long-term (>300 days from infection) responses in unvaccinated participants show a reduced efficacy against beta, but not alpha nor delta, variants. Multivariate analysis identifies the severity of primary infection as an independent determinant of higher magnitude and lower relative cross-neutralization activity of long-term neutralizing responses.

INTRODUCTION

Immune responses to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection involve an undefined balance of innate and adaptive pathways[1] resulting in the development of a seemingly long-lasting immunological memory.[2,3] Although there is a general consensus on the key role of both T and B cells in the protection against SARS-CoV-2 infection and the development of coronavirus disease 2019 (COVID-19), the specific contribution of each arm of the immune system is still unclear.[1] Neutralizing antibodies mediate their protective effect by binding to the spike (S) glycoprotein of SARS-CoV-2 and by blocking viral entry into target cells; however, additional effector functions promoting viral clearance or natural killer (NK)-mediated infected-cell killing seems to be also relevant in SARS-CoV-2 and other viral infections.[4] Nevertheless, abundant experimental and epidemiological studies on SARS-CoV-2 indicate that neutralizing antibodies can serve as surrogate markers of protection,[5–7] as they do for other viral infections.[8,9]

Given the relevance of antibodies, the early (1–3 months) and mid-term (3–12 months) humoral responses after SARS-CoV-2 infection have been thoroughly described.[10–14] Current data outline a heterogeneous scenario in which infected individuals generate a wide range of neutralizing antibodies (from no seroconversion to rapid development of high titers) with no definitive association to age, gender, or disease severity.[15–17] Various authors have also suggested complex kinetics of neutralizing activity decay.[3,18,19] This is particularly relevant in the current context of viral evolution, as several variants of concern (VOCs) have shown total or partial resistance to neutralizing antibodies and partial resistance to polyclonal humoral responses elicited by infection or vaccination.[20]

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Table 1. Characteristics of individuals included in analysis

	March 20–June 20		July 20–December 20		January 21–March 21		
	Non-hospitalized (n = 128)	Hospitalized (n = 84)	Non-hospitalized (n = 43)	Hospitalized (n = 36)	Non-hospitalized (n = 19)	Hospitalized (n = 22)	p value
Gender (female), n (%)	92 (72)	39 (46)	24 (56)	12 (33)	9 (47)	5 (23)	0.0006[a]
Age (years), median (IQR)	47 (38–54)	58 (48–67)	43 (33–53)	55 (45–63)	46 (23–52)	56 (49–62)	< 0.0001[b]
Severity							
Asymptomatic, n (%)	12 (9)	–	7 (16)	–	1 (5)	–	
Mild, n (%)	116 (91)	–	36 (84)	–	18 (95)	–	
Hospitalized non-severe, n (%)	–	31 (37)	–	9 (25)	–	2 (9)	
Hospitalized severe, n (%)	–	41 (49)	–	23 (64)	–	20 (91)	
Hospitalized (intensive care unit), n (%)	–	12 (14)	–	4 (11)	–	0 (0)	
Samples per participant median (IQR)	3 (1–3)	3 (2–4)	2 (1–2)	4 (2–4)	1 (1–2)	3 (2–4)	
Last sampling day, median (IQR)	272 (180–360)	311 (115–362)	116 (21–186)	183 (90–208)	47 (17–108)	32 (28–83)	
Length of follow up, days median (IQR)	172 (0–256)	185 (5–266)	75 (0–166)	169 (7–193)	0 (0–80)	26 (16–76)	

IQR: IQR (25th and 75th percentiles).
[a]Chi-square test.
[b]Mann-Whiney test.

To understand the dynamics of natural responses to infection, we focused on the longitudinal analysis of the neutralizing humoral response in a large cohort of mild/asymptomatic and hospitalized individuals infected by SARS-CoV-2. Our analysis includes one of the longest follow-up periods (up to 15 months) and shows that the long-term magnitude of neutralization is remarkably stable, being boosted by vaccines, and potentially threatened by VOCs. Clinical severity of primary infection was identified as the main factor determining the kinetics, magnitude, and quality of neutralizing antibodies.

RESULTS

Cohort description

Our cohort included 332 participants with confirmed SARS-CoV-2 infection who were recruited between March 2020 and March 2021 in Catalonia (Northeastern Spain). Participants were group-ed according to the epidemiological waves of the SARS-CoV-2 pandemic in Spain, defined by an early outbreak caused by the original 19B strain and the B.1/20A variant (D614G) (from March to June 2020), a second wave dominated by the 20E (EU1) variant (from July to December 2020), and a third wave associated with the emergence of the B.1.1.7/20I Alpha variant covering the January to June 2021 period, until the recent introduction of the B.1.617.2 delta variant in June 2021 (Figure S1). A total of 212 participants were recruited during the first-wave period, 128 of whom had mild or absent symptomatology (WHO progression scale[21] levels 1–3; non-hospitalized) and 84 of whom required hospitalization (WHO progression scale[21] levels 4–10) with a wide range of severity from non-severe pneumonia to intensive care unit admission/death (Table 1). Comparable proportions of disease severity were observed in patients recruited in the second (n = 79) and third (n = 41) COVID-19 waves. In all cases, the hospitalization groups showed older age and lower female frequency when compared with non-hospitalized groups (mild or asymptomatic; Table 1).

Longitudinal analysis of neutralization activity

For comparative purposes, samples from all patients prior to vaccination, irrespective of the infection wave, were assayed for their plasma neutralization capacity of the original isolate WH1 sequence in a validated pseudovirus assay.[13] Maximal follow-up periods for unvaccinated individuals infected during the first, second, and third waves were 458, 320, and 145 days, respectively. In line with previous analyses,[15,17] irrespective of the infecting virus, hospitalized patients showed a rapid development of neutralizing activity over the first month after symptom onset and a transient decrease reaching a plateau (Figures 1B, 1D, and 1F). This was observed only in the first- and second-wave participants due to the limited follow-up of

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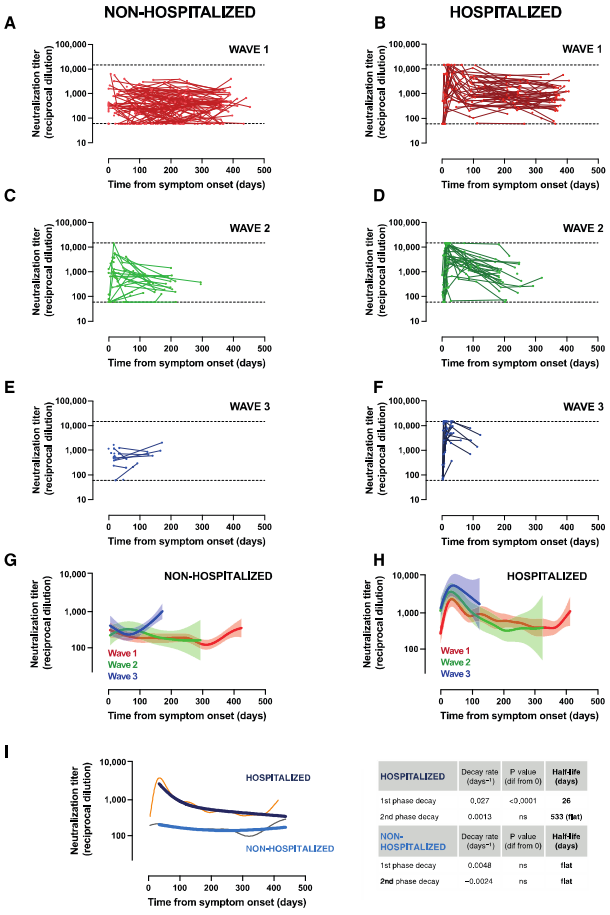

HOSPITALIZED	Decay rate (days^{-1})	P value (dif from 0)	Half-life (days)
1st phase decay	0.027	<0.0001	26
2nd phase decay	0.0013	ns	533 (flat)
NON-HOSPITALIZED	Decay rate (days^{-1})	P value (dif from 0)	Half-life (days)
1st phase decay	0.0048	ns	flat
2nd phase decay	−0.0024	ns	flat

Figure 1. Longitudinal analysis of neutralizing activity

(A–F) Neutralization titer of 332 individuals according to disease severity (non-hospitalized or hospitalized groups) and date of infection (wave 1: March–June 2020, wave 2: July–December 2020, and wave 3: January–March 2021). Dots are single determinations, and lines indicate individual follow up. Dotted lines indicate the upper and lower limits of the neutralization assay.

(G and H) Longitudinal smoothing-splines mixed-effects models for the different groups shown in (A)–(F). Solid lines indicate the best fit, and light areas indicate confidence intervals (CIs).

(I) Non-linear models of the full dataset (n = 190 for non-hospitalized and n = 142 for hospitalized groups) were analyzed by smoothing-splines mixed-effects models (gray and orange narrow lines) or fitted to a non-linear two-phase exponential decay model (light and dark blue lines). Decay rate constants are described on the right side of the figure.

recently infected patients. In contrast, mildly affected or asymptomatic individuals developed a lower maximal neutralization titer with flatter behavior (Figures 1A, 1C, and 1E), although an early peak could be observed in some of the second-wave participants who had earlier sampling (Figure 1C). Longitudinal analysis using smoothing-splines mixed-effects models showed overlapping kinetics for the different waves in each clinical group (Figures 1G and 1H), although neutralizing activity tended to reach higher values at the peak (around 30 days) in hospitalized patients from the third wave (mostly infected by the Alpha variant; Figure 1H). According to recent data,[22] we assumed the generation of early short-lived plasmablast/plasma cells and long-lived plasma and memory B cells and modeled data from all patients to a two-phase exponential decay. The longitudinal modeling revealed that hospitalized individuals had a significantly rapid first-phase decay (half-life of 26 days) and a flat slope in the second phase (half-life of 533 days; Figure 1I). Conversely, a flat slope (i.e., not significantly different from 0 in any phase) was observed in individuals with asymptomatic infection or mild disease (Figure 1I). These data confirm that, despite different individual patterns, a large fraction of infected individuals (87% in non-hospitalized and 96% in hospitalized) generate detectable long-lasting neutralizing antibodies (infective dose [ID]50 > 60).

Impact of vaccination

Massive vaccination campaigns across developed countries have positively impacted the course of the COVID-19 pandemic and have interfered with the follow-up of immune responses induced by natural infection. During routine follow-up visits, we identified 58 vaccinated individuals in our cohort. Participants showed a wide range of vaccination status in terms of type of vaccine (21% received BNT162b2 [Pfizer-BioNTech], 62% mRNA-1273 [Moderna], and 17% AZD1222 [AstraZeneca-Oxford]), number of doses (only 55% had received the full 2-dose schedule), and time from the last dose (BNT162b2 vaccinees analyzed at longer time points after vaccination). Despite these differences, vaccines boosted pre-existing neutralizing responses in all non-hospitalized (n = 40) and hospitalized (n = 18) participants (Figures 2A and 2B). A direct comparison of pre- and post-vaccination titers of neutralizing antibodies clearly confirms a highly significant increase in both

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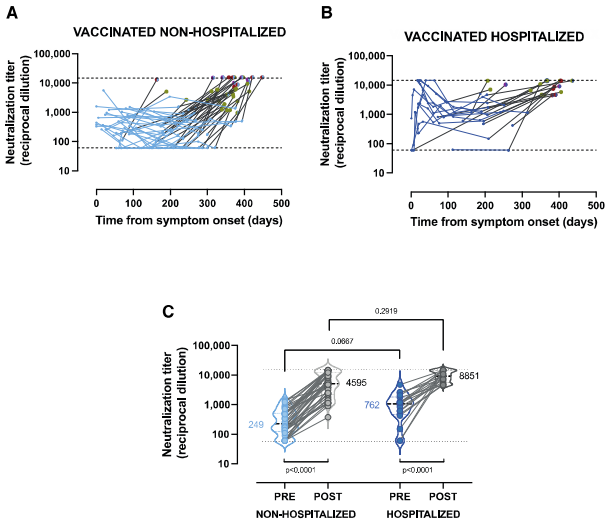

Figure 2. Impact of vaccination on convalescent plasma neutralizing activity

(A and B) Single measurements (dots) and individual evolution (lines) of the longitudinal analysis for vaccinated non-hospitalized (n = 40) and hospitalized (n = 18) individuals. Blue dots (light or dark) correspond to pre-vaccination measurements. Post-vaccine data are color-coded according to vaccine schedules: BNT162b2 (maroon), mRNA-1273 (red), and AZD1222 (purple). Full symbols indicate full schedule (two doses), while half circles indicate one single dose.

(C) Comparison of pre- and post-vaccination neutralizing antibody titers in both groups. Lower p values indicate paired comparison (Wilcoxon test) of pre- and post-values. Upper p value indicates Mann-Whitney comparison of pre-vaccination between groups. Geometric means of neutralization titer for each group are indicated. Dotted lines indicate the upper and lower limits of detection.

groups ($p < 0.0001$). Pre-vaccination titers tended to be lower in non-hospitalized than in hospitalized individuals (geometric mean, 249 and 762, respectively, $p = 0.0667$) and reached comparable levels after vaccination (4,595 and 8,851, respectively; Figure 2C; $p = 0.2919$). However, the heterogeneous vaccine schedules and sampling times prevented further analysis.

Impact of viral variants

It is well known that SARS-CoV-2 VOCs show variable degrees of resistance to neutralizing responses elicited by natural infection or vaccination.[20] Therefore, to evaluate the impact of the most relevant VOCs on long-term neutralizing activity, a subset of 60 unvaccinated individuals with follow-up periods beyond 300 days was analyzed against Alpha, Beta, and Delta variants. A global analysis showed that long-term neutralizing responses blocked the WH1 and the Alpha (B.1.1.7) variants with similar potency, while lower titers were measured against the Beta (B.1.351) variant ($p = 0.0001$), and an intermediate but not significant loss of neutralization was observed against the more transmissible Delta variant (Figure 3A). The analysis of non-hospitalized and hospitalized subgroups showed similar but not identical results. We observed a highly significant loss ($p \leq 0.0001$) of neutralizing capacity against the Beta variant in hospitalized individuals (Figure 3A) but a lower loss in non-hospitalized individuals, reaching significance when compared with the Alpha but not with the original WH1 variant ($p = 0.4020$; Figure 3A). To quantitatively assess these differences, we compared the ratio of neutralization titers between the original WH1 sequence and the tested variants as a measure of the relative loss of neutralization for each individual. The comparison of this parameter among subgroups, using only values with measurable neutralization titers for both WH1 and the variant of interest, showed no differences between non-hospitalized and hospitalized groups for the Alpha/B.1.1.7 variant (with a mean fold change around 1). On the other hand, statistically significant differences were observed for the Beta/B.1.351 variant, which induced a higher relative loss of neutralization in hospitalized patients ($p = 0.0350$; Figure 3B), while the Delta/B.1.617.2 variant showed again intermediate behavior with a more pronounced decrease in hospitalized patients that did not reach significance ($p = 0.3425$; Figure 3B). As a consequence, the median magnitudes of neutralization against WH1, Alpha, and Delta were all superior in hospitalized individuals compared with in non-hospitalized individuals ($p = 0.0005$, 0.0003, and 0.0048 respectively), while statistical significance was lost for the Beta variant ($p = 0.3107$; Figure 3A).

Following previous reports correlating protection with neutralization titers,[7,23] we estimated the frequency of individuals with undetectable, low, and high neutralization titers using a previously described cutoff value of 250.[2] The analysis showed that 33% of individuals had undetectable or low neutralization against the WH1 or the Alpha variant, increasing to 52% or 41% for the Beta and Delta variants, respectively. In all cases, the frequency of non-neutralizers and low neutralizers was higher in non-hospitalized individuals, reaching 63% against the Beta variant, compared with 36% in hospitalized patients (Figure 3C)

Factors determining long-term neutralizing activity

Despite similar long-term stability in non-hospitalized and hospitalized individuals, neutralizing activity was highly heterogeneous with the presence of non-neutralizer and highly neutralizer patients in both groups (see Figure 3). Therefore, we analyzed the factors that potentially define the magnitude of long-term neutralization (>300 days after infection) in unvaccinated infected patients. A multivariate analysis including severity group,

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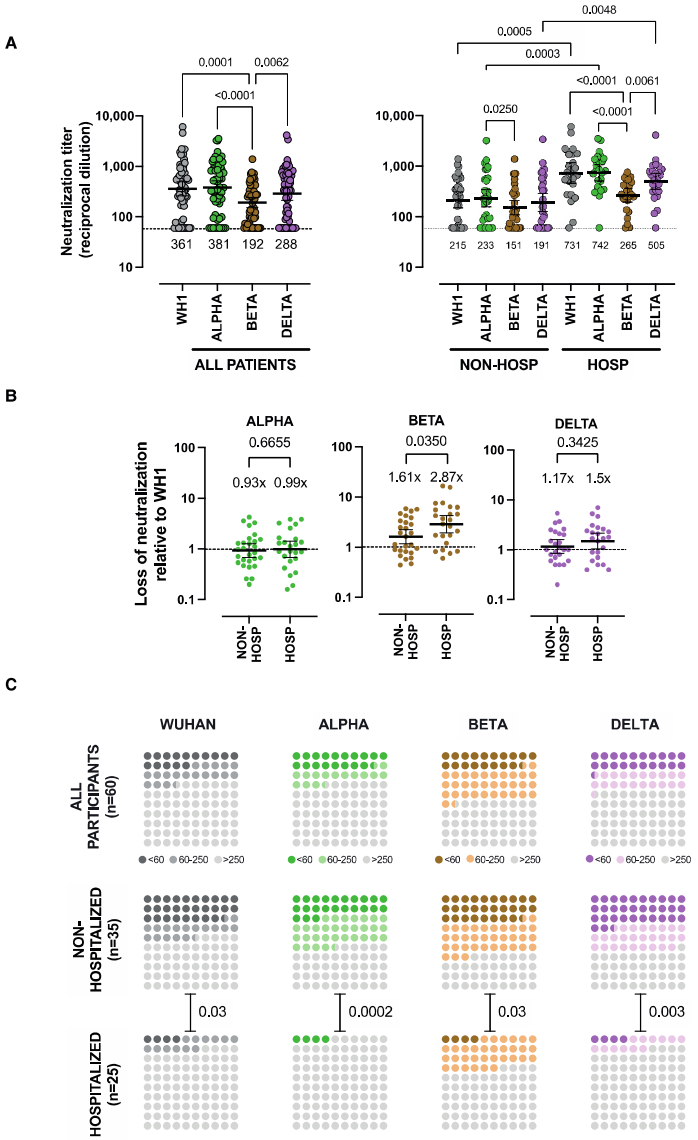

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age, and gender showed that only severity, as defined by hospitalization, was independently associated with the magnitude of responses ($p = 0.0285$; Figure 4A). Consistent with the close relationship between age and severity, age showed a significant effect in the univariate analysis that was lost in the multivariate model ($p = 0.0951$; Figure 4B), while gender had no impact (Figure 4C).

A similar approach was used to assess the impact of severity, age, and gender on variant cross-neutralization ratios (shown in Figure 3B). In this case, the multivariate analysis ruled out any impact of age and gender in the loss of neutralization against Alpha, Beta, and Delta variants, which pointed to severity as the main determinant, although it only reached significance for the Beta variant ($p = 0.0259$; Table S2).

DISCUSSION

To our knowledge, this report has analyzed the neutralizing response against SARS-CoV-2 with the longest follow-up to date, with sampling more than 1 year after symptom onset, in a large cohort with a broad spectrum of clinical disease presentation (from asymptomatic to patients requiring intensive care) over different COVID-19 outbreaks in Catalonia. Longitudinal sampling allowed us to model accurate kinetics of neutralizing activity for the different waves (associated with different viral variants). The temporal patterns for each wave appear to repeat themselves independently of the infecting variant but with a strong impact of disease severity, as previously defined.[13]

In comparison to the apparent short-lasting immunity against seasonal human coronaviruses,[24,25] the neutralizing response developed against SARS-CoV-2 shows a dynamic pattern similar to the ones described against other coronaviruses that cause severe acute respiratory illness, such as SARS-CoV and Middle East respiratory syndrome (MERS)-CoV. For these viruses, several studies detected neutralizing antibodies in the first days after diagnosis with a rapid increase peaking between 2 weeks and 1 month post-symptom onset. Thereafter, there was a decline and, subsequently, a "stabilization" that was maintained beyond 1 year after infection in most cases and was related to disease severity.[26–32] Our data demonstrate the long-term persistence of neutralizing antibodies against SARS-CoV-2 in most individuals with COVID-19. Although long-term increases were observed in qualitative spline models, biexponential fitted models confirm a flat slope and therefore predict longer stability, as has also been described for SARS-CoV and MERS-CoV.[33–38] This raises an optimistic scenario, as neutralizing antibody levels are highly predictive of immune protection,[5,7,39,40] although sporadic cases of reinfection have been reported even in the presence of neutralizing antibodies.[41,42]

Our results complement previous studies that evaluated mid-term immunity[2,17,18,43,44] and are in line with current evidence showing a long-lasting neutralizing response for at least 1 year[45–47] and the presence of receptor-binding domain (RBD)-specific memory B cells[3,48] and long-lived bone marrow plasma cells.[22] Although several mechanisms have been proposed that may lead to the long-term persistence of antibodies,[49] the presence of long-lived plasma cells has received more support in recent years,[50–52] and a biphasic model considering short- and long-lived plasma cells has been described.[53,54] On this basis and considering the neutralizing capacity of plasma as a surrogate marker of the plasma-cell lifespan, we fitted our data to a two-phase exponential decay curve, probably reflecting both short- and long-lived plasma cells. Therefore, our data point to an initial and transient generation or expansion of short-lived SARS-CoV-2-specific plasmablast/plasma cells in hospitalized patients. While the selection of high-affinity B cells into germinal centers seems to be a hallmark for the generation of long-lived plasma cells,[55] short-lived cells can be generated following an extrafollicular response,[51] which does not necessarily imply immunoglobulin evolution through somatic hypermutation nor selection of high-affinity B cells. Interestingly, hospitalized patients showed a more limited cross-neutralizing response against the Beta variant relative to WH1, suggesting that B cell responses in severe disease, despite being higher in magnitude, could be less cross-neutralizing. Although the association of severity and magnitude of neutralizing responses has been pointed out for early responses in different studies,[2,13,56] our data extend this observation to the long-term responses, also suggesting a discordant relationship between the magnitude and quality of antibodies in hospitalized individuals.

In the cohort studied, we observed that neutralizing activity is significantly boosted after vaccination, although the longevity of this response still needs to be determined. Based on our data on unvaccinated infected individuals, the vaccination of people who have overcome the SARS-CoV-2 infection should lead to a long-lasting protection. But this information must be interpreted carefully since new emerging variants of the virus could escape both natural and vaccine-induced immunity.[57]

Figure 3. Impact of SARS-CoV-2 variants on long-term neutralizing activity

(A–C) Neutralization titers, against WH1, Alpha, Beta, and Delta spike variants, measured on convalescent plasmas collected more than 300 days after symptom onset from non-hospitalized (n = 35) and hospitalized (n = 25) patients (see Table S1 for details).

(A) Neutralization titers (ID50 expressed as reciprocal dilutions) from all patients (left) or divided into non-hospitalized and hospitalized patients (right). Bars and values below symbols indicate the geometric mean titer in each group. p values show the comparison of median titers among the four viruses (Friedman test with Dunn's multiple comparison) or the comparison of the same variant between the two groups (Kruskal-Wallis test with Dunn's multiple comparison). Only significant differences are shown. Dotted lines indicate lower limits of detection.

(B) Loss of neutralization titers against variants (indicated on top) compared with WH1 pseudovirus (lower values identify maintained neutralization). Samples with undetectable titers for both WH1 and the analyzed variant were removed from the analysis. Bars and values indicate the median ratio, and p values indicate the comparison of the two patient groups (Mann-Whitney test).

(C) Frequency of long-term non-neutralizers (ID50 < 60), low neutralizers (i.e., ID50 between 60 and 250 after 300 days post-symptom onset), and high neutralizers (ID > 250, light gray) in all patients and separately in hospitalized and non-hospitalized groups. p values show the comparison of frequency between both groups for each variant (chi-square test).

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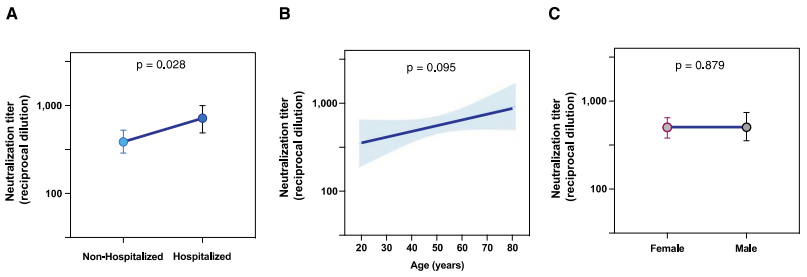

Figure 4. Factors determining long-term neutralizing titer

(A–C) Factor effects by multivariate linear regression for samples collected more than 300 days post-symptom onset from 99 participants. Estimated effect (dots) and 95% CI (bars or bands) are plotted, and the p value is shown for each predictor covariate: (A) severity, (B) age, and (C) gender. Multivariate analyses were performed with R-3.6.3 software.

To address the impact of VOCs, we tested neutralization titers against Alpha, Beta, and Delta variants. Despite showing lower titers, non-hospitalized individuals tended to have better relative cross-neutralization against all variants tested. Of note, the loss of neutralization titers observed in our study was lower than the values reported for vaccinated individuals.[58] We did not observe changes for the Alpha variant, and the ratios calculated for the Beta and Delta variants compared with the WH1 isolate below 3-fold. This fact could be related to antibody evolution after recovery from infection and is consistent with data reporting increased cross-neutralization in vaccinated or unvaccinated COVID-19 convalescent individuals.[48,58,59] Therefore, we exclusively observed a significant reduction of titers for the Beta variant, resulting in a high frequency of individuals with undetectable or low (<250) neutralizing capacities that were significantly higher in non-hospitalized individuals. When analyzing the clinical and demographic factors that could influence the long-term neutralizing antibody response, we did not observe any differences between women and men. In contrast, age shows a certain tendency (older participants present higher neutralizing activity) whose significance was evident in the univariate analysis but did not reach significance in the multivariate linear regression. This latter result could indicate that age by itself is not a determinant component but depends on other cofactors, as could be the severity of the disease, which is highlighted as the main determinant of the magnitude of long-term responses. This is in line with the evidence described so far,[44,47] although it disagrees with another study describing antibody kinetics influenced by gender.[46] Despite the clear effect of severity, there is still a high individual heterogeneity in the magnitude of neutralization achieved by participants in each group (non-hospitalized or hospitalized individuals) that needs further study to unveil additional determinants.

Limitations of the study

Our analysis provides one of the largest datasets on neutralizing activity (in number of participants and follow-up time) but is limited by the lack of parallel data on T cells and other immune-related factors. In addition, the long-term impact of vaccination is still an open question; therefore, beyond the clear boosting effect observed, we cannot draw further conclusions due to heterogeneous vaccine schedules and sampling times.

Our longitudinal analysis confirmed the early decay and long-term maintenance of neutralizing activity observed in other cohorts.[10,12] Moreover, our data identified different dynamics of short- and long-lived responses after infection. In particular, severity of primary infection is associated with the emergence of short-lived antibodies (not observed in non-hospitalized individuals) and the generation of higher titers of less cross-neutralizing long-lived antibodies (beyond 1 year).

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100523.

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AUTHOR CONTRIBUTIONS

J.B. and B.C. designed and coordinated the study. E.P, B.T., S.M., F.T.-F., R.O., C.R., J.R., J.V.-A., J.S., and N.I.-U. performed and analyzed neutralization assays. E.P., B.T., and V.U. performed statistical analysis. M.N.-J., R.P., L.M., A.C., R.T., M.M., V.G., A.V., and J.C. selected patients and coordinated data. J.B. and E.P. drafted the manuscript, and all authors have made substantial contributions to the revision of the subsequent versions. All authors approved the submitted version of the manuscript and agreed both to be personally accountable for their own contributions and to ensure the accuracy or integrity of any part of the work.

DECLARATION OF INTERESTS

Unrelated to the submitted work, J.B. and J.C. are founders and shareholders of AlbaJuna Therapeutics, SL. B.C. is founder and shareholder of AlbaJuna Therapeutics, SL, and AELIX Therapeutics, SL. The other authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
pNL4-3.Luc.R-.E-	NIH ARP	Cat#3418
SARS-CoV-2.SctΔ19	This paper	N/A
pcDNA3.1(+)	GeneArt/Thermo Fisher Scientific	Cat#810330DE
pVSV-G	Clontech	Sánchez-Palomino et al[48]
Chemicals, peptides, and recombinant proteins		
Fetal Bovine Serum	Thermo Fisher Scientific	Cat#10270106
Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific	Cat#41966052
Expi293 Expression Medium	Thermo Fisher Scientific	Cat#A1435102
Opti-MEM I Reduced Serum Medium	Thermo Fisher Scientific	Cat#31985070
ExpiFectamine 293 Transfection Kit	Thermo Fisher Scientific	Cat#A14524
Versene	Thermo Fisher Scientific	Cat#15040033
Puromycin	Thermo Fisher Scientific	Cat#A1113803
DEAE-Dextran	Sigma-Aldrich	Cat#D9885-100G
BriteLite Plus Luciferase	PerkinElmer	Cat#6066769
Experimental models: Cell lines		
Expi293F GnTI- cells	Thermo Fisher Scientific	Cat#A39240
HEK293T/hACE2 cells	Integral Molecular	Cat#C-HA101
Software and algorithms		
GraphPad Prism v9.3.1	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
R v3.6.3	R Foundation for Statistical Computing	https://www.r-project.org/
"nlme" R Package	R Foundation for Statistical Computing	https://cran.r-project.org/web/packages
"sme" R Package	R Foundation for Statistical Computing	https://cran.r-project.org/web/packages
Other		
GeneArt Gene Synthesis	Thermo Fisher Scientific	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Julià Blanco (jblanco@irsicaixa.es).

Materials availability

The plasmids pcDNA3.1 SARS-CoV-2.SctΔ19 are available upon request to the lead contact.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study overview and subjects

The study KING was approved by the Hospital Ethics Committee Board from Hospital Universitari Germans Trias i Pujol (HUGTiP, PI-20-122 and PI-20-217) and was further amended to include vaccinated individuals. All participants provided written informed consent before inclusion.

Plasma samples were obtained from individuals of the prospective KING cohort of the HUGTiP (Badalona, Spain). The recruitment period lasted from March 2020 to March 2021, thus covering the three consecutive outbreaks of COVID-19 in Catalonia (Figure S1).

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The KING cohort included individuals with a documented positive RT-qPCR result from nasopharyngeal swab and/or a positive serological diagnostic test. Participants were recruited irrespective of age and disease severity including asymptomatic status in various settings, including primary care, hospital, and epidemiological surveillance based on contact tracing. We collected plasma samples at the time of COVID-19 diagnosis and at 3, 6 and 12 months after diagnosis. Additionally, hospitalized individuals were sampled twice a week during acute infection. Viral sequences were available from a subset of participants (n = 26, 8% of the cohort) and confirmed the expected prevalence of B.1 variant during the first wave (67% of sequences), 20E(EU1) variant during the second one (70% of sequences) and alpha variant after January 2021 (80% of sequences).

Cell lines

HEK293T cells overexpressing WT human ACE-2 (Integral Molecular, USA) were used as target in pseudovirus-based neutralization assay. Cells were maintained in T75 flasks with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1 μg/ml of Puromycin (Thermo Fisher Scientific, USA).

Expi293F cells (Thermo Fisher Scientific) are a HEK293 cell derivative adapted for suspension culture that were used for SARS-CoV-2 pseudovirus production. Cells were maintained under continuous shaking in Erlenmeyer flasks following manufacturer's guidelines.

METHOD DETAILS

Pseudovirus generation and neutralization assay

HIV reporter pseudoviruses expressing SARS-CoV-2 S protein and Luciferase were generated. pNL4-3.Luc.R-.E- was obtained from the NIH AIDS Reagent Program.[60] SARS-CoV-2.SctΔ19 was generated (GeneArt) from the full protein sequence of the original WH1 SARS-CoV-2 spike (Genbank MN908947.3) with a deletion of the last 19 amino acids in C-terminal,[61] human-codon optimized and inserted into pcDNA3.1(+). A similar procedure was followed to generate expression plasmids for the alpha (69-70 del, Y144 del, N501Y, A570D, P681H, T716I, S982A and D1118H), beta (L18F, D80S, D215G, L242_244 Del, R246I, K417N, E484K, N501Y, D614G, A701V) and delta (T19R, 157-158del, L452R, T478K, D614G, P681R, D950N) variants of SARS-CoV-2 S protein[59] according to consensus data (www.outbreak.info/). Expi293F cells were transfected using ExpiFectamine293 Reagent (Thermo Fisher Scientific) with pNL4-3.Luc.R-.E- and SARS-CoV-2.SctΔ19 (WH1, alpha, beta or delta), at an 8:1 ratio, respectively. Control pseudoviruses were obtained by replacing the S protein expression plasmid with a VSV-G protein expression plasmid as reported.[62] Supernatants were harvested 48 hours after transfection, filtered at 0.45 μm, frozen, and titrated on HEK293T cells overexpressing WT human ACE-2 (Integral Molecular, USA). This neutralization assay has been previously validated in a large subset of samples and negative controls with a replicative viral inhibition assay.[13]

Neutralization assays were performed in duplicate. Briefly, in Nunc 96-well cell culture plates (Thermo Fisher Scientific), 200 $TCID_{50}$ of pseudovirus were preincubated with three-fold serial dilutions (1/60–1/14,580) of heat-inactivated plasma samples for 1 hour at 37°C. Then, $2x10^4$ HEK293T/hACE2 cells treated with DEAE-Dextran (Sigma-Aldrich) were added. Results were read after 48 hours using the EnSight Multimode Plate Reader and BriteLite Plus Luciferase reagent (PerkinElmer, USA). The values were normalized, and the ID_{50} (reciprocal dilution inhibiting 50% of the infection) was calculated by plotting and fitting all duplicate neutralization values and the log of plasma dilution to a 4-parameters equation in Prism 9.0.2 (GraphPad Software, USA).

QUANTIFICATION AND STATISTICAL ANALYSIS

Continuous variables were described using medians and the interquartile range (IQR, defined by the 25^{th} and 75^{th} percentiles), whereas categorical factors were reported as percentages over available data. Quantitative variables were compared using the Mann-Whitney test, and percentages using the chi-squared test. For the longitudinal analysis of neutralizing activity, patients were grouped into two severity groups according to the WHO progression scale[21] asymptomatic or mild (levels 1-3), and hospitalized (levels 4-10).

Longitudinal kinetics of neutralization activity for hospitalized and mild groups were analyzed by nonlinear models in two ways, parametric and non-parametric models and stratifying by severity in both cases. We fitted a non-parametric model using smoothing-splines mixed-effects model using the "sme" package of R. The final part of this model, showing an increase in neutralization activity, is unreliable due to the small sample size available in that stretch. We also analyzed the observed decrease of neutralization after 30 days by a biexponential decay model [y=P1*exp(-k1*t) + P2*exp(-k2*t)] fitting a nonlinear mixed-effects model and using "nlme" package of R. In this case three samples were excluded due to their influence in the model fitting since were samples after 350 days with and important increase of neutralization with respect the previous determinations and although we cannot rule out their veracity, they had a great impact on the proper fit of the model due to the lack of sample size in the final part of the follow-up.

Differences in neutralization between both groups after 300 days since symptoms were analyzed. We also analyzed the effect of age and gender using a multivariate linear model adjusting by severity to avoid confusion effects, especially for age that are associated with severity. Statistical analyses were performed using R-3.6.3 (R Foundation for Statistical Computing) and Prism 9.0.2 (GraphPad Software).

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Supplemental information

Clinical course impacts early kinetics, magnitude, and amplitude of SARS-CoV-2 neutralizing antibodies beyond 1 year after infection

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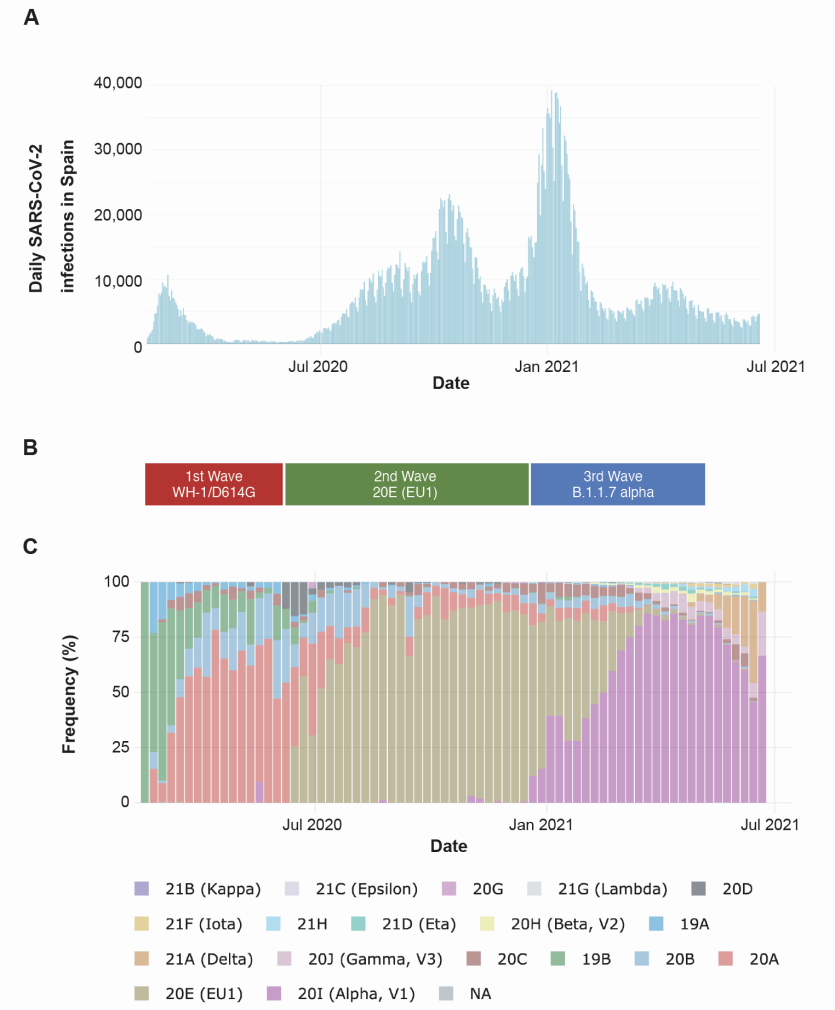
SUPPLEMENTARY FIGURE 1



Supplementary Figure 1. Temporal identification of SARS-CoV-2 waves and main viral variants in Spain. **Panel A** shows the incidence of SARS-CoV-2 infection (source: https://www.mscbs.gob.es/profesionales/saludPublica/ccayes/alertasActual/nCov/home.htm) identifying the main waves between March 2020 and June 2021 that are shown in **Panel B**. **Panel C** shows the frequency of circulating SARS-CoV-2 variants in the same time period and clearly associates wave 1 with the 19B and 20A variants, wave 2 with the 20E (EU1) variant and third wave with the 20I (alpha or B.1.1.7) variant (source: http://covidtag.paseq.org/). **Related to Table 1 and Figure 1**.

SUPPLEMENTARY TABLE 1

Subset of participants selected for variant analysis. **Related to Figure 3**

		Non-Hospitalized	Hospitalized	TOTAL
Number of cases		35	25	60
AGE	median [IQR]	44 [34.5 - 48.5]	62 [47 - 68]	46.5 [38 - 57.2]
GENDER	Female, n (%)	28 (80%)	11 (44%)	39 (65%)
GROUP, n (%)	Asymptomatic	1 (2.9%)	0 (0%)	1 (1.7%)
	Mild	34 (97.1%)	0 (0%)	34 (56.7%)
	Hospitalized non-severe	0 (0%)	7 (28%)	7 (11.7%)
	Hospitalized severe	0 (0%)	12 (48%)	12 (20%)
	Hospitalized (intensive care unit)	0 (0%)	6 (24%)	6 (10%)

SUPPLEMENTARY TABLE 2

Multiparametric analysis of loss of neutralization titers associated with alpha, beta and delta variants. Impact of age, gender and severity (Non-hospitalized / hospitalized). **Related to Figure 3.**

ALPHA variant			
	Estimate	**Std. Error**	**p-value**
Intercept	0.00067	0.20869	0.997
SEVERITY (Hosp)	0.05962	0.12506	0.636
AGE (per year)	-0.00037	0.00461	0.935
GENDER (Male)	-0.07751	0.11630	0.508
BETA variant			
	Estimate	**Std. Error**	**p-value**
Intercept	0.341029	0.220215	0.1282
SEVERITY (Hosp)	0.288541	0.132574	0.0346 *
AGE (per year)	-0.003163	0.004868	0.5191
GENDER (Male)	0.019781	0.123079	0.8730
DELTA variant			
	Estimate	**Std. Error**	**p-value**
Intercept	0.305829	0.197241	0.128
SEVERITY (Hosp)	0.173067	0.117602	0.148
AGE (per year)	-0.005322	0.004312	0.224
GENDER (Male)	-0.006291	0.108997	0.954

Part IV: Virological and clinical determinants of the magnitude of humoral responses to SARS-CoV-2 in mild-symptomatic individuals

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Virological and Clinical Determinants of the Magnitude of Humoral Responses to SARS-CoV-2 in Mild-Symptomatic Individuals

Edwards Pradenas[1†], Maria Ubals[2†], Víctor Urrea[1], Clara Suñer[2], Benjamin Trinité[1], Eva Riveira-Muñoz[1], Silvia Marfil[1], Carlos Ávila-Nieto[1], María Luisa Rodríguez de la Concepción[1], Ferran Tarrés-Freixas[1], Josep Laporte[2], Ester Ballana[1,3,4], Jorge Carrillo[1,4], Bonaventura Clotet[1,2,4,5], Oriol Mitjà[2] and Julià Blanco[1,3,4,5*]*

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Background: Evidence on the determinants of the magnitude of humoral responses and neutralizing titers in individuals with mild COVID-19 is scarce.

Methods: In this cohort study of mild COVID-19 patients, we assessed viral load (VL) by RT-qPCR at two/three time points during acute infection, and anti-SARS-CoV-2 antibodies by ELISA and plasma neutralizing responses using a pseudovirus assay at day 60.

Results: Seventy-one individuals (65% female, median 42 years old) were recruited and grouped into high viral load (VL) >7.5 Log_{10} copies/mL (n=20), low, VL ≤7.5 Log_{10} copies/mL (n=22), or as Non-early seroconverters with a positive PCR (n=20), and healthy individuals with a negative PCR (n=9). Individuals with high or low VL showed similar titers of total neutralizing antibodies at day 60, irrespective of maximal VL or viral dynamics. Non-early seroconverters had lower antibody titers on day 60, albeit similar neutralizing activity as the groups with high or low VL. Longer symptom duration and older age were independently associated with increased humoral responses.

Conclusions: In mild SARS-CoV-2-infected individuals, the duration of symptoms and age (but not VL) contribute to higher humoral responses.

Keywords: COVID-19, seroconversion, neutralizing antibodies, viral load, humoral response, symptoms

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INTRODUCTION

Large efforts to understand the Coronavirus Disease 2019 (COVID-19) pathology suggest that infected patients elicit a rapid humoral response against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Most patients seroconvert 19 days after symptom onset (1), though the kinetics of IgM and IgG antibodies is heterogeneous (2). Elicited antibodies show reactivity

against multiple viral proteins, including the outer Spike (S) protein, which is the target of neutralizing antibodies (3). These include mainly, but not exclusively, antibodies blocking the binding of the S protein to the ACE-2 receptor through interaction with different epitopes of the receptor-binding domain (RBD) (4–10).

Although there is not a clear clinical definition of protective immunity in humans, neutralizing antibodies, which are elicited in most infected individuals, are able to protect golden Syrian hamsters from the acquisition of SARS-CoV-2 infection (9, 11) and are thought to play a relevant role in viral clearance after natural infection (12). Moreover, neutralizing antibodies generated by natural infection seem to be long-lasting (13) and correlate with protection against clinical reinfection (14, 15). Paradoxically, individuals with severe COVID-19 produce high-titers of antibodies (1, 16), while mild or asymptomatic infection leads to lower antibody titers or even lack of seroconversion (17).

Most of the knowledge generated on humoral responses against SARS-CoV-2 is based on severe/hospitalized patients. However, epidemiological data indicate that up to 80% of infected individuals present with mild disease (18). Importantly, there is an undetermined number of infected individuals, up to 40% in some studies, that do not develop symptoms (19). Overall, the heterogeneity of clinical trajectories observed after SARS-CoV-2 infection has been linked to different dynamics of immune responses, being an early innate and adaptive responses associated with early control of the infection and a mild clinical course, while late appearance of antibodies could be associated with more severe disease (20). It is, therefore, essential to gain a more comprehensive understanding of the antibody response to SARS-CoV-2 infection that captures the case-mix of disease pathways, particularly in patients with asymptomatic or mild COVID-19.

Here, we hypothesized that the degree of antigenic exposure could be a major determinant of the level of the humoral immune response. To evaluate this hypothesis, we designed the CIRCUS study and determined the titer of antibodies (total and neutralizing) in samples from individuals with well-defined viral load (VL) dynamics during acute infection, recruited from a previous randomized-controlled trial of COVID-19 cases and their contacts.

MATERIALS AND METHODS

Study Design and Participants

This was an observational, prospective, and comparative pilot study: Characterizing the Immune Response to SARS-CoV-2 Under well-defined infection Settings, the CIRCUS study. The study aimed to characterize immune responses to SARS-CoV-2 among participants of the PEP CoV-2 "CQ4COV19" and Eudra CT 2020-001031-27 studies (21). Briefly, the PEP CoV-2 Study was a cluster-randomized clinical trial conducted during March and April 2020 in Catalonia (North-East Spain) to investigate the efficacy of hydroxychloroquine to treat and prevent COVID-19. The trial included two types of participants: mild confirmed cases of COVID-19 ("cases") and asymptomatic adults who had a recent history of close-contact exposure to a PCR-confirmed COVID-19 case ("contacts"). Serial oral and nasopharyngeal swab samples were obtained on days 0, 3, and 7 for cases, and days 0 and 14 for contacts. The presence of SARS-CoV-2 was investigated from nasopharyngeal swabs, and viral load was quantified by RT-qPCR as described below. For contacts, IgM and IgG antibodies were detected from fingertip blood at day 14 visit using a rapid test (VivaDiag^{TM} COVID-19 IgM/IgG) (22).

Participants of the CIRCUS study were selected among adult individuals (age ≥18 years) allocated in the control arm of the PEP CoV-2 trial and their close contacts; therefore, participants did not receive any investigational product. To characterize the impact of viral load on the magnitude of humoral responses to SARS-CoV-2, we defined four groups of patients from this cohort. The Non-early seroconverter group included 20 contacts with an acute SARS-CoV-2 infection characterized by a positive RT-qPCR test on days 0 and 14, and a negative result in the rapid antibody tests on day 14. The High VL group included 20 cases with an acute SARS-CoV-2 infection characterized by a positive RT-qPCR test, a maximum VL in nasopharyngeal swabs >7.5 Log_{10} copies/mL during acute infection (i.e., days 0, 3, or 7). The Low VL group included 22 cases with an acute SARS-CoV-2 infection characterized by a positive RT-qPCR test, a maximum VL in nasopharyngeal swabs <7.5 Log_{10} copies/mL during acute infection (days 0, 3, and 7). The control group consisted of 9 contacts without SARS-CoV-2 infection, confirmed by a negative RT-qPCR test (days 0 and 14), and a negative result in the rapid antibody tests (day 14). Cut-off VL value was selected according to virological data from the original PEP CoV-2 study (21).

A follow-up visit was scheduled 60 days after symptom onset or diagnosis by PCR (± 7 days). A blood sample and a nasopharyngeal swab were collected. Participants were interviewed about the presence of specific comorbidities and risk factors (smokers, hypertension, dyslipidemia, obesity, diabetes mellitus, respiratory or autoimmune disease). Respiratory diseases included Chronic Obstructive Pulmonary Disease (COPD) and asthma. Obesity was defined as a Body Mass Index (BMI)>30. Epidemiological and clinical data were obtained from CQ4COV19 clinical trial (age, gender, time from diagnosed infection, treatment, severity of infection, peak VL, VL follow-up, cumulative antigen exposure and time to symptom resolution). In this trial, resolution of symptoms was assessed sequentially using a symptoms questionnaire designed to gather information on the type of symptom and last day experienced; complete resolution was considered when no COVID-19–related symptoms were reported at all. Participants received telephonic interviews on days 3, 7, 14, and 28. The following symptoms were considered as COVID-19-related: dyspnea, fever, cough, sudden olfactory or gustatory loss, rhinitis, headache, thoracic pain (21). Severity of SARS-CoV-2 infection was assessed using the WHO scale (23).

SARS-CoV-2 PCR Detection and Viral Load Quantification

RNA extraction was performed using the Viral RNA/Pathogen Nucleic Acid Isolation kit (Thermo Fisher), optimized for a

KingFisher instrument (Thermo Fisher), following manufacturer's instructions. PCR amplification was based on the 2019-Novel Coronavirus Real-Time RT-PCR Diagnostic Panel guidelines and protocol developed by the American Center for Disease Control and Prevention (24). Briefly, a 20 µL PCR reaction was set up containing 5 µL of RNA, 1.5 µL of N2 or RNAseP primers and probe (2019-nCov CDC EUA Kit, Integrated DNA Technologies) and 10 µL of GoTaq 1-Step RT-qPCR (Promega). Thermal cycling was performed at 50°C for 15 min for reverse transcription, followed by 95°C for 2 min and then 45 cycles of 95°C for 10 sec, 56°C for 15 sec and 72°C for 30 sec in the Applied Biosystems 7500 or QuantStudio5 Real-Time PCR instruments (Thermo Fisher). For absolute quantification, a standard curve was built using 1/5 serial dilutions of a SARS-CoV-2 plasmid (2019-nCoV_N_Positive Control, 200 copies/µL, Integrated DNA Technologies) and run in parallel in all PCR determinations. The VL of each sample was determined in triplicate, and mean VL (in copies/mL) was extrapolated from the standard curve and corrected by the corresponding dilution factor. RNAseP gene amplification was performed in duplicate for each sample as an amplification control.

Humoral Response Determination

The humoral response against SARS-CoV-2 was evaluated with an in-house sandwich- ELISA using the following antigens (Sino Biological): S1+S2 protein, RBD (Arg319-Phe541), both potentially contributing to neutralizing activity; and whole nucleocapsid protein (NP), which is unrelated to neutralizing capacity. Nunc MaxiSorp plates were coated with 50 µL of anti-6x-His antibody clone HIS.H8 (2 µg/mL, Thermo Fisher) in PBS overnight at 4°C. After washing, plates were blocked with 1% BSA (Miltenyi Biotec in PBS) for two hours at room temperature. Antigens were added at 1 µg/mL (50 µL/well) and incubated overnight at 4°C. Plasma samples were heat-inactivated before use (56°C for 30 min) and analyzed in duplicate in antigen-coated and antigen-free wells in the same plate. Serial dilutions of a positive plasma sample were used as standard. A pool of pre-pandemic plasmas from healthy controls was used as a negative control. Standards, negative control, and plasma samples were diluted in blocking buffer and were incubated (50 µL/well) for one hour at room temperature. The HRP-conjugated F(ab')2-goat anti-human IgG (Fc specific, Jackson ImmunoResearch) was then incubated for 30 minutes at room temperature. Plates were revealed with o-Phenylenediamine dihydrochloride (Sigma-Aldrich), and the reaction was stopped using 4N of H_2SO_4 (Sigma-Aldrich). Optical density (OD) at 492 nm with noise correction at 620 nm were used to calculate specific signals for each antigen after subtracting the antigen-free well signal for each sample. Standard curves were fitted to a 5-parameter logistic curve, and data was quantitatively expressed as arbitrary units (AU) according to the standard.

Pseudovirus Neutralization Assay

HIV reporter pseudoviruses expressing SARS-CoV-2 S protein and Luciferase were generated. pNL4-3.Luc.R-.E- was obtained from the NIH AIDS Reagent Program (25). SARS-CoV-2.SctΔ19 was generated (GeneArt) from the full protein sequence of SARS-CoV-2 spike with a deletion of the last 19 amino acids in C-terminal (26), human-codon optimized and inserted into pcDNA3.4-TOPO. Expi293F cells were transfected using ExpiFectamine293 Reagent (Thermo Fisher) with pNL4-3.Luc.R-.E- and SARS-CoV-2.SctΔ19 at an 8:1 ratio, respectively. Control pseudoviruses were obtained by replacing the S protein expression plasmid with a VSV-G protein expression plasmid as reported (27). Supernatants were harvested 48 h after transfection, filtered at 0.45 µm, frozen, and titrated on HEK293T cells overexpressing WT human ACE-2 (Integral Molecular). This neutralization assay has been previously validated in a larger subset of samples (1).

Neutralization assays were performed in duplicate. Briefly, in Nunc 96-well cell culture plates (Thermo Fisher), 200 TCID50 of pseudovirus were preincubated with three-fold serial dilutions (1/60–1/14,580) of heat-inactivated plasma samples for 1 h at 37°C. Then, $2x10^4$ HEK293T/hACE2 cells treated with DEAE-Dextran (Sigma-Aldrich) were added. Results were read after 48 hours using the EnSight Multimode Plate Reader and BriteLite Plus Luciferase reagent (PerkinElmer). The values were normalized and the inhibitory dilution (ID) 50 (the reciprocal dilution inhibiting 50% of the infection) was calculated by plotting and fitting the log of plasma dilution versus response to a 4-parameters equation in Prism 8.4.3 (GraphPad Software).

Statistical Analysis

Continuous variables were described using medians and the interquartile range (IQR, defined by the 25^{th} and 75^{th} percentiles) or the mean and the standard error of the mean (SEM), whereas categorical variables were reported as percentages over available data. The different groups were compared using the nonparametric Mann-Whitney and Kruskal-Wallis with Dunn's multiple comparison tests. Correlations were assessed by Spearman test. Multivariate linear regression analyses were performed to assess independent associations of gender, age, symptoms duration and VL with the measured humoral responses at day 60 (Log_{10} transformed). Two-sided p-value ≤0.05 was considered statistically significant. All analyses were performed with GraphPad Prism 8.4.3 (GraphPad Software, Inc.) and R version 4.0 (R Foundation for Statistical Computing).

RESULTS

Study Cohort

The CIRCUS study enrolled 71 participants who had been exposed to a COVID-19 case no more than 5 days before enrollment and were asymptomatic or had mild symptoms with onset up to 3 days before enrollment. Main characteristics are described in **Table 1**. The median [IQR] age of individuals was 43 [30-52] years, and 64.8% (46/71) were female. Most of the individuals were either healthcare workers (60.6%) or nursing home workers (14.1%); the remaining were household contacts (22.5%). The main comorbidities were obesity, respiratory disease, dyslipidemia, and hypertension.

TABLE 1 | Description of participants.

	Non-early seroconverter n = 20	High VL n = 20	Low VL n = 22	Control n = 9
Gender (female), n (%)	11 (55)	12 (60)	18 (82)	5 (56)
Age (years), median [IQR]	45 [36-54]	37 [27-51]	40 [30-54]	47 [32-52]
Type of contact with index case, n (%)				
Household contact	9 (45)	1 (5)	1 (5)	5 (56)
Healthcare worker	4 (20)	18 (90)	17 (77)	4 (44)
Nursing home worker	7 (35)	0 (0)	3 (14)	0 (0)
Unknown	0 (0)	1 (5)	1 (5)	0 (0)
Coexisting disease and other factors, n (%)				
None	8 (40)	15 (75)	12 (54)	1 (11)
Smoker	5 (25)	1 (5)	1 (5)	5 (56)
HT or Dyslipidemia	4 (20)	0 (0)	5 (22)	1 (11)
Obesity or DM	3 (15)	0 (0)	5 (22)	3 (33)
Respiratory disease	3 (15)	4 (20)	1 (5)	1 (11)
Autoimmune disease	0 (0)	0 (0)	1 (5)	0 (0)
Clinical presentation				
Symptoms (yes), n (%)	5 (25)	19 (95)	19 (86)	–

IQR, interquartile range (25th and 75th percentiles).

For our analysis on determinant factors of antibody response, RT-qPCR positive individuals were categorized into 3 groups: 20 individuals (28.2%) with maximum viral load >7.5 $\log_{10}$ RNA copies/mL (High VL); 22 individuals (31.0%) with maximum viral load <7.5 $\log_{10}$ RNA copies/mL (Low VL), and 20 individuals (28.2%) with a negative rapid antibody test on day 14 after a positive PCR result (Non-early seroconverter). A group of 9 RT-qPCR negative individuals was also included as control. **Table 1** summarizes the main demographical and clinical characteristics of individuals allocated in each of the immune response groups.

When we looked at the clinical presentation in each of the three infected groups, the proportion of symptomatic disease was significantly higher in the High VL group (95%) and Low VL group (86%) compared to Non-early seroconverter individuals (25%, chi-square test $p < 0.001$, **Table 1**). No significant differences were observed between the High and Low VL groups.

Viral Dynamics and Seroconversion

The characterization of the study cohort included a virological and serological follow-up. The VL peak of Non-early seroconverter individuals was similar to that of the Low VL group and significantly lower than the High VL group (by definition >7.5 Log_{10} copies/mL, **Figure 1A**). The VL declined rapidly in the High VL group and slower in the Low VL group; Non-early seroconverter individuals showed a fast decay in VL, with only two positive samples 14 days after diagnosis (**Figure 1B**). The VL was associated with self-reported symptom duration, which was significantly lower in the Non-early seroconverter group and showed no significant differences between the Low and High VL groups (**Figure 1C**).

All individuals were tested 60 days after diagnosis to evaluate IgG humoral response by in-house ELISAs against the spike (S1+S2 protein), the RBD, and the NP. Of the 62 subjects with positive RT-qPCR for SARS-CoV-2, 49 (79.0%) had detectable IgG titers against all three antigens tested, while 5 (8.1%) individuals had no IgG antibodies against any antigen (all of them belonged to the Non-early seroconverter group). Antibodies against S1+S2 proteins and RBD were more frequently positive (89% and 90% of cases, respectively) than anti-NP antibodies (79%). The overall positivity (i.e., the proportion of individuals testing positive in at least one antigen) at day 60 was 100% for both the Low and High VL groups. The Non-early seroconverter group had a lower proportion of positive individuals to antibodies against S1+S2 proteins, RBD, and anti-NP antibodies: 70%, 70%, and 50%, respectively. The overall positivity (75%) was also lower in this group. All uninfected individuals had undetectable IgG antibodies (**Figure 1D**).

Levels of Humoral Immunity and Neutralizing Activity

The analysis of humoral responses at day 60 showed no differences between High and Low VL groups for anti-S1+S2, anti-NP, or anti-RBD responses. When comparing these two groups together with the Non-early seroconverter group, we observed significant differences with lower median IgG titer against all antigens ($p < 0.05$, **Figures 2A–C**). Using a neutralization assay with HIV-based pseudoviruses exposing the SARS-CoV-2 S or the VSV-G proteins, we analyzed all plasma samples using serial dilutions starting at 1/60 dilution (limit of detection). Specific neutralizing activity against SARS-CoV-2 was detected in 95% of RT-qPCR positive cases, including 85% in the Non-early seroconverter group. High and Low VL groups showed similar median values of neutralization titers, and the Non-early seroconverter group did not have significantly lower values compared to the other groups (**Figure 2D**).

Similar results were observed in an additional analysis in which Non-early seroconverters with High or Low VL (n=3 and n=17, respectively) were reclassified in the High and Low VL groups. These larger (n=23 High VL, n=39 Low VL) groups did not show differences in the levels of anti-S1+S2, anti-NP, anti-RBD or neutralizing antibodies at day 60 of follow-up (**Supplementary Figure 1**).

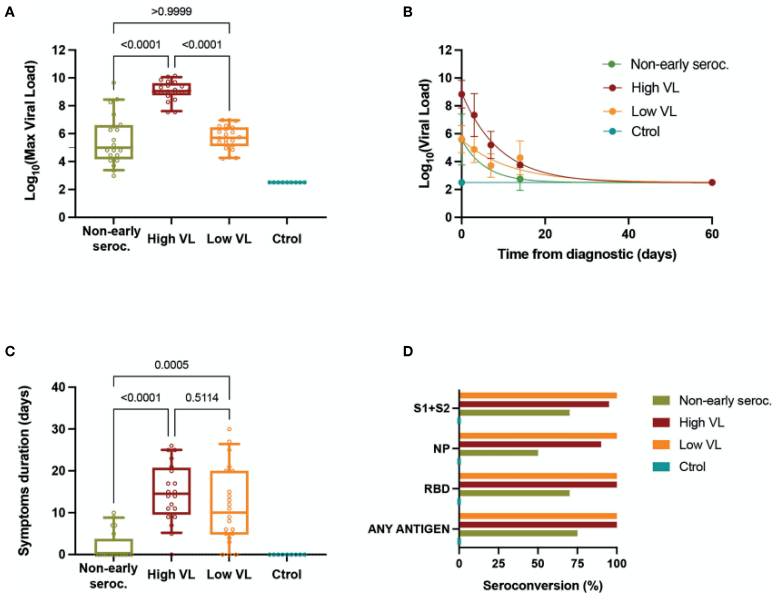

FIGURE 1 | Viral load determinations. **(A)** Maximal values of VL for each individual. Boxes show the median and the 25th-75th interquartile range and bars the 10th-90th interquartile range. P values correspond to Kruskal-Wallis test with Dunn's multiple comparisons. **(B)** VL dynamics in each group (mean ± SEM) with the best fit curve (single exponential decay). **(C)** Self-reported symptom duration for each individual. Boxes show the median and the 25th-75th interquartile range and bars the 10th-90th interquartile range. P values correspond to Kruskal-Wallis test with Dunn's multiple comparisons. **(D)** Seroconversion (frequency of positive samples for the indicated individual antigens or for any of them) in the different groups assessed by ELISA at day 60 of follow-up.

The unexpected high neutralization observed in Non-early seroconverter individuals, despite significantly lower antibody titers, suggested a good quality of neutralization in this group. To assess this possibility, we calculated the ratio of neutralizing titers and total antibodies for each individual as a proxy of antibody quality. The comparison of these ratios among groups showed significant differences for the ratio neutralization/anti-S1+S2 antibodies and anti-NP antibodies, being in both cases higher in Non-early seroconverter individuals (**Figures 2E, G**). In contrast, no impact of levels of anti-RBD antibodies on neutralization activity was observed (ratios similar among groups, **Figure 2F**).

Analysis of Determinants of Humoral Response

The multiple correlation analysis showed a positive association between the neutralizing antibody titer and the total IgG antibody levels against all antigens, with the highest association observed in anti-spike antibodies (r=0.78, **Figure 3A**). The results were similar when we performed the analysis for each group of patients separately (data not shown). In the analysis of determinants of humoral response, we adjusted for virological and demographical factors that could be associated with the presence of IgG antibodies and neutralizing activity. Gender was analyzed separately, showing no impact on neutralizing antibodies (**Figure 3B**). The maximum VL was not associated with neutralizing capacity (p = 0.17, **Figure 3C**), nor with IgG antibody titers against any antigen (anti-S1+S2: p = 0.11; anti-RBD: p = 0.12; anti-NP: p = 0.21, data not shown). In contrast, we observed that both the duration of symptoms and age were associated with the level of the humoral response. The duration of symptoms was positively associated with antibody titers (**Figure 3A**) and neutralizing activity (r = 0.33; p = 0.0095, **Figure 3D**), although the analysis by groups revealed a lack of correlation for the Non-early seroconverter group (r = 0.99; p = 0.72, data not shown), probably due to the higher frequency of asymptomatic individuals.

Older age correlated with increasing neutralizing antibody titer (r = 0.29; p = 0.023) (**Figure 3E**), but the correlation was not significant for the Non-early seroconverter group (r = 0.35; p = 0.14, data not shown). This correlation was also observed for anti-RBD antibodies (r = 0.35; p = 0.005), but not for anti-S1+S2 (r = 0.21; p = 0.10) and anti-NP (r = 0.18; p = 0.16) antibodies (**Figure 3A**).

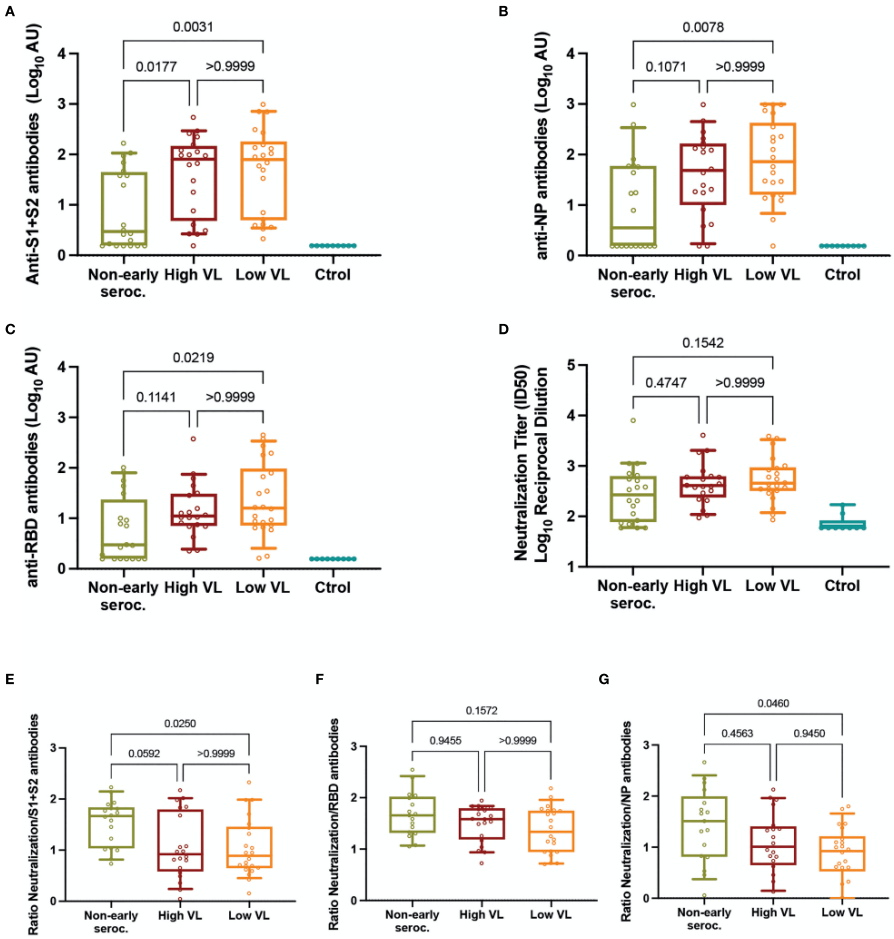

FIGURE 2 | Neutralizing activity. Individual titers of **(A)** anti-S1+S2 antibodies, **(B)** anti-NP antibodies, **(C)** anti-RBD antibodies, and **(D)** neutralization. Individual ratios of neutralization titers to **(E)** anti-S1+S2, **(F)** anti-RBD, and **(G)** anti-NP antibodies. In all panels, boxes show the median and the 25th-75th interquartile range and bars the 10th-90th interquartile range. *P* values correspond to Kruskal-Wallis test with Dunn's multiple comparisons. ID, inhibitory dilution; NP, nucleocapsid protein; VL, viral load.

We also performed multivariate linear regression analysis to identify the relationship of gender, age, duration of symptoms and VL with humoral responses. As summarized in **Supplementary Table 1**, duration of symptoms was independently associated with anti-S1+S2 and anti-NP antibody titers (p = 0.0018 and 0.0036, respectively). Both duration of symptoms and age were associated with anti-RBD antibody titers (p = 0.0062 and 0.0140, respectively), while exclusively age was significantly associated with neutralization titers (p = 0.0114, **Supplementary Table 1**). In summary, these analyses confirmed that duration of symptoms and age are the main determinants of the magnitude of humoral responses in our cohort.

To further assess the impact of asymptomatic infection on neutralization titers, we grouped patients into asymptomatic (n=19) or symptomatic (n=43), irrespective of the initial classification. Although neutralization titers tended to be lower in asymptomatic individuals, the comparison did not reach statistical significance due to the presence of high-neutralizers

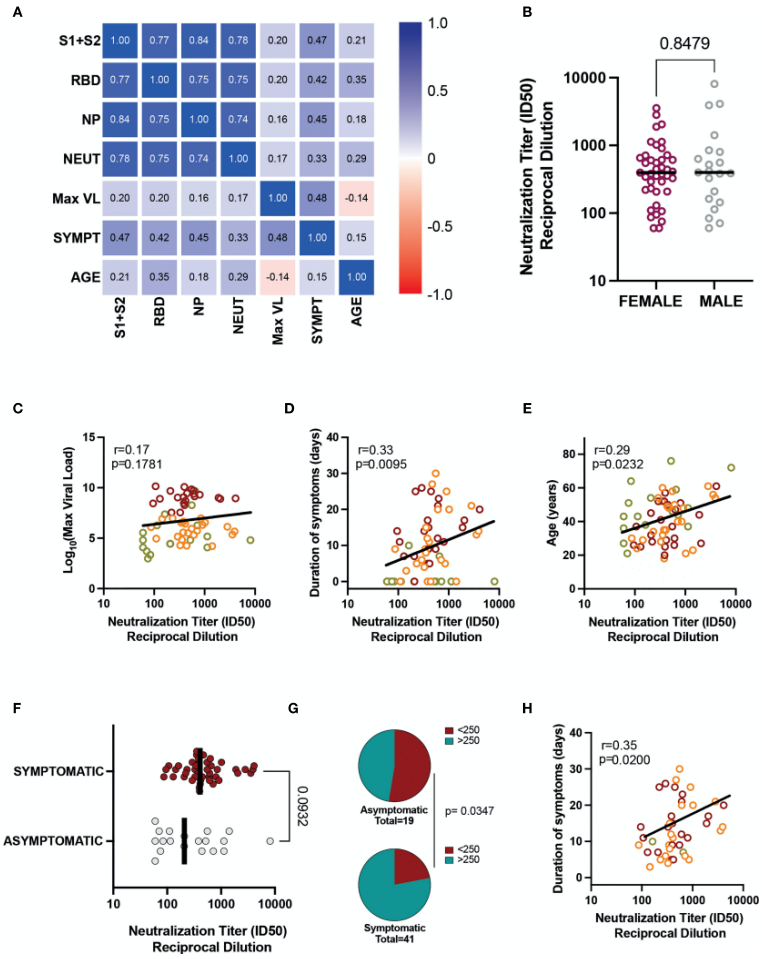

FIGURE 3 | Associations between humoral responses and clinical/demographic characteristics. **(A)** Heatmap of correlations between total IgG, neutralizing antibody titer, VL, symptom duration, and age for all patients. **(B)** Neutralization titers in female and male participants (all groups). Bar indicates median values, and the *p*-value is shown (Mann-Whitney test). Correlation of neutralization titers with **(C)** VL, **(D)** Duration of Symptoms, and **(E)** Age. Individual values are color-coded: green for Non-early seroconverter individuals at diagnosis, orange for individuals with low viral load, and red for individuals with high viral load. Lines indicate linear regression of all values for illustrative purposes. The correlation coefficient and *p*-value (Spearman correlation test) are shown. **(F)** The impact of asymptomatic infection was assessed by comparing neutralizing titers between all asymptomatic (grey dots) and all symptomatic patients (red symbols). **(G)** Frequency of low and high neutralizers (cutoff value 1/250); the Fisher exact test *p*-value is shown. **(H)** Correlation between symptom duration and neutralization titer in symptomatic individuals. The correlation coefficient and *p*-value (Spearman correlation test) is shown. ID, inhibitory dilution; NP, nucleocapsid protein; RBD, receptor-binding domain; VL, viral load.

in the asymptomatic group (**Figure 3F**). Nevertheless, the frequency of low neutralizers (using a cutoff value of 1/250) (14, 16) was significantly higher in the asymptomatic (**Figure 3G**), suggesting that asymptomatic individuals may interfere in the global analysis of the correlation of symptom duration and neutralizing responses. To avoid this potential artifact, we reanalyzed the data using only symptomatic individuals, which maintained a significant correlation (r =

0.35; p = 0.0200) between neutralization titers and symptom duration (**Figure 3H**), thus confirming the robust link between both parameters. Humoral responses (neutralizing or total antibodies) were not significantly influenced by other clinical or demographic characteristics such as gender, smoking, cardiovascular disease, obesity, respiratory disease, influenza vaccination, or residual symptoms (data not shown).

DISCUSSION

In this study of factors influencing the levels of neutralizing antibodies in asymptomatic and mildly symptomatic SARS-CoV-2-infected individuals, we observed that more than 90% of participants had detectable IgG antibodies 60 days after diagnosis. The proportion of serum antibody positivity was 100% in symptomatic individuals; however, it was lower in asymptomatic individuals, also leading to lower titers of neutralizing antibodies. This finding has already been described (1, 28, 29) and points to a relevant role of other arms of the immune system (innate immunity or cellular responses) in the early and effective control of SARS-CoV-2 infection, in the absence of detectable serum antibodies (30, 31).

Several studies have shown that humoral response is related to COVID-19 severity (1, 2, 32), which in turn is associated with age and gender (33) and has also been linked to VL (34). However, these reports analyze both hospitalized individuals and outpatients and are usually not designed to analyze these groups independently. Our study, which specifically focuses on outpatients to uncover the potential determinants of the magnitude of humoral responses, found that only age and symptom duration had a significant association. While age seems to correlate with neutralization and RBD antibodies, symptom duration showed a more consistent correlation with all humoral response parameters analyzed (anti-S1+S2, anti-RBD, anti-NP, and neutralizing antibodies). The correlation was still strong when looking at symptomatic individuals alone to avoid a potential confounding effect of asymptomatic individuals.

Finally, we did not observe an association between the elicitation of humoral responses and the different comorbidities; this should be interpreted with caution because the number of individuals with these characteristics did not allow us to perform a formal statistical analysis. Additionally, some comorbidities, such as hypertension, cardiovascular and respiratory diseases, have been found to be related to the COVID-19 severity (35); nevertheless, our study included only asymptomatic or mild symptomatic cases, explaining the fact that no differences were observed between groups with and without underlying diseases.

Our data is consistent with the notion that the early control of SARS-CoV-2 replication may be determinant in humoral responses. An effective control of infection by strong innate mechanisms or preexisting cross-reactive CTLs may limit the extent of SARS-CoV-2 replication (36, 37), and hence antigen levels and subsequent antibody development. In contrast, the failure to control viral replication may lead to sustained B cell activation and antibody generation, resulting in increased titers of humoral responses. The determinants of this early control are still unclear and involve the efficacy of both innate and adaptive responses (20). Cross-reactivity of SARS-CoV-2 and other common cold human coronaviruses have been reported not only for cellular responses but also for antibodies (38–40), mainly those directed against the S2 subunit of Spike glycoprotein (41). In our cohort, we could not analyze preexisting responses but we found surprisingly high levels of neutralizing antibodies in individuals that were seronegative 14 days after diagnosis. When examining the reasons behind this high activity, we observed that it seems to be related to a high quality of neutralizing antibodies against the S1 and S2 proteins, but not against the RBD. Whether this observation reflects the rapid expansion of preexisting memory B cell responses remains to be defined.

Our analysis is mainly limited by the reduced sample size, which is insufficiently powered to assess comorbidities as factors that could determine the humoral response. In addition, our findings are not necessarily transferable to newer SARS-CoV-2 variants that exhibit modified tissue tropism, transmissibility and potentially immune response pattern (42). These and other parameters should be investigated further, and larger studies with longer follow-ups will be needed to address this issue properly. In contrast, despite limited sample size, our data on VL dynamics clearly rule out an impact of the level of viral exposure (as determined in nasopharyngeal swabs) on humoral responses. Rather, we identified age and symptom duration, which showed a partial correlation with VL, as the main parameter determining humoral responses in mild COVID-19 patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité Ético Hospital Germans Trias i Pujol. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JB, OM and BC designed and coordinated the study. EP, BT, SM, CA-N, MLR, FT-F and JC, performed and analyzed the neutralization and ELISA assays. ER-M and EB, performed viral load quantification. VU performed the statistical analysis. MU, CS and JL selected the patients and coordinated the data. EP, OM and JB drafted the manuscript, and all authors made

substantial contributions to the revision of the subsequent versions. All authors approved the submitted version of the manuscript and agreed both to be personally accountable for their own contributions and to answer the questions related to the accuracy or integrity of any part of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.860215/full#supplementary-material

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Conflict of Interest: Outside the submitted work JB and JC are founders and shareholders of AlbaJuna Therapeutics, S.L. BC is founder and shareholder of AlbaJuna Therapeutics, S.L and AELIX Therapeutics, S.L.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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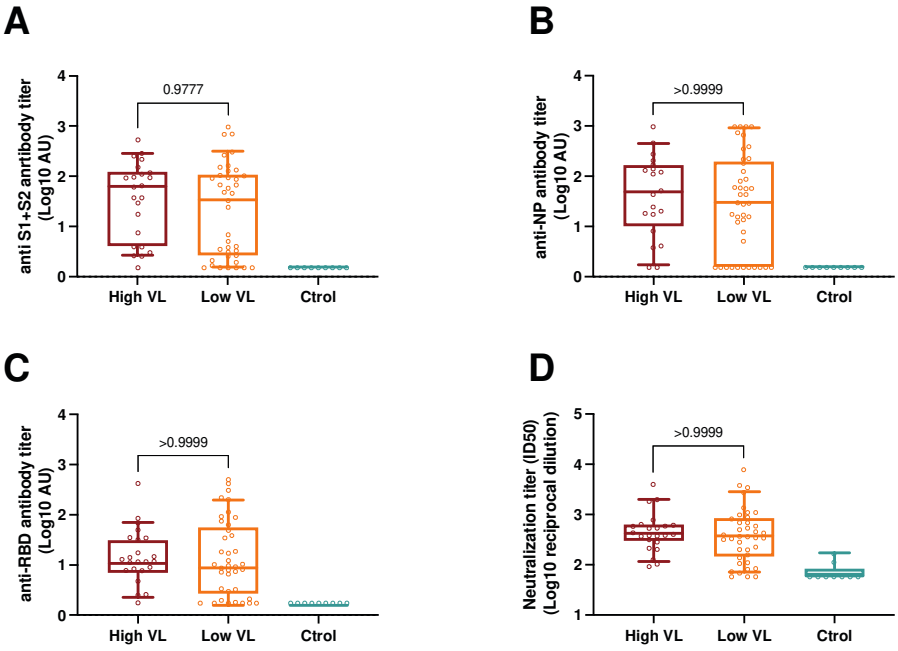
Supplementary Material



Supplementary Figure 1. Merged analysis of all participants. Participants were analyzed according to their maximal VL. Three Non-early seroconverter individuals were included in the High VL group (n=23) and 17 were included in the Low VL group (n=39). Control uninfected individuals were included for reference (n=9). The indicated quantitative parameters of humoral responses were analyzed at day 60. No statistical differences were observed (Mann-Whitney test).

Neutralization				
	Estimate	Std. Error	*p*-value	
Gender (men)	0.050094	0.129006	0.6992	
Age (year)	0.01165	0.004456	0.0114	*
Duration of symptoms (days)	0.013253	0.007762	0.0932	
Viral Load (Log10 copies/mL)	0.023485	0.033324	0.4838	

anti-S1+S2 antibody titer				
	Estimate	Std. Error	*p*-value	
Gender (men)	-0.052055	0.224112	0.81716	
Age (year)	0.010559	0.007742	0.17798	
Duration of symptoms (days)	0.044127	0.013484	0.00181	**
Viral Load (Log10 copies/mL)	0.015272	0.057891	0.79288	

anti-NP antibody titer				
	Estimate	Std. Error	*p*-value	
Gender (men)	-0.039961	0.244766	0.8709	
Age (year)	0.006731	0.008455	0.4293	
Duration of symptoms (days)	0.044728	0.014726	0.0036	**
Viral Load (Log10 copies/mL)	0.00127	0.063226	0.984	

anti-RBD antibody titer				
	Estimate	Std. Error	*p*-value	
Gender (men)	0.113802	0.173774	0.51517	
Age (year)	0.015217	0.006003	0.01402	*
Duration of symptoms (days)	0.029688	0.010455	0.00625	**
Viral Load (Log10 copies/mL)	0.001334	0.044888	0.97639	

Supplementary Table 2. Analysis of factors associated with humoral responses. A multivariate linear regression analysis was performed to identify independent association of gender, age, duration of symptoms and VL with Neutralization or the indicated antibody titers. Asterisks denote significant association (*<0.05, **<0.001).

Part V: Previous SARS-CoV-2 infection increases B.1.1.7 cross-neutralization by vaccinated individuals

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Previous SARS-CoV-2 Infection Increases B.1.1.7 Cross-Neutralization by Vaccinated Individuals

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Abstract: With the spread of new variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), there is a need to assess the protection conferred by both previous infections and current vaccination. Here we tested the neutralizing activity of infected and/or vaccinated individuals against pseudoviruses expressing the spike of the original SARS-CoV-2 isolate Wuhan-Hu-1 (WH1), the D614G mutant and the B.1.1.7 variant. Our data show that parameters of natural infection (time from infection and nature of the infecting variant) determined cross-neutralization. Uninfected vaccinees showed a small reduction in neutralization against the B.1.1.7 variant compared to both the WH1 strain and the D614G mutant. Interestingly, upon vaccination, previously infected individuals developed more robust neutralizing responses against B.1.1.7, suggesting that vaccines can boost the neutralization breadth conferred by natural infection.

Keywords: SARS-CoV-2; humoral response; pseudovirus; neutralization; B.1.1.7 variant

1. Introduction

Early in the COVID-19 pandemic, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants started to develop regionally and globally. Currently, the rapid spread of the B.1.1.7, or 501Y.V1, variant [1], first reported in the UK, casts doubts on the

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protection conferred by the neutralizing antibody response acquired during a previous immunization. Besides the D614G mutation, the B.1.1.7 variant contains six non-synonymous mutations and three deleted amino acids in the spike (S) protein (Figure 1A). The major changes are the mutation N501Y in the receptor-binding domain (RBD); the deletion 69–70 which may increase transmissibility [2] and produces a false negative in certain RT-PCR-based diagnostic assays; and the mutation P681H, next to the furin cleavage site, that could impact antigenicity and enhance viral infectivity. Recent studies indicate that B.1.1.7 is associated with a higher hospitalization risk [3] and higher mortality [4] and several reports indicate that it has a higher secondary attack rate, making this viral variant 30–50% more transmissible [5]. Importantly, this variant remains susceptible to some monoclonal and plasma antibodies from convalescent or vaccinated individuals [6–8].

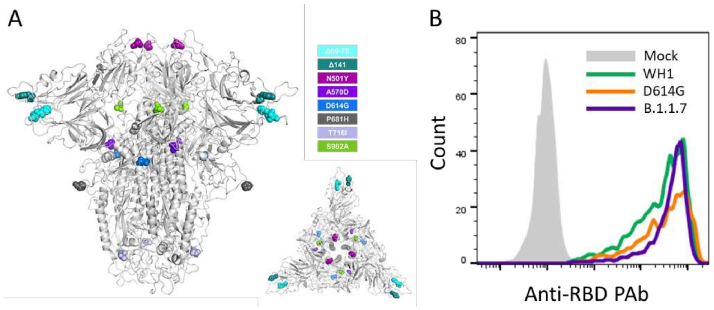

Figure 1. In vitro infectivity of SARS-CoV-2 variants. (**A**). The different mutations identified in the B.1.1.7 variant are listed and their location in the spike protein (side and top views) is shown. This variant also includes the D614G mutation. (**B**). Spike expression in pseudovirus-producing cells stained with an anti-RBD polyclonal rabbit antibody (See Methods for details).

Here we analyzed cross-neutralizing plasma antibody titers in individuals infected during both the first and second waves of COVID-19 epidemics in Catalonia (Spain), as well as in vaccinated individuals. The B.1.1.7 variant showed minimal resistance to the neutralizing capacity from both infected and vaccinated individuals, but its impact was significantly more pronounced on the latter group. Interestingly, previous infection significantly improved neutralization titers against this variant upon vaccination.

2. Materials and Methods

2.1. Study Overview and Subjects

The study was approved by the Hospital Ethics Committee Board from Hospital Universitari Germans Trias i Pujol (PI-20-122 and PI-20-217) and all participants provided written informed consent before inclusion.

Plasma samples were obtained from the prospective KING cohort of the HUGTiP (Badalona, Spain) and from Althaia (Manresa, Spain). The KING cohort included individuals with a documented positive RT-qPCR result from nasopharyngeal swab and/ or a positive serological diagnostic test.

Samples in this study were collected from March 2020 to February 2021; thus, covering the different COVID-19 outbreaks in Catalonia (dadescovid.cat). We analyzed 32 non-vaccinated individuals infected in March 2020, using plasma samples collected at a median of 48 days ($n = 16$) or 196 ($n = 16$) days after symptom onset. We also selected 16 individuals infected in August 2020 using plasma samples collected at a median of 44 days after symptom onset, and 5 patients ($n = 13$ samples) infected in January 2021 by the B.1.1.7 variant. Finally, 32 individuals having received two doses of Pfizer/ BioNTech vaccine were sampled 2 weeks after the second dose. This last group included uninfected and long-term

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previously-infected individuals. A description of the different groups and subgroups is shown in Table 1.

Table 1. Description of participants. Uninfected individuals were included as negative controls for neutralizing activity. All of them showed undetectable neutralizing activity. IQR: interquartile range.

	Uninfected *n* = 5	**Infected Non-Vaccinated** *n* = 53				**Vaccinated** *n* = 32	
Infection Status Date of Infection Strains	**Uninfected**	**Infected March 2020 D614/G614**		**Infected August 2020 20E (EU1)**	**Infected January 2021 B.1.1.7**	**Infected March 2020 D614/G614**	**Uninfected**
Sampling		**Early** *n* = 16	**Late** *n* = 16	*n* = 16	*n* = 5	*n* = 16	*n* = 16
Age (years), median (IQR)	46 (42–52)	65 (55–68)	56 (54–62)	44 (37–54)	79 (60–91)	39 (29–44)	45 (30–61)
Gender (female), *n* (%)	4 (80)	4 (25)	7 (44)	8 (50)	2 (40)	11 (69)	12 (75)
Days from symptom onset, median (IQR)	—	48 (36–57)	196 (186–207)	44 (37–54)	16 (8–20) *	324 (184–339)	—
Days from vaccination, median (IQR)	—	—	—	—	—	13 (10–14)	9 (7–12)
Hospitalized, *n* (%)	—	11 (69)	11 (69)	11 (69)	5 (100)	0 (0)	—
Severe (%)	—	6 (38)	6 (38)	5 (31)	0 (0)	0 (0)	—

* Data from 13 samples obtained from 5 participants in this group.

2.2. Cell Lines

HEK293T cells (presumably of female origin) overexpressing WT human ACE-2 (Integral Molecular, Philadelphia, PA, USA) were used as a target for a SARS-CoV-2 spike-expressing pseudovirus infection. The cells were maintained in T75 flasks with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1 µg/mL of Puromycin.

2.3. Spike Plasmid Generation

SARS-CoV-2.SctΔ19 WH1 and B.1.1.7 were generated (Geneart) from the full protein sequence of the original SARS-Cov-2 isolate Wuhan-Hu-1 (WH1) and the UK variant (B.1.1.7) spike sequences respectively, with the deletion of the last 19 amino acids in C-terminal [9], human-codon optimized and inserted into pcDNA3.1(+). The D614G spike mutant was generated by site-directed mutagenesis as previously described [10]. In brief, SARS-COV-2.SctΔ19 WH1 plasmid was amplified by PCR with Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA, ref# F-549S) and the following primers: 5′-TACCAGGgCGTGAACTGTACCGAAGTGCC-3′ and 5′-GTTCACGcCCTGGTACAGCA CTGCCAC-3′. PCR was 20 cycles with an annealing temperature of 60 °C and an elongation temperature of 72 °C. The PCR product was then treated for 3 h with the DpnI restriction enzyme (Thermo Fisher Scientific, ref# ER1705), to eliminate template DNA, and transformed into supercompetent *E. coli*. The final mutated DNA was then fully sequenced for validation.

2.4. Pseudovirus Generation and Neutralization Assay

HIV reporter pseudoviruses expressing SARS-CoV-2 S protein and Luciferase were generated using the defective HIV plasmid pNL4-3.Luc.R-.E- obtained from the NIH AIDS Reagent Program [11]. Expi293F cells were transfected using ExpiFectamine293 Reagent (Thermo Fisher Scientific) with pNL4-3.Luc.R-.E- and SARS-CoV-2.SctΔ19 (WH1, G614 or B.1.1.7), at an 8:1 ratio, respectively. Control pseudoviruses were obtained by replacing the S protein expression plasmid with a VSV-G protein expression plasmid as reported [12]. Supernatants were harvested 48 h after transfection, filtered at 0.45 µm, frozen and titrated on HEK293T cells overexpressing WT human ACE-2. Spike expression in pseudovirus-producing cells was confirmed by flow cytometry, showing comparable expression for all constructs (Figure 1B).

Neutralization assays were performed in duplicate. Briefly, in Nunc 96-well cell culture plates (Thermo Fisher Scientific), 200 $TCID_{50}$ of pseudovirus were preincubated with three-fold serial dilutions (1/60–1/14,580) of heat-inactivated plasma samples for 1 h at 37 °C. Then, 2×10^4 HEK293T/hACE2 cells treated with DEAE-Dextran (Sigma-Aldrich) were added. Results were read after 48 h using the EnSight Multimode Plate Reader and BriteLite Plus Luciferase reagent (Perkin Elmer, Waltham, MA, USA). The values were normalized, and the ID_{50} (the reciprocal dilution inhibiting 50% of the infection) was calculated by plotting and fitting the log of plasma dilution vs. response to a 4-parameter equation in Prism 8.4.3 (GraphPad Software, San Diego, CA, USA). This neutralization assay had been previously validated in a large subset of samples [13,14]. The lower limit of detection was 60 and the upper limit was 14,580 (reciprocal dilution).

2.5. Flow Cytometry

Transfected Expi293F cells were first stained extracellularly with a polyclonal rabbit anti-spike RBD antibody (Sino Biological, Beijing, China, ref# 40592-T62) and a secondary APC labeled anti-rabbit (Jackson Immuno Research, West Grove, PA, USA, ref# 711-605-152). Cells were then fixed (Life Technologies, Carlsbad, CA, USA, ref# GAS001S100) and stained in permeabilization buffer (Life Technologies, ref# GAS002S100), with a FITC-labeled mouse anti-p24Gag antibody KC57 (Beckman Coulter, Brea, CA, USA, ref# 6604665). Cells were acquired on a BD Celesta flow cytometer with DIVA software and analyzed on FlowJo vX.0.7 (Tree Star, Inc., Ashland, OR, USA).

2.6. Statistical Analysis

Continuous variables were described using medians and the interquartile range (IQR, defined by the 25th and 75th percentiles), whereas categorical factors were reported as percentages over available data. Quantitative variables were compared using the Mann–Whitney test; and percentages using the chi-squared test. The Friedman test with Dunn's multiple comparison test was used to compare neutralization of different pseudoviruses. Multiple M–W comparisons were corrected by false discovery rate. Analyses were performed with Prism 8.4.3 (GraphPad Software) and R version 4.0 (R Foundation for Statistical Computing).

3. Results

3.1. Global Analysis of Cross-Neutralizing Titers in Vaccinated and Infected Participants

As a first approach, we tested the neutralizing antibody response of all participants segregated in two main groups labeled "all vaccinated" and "all infected". "All vaccinated" included all the vaccinated participants whether they had experienced previous natural infection or not. "All infected" included all non-vaccinated participants who were infected during either the first wave, the second wave or specifically infected by the B.1.1.7 variant. We tested all the plasma samples ($n = 98$) against pseudoviruses expressing three different spike glycoproteins: a spike corresponding to the original SARS-CoV-2 virus, isolated in Wuhan, and named here WH1; a D614G mutant based on the WH1 spike and a spike including the defining mutations of the B.1.1.7 variant and named B.1.1.7.

The neutralization titers of all vaccinated individuals against B.1.1.7 and WH1 were not statistically different (according to the Friedman test with Dunn's multiple comparison test) (Figure 2A), despite WH1 expressing the spike sequence on which the vaccine was based. In contrast, vaccinated individuals showed significantly higher potency to neutralize the intermediate D614G mutant ($p < 0.0001$) (Figure 2A). A similar analysis, including all non-vaccinated infected individuals, showed similar results (Figure 2B). The highest neutralization ($p < 0.0001$) was noticed for the D614G mutant, while no significant differences were observed between WH1 and B.1.1.7. Next, we compared cross-neutralization capacities by determining fold change ratios between a spike of interest (S^x, indicated on top) and a reference spike (S^r, indicated in the Y axis of Figure 2C,D, fold change = S^r/S^x). This ratio is a measure of the loss of neutralizing capacity; a ratio inferior to one indicates a better neutralization of the spike of interest in comparison to the reference spike, and

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vice versa. When B.1.1.7 and WH1 variants were compared, median fold-change was 0.97 in the vaccinated group, a value significantly higher than the one obtained in naturally infected individuals (0.7; $p = 0.033$, M–W test, Figure 2C). Similarly, the fold change between B.1.1.7 and D614G was significantly different between the all vaccinated and the all infected groups (median values 1.9 vs 1.17, respectively; $p < 0.0001$, M–W test, Figure 2D). Altogether, these data indicate that, relative to WH1 and D614G, cross-neutralization of B.1.1.7 was worse in vaccinated individuals in comparison to infected ones.

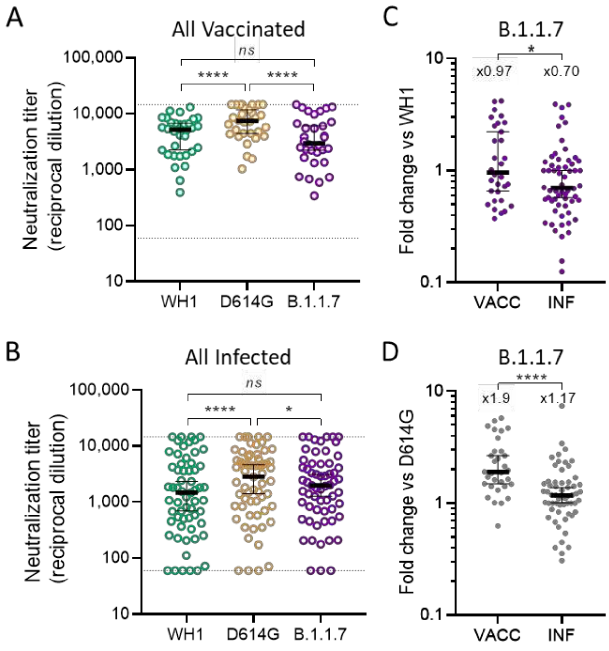

Figure 2. Global analysis of neutralization titers in SARS-CoV-2 vaccinated and infected individuals. Values of ID_{50} (as reciprocal dilution) are shown for all plasma samples from (**A**) vaccinated and (**B**) infected non-vaccinated individuals against the indicated pseudoviruses. Bars indicate median titer in each group with a 95% confidence interval and $p$values show the comparison of median titers among the three viruses (Friedman test with Dunn's multiple comparison test, * $p < 0.05$, **** $p < 0.0001$). The corresponding fold-change in neutralization titers between (**C**) WH1 and B.1.1.7 or (**D**) D614G and B.1.1.7 is shown (lower is better), comparing the vaccinated (VACC) and the infected (INF) groups. Bars indicate median in each group with 95% confidence interval and top values indicate the median fold-change between the indicated variants (variants compared are indicated in the graph title and in the Y axis). Fold change medians were compared using the Mann–Whitney test (* $p < 0.05$, **** $p < 0.0001$).

3.2. Identification of Parameters Influencing Cross-Neutralization

To better understand these differences, we analyzed infected and vaccinated subgroups. To assess the impact of sequence evolution on the immune responses, infected individuals were divided according to infection date. Individuals infected during the first wave (March 2020) in Spain were initially exposed to the original D614 virus that was rapidly displaced by the G614 variant. An evolving 85 to 22% prevalence for the original variant has been estimated [15]. Individuals infected during the second wave

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(August 2020) were almost exclusively exposed to the G614-containing 20E (EU1) lineage, which accounted for nearly 100% of new infections during the summer of 2020 (https://nextstrain.org/ncov/global, accessed on 31 March 2021). Individuals infected by the B.1.1.7 variant, identified in January 2021, were also analyzed.

First, we analyzed the neutralization response specifically in participants infected during the first wave. Individuals sampled 48 days after infection showed a small but significant decrease in neutralization capacity against the B.1.1.7 variant when compared with the D614G mutant (median fold change of 1.53, $p = 0.031$, Friedman test, Figure 3A,B). When compared to WH1, no significant difference was observed (median = 1.20-fold, $p = 0.155$). Individuals infected on March 2020 and sampled 6 months later also showed a general decay of the neutralization response as previously reported by us and many others [14,16]. Interestingly though, in these limited cohorts, decay of the neutralization response was only significant when measured against WH1 ($p = 0.0374$, K-W, Figure 3A) and not against D614G or B.1.1.7. In fact, we observed a general trend of improving cross-neutralization capacities against both D614G and the B.1.1.7 variant when compared with WH1, although it was only significant for the latter (fold change evolving from 1.2 to 0.6, $p = 0.024$, M–W, Figure 3B). These data suggest that the neutralization response induced by natural infection continued to evolve several months after the initial symptoms.

We then looked at the neutralization responses induced during the subsequent waves of infection. Individuals infected in August 2020 or infected by the B.1.1.7 variant behaved differently from those infected in March 2020, in that no significant differences were detected between neutralizing titers against all three tested spikes (Figure 3C). When comparing ratios with first wave participants, B.1.1.7 infected individuals showed a significant increase in the neutralization of B.1.1.7 vs D614G (fold change going from 1.53 to 1, $p = 0.0006$) (Figure 3D). These data illustrate the progressive evolution of the virus infecting the population and its impact on the induced cross-neutralization response. Importantly, individuals infected by the B.1.1.7 variant were still able to cross-neutralize the original WH1 spike and the D614G mutant.

Finally, we analyzed vaccinated individuals. Vaccinated individuals were sub-classified according to previous COVID-19 evidence into infected or uninfected. We compared three groups: vaccinated non-infected, vaccinated previously infected during the first wave and the group of first wave infected participants (late plasma sampling) not vaccinated. As we did not have the neutralization levels of vaccinated previously infected individuals prior to vaccination, we used the group of first wave infected (late plasma) individuals as a surrogate.

When analyzing specifically vaccinated individuals not previously infected, the decrease in B.1.1.7 cross-neutralization already observed for the all vaccinated group (Figure 2A) was even more clear (Figure 4A). This was significant in comparison to both WH1 (fold change of 2.04, $p = 0.0021$) and D614G (fold change of 2.65, $p = <0.0001$). Vaccinated individuals previously infected did not show such a decrease; in fact, B.1.1.7 neutralization was increased compared to WH1 (fold change of 0.60, $p = 0.0034$). B.1.1.7 neutralization was still reduced when compared to D614G (fold change of 1.55, $p = 0.0027$) but, overall, the response in the vaccinated previously infected group was improved in comparison to vaccinated only individuals (Figure 4A,B).

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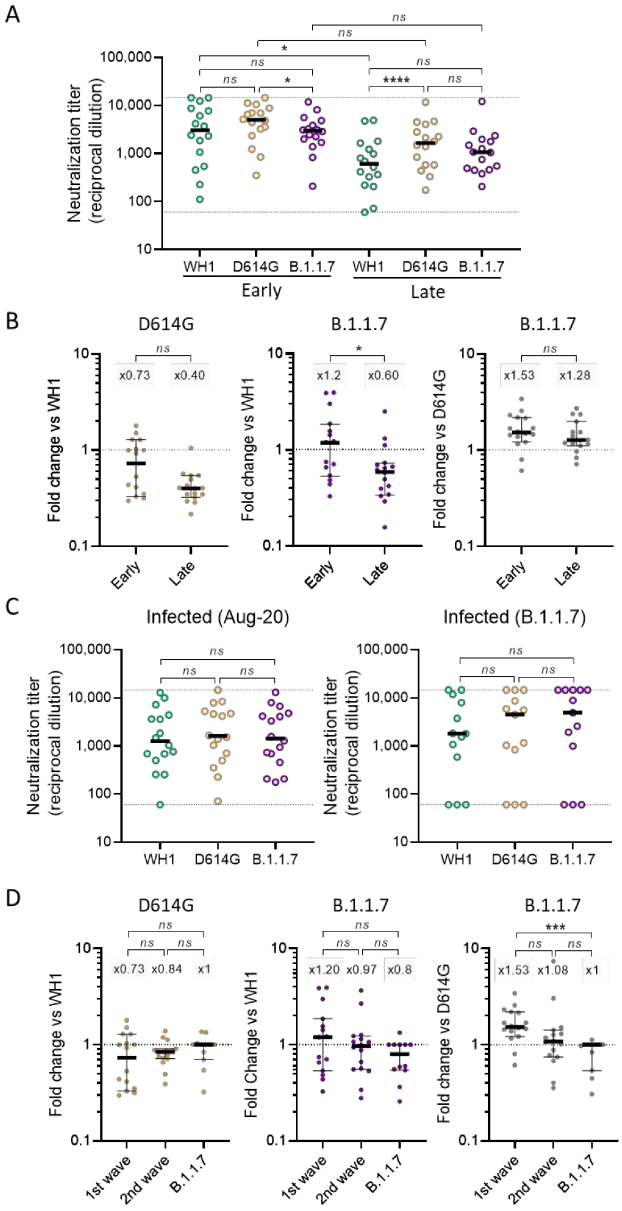

Figure 3. Subgroup analysis of neutralization titers in infected individuals. (**A**,**B**). Comparison of plasmas collected at 48 days (early sampling) and >6 month (late sampling) after infection from

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2 independent groups of participants infected in March 2020 (first wave). Neutralization titers (ID_{50} expressed as reciprocal dilutions) are shown in (**A**), and the corresponding ratios between variants (lower is better) are shown in (**B**). (**C**,**D**) Neutralizing titer of individuals infected during the second wave (August 2020) or specifically by the B.1.1.7 variant. Neutralization titers (ID_{50} expressed as reciprocal dilutions) are shown in (**C**), and the corresponding ratios between variants (lower is better) are shown in (**D**). In (**D**), we also included ratios from the first wave infected individuals (early sampling) for comparison. In (**A**,**C**), bars indicate median titer in each group and p values show the comparison of median titers among the three viruses (Friedman test with Dunn's multiple comparison test; * $p < 0.05$, **** $p < 0.0001$). Specifically in (**A**), p values are also indicated for the comparison of neutralization titers against the same spike between the 2 groups (Kruskal–Wallis test; * $p < 0.05$). In (**B**,**D**), bars indicate the median in each group with a 95% confidence interval and top values indicate the median fold-change between the indicated variants (variants compared are indicated in the graph title and in the Y axis). p values show the comparison of the group sampled at 48 days vs the group sampled at 6 months (Mann–Whitney test; * $p < 0.05$). In (**D**), p values show the comparison between individuals from the different infection waves (Kruskal–Wallis with Dunn's multiple comparison test; *** $p < 0.001$).

Neutralization titers in infected only individuals were generally lower than in the vaccinated groups (Figure 4A) as the former group experienced a decay in the neutralizing response over time (Figure 3A) [14,16] while vaccinated participants were sampled between 1 and 2 weeks after the second shot, when the neutralizing response was maximized [17]. However, in comparison to vaccinated only individuals, the cross-neutralizing response against both D614G and B.1.1.7 was superior to WH1 and the response against B.1.1.7 was only marginally reduced compared to D614G (fold change of 1.28, $p = 0.034$). Interestingly, the cross-neutralization profile of vaccinated previously infected individuals was very similar to infected only participants as there was no significant difference between the two groups when looking at the ratios of the neutralizing response between variants (Figure 4B). This suggests that vaccination was able to boost the full cross-neutralization breadth of the response previously established by natural infection during the first wave. In fact, in these limited cohorts, the averages of the titers against each variant were all increased by a factor of four when considering the average titers of vaccinated and non-vaccinated previously infected groups (Figure 4A).

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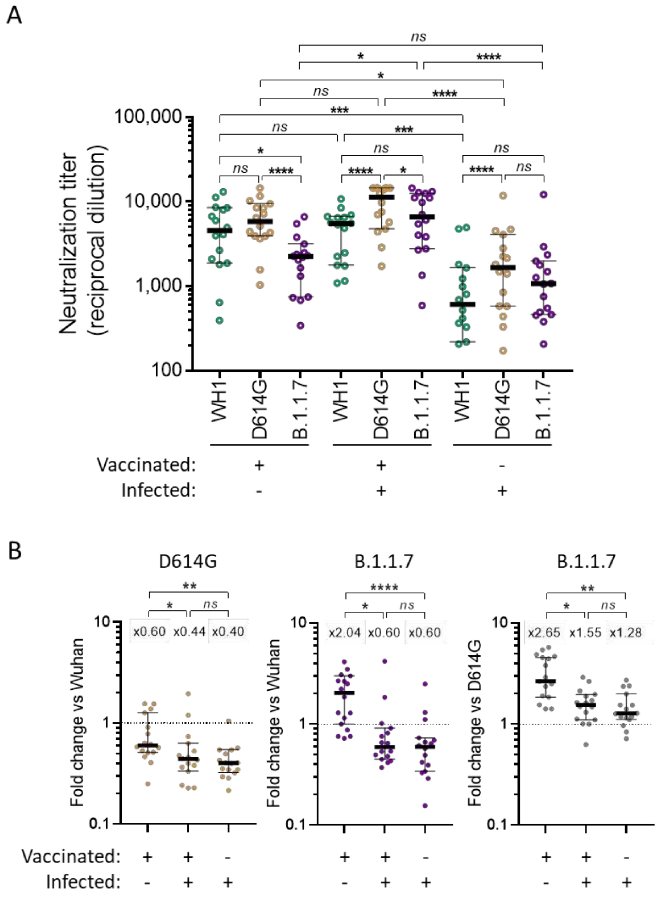

Figure 4. Subgroup analysis of neutralization titers in vaccinated individuals. Comparison of plasma from vaccinated individuals, previously infected or not during the first wave, as well as late plasma from first wave infected individuals. (**A**) Neutralization titers (ID_{50} expressed as reciprocal dilutions). Bars indicate the median titer in each group with a 95% confidence interval. **p**values show the comparison of median titers against the three variants in the same group (Friedman with Dunn's multiple comparison test ; * **p**< 0.05, **** **p**< 0.0001) and the comparison of the response against the same spike between groups (Kruskal–Wallis with Dunn's multiple comparison test * **p**< 0.05, *** p < 0.001, **** **p**< 0.0001) (**B**) Corresponding ratios between variants (lower is better). Bars indicate the median in each group with a 95% confidence interval and top values indicate the median fold-change between the indicated variants (variants compared are indicated in the graph title and in the Y axis). **p**values show the comparison of median ratios between each group (Kruskal–Wallis with Dunn's multiple comparison tests; * p < 0.05, ** p < 0.01, **** p < 0.0001).

4. Discussion

D614G neutralization was significantly increased in both vaccinated and infected, compared to WH1. This is in agreement with recent reports showing that D614G mutation is associated with a more open (one-up) conformation which increases access to the RBD

and results in both an increase in infectivity [18] and an increase in sensitivity to neutralization [19,20]. Interestingly, this effect was increased when testing the plasma at 6 months from infected individuals but reduced when testing plasma from the second wave (largely G614) as well as B.1.1.7 infected participants.

Overall, we show that the B.1.1.7 variant minimally impacted sensitivity to neutralizing immune responses from individuals infected during the first wave and sampled between 1 and 2 months after symptoms, in line with other reports [6,21]. The neutralizing response associated with the B.1.1.7 variant only showed a significant decrease when compared to the D614G mutant but not with the original WH1 spike. Importantly, these small differences faded in first wave participants tested 6 months after infection, despite a general decay in the neutralization response. This observation supports the positive evolution over time of neutralizing responses in infected individuals suggested by different authors [14,22].

No significant impact of the B.1.1.7 variant was observed when analyzing the neutralizing activity of participants infected during the second wave, in August 2020. During the second wave in Spain, G614 genotype was highly prevalent but the B.1.1.7 variant was still absent. This suggests that the intermediate evolution of the virus spike sequence was sufficient to increase the quality of the neutralizing response against the B.1.1.7 variant. The second wave participants' plasma was collected between 1 and 2 months following symptoms. Considering our observations on the evolution of the cross-neutralization capacities in first wave participants, it will be interesting to perform a similar follow up on second wave participants. As expected, participants specifically infected by the B.1.1.7 variant, in January 2021, demonstrated the best cross-neutralization capacity against the B.1.1.7 variant in comparison to other spikes. Their plasma was collected earlier than the other groups, between 1 and 3 weeks following symptom onset. Once again, additional follow ups will refine our data. Finally, in line with other reports [23], we demonstrate that infection by the G614-containing 20E (EU1) and B.1.1.7 variants elicits cross-neutralizing responses against former viral variants.

Finally, we show here that vaccinated individuals, as a whole, suffered a small reduction of cross-neutralizing activity against B.1.1.7 in comparison to D614G, as previously reported [24–26]. This loss of neutralization was more evident when excluding vaccinated individuals who had experienced a previous SARS-CoV-2 infection. Indeed, previously infected vaccinated participants demonstrated a much-improved neutralization of the B.1.1.7 variant. The capacity of this group to neutralize B.1.1.7 in comparison to both WH1 and D614G was not statistically different from infected individuals tested after 6 months. This suggests that, even though the vaccine alone did not generate an optimal B.1.1.7 neutralization response (compared to natural infection), it was capable of boosting the full cross-neutralization response pre-established by natural infection, in line with other reports [27,28]. Importantly, vaccinated individuals were all sampled at least 7 days after the second dose of vaccine, at a time when both humoral and neutralizing responses have reportedly reached a plateau [29,30]. However, another study suggests that the kinetics of neutralization following vaccination might vary depending on the variant [31]. Moreover, our data do not allow us to draw any conclusion on the long-term evolution of immune responses elicited by vaccines, in terms of the quality or durability of antibodies.

In conclusion, the neutralizing response from infected individuals, while slowly decaying in magnitude, seems to show a good qualitative evolution which can be fully recalled upon vaccination. Importantly, our data suggest a better cross-neutralizing quality of antibodies induced by natural infection compared to those induced exclusively by vaccination. It will be interesting to analyze how neutralization breadth evolves over time in non-infected vaccinated individuals to define if new vaccines will be necessary to further enrich it.

Author Contributions: B.T., J.B. and B.C. designed and coordinated the study. E.P., S.M., C.R., F.T.-F., R.O., J.R., J.V.-A., J.S. and N.I.-U. performed and analyzed the neutralization assays. V.G., R.L. and V.U. performed structural and statistical analysis. G.T., J.T., C.G.-F., A.F., R.P.-V., R.T., A.C., R.P., I.B., E.G., M.M. and J.C. selected patients and coordinated data. J.B. and B.T. drafted the manuscript and all authors have made substantial contributions to the revision of the subsequent versions. All authors approved the submitted version of the manuscript and agreed both to be personally accountable for their own contributions and to answer any questions related to the accuracy or integrity of any part of the work.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Hospital Germans Trias i Pujol (protocol code PI-20-122 and PI-20-217).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are not publicly posted. They can be shared upon request to corresponding authors.

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Conflicts of Interest: Unrelated to the submitted work, J.B. and J.C. are founders and shareholders of AlbaJuna Therapeutics, S.L. B.C. is founder and shareholder of AlbaJuna Therapeutics, S.L. and AELIX Therapeutics, S.L. The other authors do not declare any conflict of interest.

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Part VI: Impact of hybrid immunity, booster vaccination and Omicron breakthrough infection on SARS-CoV-2 VOCs cross-neutralization

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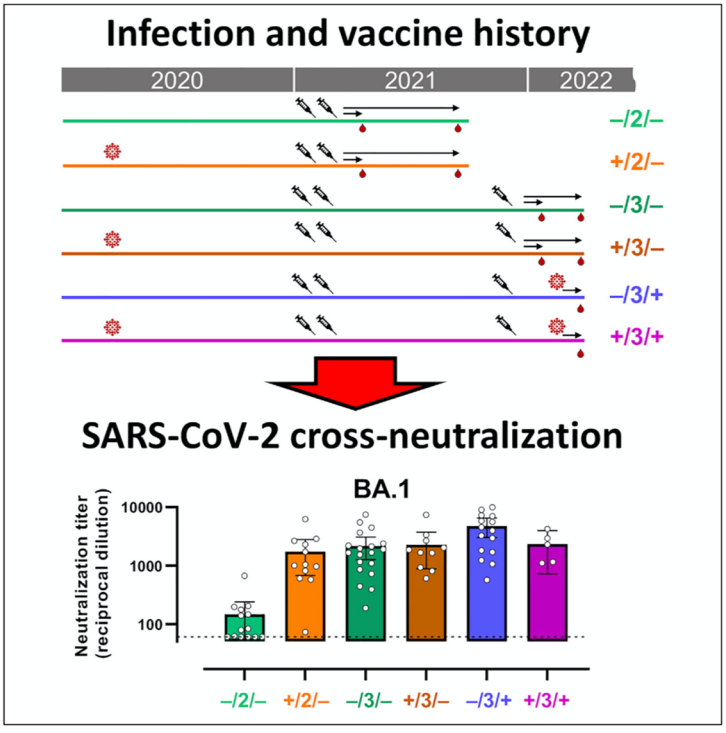

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Highlights

Initial mRNA vaccine schedule achieved limited cross-neutralization of Omicron VOCs

Previous infection increased Omicron neutralization in vaccinated individuals

Booster vaccine had a critical impact on broadening cross-neutralizing responses

Omicron breakthrough infection further improved Omicron cross-neutralization

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Impact of hybrid immunity, booster vaccination and Omicron breakthrough infection on SARS-CoV-2 VOCs cross-neutralization

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SUMMARY

The elicitation of cross-variant neutralizing antibodies against SARS-CoV-2 represents a major goal for current COVID-19 vaccine strategies. Additionally, natural infection may also contribute to broaden neutralizing responses. To assess the contribution of vaccines and natural infection, we cross-sectionally analyzed plasma neutralization titers of six groups of individuals, organized according to the number of vaccines they received and their SARS-CoV-2 infection history. Two doses of vaccine had a limited capacity to generate cross-neutralizing antibodies against Omicron variants of concern (VOCs) in uninfected individuals, but efficiently synergized with previous natural immunization in convalescent individuals. In contrast, booster dose had a critical impact on broadening the cross-neutralizing response in uninfected individuals, to level similar to hybrid immunity, while still improving cross-neutralizing responses in convalescent individuals. Omicron breakthrough infection improved cross-neutralization of Omicron subvariants in non-previously infected vaccinated individuals. Therefore, ancestral Spike-based immunization, via infection or vaccination, contributes to broaden SARS-CoV-2 humoral immunity.

INTRODUCTION

One of the main features of the coronavirus disease 2019 (COVID-19) pandemic is the continuous evolution of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) fueled by the high rate of viral transmission (as illustrated by the > 759 million confirmed cases worldwide to date, World Health Organization (WHO) COVID-19 Dashboard; https://covid19.who.int/ accessed on March 9th 2023). Over the course of the pandemic, the WHO has considered five variants of SARS-CoV-2 (Alpha, Beta, Gamma, Delta, and Omicron) as variants of concern (VOCs), but currently four of them have been de-escalated. At the end of 2021, the world saw the rise of the Omicron (B.1.1.529) family of SARS-CoV-2 VOCs.[1] First identified in Botswana and South Africa,[2] Omicron BA.1 (B.1.1.529.1) and its derivative BA.1.1 achieved global prevalence, like the Delta (B.1.617.2) VOC before it. BA.1 shows low fusogenicity, shifted tropism, reduced pathogenicity[3–5] and roughly 3-fold increased transmissibility compared to Delta.[6] In addition, Omicron was shown to evade the action of previously acquired or therapeutic neutralizing antibodies[7–15] by adding mutations in the coding region of the Spike glycoprotein, particularly in the receptor binding domain (RBD), and the N-terminal domain (NTD). In March 2022, a new—incrementally more transmissible[16]—Omicron VOC, BA.2 (B.1.1.529.2) took the lead and became highly prevalent worldwide.[17] While considered to be part of the Omicron lineage and showing similar resistance to neutralization,[12] BA.2 carries a different NTD mutation landscape and several additional mutations in the RBD.[18] Finally, since June 2022 the Omicron BA.4 (B.1.1.529.4) and mainly the Omicron BA.5 (B.1.1.529.5) replaced BA.2 as the most prevalent VOCs and—at the time of this writing—are associated with a global increase in infection cases.[17] BA.4 and BA.5 share an identical Spike glycoprotein that is close to BA.2 although it contains the 69–70 deletion described for BA.1, and the RBD mutation L452R previously detected in the Delta VOC.

Remarkably, COVID-19 vaccination campaigns started less than a year after the beginning of the pandemic. While the precise impact of vaccines on the population worldwide is still evaluated,[19] their

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effectiveness in preventing infection and reducing disease severity at the individual level was clearly demonstrated.[20,21] However, while the effect on fatality rate seemed longer lasting,[22] protection against symptomatic disease started to wane progressively after 3 months[23] due to the combination of both a progressive decrease in the titer of circulating neutralizing antibodies, and the emergence of neutralization escape variants. Not coincidently, the Omicron waves fueled an unprecedented surge of infection worldwide with a large number of mostly mild breakthrough infections.[24] Interestingly, the administration of a booster vaccine based on the ancestral Spike, which was initiated concomitantly with rising Omicron cases, showed a remarkable effect in restoring protection against Omicron, although not to optimal levels and probably only for a limited time. This was visible in a large efficacy study[25] but also through the measurement of neutralizing antibody titers,[10,26,27] which are considered a strong surrogate marker of protection against symptomatic disease.[28–32] Indeed, booster vaccine doses based on the ancestral variant have shown a highly positive impact on cross-neutralization of new variants.

Immunity against SARS-CoV-2 is also acquired from natural infection. As a considerable percentage of the global population has already been infected by SARS-CoV-2 at least once especially since the emergence of Omicron variants[24] immunity acquired through infection with previously circulating variants becomes an important parameter to understand and predict the level of protection against currently circulating ones. Indeed, immune responses induced by natural infection present some advantages over mRNA vaccines; natural infection was shown to trigger a more sustained B cell evolution leading over time to more potent and more broadly neutralizing antibodies.[24] Better yet, previous infection can combine with vaccination, in so-called hybrid immunity, to improve responses in vaccinated convalescent individuals.[33–37] Hybrid immunity was also observed following breakthrough infection which improved vaccine induced cross-neutralizing responses.[38] Finally, Omicron breakthrough infection was shown to induce poor cross-neutralizing responses in non-vaccinated individuals but this was improved with previous vaccination.[39]

To measure the impact of booster vaccines and hybrid immunity on the cross-neutralization of VOCs, we followed a cohort of vaccinated individuals (mRNA vaccine BNT162b2 from Pfizer-BioNTech or mRNA-1273 Moderna) who received or not an mRNA booster dose and who experienced or not mild infection before vaccination during the first year of the pandemic. In addition, we analyzed individuals who all received a booster dose and subsequently experienced breakthrough infection during the Omicron VOCs onset. We evaluated their neutralizing antibody responses against the ancestral SARS-CoV-2, Delta, and Omicron BA.1, BA.2, and BA.4/5 variants. Our data suggest that the number of vaccine doses and/or infection exposures similarly contribute to cross-neutralization activity.

RESULTS

A total of 76 participants were included in this study and organized in 6 groups (Table 1) according to 3 parameters: status of SARS-CoV-2 infection before all mRNA-based vaccine, status of vaccination (full schedule only or additional booster) and infection following the booster vaccine. All participants were vaccinated with mRNA vaccines (BNT162b2 [Pfizer-BioNTech] or mRNA-1273 [Moderna]) and at the time of sampling, had received either a full vaccine schedule (2 doses with 3–4 weeks interval between first and second dose) or also an additional booster dose (six months interval from full schedule). Notably, 5 participants, under 55 years of age, who experience SARS-CoV-2 infection before vaccination only received 1 mRNA vaccine dose that was considered a full vaccine schedule by the Spanish health authority. However, for simplicity, throughout the manuscript, full vaccine schedule is referred to "2 doses vaccination" while vaccination that included booster are referred to "3 doses vaccination". Booster vaccine was in large majority mRNA-1273 (Moderna) including for most participants who had received BNT162b2 (Pfizer-BioNTech). The rest of the booster was BNT162b2.

Infections before vaccination occurred a median of 277 (IQR = 87–296) days before vaccination (Table 1), during the first year of the pandemic, between March 2020 and February 2021, which, in Catalonia Spain, involved mainly ancestral Wuhan-Hu-1 (WH1), B.1, B.1.177, and Alpha (B.1.1.7) variants (Figure S1A, http://covidtag.paseq.org/). Infections after the third vaccine dose happened, from December 2021 to March 2022, when, in Catalonia Spain, Omicron (BA.1 and BA.2) displaced Delta and became largely prevalent (Figure S1B). Breakthrough Infections occurred a median of 39 (IQR = 24–54) days after last vaccine dose (Table 1). All samples from boosted individuals were collected around 4 weeks after the latest event (either vaccination or infection, Table 1). The uninfected status of participants was verified by testing their plasma

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Table 1. Characteristics of individuals included in the analysis

	Infection/Vaccine status					
	−/2/−	+/2/−	−/3/−	+/3/−	−/3/+	+/3/+
	n = 14	n = 12	n = 19	n = 10	n = 16	n = 5
Age (years), median [IQR]	45 [28–52]	43 [35–51]	42 [26–48]	37 [24–49]	49 [35–51]	43 [38–49]
Sex (% female)	78.6	66.7	73.7	70.0	75.0	100.0
Time between the first plasma sample collection and the indicated event (median days [IQR]):						
Last vaccine dose	34 [32–38]	34 [14–46]	34 [31–37]	33 [31–41]	90 [60–92]	35 [32–70]
Pre-vaccine infection	NA	336 [153–360]	NA	588 [459–662]	NA	621 [424–677]
Post-vaccine infection	NA	NA	NA	NA	35 [18–43]	25 [13–38]
Time between the second plasma sample collection and the indicated event (median days [IQR]):						
Last vaccine dose	183 [177–197]	200 [176–223]	92 [91–99]	92 [91–104]	NA	NA
Pre-vaccine infection	NA	488 [333–529]	NA	697 [502–725]	NA	NA

Groups are named according to their SARS-CoV-2 infection status prior to infection (+ or –), the number of vaccines received (2 or 3) and the occurrence of breakthrough infection (+ or –).

sample for the absence of anti-nucleocapsid protein antibodies by ELISA (Figure S1C). No significant differences among groups were observed in age or sex (Table 1).

Hybrid immunity is associated with increased and more stable cross-variant neutralization responses

First, we tested the effect of previous infection in combination with 2 doses of mRNA vaccines on the neutralization of WH1, Delta, Omicron BA.1, BA.2, and BA.4/5 variants. In plasma samples collected from uninfected participants 1 month after second dose of mRNA vaccine, neutralization titers (50% inhibitory dilution, ID_{50}) were the highest against WH1 (Figures 1B and S2A: geometric mean titer [GMT] = 2319), decreased, although not significantly, against Delta (GMT = 1000, $p = 0.943$) and were highly reduced against Omicron subvariants BA.1 (GMT = 108, $p < 0.0001$), BA.2 (GMT = 151, $p < 0.0001$), and BA.4/5 (GMT = 326, $p = 0.0053$).

By comparison, in vaccinated participants who were previously infected (and therefore experienced 3 immunogenic impacts), neutralization titers, 1 month after second vaccine dose, were similar against WH1 (Figure 1B: x 1.4, GMT = 3356, $p = 0.1759$) but significantly increased against Delta (x 5.2, GMT = 5181, $p < 0.0001$), BA.1 (x 10.4, GMT = 1120, $p < 0.0001$), BA.2 (x 8.2, GMT = 1236, $p < 0.0001$), and BA.4/5 (x 3.2, GMT = 1045, $p = 0.0003$). While Delta neutralization of previously infected individuals was above but not significantly different from the ancestral Spike (Figure S2B: Delta = 5181 vs WH1 = 3356, $p > 0.9999$), the improved neutralization titers against Omicron subvariants were still about 3-fold reduced compared to WH1 (BA.1 = 1120, $p = 0.0368$; BA.2 = 1236, $p = 0.0241$; BA.4/5 = 1045, $p = 0.0008$).

VOCs cross-neutralization was also assessed by calculating ratios between each VOC and the corresponding WH1 titers from the same individuals (Figure 1C). This approach gives a measurement of the cross-neutralization potential using WH1 response as a reference and provides an alternative analysis with reduced inter-individual variability, which can bias group comparisons when analyzing only raw titers. An increased ratio value indicates increased cross-neutralization. A ratio superior to 1 indicates a better neutralization in comparison to WH1, while a ratio inferior to 1 indicates a worsen neutralization compared to WH1. As suggested by titer comparisons, the cross-neutralization potential against Delta that was inferior in vaccinated-only participant (ratio = 0.43) was increased to an apparent optimal level in previously infected individuals (ratio = 1.54, $p < 0.0001$). In the case of Omicron BA.1, BA.2, and BA.4/5, cross-neutralization potential was highly reduced in vaccinated-only participants (fold reduction for BA.1 = 0.05, BA.2 = 0.06, BA.4/5 = 0.14, Figure S2C) and sensibly improved in the previously infected group (fold reduction for BA.1 = 0.33, $p = 0.0004$; BA.2 = 0.37, $p = 0.0004$; BA.4/5 = 0.31, $p = 0.001$) albeit still lagging behind the response against Delta (Figure S2D). Importantly, in the non-previously infected group, some individuals showed undetectable neutralization titers against BA.1 (Figure 1B; 64% responders) and BA.2 (86%

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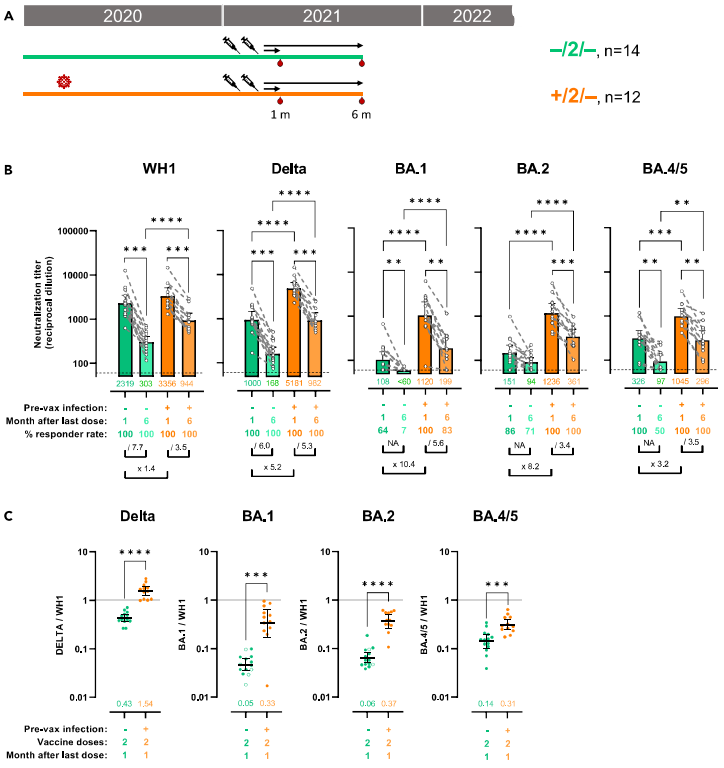

Figure 1. Hybrid immunity is associated with increased and more stable cross-variant neutralization responses

(A) Schematic representation of plasma samples collection from individuals previously infected or not who received 2 doses of mRNA vaccine were tested for neutralization activity against pseudo viruses expressing the WH1, B.1.617.2 (Delta), BA.1, BA.2, and BA.4/5 SARS-CoV-2 Spike proteins. Plasma samples were collected at about 1 or 6 months after last vaccination.

(B) Raw ID_{50} (reciprocal dilutions) titers against the indicated variant Spikes. Horizontal bars and numbers indicate ID_{50} geometric means (GMT) for each group and error bars indicate 95% confidence intervals. At the bottom are fold changes for the indicated comparisons. For the comparison between 1- and 6-month plasma samples (intragroup), we applied a Wilcoxon paired test or a Prentice Z test when undetectable titers were observed. For intergroup comparison we applied a Mann-Whitney test or a Peto-Peto test when undetectable titers were observed. Significant p values are indicated and were corrected for multiple comparison (**p < 0.01, ***p < 0.001, ****p < 0.0001). Dashed lines indicate the assay lower limit of detection (60 reciprocal dilutions). Non-responder rate was defined by undetectable neutralization titers for a specific variant at an initial serum dilution of 1:60 and are indicated.

(C) Ratio of VOC ID_{50} over the indicated WH1 ID_{50} calculated for the groups with plasma samples at 1 month (shown in 1B) after the second vaccine dose. Horizontal bars indicate ratio geometric means for each group and error bars indicate 95% confidence intervals. Bottom numbers indicate the geometric mean of the ratio for each group. Significant p values are indicated for comparisons between uninfected and previously infected groups (Mann-Whitney test, ***p < 0.001, ****p < 0.0001). Non-responder samples are indicated by an open circle. See also Figure S2.

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responders). Therefore, we also analyzed cross-neutralization ratios after removal of undetectable samples, confirming the initial results with slightly weaken statistics (Figures S2E and S2F).

To evaluate the stability of these responses at mid-term, the same participants were tested 5 months later (6 months after second dose). Non-infected participants showed a major decrease in their neutralizing titers against all variants tested (Figure 1B): while titers against WH1 and Delta were reduced 7.7 times ($p < 0.0001$) and 6 times ($p = 0.0016$) respectively, many participants reached non-detectable levels ($ID_{50} < 60$) against Omicron BA.1, BA.2 and BA.4/5 in our assay. In contrast, previously infected participants showed a more robust stability of their neutralizing response against all variants (WH1 =/3.5; Delta =/5.3; BA.1 =/5.6; BA.2 =/3.4; BA.4/5 =/3.5). Temporal changes in ratios between VOC and WH1 neutralization could not be calculated after six months due to high number of undetectable measurements. We considered that a level superior to 25% undetectable samples could bias the results.

Overall, these results indicate that hybrid immunity greatly improves quantitatively and qualitatively the neutralization capacities against Delta and Omicron. However, neutralization against Omicron variants was still severely reduced after previous infection and 2 vaccine doses.

Booster shot of mRNA vaccine in non-infected individuals generates hybrid immunity-like benefits 1 month after last immunization

Next, we evaluated the effect of a third vaccine dose, which was administered with a median of 9.2 (IQR = 8–10.3) months after the second dose, comparing participants previously infected or not (Figure 2A). In this analysis, we focused on plasma samples collected with a median of 34 (IQR = 16–40) days after last vaccine dose and, for comparison, we included data on participants who only received 2 vaccine doses, already shown in Figure 1.

When comparing neutralization titers at similar time (1 month) after last vaccination, the effect of the third dose on WH1 neutralization was not statistically different from the 2 doses groups respectively, whether the participants had been previously infected or not (Figure 2B). The third vaccine dose (booster) did, in fact, increase WH1 neutralization titers when compared with decayed neutralization response at 6 months post second dose (2817 vs 303 in Figures 1B and 2B), but it only brought back WH1 neutralization to an apparent maximal activity already reached after 2 doses. In sharp contrast, third dose vaccine increased protection against Delta (x 4.2, $p < 0.0001$), BA.1 (x 14.1, $p < 0.0001$), BA.2 (x 4.9, $p = 0.0002$) and BA.4/5 (x 2, $p = 0.0187$) to levels not previously reached in vaccinated-only individuals (Figures 2B and S3A).

Of note, we observed a slightly decreased neutralization against WH1 in previously infected individuals compared to vaccinate only after booster; however, the difference was not significative and may result from the limited size of this group. This reduction was not observed against other variants.

When considering raw neutralization titers, previously infected participants who received a third vaccine dose did not show any significant increase of neutralization activity against any of the variant tested when compared to previously infected individuals who received only 2 doses of vaccine (Figures 2B and S3B). However, when considering cross-neutralization ratios (Figures 2C, S3C, and S3D), a trend for a better response against all VOCs was visible for the previously infected group who received the third vaccine dose compared to previously infected group who received 2 doses, but did not reach significance except for BA.1 (+/2/− = 0.33 and +/3/− = 0.89, $p = 0.04711$). Finally, the benefit of previous infection in the context of boosted vaccination (3 doses) was not visible when considering raw neutralization titers (Figure 2B), although there was still a trend for a better response quality against Omicron variants when considering cross-neutralization ratios (Figure 2C), which only reached significance against BA.2 ($p = 0.0314$) and BA.4/5 ($p = 0.0352$).

Of note, the group of individuals, who were previously infected and received booster vaccine, contained 3 individuals who had only received a single vaccine dose as part of the full vaccine schedule. To verify the impact of these individuals on our results, we re-analyzed both neutralization titers and ratios excluding them (Figures S3C, S3F, S4A, and S4B). No clear difference was observed although some statistical significance was lost as a result of group reduction and lower statistical power. We also verified the impact of BA.1 and BA.2 non-responders on the analysis of the cross-neutralization responses (Figure S3G). Results were almost identical.

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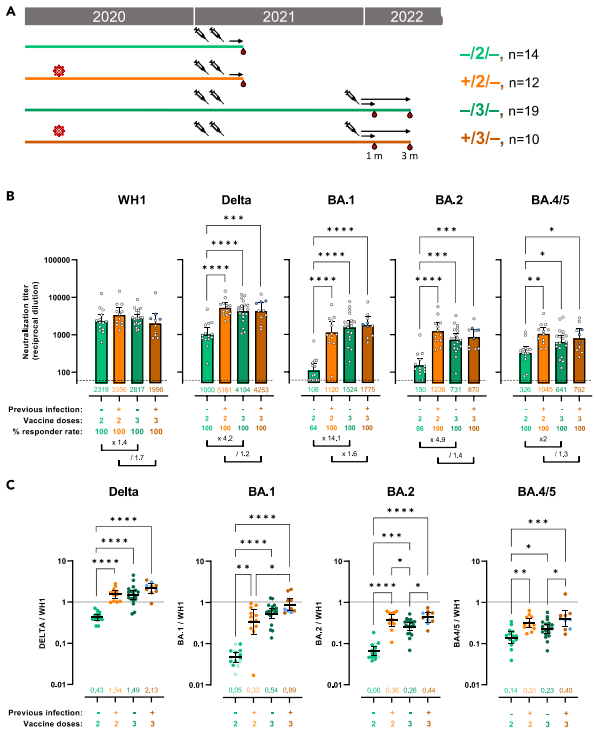

Figure 2. Booster shot of mRNA vaccine in non-infected individuals generates hybrid immunity-like benefits 1 month after last immunization

(A) Schematic representation of plasma samples collection from individuals previously infected or not, who received 2 or 3 doses of mRNA vaccine were tested for neutralization activity against pseudo viruses expressing the WH1, B.1.617.2 (Delta), BA.1, BA.2, and BA.4/5 SARS-CoV-2 Spike proteins. Plasma samples were collected at about 1 month after last vaccination.

(B) Raw ID_{50} (reciprocal dilutions) titers against the indicated variant Spikes. Horizontal bars and numbers indicate ID_{50} geometric means (GMT) for each group and error bars indicate 95% confidence intervals. At the bottom are fold changes for the indicated comparisons. Significant p values are indicated for comparisons between all groups (Kruskal-Wallis test with two-stage step-up multiple comparison, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Dashed lines indicate the assay lower limit of detection (60 reciprocal dilutions). Non-responder rate was defined by undetectable neutralization titers for a specific variant at an initial serum dilution of 1:60 and are indicated. Participants (3 from the +/3/− group) who only received 1 dose as part of the full vaccine schedule are indicated in blue.

(C) Ratio of WH1 ID_{50} over the indicated VOC ID_{50} calculated for the groups with plasma samples at 1 month (shown in 2A) after last vaccine dose. Horizontal bars indicate ratio geometric means for each group and error bars indicate 95% confidence intervals. Bottom numbers indicate fold reduction compared to WH1. Significant p values are indicated for comparisons between all groups (Kruskal-Wallis test with two-stage step-up multiple comparison, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Non-responder samples are indicated by an open circle. Participants (3 from the +/3/− group) who only received 1 dose as part of the full vaccine schedule are indicated in blue. See also Figures S3 and S4.

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Finally, we performed a follow up on these individuals at around 3 months after booster vaccine (Figure S4C). Neutralization titers showed an encouraging limited decay for all variants. Additionally, we could observe a trend for an improved stability in previously infected individuals; however, it did not reach significance.

All together these results indicate that hybrid immunity conferred by previous infection provides clear benefits increasing protection against Delta and Omicron variants, but third vaccine dose could provide non-infected participants with a level of protection similar to previously infected individuals.

Omicron breakthrough infection specifically increases neutralization activity against Omicron

Finally, we tested the effect of breakthrough infection in boosted individuals previously infected or not (Figure 3A). All breakthrough infections happened between December 2021 and March 2022 and probably include mostly Omicron BA.1 and BA.2 infections (Figure S1B). Plasma samples were collected with a median of 35 days (IQR = 18–43) after last infection in the case of participants only infected after the third dose (Table 1) and 25 days (IQR = 13–38) in the case of individuals infected before and after full vaccination. We included for comparison the groups of participants, who received 3 vaccine doses but did not undergo breakthrough infection, already shown Figure 2.

Omicron breakthrough infection had no effect on neither WH1 nor Delta neutralization titers, whether participants were or not previously infected (Figures 3B, S5A, and S5B). Delta cross-neutralization ratio did not change, either, compared to other 3 vaccine dose groups (Figures 3C, S5D, and S5E). The absence of increased neutralization against WH1 and Delta suggests that recent breakthrough infection had limited cross-variant immunity toward these variants because of a more pronounced antigenic disparity between pre-Omicron and Omicron variants. Alternatively, as the participants had received a booster vaccine soon before breakthrough infection (median of 45 days in between), it is possible that neutralization responses against WH1 and Delta were still at a peak after vaccine. BA.1, BA.2, and BA4/5 raw neutralization titers all showed trends of improvement following breakthrough infection in non-previously infected participants (Figure 3B) but statistical significance was only met for BA.1 (x 2.3, p = 0.0095). When looking at cross-neutralization ratios (Figure 3C), BA.1, BA.2, and BA.4/5 all showed significant improvement when comparing the breakthrough infection groups (−/3/+ and +/3/+) to the 3 doses uninfected group (−/3/−) but not the 3 doses previously infected group (+/3/−).

Of note, the group of individuals who experienced 2 infections (+/3/+) included 2 individuals who received a single dose as part of the full vaccine schedule. To verify their impact on the overall results, we re-analyzed both titers and ratios excluding them (Figures S5C, S5F, S6A, and S6B). Titers and ratios were both increased as a result but statistical analyses were hampered by the group reduction.

Taken together these results show that BA.1/BA.2 breakthrough infection contributed to improve cross-neutralization against all Omicron variants but not Delta. This effect was more evident when analyzing participants who did not experience previous infection with pre-Omicron variants.

DISCUSSION

We face a seemingly relentless race between COVID-19 vaccinations and viral evolution, giving rise to more transmissible and immunity-escaping variants, leading to successive COVID-19 waves. As more and more vaccinated people experience SARS-CoV-2 breakthrough infection, especially since the onset of the Delta and Omicron variants,[24,40] individual and global levels of protection get redefined. In line with previous reports,[33,37] we show that hybrid immunity, based on previous infection with non-Omicron variants, increased protection against SARS-CoV-2 VOCs following 2 doses vaccine. Importantly, these benefits were observed against all Omicron variants although, the levels of protection obtained were still reduced about 3-folds compared to the ancestral variant. Interestingly, after booster vaccine, hybrid immunity still showed a trend for improving cross-neutralization: This was visible against all the VOCs tested including significative difference against BA.2 and BA.4/5. The waning neutralizing responses following mRNA vaccine is a major concern.[41–43] We observed an improved stability of neutralization titers at mid-term associated with previous infection status following 2 doses vaccination. Additionally, following booster vaccine, titers showed an encouraging limited decay.

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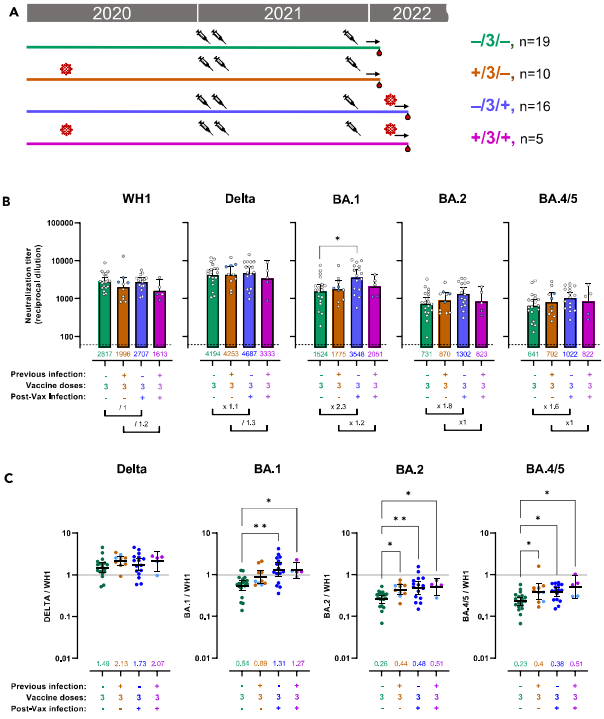

Figure 3. Omicron breakthrough infection specifically increases neutralization activity against Omicron

(A) Schematic representation of plasma samples collected from individuals previously infected or not, who received 3 doses of mRNA vaccine and were affected or not by Omicron breakthrough infection were tested for neutralization activity against pseudo viruses expressing the WH1, B.1.617.2 (Delta), BA.1, BA.2, and BA.4/5 SARS-CoV-2 Spike proteins. Plasma samples were collected at about 1 month after last vaccination or 1 month after breakthrough infection if the latter happened.

(B) Raw ID_{50} (reciprocal dilutions) titers against the indicated variant Spikes. Horizontal bars and numbers indicate ID_{50} geometric means (GMT) for each group and error bars indicate 95% confidence intervals. At the bottom are fold changes for the indicated comparisons. Significant p values are indicated for comparisons between all groups (Kruskal-Wallis test with two-stage step-up multiple comparison, *$p < 0.05$). Dashed lines indicate the assay lower limit of detection (60 reciprocal dilutions). Responder rates were all 100% ($ID_{50} > 60$). Participants (3 of the +/3/− group and 2 of the +/3/+ group) who only received 1 dose as part of the full vaccine schedule are indicated in blue.

(C) Ratio of WH1 ID_{50} over the indicated VOC ID_{50} calculated for the groups with plasma samples at 1 month (shown in 3A) after last event (infection or vaccination). Horizontal bars indicate ratio geometric means for each group and error bars indicate 95% confidence intervals. Bottom numbers indicate fold reduction compared to WH1. Significant p values are indicated for comparisons between all groups (Kruskal-Wallis test with two-stage step-up multiple comparison, *$p < 0.05$, **$p < 0.01$). Participants (3 of the +/3/− group and 2 of the +/3/+ group) who only received 1 dose as part of the full vaccine schedule are indicated in blue. See also Figures S5 and S6.

We evaluated the effect of Omicron BA.1 and BA.2 breakthrough infection on neutralization titers. While a modest but significant increase of BA.1 neutralizing titer was detected, most of the qualitative benefits observed after Omicron breakthrough infection on cross-neutralization ratios in uninfected individuals were comparable to the values observed in individuals previously infected with non-Omicron variants. In

addition to the strong antigenic differences between Omicron and ancestral variants (particularly in the RBD), this apparent low impact of Omicron breakthrough infections could be explained by intrinsic viral factors of this VOC (tropism, fusogenicity)[3,4] or by the time interval and order of antigenic exposures. Certainly, pre-vaccination infections were separated by a median of 277 days from vaccination, while only a median of 39 days separated booster vaccination and breakthrough infection. Timing between infection and vaccination seems to be a major determinant on the extent of boosting of humoral responses with shorter time intervals resulting in lower suboptimal boost.[44]

Finally, in line with other studies,[10,26,33,45,46] we saw a remarkable effect of booster vaccines in improving neutralization titers against Omicron VOCs to similar levels as the ones obtained with hybrid immunity. These and others' results suggest that the number of antigenic exposures is a critical parameter for the improvement of cross-neutralizing responses. Indeed, it has been shown that booster mRNA vaccines can mobilize additional memory B cells, including Omicron specific B cells.[47,48] In addition, recent studies now propose that the generation of more broadly neutralizing antibodies, following immunization with ancestral Spike, requires multiple and sequential antigenic exposures to achieve the activation, differentiation, and expansion of subdominant memory B cells.[49,50]

Our data indicate that the benefits of Omicron over non-Omicron-based hybrid immunity on the neutralization Omicron VOCs was marginal. The scientific community and the pharmaceutical industry have developed second-generation COVID-19 vaccines that can boost acquired immunity more specifically against new variants and reduce their transmission.[51–55] A recent study not yet peer-reviewed predicts a limited added benefits of new Omicron specific COVID-19 mRNA vaccines.[56] These data suggest that for the time being ancestral Spike-based immunization remains a valuable strategy to improve and maintain protection against SARS-CoV-2 and limit transmission globally since the number of immunological exposures to the Spike, rather than the specific sequence, seems to be a major determinant of VOC cross-neutralization of humoral immune response.

Limitations of the study

The present work is limited mainly by the number of individuals included in each group, and the lack of parallel data on $CD4^+$ and $CD8^+$ T cells. In addition, there are many variables that have been detailed but not analyzed due to the complexity and limited power of the analyses, including the time elapsed between previous infections and vaccination and between the third dose and breakthrough infection, that could influence on the neutralizing response in the short, mid, and/or long term.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106457.

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AUTHOR CONTRIBUTIONS

J.B., B.T., and B.C. designed and coordinated the study. E.P., S.M., M.T., T.P., A.P.G., R.O., C.R, F.T.-F., C.A-G. performed and analyzed experimental assays. V.U. and E.P. performed statistical analyses. R.T., A.C., M.N.-J., L.M., I.B., E.G., M.M., and J.C. selected patients and coordinated data. J.B., B.T., and E.P. drafted the manuscript, and all authors have made substantial contributions to the revision of the subsequent versions. All authors approved the submitted version of the manuscript and agreed both to be personally accountable for their own contributions, and to ensure the accuracy or integrity of any part of the work.

DECLARATION OF INTERESTS

Unrelated to the submitted work, J.B. and J.C. are founders and shareholders of AlbaJuna Therapeutics, SL. B.C. is founder and shareholder of AlbaJuna Therapeutics, SL, and AELIX Therapeutics, SL. The other authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Peroxidase AffiniPure F(ab')₂Fragment Goat Anti-Human IgG, Fcγ Fragment Specific	Jackson ImmunoResearch	Cat# 109-036-098; RRID: AB_2337596
Anti-6x-His clone HIS.H8	Thermo Fisher Scientific	Cat#MA1-21315; RRID: AB_557403
Bacterial and Virus Strains		
pNL4-3.Luc.R-.E-	NIH ARP	Cat#3418
SARS-CoV-2.SctΔ19	This paper	N/A
pcDNA3.1(+)	GeneArt/Thermo Fisher Scientific	Cat#810330DE
pVSV-G	Clontech	Sánchez-Palomino et al.[57]
Biological Samples		
Pre-pandemic Plasma Samples	This paper	N/A
Chemicals, Peptides, and Recombinant Proteins		
Fetal Bovine Serum	Thermo Fisher Scientific	Cat#10270106
Dulbecco's Modified Eagle Medium	Thermo Fisher Scientific	Cat#41966052
Expi293 Expression Medium	Thermo Fisher Scientific	Cat#A1435102
Opti-MEM I Reduced Serum Medium	Thermo Fisher Scientific	Cat#31985070
ExpiFectamine 293 Transfection Kit	Thermo Fisher Scientific	Cat#A14524
Versene	Thermo Fisher Scientific	Cat#15040033
Puromycin	Thermo Fisher Scientific	Cat#A1113803
DEAE-Dextran	Sigma-Aldrich	Cat#D9885-100G
BriteLite Plus Luciferase	PerkinElmer	Cat#6066769
SARS-CoV-2 (2019-nCoV) Nucleocapsid-His Recombinant Protein	Sino Biological Inc	Cat# 40588-V08B
MACS BSA Solution	Miltenyi Biotec	Cat# 130-091-376
Phosphate Buffered Saline	Thermo Fisher Scientific	Cat# 10010015
Phosphate-Citrate Buffer with Sodium Perborate	Merck Life Science SLU	Cat# P4922-50CAP
o-Phenylenediamine Dihydrochloride	Merck Life Science SLU	Cat# P8787-100TAB
H_2SO_4	Sigma-Aldrich	Cat# 258105-1L-PC
Deposited Data		
Group Geo Means and Raw Neutralization Titers	https://data.mendeley.com/	https://doi.org/10.17632/3572bvtgsb.1
Experimental Models: Cell Lines		
Expi293F GnTI- cells	Thermo Fisher Scientific	Cat#A39240
HEK293T/hACE2 cells	Integral Molecular	Cat#C-HA101
Software and Algorithms		
GraphPad Prism v9.3.1	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
R v3.6.3	R Foundation for Statistical Computing	https://www.r-project.org/
Other		
GeneArt Gene Synthesis	Thermo Fisher Scientific	N/A

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RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Benjamin Trinité (btrinite@irsicaixa.es).

Material availability

The plasmids pcDNA3.1 SARS-CoV-2.SctΔ19 of each variant described in this work are available upon request to the lead contact.

Data and code availability

- De-identified human neutralization data have been deposited at Mendeley Data and are publicly available as of the date of publication. DOIs are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study overview and subjects

This work includes plasma samples from participants of 2 different cohorts: the KING cohort extension and the KING VAX study. Both studies were approved by the Hospital Ethics Committee Board from Hospital Universitari Germans Trias i Pujol (HUGTiP, PI-20-217 and PI-21-351). This is an observational cohort; no blinding or randomization was applied. All participants provided written informed consent. Both uninfected and infected individuals were included in both studies. Infected ones had a documented positive RT-qPCR result from nasopharyngeal swab, positive antigen test and/or a positive serological diagnostic test. Plasma samples from Uninfected individuals were negative for SARS-CoV-2 specific Nucleocapside antibodies (Figure S1C). At the time of sampling, all participants had received either complete vaccine schedule (2 doses or 1 dose in case of previously infected individuals < 55 years) or booster dose of mRNA vaccines BNT162b2 (Pfizer-BioNTech) and or mRNA-1273 (Moderna). Samples in this study were collected between February 2021 and March 2022, about one month after complete vaccine schedule or booster vaccines or about one month after breakthrough infection. Participants were recruited irrespective of age and all the infected groups included only mild disease cases. Information on sex and age is available in Table 1.

Cell lines

HEK293T cells (presumably of female origin) overexpressing wild type (WT) human ACE-2 (Integral Molecular, USA) were used as target in pseudovirus-based neutralization assay. Cells were maintained in T75 flasks with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1 μg/ml of Puromycin (Thermo Fisher Scientific, USA).

Expi293F cells (presumably of female origin, Thermo Fisher Scientific) are a HEK293 cell adapted for suspension culture that were used for SARS-CoV-2 pseudovirus production. Cells were maintained in Expi293 expression medium (Thermo Fisher Scientific) under continuous shaking at 125 rpm in Erlenmeyer flasks following manufacturer's guidelines.

METHOD DETAILS

Pseudovirus generation and neutralization assay

HIV reporter pseudo viruses expressing SARS-CoV-2 S protein and Luciferase were generated. pNL4-3.Luc.R-.E- was obtained from the National Institut of Health (NIH) acquired immune deficiency syndrome (AIDS) Reagent Program.[58] SARS-CoV-2.SctΔ19 was generated (GeneArt) from the full protein sequence of the original WH1 SARS-CoV-2 Spike (Genbank: MN908947.3) with a deletion of the last 19 amino acids in C-terminal[58], human-codon optimized and inserted into pcDNA3.1(+).[58] A similar procedure was followed to generate expression plasmids for the Delta, BA.1, BA.2 and BA.4/5 variants of SARS-CoV-2 S protein according to consensus data (www.outbreak.info/) (see Figure S1A for a detail of the mutations). Expi293F cells were transfected with pNL4-3.Luc.R-.E- and the different SARS-CoV-2.SctΔ19 Spike

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plasmids using ExpiFectamine 293 Reagent (Thermo Fisher Scientific). Control pseudo viruses were obtained by replacing the S protein expression plasmid with a VSV-G protein expression plasmid as reported.[57] Supernatants were harvested 48 hours after transfection, filtered at 0.45 μm, frozen, and titrated on HEK293T cells overexpressing WT human ACE-2 (Integral Molecular, USA).

Neutralization assays were performed in duplicate as previously described.[59] Briefly, in Nunc 96-well cell culture plates (Thermo Fisher Scientific), 200 TCID50 of pseudovirus were preincubated with three-fold serial dilutions (1/60–1/14,580) of heat-inactivated (incubated at 56°C for 30 minutes) plasma samples for 1 hour at 37°C. Then, $1x10^4$ HEK293T/hACE2 cells treated with DEAE-Dextran (Sigma-Aldrich) were added. Results were read after 48 hours using the EnSight Multimode Plate Reader and BriteLite Plus Luciferase reagent (PerkinElmer, USA). The values were normalized, and the ID50 (reciprocal dilution inhibiting 50% of the infection) was calculated by plotting and fitting all duplicate neutralization values and the log of plasma samples dilution to a 4-parameters equation in Prism 9.0.2 (GraphPad Software, USA). This assay has been previously validated with a replicative viral inhibition assay.[60]

ELISA

Confirmation of SARS-CoV-2 serostatus was performed by evaluating anti-N protein IgG responses.[61] Nunc MaxiSorp plates were coated with 50 μL of anti-6x-His antibody clone HIS.H8 (2 μg/mL, Thermo Fisher Scientific) in PBS overnight at 4°C. After washing, plates were blocked with 1% BSA in PBS (Miltenyi Biotec, Germany) for two hours at room temperature. Whole nucleocapsid protein (NP, Sino Biological, Germany) were added at 1 μg/mL concentration (50 μL/well) and incubated overnight at 4°C. Plasma samples were heat-inactivated before use (56°C for 30 minutes) and analyzed in duplicate in antigen-coated and antigen-free wells in the same plate. A positive plasma sample and a pool of pre-pandemic plasmas from healthy controls was used as positive and negative controls, respectively. Standards, negative control, and plasma samples were diluted (1/100) in blocking buffer and were incubated (50 μL/well) for one hour at room temperature. The HRP-conjugated (Fab)2 goat anti-human IgG (Fc specific, Jackson ImmunoResearch, UK) was then incubated for 30 minutes at room temperature. Plates were revealed with o-Phenylenediamine dihydrochloride (Sigma-Aldrich, USA) and reaction was stopped using 4N of H_2SO_4 (Sigma-Aldrich). Optical density (OD) at 492 nm with noise correction at 620 nm were used to calculate specific signal for each antigen after subtracting the antigen-free well signal for each sample.

QUANTIFICATION AND STATISTICAL ANALYSIS

For cohort analyses (Table 1), continuous variables were described using medians and the interquartile range (IQR, defined by the 25^{th} and 75^{th} percentiles), whereas categorical factors were reported as percentages over available data. Values of n are indicated in Table 1.

Neutralization titers and ratios were shown as geometric means. The Friedman test with Dunn's multiple comparison was used to compare neutralization of different pseudo viruses (paired comparison). A Wilcoxon paired test or a Prentice Z test (when undetectable titers were observed) were used for longitudinal comparisons. The Mann-Whitney or Kruskal-Wallis test with Dunn's multiple comparison were used to compare neutralization titers and ratio when comparing 2 groups or multiple groups, respectively. A Peto-Peto test was used when undetectable titers were observed. Comparison involving multiple tests were corrected by false discovery rate. In all analyses, statistical significance was indicated on graphs as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Analyses were performed using R-3.6.3 (R Foundation for Statistical Computing) and Prism 9.4.1 (GraphPad Software).

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Supplemental information

Impact of hybrid immunity booster vaccination and Omicron breakthrough infection on SARS-CoV-2 VOCs cross-neutralization

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Figure S1

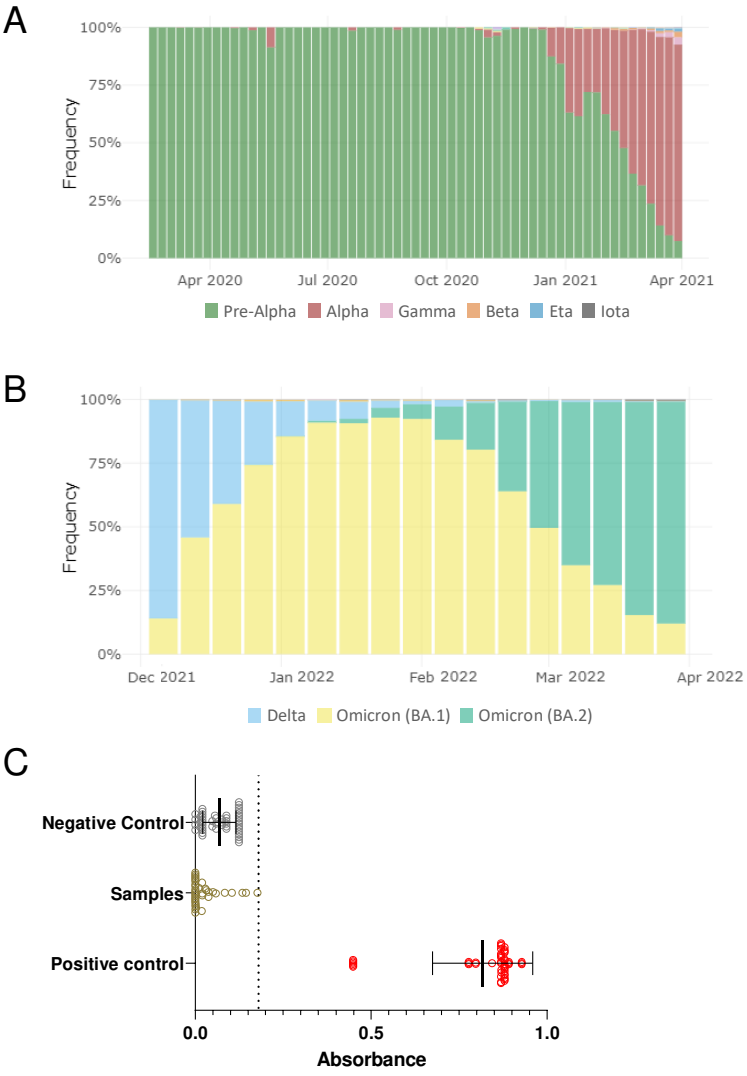

Figure S1. Participants infection history and nucleocapsid test (related to STAR Methods: study overview and subjects).
A. Temporal identification of circulating SARS-CoV-2 variants and their frequency in Catalonia Spain between March 2020 and March 2021 (source: http://covidtag.paseq.org/).
B. Temporal identification of circulating SARS-CoV-2 variants and their frequency in Catalonia Spain between December 2021 and March 2022 (source: http://covidtag.paseq.org/).
C. Anti-nucleocapsid protein IgG quantification for the status confirmation of uninfected participants. Threshold for positivity was calculated as the mean of negative controls + 2 standard deviations. Error bars indicate standard deviations. All uninfected participants were below threshold.

Figure S2

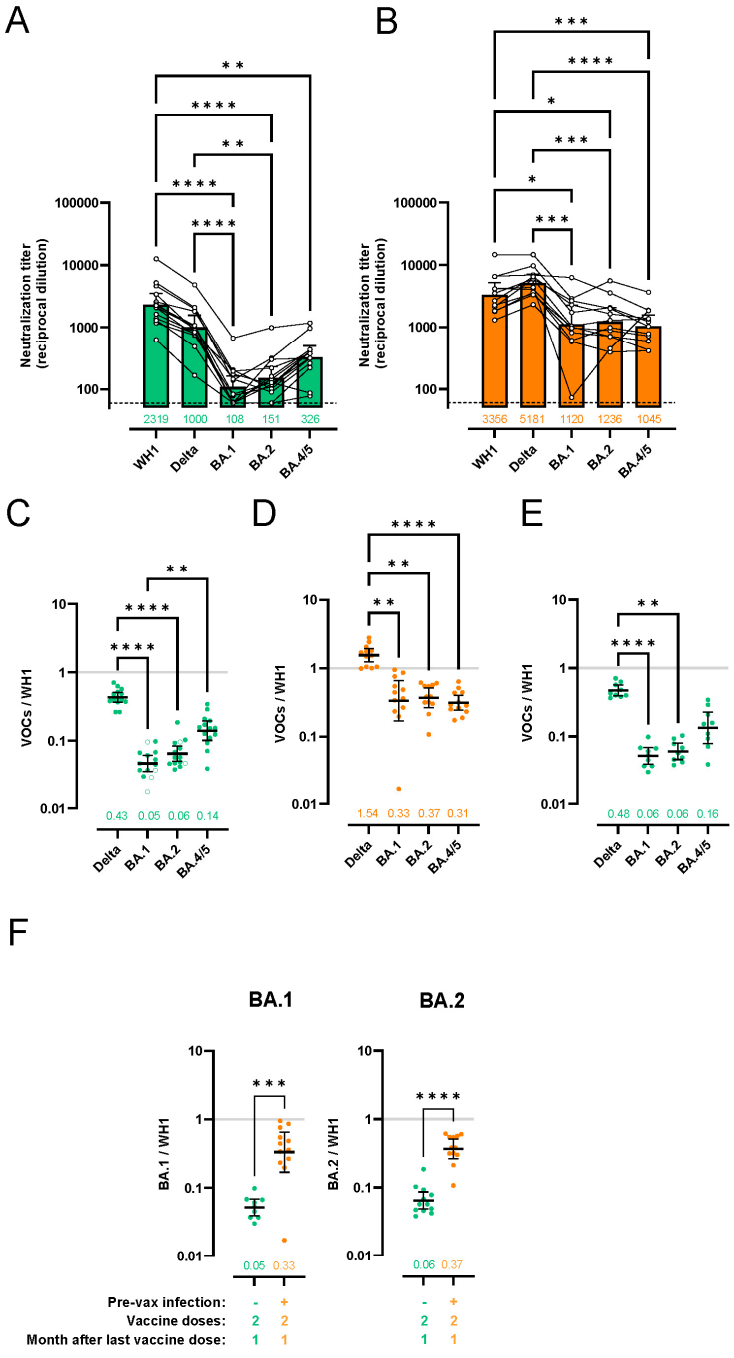
A
B
Neutralization titer (reciprocal dilution)
100000
10000
1000
100
2319 1000 108 151 326
3356 5181 1120 1236 1045
WH1
Delta
BA.1
BA.2
BA.4/5
C
D
E
VOCs / WH1
10
1
0.1
0.01
0.43 0.05 0.06 0.14
1.54 0.33 0.37 0.31
0.48 0.06 0.06 0.16
F
BA.1
BA.2
BA.1 / WH1
BA.2 / WH1
0.05 0.33
0.06 0.37
Pre-vax infection: - +
Vaccine doses: 2 2
Month after last vaccine dose: 1 1

Figure S2. Direct comparison of variants neutralizing titers and ratios within -/2/- and +/2/- groups at 1 month after last vaccine dose (Related to Figure 1).
A/B. raw ID_{50} (reciprocal dilutions) titers against the indicated variant spikes for -/2/- (A.) and +/2/- (B.) participants. Horizontal bars and numbers indicate ID_{50} geometric means (GMT) for each variant and error bars indicate 95% confidence intervals. At the bottom are fold changes for the indicated comparisons. Only significant *p* values are indicated for comparisons between all variants (Friedman test with Dunn's multiple comparison, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Dashed lines indicate the assay lower limit of detection (60 reciprocal dilutions).
C/D/E. Ratio of WH1 ID50 over the indicated VOC ID50 calculated for spikes for -/2/- (C.) and +/2/- (D.) participants. E. is a reanalysis of C. excluding non-responders. Horizontal bars indicate ratio geometric means for each variant and error bars indicate 95% confidence intervals. Bottom numbers indicate fold reduction compared to WH1. Significant p values are indicated for comparisons between all variants (Friedman test with Dunn's multiple comparison, ** $p < 0.01$, **** $p < 0.0001$)
F. Re-analysis of Figure 1C for variant BA.1 and BA.2 excluding non-responders.

Figure S3

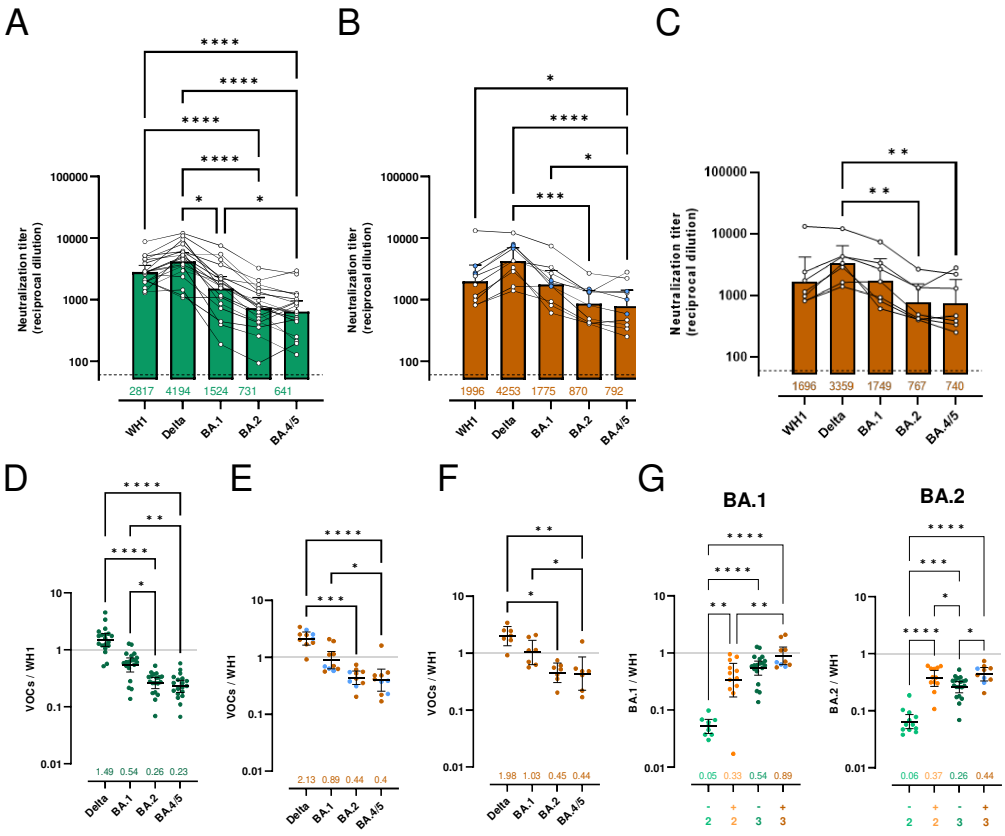

Figure S3. Direct comparison of variants neutralizing titers and ratios within -/3/- and +/3/- groups (related to Figure 2).

A/B/C. raw ID_{50} (reciprocal dilutions) titers against the indicated variant spikes for -/3/- (A.) and +/3/- (B.) participants. C. is a reanalysis of B. excluding participants who received only 1 dose as part of full vaccine schedule (highlighted in blue in B.). Horizontal bars and numbers indicate ID_{50} geometric means (GMT) for each variant and error bars indicate 95% confidence intervals. At the bottom are fold changes for the indicated comparisons. Significant *p* values are indicated for comparisons between all variants (Friedman test with Dunn's multiple comparison, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$). Dashed lines indicate the assay lower limit of detection (60 reciprocal dilutions).

D/E/F. Ratio of WH1 ID_{50} over the indicated VOC ID_{50} calculated for spikes for -/3/- (D.) and +/3/- (E.) participants**.** F. is a reanalysis of E. excluding participants who received only 1 dose as part of full vaccine schedule (highlighted in blue in D.). Horizontal bars indicate ratio geometric means for each variant and error bars indicate 95% confidence intervals. Bottom numbers indicate fold reduction compared to WH1. Significant *p* values are indicated for comparisons between all variants (Friedman test with Dunn's multiple comparison, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

G. Re-analysis of Figure 2C for variant BA.1 and BA.2 excluding non-responders.

Figure S4

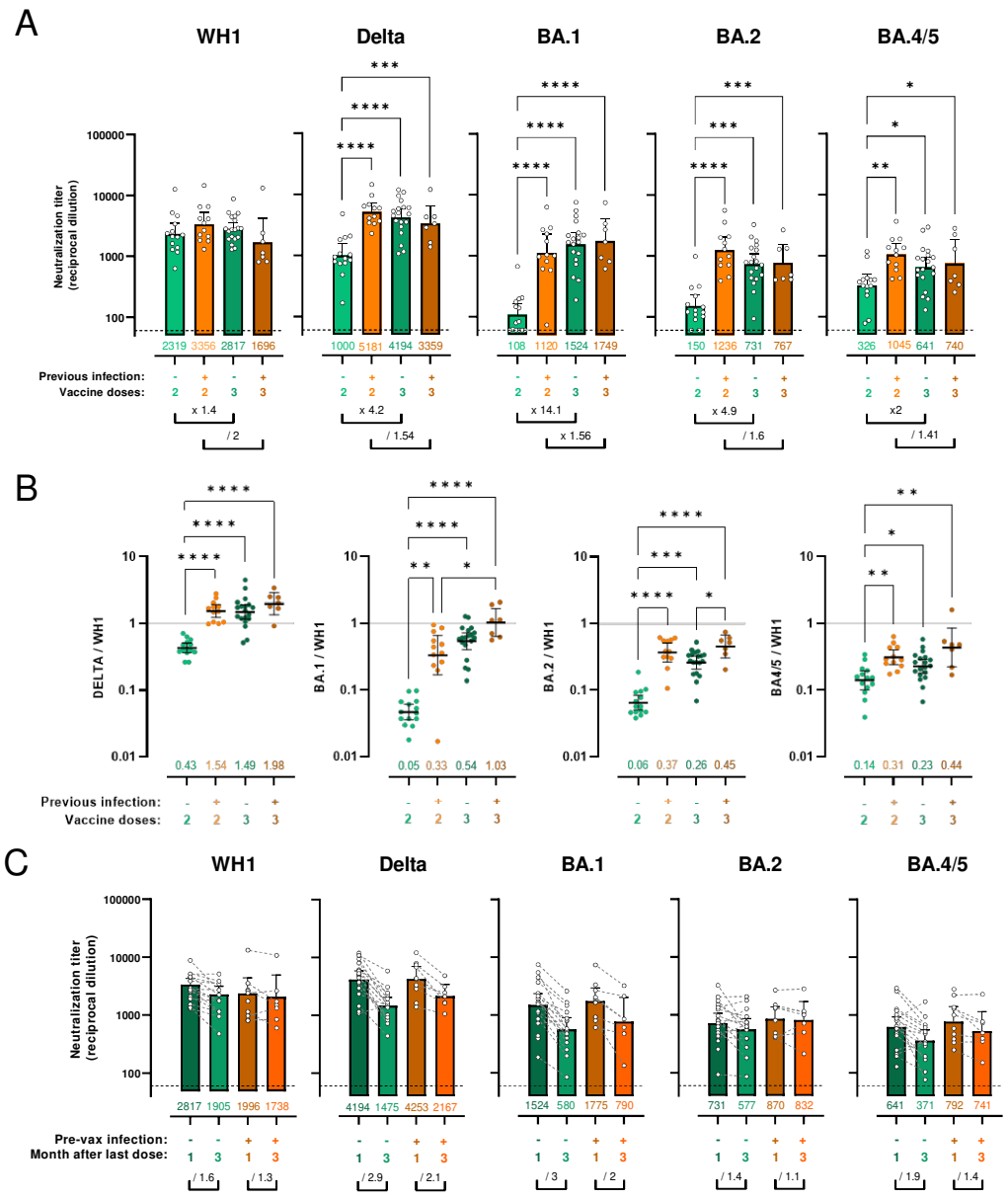

Figure S4. Additional stratified analyses and 3 months follow up on participants who received a booster vaccines (related to Figure 2).

A/B. Reanalyses of Figure 2B and C, respectively, excluding participants who only received 1 dose as part of full vaccine schedule.

C. Follow-up neutralization titers about 1 month (=Figure 2B) and 3 month after booster vaccine.

Figure S5

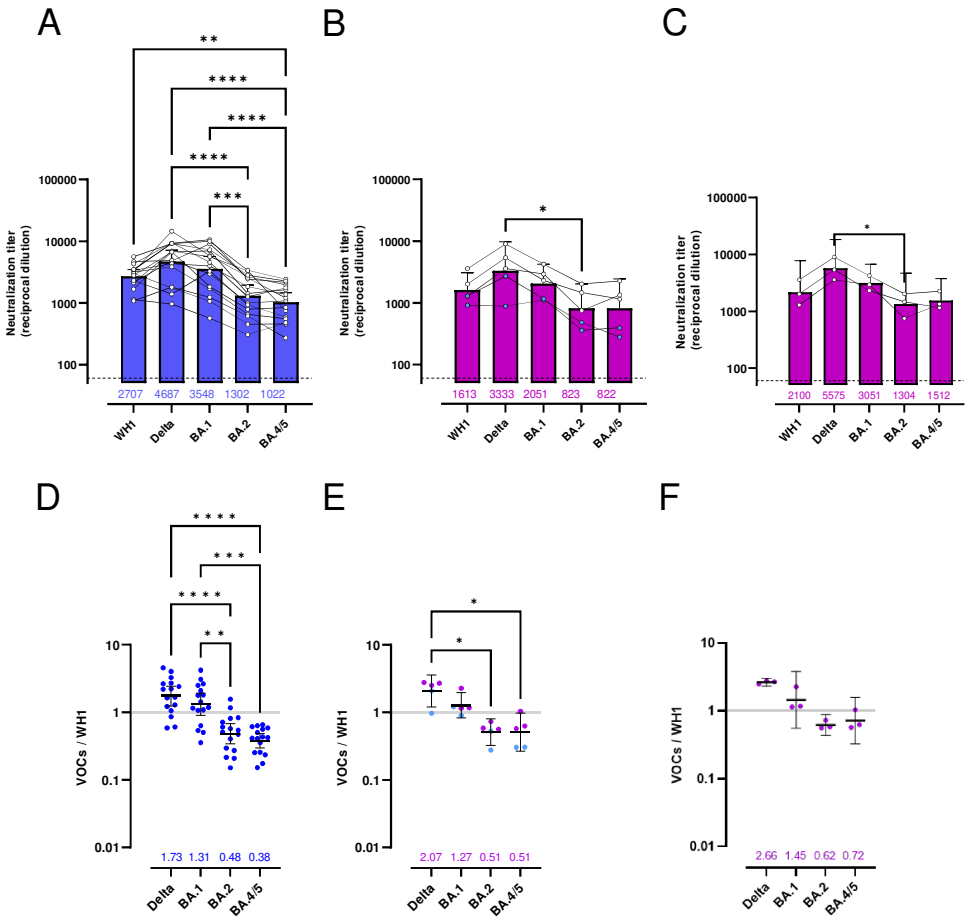

Figure S5. Direct comparison of variants neutralizing titers and ratios within -/3/+ and +/3/+ groups (related to Figure 3).

A/B/C. raw ID_{50} (reciprocal dilutions) titers against the indicated variant spikes for -/3/+ (A.) and +/3/+ (B.) participants. **C.** is a reanalysis of B. excluding participants who received only 1 dose as part of full vaccine schedule (highlighted in blue in B.). Horizontal bars and numbers indicate ID_{50} geometric means (GMT) for each variant and error bars indicate 95% confidence intervals. At the bottom are fold changes for the indicated comparisons. Significant *p* values are indicated for comparisons between all variants (Friedman test with Dunn's multiple comparison, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Dashed lines indicate the assay lower limit of detection (60 reciprocal dilutions).

D/E/F. Ratio of WH1 ID_{50} over the indicated VOC ID_{50} calculated for spikes for -/3/+ (D.) and +/3/+ (E.) participants**. F.** is a reanalysis of E. excluding participants who received only 1 dose as part of full vaccine schedule (highlighted in blue in E.). Horizontal bars indicate ratio geometric means for each variant and error bars indicate 95% confidence intervals. Bottom numbers indicate fold reduction compared to WH1. Significant *p* values are indicated for comparisons between all variants (Friedman test with Dunn's multiple comparison, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Figure S6

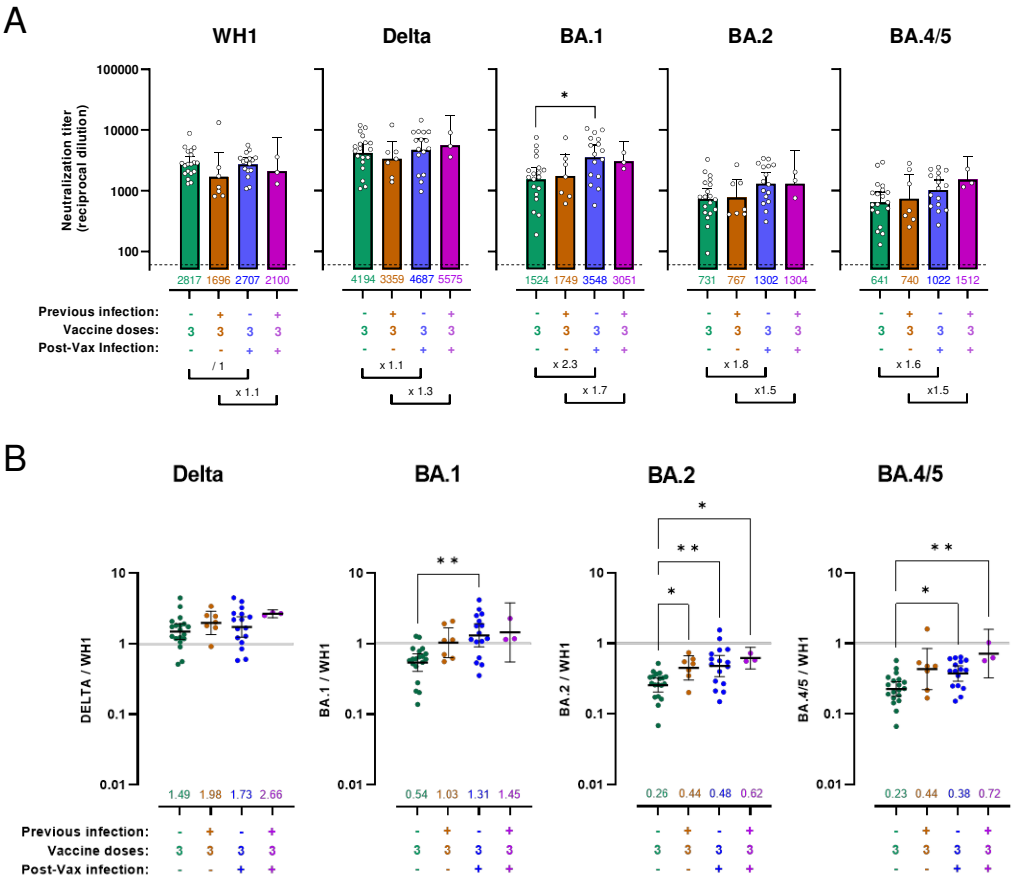

Figure S6. Additional stratified analyses (related to Figure 3).
A/B. Reanalyses of Figure 3B and 3C, respectively, excluding participants who only received 1 dose as part of full vaccine schedule.

DIRECTORS' REPORT

As supervisors of the present Doctoral Thesis, Dr. Julià Blanco Arbués and Dr. Jorge Carrillo Molina hereby state that Edwards Pradenas Saavedra has actively participated during the last five years in the work done at the group of Cell Virology and Immunology of the IrsiCaixa AIDS Research Institute. This work is summarized in this Thesis.

When Edwards Pradenas joined IrsiCaixa the main research focus of our group was the analysis of humoral responses against HIV, therefore the candidate started his PhD work on the identification of individuals with high titers of HIV-neutralizing antibodies targeting a specific region of the Envelope Glycoprotein. However, the COVID-19 pandemics completely changed the research focus of our team and hence the scheduled PhD activities of the candidate. As a result of the strong commitment of IrsiCaixa in COVID-19 research, and taking advantage of the technical skills that Edwards had gained on the development of neutralization assays, most of the work performed by Edwards Pradenas since February 2020 has been focused on the optimization of a robust neutralization assay for SARS-CoV-2 and in its application to the analysis of infection and/or vaccination-induced humoral responses. The candidate had an essential role in the development and execution of the experimental design, data acquisition and analysis, and in the discussion and publication of the main results and conclusions. Importantly, the candidate has published 26 manuscripts during his PhD. A selection of these studies, in which the candidate was more deeply involved, are included in this Doctoral Thesis. The description of these publications and the active participation of the candidate is as follows:

PAPER I

Title: SARS-CoV-2 infection elicits a rapid neutralizing antibody response that correlates with disease severity.

Authors: Trinité B, Tarrés-Freixas F, Rodon J, **Pradenas E**, Urrea V, Marfil S, Rodríguez de la Concepción ML, Ávila-Nieto C, Aguilar-Gurrieri C, Barajas A, Ortiz R, Paredes R, Mateu L, Valencia A, Guallar V, Ruiz L, Grau E, Massanella M, Puig J, Chamorro A, Izquierdo-Useros N, Segalés J, Clotet B, **Carrillo J**, Vergara-Alert J, **Blanco J**.

Journal: Scientific Reports

Year: 2020

DOI: 10.1038/s41598-021-81862-9

JCR IF (2021): 4.997

Rank: Q2 Multidisciplinary Sciences

Doctoral student participation: The candidate carried all neutralization assays involving pseudoviruses and validated the assay with data from collaborators (IRTA-CReSA) using replicative virus, discussed the results, and contributed to the writing of the manuscript.

PAPER II

Title: Stable neutralizing antibody levels 6 months after mild and severe COVID-19 episodes.

Authors: **Pradenas E**, Trinité B, Urrea V, Marfil S, Ávila-Nieto C, Rodríguez de la Concepción ML, Tarrés-Freixas F, Pérez-Yanes S, Rovirosa C, Ainsua-Enrich E, Rodon J, Vergara-Alert J, Segalés J, Guallar V, Valencia A, Izquierdo-Useros N, Paredes R, Mateu L, Chamorro A, Massanella M, **Carrillo J**, Clotet B, **Blanco J**.

Journal: Med

Year: 2021

DOI: 10.1016/j.medj.2021.01.005

JCR IF (2021): >18

Rank: D1 Medicine, Research & Experimental

Doctoral student participation: The candidate carried out all the neutralization assays in the longitudinal follow-up, analyzed these data and performed the graphs and tables in collaboration with the team, discussed the results, and contributed to the writing of the manuscript.

PAPER III

Title: Clinical course impacts early kinetics, magnitude, and amplitude of SARS-CoV-2 neutralizing antibodies beyond 1 year after infection.

Authors: **Pradenas E**, Trinité B, Urrea V, Marfil S, Tarrés-Freixas F, Ortiz R, Rovirosa C, Rodon J, Vergara-Alert J, Segalés J, Guallar V, Valencia A, Izquierdo-Useros N, Noguera-Julian M, **Carrillo J**, Paredes R, Mateu L, Chamorro A, Toledo R, Massanella M, Clotet B, **Blanco J**.

Journal: Cell Reports Medicine

Year: 2022

DOI: 10.1016/j.xcrm.2022.100523

JCR IF (2021): 16.988

Rank: D1 Medicine, Research & Experimental

Doctoral student participation: The candidate carried out all the neutralization assays in the longitudinal follow-up, analyzed these data and performed the graphs and tables in collaboration with the team, discussed the results, and contributed to the writing of the manuscript.

PAPER IV

Title: Virological and clinical determinants of the magnitude of humoral responses to SARS-CoV-2 in mild-symptomatic individuals.

Authors: **Pradenas E**, Ubals M, Urrea V, Suñer C, Trinité B, Riveira-Muñoz E, Marfil S, Ávila-Nieto C, Rodríguez de la Concepción ML, Tarrés-Freixas F, Laporte J, Ballana E, **Carrillo J**, Clotet B, Mitjà O, **Blanco J**.

Journal: Frontiers in Immunology

Year: 2022

DOI: 10.3389/fimmu.2022.860215

JCR IF (2021): 8.787

Rank: Q1 Immunology

Doctoral student participation: The candidate carried out all the neutralization assays, analyzed these data and performed the graphs and tables in collaboration with the team, discussed the results, and wrote the manuscript.

PAPER V

Title: Previous SARS-CoV-2 infection increases B.1.1.7 cross-neutralization by vaccinated individuals.

Authors: Trinité B, **Pradenas E**, Marfil S, Rovirosa C, Urrea V, Tarrés-Freixas F, Ortiz R, Rodon J, Vergara-Alert J, Segalés J, Guallar V, Lepore R, Izquierdo-Useros N, Trujillo G, Trapé J, González-Fernández C, Flor A, Pérez-Vidal R, Toledo R, Chamorro A, Paredes R, Blanco I, Grau E, Massanella M, **Carrillo J**, Clotet B, **Blanco J**.

Journal: Viruses

Year: 2021

DOI: 10.3390/v13061135

JCR IF (2021): 5.818

Rank: Q2 Virology

Doctoral student participation: The candidate carried out all the neutralization assays, analyzed these data and performed the graphs and tables in collaboration with the team, discussed the results, and contributed to the writing of the manuscript.

PAPER VI

Title: Impact of hybrid immunity, booster vaccination and Omicron breakthrough infection on SARS-CoV-2 VOCs cross-neutralization.

Authors: **Pradenas E**, Marfil S, Urrea V, trigueros M, Pidkova T, Pons-Grífols A, Ortiz R, Rovirosa C, Tarrés-Freixas F, Aguilar-Gurrieri C, Toledo R, Chamorro A, Noguera-Julián M, Mateu L, Blanco I, Grau E, Massanella M, **Carrillo J**, Clotet B, Trinité B, **Blanco J**.

Journal: iScience

Year: 2023

DOI: 10.1016/j.isci.2023.106457

JCR IF (2021): 6.107

Rank: Q1 Multidisciplinary Sciences

Doctoral student participation: The candidate carried out all the neutralization assays, analyzed these data and performed the graphs and tables in collaboration with the team, discussed the results, and contributed to the writing of the manuscript.

INTEGRATIVE SUMMARY OF RESULTS

A high-sensitivity pseudovirus-based neutralization assay to determine the titer of neutralizing antibodies against SARS-CoV-2

Neutralizing antibodies represent one of the best correlates of protection against infections. For this reason, we considered designing an *in vitro* assay that could be useful for assessing the levels of neutralizing antibodies in biological samples from SARS-CoV-2 infected and/or vaccinated individuals.

Our pseudovirus-based neutralization assay (PBNA) was conceptualized and adapted from a standardized HIV neutralization assay and the neutralizing activity was measured as a function of reduction in luciferase reporter gene expression[1326–1328]. Thereafter, it was optimized and validated.

First, we conducted a validation by comparing our PBNA with a neutralization assay based on replicative viruses (genuinely infectious) performed by an independent laboratory (IRTA-CReSA) against WH1 virus. For this, we analyzed 122 plasma samples by both methods and observed a significant correlation between them (**Figure 1B, Results Part I**, r=0.865)[1329]. This results additionally confirms that plasma-mediated inhibition of fully replicative virus is primarily associated with the presence of neutralizing anti-S-glycoprotein antibodies.

Next, we used a collection of samples with different neutralization capacity to check several validation parameters. Four samples came from a pool of human sera at different dilutions and ranged from a high (ID_{50}=6,000) to a low (ID_{50}=94) neutralization titer. The fifth sample corresponded to a pre-pandemic human serum (Sigma-Aldrich) with no detectable neutralizing activity (ID_{50}<20). In addition, we added a positive internal control hACE2-mIgG (Fc-fusion protein containing the globular extracellular domain of human ACE2 fused to a murine IgG1 F_c molecule) to each round of neutralization performed. The validation process was carried out by 2 analysts for 2 days.

All parameters analyzed comply with the acceptance criteria (**Table 3**), therefore, PBNA was validated, concluding that this method is consistent, reliable, and reproducible.

In addition, our in-house internal positive control complies with Westgard quality control rules[1330] against all SARS-CoV-2 variants tested (data not shown).

Table 3 | PBNA validation parameters

Parameter	Results
Accuracy (recovery)	98%
Repeatability (CV)	2.4%
Intermediate Precision (CV)	2.9% (day) and 3.1% (analyst)
Specificity	100%
Limit of Quantification (ID_{50})	20
Linearity (R^2)	0.993
Robustness (CV)	3.6%

CV: coefficient of variation

Our PBNA was also subjected to a blind external validation with a panel of 15 SARS-CoV-2 convalescent plasma samples—from the National Institute for Biological Standards and Control (NIBSC), UK—whose dilutions have been calibrated into international units (IU/ml)[1331]. Our results showed a strong correlation (R^2=0.995) with live virus-based neutralization assays from 12 European laboratories (from nine countries) that participated in the study (**Figure 28**). Interestingly, our PBNA is one of the most sensitive assays evaluated. It is important to note that our PBNA includes a negative control (uninfected cells) and a positive control (in-house soluble recombinant hACE2-mIgG) for each round performed, and a specificity control (VSV pseudovirus, built under the same HIV-1 backbone of SARS-CoV-2 pseudoviruses, but bearing the G-protein of VSV instead of the S-glycoprotein of SARS-CoV-2) for each sample tested.

All of the above described led us to validate and make reliable our PBNA to evaluate the levels of anti-SARS-CoV-2 neutralizing antibodies. Additionally, this assay received EMA approval to evaluate the levels of neutralizing antibodies in the PHH-1V vaccine (HIPRA).

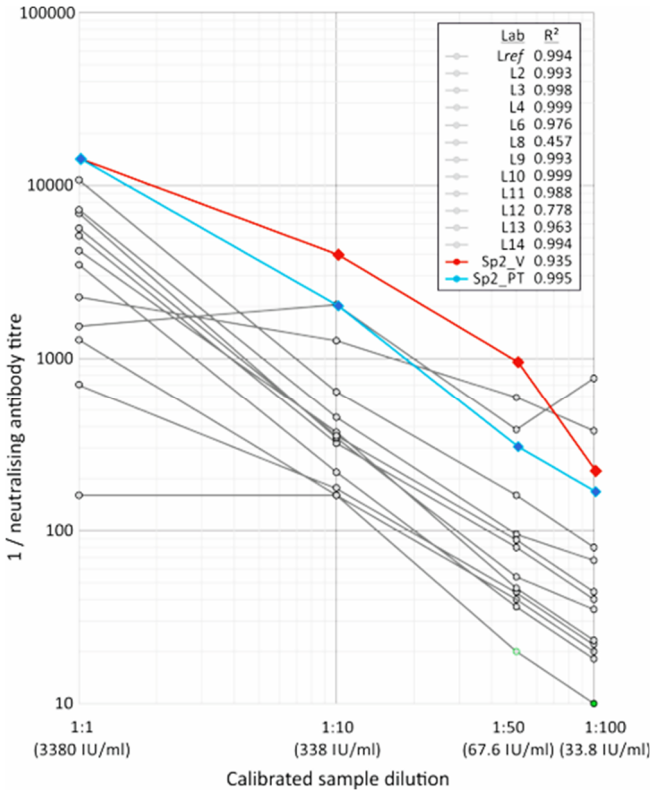

Figure 27 | Linearity of neutralizing antibody quantification. Representation of results obtained from virus- and pseudovirus-based neutralization assays developed at IrsiCaixa in comparison to 12 European laboratories. Serial dilutions were tested from a single sample with high neutralizing antibody titers. L*ref*: reference laboratory; L2-L14: other European laboratories; Sp2_V: IrsiCaixa virus neutralization assay; Sp2_PT: IrsiCaixa pseudovirus-based neutralization assay.

Kinetics of neutralizing antibodies acquired by natural infection at short, mid, and long-term

The evolution over time of the neutralizing response and the lifespan of antibodies are a cornerstone for assessing current—and forecast future—levels of protection. For this reason, we evaluated the early, mid-term, and long-term neutralizing antibody kinetics.

In a first instance (May-July 2020) we characterized the early neutralizing response using a virus-based neutralization assay (results were highly correlated subsequently with a PBNA, see above). The initial cohort (KING) included 72 SARS-CoV-2-positive individuals with a wide range of clinical presentations of COVID-19 (from asymptomatic to patients requiring intensive care [ICU])[1329]. We analyzed 128 samples, which showed a broad spectrum of neutralization, from undetectable neutralizing activity to neutralizing titers (ID_{50}) >5,000 (**Figure 1C, Results Part I**).

The median neutralization activity showed a gradual increase according to the clinical severity of the patients. The subgroups of hospitalized patients (non-severe, severe and ICU) did not show significant differences among them, but did when compared with asymptomatic individuals and patients with mild symptomatology. However, both subgroups of non-hospitalized patients (asymptomatic and mild symptomatic) did not show statistically significant differences between them (**Figure 1D, Results Part I**). These results indicated that non-hospitalized individuals had significantly lower levels of neutralizing activity compared to hospitalized ones, and both groups should be handled independently in the modeling of antibody kinetics to minimize inaccuracies in the analyses and prevent potential misinterpretation of the results.

To determine the kinetics of the neutralizing response, we used nonlinear mixed-effect models. Due to the limited number of time points in asymptomatic and mildly symptomatic individuals, and the impossibility of determining the day of symptom onset in asymptomatic individuals, we were only able to appropriately adjust the early kinetics in hospitalized patients. Considering all datasets, we detected neutralizing activity during the first days PSO, which reached half of the maximum neutralizing activity at day ≈11, and 80% of the maximum neutralizing activity at day ≈17, which subsequently reached a sort of plateau (**Figure 2A, Results Part I**).

Although we were unable to fit a curve in the non-hospitalized infected, in this group, the mean level of neutralization over time was ID_{50}=234 (**Figure 2B, Results Part I**). This value showed a highly significant difference compared to the plateau reached by the group of hospitalized patients (ID_{50}=1,584).

Of note, the distribution of neutralization titers was different between the two groups, with nearly 40% of non-hospitalized individuals presenting an ID_{50}<100 (with a significant proportion of individuals with undetectable neutralizing activity), while ≈50% of hospitalized patients had an ID_{50}>1,000 (**Figure 2C, Results Part I**).

By increasing the size of the KING cohort (from n=72 to n=210) and the follow-up time (up to more than 200 days PSO), we were able to fine-tune the early kinetics, compare the behavior to the first and second COVID-19 waves, and characterize the mid-term neutralizing antibody response[1332].

The group of hospitalized patients (well characterized in the acute phase of the disease due to the large number of samples obtained in this time frame), showed a sharp increase in neutralizing activity irrespective of the COVID-19 wave (with no significant difference between the two waves). Using this set of data, and in line with the initial findings (see above), we observed that half-maximal neutralizing activity (ID_{50}=5,833) was reached on day 10, and 80% of the maximum response (ID_{50}=9,333) on day 14. After that, the neutralizing titers reached a temporary plateau until day 30 PSO (**Figure 1D, Results Part II**).

In outpatients, the plateau reached at day 30 remained practically unchanged (almost flat slope, ID_{50}=525) with a half-life of 2134 days. On the contrary, in hospitalized patients, after day 30 PSO, a decrease in neutralizing activity was observed, characterized by a two-phase pattern: 1) a rapid decrease until day 80, and 2) a slower decline thereafter (significantly less steep slope, ID_{50}=1,445), with a half-life of 753 days (**Figure 2A-B, Results Part II**). According to our results, 91% of hospitalized patients had a neutralization titer ID_{50}>250 beyond 135 days PSO, while only 58% of asymptomatic or mildly symptomatic patients showed these levels after 4.5 months PSO (**Figure 2D, Results Part II**).

In parallel with the assessment of neutralizing activity, we determined the IgG antibody titer (anti-RBD, anti-S2 and anti-NP) in a subset of 28 individuals (14 asymptomatic/mild disease and 14 hospitalized patients) with the longest follow-up. Surprisingly, the longitudinal analysis revealed a 1-phase steady decay pattern in all cases (hospitalized patients showed higher titers of anti-S2 antibodies). The calculated half-lives for anti-RBD, anti-S2 and anti-NP antibodies were 86, 108 and 59 days, respectively (**Figure 3, Results Part II**). These kinetics and half-lives demonstrate that, at least at mid-term, IgG antibody titers generated by natural SARS-CoV-2 infection are not a mirror of neutralizing activity.

Finally, our most recent published analysis about the kinetics of the neutralizing humoral response in non-vaccinated subjects, included 332 individuals (KING cohort) who had a positive diagnosis of SARS-CoV-2 and had a follow-up beyond 1-year PSO[1333]. The enrollment period encompassed three COVID-19 waves in Spain, characterized by the prevalence of the ancestral virus (WH1) co-occurring with D614G, the dominance of the 20E variant (EU1), and the predominance of the first variant of concern B.1.1.7 (Alpha), during the first, second and third COVID-19 waves, respectively.

The results obtained in the modeling of neutralizing antibody kinetics confirmed our previous findings. In hospitalized patients, a rapid generation of neutralizing response was observed during the first month PSO and a transient decay towards month 3, until reaching a relative stabilization that lasted more than one year and projected a half-life of 533 days. The kinetic pattern remained invariable when the 3 waves were analyzed independently (and consequently irrespective of the SARS-CoV-2 variant dominant in each of them), although the neutralizing activity in the third wave was marginally higher than in the first two waves. In contrast, non-hospitalized patients developed a lower maximum neutralizing titer with a flat slope (**Figure 1, Results Part III**).

The behavior of the neutralizing response evidenced by the group of patients requiring SARS-CoV-2 hospitalization suggests the rapid generation of specific B cells, followed by the presence of short-lived plasma cells, until the generation of long-lived plasma cells that probably maintain stable circulating levels of neutralizing antibodies.

Thanks to all the data, we were able to determine the short, mid and long-term kinetics of the neutralizing humoral response against the ancestral SARS-CoV-2 virus (WH1). In outpatients, we were able to sense a phase of increased neutralizing response that reached a sort of plateau and lasted for more than 1 year, estimating a half-life near 6 years. While in hospitalized patients, the kinetics were clearly defined by 3 phases, a first acute phase with an exponential increase in neutralizing activity, a second convalescent phase with a sharp decrease in the neutralizing antibody titer, and a post-convalescent phase characterized by a slowed (with almost stable levels) and long-term (>12 months) decline in the neutralizing response, with a projected half-life of 533-753 days according to our modeling.

Factors associated with the magnitude of the humoral response acquired by infection

To unravel the factors associated with the magnitude of the antibody response, we considered two independent cohorts. 1) The KING cohort initially constituted by 72 participants and achieved a final enrollment of 332 COVID-19 individuals; these participants showed a wide range of clinical presentation of the disease, from asymptomatic individuals to ICU patients[1329,1332,1333]. 2) The CIRCUS cohort, formed by 62 SARS-CoV-2 positive subjects (plus 9 uninfected controls) with asymptomatic or mild infection[1334].

Firstly, as described above, in the KING cohort there was a strong influence of disease severity on neutralizing antibody titer, with higher levels in hospitalized patients compared to non-hospitalized individuals. The differences between both groups were statistically significant and multivariate analysis confirmed this association. This behavior was evident during the acute phase, the early decay, and the subsequent "stabilization" stage (**Results Part I, II and III**). In fact, infected asymptomatic or with mild disease developed a 10-fold lower maximum neutralization titer compared to patients who required hospitalization, although heterogeneity within each group remained high. Analyses in the CIRCUS cohort led to similar results, with a significantly lower proportion of neutralizing levels above 250 (reciprocal dilution) in asymptomatic individuals compared to patients with mild symptomatology (**Figure 3G, Results Part IV**).

In parallel, we explored the factors determining neutralizing responses in hospitalized patients. During the analysis of early kinetics, we failed to identify the potential impact of antiviral or immunomodulatory treatment on neutralizing titers (all hospitalized patients except for one outpatient). No association was observed between neutralizing antibody levels and treatment with corticosteroids, tocilizumab (or other anti-IL-6 drugs), type I IFN (mainly IFN-β) or protease inhibitors (mainly Lopinavir) (**Figure 4, Results Part I**). However, this analysis was limited by the number of participants in each treatment group, which prevented us from drawing clear conclusions.

In addition, we also explored the factors that determine neutralizing responses in outpatients. In the CIRCUS cohort (encompassing only asymptomatic or mildly ill patients), we were able to identify the length of symptom duration as a factor associated with the magnitude of humoral responses (binding antibodies to S1+S2, RBD and NP, and neutralizing antibodies). However, viral load (levels and viral dynamics), although it had some association with the duration of self-reported symptoms, had no impact on any of the parameters of the humoral response (**Figure 3, Results Part IV**).

Concurrently, to gain a more comprehensive understanding of the humoral response, we assessed the IgG antibody response against specific regions of SARS-CoV-2 (**Figure 2, Results Part IV**). The findings at day 60 revealed a positive correlation between neutralizing antibody titers and total IgG levels against RBD, S1+S2, and NP. Notably, the group of early non-seroconverters exhibited a surprising neutralizing capacity that was comparable to that of early seroconverter individuals with high and low viral loads. To explain this behavior, we calculated the ratio of neutralizing titers and total IgG antibodies specific to each antigen as a proxy of antibody quality. The results indicated significant differences in the ratios for anti-S1+S2 and anti-NP antibodies, with higher ratios observed in the non-early seroconverter group, suggesting the generation of a better quality of neutralizing antibodies compared to the other two groups.

Moving on to demographic characteristics. In the KING cohort there were statistical differences in the distribution of gender (used in this thesis indistinctly with sex) and age between non-hospitalized and hospitalized patients. Hospitalized patients were mostly male and significantly older. To evaluate the impact of these factors on the neutralizing response, we performed univariate and multivariate analyses.

During the early phase of the disease, although a first analysis showed a moderately positive correlation ($r=0.25$, $p=0.03$) between age and neutralizing titer when all participants were analyzed pooled, the correlation was lost in a subsequent analysis when each group (hospitalized and non-hospitalized) was analyzed independently (**Figure 3, Results Part I**). A two-factor regression model (including age and hospitalization status) showed a strong correlation of neutralizing titers with hospitalization ($p=0.0001$) and a non-significant contribution of age ($p=0.523$) (**Table 2, Results Part I**).

Similarly, during the late post-convalescent phase (>300 days PSO), a univariate analysis showed a significant effect of age on the neutralizing response, but this effect was lost in the multivariate model ($p=0.095$) (**Figure 4B, Results Part III**). This suggests that age (although it showed some tendency, older participants exhibited higher neutralization titers) by itself is not a determinant component of neutralizing capacity, but depends on other cofactors, such as the severity of the disease.

Additionally, we did not observe any impact of gender on neutralizing antibody titer, either in the short- or long-term; and longitudinal kinetics proved to be similar between females and males; only showing a significant difference between the two genders during the first decay phase (day 30-80 PSO), but not in the long-term (**Figure S2, Results Part II**).

On the other hand, when we analyzed the potential association of these parameters in the CIRCUS cohort, multivariate linear regression analysis evidenced that only age correlated with incrementally neutralizing antibody titers ($r=0.29$, $p=0.023$) (**Figure 2E, Results Part**

IV), but was not statistically significant in the non-early seroconverter group; probably due to the high prevalence of asymptomatic individuals, who had mostly neutralizing titers below 250. It indicated that older age was also correlated with anti-RBD IgG antibody titer, the most immunogenically neutralizing region of SARS-CoV-2, but not with anti-S1+S2 or anti-NP antibodies.

Furthermore, the multiple correlation analysis revealed a strong association between the magnitude of the neutralizing response and IgG antibody levels against all antigens (anti-S1+S2, anti-RBD and anti-NP). Age was only associated with anti-RBD IgG antibody titers, whereas gender was not correlated with any antigen-specific IgG or neutralizing antibody levels (**Table S1, Results Part IV**).

In general, the multiple analyses in the studies conducted in different COVID-19 cohorts identified a correlation between the magnitude of the neutralizing response with the severity of the disease, duration of symptoms and age (the latter two factors were assessed only in asymptomatic or mildly ill individuals); and showed that humoral responses were not significantly influenced by any other clinical or demographic characteristics such as gender, viral load, smoking, cardiovascular disease, obesity, respiratory disease, influenza vaccination, residual symptoms, antiviral treatments or immunomodulatory therapies. However, these results should be interpreted with caution, since the fact that our studies found no association with the latter factors does not mean that there is none, and the limitations of our cohorts should be taken into consideration.

Cross-neutralization by infection against different SARS-CoV-2 variants

The emergence of more transmissible and immuno-evasive variants early during the pandemic raised the urgent need to evaluate the protection conferred by previous infection. The first variant contained the D614G mutation in the S-glycoprotein. Later, the B.1.1.7 (Alpha) variant emerged, which in addition to the D614G mutation, contained other mutations of interest. All these mutations gave it an advantage over the previous lineages, making it prevalent around the world.

To evaluate the impact of both variants on the neutralizing response conferred by distinct infective variants, we selected a subset of 53 unvaccinated individuals from the KING cohort[1335]. The participants were divided into different groups according to the date of infection that corresponded to three different COVID-19 waves (WH1+D614G, 20E (EU1) and Alpha). 16 participants were infected during the first wave and each of them had an early sample (median 48 days PSO) and a late sample (median 196 days PSO) (**Table 1, Results Part V**).

In an analysis considering the pool of infected individuals (irrespective of the group to which they belonged), we were able to observe significantly higher neutralizing sensitivity to D614G, with no major differences between WH1 and the Alpha variant (**Figure 2B, Results Part V**).

Later, in an analysis restricted to early (median 48 days PSO) and late (median 196 days PSO) neutralizing activity (participants infected during the first COVID-19 wave in Spain), we evidenced that although a higher neutralization sensitivity to D614G was maintained at both time-points, the levels of neutralizing antibodies decreased over time against all viruses evaluated (WH1, D614G and Alpha), but it was only significant against the ancestral WH1 virus (**Figure 3A, Results Part V**). However, when we explore the fold change ratio, as an approximation that gives a measure of the cross-neutralization potential by using one virus as a reference and providing an alternative analysis by reducing interindividual variability, which could bias group comparisons when only the raw data are analyzed, we observed an improvement of the cross-neutralizing capacity in the late response compared to the early response (**Figure 3B, Results Part V**). These differences became significantly between WH1 and Alpha variant, indicating that the neutralizing response induced by natural infection continues to evolve for several months after infection.

Next, independent analyses of cross-neutralization, on the one hand individuals infected during the second COVID-19 wave (exposed almost exclusively to the 20E (EU1) variant) and, on the other hand, those infected with Alpha variant (third COVID-19 wave), did not show statistically significant differences in both groups against the different viruses tested. However, individuals infected during the third COVID-19 wave tended to have higher neutralization titers, especially against the D614G mutant and the Alpha variant (**Figure 3C, Results Part V**). Comparison of the fold change between the different COVID-19 waves did not show significant differences. An exception was the cross-neutralization capacity between individuals infected in the first vs. the third wave, where the former lost neutralization levels against the Alpha variant compared to D614G (fold change x1.53), while the latter were invariable (fold change x1.0) (**Figure 3D, Results Part IV**). These data illustrate the progressive evolution of the virus infecting the population and its impact on the induced cross-neutralization response.

COVID-19 waves continued, and newly emerging variants of concern challenged the protective immunity conferred almost a year ago on those infected during the first COVID-19 wave. For this reason, we evaluated the long-term neutralizing response in samples from 60 patients obtained after 300 days PSO. Cross-neutralization levels were evaluated against Alpha, Beta and Delta variants, and compared to the ancestral WH1 virus[1333].

The global analysis indicated that the neutralizing levels against the Alpha variant were similar to the response against WH1, decreasing marginally against the Delta variant and being significantly lower against the Beta variant (**Figure 3A, Results Part III**).

Analyses performed by group (hospitalized and non-hospitalized) showed similar but not identical results, with Beta always being the most neutralization resistant variant. Additionally, hospitalized patients presented significantly higher neutralization titers against most viruses (WH1, Alpha and Delta) compared to outpatients, but did not reach statistical significance against the Beta variant (non-hospitalized GMT=151 vs. hospitalized GMT=265) (**Figure 3A, Results Part III**).

To analyze the differences in more detail, we used ratios to compare the relative loss of neutralization for each individual with reference to the neutralizing capacity against the ancestral WH1 virus. To reduce misinterpretation, censored values (undetectable: ID_{50}<60) were not considered in the analyses. No significant differences were observed between hospitalized

and non-hospitalized patients for the Alpha and Delta variants, while the loss of neutralization to the Beta variant was significantly higher in hospitalized patients (fold change loss of neutralization relative to WH1: x1.61 vs. x2.87 in non-hospitalized and hospitalized patients, respectively) (**Figure 3B, Results Part III**).

On the basis of neutralization titer as surrogate marker of protection[600,1336] and assuming a cutoff value of ID_{50}=250 as indicated by the FDA (EUA 26382) to differentiate between a convalescent plasma with low and high neutralization titers[1337], we estimated that 33% of individuals had undetectable or low neutralization against the ancestral virus WH1 or the Alpha variant, increasing to 52% or 41% for the Beta and Delta variants, respectively (**Figure 3C, Results Part III**). In any case, for all viruses tested, the frequency of individuals with low or undetectable neutralization titers was significantly higher in non-hospitalized patients, suggesting that this group of patients has a higher hypothetical risk of reinfection (considering only the neutralizing response, in the absence of other factors of humoral, cellular, and innate immune response) compared to patients who required hospitalization.

In summary, all the data described above indicate that the emerging variants do not elude completely the polyclonal neutralizing humoral response, and that irrespective of the infecting variant, it confers cross-neutralization against other never exposed variants (with the Beta variant being the most resistant to neutralization), and this capacity is progressively reduced over time.

Impact of COVID-19 vaccination, hybrid immunity, booster dose and breakthrough infection on cross-neutralization

WHO estimates that about 90% of the world's population is immunized against SARS-CoV-2 as a result of vaccination and/or infection[1338]. However, within this 90% "there is still a lot of ground to cover" and the level of protection could be highly heterogeneous. The value of immunization obtained by different routes, and in different numbers and/or sequence, may influence protective immunity. We therefore decided to evaluate the nature and number of antigenic exposures in participants from the KING cohort who had different immunization profiles[1333,1335,1339].

Six months after the vaccination campaign began in Spain, we observed that vaccination reinforced the pre-existing neutralizing response, even raising a part of the values to the upper limit of detection of our assay (ID_{50}>14,580). Post-vaccination neutralizing antibody titers (against WH1) were significantly higher in individuals with previous infection in both groups (hospitalized and non-hospitalized). The GMT of non-hospitalized individuals increased from 249 to 4,595, while in hospitalized patients the GMT increased from 762 to 8,851, which tended to balance the neutralizing responses between outpatients and hospitalized (p=0.292) (**Figure 2, Results Part III**). Potential further analyses were hampered by the large heterogeneity of vaccination status in terms of type of vaccine, number of doses, and time from the last dose.

Moreover, we compared the cross-neutralization, considering WH1, D614G and the Alpha variant, between vaccinated uninfected (sampled approx. 9 days post 2nd dose BNT162b2),

vaccinated individuals infected during the first COVID-19 wave (sampled approx. 13 days post 2nd dose BNT162b2), and the group infected during the first COVID-19 wave (late plasma sampling, median 196 days PSO) not vaccinated (**Table 1, Results Part V**).

Participants with hybrid immunity exhibited relatively higher levels of neutralization against all viruses (WH1, D614G and Alpha) compared to uninfected vaccinees, but this difference was only significant for the Alpha variant. In contrast, unvaccinated individuals who had experienced a previous infection (approximately 196 days ago) exhibited a markedly lower global neutralizing response than the other two groups, especially patients with hybrid immunity, showing the greatest statistical differences across all viruses tested (**Figure 4A, Results Part V**). Importantly, these differences became so evident because of the time point of sample collection from the unvaccinated infected individuals, where the overall response had decreased as we had previously observed (see above).

In terms of cross-neutralization (fold change referring to the loss of neutralizing capacity; ratio inferior to 1 and closer to 0 indicates better cross-neutralization), the differences narrowed. In all three groups, the neutralizing response against D614G relative to WH1 improved, although it was significantly higher for those infected vaccinated (x0.44) and infected unvaccinated (x0.40), compared to individuals only vaccinated (x0.60). Something similar occurred when analyzing the Alpha variant, the neutralization ratios improved (compared to WH1) against the Alpha variant for the vaccinated infected group (x0.60) and the unvaccinated previously infected individuals (x0.60), while for the vaccinated-only group the fold change was x2.04, indicating that neutralizing potency was lost against this variant (**Figure 4B, Results Part V**).

Overall, this subset of data indicates that infection alone shows similar cross-neutralization to hybrid immunity (infection plus vaccination), and both are better than immunity induced by vaccination alone (in terms of cross-neutralization). However, although cross-neutralization is similar between the two groups, vaccination boosts pre-existing immunity and improves the magnitude of the neutralizing response.

In our most recent study (March 2023), we analyzed a total of 76 participants from the KING cohort who were categorized into 6 groups according to 3 parameters: status of SARS-CoV-2 infection pre-vaccination (mild disease during the first year of COVID-19 in Spain), status of vaccination (initial schedule of vaccination only or with booster shot, all of them with BNT162b2 or mRNA-1273 vaccines), and infection following the booster dose (mostly by BA.1 and BA.2, without ruling out infections by the Delta variant)[1339].

In this way, and to simplify the description of the results, the different groups will be identified by signs and numbers (the description of the cohort is summarized in **Table 1, Results Part VI**):

1. No pre-vaccination infection, two doses of vaccine, and no post-vaccination infection: **-/2/-**
2. Pre-vaccination infection, two doses of vaccine, and no post-vaccination infection: **+/2/-**
3. No pre-vaccination infection, three doses of vaccine, and no post-booster infection: **-/3/-**
4. Pre-vaccination infection, three doses of vaccine, and no post-booster infection: **+/3/-**

5. No pre-vaccination infection, three doses of vaccine, and post-booster breakthrough infection: **-/3/+**
6. Pre-vaccination infection, three doses of vaccine, and post-booster breakthrough infection: **+/3/+**

For comparative purposes, all samples were collected at a median of 25-35 days after the last antigenic event (vaccination or infection) and in special cases, the neutralizing response was evaluated at mid-term (3 or 6 months) after the last dose (third or second). We evaluated the neutralizing activity against WH1, Delta, BA.1, BA.2 and BA.4/5 (so named because BA.4 and BA.5 have the same mutations in the S-glycoprotein).

The analyses indicated that in all groups the neutralizing capacity against the Omicron subvariants (BA.1, BA.2 and BA.4/5) decreased, evidencing a greater proportion of significant differences (with respect to WH1 and Delta) in the -/2/-, +/2/- and -/3/- groups, although the latter two showed a higher magnitude of neutralization across the different viruses tested, compared to individuals who had only received the initial vaccination schedule (**Figure S2-S6, Results Part VI**).

Two doses of vaccine were not able to induce adequate cross-neutralization and the neutralizing activity was severely affected by Omicron subvariants. Furthermore, the neutralizing response at mid-term (6 months) of this group experienced a significant reduction in most cases compared to the response at short-term (1 month) (**Figure 1B, Results Part VI**).

When we analyzed fully vaccinated (without booster dose) but previously infected (+/2/-) individuals, the magnitude of the neutralizing response was greater than the -/2/- group across all viruses, although the greatest differences were observed for Delta, BA.1, BA.2 and BA.4/5 variants. Similar to -/2/- individuals, the mid-term response was significantly affected in all cases, with a 3.4 to 5.6-fold reduction among the viruses tested (**Figure 1B, Results Part VI**).

Moreover, the booster dose (-/3/-) maximized the short-term neutralizing response against all viruses (although it was not significantly higher for WH1) compared to the -/2/- group, but achieved neutralizing titers similar to those obtained by hybrid immunity (+/2/-) (**Figure 2B, Results Part VI**). Previous infection plus three doses of vaccine did not improve neutralizing activity relative to -/3/- and +/2/- (**Figure 2B, Results Part VI**); however, patients with the booster shot with or without previous infection were not significantly impaired in their mid-term (3 months) neutralization for WH1, nor any of the SARS-CoV-2 variants analyzed (**Figure S4, Results Part VI**). Nevertheless, it is worth pointing out that there was a slight downward trend, which could lead to greater differences if the response were reanalyzed at 6 months, as we did for the groups of patients with two doses of vaccine.

Finally, both groups of individuals with three doses of vaccine and a subsequent breakthrough infection, regardless of whether or not they experienced a pre-vaccination infection (-/3/+ and +/3/+), in general showed similar levels of neutralization to the other two groups with booster doses in the absence of breakthrough infection (-/3/- and +/3/-); however, breakthrough infection tended to improve cross-neutralization specifically against Omicron subvariants (**Figure 3, Results Part VI**).

In summary, the overall data indicate that two vaccine doses induce neutralizing antibodies levels that are severely affected by both emerging variants (except D614G) and over time,

with the Omicron subvariants exhibiting the strongest resistance to neutralization. Vaccination potentiates pre-existing immunity, and hybrid immunity improves the quantity (magnitude) and quality (breadth of neutralization) of the neutralizing response. The third vaccine dose boosts the neutralizing immunity (understood in this thesis as the neutralizing antibody-mediated immune response) induced by vaccination and mimics the neutralizing response acquired by hybrid immunity. Lastly, Omicron breakthrough infection (after three vaccine doses) improves the magnitude of the neutralizing response (at least in the short-term), albeit modestly, specifically against Omicron subvariants, but does not appear to be a determining factor in cross-neutralization.

4

DISCUSSION

As mentioned in the introductory chapter, immunity to SARS-CoV-2 is complex and we have not yet succeeded in elucidating the multiple host immune mechanisms involved. To date, it is known that both innate and adaptive immunity (cellular and humoral) work collectively to respond to SARS-CoV-2 infection[342,426,1340–1342].

Antibodies are one of the key components of the immune response to viral infections, including SARS-CoV-2. As such, antibodies can be a useful starting point for assessing protective immunity to the virus. However, antibodies generated following the antigenic encounter exhibit a great variety of peculiarities: they may have several effector functions (e.g., neutralizing, ADCC, ADCP, among others), they may interfere with the functions or recognition of other antibodies, or may be dysfunctional due to structural alterations, improper binding to epitopes or recognition of viral debris[583,586–588,596,953,1343,1344]. Eventually, they may also play a pathogenic role[962,1345].

Neutralizing antibodies are one of the major correlates of protection from infection and their elicitation is a major goal of antiviral vaccines[589–597]. Numerous scientists have studied their behavior in response to SARS-CoV-2 infection and/or vaccination; however, more research is still needed to define which factors determine the development of neutralizing antibodies, how long is the protective response or how it behaves against the new variants that are spreading rapidly around the world. Moreover, to determine the relationship between the magnitude and quality of the immune response and the disease severity may open the gateway to understand protective mechanisms and improve preventive strategies and treatments that can be helpful to reduce the morbidity and mortality associated with COVID-19.

In an attempt to contribute with answers to these issues, our studies were focused on the understanding of the neutralizing immune response to infection and vaccination, which is one of the cornerstones for assessing protective immunity.

In this sense, two factors allowed us to evaluate in detail the antibody-mediated neutralizing response to SARS-CoV-2. Firstly, the rapid development of a reliable neutralization assay

based on HIV-pseudoviruses that has been adapted to emerging viral variants. And secondly, the fact that we have been able to establish a cohort early during the initial phases of the pandemic in Spain. The longitudinal sample collection from a large number of individuals allowed us to evaluate the short, mid and long-term kinetics of neutralizing antibodies against the ancestral virus detected in Wuhan and the cross-neutralization activity against the major variants emerging during this period of time (from April 2020 to March 2023).

Thanks to our expertise in HIV neutralization assays based on pseudoviruses[1328], we were able to quickly and successfully adapt this assay to the new SARS-CoV-2. For validation purposes, we compared the results obtained with two different neutralization assays: true replicative viruses and HIV-based pseudoviruses carrying the SARS-CoV-2 S-glycoprotein. Both assays showed a high degree of correlation, confirming that pseudoviruses can be used for evaluating neutralizing activity of plasma samples, and that the S-glycoprotein was the main target of neutralizing humoral responses, as has been demonstrated in multiple studies[620–622,1346,1347]. Thus, we opted for the pseudovirus-based neutralization assay in the subsequent studies for several reasons: 1) due to its versatility for adapting to new viral variants, 2) it is less laborious, 3) pseudoviruses carry a luciferase reporter gene that facilitates the detection of infection and, 4) pseudovirions only generate a single cycle of replication, therefore they do not require a high level of biosafety (e.g. BSL-3 laboratories). Despite these advantages, the pseudovirus based neutralization assay have two main limitations. First, it is not compatible with samples from HIV infected individuals, because antiretroviral drugs present in plasma or serum samples could interfere with the assay (spikes were assembled on an HIV backbone). Second, if the pseudoviral particles potentially express a large number of molecules on their surface, the sensitivity of the assay could decrease, because the amount of neutralizing antibodies would be insufficient for binding to all epitopes. This needs to be studied further and it might be necessary to optimize the assay and/or pseudovirus production conditions. Despite this potential limitation, our technique resulted to be one of the most sensitive when assessed with other European laboratories[1331].

Regarding this last point, it should be noted that neutralization results vary depending on the technique used (cell line, replicative virus or pseudovirus, assay sensitivity, etc.). Nevertheless, this validation (performed in mid-2021) allows us to convert our results to IU/ml, which offers greater data transparency, but most importantly, allows us to standardize results and compare our neutralization values with other laboratories. This is relevant considering that assay standardization is an essential aspect of assessing the immune response to SARS-CoV-2 and COVID-19 vaccines[1348]. However, external validation should be approached with caution, as it has only been done with the ancestral virus WH1 and has not been analyzed against other variants. Neutralizing activity varies widely among the different viral variants, which could affect the IU/ml conversion factor due to the intrinsic characteristics of the viral isolate and/or the presentation of the S-glycoprotein in pseudoviruses.

Finally, compliance with parameters such as accuracy, repeatability, intermediate precision, specificity, limit of quantification, linearity and robustness, allowed us to fully validate our assay and use it in preclinical and clinical trials for the PHH-1V vaccine (HIPRA)[552,1213,1349].

Once the PBNA has been optimized, we evaluated the early neutralizing humoral response to SARS-CoV-2 infection and observed that most individuals presented neutralizing antibodies during the first days of infection, reaching 50% and 80% maximal neutralizing responses between days 10-11 and 14-17 PSO, respectively (specifically analyzed in hospitalized patients). These data where confirmed in other cohorts[1350,1351] and are consistent with the time frame of seroconversion[1352–1354], suggesting that the early humoral response already involves neutralizing antibodies. This fact was independent of the COVID-19 wave analyzed—ergo irrespective of the infecting variant—and was also observed by other authors[1351,1354,1355].

Importantly, we described that a proportion of participants (most of them individuals with mild or asymptomatic disease) did not generate detectable neutralizing antibodies or their levels were very low compared to the global median. Although the minimum level of neutralizing antibodies necessary for protection against infection and reinfection remains undefined, these individuals with undetectable or low levels of neutralizing antibodies could be at higher risk of reinfection[601,645,647,1356]. However, sporadic cases of reinfection have been reported even in the presence of a high titer of neutralizing antibodies[1357,1358]. Therefore, this suggests that measuring neutralizing antibody levels alone does not provide a complete picture of an individual's immune status, and points to a relevant role of other immune mechanisms, such as non-neutralizing antibodies and cell-mediated immunity, that should be considered to assess the state of protection against SARS-CoV-2[296,1359].

The longitudinal analysis revealed mid-term (6 months) and long-term (>12 months) persistence of neutralizing activity, consistent with other studies[1360,1361] and with reports about the presence of RBD-specific memory B cells and long-lived bone marrow plasma cells after SARS-CoV-2 infection[427,459,464]. Of note, the magnitude and kinetics of neutralizing responses were different between hospitalized and non-hospitalized individuals. While the former ones presented the highest levels of neutralization, with a sharp decline at month 1 PSO, and a slower decay (flatter slope) from day 80 PSO onwards; asymptomatic or mildly symptomatic patients showed lower but stable levels of neutralizing activity over time[526,550,1362]. Notably, and supported by other studies[1362–1365], we observed no significant differences in the neutralizing response between asymptomatic and mildly ill individuals, despite a higher fraction of non-neutralizers in the group without symptomatology, reflecting the broad spectrum and heterogeneity in the nature of the neutralizing response.

The behavior of neutralizing antibodies and their durability leads us to hypothesize that long-lived plasma cells are responsible for the long-term maintenance of anti-SARS-CoV-2 neutralizing antibodies in peripheral blood[440,1366–1370]. A pattern that was proposed early on during the pandemic[1371].

In summary, our statistical models predict a half-life of neutralizing antibodies between 533 and 753 days for hospitalized patients, and nearly 6 years for asymptomatic or mildly ill individuals; however, some subjects may have different neutralization kinetics over time[1372], and accordingly different half-lives of the humoral immune response. These results are in line with the one described for other coronaviruses causing severe acute respiratory disease (SARS-CoV and MERS-CoV), for which the neutralizing activity remains detectable for several years[1312–1316], and diverge from the apparent short-lasting protective immunity against seasonal "common cold" coronaviruses[1020,1317,1318,1373], although a recent study showed certain stability of the antibody response against endemic coronaviruses[428]. The exact reasons why

some pathogens induce long-lasting immune responses while others induce short-lived responses are not fully understood. However, there are several factors that are thought to contribute to the duration and robustness of the immune response, including antigen persistence, antigen complexity, host factors, pathogen evasion strategies, etc.[1374–1376].

To have a more global view of the humoral response, we evaluated IgG antibodies against RBD, S2 subunit and NP. Interestingly, we observed a different behavior between neutralizing and specific IgG antibodies over time. This has also been observed by other research groups[502,1377,1378]. A steady longitudinal decay pattern was perceived for all binding antibodies, which contrasted with the kinetics observed for neutralizing response. This might have been expected for anti-NP IgG antibodies, which, being directed toward unexposed virion proteins, are not related to neutralizing activity; however, it was also evident in anti-RBD IgGs, the main target of SARS-CoV-2-specific neutralizing antibodies[620,622], and anti-S2, which may also contribute to neutralizing activity and is more cross-reactive with other coronaviruses[584]. These results suggest a continuous evolution of the humoral response, and that affinity maturation of anti-Spike antibodies may play a key role maintaining the neutralizing activity, despite the decrease in specific IgGs antibody titers[763,1379]. Why the immune response continues to evolve after infection remains to be elucidated. One possible explanation could be the persistence of residual SARS-CoV-2 nucleic acid and proteins in some tissues even months after resolution of symptoms[262,1379,1380], which could fuel humoral evolution. Another explanation could be due to immune complexes present in follicular dendritic cells, which retain the antigen for long periods of time and periodically display it on the cell surface promoting somatic hypermutation and affinity maturation[1381,1382].

Other authors have reported a longer half-life of antibodies[459,1377,1379] compared to our findings, which could explain the long-term stability of the neutralizing activity (because the neutralizing antibodies are a subset of the binding antibodies). These differences may be attributable to limitations in our cohort, the lack of evaluation of anti-NTD antibodies, which although less prevalent, also contribute to neutralization[584,625], the non-consideration of other immunoglobulin isotypes with neutralizing properties[526,1383,1384], or the absence of determination of total antibodies against a native trimeric conformation of S-glycoprotein, that could expose additional binding sites[1377,1385,1386].

Moreover, in the CIRCUS cohort we observed that non-early seroconverters had considerable levels of neutralizing antibodies at 60 days PSO. A more detailed analysis suggested to us that the quality of the neutralizing response was a determining factor in this regard. Thus, non-early seroconverter individuals have lower levels of specific IgG antibodies (anti-S1+S2, anti-RBD and anti-NP), but are of superior quality, especially anti-S1+S2 antibodies, compared to early seroconverters with high or low viral load. Whether this is a cause or a consequence of preexisting immunity is unknown. An effective control of infection by strong innate mechanisms or preexisting cross-reactive cellular response may limit the extent of SARS-CoV-2 replication[1387,1388], and hence antigen levels and subsequent antibody development. In contrast, the failure to control viral replication may lead to sustained B cell activation and antibody generation, resulting in increased titers of humoral responses. Cross-reactivity of SARS-CoV-2 and other common cold human coronaviruses have been described not only for cellular responses but also for antibodies[399,400,975], mainly those directed against the S2 subunit of the

S-glycoprotein[1389]. If what we report is a consequence of the latter, it leaves an outstanding open question that needs to be studied further.

Globally, all these data suggest that the binding antibodies targeting specific epitopes are not a mirror of the neutralizing response and their association may be compromised over time. Therefore, beyond the magnitude, the quality of the response must be considered. In a nutshell, while IgG binding antibodies can be a useful tool for monitoring immune responses, it is important to also consider other factors, such as neutralizing antibody levels and cellular response, when evaluating a person's immunity to SARS-CoV-2.

Considering that neutralizing antibodies are highly predictive of immune protection from SARS-CoV-2 infection[601,602], and the fact that some patients show a low neutralizing activity (mostly patients with mild or asymptomatic disease)[1365,1390], we evaluated which clinical-demographic factors might be associated with their elicitation. Despite the large heterogeneity observed in the neutralizing titers, multivariate analyses identified disease severity (hospitalized versus non-hospitalized), and age (in mild-symptomatic individuals) as factors associated with the magnitude of the neutralizing humoral response. These results were confirmed by other research groups[1365,1391–1393]. Thus, patients with severe disease (i.e. requiring hospitalization), showed higher neutralization titers compared to outpatients or asymptomatic individuals[1394–1396]. Similarly, older patients presenting with mild disease, but longer duration of symptoms showed a greater magnitude of humoral responses. This finding was also reported in other cohorts, where neutralizing anti-SARS-CoV-2 antibody levels in sera correlate positively with symptom duration[1390,1397].

Our study in patients with mild disease did not identify viral load levels as a factor influencing the generation of neutralizing antibodies[1398,1399]. These results contrast with other studies based on cohorts covering a broader spectrum of COVID-19 severity, in which viral load was associated with greater neutralizing responses[1400,1401]. Some studies indicate that viral load is associated with more severe disease[1402,1403], which could partly explain the observed differences; however, this is not always the case, and this point remain unclear[1404–1407]. In addition, other potential players, such as SARS-CoV-2 variants, may be determinant in this regard[1408,1409]. All these apparent inconsistencies point to other factors being involved—beyond the neutralizing response, such as specific T-cells, in the early control and clearance of SARS-CoV-2[342,345].

Some studies suggested that gender plays a role in the levels of SARS-CoV-2 neutralizing activity[1393,1410]. However, and according to other publications[1372,1411], in neither of our multiple analyses, did we find an association between gender and neutralizing antibody titers. This could be a consequence of limited sample size, therefore, further investigation will be needed to clarify this apparent discordance.

Lastly, we did not find any significant link between the elicitation of humoral responses and various comorbidities. However, again, the limited number of individuals with these medical conditions prevented us from conducting a formal statistical analysis, and caution is advised when interpreting this finding. It is worth noting that certain comorbidities, such as hypertension, cardiovascular and respiratory diseases, have been associated with more severe cases of COVID-19[1412]; nevertheless, our study included only asymptomatic or mild symptomatic

cases, explaining the fact that no discernible differences were observed between groups with and without underlying medical conditions.

Our results may shed light on the different neutralizing responses. Hospitalized patients take longer to recover[1390,1397]. As a result, the immune system is longer in contact with the virus, which could lead to a greater magnitude and breadth of the immune response. This in turn would be a logical reasoning to explain the antibody kinetics between hospitalized and non-hospitalized individuals. Hospitalized patients generate a robust extrafollicular B cell polyclonal response with higher titers of neutralizing antibodies[443] that, after successive maturation processes, leads to a decrease in total levels to maintain only the neutralizing antibodies of higher affinity over time. For this reason, it has been observed that antibodies expressed from specific memory B cells with higher somatic hypermutation exhibit stronger antigen binding, potency and breadth of neutralization compared to antibodies obtained from memory B cells at earlier time points[464,1379,1413,1414]. Based on our findings, it appears that a specific threshold of disease severity and/or duration of symptoms may be necessary to produce a more effectively neutralizing anti-SARS-CoV-2 response.

The emergence of highly transmissible and immune-evading variants posed a challenge to the immunity produced by previous infection, prompting us to recognize the importance of assessing the potential for cross-neutralization.

The fact that the neutralization assay developed is based on pseudoviruses, allowed us to adapt it to the new viral variants. We did this during the first months of the pandemic in Spain, when the D614G mutation (more transmissible than the ancestral virus) in the S-glycoprotein started to become predominant in Europe; and later with the emergence of the Alpha variant (the first variant of concern, more transmissible and resistant to antibodies than the previous ones). In our initial analyses, we observed that the D614G mutation is more sensitive to neutralization in vaccinated and infected individuals compared to the ancestral WH1 virus. This is in line with the fact that the substitution of aspartic acid by glycine at residue 614 confers to the SARS-CoV-2 spike protein a more open configuration that better exposes the RBD[699,705–708], which is the immunodominant region of the S-glycoprotein[620,622].

The Alpha variant was modestly more resistant to neutralization than WH1, as indicated by other studies[1415–1417], particularly in vaccinated participants, but it was not evident in infected individuals. These subtle differences suggest that cross-neutralization elicited by infection is potentially better than that induced by vaccination (2 doses), although the size of the cohort prevents us from drawing a clear conclusion.

The global analysis showed that the neutralization levels decreased against all viruses tested (WH1, D614G and Alpha) in samples collected 6 months PSO, in line with other reports[464,1418,1419]. Considering that almost 70% of the cohort required COVID-19 hospitalization, these results are expected since we compared samples at 1 month (peak of neutralizing activity) vs samples at month 6 (phase of relative stabilization, after the decrease in the levels of neutralizing antibodies generated during the acute phase). However, the cross-neutralization against D614G and Alpha relative to WH1 was improved in late sampling in comparison to early sampling, suggesting an evolution in humoral response over time[529,1379].

Individuals infected during the third COVID-19 wave in Spain tended to show higher neutralizing titers against D614G and the Alpha variant, compared to those infected during the first (WH1+D614G) and second waves (20E variant). This finding is in line with what is expected, as individuals who have been infected with a particular variant are likely to have developed a strong immune response specific to that variant. The fact that Alpha variant contain the D614G mutation helps to explain this behavior.

When we evaluated the long-term cross-neutralization capacity against several variants of concern (Alpha, Beta and Delta), we observed that hospitalized individuals showed higher levels of neutralization against each variant, but lower cross-neutralization than non-hospitalized individuals. This findings extend previous information that has related disease severity to the magnitude of early neutralizing responses[1362,1365,1394] by demonstrating that this association may also apply to long-term responses, but also suggest that there may be a discordant relationship between the quantity and quality of antibodies in hospitalized patients.

By contrast, asymptomatic or mildly symptomatic individuals develop fewer antibodies, but are of better quality (in terms of cross-neutralization) compared to patients who experienced more severe COVID-19. These results again pointed out the potential role of immune response evolution[529,1379]. Further investigation is required to gain a better understanding of the unclear reasons behind this differential cross-neutralization profile as a function of disease severity.

The rapid development of vaccines with high protective efficacy against severe COVID-19 gave hope of controlling the pandemic[1420–1423]. However, there are at least two key factors that limit the effectiveness of vaccines: the emergence of immune-resistant viral variants and the decline of neutralizing antibodies over time. For this reason, we analyzed the impact of COVID-19 vaccination on the neutralizing response. In hospitalized and non-hospitalized patients who already had an immune response induced by natural infection; we observed, similar to other reports[464,1424], that vaccinated persons boosted the pre-existing neutralizing response, independent of the COVID-19 clinical outcome, which could lead to long-term protection[550,1425]. But this information must be interpreted carefully since new emerging variants of the virus could escape both natural and vaccine-induced immunity[1426].

To evaluate cross-neutralization of variants, we considered different immunization profiles: initial vaccination schedule (2 doses of mRNA vaccine), hybrid immunity due to previous infection, booster shot, and hybrid immunity due to breakthrough infection and/or previous infection (considering 3 doses of mRNA vaccine).

In the first study, we found that vaccinated previously infected subjects showed a better neutralization against the Alpha variant compared to both only vaccinated with early sampling and only infected with late sampling, demonstrating that vaccination boost the response offered by the pre-existing natural immunity[578,1307,1427], even against antigenically distinct variants to which individuals have not been exposed (infection occurred during the first COVID-19 wave in Spain with the prevalence of the ancestral WH1 and D614G virus, and the first mRNA vaccines were based on the original WH1 strain). Cross-neutralization (considering D614G and the Alpha variant) achieved by hybrid immunity was comparable to that generated by infection alone and better than that induced by vaccination; but the vaccination in previously infected individuals restored neutralization titers obtained by natural infection that were

decreasing over time. These data suggest that vaccination restores the magnitude and quality of the neutralizing response.

In our most recent study, the findings are consistent with other independent publications[578,1428]. Hybrid immunity confers higher levels of neutralizing antibodies and better cross-neutralization than the two-dose vaccination schedule, which may result in increased protection from SARS-CoV-2 infection[1429–1431]. This was not as evident in our first study of infected and/or vaccinated individuals, probably because the Alpha variant is less resistant to neutralization than the Delta and Omicron variants[757,762,1432–1434].

Additionally, we observed that the booster dose broadened the cross-neutralizing response (Delta, BA.1, BA.2 y BA.4/5) in individuals not previously infected, at similar levels and quality achieved by the hybrid immunity; this was also confirmed by other authors[555,628], even when considering different age groups and environmental virological pressure[1435,1436]. This indicates that a re-exposure to the same ancestral antigen has a positive effect not only on the magnitude of the neutralizing response, but also impacts on its ability to act against new variants, possibly due to the reactivation, expansion, and evolution of specific memory B cells[1437,1438].

Finally, in line with other studies[1439–1441], the effect of Omicron BA.1 and BA.2 breakthrough infection—in individuals boosted with a third vaccine dose previously infected or uninfected—tended to improve cross-neutralization against Omicron subvariants (BA.1, BA.2, and BA.4/5), however, this same effect was seen in persons previously infected with 3 doses of vaccine, who did not experience post-vaccination reinfection. This data might suggest that, at least in the short term, four antigenic exposures lead to an adequate cross-neutralizing response even against the Omicron subvariants without previous exposure to any of them. However, it is important to note that, aside from the marked antigenic differences, especially in RBD[768,1442], between Omicron and earlier variants, the apparent limited impact of breakthrough Omicron infections on the neutralizing response could be due to intrinsic viral factors of this variant of concern (such as tropism and fusogenicity)[833,1443] or by the timing and sequence of antigenic exposures. Specifically, the median interval between pre-vaccination infections and vaccination was 277 days, whereas only a median of 39 days separated booster vaccination and breakthrough infection. Timing between infection and vaccination is likely to be a crucial determinant on the extent of boosting of humoral responses, with shorter intervals resulting in lower suboptimal boost[1444,1445].

In summary, these data suggest that multiple re-exposures contribute to shape a broad neutralizing humoral response and highlights that ancestral spike-based immunization remains a valuable strategy to improve and maintain protection against SARS-CoV-2 and limit transmission globally. Remarkably, the number of immunological exposures to the spike, rather than the specific sequence, may be a major determinant of VOC cross-neutralization of humoral immune response. However, this behavior should be studied more thoroughly and longitudinally to see the possible implications for new generations of bivalent vaccines and to establish suitable vaccination schedules in persons at higher risk.

Overall, the impact of COVID-19 vaccination, hybrid immunity, booster dose, and breakthrough infections on cross-neutralization is complex and may vary depending on several factors, including the type of vaccine, the timing between infection and vaccination, infecting viral variant, and host immune system. Further research is needed to better understand the extent and duration of cross-neutralization in different populations and under different conditions. However, in light of our findings, we conclude that there must be a balance between the duration, magnitude, and quality of cross-neutralization.

Value of the study

The humoral immune response, collectively with innate immunity and cell-mediated responses, are key players in the control and protection from SARS-CoV-2 infection. Antibodies are important in several aspects related to immunity. They are useful as a method for indirect detection of active or past infection by serological assay, they are the basis for antigen tests, they are used for seroprevalence and surveillance studies, they are predictors of protection from infectious diseases and therefore their generation is one of the main goals of viral vaccines, and they are used as therapeutic and/or preventive agents.

Analysis of the longitudinal behavior of neutralizing antibodies induced by natural infection, the impact of vaccination, hybrid immunity, and breakthrough infections, the cross-reactivity against variants of concern, and the factors associated with the magnitude of neutralizing response, are one of the cornerstones that will inform us about the current immunological context and would provide us with a solid basis on which to establish future immunization schedules or new vaccination strategies.

At the beginning of the COVID-19 pandemic, knowledge about the kinetics and nature of immune response to SARS-CoV-2 infection was limited. Early perspectives were based on previous knowledge about SARS-CoV, MERS-CoV, and seasonal coronaviruses[1373,1446–1450], but there was no certainty that the immune response to the new coronavirus could be similar.

Additionally, the first authorized COVID-19 vaccines were based on mRNA technology that had never been used on a large scale, so understanding the response generated after immunization with this and other vaccine platforms was—and remains—vital to define possible pandemic scenarios.

The emergence of variants was another major cause for concern. The new viral variants were more transmissible and challenged the immunity acquired by infection or induced by vaccination, which led to different waves, reinfections, and the maintenance of public health measures, such as the use of masks and COVID-19 vaccination certificates.

All of the above prompted us to act immediately and evaluate the main—but not exclusive—correlate of protection, anti-SARS-CoV-2 neutralizing antibodies.

Longitudinally monitoring the neutralizing response over time is important because it provides insight into the duration of protection and lays the groundwork for determining public

health policies and whether a booster dose or a different vaccination schedule is needed to keep us protected.

The large KING cohort analyzed, the follow-up time, the consideration of different epidemic waves in Spain dominated by different viral variants, and the broad spectrum of clinical disease presentation (from asymptomatic to patients requiring intensive care), allowed us to model an accurate kinetics of the neutralizing humoral responses, complementing other studies.

Assessing neutralizing response and cross-neutralization from natural infection, vaccines, booster dose and hybrid immunity contribute to explain the current immunological status of the population; however, the immunological context is continuously changing due to new vaccine doses, the emergence of viral variants and repeated exposures in different numbers, nature and time frame. These factors can have a significant impact on the immune response and how effectively the body can protect itself against the virus. Therefore, it is important to continuously monitor and adapt to these changes to maintain effective protection against COVID-19.

Limitations of the study

Our analyses are mainly limited by a lack of consideration of spike-specific B cells and cell-mediated response, which has also been shown to play a central role in the severity of the disease, preventing complications after reinfections, and in the protection from SARS-CoV-2. In addition, some analyses were not statistically powerful, especially due to the reduced sample size. However, other analyses, such as the modeling of the kinetics of antibodies to natural infection, were performed on the basis of a substantial dataset.

It is worth noting that the lower (ID_{50}<60) and upper (ID_{50}>14,580) limits of quantitation established in our PBNA could restrict our results; however, although this possibility exists, the probability is very low due to the small amount of data that reach these limit values. Additionally, in the cases where they probably could have influenced, these data were treated as censored values in the statistical analyses, and it has been explicitly indicated in the different published studies described in this thesis.

Another possible limitation is the fact that most of the cohort analyzed has been vaccinated mainly with COVID-19 mRNA vaccines, which may limit the extrapolation of our results and conclusions to individuals who have received vaccines based on other technologies. It is worth adding that our studies were conducted in Catalonia (Spain), whether the full findings can be replicated in regions where other variants were temporally prevalent (e.g., South Africa, Brazil, China) remains an open question.

Finally, caution should be exercised in interpreting the results and avoid extrapolating them to other population groups with different clinical and demographic characteristics. For example, individuals with a significant number of comorbidities and risk factors, immunosuppressed patients (chemotherapy, anti-CD20, HIV, transplanted patients, etc.) or with immune

pathologies, at very early or elderly ages, with a different genetic imprint, subjected to different virological pressure, etc. These and other potential caveats should be borne in mind.

Future perspectives and open questions

Our analyses have provided valuable insights into the neutralizing response acquired through SARS-CoV-2 infection. However, several important questions and future directions remain to be addressed. One key aspect is determining the actual duration of this neutralizing response and understanding how it may be impacted by new emerging viral subvariants (e.g., BQ.1, XBB.1.5, XBB.1.16).

Additionally, we have explored the impact of vaccination, including two doses and the first booster dose, as well as hybrid immunity resulting from prior and/or breakthrough infections, on cross-neutralization. However, further investigations are needed to better understand the long-term effects and durability of these immune responses. Moreover, in the current immunization context, evaluating the neutralization profile after a second booster dose and the impact of bivalent or other variant-based vaccines (e.g., PHH-1V vaccine) compared to monovalent ones, is a fundamental point to address.

Given the diverse immunization profiles within our large cohort, we plan to continue our studies to assess the mid- and long-term neutralizing response, considering various antigenic exposures in terms of nature and quantity. This will enable us to evaluate the influence of new variants/subvariants on the cross-neutralizing response over time. The continuation of our prospective cohort, consisting of individuals who have recovered from SARS-CoV-2 infection and/or received vaccination, will provide valuable insights into the long-term kinetics of the neutralizing humoral response.

Furthermore, we are committed to deepening our collaborations and expanding our ongoing projects, including surveillance studies, vaccine development, and monoclonal antibody research.

Overall, our future research endeavors aim to address these open questions, expand our knowledge about SARS-CoV-2 immune responses, and contribute to the global efforts in the fight against COVID-19. By pursuing these avenues, we hope to provide useful data that will inform public health policies and build a precedent to mitigate the risk of future outbreaks, guiding vaccination and therapeutics strategies to optimize immune protection in the face of evolving viral dynamics and for the betterment of human health worldwide.

5

CONCLUDING REMARKS

Conclusions

Objective 1: To develop a SARS-CoV-2 pseudovirus-based neutralization assay.

1.1 Our laboratory successfully developed a highly sensitive, consistent, reliable and reproducible pseudovirus-based neutralization assay to evaluate the levels of neutralizing anti-SARS-CoV-2 antibodies.
1.2 Our PBNA was clinically validated (EMA), making it a useful tool for characterizing the neutralizing humoral response in observational and clinical trials, including those involving the PHH-1V vaccine (HIPRA).

Objective 2: To longitudinally evaluate the SARS-CoV-2 neutralizing humoral response induced by vaccination, infection, and the combination of both events.

2.1 SARS-CoV-2 infection induces a rapid and stable neutralizing response (beyond 1 year), with high interindividual heterogeneity.
2.2 Short, mid, and long-term levels of neutralizing antibodies are associated with COVID-19 severity.
2.3 COVID-19 vaccines (2 doses) induce neutralizing antibodies, that decay faster after 6 months in uninfected compared to infected individuals (hybrid immunity).

Objective 3: To identify the factors associated to the magnitude of the neutralizing response after infection.

3.1 In outpatients, the magnitude of the neutralizing response is associated with duration of symptoms and age. Gender and viral load do not appear to be relevant factors.
3.2 Severity of infection determines the magnitude and quality of the neutralizing humoral response.
3.3 Hybrid immunity (including Omicron breakthrough infection) boosts the magnitude of the neutralizing antibody-mediated response.

Objective 4: To evaluate the cross-neutralizing responses against different SARS-CoV-2 variants.

4.1 SARS-CoV-2 infection induces a limited long-term cross-neutralizing response associated with disease severity.
4.2 Time from infection and nature of the infecting variant determined cross-neutralization.
4.3 Two doses of mRNA COVID-19 vaccines induce a poor cross-neutralizing response in uninfected individuals.
4.4 The third dose of vaccine and hybrid immunity have a positive impact on cross-neutralization.

Final thoughts

Since the discovery of those microscopic beings that caused unexplained diseases, and more specifically, at the end of the 19th century, when viruses were discovered[1451], science has advanced relentlessly and by leaps and bounds. The most obvious proof is the enormous and practically inexhaustible scientific knowledge generated around SARS-CoV-2 in a short period of time. For example, it took 2 years from the first reports of AIDS to the discovery of HIV, by comparison, it took only 2 weeks to identify and sequence the causative agent of COVID-19, which would become the worst pandemic in recent times.

The COVID-19 pandemic has left an everlasting mark on humanity, having a great impact on our lives. SARS-CoV-2 paralyzed our society and showed us how fragile we are to new threats. However, it had a positive impact on science that was reflected in the collaboration, dissemination, quick response, economic support, and in the enormous number of people who volunteered for the different clinical trials and observational studies.

Thanks to an unprecedented international collaboration, as a society we were able to provide an effective response to the pandemic that, despite the obstacles and its consequences (health, social, economic, scientific, etc.), can be considered successful, and as a result, today humanity has returned to the so-called "new normality", with many lessons learned during these years. However, there are still many pending issues that need to be addressed in a better way, such as vaccination hesitancy, misinformation, inequity in access to prevention measures, therapies and vaccines, stigmas and lack of empathy and responsibility towards others.

Understanding the origins, epidemiology, virology, molecular biology, physiopathology, and immunology around SARS-CoV-2 are fundamental aspects that must be addressed in order to be better prepared for (or prevent) future pandemics. Early detection, rapid response, collaboration, research and development, communication, and transparency are critical factors in controlling the spread of infectious diseases and reducing their impact on society.

During these three years, our scientific contribution was focused on evaluating the neutralizing antibody-mediated response (a surrogate of protection) acquired by SARS-CoV-2 infection and induced by COVID-19 vaccination. We have seen the great heterogeneity in the immune response and how it is affected over time and by new viral variants, as well as the positive effect that vaccines have on it, both in magnitude and quality. These results support the call to get vaccinated, which is not only important for individual health but also for the health of the community as a whole.

Finally, I would like to say that we have witnessed the fundamental role of science in society. Although science does not always provide definitive answers and sometimes it can be wrong, it continually seeks to expand our understanding and shed light on the mysteries that lie ahead. The pursuit of knowledge and comprehension is essential for progress. At the end of the day, we must reflect on this pandemic, on what we did right, what we did wrong, what we could have done and did not do, what we should do today and tomorrow, on our limitations and our achievements. It is through this introspection that we can build a stronger society and a better future, one that is more resilient and prepared to face adversities such as the COVID-19 pandemic. **Trust in science!**

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RESOURCES AND TERMINOLOGY

Abbreviations and Acronyms

aa	Amino acids
ACE2	Angiotensin-Converting Enzyme 2
ADCC	Antibody-Dependent Cellular Cytotoxicity
ADCD	Antibody-Dependent Complement Deposition
ADCP	Antibody-Dependent Cellular Phagocytosis
ADE	Antibody-Dependent Enhancement
APC	Antigen-Presenting Cell
ARDS	Acute Respiratory Distress Syndrome
BCR	B-Cell Receptor
bNAbs	Broadly Neutralizing Antibodies
CCR	CC Chemokine Receptor
CD	Connecting Domain
CDC	U.S. Centers for Disease Control and Prevention
CDR	Complementarity-Determining Region
CFR	Case Fatality Rate
CH	Central Helix
COVID-19	Coronavirus Disease 2019
CROI	Conference on Retroviruses and Opportunistic Infections
CT	Cytoplasmatic Stalk
CTD	Carboxy-Terminal Domain
DC	Dendritic Cell
E	Envelope
ECDC	European Centre for Disease Prevention and Control
EMA	European Medicines Agency
ER	Endoplasmic Reticulum

ERGIC	Endoplasmic Reticulum–Golgi Intermediate Compartment
F_{ab}	Fragment Antigen-binding
F_c	Fragment Crystallizable
F_cR	Fc Receptor
FDA	U.S. Food and Drug Administration
FP	Fusion Peptide
GMT	Geometric Mean Titer
hACE2	Human ACE2
HEK293T/hACE2	Human Embryonic Kidney 293T cells ACE2-overexpressing
hIG	Hyperimmune Immunoglobulin
HIV	Human Immunodeficiency Virus
HR	Heptad Repeat
IC_{50}	Half Maximal Inhibitory Concentration
ICU	Intensive Care Unit
ID_{50}	Half Maximal Inhibitory Dilution
IDSA	Infectious Diseases Society of America
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISG	Interferon-Stimulated Gene
k	Dispersion parameter
M	Membrane
mAb	Monoclonal Antibody
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
MHC	Major Histocompatibility Complex
MIS-C	Multisystem Inflammatory Syndrome in Children
mRNA	Messenger Ribonucleic Acid
N	Nucleocapsid

NAbs	Neutralizing Antibodies
NIBSC	U.K. National Institute for Biological Standards and Control
NIH	U.S. National Institutes of Health
NK	Natural Killer
NMPA	National Medical Products Administration (China)
NP	Nucleoprotein
NSP	Non-Structural Protein
NTD	N (amino)-Terminal Domain
ORF	Open Reading Frame
PAHO	Pan American Health Organization
PBNA	Pseudovirus-Based Neutralization Assay
PHEIC	Public Health Emergency of International Concern
PSO	Post-Symptom Onset
R_0	Basic Reproduction Number
RBD	Receptor Binding Domain
RBM	Receptor Binding Motif
R_e	Effective Reproduction Number
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
S	Spike
S1 and **S2**	Subunit 1 and 2
SARS-CoV(-1)	Severe Acute Respiratory Syndrome Coronavirus (1)
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
T_{FH}	T Follicular Helper
TM	Transmembrane Domain
TMPRSS2	Transmembrane Serine Protease 2
TNF	Tumor Necrosis Factor
VNA	Virus Neutralization Assay

VOC	Variant of Concern
VOI	Variant of Interest
VSV-G	Vesicular Stomatitis Virus Glycoprotein
VUM	Variant Under Monitoring
WH1	Wuhan-Hu-1 virus
WHO	World Health Organization

Glossary

Amino acid substitution: a change in a specific amino acid of a protein. This is caused by non-synonymous mutations. To indicate a substitution of this type, conventional notation is usually used. For example: N501Y, where "N" denotes the wild-type amino acid (asparagine), "501" refers to the position of the amino acid in the protein sequence, and "Y" represents the substituted amino acid (tyrosine).

Ancestral virus: the original form of SARS-CoV-2 identified in December 2019; often referred to as the “original” or “Wuhan” virus.

Asymptomatic: having no signs or symptoms of disease.

Basic reproduction number (R_0): the average number of secondary infections caused by a single infectious individual introduced into a completely susceptible population. R_0 is defined in the absence of countermeasures and immunity.

Booster dose: dose given after a previous vaccination. A booster reactivates pre-existing immunity and helps maintain or increase a protective immune response.

Breakthrough infection: infection following vaccination against a specific infectious agent.

Case fatality rate: proportion of fatalities out of total diagnosed cases in a given period.

Cluster: a group of cases of a relatively rare event in a circumscribed area or period in an amount that is perceived or assumed to be greater than would be expected by chance.

Comorbidity: the condition of having two or more diseases at the same time.

Contagious period: the period during which an infectious agent can be transferred, directly or indirectly, from one person to another, or from an infected animal to a human being, or from an infected person to an animal, including arthropods.

Cross-neutralization: ability to neutralize variants of the same virus or other related or unrelated viruses.

Dispersion parameter (k): a useful measure to describe how the number of infections generated by an individual is distributed around the mean. Lower values of k correspond to a broader distribution.

Effective reproduction number (R_e): also known as R_t (net reproductive number), is the average number of secondary infections generated by a single infectious individual over an infectious period in a partially immune population. Unlike R_0, R_e does not assume a completely susceptible population and, consequently, will vary depending on a population’s current immune state.

Emergency use authorization: an authorization granted by drug regulatory agencies during a public health emergency that allows for the use of a drug or other medical product prior to its full approval.

Emerging disease: an unknown or newly appeared disease, usually of the infectious or communicable type.

Endemic: constant presence and/or usual prevalence of a disease or infectious agent in a population within a geographic area.

Epidemic: an unusual increase in the number of cases of a given disease in a specific population during a particular period. In general, an epidemic can be considered to be the simultaneous consolidation of multiple outbreaks over a wide geographical area.

Flattening the curve: a term used to describe a strategy aimed at slowing the spread of a pandemic disease so that the healthcare system is not overwhelmed with an excessive number of cases. This strategy involves implementing public health measures to reduce the number of new cases, thus enabling the health system to manage the situation more effectively.

Generation time: the time between the infection of a primary case and one of its secondary cases is called a generation time.

Glycan shielding: the process by which a virus can cloak underlying protein, impeding antibody binding. This is mediated by glycans, bulky sugar molecules that are covalently attached to amino acid side chains of the viral protein.

Glycoprotein: a protein with oligosaccharide chains (glycans) covalently attached to amino acid side chains. Virus surface glycoproteins embedded in the membrane often have a role in interactions with host cells, including receptor binding, and are also commonly targeted by host antibodies.

Half maximal inhibitory concentration (IC_{50}): represents the concentration of a drug or compound required to inhibit 50% of a given biological activity or response, e.g., the amount of antibody required to reduce 50% of viral infectivity. A lower IC_{50} value corresponds to a higher level of neutralizing activity.

Half maximal inhibitory dilution (ID_{50}): not to be confused with median infective dose. Represents the dilution of an analyte or sample to inhibit 50% of a given biological activity or response, e.g., the dilution of a serum or plasma sample required to reduce 50% of viral infectivity. A higher ID_{50} reflects better neutralization.

Half-life: is the time required for the quantity of a substance to be reduced to half of its initial value.

Health: state of complete physical, mental and social well-being and not merely the absence of disease or infirmity.

Herd immunity: the indirect protection from infection conferred to susceptible individuals when a sufficiently large proportion of immune individuals exist in a population.

Hybrid immunity: immune protection in individuals who have received one or more doses of a vaccine and experienced at least one infection before or after the initiation of vaccination.

Immune escape: the ability of a virus to partially or fully evade immune recognition or neutralization.

Immunodominance: the phenomenon by which the host immune response against a viral particle is mostly focused on a few antigens and mediated by potently neutralizing antibodies.

Incidence: the number of new cases of a disease in a population in a given period.

Incubation period: the period of time from initial exposure to an infectious agent to the onset of signs or symptoms of the disease it produces.

Infection fatality rate: proportion of fatalities out of total infections, including all asymptomatic and undiagnosed cases.

Infectious period: period of time during which a person can transmit a disease.

Infectivity: ability of an infectious agent to survive and multiply in a host.

Isolation: physical separation of infected individuals from healthy individuals.

Lineage: group of closely related viruses with a common ancestor.

Lockdown: intervention applied at the community level when other health measures have been insufficient to contain the spread of an infectious disease.

Long COVID: continuation or development of new symptoms 3 months after the initial SARS-CoV-2 infection, with these symptoms lasting for at least 2 months with and cannot be explained by an alternative diagnosis.

Monoclonal antibody: antibody recognizing a single epitope on an antigen. It is produced artificially from a single cell clone and therefore consists of a single type of immunoglobulin.

Mortality rate: percentage of people in a population who die out of the total population.

Mutation: mutation refers to the substitutions, insertions or deletions of one or more nucleotides in the virus RNA genome. Non-synonymous nucleotide substitutions in protein-coding sequence result in a change in amino acid (referred to as a substitution or replacement), whereas synonymous nucleotide substitutions do not change the amino acid.

Mutation rate: the intrinsic rate at which genetic changes emerge per replication cycle, a biochemical property determined by the replication fidelity of a virus' polymerase enzyme.

Natural history of disease: the course of a disease in a person from onset to resolution in the absence of any mediating interventions.

Neutralization assay: *in vitro* assay useful for assessing neutralizing activity in human or animal samples and antibodies.

Neutralizing antibodies: antibodies that interfere with the ability of a virus to infect a cell.

Non-neutralizing antibodies: antibodies that bind to viral antigens but do not impede the ability of the virus to infect a cell.

Outbreak: two or more cases linked epidemiologically to one another. The existence of a single case under surveillance in an area where the disease did not exist is also considered an outbreak. An outbreak occurs when there is an unusual increase in the number of cases of a disease beyond what would normally occur.

Pandemic: disease that experiences an exponential growth of cases, and spreads over a sprawling region, affecting several countries across multiple continents or worldwide.

Physical distancing: prevention method to slow the person-to-person transmission of the disease. Distancing needs to be physical, but not necessarily social, as people can use technology to continue to socialize.

Preprint: version of a manuscript that precedes publication in a peer-reviewed journal.

Prevalence: the total number of people who have a disease (new and existing cases) in a population or in a given place at a given time.

Protective immunity: the relative ability to resist infection or reinfection or attenuate an infectious disease or its clinical presentation.

Pseudoviruses: laboratory tools used to study and compare viral entry processes and their inhibition. Pseudovirus particles are formed by the structural proteins of safe, non-replicative forms of viruses coated (pseudotyped) with the entry glycoprotein of a heterologous virus.

Quarantine: restriction of movement of individuals who have been exposed to potential contagion and who are possibly infected.

Randomized controlled trial: a study in which the participants are divided by chance into separate groups (experimental and control) that compare different treatments or other interventions.

Recombination: the combining of genetic material from two different viruses during replication, producing an offspring virus carrying a portion of the genetic material from either parent.

Reemerging disease: resurgence or increase in the incidence of infectious or communicable diseases that were considered to be already under control.

Reinfection: infection by the same pathogen, after recovery from or during the course of a primary infection.

Reservoir: any person, animal, arthropod, plant, soil, or substance, or combination of these in which an infectious agent normally lives and multiplies, on which it depends for its survival, and where it reproduces itself in such a manner that it can be transmitted to a susceptible host.

Seroconversion: change from negative to positive specific antibodies in response to an antigen.

Sterilizing immunity: a unique immune status, which prevents effective virus infection into the host.

Sublineage: a term used to define a lineage as it relates to being a direct descendent of a parent lineage. For example, BA.2 is an Omicron sublineage. This term is sometimes used interchangeably with "subvariant".

Substitution rate: the rate at which new mutations accumulate in a viral population, usually measured per nucleotide site per year (also known as evolutionary rate).

Subvariant: a subgroup of variants categorized based on genomic changes that they have in common and the time frame in which those changes appeared in tandem. This term is sometimes used interchangeably with "sublineage".

Superspreading event: is an event in which an infectious disease is spread much more than usual, due to one or a few individuals infecting a large number of other people.

Transmissibility: ability or potential of an infectious agent to be transmitted from one organism to another. Besides intrinsic transmissibility of the pathogen, this includes immune evasion as well as temporal components such as duration and time of onset of infectiousness.

Vaccine effectiveness: the ability of a vaccine to prevent outcomes of a disease in the "real world", not just under optimal conditions. In other words, it measures how well the vaccine works in practice.

Vaccine efficacy: the percentage reduction of disease in a vaccinated group of people compared to an unvaccinated group under the most favorable conditions or ideal circumstances. For example, in randomized controlled trials.

Vaccine hesitancy: refers to delay in acceptance or refusal of vaccination despite availability of vaccination services and supporting evidence.

Vaccine nationalism: is an economic strategy to hoard vaccinations from manufacturers and increase supply in their own country. It occurs when governments sign deals with pharmaceutical companies for the supply of vaccines for their own citizens and prioritizing the same before that of other countries.

Variant: virus that has mutations due to changes in the genetic sequence with respect to its ancestral virus.

Variant of concern: variant that meets the definition of a VOI (see below) and, through a risk assessment, conducted by WHO TAG-VE, and determined to be associated with a moderate or high level of confidence, meets at least one of the following criteria when compared with other variants: detrimental change in clinical disease severity; or change in COVID-19 epidemiology causing substantial impact on the ability of health systems to provide care to patients with COVID-19 or other illnesses and therefore requiring major public health interventions; or significant decrease in the effectiveness of available vaccines in protecting against severe disease.

Variant of interest: variant with genetic changes that are predicted or known to affect virus characteristics such as transmissibility, virulence, antibody evasion, susceptibility to therapeutics and detectability; and identified to have a growth advantage over other circulating variants in more than one WHO region with increasing relative prevalence alongside increasing number of cases over time, or other apparent epidemiological impacts to suggest an emerging risk to global public health.

Variant under monitoring: variant with genetic changes that are suspected to affect virus characteristics and early signals of growth advantage relative to other circulating variants (e.g. growth advantage which can occur globally or in only one WHO region), but for which evidence of phenotypic or epidemiological impact remains unclear, requiring enhanced monitoring and reassessment pending new evidence.

Viral fitness: it is a complex parameter aimed at describing the replicative adaptability of a virus in a given environment, referred to more specifically as replicative fitness.

Virulence: the ability of an infectious agent to produce severe and fatal cases. The measure of virulence is the ratio of the number of severe and fatal cases to total overt cases.

Zoonosis: any disease or infection that is naturally transmissible between animals and humans via direct or indirect contacts.

Note: *the terms have been described in the context of this thesis and do not necessarily represent an extended definition of themselves. Some of the terms listed are not explicitly mentioned in the main text of the thesis, but they may prove helpful in comprehending other related concepts.*

Source: *definitions were obtained integrally and/or adapted from official sources (PAHO, WHO, CDC, ECDC, IDSA) and from the scientific literature cited in this thesis.*

Recommended web resources

https://search.bvsalud.org/global-literature-on-novel-coronavirus-2019-ncov

https://www.ncbi.nlm.nih.gov/research/coronavirus

https://outbreak.info

https://viralzone.expasy.org

https://covariants.org

https://cov-lineages.org

https://ourworldindata.org

https://covid19.who.int

https://www.ecdc.europa.eu/en/covid-19

https://www.cdc.gov/coronavirus/2019-ncov

https://covid.cdc.gov/covid-data-tracker

https://worldhealthorg.shinyapps.io/euro-covid19

https://sivic.salut.gencat.cat

http://covidtag.paseq.org

https://coronavirus.jhu.edu/map.html (website archived and no longer updated)

https://www.nytimes.com/interactive/2021/world/covid-cases.html and its related websites (website archived and no longer updated)

https://www.covid19treatmentguidelines.nih.gov

https://www.idsociety.org/practice-guideline/covid-19-guideline-treatment-and-management

https://app.magicapp.org/#/guideline/nBkO1E

https://covid19.seimc.org

https://euclinicaltrials.eu

https://www.clinicaltrials.gov

https://trialsearch.who.int

https://www.who.int/activities/tracking-SARS-CoV-2-variants

https://www.finddx.org/covid-19

https://covid-19-diagnostics.jrc.ec.europa.eu

https://www.covid19-druginteractions.org

https://serotracker.com/en/unity

https://cov-spectrum.org

https://opendata.ncats.nih.gov/covid19

https://github.com/MurrellGroup/lineages

https://gisaid.org

https://nextstrain.org

https://jbloomlab.github.io/SARS2_RBD_Ab_escape_maps and https://jbloom-lab.github.io/SARS2-RBD-escape-calc and https://jbloomlab.github.io/SARS2-mut-fitness

http://metrics.covid19-analysis.org

https://covdb.stanford.edu

https://sars2.cvr.gla.ac.uk/cog-uk

https://cov.lanl.gov/content/index

https://coronavirus3d.org

https://www.cdc.gov/vaccines/acip/index.html

https://www.healthdata.org/covid and https://covid19.healthdata.org/global

https://www.isciii.es/QueHacemos/Servicios/VigilanciaSaludPublicaRENAVE/EnfermedadesTransmisibles/Paginas/InformesCOVID-19.aspx

https://www.covid19dataportal.org/

https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/conditionsanddiseases

https://www.idsociety.org/covid-19-real-time-learning-network/

Note: *the websites indicated were consulted during the writing of this thesis but some of them were not explicitly included in the final content. These web bookmarks reflect only my own recommendations and were not listed in any priority order.*

Disclaimer

The data and information contained in this thesis does not represent a systematic review, and it was written with the purpose of being submitted to the University of Barcelona to qualify for the academic degree of Doctor in Biomedicine. The studies cited in this thesis, constitute just 0.4% of the COVID-19 publications included in the LitCovid database and 0.15% of the WHO COVID-19 research database.

All the results included in this thesis have been published in scientific journals after formal peer review process.

The data indicated correspond mostly to those published most recently at the date of writing this manuscript; however, during the time of writing, some web pages were no longer updated periodically, therefore the information may not correspond to the date of availability of the thesis. In addition, the data and web references indicated here may vary over time and some figures are only estimates and do not necessarily reflect the current reality. Medicine is a field in constant change and development, and data can quickly become obsolete and change radically. Many data provided are from analyses performed by the authors of the studies and are generic (unless otherwise specified), therefore, some data such as incubation period, serial interval, basic reproduction number, etc. may vary significantly between different SARS-CoV-2 variants/subvariants.

The treatments and therapies section is purely informative and has been written based on information available online (treatment guidelines from different agencies), published studies (including preprints), and results presented at CROI 2023. It has not been reviewed by an expert panel and is therefore not intended to influence COVID-19 treatments. It is strongly recommended to consult updated treatment guidelines suggested and/or applied by the health authorities in the areas under their jurisdiction and follow local standards of care.

Although few references hosted on preprint servers have been used, the methodology, results, and conclusions of the authors should be interpreted with caution, pending formal peer review.

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El mundo parecía amenazante hasta que suave como pluma clara,
o dulce como pétalo de azúcar,
de labio en labio pasa gracias,
grandes a plena boca o susurrantes,
apenas murmulladas,
y el ser volvió a ser hombre y no ventana,
alguna claridad entró en el bosque,
fue posible cantar bajo las hojas.

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la luz contra el altar de la dureza.

Tal vez también tapiz entre los más distantes hombres fuiste.
Los pasajeros se diseminaron en la naturaleza y entonces en la selva de los desconocidos,
merci, mientras el tren frenético cambia de patria,
borra las fronteras, spasivo,
junto a los puntiagudos volcanes, frío y fuego, thanks,
sí, gracias, y entonces se transforma la tierra en una mesa.
Una palabra la limió, brillan platos y copas,
suenan los tenedores y parecen manteles las llanuras.

Gracias, gracias,
que viajes y que vuelvas,
que subas y que bajes.
Está entendido, no lo llenas todo, palabra gracias,
pero donde aparece tu pétalo pequeño se esconden los puñales del orgullo,
y aparece un centavo de sonrisa.

Pablo Neruda

Moltes gràcies a tots!

Neutralizing antibodies play a crucial role in protecting against viral infections and are a major goal of vaccine development. This work provides valuable insights into the humoral immune response to SARS-CoV-2 infection and vaccination over time, including the efficacy of this response against multiple viral variants. Scientists found that the body produces neutralizing antibodies early after infection at different levels between mild or asymptomatic disease and hospitalized patients. These antibodies persist beyond one year, but are not highly effective against newly emerging variants. However, a combination of infection and vaccination boosts immunity and provides better protection against the major SARS-CoV-2 variants. The study highlights the complexity of the immune response and the influence of various factors on its antiviral efficacy.

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