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APPROACHES TO CONTROL THE COVID-19 PANDEMIC

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"*All our dreams can come true if we have the courage to pursue them*"

Walt Disney

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SUMÁRIO

O coronavírus da síndrome respiratória aguda grave 2 (SARS-CoV-2), é o agente patogénico responsável pela COVID-19 (do inglês *coronavírus disease 2019*), a pandemia que já causou mais de 250 milhões de infecções e 5 milhões de mortes em todo o mundo. O SARS-CoV-2 foi detectado e identificado pela primeira vez na cidade de Wuhan, na China, nos finais de 2019. Devido à sua rápida disseminação em vários países, a Organização Mundial de Saúde (OMS) declarou a COVID-19 como Emergência de Saúde Pública de Interesse Internacional (ESPII) e pandemia a 30 de Janeiro e 11 de Março de 2020, respetivamente.

Esta dissertação de mestrado foi elaborada no âmbito de dois projetos que exploraram questões pertinentes e metodologias cruciais para o controlo da atual pandemia. O primeiro projecto teve como principal objectivo determinar a capacidade de anticorpos gerados em resposta à infecção pelo SARS-CoV-2 em neutralizar as variantes do vírus. A evolução do novo coronavírus tem sido marcada pelo aparecimento de diversas variantes, em diferentes países, alguns com rápida disseminação pelo mundo, o que provocou sérias preocupações no seio da comunidade científica internacional devido ao seu potencial de maior transmissibilidade, evasão ao sistema imunitário e severidade da doença relativamente ao vírus original. Como consequência, a OMS classificou quatro dessas variantes como variantes de preocupação (*variants of concern* - VOCs): Alfa, Beta, Gama e Delta, detectadas pela primeira vez no Reino Unido, África do Sul, Brasil e Índia, respectivamente. Estas variantes possuem e partilham várias mutações na proteína espícula (*spike*), a proteína responsável pela entrada do vírus nas células (através da ligação ao receptor celular, a enzima conversora da angiotensina 2, conhecida como ACE2) e reconhecimento por anticorpos, sendo por isso o principal alvo das vacinas contra a COVID-19. A monitorização das VOCs e das suas mutações, bem como a compreensão dos mecanismos que conferem ao vírus resistência à acção dos anticorpos neutralizantes gerados por infecção natural e pela vacinação é crucial para a gestão e controlo da pandemia.

Neste sentido, foram feitos ensaios de neutralização *in vitro*, com soro de pacientes recuperados da COVID-19, de partículas pseudovirais que expressam a proteína *spike* contendo diferentes combinações das mutações mais prevalentes e reportadas nas VOCs Alfa, Beta e Gama, assim como da variante Kappa, que partilha a mesma linhagem com a variante Delta. Através desta análise pode observar-se que os pseudovírus contendo as mutações das variantes Beta e Gama diminuíram significativamente a capacidade dos anticorpos de neutralizarem a sua entrada nas células comparativamente aos pseudovírus da variante Alfa e da variante selvagem (*wild type*, WT), confirmando resultados descritos na literatura. Foram também confirmadas e identificadas duas mutações (E484K e S494P) no domínio de ligação da proteína *spike* ao receptor (*receptor binding domain*, RBD), capazes de reduzir a capacidade neutralizante dos anticorpos. Observou-se uma diminuição ainda mais acentuada da capacidade neutralizante quando estas mutações foram complementadas com as mutações K417N e N501Y, conhecidas por aumentar a afinidade ao receptor celular ACE2. Este efeito aditivo sugere a existência de interacções sinérgicas entre mutações, não só pela regulação da ligação de anticorpos, como também da capacidade de entrada do vírus. O ensaio de neutralização com pseudovírus descrito neste projecto é um ensaio de alto rendimento e adequado para experiências em contexto de Laboratórios de Biossegurança de Nível 2 (BSL-2), útil para a monitorização continuada da evolução das variantes do SARS-CoV-2, mas que também pode ser usado para o estudo de outros vírus altamente patogénicos num contexto envolvendo menos riscos biológicos.

No segundo projecto avaliaram-se metodologias alternativas de diagnóstico da infecção pelo SARS-CoV-2, tendo como principal objectivo determinar a sensibilidade analítica da testagem molecular por transcrição reversa do RNA viral seguida de reacção em cadeia da polimerase quantitativa (*Reverse*

Transcription – quantitative Polymerase Chain Reaction, RT-qPCR) do SARS-CoV-2, usando a saliva de uma população pediátrica. O método de diagnóstico padrão para a COVID-19 é a detecção do RNA viral por RT-qPCR em amostras do tracto respiratório superior. As zaragatoas oro e nasofaríngeas são as amostras biológicas de eleição para a detecção do SARS-CoV-2, no entanto a sua colheita é invasiva para crianças, envolvendo riscos acrescidos de biossegurança. A saliva tem sido apontada como uma amostra alternativa em vários estudos, e que poderá ter um grande potencial na testagem e monitorização de crianças, principalmente em contexto escolar.

A campanha global de vacinação contra a COVID-19 é uma das estratégias mais promissoras para o controlo e erradicação da atual pandemia. No entanto as crianças abaixo dos 12 anos ainda não são um grupo elegível para a vacinação, o que suscita questões relativamente ao seu papel na transmissão do SARS-CoV-2 e das suas variantes. Apesar da maioria não apresentar sintomas clínicos da doença, são susceptíveis à infecção podendo transmitir o vírus para a comunidade. Neste sentido, metodologias de testagem que sejam facilmente implementadas e não invasivas são cruciais para identificar, conter e controlar os casos de infecção na população pediátrica.

Para tal, validou-se o método de testagem molecular do SARS-CoV-2 usando a saliva de uma população adulta hospitalizada, simptomática para a COVID-19 e com amostras emparelhadas de zaragatoa nasofaríngea. Uma vez validado o método, este foi aplicado numa população pediátrica de 85 crianças com idade até 10 anos, tendo sido avaliados dois protocolos: com e sem extracção de RNA viral. Nos adultos, a sensibilidade do teste foi de 100%, tendo sido identificados eficientemente todos os casos de COVID-19, quer no protocolo com extracção, quer no protocolo sem extracção de RNA. Ainda na análise referente aos adultos, obteve-se uma exatidão do método de 98.0% para o protocolo com extracção e 97.9% para o protocolo sem extracção. Nas crianças, quando se comparam os resultados da saliva com os da zaragatoa nasofaríngea, obtiveram-se valores de sensibilidade, especificidade e exatidão de 84.8%, 100% e 91.8%, respectivamente, no protocolo em que se procedeu à extracção de RNA. Relativamente ao protocolo sem extracção de RNA, os mesmos parâmetros foram de 81.8%, 100% e 90.4%. Estes resultados indicam que o método é eficiente em diagnosticar casos de infecções activas do SARS-CoV-2 em crianças até 10 anos. O método revelou-se igualmente eficaz no protocolo em que não se procedeu à extracção de RNA, o que pode ser extremamente vantajoso em contextos com limitações de recursos. A aplicação deste método, bem como a complementaridade com outros testes como os testes de antigénio depende do contexto, recursos e situação epidemiológica de cada região/país. Neste sentido, a saliva surge como uma amostra promissora na monitorização da população e crianças em contexto escolar.

Em suma, os dois projectos desenvolvidos nesta dissertação permitiram gerar conhecimento e desenvolver metodologias cruciais no contexto da pandemia da COVID-19. Por ainda ser um problema de saúde pública e ter ainda muitas questões em aberto, as abordagens deste trabalho são pertinentes para o futuro.

Palavras-chave: SARS-CoV-2, spike, anticorpos neutralizantes, saliva, diagnóstico

ABSTRACT

The novel human coronavirus SARS-CoV-2 is the causative agent of the COVID-19 pandemic that, to date, resulted in ~250 million infections and over 5 million fatalities. Despite prevention measures and vaccination efforts, the virus remains a public health concern with many outstanding questions related to viral control, evolution, disease understanding and therapy development.

We explored two approaches in this study for providing knowledge and tools to better respond to the pandemic. First, we engineered spike-pseudotyped particles to analyse how single and multiple mutations in the spike protein of the different SARS-CoV-2 variants of concern impaired the neutralizing potential of antibodies elicited by natural infection. Pseudoviruses with Alpha (UK) variant and WT spike were efficiently neutralized by convalescent sera antibodies, but those mimicking mutations in Beta (SA) and Gamma (Brazil) variants escaped neutralization significantly. We identified mutations (E484K and S494P) in the receptor-binding domain (RBD) of spike that reduced antibody neutralizing capacity and, when combined with mutations known to increase receptor binding (K417N and N501Y), exacerbated host immune escape.

The second project involved using easier, cheaper, but sensitive diagnostic methods for detecting infected people to substitute nasopharyngeal (NP) swabs. We implemented an 98% accurate saliva molecular testing method in adults and asked whether the method was suitable for children up to 10 years old. In children, the sensitivity, specificity, and accuracy were, respectively, 84.8%, 100%, and 91.8% for a protocol with RNA extraction, and 81.8%, 100%, and 90.4% for a protocol without. Thus, saliva molecular testing is suitable to diagnose SARS-CoV-2 infected children up to 10-years-old, even bypassing RNA extraction.

In summary, the two projects generated critical tools addressing scientific questions regarding immunity and diagnostic. As SARS-CoV-2 is still a public health challenge with many unanswered questions, the tools developed in this study are pertinent for the future.

Keywords: SARS-CoV-2, spike, neutralizing antibodies, saliva, diagnostic

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ABBREVIATIONS

ACE2	Angiotensin converting enzyme 2
Ag-RDTs	Antigen-detection rapid diagnostic tests
ALI	Acute lung injury
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ARDS	Acute respiratory distress syndrome
BatCoV	Bat coronavirus
bp	Base pairs
CDC	Centers for Disease Control and Prevention
COVID-19	Coronavirus disease 2019
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRP	C-reactive protein
CT	Cycle Threshold
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERGIC	Endoplasmic Reticulum Golgi Intermediate Compartment
FBS	Fetal Bovine Serum
HCOV	Human coronavirus
ICTV	The International Committee on Taxonomy of Viruses
IFN	Interferon
LB	Luria-Bernati
LDH	Lactate dehydrogenase
MERS	Middle East respiratory syndrome
MERS-CoV	Middle East respiratory syndrome coronavirus
MOI	Multiplicity of infection
mRNA	Messenger RNA
NAATs	Nucleic acid amplification tests

NGS	Next generation sequencing
nsps	Non-structural proteins
NT50	50% neutralization titer
OE-PCR	Overlap-extension polymerase chain reaction
ORF	Open reading frame
PCR	Polymerase chain reaction
POC	Point of care
PRNT	Plate reduction neutralizing test
SARS-CoV-2	Severe acute respiratory syndrome associated coronavirus 2
SARS	Severe Acute Respiratory Syndrome
SD	Standard deviation
ssRNA	Single stranded RNA
TMPRSS	Transmembrane Serine Protease
TAE	Tris-acetate-EDTA
TNF	Tumor Necrosis Factor
RNA	Ribonucleic acid
RdRp	RNA-dependent RNA-polymerase
RBD	Receptor Binding Domain
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RT-LAMP	Reverse transcription loop-mediated isothermal amplification
VLPs	Virus like particles
VOC	Variant of concern
VOI	Variant of interest
VUM	Variant under investigation
WHO	World Health Organization
WT	Wild type

Chapter 1 - General Introduction

1.1 INTRODUCTION

1.1.2 The COVID-19 pandemic

Another deadly pandemic is ravaging the world since late December 2019. The global economy, our society and public health have been severely affected by the spread of an emerging virus whose first associated cases of pneumonia were reported in a seafood market in Wuhan, Hubei Province, China [1–3].

The outbreak gradually got out of control as the number of cases and related deaths increased. Chinese health authorities notified the World Health Organization (WHO) of the eminent outbreak on December 31, conducting a large-scale joint effort to identify the causative pathogen [4–6]. Following an unprecedented analysis of respiratory samples from the patients with history contact to the seafood market, the unknown pathogen was isolated and determined to be a new type of beta coronavirus from the *Coronaviridae* family and order *Nidovirales*. In addition, the viral genome was released quickly and efficiently on January 10, 2020, accelerating all research on the new virus [4,7–9].

The first phylogenetic comparisons indicated genetic similarities to severe acute respiratory syndrome coronavirus (SARS-CoV), suggesting that both viruses belong to the same species of SARS-related coronaviruses [1,9]. Originally called 2019-nCoV, it was renamed SARS-CoV-2 on February 11, 2020, and its associated disease was officially known as Coronavirus Disease 2019 (COVID-19). According to this new evidence and based on the International Committee on Taxonomy of Viruses (ICTV) summaries, SARS-COV-2 was included in the *Coronaviridae* family [10]. *Orthocoronavirinae*, a major subfamily of the *Coronaviridae* family, consists of four genera: *Alpha-*, *Beta-*, *Gamma- and Delta* coronaviruses, which represents an increasing public health concern at both human and veterinary levels. In addition to infection in humans, infection in several animals, including bats, birds, pigs, and cats is well-documented [11,12].

Reports on coronavirus disease date back to 1930 [13–15]. Almost 40 years later, in the late 1960s, collaborative research from two independent groups allowed the identification and characterization of two human coronaviruses (HCoVs) associated with "common cold" and mild disease in humans, HCoV-229E and HCoV-OC43 [16,17]. These viruses were described and formally named coronaviruses (from the Latin "corona") due to its spherical shape with spike-like projections similar to a solar corona.

Along with the HCoV-NL63 and HCoV-HKU1, discovered in 2004 and 2005, respectively, the four identified viruses are responsible for 5-10% of common cold cases and generally cause mild disease, except in people with compromised immunity. However, during the past two decades, three highly pathogenic HCoVs emerged and caused epidemics, drawing our attention to the fact that coronaviruses must be taken more seriously [18].

SARS and Middle East Respiratory Syndrome (MERS) were the first recorded epidemics caused by HCoVs, spreading from Guangdong Province, China, and Saudi Arabia to other countries in 2002 and 2012, respectively [18,19]. The current pandemic, COVID-19, has caused millions of deaths worldwide [20], most of them elderly and people with comorbidities such as diabetes, chronic kidney disease, heart conditions, immunocompromised state, and many more listed in CDC guides for health care providers [21].

Initial attempts to contain SARS-CoV-2 within China's borders were unsuccessful mainly due to insufficient knowledge about the virus and its transmission. In a globalized and increasingly connected world, it has spread rapidly to many countries, leading WHO to declare it a public health emergency of international concern (PHEIC) and a pandemic on January 30 and March 11, 2020, respectively [8].

As the pandemic unfolded, world leaders and international health entities mobilized and established a set of rules and guidelines for use on a global scale. Drastic measures such as lockdown, border closures, massive testing, identification and quarantine of infected people were adopted worldwide to stop the pandemic and related deaths [5,22].

Despite all the preventive measures mentioned above, as of the date it was declared as a pandemic, the disease had infected approximately 118 000 people in 114 countries, with 4291 reported deaths, confirming its human-to-human transmission. Those data contrast sharply with the current 265 681 353 cumulative cases and 5 254 677 deaths reported as of 05 of December 2021 [20] .

We are currently still facing a serious pandemic, and vaccinating people for the first time against a coronavirus. SARS-CoV-2 is thus a novel pathogen in humans, for which we need to identify its origin, how it replicates inside humans and the disease it provokes, better explain how the virus is transmitted, and which type of immunity is developed, to be able to manage and eradicated it.

1.1.3 Severe Acute Respiratory Coronavirus 2 - SARS-CoV-2

1.1.3.1 Origin

There are two coronavirus genera that infect humans, Alpha (HCoV-229E; HCoV- NL63) and Beta coronaviruses (HCoV-OC43; HCoV-HKU1, SARS-CoV; MERS-CoV; SARS-CoV-2). These two genera include the seven HCoVs currently described, which acquired the ability to infect humans upon zoonotic spillover from animals [11,17,23].

Infectious pathogens are transmitted from animals to humans very frequently. According to research on emerging infectious diseases [24], six out of ten human infections are transmitted through animals, accounting for 60% of all emerging infectious diseases [25]. Zoonoses occur when animal pathogens such as bacteria, fungi, parasites and viruses infect humans, crossing the species barrier.

Bats are ancient mammals with a well-recorded history as a natural reservoir for viral pathogens, namely Lyssavirus, Ebola and Hendra virus [26]. Bat's coronaviruses were discovered in 2005 after epidemiological research into the SARS epidemic [13,26,27]. Since then, research on the genetics and biodiversity of coronaviruses has increased significantly. More than 200 coronaviruses have been identified in bats so far and according to several evolutionary models, bats are a natural reservoir of human coronaviruses. Other reservoirs include rodents, which are considered the origin of HCoV-OC43 and HCoV-HKU1[13,27,28].

Investigating the origin of a virus is a complex process involving the intersection of data such as phylogenetic analysis, genetic comparisons, and fieldwork. For instance, epidemiological studies of SARS and MERS epidemics have revealed that the viruses spread to humans from masked palm civets or dromedary camels, respectively, after being acquired from bats [18,19].

Although the origin of SARS-CoV-2 remains unclear, potential pathways by which the virus may have infected humans are under investigation. Apart from the genetic similarity to the SARS-CoV (80%) and MERS-CoV (50%) viruses [29], SARS-CoV-2 may also originate from bats. It is also possible that SARS-CoV-2 adaptation to humans required an intermediate host. Studies have shown that it is also genetically related to bat coronaviruses (BatCoV) of the *Rhinolophus* genus [30]. BatCoV RaTG13, found in the species *Rhinolophus affinis* in Yunnan province, is 96.2% similar and BatCoV RmyNO2 from the species *Rhinolophus malayanus* is 93.0% identical at the genomic level [1,7,9].

A similar virus has also been found in pangolins [31]. A study showed that a coronavirus recovered from lung samples of two dead rescued pangolins in Guangdong, China, shared 91.02% of its genome with SARS-CoV-2 [31]. Interestingly, while bat viruses do not bind the SARS-CoV-2 human receptor, angiotensin-converting enzyme 2 (ACE2) [32], the pangolin virus has signatures more similar to SARS-CoV-2 receptor-binding domain (RBD), enabling binding to ACE2. These findings provided some support to the possibility that SARS-CoV-2 was transmitted to humans via an intermediate host. However, the source of SARS-CoV-2 remains unknown and is one of the pressing questions to resolve [30].

With the emergence of two epidemics, SARS and MERS, less than 20 years ago and SARS-CoV-2 pandemic, it is clear that strategies to prevent and control host species jumps of zoonotic coronaviruses are critical to anticipate and prepare for future outbreaks. This requires surveillance methodologies, as those used for influenza and developing new control measures and therapies to block the spread of pathogens from animals to humans [33].

1.1.3.2 Virion structure and virus life cycle

The scientific community has fully mobilized to generate relevant data regarding the emergent virus to respond to the pandemic. Shortly after the identification of the SARS-CoV-2, its genome was fully sequenced and data regarding its structure and lifecycle became available [1,34–36].

The primary structural organization of SARS-CoV-2 is a common feature of all members of the *Coronaviridae* family [37]. Coronaviruses are enveloped viruses with the largest RNA genomes known to date. SARS-CoV-2 virion is a spherical particle (~100 nm) coated with a lipid bilayer containing the structural and surface proteins spike (S), membrane (M), and envelope (E), as represented in Fig.1.1 [36]. Under the envelope is the genome, a positive-sense, unsegmented single-stranded RNA (+ssRNA) bound to several copies of the nucleocapsid protein (N), forming the viral ribonucleoprotein complex (vRNP) [29,34,38]. The M and E proteins, alongside N protein are the major regulators of viral assembly. The spike protein acts as the primary interface between SARS-CoV-2 and the host cell receptor, promoting cell entry and infection [35,39].

The viral genome has ~30 kb encompassing at least 14 open reading frames (ORFs), which encode 29 identified proteins [39]. The single-stranded RNA is oriented in a 5’ to 3’ direction and delimited by two untranslated regions (UTR) of 265 and 345 nucleotides. The SARS-CoV-2 genome has a 5’ methylated cap and 3’ polyA-tail mRNA, enabling it to function as a host mRNA and be translated by cellular ribosomes [19,29,41].

The initial two-thirds of the genome comprise the transcriptional regulatory sequence leader (TRS-L) and two ORFs: ORF1a (4382 amino acids (aa)) and ORF1b (7073 aa), overlapped by a ribosomal frameshift [37,39]. This biological mechanism enables the translation of several distinct proteins joining one or more ORFs. In addition, it also helps the virus to maintain the precise stoichiometry of both polyproteins used for replication and infectivity [36,39]. Immediately downstream of ORF1b set the remaining overlapping ORFs, which encode both structural proteins S, E, M, N, and accessory proteins, whose function is not yet fully elucidated. ORF1a and ORF1b encode for 16 non-structural proteins (nsps) via two polyproteins, pp1a (nsp1-11) and pp1ab (nsp1-16) [41]. These polyproteins are proteolytically cleaved by two viral proteases, a papain-like protease PLpro or 3CLpro and a chymotrypsin-like cysteine protease Mpro, to release the nsps [37,42].

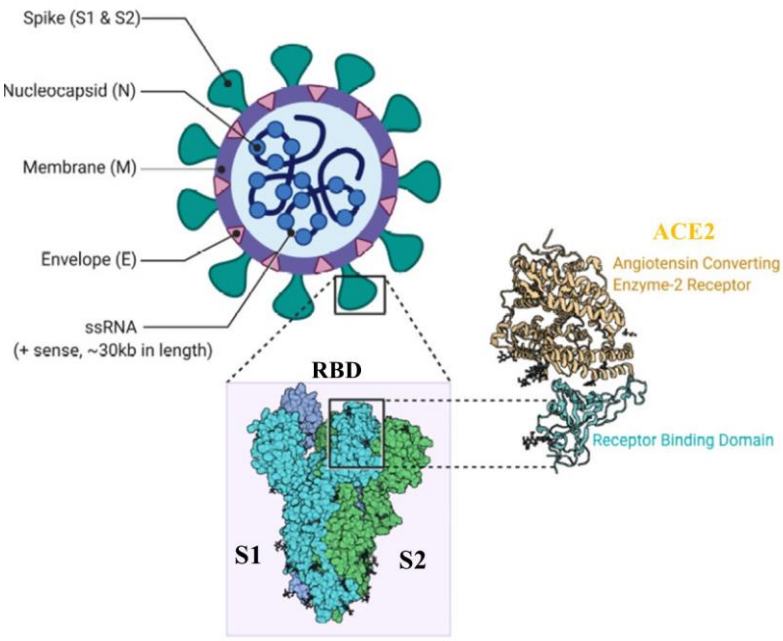

Figure 1.1 | Representation of SARS-CoV-2 virion structure. The viral particle contains the structural proteins spike (S), membrane (M), and envelope (E), embedded in the lipid bilayer, and nucleocapsid (N) which assembles with the viral genome (+ssRNA) beneath the lipid bilayer. The spike protein has two subunits: S1 and S2. The S1 subunit comprises the receptor-binding domain (RBD), which binds to the host cell receptor angiotensin-converting enzyme 2 (ACE2), and the S2 subunit that facilitates spike fusion with the host cell membrane. Image adapted from [40].

Viruses are obligate intracellular parasites with a striking ability to replicate and perpetuate progeny using the host cellular machinery. To establish in humans, SARS-CoV-2 must infect cells and be transmitted to neighbouring cells and tissues [11,43].

The first stages of SARS-CoV-2 life cycle (Fig.1.2) begin with the viral entry, encompassing specific and highly coordinated events between the spike protein and host cell factors. The spike protein, a glycosylated trimeric protein that contains two subunits, S1 and S2, regulates the attachment and fusion of the virus with the host cell membrane [36,44]. The S1 subunit contains the RBD that specifically binds to the host cell receptor ACE2, which is also the receptor recognizing HCoV-NL63 and SARS-CoV (of alpha and betacoronavirus genera, respectively) [1,45]. ACE2 is a transmembrane proteinase widely distributed in the human body, including, the respiratory and gastrointestinal tracts, heart, kidneys, brain, etc. It is also a central component of the Renin-Angiotensin System, a highly dynamic and a critical regulator of blood and fluid pressure [46,47]. The subunit S2 contains two heptad repeats (HR1 e HR2) and a fusion peptide crucial for the conformational rearrangement that enables fusion with the cell membrane.

Binding of spike to ACE2 alone does not render the virus apt to enter the cell [39,48,49]. Host proteases must first activate the spike protein for the viral lipidic envelope to fuse with the host cell membrane and deliver viral contents inside the cell [37,51,52]. The release and insertion of the N-terminus of S2 into the host cell membrane is enabled by host proteolytic cleavage between the S1 and S2 domains and S2' cleavage site of the SARS-CoV-2 spike protein [53]. Research on coronavirus cell entry pathways [52–55] has uncovered several host proteases that can activate spike at specific stages during viral infection: during virion assembly in infected cells, by protein convertases (*e.g.*, furin); after the virus release from the infected cell, by extracellular proteases (*e.g.*, elastase); after viral engagement with target cells, by cell

surface proteases (*e.g.*, TMPRSS2) and after viral entry by endocytosis, by lysosomal proteases (*e.g.*, Cathepsin L) [56].

The usage of specific host proteases regulates where SARS-CoV-2 fusion occurs: at the cell surface by serine proteases such as TMPRSS2 (after furin priming), or in endosomal lysosomal compartments by lysosomal proteases such as cathepsin L. This biphasic nature suggests that the mechanism of SARS-CoV-2 spike activation and cell entry depends on the presence of these factors as well as cell-type specificities [53,55]. For instance, the pathway that allows SARS-CoV-2 to fuse with the host cell membrane is activated whenever factors such as TMPRSS2 and furin [57], TMPRSS4 [58], or human airway trypsin-like protease (HAT) are expressed [52]. On the other hand, in its absence, the endocytic pathway is preferred.

Recently, another factor, Neuropilin1 (NPR1), was highlighted as promoting the entry of SARS-CoV-2 into cells [59]. NPR1 facilitated the entry of SARS-CoV-2 into ACE2-expressing cells, and to a lesser extent, in the absence of ACE2 and TMPRSS2. Despite the substantiated evidence of all these factors, researchers have shown that the type II transmembrane protease TMPRSS2 is critical for priming the spike protein on the host cell surface. Known inhibitors of several of these proteases have been tested and found to have antiviral potential, raising awareness for the importance of selecting the most physiological models for drug screening purposes. [55,57].

Immediately after fusion, genomic RNA dissociates from the nucleocapsid and is directly translated by host ribosomes in the cytoplasm (Fig.1.2). The translation of ORF1a and ORF1b from the genomic positive-sense RNA produces the polyproteins pp1a and pp1ab, described above. The cleavage of these two polyproteins produces 16 nsps, which are regulators of a range of functions in the SARS-CoV-2 life cycle, acting, for example, as the central core of the replication and transcription viral machinery [37,39,41,60].

The RNA-dependent RNA-polymerase (RdRp) starts viral replication by synthesizing a full-length negative-strand intermediate (-gRNA) that can act as a template to generate new genomic RNA (+gRNA) or be used to replicate a nested set of subgenomic mRNAs (+sgRNA). The sg mRNAs are then used to translate the structural and accessory proteins [37,38].

The viral RNA and structural proteins must assemble into a functional virion to propagate the infection to other cells and ultimately from person-to-person. Thus, the final steps in the SARS-CoV-2 life cycle are determined by the viral assembly and exocytosis of the newly formed virion. First, the structural proteins S, M, and E, translated in the Endoplasmic Reticulum (ER), are translocated to the ER-to-Golgi intermediate compartment (ERGIC), the primary coronaviruses assembly site [23,35]. Then the +gRNA is encapsulated by N protein, budding into membranes of the ERGIC, and associates with the surface proteins S, M, and E to form a new virion. Following assembly, the virion exits the infected cell in double-membrane vesicles through exocytosis to infect neighbouring cells and tissues [39,42].

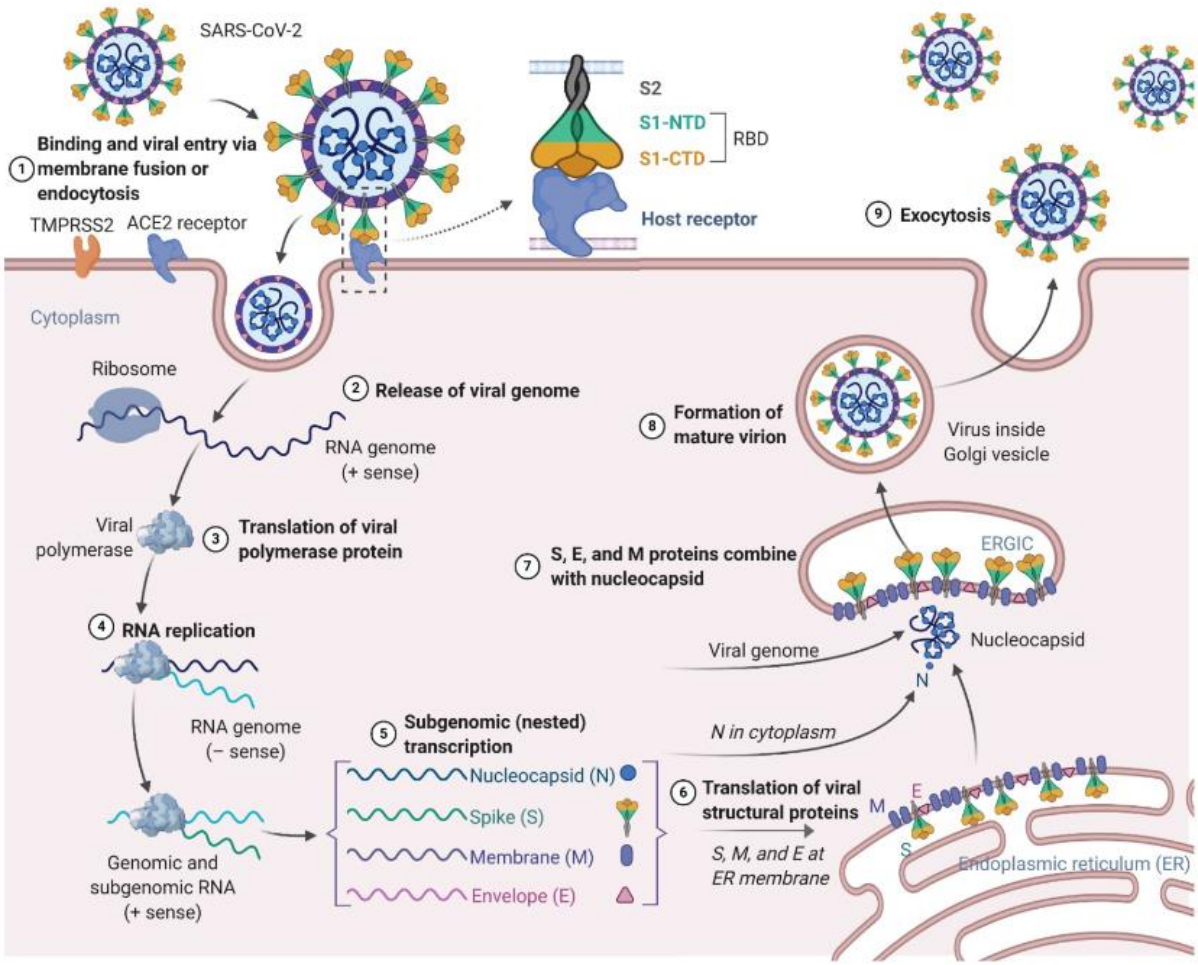

Figure 1.2 | SARS-CoV-2 life cycle. SARS-CoV-2 enters the cell through the binding of the spike (S) protein receptor-binding domain (RBD) and the host cell receptor angiotensin-converting enzyme 2 (ACE2). After fusion with the cell membrane, the viral RNA genome is released into the cytoplasm and translated into a large polyprotein that contains the viral polymerase. The RNA-dependent RNA polymerase (RdRp) synthesizes a full-length negative-strand intermediate (-gRNA) that acts as a template for genomic RNA (+gRNA) and a nested set of subgenomic RNAs (+sgRNAs) synthesis. The structural proteins and the viral ribonucleoprotein associate in the ER-Golgi intermediate compartment (ERGIC) to form a functional virion that exits the cell through exocytosis. Image from [50].

1.1.3.3 SARS-CoV-2 transmission

Understanding the pathways of viral transmission is critical to prevent its spread in the population. It is also important to understand the biological aspects and the type of virus to develop and implement effective prevention strategies [61]. The dramatic increase of reported cases in Wuhan and the subsequent global spread of COVID-19 confirmed the person-to-person transmission of SARS-CoV-2 [3,4]. Apart from direct contact, the most well-recognized transmission pathways include airborne and fomite transmission [62].

The virus can be directly transmitted if a healthy individual comes into close contact with an infected person's respiratory and/or saliva droplets/aerosols. These secretions can reach the eyes, mouth, or nose of the vulnerable person and cause infection. Of note, the viral transmission will depend on many factors such as the individual's stage of infection, the amount of virus they shed and proper ventilation systems [61].

From an epidemiological perspective, airborne transmission is one of the most concerning routes of viral transmission. The respiratory system enhances viral entry and transmissibility due to its large area of direct contact with the environment [63,64]. Because SARS-CoV-2 is a respiratory virus, it can spread quickly through droplets and aerosols generated when an infected person speaks, sneezes, coughs, or sings [2,3].

Therefore, the physical factors underlying droplet and aerosol formation, as well as their dispersion, must be understood. Research in this field [63,65,66] resulted in a better knowledge of SARS-CoV-2 transmission dynamics through droplets and aerosols, allowing health authorities to adjust the preventative measures. Droplets and aerosols are defined as particles with diameters of > 5-10 μm and ≤ 5 μm, respectively, and due to their small dimensions, aerosols remain in the air a long time and can reach up to 2 meters [61,63]. In this scenario, complying with the procedures recommended by health authorities becomes imperative. Wearing a mask, avoiding crowded spaces without ventilation systems, and keeping the required safety distance of 2 meters are necessary precautions to prevent viral transmission [61,63].

Early in the epidemic, there was much discussion about airborne transmission and the widespread usage of masks. However, more than 200 scientists signed a letter warning the international community to consider this transmission type and include it in pandemic preparedness measures [65].

Public and surgical masks has become mandatory in confined and crowded spaces such as shopping centers, public transport, and gyms [67]. The number of droplets produced when individuals talk, for example, is reduced through mask-wearing. Consequently, these are effective strategies to prevent the transmission of SARS-CoV-2 when combined with social distance, frequent hand washing, and cleaning public places and commonly touched surfaces [68,69].

Regarding fomite transmission, it is believed to occur when susceptible individuals directly contact contaminated surfaces (fomites). Infected people spread the virus mostly by aerosols and droplets that can remain on surfaces or contact objects [70]. Under controlled laboratory conditions, SARS-CoV-2 is more stable on plastic and stainless steel than on paper and tissue paper, surviving on these surfaces for up to four days [71]. The viral viability on-air or surfaces is also affected by environmental factors, such as gravity, temperature, and humidity [71]. The same study has shown that the virus is temperature sensitive. It may survive at 4ºC for up to 14 days, and it can be inactivated after 30 minutes at 56ºC and 5 minutes at 70ºC. Thus, even though fomites are acknowledged as a transmission pathway, the CDC and WHO believe they are not the preferred route of contagion, posing minimal risk to the population [61,70,71]. Even so, it is important to avoid touching our eyes, mouth, or ears as much as possible and to wash or disinfect hands frequently.

The SARS-CoV-2 virus particles are coated with a lipid bilayer, which means that they can be easily degraded by the surfactants found in many common detergents such as bleach, soap, hydrogen peroxide, and ethanol (70%) [71]. As a result of the pandemic, hundreds of formulations are now authorized for usage and disinfection in domestic, institutional, and medical settings [72].

1.1.4 COVID-19 clinical symptoms and treatment

The clinical manifestations of COVID-19 are very heterogeneous and vary according to the disease severity [1,18]. Health organizations have established medical criteria to classify COVID-19 as asymptomatic (no clinical signs of infection), mild (no clinical signs of viral pneumonia and hypoxia), moderate (with clinical signs of viral pneumonia but no signs of hypoxia), severe (cases of dyspnea,

hypoxemia, or lung infiltrates >50%), or critical disease (septic shock, respiratory distress syndrome-ARDS, and multi-organ failure) [40,73,74].

Symptomatic cases develop disease-specific symptoms after the virus has gone through an incubation period, i.e., the time from viral exposure to the symptom onset, which is estimated to be 4 to 5 days but can last up to 14 days [4,18].

Current evidence reports that the most frequent and characteristic symptoms of the infection are fever (83-99%), dry cough (59-82%) and fatigue (44-70%). Several studies also recorded symptoms such as shortness of breath, nasal congestion, sore throat, and taste (ageusia) and smell (anosmia) alterations [75–77]. Also associated with these symptoms, but reported less frequently, are non-specific symptoms such as diarrhea, vomiting, myalgias and headaches [78,79].

Of all people susceptible to be infected by SARS-CoV-2, there is a greater risk of developing severe disease in the elderly, and in people with associated comorbidities. Diabetes, hypertension, cardiovascular disease, cancer, kidney disease, obesity are comorbidities that are described as risk factors for development of serious complications [21,74,80]. Of note, a study including more than 72,314 confirmed cases of COVID-19 showed that the overall case fatality rate in those patients was 2.3%, however, for the elderly aged between 70 and 79 years, it rose to 8%, and for the elderly of > 80 years rose to 14.8% [80].

Although COVID-19 is a disease that preferentially affects the respiratory tract [2,39,76], it can affect other organs and, in the most severe cases, it may cause multi-organ failure, sometimes leading to death. The infection can affect the kidneys, heart, blood, central nervous system, liver, intestinal tract, etc. Cases of acute kidney injury, neurological disorders, myocardial ischemia, hematologic alterations are well-documented in patients with severe and critical disease (Fig.1.3) [18].

The most severe cases are also associated with common laboratory parameters alterations that include lymphopenia, thrombocytopenia, elevated liver enzymes (Aspartate aminotransferase-AST and alanine aminotransferase-ALT), elevated lactate dehydrogenase (LDH), elevated inflammatory markers and cytokines (*e.g.*, C-reactive protein-CRP, interleukin 6 -IL-6 and tumor necrosis factor -TNF) [75,76,79].

Persistent symptoms have been reported after patient's recovery. Many COVID-19 survivors continue to experience symptoms after healing, which are referred to as "post-acute COVID-19". Hair loss, cognitive problems (brain fog), anxiety/depression, muscle weakness, chest pain, dyspnea, and fatigue are some of the most reported symptoms. According to reports, approximately 20% of the survivors experienced hair loss and 30-40% experienced depression, anxiety, and sleep alterations. This highlights the need for follow-up in recovered patients with persistent symptoms [81].

The potential therapies and drugs available to treat COVID-19 continue to be closely monitored through clinical trials overseen by international health authorities. An example of such initiatives is the Solidarity trial, established by WHO and its partners to assess the efficacy and safety of current and novel drugs in COVID-19 therapy. Drugs are being evaluated in over 116 countries to find effective treatments that reduce mortality, hospitalization, and the need for supplemental oxygen [82].

Pharmacological therapies approved and/or under evaluation by the US Food and Drug Admnistration (FDA) and international entities in the treatment of COVID-19 currently include antiviral therapy (*e.g.*, remdesivir, favilavir), anti-inflammatory drugs (*e.g.*, dexamethasone), anti-SARS-CoV-2 monoclonal antibodies (*e.g.*, sotrovimab, casirivimab, indemivab), immunomodulators agents (*e.g.*, IL-6 receptor agonists - tocilizumab) and convalescent sera [40,83].

The use of such therapies is reliant on disease severity and the approval of drug regulatory agencies after evaluation its safety and effectiveness. Drugs such as dexamethasone, indevimab, and tocilizumab, for example, are authorized for emergency use in severe and critical cases. However, most of the new drugs are approved only in the context of a clinical trial [84].

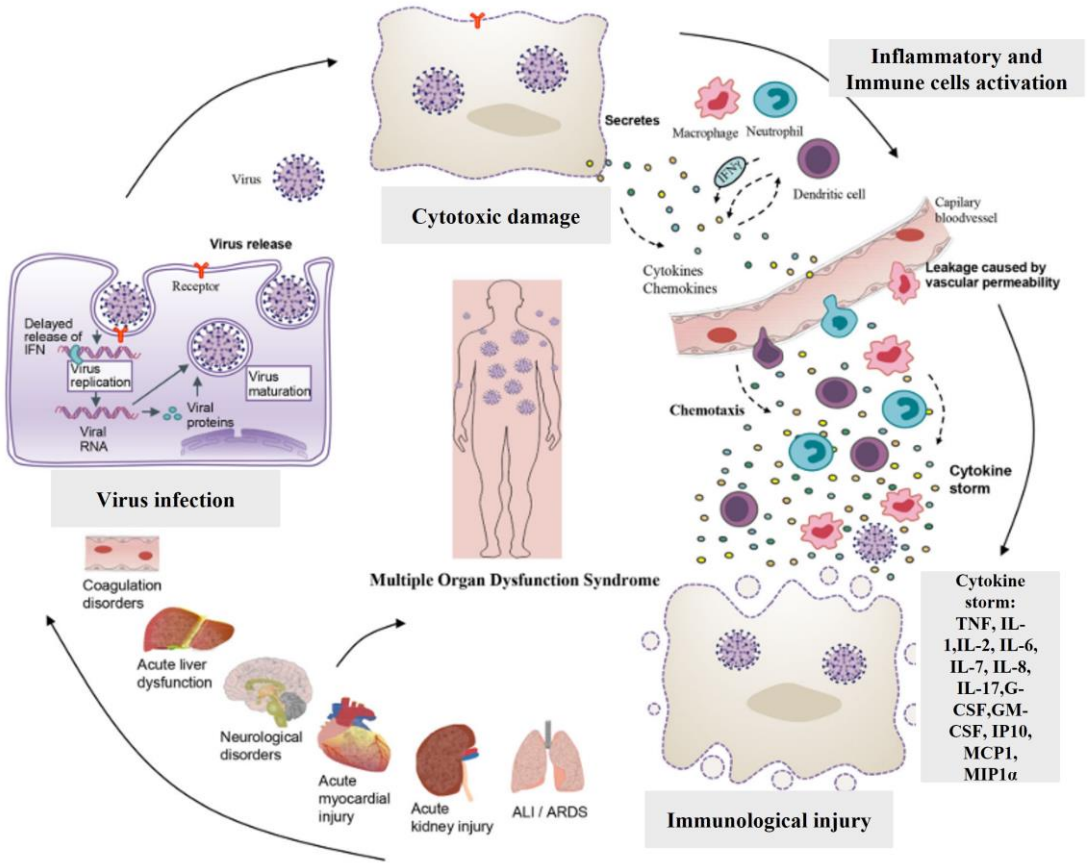

Figure 1.3 | Schematic representation of cytotoxic and immunological damage from SARS-CoV-2 infection. In addition to causing damage to cells and lungs, SARS-CoV-2 infection can also affect several organs such as the kidneys, brain, heart, and liver. Furthermore, a well-documented cytokine storm in severe and critical COVID-19 patients can lead to a hyperactivation of the immune system, often resulting in tissue damage, acute respiratory distress syndrome (ARDS), acute lung injury (ALI), and multiple organ failure. Image from [18].

The majority of those infected with SARS-CoV-2, about 80%, represent cases ranging from asymptomatic to mild that normally recover from the disease without needing medical care and hospitalization. In case of disease progression and in more severe cases, where symptoms tend to last for a long time [74], treatment and hospitalization are necessary to manage the disease and prevent worst outcomes. In cases of hypoxemia and depending on its severity, oxygen support is given to patients. The decision on how to treat patients is based on symptoms, oxygenation index that allows the assessment of respiratory function, radiographic and laboratory alterations [76,79].

Early in the pandemic, chloroquine and hydroxychloroquine, which are used to treat malaria and rheumatoid arthritis, were identified as potential drugs for COVID-19 treatment [85,86]. However, evidence from clinical trials revealed that the two drugs did not improve the health of hospitalized patients, causing a lot of debate. Due to the lack of consensus on their effectiveness, the CDC suspended its emergency use authorization in June 2020 [40,78].

Remdesivir (Veklury), an antiviral drug initially developed by Gilead Sciences to treat Ebola [87], was authorized by the FDA in October 2020 to treat hospitalized adults and children > 12 years of age. It was also authorized in the European Union after showing a slight improvement in the recovery time of hospitalized patients [84]. In addition to steroid treatment, the WHO has recently recommended IL-6 receptor blockers in the treatment of COVID-19. An analysis of data from 27 clinical trials involving over 10,000 patients revealed a 13% reduction in mortality in severe and critical cases [88].

Currently, hundreds of therapies are being evaluated in clinical trials [82], with the expectation that safe and effective treatments for COVID-19 may be accessible shortly. In this way, hospitalizations can be reduced, and national health systems can be relieved of the burden caused by thousands of daily cases and fatalities.

1.1.5 COVID-19 diagnostic methods

The absence of effective COVID-19 therapies and preventive measures at the beginning of the pandemic [1] led to a sharp increase in infections and fatalities throughout the world. Therefore, it became essential for epidemiological control of the pandemics to identify and isolate infected people and their close contacts [22]. A major challenge in COVID-19 management is the fact that asymptomatic people can infect others [89]. In contrast to SARS and MERS [37], this silent feature of SARS-CoV-2 has been a serious problem that accelerated worldwide viral transmission with severe impact in the most vulnerable groups *i.e.,* the elderly and those with underlying comorbidities [4,74]. In this sense, mass testing had to be implemented as a strategy to identify SARS-CoV-2 infected individuals, symptomatic and asymptomatic, to prevent viral spread and protect the most susceptible groups [90].

The identification of an infected person is achieved by several methodologies and using several types of sampling. The most sensitive methods rely on the amplification and detection of the genetic material of the virus, in this case RNA. [39,91]. Therefore, these molecular methods, *i.e.,* nucleic acid amplification assays (NAATs) are the preferred methods for detecting SARS-CoV-2 infections as they are the methods with the greatest sensitivity and specificity. SARS-CoV-2 molecular detection techniques have advanced significantly, with many methods now on the market, such as RT-qPCR, reverse-transcription loop-mediate isothermal amplification (RT-LAMP) assay; CRISPR-based assays and next generation sequencing (NGS) [91–93].

These techniques are constantly being updated. The golden standard is the detection of viral RNA in oral and nasopharyngeal swabs as well as bronchial aspirates, through the molecular technique of RT-qPCR. However, it involves very high costs and complicated logistics. Sample collection is an invasive procedure that requires skilled and qualified experts, and due to the proximity required it increases the probability of infection for health professionals [78,94]. Due to all these constraints, there has been the need to test alternative sampling, including saliva, to increase the testing capacity and contain the virus.

The other types of tests used are antigen-detection rapid diagnostic tests (Ag-RDTs) These are now widely used and are a less expensive approach for diagnosing COVID-19., despite being much less sensitive and only detecting infected people with very high viral load. These are single-use tests that can be used outside of a clinical setting and can take as little as 15 minutes to get results. However, because they are not as sensitive as molecular tests, they may produce false negatives [95].

In addition, there are also methods for detecting people who have contracted the virus, at least 14 days before the test. These are serological tests, which are based on our body's immune response to pathogens (in this case, SARS-CoV-2). Although they are not suitable for identifying patients with acute

infections, they are critical for understanding the immune protection conferred by COVID-19. They are also being used to assess the population's immune responses after natural viral exposure or by vaccination [96,97].

Aside from the different diagnostic tests available, there is extensive research for alternative biological samples for SARS-CoV-2 detection. For example, the virus has also been found in stool, sputum, and saliva samples, and several kits are now using saliva molecular sampling to identify SARS-CoV-2 since it is less invasive and can be self-collected. In addition, several studies have demonstrated that saliva is a suitable alternative for diagnosing actively infected individuals [77,98–100].

Mass testing is a significant challenge, particularly in low-income countries with fewer resources distributions. As a result, experts advocate combining quicker testing that doesn't require laboratories settings with molecular methods to diagnose SARS-CoV-2 infections accurately [95].

1.1.6 Immune responses against SARS-CoV-2

SARS-CoV-2 infection triggers innate and adaptive immune responses that target the virus. Innate immunity involves the initial responses to infection, including type I interferon (IFN-I) expression as explained below, to prevent or decrease viral replication in cells. In addition, they stimulate the recruitment of effector cells such as macrophages, neutrophils, dendritic cells, and Natural Killer cells (NK) to the sites of infection. Consequently, an adaptive immune response is activated at a more advanced stage of the disease, which involves immune responses from B and T lymphocytes [101].

Dendritic cells and macrophages stimulate IFN-I production and the initial response of T-cells (Helper T cells-$CD4^+$ and cytotoxic T cells-$CD8^+$) through antigen presentation. Ideally, the innate immune system recognizes the infection after a few hours and activates the expression of IFN-I, which is one of the first lines of defense against viral infections [102]. However, an ineffective IFN-I response and dysregulation of interferon-stimulated gene (ISG) expression have been associated with COVID-19 severe and critical cases. It may lead to a delay in the immune response and, consequently, in the adaptive immune response, prolonging the infection and resulting in the worst outcomes [103].

T cells are key players in antiviral host responses. $CD4^+$ T cells are essential to stimulate B cells, which produce viral-specific antibodies, and $CD8^+$ T cells target and destroy infected cells. The T cell population is strongly affected in patients with severe and critical COVID-19, the majority presenting with lymphopenia, *i.e.*, depletion of lymphocytes. Therefore, initial lymphopenia has been used as a predictive marker of severe disease [3,75,101,104]. In addition, Grifoni *et al.* demonstrated strong T cell specific responses against SARS-CoV-2 spike protein in 20 COVID-19 recovered patients, suggesting that T cells responses are protective and confer immunity [105].

The humoral response (antibody-mediated response) is a critical component of the adaptive immunity, required for viral clearance. Viruses must move through body-extracellular fluids to infect cells and spread the infection to tissues [106]. Antibodies, also known as immunoglobulins (Ig), are glycoproteins produced by plasma cells derived from B lymphocytes. There are five Ig classes that promote the elimination of extracellular pathogens preventing cell invasion and subsequent infection: IgA, IgD IgE, IgG, and IgM.

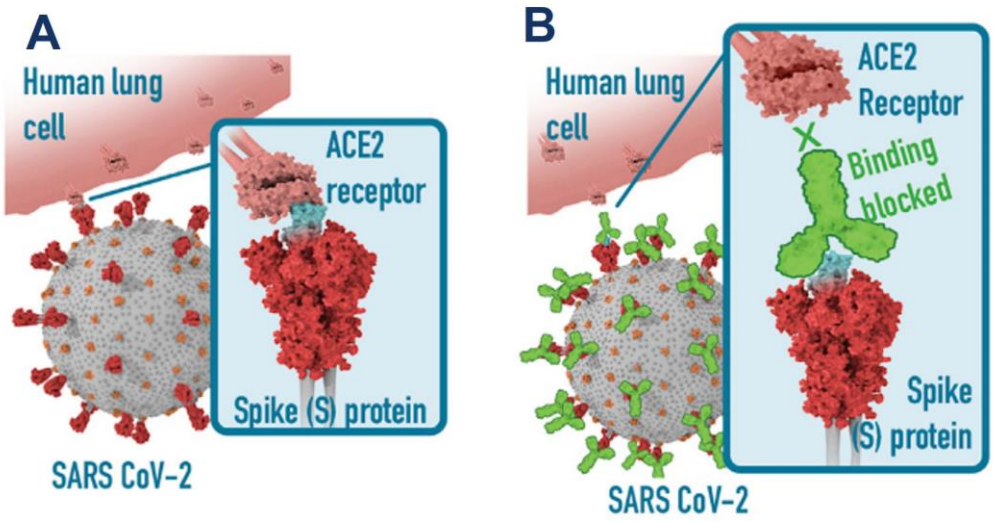

Figure 1.4 | SARS-CoV-2 neutralization by antibodies. **A:** SARS-CoV-2 spike protein binding to ACE2 receptor in a human lung cell. **B:** Neutralizing antibodies block and prevent spike from engaging with ACE2. Image from [97].

Antibodies can eliminate pathogens in several ways. They can act as neutralizing antibodies, blocking the binding of pathogens to cellular receptors; by binding to pathogen surfaces, promoting opsonization, or activating complement system proteins [97,106]. Not only are they involved in the primary response to infections, but they also play a critical role in the defense against re-infection and breakthrough infections [106,107]. Virus-neutralizing antibodies are essential for virus clearance. In the case SARS-CoV-2 infection, neutralizing antibodies bind to the spike protein, preventing it from binding to the ACE2 receptor [97,101,107,108].

The IgM antibodies are the first antibodies to be produced and act in primary responses to viral infections [106,108]. After isotype switching, high-affinity IgG and IgA antibodies are produced and play a critical role in virus neutralization [106,109]. IgG antibodies are the most important neutralizing antibodies found in human sera and, IgA antibodies act mainly on the mucous membranes of the gastrointestinal and respiratory tracts [106]. The presence of specific antibodies against SARS-CoV-2 is one of the best indicators of protection against re-infections and is also an informative biomarker of previous viral exposure [96,101]. In fact, several studies have shown a direct relationship between the capacity of serum neutralizing antibodies and the anti-spike IgG content [97,107,110].

Most infected individuals develop SARS-CoV-2 specific antibodies within 5-15 days after symptom onset [97,101,107]. According to several studies, the IgM response develops in the initial weeks after symptoms onset and subsequently declines throughout the infection. In most cases, this reduction is accompanied by an increase in IgG and IgA titers, which remain relatively high after infection. Of note, Jun Wu and colleagues [107] showed that SARS-CoV-2 IgG specific antibodies levels remained relatively high during a six-month observation period. However, the lifespan of such protection remains an open question. About 90% of SARS-CoV-2 neutralizing antibodies in COVID-19 patients target the spike protein, namely its RBD and N-terminal domain. More specifically neutralizing epitopes on the RBD domain are highly immunogenic and easily recognized by antibodies [97,101].

Understanding how long immunity to SARS-CoV-2 persists and how protective that immunity is against possible reinfections is crucial for vaccine development and pandemic management decisions [107].

1.1.7 COVID-19 Vaccination

The urgency to develop therapies or vaccines for COVID-19 became evident with increasing cases and deaths worldwide. World leaders, international health organizations, scientists, biotech companies, and manufacturers have engaged all available funds and resources to control the pandemic. The rapid exchange of data on SARS-CoV-2 structure and genome and all the scientific and technological advances to date have allowed researchers to accelerate the vaccine development process [34,36,44]. Almost ten months after the initial outbreak, more than 150 vaccine candidates were available. Of those, nearly 50 were approved for clinical trials [111].

Vaccination is one of the best available approaches to prevent and control infectious diseases by stimulating our immune response against infectious agents. The surface SARS-CoV-2 spike protein, more precisely its RBD, has been demonstrated to elicit a robust immune response through neutralizing antibodies [97,112,113]. Although some vaccine technologies target the whole virus, most COVID-19 vaccines target the spike protein [92].

COVID-19 vaccines are based on different platforms: traditional and well-established (*e.g.*, live attenuated, inactivated vaccines) and innovative (*e.g.*, mRNA vaccines). Currently, the approved vaccines with scientifically proven effectiveness and safety include approaches based on non-replicating viral vectors (Jansen-Ad26.CoV.2.S-Johnson&Johnson; Vaxzevria-AZD1222-Astrazeneca/University of Oxford; SputnikV-Gamaleya; Canvidicea-Ad5-nCoV-CanSino Biologics), mRNA (Spikevax-mRNA-1273-Moderna/NIAID; Comirnaty-BNT162b2-BioNTech/FosunPharma/Pfizer), protein subunit (EpiVacCorona-Vector Institute, Russia) and inactivated vaccines (Coronavac-Sinovac Biotech; BB1BP CorV-Sinopharm Beijing) [114–116]. The remaining candidate vaccines under evaluation also include DNA-based vaccines, replicating viral vectors, virus-like particles (VLPs), plant-based vectors, among others [117]. Specific information on all clinical trials is updated weekly by WHO, and it is available on the COVID-19 vaccine tracker platform [118].

By December 05, 2021, a total of 8 169 754 695 people had been vaccinated, reflecting the global effort and commitment to stop the pandemic [20]. Israel is one of the leading countries in the COVID-19 vaccination campaign, having 54% of its total population fully vaccinated (9.1 million people) between December 2020 and April 2021. The positive impact that has already been noticed in several countries shows that immunization is one of the most promising and powerful allies in the global fight against the pandemic. For example, the administration of two doses of the Comirnaty (BNT162b2) vaccine lead to a decrease of 45% in the number of cases and 68% in the number of hospitalizations of people over 60 years of age [119].

Currently, there are 8 authorized vaccines approved worldwide by WHO and more than 100 vaccines in clinical development stages. Of those approved, only 4 are authorized for use in the European Union: Cominarty, Spikevax, Vaxzevria and Jansen COVID-19 vaccines [118]. The licensed vaccines were shown to be effective and safe, some of which providing 95% protection against COVID-19 [114]. However, clinical trials will continue to evaluate the long-term efficacy of vaccines, the amount and length of the immune response, and the potential for side effects [115,120]. It is also necessary to

overcome logistics-related constraints such as vaccine production, distribution, and storage for equal vaccination campaigns.

1.2 GOALS OF THE PROJECT

This project was developed under the scope of the COVID-19 Task Force established by the Cell Biology of the Viral Infection Laboratory (CBV) at Instituto Gulbenkian de Ciência and I was involved in two projects that, despite being unrelated, concentrated on answering outstanding questions regarding the pandemic.

One of the most pressing questions regarding this novel virus when I started developing my MS.c. project was determining the type of immunity developed after infection to understand if people were protected from re-infection. As the pandemic unfolds, it is critical to assess how circulating and emerging variants of SARS-CoV-2 can affect viral transmissibility, infectivity, host immune evasion, and ability to cause disease. Extensive studies have been carried out on these variants since their emergence, but due to their rapid evolution and the appearance of several significant mutations, it is crucial to maintain close surveillance of these variants and their defining mutations. In practice, this translates into predicting and assessing the effects of viral structural changes that may increase its transmissibility or evade immune responses, thus providing relevant information to the development of vaccines, therapies, and pandemic control policies.

The other pressing question is the development of easy and fast methods of diagnosing SARS-CoV-2 infected people. Alternative methods for detecting SARS-CoV-2, including molecular methods based on saliva sampling, are being widely investigated. Most studies, however, have focused only on the adult population. There is little research on this topic concerning children and it is vital to establish less invasive approaches that can be used in several contexts, such as schools. Furthermore, most children exhibit asymptomatic or moderate symptoms, so their actual contribution to the spread of SARS-CoV-2 remains unknown. In this sense, it is imperative to develop simple and fast methods for closely monitoring the infant population, mainly because this is a group for which vaccination has not yet been approved.

As such, the aims of this project are:

- To assess the antibody neutralizing capacity of COVID-19 patient's convalescent sera to circulating SARS-CoV-2 variants and different mutations in spike.

- To compare SARS-CoV-2 molecular detection by RT-qPCR in children's nasopharyngeal swabs and saliva samples, to understand if saliva molecular testing is a viable alternative method for children diagnostics of SARS-CoV-2 infection.

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Chapter 2 - Neutralizing capacity of antibodies developed by COVID-19 patients to SARS-CoV-2 variants

2.1 AUTHOR CONTRIBUTIONS

The experiments presented in this chapter were designed and planned by Marta Alenquer, Filipe Ferreira and Maria João Amorim. Experiments and data generated for Fig. 2.4-2.8 were performed by Marta Alenquer, Filipe Ferreira and **Mónica Medina**, as they involved a serious amount of sample analysis and processing. The candidate of this M.Sc. thesis has engineered the plasmids with the mutations: E484K; S494P; S494P/N501Y; E484K/S494P; E484K/S494P/N501Y and L452R/E484Q, as well as the plasmids with all defining mutations of Alpha, Beta and Gamma variants of concern (VOCs), together with Filipe Ferreira. The candidate made, with Filipe Ferreira, the spike-pseudotyped particles S494P; S494P/N501Y; E484K/S494P and E484K/S494P/N501Y and, performed the neutralization assay with the spike-pseudotyped particle bearing L452R/E484Q mutations. The neutralization assays for all others spike-pseudotyped particles listed in table 2.5 were made with Marta Alenquer.

This chapter resulted in a published manuscript with the reference: Alenquer, M.; Ferreira, F.; Lousa, D.; Valério, M.; **Medina-Lopes, M**.; Bergman, M.-L.; Gonçalves, J.; Demengeot, J.; Leite, R.B.; Lilue, J., Ning Z, Penha-Gonçalves C, Soares H., Soares C. M., Amorim M. J. Amino acids 484 and 494 of SARS-CoV-2 spike are hotspots of immune evasion affecting antibody but not ACE2 binding. ***2021***, ***PLOS pathogens***, 17(8): e1009772. https://doi.org/10.1371/journal.ppat.1009772. The manuscript may also be assessed in **bioRxiv,** 2021.2004.2022.441007, https://doi.org/10.1101/2021.04.22.441007.

2.2 INTRODUCTION

The fast spread and rapid evolution of SARS-CoV-2 infection from China in late 2019 led to the emergence of multiple variants (Fig.2.1) throughout the world [1]. Several viral mutations, particularly in the spike protein, have provoked global alarm due to the possibility of viral escape from host immunity upon natural infection and vaccination, rendering the strategies to control the virus ineffective [2–4].

SARS-CoV-2 infects cells after attaching its spike protein RBD to the ACE2 receptor on host cell surface. This means that the interaction of viral spike protein-host ACE2 receptor plays a central role in viral infectivity and transmissibility. In addition, the spike protein is the viral protein that elicits the strongest immune response and, therefore, is the target of COVID-19 vaccines [5–7]. Due to its essential functions, mutations in the spike protein, particularly in the RBD, may affect viral interactions with the host. These include viral interactions with the host receptor, recognition by neutralizing antibodies and escaping immunity elicited by natural infection or vaccines, transmissibility, and treatment strategies [4,8–10]. Therefore, maintaining a close surveillance on circulating and novel variants is essential to understand the impact and the dynamics of SARS-CoV-2 evolution in the population.

Epidemiological and genetic studies combined enable real-time tracking of SARS-CoV-2 genetic diversity and evolution [11–14]. This effort has been possible by advanced sequencing technologies and global data-sharing via deposition on online open-source platforms such as the Global Initiative on Sharing All Influenza Data (GISAID), NEXSTRAIN, and Phylogenetic Assignment of Named Global Outbreak Lineages (PANGOLIN) [15–17].

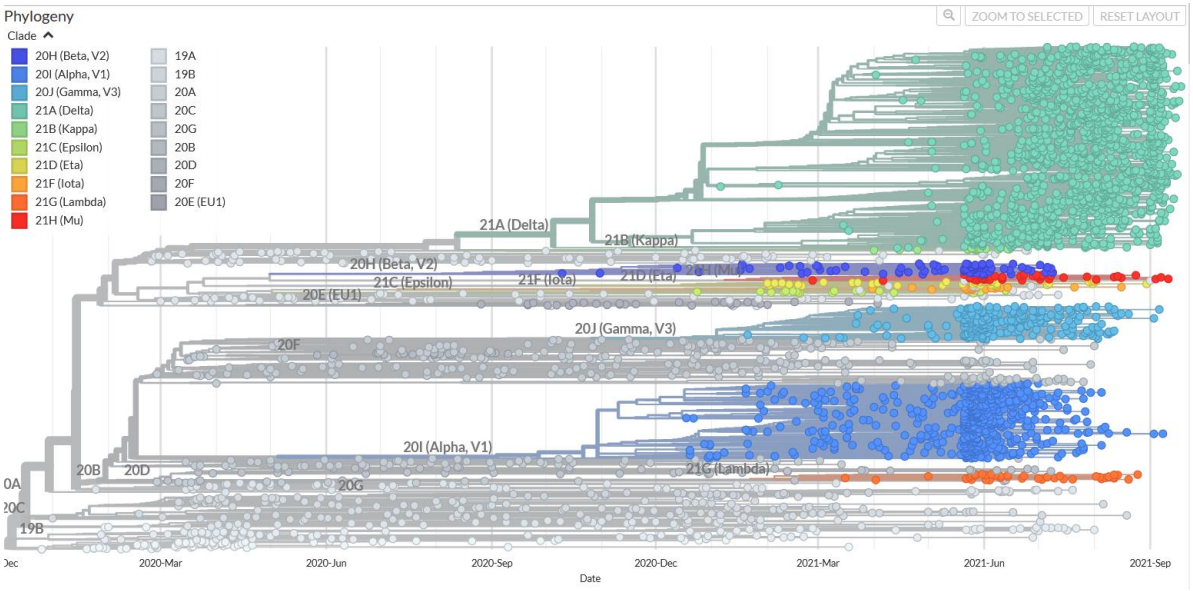

Figure 2.1 | Genomic epidemiology of SAR-CoV-2 variants. Global sequencing data from the initial outbreak back in December 2019 to the current date. From December 2020, the rapid expansion of variants of concern (VOCs) is evident, mainly the Alpha variant (B.1.1.7) and, more recently, the Delta variant (B.1.617.2). Graphic downloaded from NEXSTRAIN and available at https://nextstrain.org/ncov/gisaid/global [16].

By mid-2020, the original virus first detected in China was replaced by a new variant that encompasses a non-synonymous mutation at position 614 of the spike protein due to the substitution of aspartic acid (D) for glycine (G) (D614G). This variant, which is now considered the wild type, is present in every new lineage's genome and has caused increased viral transmissibility among the population [9,18], without increasing mortality or COVID-19 severity [4,8,18].

Reports of variants with characteristics enabling higher transmissibility and/or immune escape began to fast spread in late 2020. So far, four variants were classified as VOCs and include: Lineages Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and the recently added Delta (B.1.617.2), which were first detected in the United Kingdom (UK), South Africa (SA), Brazil, and India, respectively [14,19,20].

The Alpha (B.1.1.7) variant spread from the UK [21] to the rest of the world and became prevalent in more than 150 countries [17]. Of note, this variant contains mutations in the spike protein RBD, including N501Y, which is associated with increased transmissibility and higher binding affinity with ACE2 [14,22]. The Beta (B.1.351) and Gamma (P.1) variants, discovered in South Africa and Brazil, respectively, were alarming variants reported in late 2020 [2,3,19]. They share the N501Y mutation with the Alpha variant (B.1.1.7) and comprise two additional significant mutations at positions 417 (K417N for Beta variant and K417T for Gamma variant) and 484 (E484K present in both variants) of the spike protein. The E484K mutation is also shared by several lineages, including the former variants of interest (VOIs) Eta (B.1.525) and Iota (B.1.526) [23]. The great concern around this mutation relies on its potential to reduce the neutralization capacity of antibodies elicited by natural infection or immunization, which was demonstrated in several studies [10,24–27]. Mutations of interest on spike protein of each variant of concern or interest are shown in Table 2.1.

Table 2.1 | Summary of currently described (as of July 26, 2021) SARS-CoV-2 VOCs and VOIs. Table from [1].

Pango lineage	Nextstrain clade	First detection location	VOI or VOC	WHO designation	Mutations of interest on S protein
B.1.1.7	20I/501Y.V1	United Kingdom	VOC	Alpha	N501Y, E484K , P681H, D614G
B.1.351	20H/501Y.V2	South Africa	VOC	Beta	N501Y, K417N, E484K, D614G
P.1	20J/501Y.V3	Brazil/Japan	VOC	Gamma	N501Y, K417T, E484K, D614G
B.1.427 and B.1.429	21C/S:452R	United States (California)	VOI	Epsilon	L452R, D614G
B.1.525	21D	United States (New York)/Nigeria	VOI	Eta	A67V, E484K, D614G, Q677H, F888L
B.1.526	21F	United States (New York)	VOI	Iota	L5F , T95I, D253G, S477N , E484K, D614G, A701V
B.1.617.1	21B/S:154K	India	VOI	Kappa	(T95I), G142D, E154K, L452R, E484Q, D614G, P681R, Q1071H
B.1.617.2	21A/S:478K	India	VOC	Delta	T19R, G142D , 156del, 157del, R158G, L452R, T478K, D614G, P681R, D950N

Another mutation of potential interest, L452R, associated with former VOI Epsilon (B.1.427/B.1.429 lineages), caused a significant increase in cases in the State of California, USA, and is associated with a moderate reduction in the neutralization of antibodies from convalescent serum [28]. This mutation is also associated with the Delta variant (B.1.617.2 - which contains additional mutations in the RBD, T478K and P681R), and Kappa variant (B.1.617.1 – with additional mutations L452R and E484Q) declared as VOC and VOI on May 11 and 31, 2021, respectively [29]. They are responsible for the exponential increase of thousands of cases and deaths in India since March 2021 [29,30]. The prevalence of the Delta variant has been increasing exponentially and is currently the dominant variant, present in more than 180 countries, replacing the global prevalence of the Alpha variant (https://sarscoverage.org/).

By now, it is well clear that SARS-CoV-2 evolves fast, which is evidenced by the establishment of several additional lineages worldwide such as: Epsilon (represented by B.1.427 and B.1.429 lineages first detected in California, USA), Eta (B.1.525 first detected in UK and Nigeria), Iota (B.1.526, first detected in New York, USA) [31], Kappa (B.1.617.1, first detected in India) [32,33], Delta (B.1.617.2, first detected in India) [29]and Theta (P.3, first detected in Brazil), and many others [1,23]. Given their potential in rendering vaccination and therapies ineffective, there is the need for close surveillance of viral evolution and determining its effect on host immunity upon vaccination and natural infection, disease severity and transmission.

With millions of people recovered and the efforts of vaccination worldwide to control the disease, it is essential to understand how these variants overcome host immunity. Answering this question will demonstrate how mutations could result in reinfections and/or reduce vaccine efficacy and viral circulation. High transmissibility (*e.g.*, D614G and N501Y) [9,14,18,21] and immune evasion capacity (*e.g.*, E484K) [26,27,34,35] are now the most evident impacts of the most worrying variants, leading to international policies being adjusted to manage the pandemic.

In this sense, it is necessary to keep track of SARS-CoV-2 genetic diversity, evolution, and epidemiology to determine how these mutations (mainly in the spike RBD) affect viral entry and replication, infectivity, and transmissibility, as well as the potential to reduce antibody neutralizing capacity from natural infection or vaccination.

In this study, individual and combined SARS-CoV-2 spike protein mutations with epidemiological significance and reported globally in VOCs and VOIs were analyzed and used to engineer spike-pseudotyped lentiviral particles to evaluate their individual or synergistic effect on neutralizing capacity of antibodies elicited by natural infection. The manuscript has, in addition, included the analysis of sera of vaccinated individuals, however as the M.Sc. candidate did not contribute to these figures of the manuscript, these were not included in the present chapter.

2.3 MATERIALS AND METHODS

2.3.1 Patients and human convalescent sera

Sixteen healthcare professionals who were infected with SARS-CoV-2 in mid-2020 and diagnosed by RT-qPCR on nasopharyngeal swabs were enrolled in this project. Peripheral blood was collected by conventional phlebotomy. The serum was processed following standard procedures and kept at -20°C until use. All patients signed an informed consent authorizing their biological samples for research purposes, and international and national guidelines were used for patient data protection. The Ethics committees of Centro Hospitalar Lisboa Ocidental and Centro Hospitalar Lisboa Central authorized this study, which was implemented in agreement with the Declaration of Helsinki and good clinical practice.

2.3.2 Cells and Plasmids

The cells and plasmids used in this study are shown in Table 2.2. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, 21969035) supplemented with 10% fetal bovine serum (FBS, Gibco, 10500064), 1% penicillin/streptomycin solution (Biowest, L0022) and 2mM L-glutamine (Thermo Fisher, 25030024), at 37 ºC and 5% CO_2 atmosphere. Competent XL10 Gold *E. coli* cells were kept at -80ºC and tawed on ice before use.

Table 2.2 | Cells and plasmids used in this study.

		Source
Cells	Human Embryonic Kidney 293T	Prof Paul Digard, Rolsin Institute, UK
	293ET	Dr Colin Adrain, IGC, Portugal
	Competent XL10 Gold *E. coli* cells	Homegrown (CBV Lab)
Plasmids	pLEX.MSC	Thermo Fisher Scientific
	psPAX2	Dr Luís Moita, IGC, Portugal
	pCAGGS-SARS-CoV-2-S, NR-52310	BEI Resources

2.3.3 Spike site-directed mutagenesis

The SARS-CoV-2 spike mutations and variants were selected based on their global frequency and distribution, reported in the online open-source platform GISAID [17], between December 2019 and March 2021. The site-directed mutagenesis (SDM) was the technique used in this study to introduce the selected mutations into the plasmid pCAGGS encoding the spike protein of SARS-CoV-2. The C-terminal 19 amino acids encompassing the ER-retention domain of the cytoplasmic tail of spike were deleted by Marta Alenquer and Filipe Ferreira to facilitate incorporation of spike protein into lentiviral pseudovirions.

The SDM methods are used to alter nucleotide sequences of a specific gene by introducing point mutations, such as substitutions or deletions [36]. These alterations are used to evaluate the function/effect of a particular gene and the respective protein for which it codifies. The method allows the amplification

of specific DNA (deoxyribonucleic acid) sequences through the polymerase chain reaction (PCR) technique.

2.3.3.1 PCR and Overlap PCR (OE-PCR)

PCR is a powerful technique used in molecular biology, developed by Kary Mullis in 1983 and used to produce high amounts of synthetic DNA [37]. It is a fast and efficient method that involves the artificial and exponential amplification of a target DNA sequence by the specific binding of complementary and smaller DNA sequences (primers) [36]. Marta Alenquer and Filipe Ferreira optimized the site-directed mutagenesis protocol, using a well-established adapted version of PCR, known as overlap extension PCR (OE-PCR) and represented in Fig.2.2 [36,38]. The OE-PCR technique was used to engineer recombinant plasmids containing single, double, triple up to all defining mutations of SARS-CoV-2 variants selected for this study.

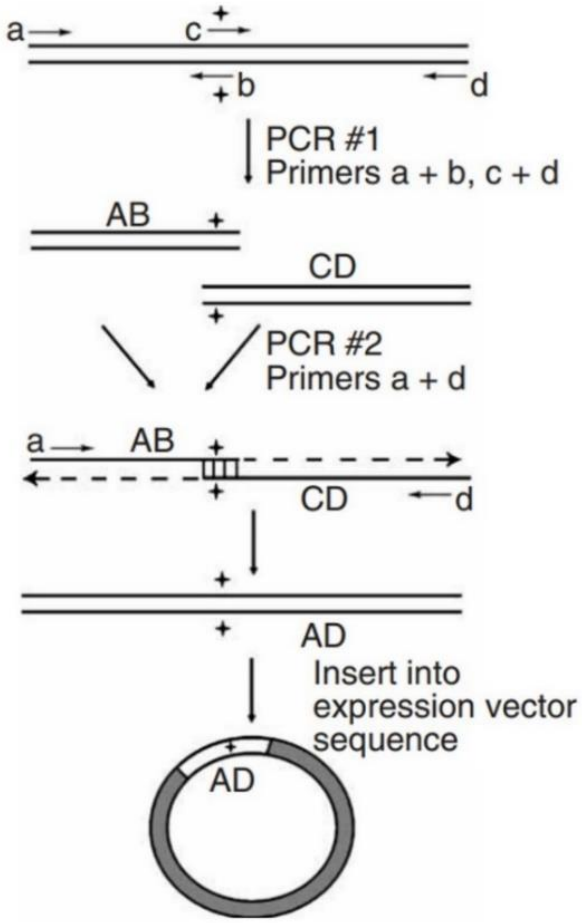

Figure 2.2 | Schematic illustration of the OE-PCR method. Briefly, two intermediate PCR products (AB and CD) are first amplified using two outer primers with restriction sites (a+d) that are used to flank the 5' end of AB and the 3' end of CD, and two inner mutagenic primers (c+b) with overlapping sequences that are used to introduce the selected mutations (cross regions). Thus, the two intermediate PCR products with the selected mutation and overlapping segments are generated and used as template DNA to amplify, by OE-PCR, the full-length segment (AD) encompassing the target mutations. The recombinant sequence is then inserted into an expression vector plasmid and used to transform *E. coli* cells. Figure from [38].

Mutagenic primers encompassing the selected mutations were designed in the online program http://www.genomics.agilent.com/primerDesignProgram.jsp using as template the codon optimized spike plasmid (pCAGGS-SARS-CoV-2-S, NR-52310) (Table S1 of supplementary material). Primers were ordered from Integrated DNA Technologies-IDT. The DNA fragments containing the selected mutations were generated in a 50µL reaction containing the template plasmid pCAGGS SARS-CoV-2-spike protein, forward and reverse primers, and a master mix containing the reagents and concentrations detailed in Table 2.3.

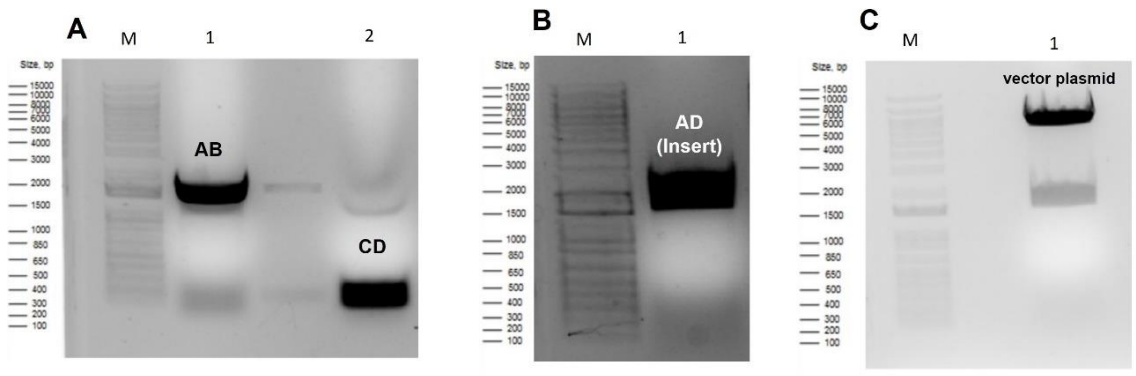

Figure 2.3 | Agarose gel electrophoresis of PCR products from Kappa variant (double mutation: L452R/E484Q) site-directed mutagenesis (SDM) as a representative SDM of all spike mutants engineered in this study.
A: Intermediate PCR products (**Lanes 1-2):** Two DNA intermediate fragments (AB-1574bp; CD-129bp) generated by PCR using mutagenic primers containing selected mutations. After gel excision and purification, the two fragments were used as a template to generate the full-length fragment AD, represented in lane 1 of image B.
B: Overlap PCR (Lane 1): Fragment AD (1668bp), generated by overlap extension PCR of AB and CD intermediate PCRs from image A.
C: Expression vector digestion (Lane 1): Digested plasmid pCAGGS (vector-6860bp) encoding the spike protein. Both insert of image B and plasmid of image C, are digested with the same restriction enzymes before the ligation and transformation reactions (in this case, *Xba*I and *Sph*I). **Lane M:** Molecular marker weight (1kb plus DNA ladder, Invitrogen, USA).

PCR reactions were performed in a BioRad T100^{TM} thermal cycler, and the PCR program is detailed in Table 2.4. Following a double-strand DNA denaturation step, the PCR reactions went through 30 cycles of denaturation, primer annealing to template DNA, and complementary strand extension. The final extension step was carried out at 72°C for 5 minutes. For the OE-PCR reactions, fragments generated in separate PCR reactions were combined and used as template DNA to have a final concentration of 50 ng/µL and 1 µL was added to the reaction.

Table 2.3 | PCR reaction reagents.

PCR mix	Final concentration
Template plasmid DNA	50ng
Forward primer	0.5µM
Reverse primer	0.5µM
dNTPs	200µM
5X Phusion HF Buffer	1X
DMSO	3%
Phusion DNA polymerase	1.0 units
Double distilled water	-
Total	Up to 50µL

Table 2.4 | PCR Thermocycler program.

Step	Cycles	Temperature	Time
Initial denaturation	1	98 ºC	2 minutes
Denaturation	30	98 ºC	10 seconds
Annealing	30	70 ºC	30 seconds
Extension	30	72 ºC	60 seconds
Final extension	1	72 ºC	5 minutes
Infinite hold	-	4 ºC	∞

After the amplification cycles, the PCR reactions were cleaned up using the NZYGelpure kit (Nzytech, genes and enzymes, Portugal) according to manufacturer's instructions. The DNA was eluted from the spin membrane after centrifugation and stored at -20°C until use.

2.3.3.2 Electrophoresis

PCR and OE-PCR amplified products were confirmed and separated by 0.8% agarose gel electrophoresis, stained with Xpert Green DNA Stain (1:20'000-Grisp Research Solutions), visualized and digitally documented under ultraviolet (UV) light on the Gel Doc transilluminator (Bio-Rad, TX, USA). To visualize the migration of the DNA fragments on the agarose gel, 10µL of loading dye (6x, New England BioLabs) was added to the total volume (50µL) of each PCR reaction. After loading the PCR products onto the agarose gel, electrophoresis was carried out in a horizontal electrophoresis tank (Bio-Rad, TX, USA) containing 1x TAE (Tris-acetate-EDTA) for 30 minutes at 100 V. Determination of molecular size (base pairs-bp) of the PCR products was made using the 1Kb plus DNA ladder molecular marker purchased from Invitrogen, Thermo Fisher, USA.

Once the correct sizes of the PCR products were separated and confirmed, they were purified. The DNA bands were excised from the agarose gel with a sharp scalpel, under UV light and following the instructions of the NZYGelpure kit (Nzytech, genes and enzymes, Portugal). After DNA elution, its concentration was measured spectrophotometrically and stored at -20°C.

2.3.3.3 Overlap PCR and plasmid pCAGGS enzymatic digestion

The OE-PCR products and plasmid were both subjected to enzymatic digestion with the same restriction enzymes to yield the same cohesive ends for further insert and vector ligation. First, the OE-PCR products were cleaned up with the NZYGelpure kit (Nzytech, genes and enzymes, Portugal), following the manufacturer's instructions. The tubes containing the digestion reactions were incubated in a water bath for 1h30 minutes at 37°C. Electrophoresis was performed after the incubation (described above in section 2.3.3.2) to separate the DNA fragments. Then, the excised bands were purified with the NZYGelpure kit (Nzytech, genes and enzymes, Portugal), following the manufacturer's instructions and the DNA concentration was measured.

2.3.4 Ligation

The ligation of the insert generated by OE-PCR and the plasmid vector pCAGGS was performed using the Quick Ligation™ kit (New England Biolabs – NEB, USA) with a molar ratio of 1:3 for vector to insert. Then, the reactions were incubated at room temperature (21°C) for five minutes and cleaned up with the DNA clean & concentrator kit (Zymo Research, USA) according to manufacturer's instructions. The ultra-pure DNA was used immediately for transformation protocol or stored at -20ºC.

2.3.5 Transformation of competent XL10 Gold *E. coli* cells

Competent XL10 Gold *E. coli* cells were transformed with pCAGGS-SARS-CoV-2-S plasmid by heat shock after ligation reactions. The transformation was performed using the competent X10 Gold as follows: cells were thawed on ice, and 100 µL of these cells were added to 9 µL of ligation per microcentrifuge tubes. Reactions were gently mixed and incubated on ice for 30 minutes. The transformation reactions were heat pulsed for 30 seconds at 42ºC and then placed on ice for 5 minutes. Then, 0.5 mL of Super Optimal Broth (SOC: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose), previously preheated to 37ºC, was added to each microcentrifuge tube incubated at 37°C for 1 hour, shaking at 225–250 rpm. The reactions were then spined down, plated into Luria-Bertani broth (LB: 10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L

of NaCl, pH to 7.0 adjusted with 1 N NaOH) supplemented with ampicillin (50µg/mL), and incubated overnight at 37ºC.

2.3.6 Plasmid isolation and diagnostic digestion

To isolate the recombinant plasmids, single colonies were picked from a fresh selective agar plate and cultured t in 1-5 mL of LB medium containing ampicillin. The cultures were incubated for 12–16 h at 37 °C with vigorous shaking. In the next day, the NZYMiniprep kit (Nzytech, genes and enzymes, Portugal) was used to isolate plasmid DNA from the *E. coli* cells by alkaline lysis followed by adsorption of DNA onto silica in the presence of high salt concentration. Restriction enzyme digestion following agarose gel electrophoresis (as described in section 2.3.3.2) was made to confirm the correct size of plasmids and insert with the selected mutations.

2.3.7 Sequencing

Confirmation and screening of positive clones, *i.e.*, containing the desired DNA mutations and no unwanted mutations was done through Sanger sequencing service from https://eurofinsgenomics.eu/, using the primers in table S1 of supplementary material. The sequences obtained were aligned using Benchling program available at https://www.benchling.com/.

2.3.8 Production and titration of spike-pseudotyped lentiviral particles

The spike-pseudoviruses harboring SARS-CoV-2 spike mutations were produced through a 2nd generation lentiviral approach using three plasmids: pLex-GFP lentiviral reporter, psPAX2 packaging vector, and pCAGGS-SARS-CoV-2-S WT, or mutants. A 293ET cell line was used as the packaging cells to produce the spike-pseudotyped lentiviral particles as follows: 3x10^6 293ET cells were co-transfected with 8.89µg of pLex-GFP reporter, 6.67µg of psPAX2, and 4.44µg of pCAGGS-SARS-CoV-2-S WT or mutants, using jetPRIME and following manufacturer's instructions.

After three days, the lentivirus-containing supernatant was harvested and concentrated 20-fold with Lenti-XTM Concentrator (Takara, 631231). Lentiviral particles were aliquoted and stored at -80ºC until use. To determine pseudovirus titers, 5x10^4 293T cells expressing SARS-CoV-2 ACE2 receptor (293T-ACE2) were infected with serial dilutions of pseudovirus stocks. Marta and Filipe used flow cytometry to quantify the percentage of cells expressing GFP 24 hours after transduction and the number of transduction units per mL.

2.3.9 Neutralization assays

In vitro neutralization assays using spike-pseudoviruses are immunoassays widely used to assess human antibodies responses against SARS-CoV-2 variants [27,39–41]. We performed *in vitro* neutralization assays to evaluate the neutralizing capacity of convalescent sera antibodies against the spike-pseudotyped lentiviral particles engineered in this study. 1x10^4 293T-ACE2 cells were seeded on 96 well plates previously treated with 0.1 mg/mL of poly-d-lysine. The next day, heat-inactivated sera were four-fold serially diluted over seven dilutions, starting with a 1:30 initial dilution. 4x10^3 transduction units of spike-pseudotyped lentiviral particles were added to each serum dilution (for a final MOI of 0.2), and incubated for 1hr at 37°C. The mixtures of spike-pseudovirus particles and serum dilutions

were added to the pre-seed 293T-ACE2 cells plate, in triplicate, and were incubated for 48h. The fluorescence signal from the 96 well plates was measured using the Glowmax Explorer System (Promega). The relative fluorescence units were normalized to those derived from the virus control wells (cells infected in the absence of plasma or serum) after subtracting the background in the control groups with cells only.

2.3.10 Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 9. The half-maximal neutralization titer (NT_{50}), defined as the reciprocal of the dilution at which the infection was decreased by 50% was determined using a four-parameter nonlinear regression in GraphPad Prism 9. A two-tailed Wilcoxon matched-pairs signed-rank test was used to compare the NT_{50} of WT and mutant pseudoviruses. Differences were considered statistically significant for values of *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

2.4 RESULTS

2.4.1 Spike site-directed mutagenesis and construction of spike-pseudotyped viruses

The first phase of this project involved selecting high-prevalent mutations with a significant impact on the pandemic's evolution, followed by the production of spike-pseudotyped lentiviral particles harboring those mutations. The third and final phase involved performing *in vitro* neutralization assays on engineered spike-pseudoviruses to evaluate their potential to escape neutralizing antibodies of human convalescent sera.

The most significant SARS-CoV-2 mutations documented through reports and genetic epidemiological research are those in the spike protein [4,8,10,42,43]. Its RBD interacts specifically with the host cell receptor ACE2 to promote viral entry, and it is well documented that it is the protein that elicits an enhanced immune response in the host [6,44–46]. Thus, mutations in spike protein, especially its RBD, can lead to potential structural changes affecting viral entry and antibody recognition dynamics [18,22,47,48].

Therefore, only mutations described in spike protein were selected for this study. Mutations were selected in the open-source platform GISAID between December 2019 and March 2021 and based on their frequency and global distribution. Table 2.5 shows the high prevalent mutations shared by the VOCs Alpha (B.1.1.7), Beta (B.1.351) and Gamma (P.1) and the former VOI Kappa (B.1.617.1).

Point mutations were first introduced in plasmids encoding the SARS-CoV-2 spike protein. The cloning strategy was achieved by SDM using an overlap extension PCR (OE-PCR) approach [36,38], followed by ligation and transformation protocols. In this way, nine recombinant plasmids were engineered (Table 2.5), containing single, double, and multiple defining mutations of Alpha, Beta, Gamma and Kappa (B.1.617.1) variants.

After confirming positive clones by sequencing methods, each plasmid encoding spike mutations was used alongside two additional plasmids for spike-pseudotyped lentiviral production. The spike-pseudoviruses were produced through a 2nd generation lentiviral system that divides the components of lentiviral particles production in three plasmids. Aside from the plasmid expressing the mutated SARS-CoV-2 protein, it also encompasses packaging plasmid comprising Gag and Pol genes and a plasmid encoding the lentiviral genome and a green fluorescence protein (GFP) reporter. This way, the chimeric viruses engineered comprise the lentiviral genome depleted of packaging and surface protein coding sequences and encoding a GFP reporter, as well as the SARS-CoV-2 spike glycoprotein integrated into its envelope so they can engage with the host cell ACE2 receptor and mimic SARS-CoV-2 viral entry stages. Because cells infected by these lentiviruses express GFP, the infection rates, expressed as relative fluorescent units, were quantified in a plate fluorometer (GloMax Explorer System, Promega).

Table 2.5 | Detailed list of mutations and engineered spike-pseudotyped lentiviruses for *in vitro* neutralization assays. The candidate of this M.Sc. thesis has engineered the plasmids with the mutations: **E484K; S494P; S494P/N501Y; E484K/S494P; E484K/S494P/N501Y and L452R/E484Q 4-9)** and also the plasmids with all defining mutations of Alpha, Beta and Gamma variants of concern (VOC) with Filipe Ferreira (1-3).

#	Spike protein amino acid substitutions/deletions	Mutation's description
1	**del69H-70V/del144/ N501Y / A570D / P681H /T716I/ S982A / D1118H**	**VOC: Alpha (α) variant** – Lineage B.1.1.7 (20I/501Y.V1) – It was announced for the first time in 14 December 2020 in United Kingdom [21,49,50].
2	**D80A / D215G / K417N /E484K/N501Y /A701V**	**VOC: Beta (β) variant** – Lineage B.1.351/ (20H/501Y.V2) – It was first detected in December 2020 in South Africa [27,34,43,51].
3	**L18F / T20N / P26S / D138Y / R190S/ K417T /E484K/N501Y/H655Y / T1027I**	**VOC: Gamma (γ) variant** – Lineage P.1/ (20J/501Y.V3) – Announced for the first time in January 2021 in Manaus, Brazil [2,3].
4	**E484K**	E484K is located in the spike RBD and is part of the Beta and Gamma variants. It has been implicated with the attenuation of neutralizing antibodies generated by human convalescent sera and immunization [10,24,26,51].
5	**S494P**	S494P is also in the spike RBD. S494P mutation was first detected in the UK and associated with the Alpha variant (B.1.1.7). It is being monitored in the UK, and studies have shown it to be a residue with a high binding affinity to ACE2 receptor and the potential to escape antibody binding.[47,49].
6	**S494P/N501Y**	N501Y is a spike RBD mutation found in three variants of concern: Alpha (B.1.1.7), Beta (B.1.351) and Gamma (P.1). It is associated with high binding affinity to ACE2 receptor and infectivity [14,21,22,52].
7	**E484K/S494P**	E484K is located in the spike RBD and is part of the Beta and Gamma variants. It has been implicated with the attenuation of neutralizing antibodies generated by human convalescent sera and immunization [10,24,26,51,53].
8	**E484K/S494P/N501Y**	This triple mutation has not yet been detected in existing variants.
9	**L452R/E484Q**	L452R – A spike RBD mutation found in the Epsilon variant (B.1.427/9/ 20C/S, California, USA), variant of concern Delta (B.1.617.2) and Kappa variant (B.1.617.1), first detected in India. Associated with increased infectivity and escape antibody binding. E484Q is found alongside L452R in Kappa variant (B.1.617.1)[29,30]. Associated with attenuation of neutralizing antibodies [32,51,54,55]

2.4.2 Spike-pseudotyped lentiviral particles neutralization assays

In vitro neutralization assays using spike-pseudoviruses are immunoassays widely used to assess human antibodies responses against SARS-CoV-2 variants [27,39–41]. In brief, *in vitro* neutralization assays were performed incubating in a 96 well plate pre-seeded with 293T cells expressing the human ACE2 receptor, a mixture of four-fold serial dilutions of sera from recovered COVID-19 health professionals and the engineered spike-pseudotyped viruses. The 96 well plates were read on a fluorescence reader plate after 48 hours of incubation to evaluate the efficacy of convalescent sera antibodies in neutralizing spike-pseudotyped viruses cell entry.

The 293T cell line expressing the human ACE2 receptor and the assay specificity was developed and tested by Marta Alenquer and Filipe Ferreira. For this study, sera from sixteen health professionals infected by SARS-CoV-2 in mid-2020 were used alongside one pre-pandemic and three contemporary sera as negative controls.

Collaborators in this study (Jocelyne Demengeot, IGC, and Helena Soares, CEDOC, NOVA) characterized the sera used in this study using the Enzyme-Linked Immunosorbent Assay (ELISA) technique and divided them into four categories according to their anti-spike IgG titers: low (1:150), medium (1:450), and high (1:1350). The analysis of the neutralization curves of each serum for the WT virus in Fig.2.4 revealed that sera with higher content of anti-spike IgG were able to neutralize spike-pseudoviruses mutants more efficiently than lower titer sera. This consistency between the anti-spike IgG content of the different categories of serum used and its respective neutralization titer is in accordance with research published in the literature [41,56–58].

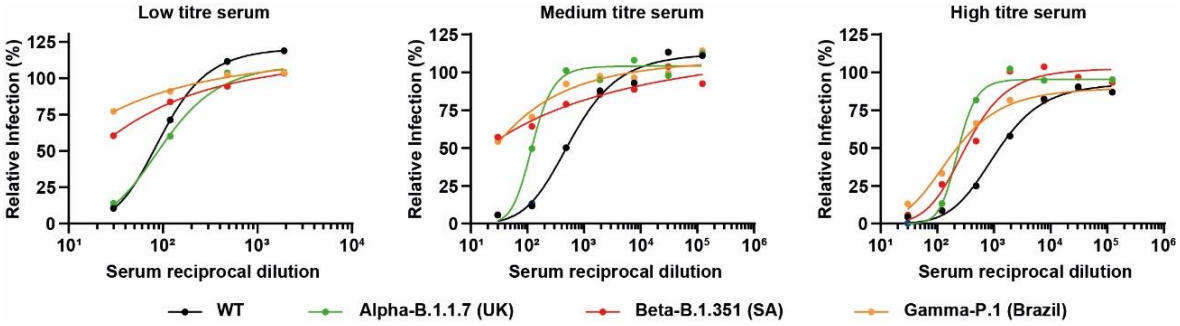

Figure 2.4 | Neutralization curves of SARS-CoV-2 spike-pseudotyped variants of concern by human convalescent sera. The neutralizing activity of the WT, Alpha (B.1.1.7 – United Kingdom), Beta (B.1.351 – South Africa) and Gamma (P.1 – Brazil) variants of concern by low, medium, and high titer convalescent sera is represented in different colors. The relative infection is represented as the percentage of infection relative to cells infected in the absence of serum (virus only).

Regarding the relative infection of the VOCs, the viral entry of the Alpha variant (B.1.1.7) suffered a modest decrease when compared to the WT pseudovirus. Beta (B.1.351) and Gamma (P.1) variants were far more resistant to neutralization, displaying higher percentages of relative infection with low sera dilutions (Fig.2.4).

A non-parametric Wilcoxon statistical test was used to evaluate the differences between the neutralization titer NT_{50} of variants and WT pseudoviruses (Fig. 2.5). The NT_{50} titer was calculated for each neutralization curve, and it was defined as reciprocal dilution of serum that decreased the infection by 50%. Additionally, an approach based on the 4-fold criteria used for update of vaccines against influenza viruses[59,60] was used to test the ability of spike pseudotyped mutants to evade neutralization of antibodies elicited by convalescent sera (Fig. 2.7).

As illustrated in Fig.2.5, the analysis of neutralization titers of pseudoviruses with all defining mutations of the Alpha variant (B.1.1.7) showed a moderate reduction of neutralization by convalescent sera when compared to WT spike ($p<0.01$) but did not surpass the stipulated 4-fold criteria, being efficiently neutralized by the convalescent sera (Fig.2.7, A). A different pattern was observed for the spike-pseudoviruses encompassing all the defining mutations of Beta (B.1.351) and Gamma (P.1) VOCs. In addition to having neutralizing titers significantly different from the WT (Fig.2.5), these spike-pseudoviruses also reduced the neutralization titers capacity over the 4-fold threshold criteria (Fig.2.7, B).

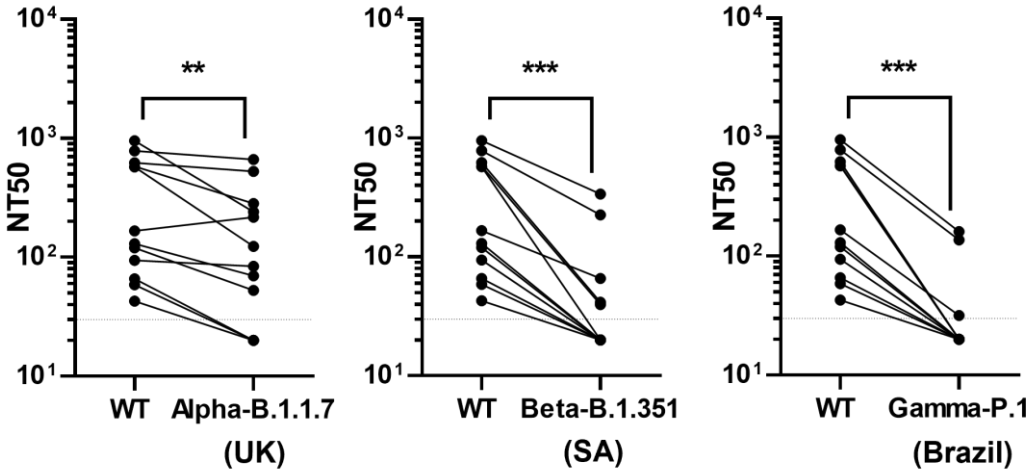

Figure 2.5 | Neutralization titers of spike-pseudotyped variants of concern by human convalescent sera. The neutralizing activity of each serum is represented as a paired analysis between the WT strain and each variant of concern: Alpha (B.1.1.7), Beta (B.1.351) and Gamma (P.1), originally detected in United Kingdom, South Africa, and Brazil, respectively. Neutralizing titer (NT_{50}) is defined as the reciprocal dilution that reduced the infection by 50%. The limit of detection of the assay is represented by dashed lines (NT_{50}=30). $^{**}p<0.01$, $^{***}p<0.001$ by two-tailed Wilcoxon matched-pairs signed-rank test.

Some of the mutations described in the literature as evading immune responses elicited by prior SARS-CoV-2 infection are located in the RBD region of spike. These mutations must be able to evade neutralizing antibodies without affecting spike's affinity for the ACE2 receptor. To understand which amino acid residues in spike's RBD contact more with antibodies or ACE2, a group that participated in this project (Cláudio Soares Lab, ITQB, NOVA) performed Molecular Dynamics (MD) simulations using Protein Data Bank (PDB) structures available containing the spike protein bound to antibodies or to ACE2. This analysis revealed two relevant RBD amino acid residues with high affinity for antibody binding and low affinity for ACE2 binding: residues E484 and S494.

Given the above results, five spike-pseudotyped harboring single, double and triple mutations (S494P, E484K, S494P/N501Y, E484K/S494P and E484K/S494P/N501Y) were engineered. The mutation N501Y, described as a mutation with a higher affinity binding to ACE2 receptor, was engineered alongside E484K/S494P to understand if there are synergic interactions between relevant mutations for ACE2 binding and neutralizing antibodies evasion. As a result, as seen in Fig.2.6, all spike-pseudotyped mutants were significantly different from the wild type in reducing the neutralization by convalescent sera.

Regarding the ratio NT_{50} mutant/NT_{50} WT, which allows us to define whether there was evasion of neutralizing antibodies, spike pseudotyped particles bearing E484K or S494P did not reach the 4-fold criteria (Geometric mean (SD) 0.28±0.37 and 0.39±0.28, respectively) as shown in Fig.2.7, B. However,

when S494P mutation was associated with the N501Y and E489K mutations, a more accentuated reduction was observed, with values of 0.28±0.21 and 0.25±0.32, respectively (Fig.2.7, A). The triple mutant E484K/S494P/N501Y, in addition to being significantly different from the WT spike pseudotyped virus regarding neutralization by sera convalescent (Fig.7, B), presents a decrease in neutralization greater than the 4-fold criteria (0.17±0.34).

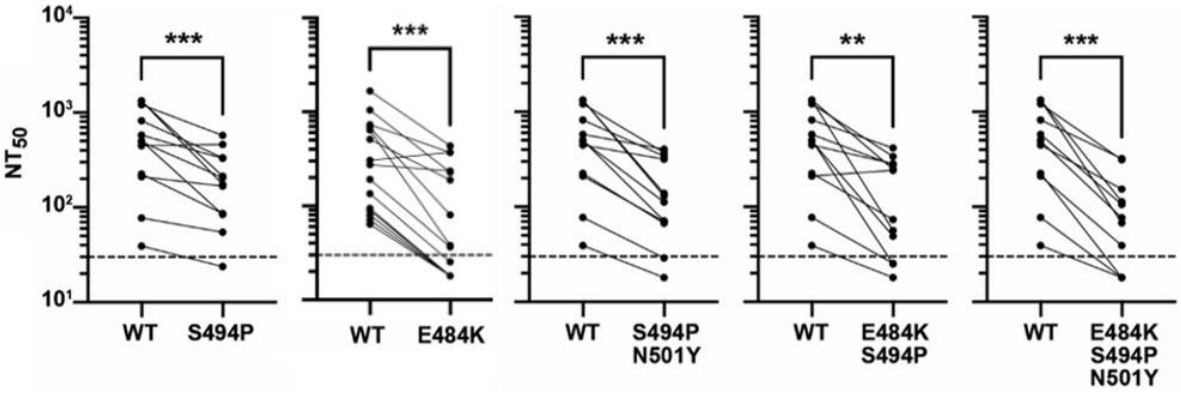

Figure 2.6 | Neutralization titers of spike-pseudotyped mutants by human convalescent sera. The neutralizing activity of each serum is represented as a paired analysis between the WT strain and single, double, and triple mutants: S494P, E484K, S494P/N501Y, E484K/S494P and E484K/S494P/N501Y. The limit of detection of the assay is represented by dashed lines (NT_{50}=30). $^{**}p<0.01$, ***$p<0.001$ by two-tailed Wilcoxon matched-pairs signed-rank test.

The lineage B.1.617, first detected in India in early 2021, is represented by the former variant of interest Kappa (B.1.617.1) and the variant of concern Delta (B.1.617.2) [30,54,55]. This lineage led to an exponential increase of COVID-19 cases in India and the UK, surpassing the frequency of the Alpha variant (B.1.1.7) in both countries and following a similar pattern in several other countries [17,29,30]. Therefore, two of the most relevant spike RBD mutations described for this lineage, L452R and E484Q [29,55], were engineered to understand the synergic effect of these two mutations on neutralization titers.

The neutralization curves of the spike pseudotyped virus bearing the double mutation L452R/E484Q are represented in Fig.2.8. As observed in Fig.2.4 for neutralizing curves of WT and variants of concern, Alpha, Beta, and Gamma, the neutralization curves of Kappa variant of the low titre sera have higher relative infection percentages because they have a reduced anti-IgG titer and therefore do not efficiently neutralize the viral entry. In contrast, the medium and high titer sera could efficiently neutralize the viral entry compared to low titer sera.

Data in Fig.2.8, B revealed that the spike-pseudoviruses harboring the double mutation L452R/E484Q behaved like the WT regarding the neutralization titers and did not escape neutralization according to the NT_{50} ratio analysis (1.8±0.68) as shown in Fig.2.7, A.

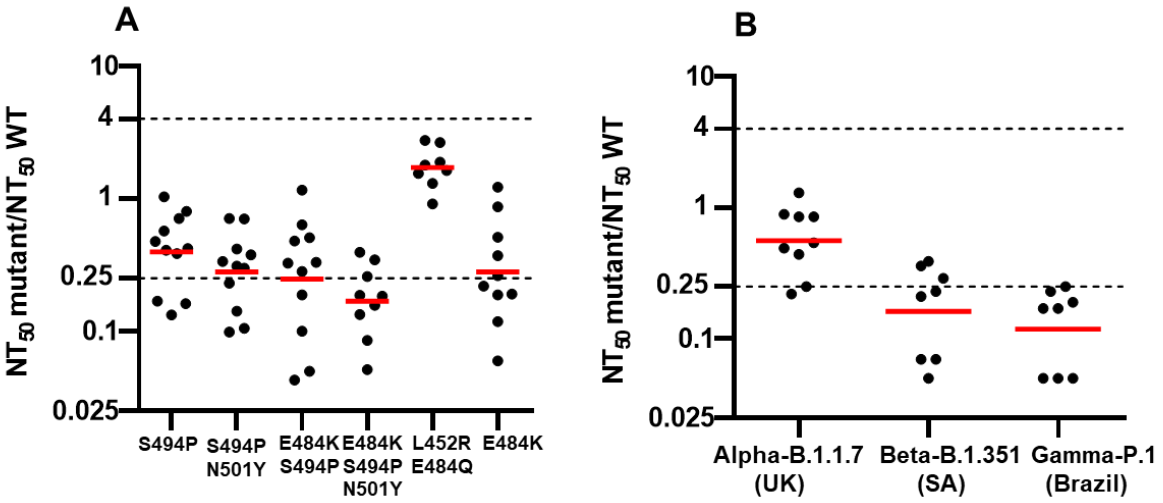

Figure 2.7 | Neutralization (NT_{50}) ratios between WT strain and spike pseudotyped viruses. **A**: Neutralization ratios of spike pseudotyped viruses harboring S494P, S494P/N501Y, E484K/S494P, E484K/S494P/N501Y, L452R/E484Q and E484K mutations. **B**: Neutralization ratios of spike pseudotyped viruses harboring all defining mutations of variants of concern: Alpha (B.1.17), Beta (B.1.351) and Gamma (P.1). Red bars indicate the geometric mean.

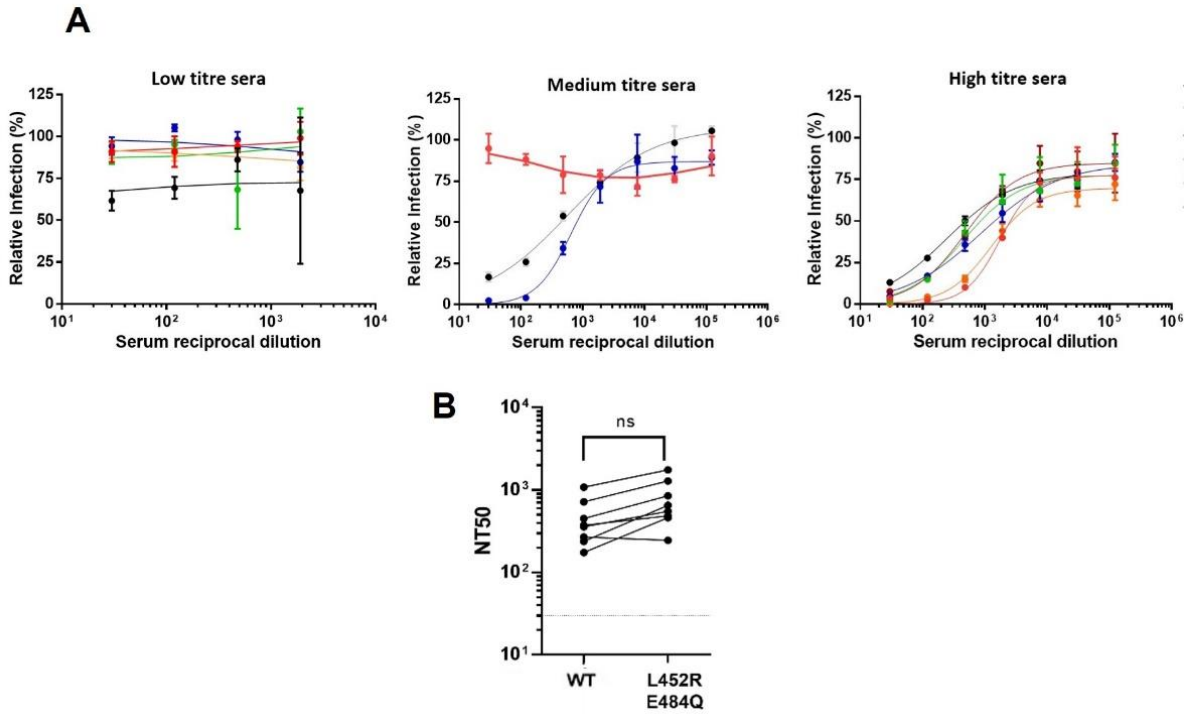

Figure 2.8 | Neutralization of spike-pseudotyped viruses harboring a double mutation of the Kappa variant (B.1.617.1) by human convalescent sera. A: Neutralization curves of spike pseudoviruses with double mutant L452R/E484Q by low, medium, and high titer of anti-spike IgG antibodies sera. The relative infection is represented as the percentage of infection relative to cells infected in the absence of serum. **B:** Neutralizing activity of each serum category, represented as a paired analysis between the WT strain and the spike-pseudotyped double mutant L452R/E484Q of Kappa variant. Neutralizing titer (NT_{50}) is defined as the reciprocal dilution that reduced the infection by 50%. The limit of detection of the assay is represented by dashed lines (NT_{50}=30). ns-non-significant, $^{**}p<0.01$, ***$p<0.001$ by two-tailed Wilcoxon matched-pairs signed-rank test.

2.5 DISCUSSION

One of the most crucial questions to be answered regarding the current COVID-19 pandemic is the evolution of SARS-CoV-2 and its impact on immunity acquired through vaccination and natural infection [61–63]. Understanding the dynamics of SARS-CoV-2 and its emerging variants is vital for identifying characteristics that allow the virus to evade neutralizing antibodies, critical for viral clearance.

The classic and gold standard plate reduction neutralizing test (PRNT) [64] is used to determine immune protection from viral infections, but it is a restrictive technique with limited throughput, not suitable to use on a wide scale. The pandemic's rapid spread and evolution prompted the development of novel approaches capable of testing the functionality of neutralizing antibodies generated by SARS-CoV-2-vaccinated and naturally infected individuals on a large scale [40,41,64].

In this regard, spike-pseudotyped lentiviral particles harboring the most significant SARS-CoV-2 mutations described to date [2,24,33,65] were engineered in this study to assess the antibody neutralization capacity of sera from recovered COVID-19 individuals using a high-throughput neutralization assay. The method developed in this project is a method that allows the quantification of fluorescence signals in 96 wells with high throughput since GFP-positive cells are used as a proxy for infection. Alongside other neutralization assay protocols that have been developed [41,42,64,66], the accessible and easily readable fluorescence in the GloMax Explorer system developed in this project allows for broader use of neutralization assays. In addition, it can be used to assess the action of antiviral drugs and monoclonal antibody that block viral entry.

In addition to the advantages of the method described above, the use of the lentiviral system to produce spike pseudotyped lentiviruses is a well-established system used to engineer replication-defective chimeric viruses, suitable and safe to use in biosafety level 2 facilities [41,42,67,68]. Given that SARS-CoV-2 propagation experiments can only be performed in biosafety level 3 (BSL-3) setting, this approach proves to be very advantageous given that BSL-3 facilities are scarcer in several countries. In addition, a study [41] comparing four neutralization assays using competent replication SARS-CoV-2 and replication-defective lentiviral particles showed that the methods shared high reproducibility and low variability, reinforcing the confidence in the use of this type of system to evaluate the viral infections.

The anti-spike content of the four human convalescent sera categories was assessed by ELISA, and the results shown in Fig.2.4, and Fig.2.8 (Graph A) are in line with the concordance found between the anti-spike IgG content of different sera and its respective neutralizing titer in several studies [41,56–58]. The neutralizing capacity of sera antibodies increased proportionally with the respective anti-spike IgG content.

Our results regarding the analysis of Beta (B.1.351) and Gamma (P.1) variants (Fig.2.7,A) showed a significant reduction in neutralization compared to Alpha (B.1.1.7) variant and the WT, which agrees with previous published data [10,34]. The Beta and Gamma variants share the E484K mutation, which is a well document mutation promoting immune evasion [10,26,42,51], and findings from this study showed that this mutation alone was able to decrease the neutralizing capacity significantly by 3.6-fold (Fig.2.7, B). Marta Alenquer and Filipe Ferreira performed a neutralization assay with spike pseudotyped lentiviruses bearing triple mutations E484K/K417N/N501Y and a more pronounced decrease was observed, suggesting that these mutations may have synergistic effects and that it is the combination of the mutations that provides an efficient evasion capacity of neutralizing antibodies as well as a good binding affinity to the ACE2 receptor. These results agree with the results recently published by [10] and [13] where

pseudoviruses bearing mutations N501Y/K417N/E484K exhibited marked resistance to neutralization compared to WT SARS-CoV-2 pseudoviruses. In the later study also using spike-pseudoviruses, N501Y mutation displayed a 13-fold increase in transduction units compared to WT pseudoviruses, while K417N and E484K alone displayed a 2-fold increase. Thus, the authors hypothesize that the emergence and spread of these SARS-CoV-2 variants is due mainly to their increased infectivity capacity, rather than neutralizing antibodies' evasion. This hypothesis makes sense considering the rapid spread and fitness advantage of viruses harboring D614G mutation [9,18,69], which was then gradually replaced worldwide by the Alpha variant containing the N501Y mutation [21,50]. These mutations are known for their high transmissibility and high worldwide prevalence, in contrast to the Beta and Gamma variants that since its emergence, despite having caused outbreaks and resurgence of infections in regions with high levels of seroprevalence, mainly in countries where they were identified for the first time [2,43,70], have not reached frequencies comparable to the Alpha variant [17]. However, monitoring these variants remains critical, mainly due to their immune evasion ability.

It is critical to develop methodologies for identifying the amino acid residues that allow SARS-CoV-2 to evade immunity. In this sense, this study also performed structural analysis of the interaction of spike-antibodies and spike-ACE2 complexes to determine which amino acid residues could evolve antibody escape mutants without affecting viral entry. Through this approach, two residues were identified at positions 484 and 494 of spike's RBD. The E484K mutation, as described above, is a well-known mutation for evading neutralization, and our findings demonstrated in Fig.2.6 and Fig.2.7, B, support this observation. According to our findings, the S494P mutation further decreases the neutralizing capacity of convalescent sera when combined with E484K and N501Y (Fig.2.7, B). The S494P mutation was first detected in the UK and associated with the Alpha variant (B.1.1.7) [49], and studies have shown that it is a residue with a high binding affinity to the ACE2 receptor and with potential to escape antibody binding [47,49]. Of note, these two amino acid residues were identified as potential vaccine escape mutations in a large scale study of 506,768 SARS-CoV-2 sequences [62] and must therefore be closely monitored.

The emergence and increase in global frequency of Alpha, Beta, and Gamma variants in late 2020 marked the beginning of the antigenic drift of SARS-CoV-2 [1,61], with several mutations accumulating in the spike protein. Since then, several variants have emerged (Table 2.1) and their circulation, especially those containing mutations that allow immune evasion, can lead to scenarios similar to influenza virus infections. In this case, rapid evolution of circulating strains leads to yearly epidemics and necessity to update vaccines.

Currently, the Delta variant became dominant [17]. It began by causing outbreaks in India, United Kingdom, and E.U.A, where several cases, hospitalizations, and deaths forced borders to close and tighten with more restrictive measures [29,30,71].

The Delta variant has been related to higher transmissibility and disease severity, as evidenced by increased hospital admissions, hospitalizations, and deaths [71]. It is also the variant responsible for significant number of breakthrough infections, *i.e.*, infections in people who have been fully vaccinated. The causes underlying the rapid dissemination of the Delta variant are incompletely defined. The Delta variant has nine mutations in the spike protein, the most notable of which are the L452R, T478K, and P681R mutations. Findings reported by Yang Liu and colleagues[72] showed that the P681R mutation, located at the furin cleavage site of the spike protein, may be the mutation that allows the Delta variant to have greater fitness compared to the Alpha variant, increasing viral replication through greater efficiency in S1/S2 cleavage. To back up their conclusions, the authors cite evidence of a more significant viral RNA load in patients infected with the Delta variant, up to 1000-fold higher than in

individuals infected with the prior variants. However, the Delta variant has additional mutations in its genome, that may be responsible for innate immune escape and/or higher capacity to infect cells and the host.

The L452R mutation, associated with the old VOI Epsilon, and the E484Q mutation are mutations in the spike RBD and present in the variant under monitoring (VUM) Kappa [54]. Of note, Kappa variant was a variant of interest (VOI) when this study was performed. A spike-pseudotyped lentiviral particle was engineered as a double mutant to understand the effect of these two mutations on the neutralizing capacity of antibodies elicited by natural infection and vaccination. According to our findings (Fig.2.8, B), the spike-pseudotyped particles harboring those two mutations were efficiently neutralized by convalescent sera, which agrees with the results reported by [54] that those two mutations are not synergic for immune evasion. At this moment, the Kappa variant, analyzed in this study, is still a VUM [31]; however, it is crucial to maintain the surveillance of these variants and especially these mutations that seem to confer some selective advantage, appearing independently in several strains through convergent evolution around the world [1]. Of note, the Delta Plus variant (B.1.617.2.1) is also being monitored and acquired the K417N mutation present in the Beta variant [73].

There are currently two variants of interest being monitored by WHO, the Mu variant (B.1.621) and the Lambda variant (C.7), first identified in Peru and Colombia and designated VOI on 06/14/2021 and 30/08/2021, respectively [31]. Interestingly, the Mu variant contains the N501Y and P681H mutations present in the Alpha variant, the D950N found in the Delta variant [23], and the E484K mutation present in the Beta and Gamma variants and identified by previous studies and by the present work as a hotspot for immune evasion [10,26,51,62]. The VOCs, VOIs, and VUMs are and will continue to be monitored by health authorities. The methodologies such as those presented in this study are crucial to help understanding the dynamics SARS-CoV-2 evolution and to be able to act accordingly to control the pandemic.

Many questions remain to be answered relative to this virus. One relates to how removing different control measures will affect viral circulation even in countries with vaccination rates above 85%. Another relates to the duration of immunity generated by the different vaccines. A third one concerns how vaccines will affect SARS-CoV-2 evolution. Emergence of variants are expected to decrease with reduced viral circulation. However, the answers to these concerns will take shape by close monitoring viral circulation. The techniques and approaches developed in this project are crucial to evaluate the prevalence and impact of mutations over time, and how they impact the immunity induced by natural infection and vaccination.

This study has some limitations. It should be highlighted that, while the spike protein is the protein that induces a more robust immune response [6,74], other SARS-CoV-2 proteins may also contribute to *in vitro* neutralization, and the use of spike-pseudotyped particles prevents its detection. In addition, the examination of sera taken from infected patients at different time intervals and geographical areas, as well as with known genomes, is advised for future investigations. Despite these limitations, this study allowed the wide scale evaluation of multiple mutations in the spike protein for their capacity to evade neutralizing antibodies produced by COVID-19 infection. Thus, the findings from this study are pertinent to the knowledge on SARS-CoV-2 and its variants evolution.

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2.7 SUPPLEMENTAR MATERIAL

Table S1- Primers used in this study.

Primer name	Sequence
INNER PRIMERS	
Spike_L18F_Fw	5'- CAGCCAGTGTGTGAACTTCACCACAAGAACCCAGC-3'
Spike_L18F_Rv	5'- GCTGGGTTCTTGTGGTGAAGTTCACACACTGGCTG-3'
Spike_T20N_Fw	5'-CAGTGTGTGAACCTGACCAATAGAACCCAGCTGCCTC-3'
Spike_T20N_Rv	5'-GAGGCAGCTGGGTTCTATTGGTCAGGTTCACACACTG-3'
Spike_P26S_Fw	5'-GAACCCAGCTGCCTTCAGCCTACACCAAC-3'
Spike_P26S_Rv	5'-GTTGGTGTAGGCTGAAGGCAGCTGGGTTC-3'
Spike_Δ69-70_Fw	5'-GGTTCCACGCCATCTCCGGCACCAATGG-3'
Spike_Δ69-70_Rv	5'-CCATTGGTGCCGGAGATGGCGTGGAACC-3'
Spike_D80A_Fw	5'-CACCAAGAGATTCGCCAACCCCGTGCTGC-3'
Spike_D80A_Rv	5'-GCAGCACGGGGTTGGCGAATCTCTTGGTG-3'
Spike_D138Y_Fw	5'-GTTCCAGTTCTGCAACTATCCCTTCCTGGGCGTCT-3'
Spike_D138Y_Rv	5'-AGACGCCCAGGAAGGGATAGTTGCAGAACTGGAAC-3'
Spike_Δ144_Fw	5'-CCCCTTCCTGGGCGTCTATCACAAGAACAACAA-3'
Spike_Δ144_Rv	5'-TTGTTGTTCTTGTGATAGACGCCCAGGAAGGGG-3'
Spike_R190S_Fw	5'-GCAACTTCAAGAACCTGAGCGAGTTCGTGTTCAAG-3'
Spike_R190S_Rv	5'-CTTGAACACGAACTCGCTCAGGTTCTTGAAGTTGC-3'
Spike_D215G_Fw	5'-ACCTCGTGCGGGGTCTGCCTCAGGG-3'
Spike_ D215G_Rv	5'-CCCTGAGGCAGACCCCGCACGAGGT-3'
Spike_K417N_Fw	5'-CCCTGGACAGACAGGCAATATCGCCGACT-3'
Spike_K417N_Rv	5'-AGTCGGCGATATTGCCTGTCTGTCCAGGG-3'
Spike_K417T_Fw	5'-CTGGACAGACAGGCACGATCGCCGACTACAA-3'
Spike_K417T_Rv	5'-TTGTAGTCGGCGATCGTGCCTGTCTGTCCAG-3'
Spike_N439K_Fw	5'-GATTGCCTGGAACAGCAAGAACCTGGACTCCAAAG-3'
Spike_N439K_Rv	5'-CTTTGGAGTCCAGGTTCTTGCTGTTCCAGGCAATC-3'
Spike_L452R_Fw	5'-GCGGCAACTACAATTACCGGTACCGGCTGTTC-3'
Spike_L452R_Rv	5'-GAACAGCCGGTACCGGTAATTGTAGTTGCCGC-3'
Spike_E484K_Fw	5'-CCCTTGTAACGGCGTGAAAGGCTTCAACTGCTA-3'
Spike_E484K_Rv	5'-TAGCAGTTGAAGCCTTTCACGCCGTTACAAGGG-3'
Spike_E484Q_Fw	5'-CCCTTGTAACGGCGTGCAGGGCTTCAACTGCTAC-3'
Spike_E484Q_Rw	5'-AGTAGCAGTTGAAGCCCTGCACGCCGTTACAAGGG-3'
Spike_S494P_Fw	5'-TACTTCCCACTGCAGCCCTACGGCTTTCAGC-3'
Spike_S494P_Rv	5'-GCTGAAAGCCGTAGGGCTGCAGTGGGAAGTA-3'
Spike_N501Y_Fw	5'-GGCTTTCAGCCCACATATGGCGTGGGCTATC-3'
Spike_N501Y_Rv	5'-GATAGCCCACGCCATATGTGGGCTGAAAGCC-3'
Spike_A570D_Fw	5'-TGGCCGGGATATCGACGATACCACAGACG-3'
Spike_A570D_Rv	5'-CGTCTGTGGTATCGTCGATATCCCGGCCA-3'
Spike_H655Y_Fw	5'-TGTCTGATCGGAGCCGAGTATGTGAACAATAGCTACGAG-3'
Spike_H655Y_Rv	5'-CTCGTAGCTATTGTTCACATACTCGGCTCCGATCAGACA-3'
Spike_Q675H_Fw	5'-CATCTGTGCCAGCTACCATACACAGACAAACAGCC-3'
Spike_P681H_Fw	5'-CACAGACAAACAGCCACAGACGGGCCAGATC-3'
Spike_P681H_Rv	5'-GATCTGGCCCGTCTGTGGCTGTTTGTCTGTG-3'
Spike_A701V_Fw	5'-AATGTCTCTGGGCGTCGAGAACAGCGTGG-3'
Spike_A701V_Rv	5'-CCACGCTGTTCTCGACGCCCAGAGACATT-3'
Spike_T716I_Fw	5'-CTCTATCGCTATCCCCATCAACTTCACCATCAGCG-3'
Spike_T716I_Rv	5'-CGCTGATGGTGAAGTTGATGGGGATAGCGATAGAG-3'
Spike_S982A_Fw	5'-TGCTGAACGATATCCTGGCCAGACTGGACAAGGTGG-3'
Spike_S982A_Rv	5'-CCACCTTGTCCAGTCTGGCCAGGATATCGTTCAGCA-3'
Spike_T1027I_Fw	5'-CCAATCTGGCCGCCATCAAGATGTCTGAGTG-3'
Spike_T1027I_Rv	5'-CACTCAGACATCTTGATGGCGGCCAGATTGG-3'
Spike_D1118H_Fw	5'-CCAGATCATCACCACCCACAACACCTTCGTGTC-3'
Spike_D1118H_Rv	5'-GACACGAAGGTGTTGTGGGTGGTGATGATCTGG-3'
Spike_Q1208H_Fw	5'-GGGGAAGTACGAGCATTACATCAAGTGGCCC-3'
Spike_Q1208H_Rv	5'-GGGCCACTTGATGTAATGCTCGTACTTCCCC-3'
OUTER PRIMERS	
Spike_*SphI*_Fw	5'-GCTGCATGCCCCTGCCACAG-3'
Spike_*SphI*_Rv	5'-GGGGCATGCAGCAGTTCGAAG-3'
Spike_*XbaI*_Fw	5'-GGCTCTAGAGCCTCTGCTAAC-3'

Spike_*HindII*I_Rv	5’-CGCCAAGCTTGGGCTGCAG-3’
SEQUENCING PRIMERS	
Spike_*SphI*_Fw	5’-GCTGCATGCCCCTGCCACAG-3’
Spike_*SphI*_Rv	5’-GGGGCATGCAGCAGTTCGAAG-3’
Spike_*XbaI*_Fw	5’-GGCTCTAGAGCCTCTGCTAAC-3’
Spike_*AgeI*_Fw	5’-TCGAACCGGTTCAACGGCATCGGA-3’
Spike_H49Y_Fw	5’-CAGATCCAGCGTGCTGTATTCTACCCAGGACCTGT-3’
Spike_D215G_Rv	5’-CCCTGAGGCAGACCCCGCACGAGGT-3’
Spike_A222V_Fw	5’-TCAGGGCTTCTCTGTTCTGGAACCCCTGG-3’
Spkie_A570D_Rv	5’-CGTCTGTGGTATCGTCGATATCCCGGCCA-3’
Spike_H655Y_Fw	5’-TGTCTGATCGGAGCCGAGTATGTGAACAATAGCTACGAG-3’
Spike_T716I_Rv	5’-CGCTGATGGTGAAGTTGATGGGGATAGCGATAGAG-3’
Spike_S982A_Rv	5’-CCACCTTGTCCAGTCTGGCCAGGATATCGTTCAGCA-3’
Spike_T1027I_Fw	5’CACTCAGACATCTTGATGGCGGCCAGATTGG-3’

Chapter 3 - Saliva as an alternative specimen for COVID-19 diagnosis in a pediatric population

3.1 AUTHOR CONTRIBUTIONS

The experiments presented in this chapter were designed and planned by Marta Alenquer, Filipe Ferreira and Maria João Amorim. Experiments and data generated for Fig. 3.2-3.4 were performed by Marta Alenquer, Filipe Ferreira and **Mónica Medina**, as they involved analysis and processing of a large number of samples. Tables for this study were prepared by Marta Alenquer and Tiago Milheiros. The candidate of this MSc. thesis has contributed with saliva sample processing and analysis.

This chapter resulted in a manuscript that is under consideration on BMC pediatrics and was published as a pre-print with the reference: Alenquer M, Milheiro Silva T, Akpogheneta O, Ferreira F, Vale-Costa S, **Medina-Lopes M**, Batista F, Garcia AM, Barreto VM, Paulino C, Costa J, Sobral J, Diniz-da-Costa M, Ladeiro S, Alves JD, Leite RB, Demengeot J, Brito MJR, Amorim MJ. Saliva molecular testing bypassing RNA extraction is suitable for monitoring and diagnosing SARS-CoV-2 infection in children. *medRxiv*. 2021:2021.2008.2011.21261899. https://doi.org/10.1101/2021.08.11.21261899.

3.2 INTRODUCTION

The management and the control of the COVID-19 pandemic caused by the novel coronavirus SARS-CoV-2 remain a major challenge. Despite widespread immunization of the world's population, which has been underway since the beginning of 2021 [1,2], the number of new cases and deaths continues to be a source of concern for international health authorities [2].

Immunization-eligible population, *i.e.*, adults and children over 12 years of age, are being vaccinated in several countries, however, a vaccine for children under 12 years old is not yet approved [3]. Currently, vaccines for this age range are undergoing phase II/III clinical trials, such as the KidCove clinical trial conducted by the biotech company Moderna [4,5]. However, until its approval, unvaccinated children will continue vulnerable to SARS-CoV-2 infections.

Even though most children are asymptomatic or have minor symptoms, there have been reports of children with severe medical conditions and hospitalizations [6,7]. In addition, several countries have reported severe complications in children infected with SARS-CoV-2 who develop symptoms of multiple systemic inflammatory syndromes that are characterized by systemic hyper inflammation with dermatological, gastrointestinal and cardiac symptoms [8,9]. Although these complications are uncommon compared to those reported in adults [10], children are still vulnerable to SARS-CoV-2 infection [7,8,11]. In fact, further research is needed to understand the significance of children in virus transmission in the community and at school settings, and how infections in children may contribute to the evolution of SARS-CoV-2 variants.

Some studies have shown that children who have clinical symptoms of COVID-19 have higher viral loads and, therefore, may be at increased risk of virus transmission [12,13]. Furthermore, there is evidence for significant viral load levels in the upper respiratory tract of asymptomatic children, suggesting that children are efficient in transmitting the virus [12,13]. Children, however, are much less prone to develop symptoms and hence initial outbreaks of SARS-CoV-2 infection in school settings may go undetected if diagnosed merely based on symptoms [14]. It is, therefore, essential to maintain regular surveillance, through testing approaches, in school settings.

Molecular testing techniques to rapidly identify actively infected individuals and their close contacts were one of the first strategies adopted to control the pandemic and remain one of the best ways to break SARS-CoV-2 transmission chains [15–18]. Nasopharyngeal (NP) swabs have become the preferred specimen for SARS-CoV-2 detection [15,19], however sampling alternatives have been explored due to the invasiveness of its collection, requirement of certified and trained health workers, personal protective equipment (PPE) and complex logistics [20].

Alternative specimens for SARS-CoV-2 molecular detection have emerged as a solution to overcome constraints of the gold standard testing method. Saliva appears as an obvious substitute if we consider that respiratory and saliva droplets and aerosols are the main pathways of human-to-human transmission of SARS-CoV-2 [21–23]. In addition to the non-invasive sampling, it can be self-collected without increasing the risk of aerosol formation and potential transmission to healthcare professionals and the community. In fact, saliva molecular testing has been shown in several studies to be effective in detecting cases of COVID-19 infection [24–27].

Despite the advantages mentioned above, RT-qPCR using NP swabs remains the standard method of SARS-CoV-2 detection [15,24,29]. Most studies evaluating saliva as an alternative specimen for COVID-19 diagnosis have focused on the adult population [24,26,27,30]. Even with emerging studies in the pediatric population [13,30,31], further research is needed to understand the dynamics of the viral load in saliva

compared with NP swabs, as well as to develop more straightforward and faster methodologies for SARS-CoV-2 detection in children.

Figure 3.1 – **SARS-CoV-2 golden standard detection by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR).** RNA extraction is performed in patient's nasopharyngeal swabs for amplification and detection of SARS-CoV-2 genetic material. Illustration adapted from "COVID-19 Fact sheet" by BioRender.com (2021) and [28].

The primary goal of this study was to evaluate the suitability of SARS-CoV-2 detection by RT-qPCR using saliva in a pediatric population aged up to ten years old admitted to Dona Estefânia Hospital, the pediatric reference center in southern Portugal.

Another important goal set for this study was to determine the efficiency of SARS-CoV-2 detection in saliva specimens bypassing the RNA extraction steps.

3.3 MATERIALS AND METHODS

3.3.1 Patients and Samples

The present study enrolled hospitalized patients with positive and negative SARS-CoV-2 laboratory diagnoses (by RT-qPCR on NP swabs) from two hospitals in the Lisbon area: 49 adults from the Hospital Professor Doutor Fernando Fonseca and 85 children (age up to 10 years) from the Hospital Dona Estefânia, with and without clinical COVID-19 symptoms. NP swabs and saliva samples were collected during patient's hospitalization from August 25, 2020, to June 20, 2021, at intervals of up to 24 hours for adults and up to 48 hours for children.

All patients involved in this research provided written and informed consent, and for children, the consent was provided by parents or legal representatives. This research was performed according to the principles established by the Helsinki Declaration and good clinical practice and was authorized by the Ethics Committee of Centro Hospitalar Lisboa Central and Hospital Professor Doutor Fernando Fonseca.

3.3.2 Saliva Collection and Transport

Saliva collection was performed at both hospitals with the help of health care professionals according to all safety guidelines. During this process, participants were instructed not to eat, drink, or brush their teeth for 30 minutes immediately before saliva collection. A sterile container was used to collect approximately 1 mL of saliva and a suction tube was used to aspirate saliva from the mouth of children under one year of age.

All samples were considered potentially infectious and packaged in a triple-packaging system to ensure maximum safety during transport and processing. Therefore, the collected samples were placed into a 50 mL sterile primary container, sealed with parafilm, and packed into a sealed leak-proof plastic bag. Finally, an isothermal box with refrigerated plates was used as a tertiary container for transport to the IGC to guarantee sample's refrigeration, conservation, and safety. Hospitals and IGC ensured samples transport which were kept at 4ºC from collection to processing. All saliva samples were processed within a maximum of 72 hours after collection.

3.3.3 Saliva Reception and Processing

Upon arrival at the IGC, samples were collected in the isothermal box and transferred to a Biosafety level 2 (BSL-2) facility. Two operators, equipped with PPE, performed sterilization and processing of all samples inside a biosafety cabinet in the BSL-2. Sterilization comprised wiping with 10% bleach and/or 70% ethanol, following by labelling in which tubes were assigned an internal code. The code ensured protection of personal information.

After disinfecting the containers and assigning internal codes to samples, the saliva was aliquoted into two 1.5 mL microcentrifuge tubes, one for immediate processing and the other to store at -80ºC. Viscous saliva samples were 1:2 diluted in TBE 2X according to [27] to facilitate pipetting before Proteinase K (645 µg/mL in 160 nM SDS) treatment. Next, two PCR tubes were prepared and identified: one for the RNA extraction protocol (Fig.3.2-A), containing 6.7µL of Proteinase K (Sigma, P6556-10MG) and 200µL of saliva, and one for the protocol without extraction (Fig.3.2-B), containing 1µL of

Proteinase K and 30µL of saliva. Finally, the mixture was slightly pipetted up and down six times to homogenize the sample and the Proteinase K solution.

After a brief centrifugation in a tabletop mini centrifuge, the PCR tubes were placed in the thermocycler (Verity, Applied Biosystems) for 30 min at 50ºC, followed by heat inactivation for 10 min at 98ºC (Table 3.1). After the final step of the thermocycler programme, samples for the RNA extraction protocol were transferred to the interior of the biosafety cabinet for RNA extraction while samples with no RNA extraction steps were kept at 4ºC for further analysis by RT-qPCR by the IGC Genomics Unit.

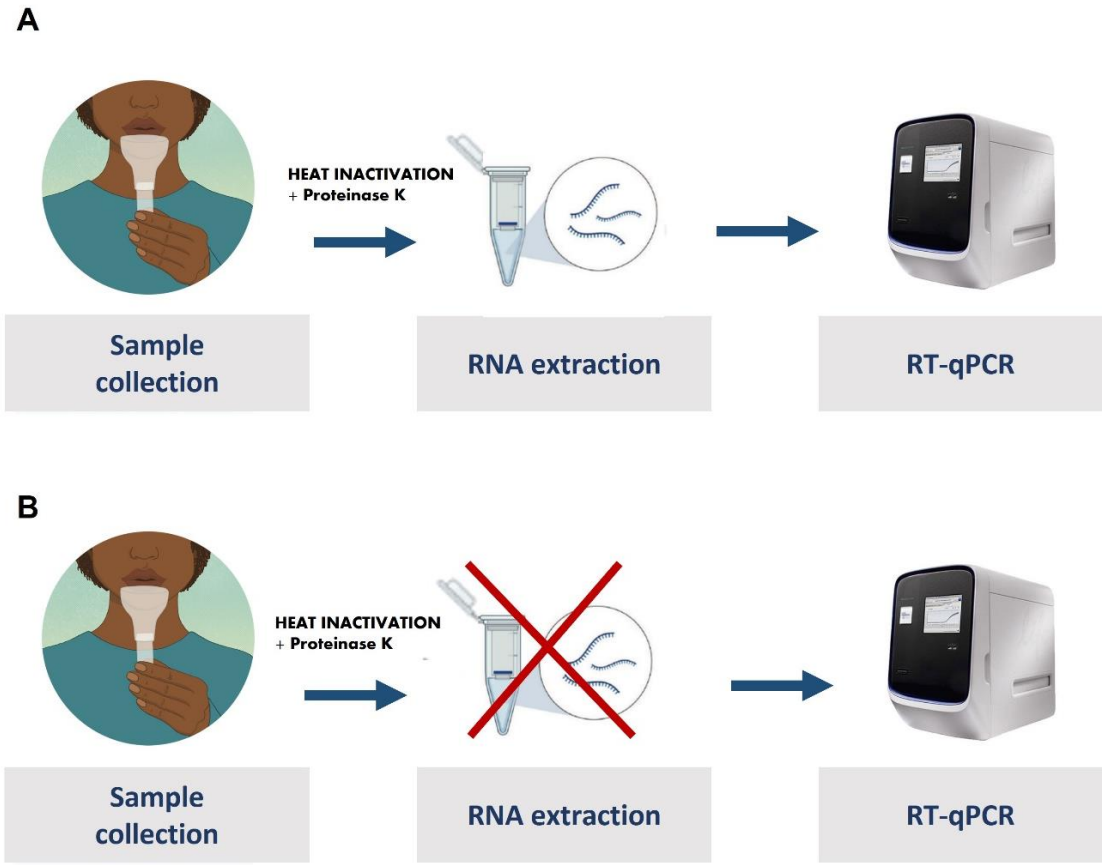

Figure 3.2 | Protocols for SARS-CoV-2 detection by saliva molecular testing used in this study. A: Protocol with RNA extraction; **B**: Protocol without RNA extraction steps. Illustration adapted from "COVID-19 Fact sheet", by BioRender.com (2021) and Joana Carvalho illustration (IGC).

Table 3.1 | Thermocycler program for Proteinase K treatment.

Step	Cycles	Temperature	Time
1	1	50ºC	30 minutes
2	1	98ºC	10 minutes
3	1	4ºC	∞

3.3.4 RNA extraction

The saliva samples (200µL) for the RNA extraction protocol from the thermocycler were immediately processed inside the biosafety cabinet. RNA extraction was performed using the commercial QIAamp Viral RNA Mini Kit (QIAgen, 52906) according to the manufacturer's instructions.

After extraction, RNA was eluted in 50µL RNase-free water and immediately transported to the Genomic Unit (IGC) kept for subsequent detection of viral RNA by RTq-PCR, together with the sample that has not been subjected to RNA extraction. The remaining RNA was kept at -80ºC.

3.3.5 SARS-CoV-2 detection by RT-qPCR

SARS-CoV-2 detection in saliva samples was performed by the Genomic Unit, at IGC. The method was as follows: 1 µL of extracted RNA or direct saliva were used for viral detection by RT-qPCR. The cDNA synthesis and the amplification were performed in a single step assay using iTaq Universal Probes One-Step Kit (BioRad, #12013250). A master mix was prepared for each set of amplifications using the following primers: 0.5 nM CDC_N1, CDC_N2 and Hs_RPP30, 1x iTaq Universal Probes reaction mix, 2.5% (V/V) iScript Reverse Transcriptase, 125nM kit probe. 10% (V/V) of sample was added in triplicate.

Two positive controls were performed separately per experiment (SARS-CoV-2 and Human) for N1, N2 and RP primers using 200 synthetic copies of nucleocapsid region of the virus or 200 synthetic copies of the Human single copy RPP30 gene. One negative control was performed without RNA/DNA template.

All the primers and probes (2019-nCoV RUO Kit, 500 rxn, #10006713 were purchased from Integrated DNA Technologies as well the sequences used for positive virus detection control (2019-nCoV_N_Positive Control, #10006625) and positive human sample control (Hs_RPP30 Positive Control, #10006626|). Reactions were performed in 384 wells plates (ThermoFisher, #TF-0384) in a QuantStudio 6 system (Applied Biosciences), using the standard mode, consisting of a hold stage at 50°C for 10 min, and 95°C for 3 min, followed by 45 cycles of a PCR stage at 95°C for 10 sec then 60°C for 30 sec.

Positive cases were considered when the two probes were amplified with a cycle threshold (CT) below 37. Negative detection was established as having no amplification or amplification of one probe above 37. Inconclusive results were considered with one probe being amplified with a CT less than 37. The limit of detection (LOD) of the saliva assay was performed by serial dilution of IDT synthetic copies (20000 to 20) of SARS-CoV-2 in fresh saliva samples (non-positives).

3.3.6 Statistical analysis

The NP swab results were used as reference standard to estimate the values of sensitivity, specificity, and accuracy. The Wilson method, recommended for small sample sizes, was used to calculate the 95% confidence intervals (CI) [32]. The analytical sensitivity of SARS-CoV-2 RNA detection between saliva and nasopharyngeal swabs from SARS-CoV-2 positive adults and children was compared using a Wilcoxon matched-pairs signed-rank test, in GraphPad Prism 9.1.2.

3.4 RESULTS

3.4.1 Method validation using saliva specimens from COVID-19 symptomatic adults

The saliva molecular testing method, used in this project, was first validated using saliva specimens from adult patients with COVID-19, or without SARS-CoV-2 infection as negative controls, to answer our question regarding the reliability of using saliva molecular testing based on RT-qPCR to diagnose children with SARS-CoV-2 infection.

NP swabs and saliva specimens from 49 adults (with and without SARS-CoV-2 infection) over 18 years of age from the Hospital Professor Doutor Fernando Fonseca were used to validate the method described in this study. First, SARS-CoV-2 infection was assessed by the hospital laboratory through RT-qPCR on RNA extracted from NP swabs. Next, approximately 1 mL of a saliva specimen was collected within the following 24 hours and sent for processing at the Instituto Gulbenkian de Ciência. In addition, the result of the CT values of each patient was sent later, by the hospital, for comparative analysis.

The saliva molecular testing involved processing saliva in two protocols. RNA was extracted in one protocol, while saliva specimens went directly to SARS-CoV-2 detection in the other, skipping the extraction steps. All saliva samples were promptly treated with Proteinase K before processing and, after the extraction, the samples were transferred to the Genomic group for SARS-CoV-2 detection by RT-qPCR.

Table 3.2 | Comparative results of saliva processing with and without RNA extraction in adults coupled with NP swab results.

NP swab No.	**Saliva No.**					
	With RNA extraction			**Without RNA extraction**		
	Positive	**Negative**	**Total**	**Positive**	**Negative**	**Total**
Adults > 18y						
Positive	36	0	36	34	0	34
Negative	1	12	13	1	12	13
Total	37	12	49	35	12	47
Sensitivity (95%CI)	100% (90.4%-100%)			100% (89.8%-100%)		
Specificity (95%CI)	92.3% (66.7%-98.6%)			92.3% (66.7%-98.6%)		
Accuracy (95%CI)	98.0% (89.3%-99.6%)			97.9% (88.9%-99.6%)		

Regarding the validation of the method, as shown in Table 3.2, all adult saliva specimens from the Hospital Professor Doutor Fernando Fonseca and with laboratory-confirmed SARS-CoV-2 infection using NP swab were also confirmed as positive using saliva molecular testing. Regardless of RNA extraction, it was possible to confirm all positive results in samples with RNA extraction (36) and all submitted to the protocol without extraction (34).

Taking these data into account, the sensitivity of the method, *i.e.*, the ability of the test to correctly diagnose the true positives, for adults was 100% (36/36) for the protocol with RNA extraction and 100% (34/34) for the protocol without extraction. One negative case was detected in NP swabs that was positive for SARS-CoV-2 infection in molecular saliva testing; therefore, the specificity of the method was 93.2% (66.7%-98.6%) either with extraction or without extraction. The method's accuracy was 98%

(89.3%-99.6%) and 97.9% (88.9%-99.6%) for the protocols with and without extraction, respectively (Table 3.2).

3.4.2 Cycle Threshold analysis in adults infected with SARS-CoV-2

To compare whether RNA extraction was an essential step, a Wilcoxon matched-pairs signed-rank test was performed to assess the differences between CT values of saliva with and without RNA extraction and NP specimens. When the CT values of NP swabs and saliva samples from adults with COVID-19 diagnostic were compared (Fig.3.3), no significant differences were observed. In addition, SARS-CoV-2 RNA was detected in all samples below the detection cut-off (37). According to this comparative analysis, the CT values of saliva samples with and without RNA extraction were significantly different. Regarding the protocol without RNA extraction, the CT values were slightly higher than the extraction protocol (Fig.3.3).

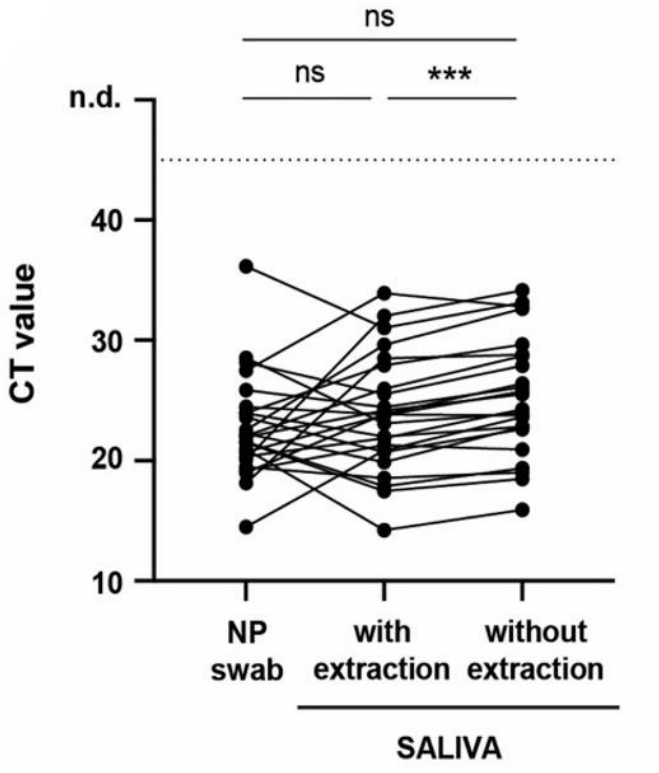

Figure 3.3 | Cycle thresholds values from SARS-CoV-2 detection by RT-qPCR in diagnosed COVID-19 adults. The CT values of NP swabs and the two saliva protocols were compared by a Wilcoxon matched-pairs signed-rank test. The CT comparison for a paired NP swab specimen and saliva with and without RNA extraction is represented in each line. n.d, not detected, ns, not significant; ***p<0.001.

3.4.3 Saliva molecular testing in children up to 10 years old

After validation in adult samples, the methods were analysed in the pediatric population, which involved 85 children aged between 0 and 10 years old, who were hospitalized at Hospital Dona Estefânia between August 2020 and June 2021. Children recruited for this study were admitted to the hospital with and without clinical symptoms for COVID-19 and the diagnosis was performed by RT-qPCR using NP swabs.

Table 3.3 | Demographic and clinical characteristics of the pediatric group recruited for this study.

Characteristic	**No. (%)**		
	Total sample	**Negative**	**Positive**
Total	85	39 (45.9)	46 (54.1)
Sex			
Female	39 (45.9)	20 (51.3)	19 (41.3)
Male	46 (54.1)	19 (48.7)	27 (58.7)
Age (y)			
0	28 (32.9)	5 (12.8)	23 (50)
1-5	25 (29.4)	15 (38.5)	10 (21.7)
6-10	32 (37.7)	19 (48.7)	13 (28.3)
COVID-19 sign or symptom			
None	17 (37.0)	NA	17 (37.0)
Fever	23 (50.0)	NA	23 (50.0)
Cough	13 (28.3)	NA	13 (28.3)
Dyspnoea	5 (10.9)	NA	5 (10.9)
Coryza	13 (28.3)	NA	13 (28.3)
Odynophagia	4 (8.7)	NA	4 (8.7)
Cephalalgia	1 (2.2)	NA	1 (2.2)
Abdominal pain	1 (2.2)	NA	1 (2.2)
Nausea/Vomit	3 (6.5)	NA	3 (6.5)
Diarrhoea	3 (6.5)	NA	3 (6.5)
Concurrent conditions			
0	11 (12.9)	1	10
1	69 (81.2)	35	34
>1	5 (5.9)	3	2
Another Infection	12 (14.1)	1	11
Cardiovascular disease	5 (5.9)	0	5 (10.9)
Urinary tract disease	13 (15.3)	6	7
Digestive tract disease	10 (11.8)	5	5
Another respiratory disease	5 (5.9)	0	5
Oral surgery	4 (4.7)	4	0
Facial congenital anomalies	3 (3.5)	3	0

NA= Not applicable

The interval between NP swab and saliva collection was limited to up to 48 hours for not submitting the children to a second swab collection. Table 3.3 summarizes the demographic and clinical characteristics of children admitted to the hospital. Fever (50%), cough (28.3%) and coryza (28.3) were the most prevalent symptoms in infected children that represented 54.1% of the total sample (46).

After saliva processing and subsequent detection of SARS-CoV-2 in children up to 10 years old, the extraction protocol managed to diagnose 84.8% of (39/46) of the positive NP swab specimens. Regarding the protocol without extraction, the sensitivity slightly decreased, being able to detect 81.8%

(36/44) of patients with a confirmed diagnosis using NP swabs. The specificity for both protocols was 100%, identifying all negative samples accurately for SARS-CoV-2 infection. The accuracy between the NP swab and the saliva method was 91.8% (78/85) for the extraction and 90.4% (75/83) for the protocol without extraction (Table 3.4).

In a separate analysis of the group of children under 1 year of age, the sensitivity of the method increased to 87.0% (20/23) in the protocol with RNA extraction and 86.4% (19/22) for the protocol without extraction (Table 3.4).

Table 3.4 | Comparative results of saliva processing with and without RNA extraction in children coupled with NP swab results.

NP swab No.		**Saliva No.**					
		With RNA extraction			**Without RNA extraction**		
		Positive	**Negative**	**Total**	**Positive**	**Negative**	**Total**
Children < 10y							
	Positive	39	7	46	36	8	44
	Negative	0	39	39	0	39	39
	Total	39	46	85	36	47	83
Sensitivity (95%CI)		84.8% (71.8%-92.4%)			81.8% (68.0%-90.5%)		
Specificity (95%CI)		100% (91.0%-100%)			100% (91.0%-100%)		
Accuracy (95%CI)		91.8% (84.0%-96.6%)			90.4% (82.1%-95.0%)		
Children < 1y							
	Positive	20	3	23	19	3	22
	Negative	0	5	5	0	5	5
	Total	20	8	28	19	8	27
Sensitivity (95%CI)		87.0% (67.9%-95.5%)			86.4% (66.7%-95.3%)		
Specificity (95%CI)		100% (56.6%-100%)			100% (56.6%-100%)		
Accuracy (95%CI)		89.3% (72.8%-96.3%)			88.9% (71.9%-96.1%)		

CI= Confidence interval

When the CT values of NP swabs were compared to those of saliva protocols in children, significant differences were observed, as shown in Fig.3.4 (A). The CT values of saliva protocols, with and without extraction, were higher than those of NP swabs, with a mean CT (SD) of 26.1 (5.1), 27.9 (4.7), and 22.9 (6.2), respectively. No correlation was found regarding the ability of the method in detecting SARS-CoV-2 in saliva and the CT value of the NP swab or the age of the patient (Fig.3.4, B).

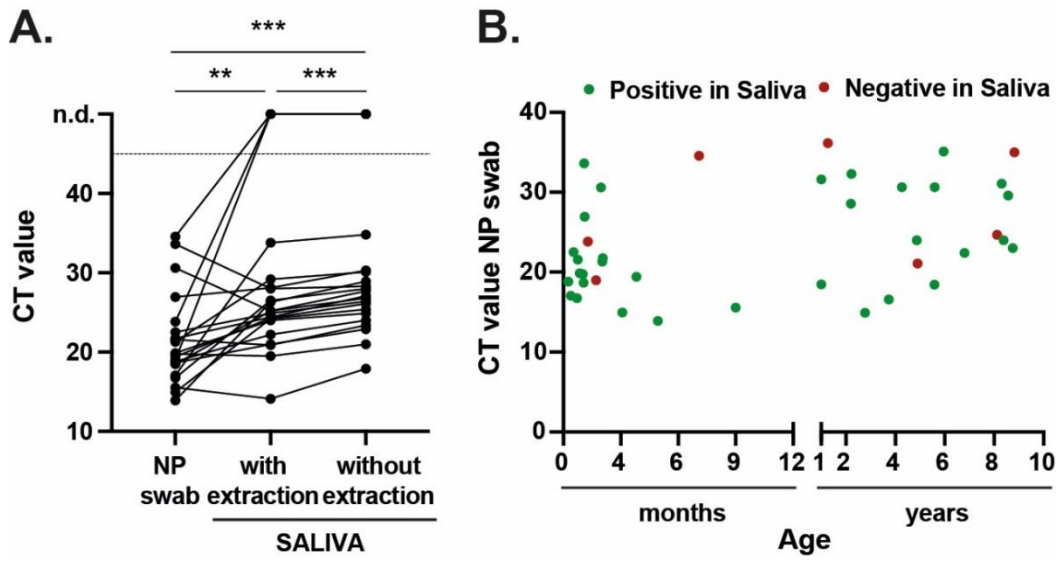

Figure 3.4 | Cycle thresholds values of SARS-CoV-2 detection by RT-qPCR in saliva samples from the pediatric group. **A**: The CT values of NP swabs and the two saliva protocols from children under 10 years old were compared by a Wilcoxon matched pairs signed rank test. The comparison for a paired NP swab specimen and saliva sample with and without RNA extraction is represented in each line. n.d,,**p<0.01, ***p<0.001. **B**: CT values from RNA extracted from NP swabs from children with confirmed SARS-CoV-2 infection. The CT values are distributed across two age groups: under 1 year old and between 1 and 10 years old.

3.5 DISCUSSION

Since its emergence in China in late 2019 [33,34], and up to now (December 5, 2021), the novel coronavirus SARS-CoV-2 has infected 265 681 353 people and caused 5 254 677 fatalities globally, according to data published by Johns Hopkins University [2].

Population screening is used to identify individuals with active SARS-CoV-2 infection and their contact networks, thereby preventing community transmission of the virus [19,22]. Currently, the gold standard method for detecting SARS-CoV-2 is the RT-qPCR molecular test on upper respiratory tract specimens, particularly NP swabs. However, NP swab testing for the mass population has several bottlenecks, including the complex logistics associated with sample collection. As a result, several studies have looked at several alternatives including saliva as a substitute to NP swabs.

This study filled in the gap to understand the potential of using saliva as a specimen for SARS-CoV-2 detection in a pediatric population, by pairing saliva samples with NP swabs. To simplify the methodology, one of the protocols involved skipping the RNA extraction steps and detecting SARS-CoV-2 by RT-qPCR directly from the saliva specimens. The method used for this project was first validated in symptomatic adult patients with a confirmed COVID-19 diagnosis. Saliva molecular testing allowed the detection of all positive NP swab cases (36/36) with an overall agreement of 98.0% for the RNA extraction protocol and 97.9% for the protocol without RNA extraction (Table 3.2). These results align with previous studies reporting the effectiveness of using saliva as an alternative sample for detecting SARS-CoV-2 in adults [25,26,35,36]. It also corroborates findings in which no significant differences were observed between the CT values between saliva and NP swabs in adults [36].

One of the significant obstacles to the widespread of molecular assays is the RNA extraction step, because it employs kits that are largely used worldwide and there is shortage of supply, and also because they are expensive and time consuming. One of the protocols in this study simplified the detection process by skipping the RNA extraction steps. According to the results, the protocol without RNA extraction, previously described by [27], had a sensitivity of 100% in the adult population and 97.3% in children up to 10 years of age, when we compared the protocol without RNA extraction with the values of the protocol with RNA extraction. In addition, in low and middle-income countries (LMICs), simplifying testing methods cand be critical as these countries face several challenges in large scale population testing [37]. While less sensitive, point of care methods can serve complementary diagnostic methods and can act as powerful allies against the pandemic.

In addition, findings from this research reinforce the evidence showed by studies regarding the efficacy of molecular saliva testing of children with infected with SARS-CoV-2 [31,38]. Interestingly, the overall concordance of the saliva and NP swab samples in the pediatric population was 91.8% (78/85) for the RNA extraction protocol and 90.4% (75/83) without RNA extraction from saliva specimens (Table 3.4). In addition, the method described in this study was also effective in diagnosing infants up to 1 year of age whose saliva was aspirated by healthcare professionals (Figure 3.4, A). These findings suggest that saliva may be applied in school settings for monitoring infection, even in a self-collection manner or collection with the assistance of parents.

Carmagnola and colleagues conducted a six-week SARS-CoV-2 surveillance study at two schools in Milan with 401 students aged 6 to 11 and 12 teachers using saliva molecular testing [31]. This approach allowed them to identify five infected children, all asymptomatic. In this study, Marta Alenquer and Filipe Ferreira also showed that infectious virus could be recovered from saliva samples with CT values up to 26, suggesting that children with CT values equal to or lower than 26 can

spread viral infection. This indicates that either very frequent antigen tests or less frequent saliva molecular tests may be used to block the active transmission of SARS-CoV-2 by children.

An additional concern relates to emerging variants and VOCs, especially the Delta variant (B.1.617.2) [39,40]. A recent report from the European Centre for Disease Prevention and Control [5], assessing current knowledge on the role of children and adolescents in SAR-CoV-2 transmission, warns for the potential of unvaccinated children to transmit the virus.

Despite being the group with the fewest cumulative cases and deaths of COVID-19 globally, the removal of restrictions and reopening of schools leads to questions about children's role in the evolution of SARS-CoV-2 variants. As a result, it is critical to develop fast, effective, and non-invasive screening approaches, such as the method described in this work, that can serve as powerful tools in the ongoing monitoring and subsequent control of SARS-CoV-2 transmission.

Importantly, this study has several caveats. The 48-hour period between the collection of the NP swab and the saliva sample in children was the most significant limitation of this study. The approach was less effective (Fig.3.4) in the pediatric population; however, it is unclear whether the results are due to the method's efficacy or a reduction in viral load in children during the 48-hour gap between NP swab and saliva collection. Thus, despite the sample collection time constraints, the method described in this study allowed for the efficient diagnosis of symptomatic and asymptomatic children with SARS-CoV-2 infection using saliva as an alternative to NP swabs.

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Chapter 4 - General Discussion and Future Perspectives

4.1 GENERAL DISCUSSION

The current COVID-19 pandemic continues to threaten human health and global economic stability, despite the best efforts of health authorities and vaccination campaigns. In less than two decades, SARS-CoV-2 along with two highly pathogenic HCoVs, SARS-CoV and MERS-CoV, have already caused three epidemics [1,2]. The millions of deaths and infections caused by SARS-CoV-2 [3] pressured the international scientific community to work together in a coordinated and timely manner to bring the pandemic under control. Traditional measures such as population screening and quarantining infected people [4], combined with the advancement and innovation of technologies such as the mRNA vaccines [5], have been critical to mitigate the burden of the current pandemic.

The emergence of SARS-CoV-2 variants, represented primarily by VOCs Alpha (first detected in the United Kingdom), Beta (first detected in South Africa), Gamma (first detected in Brazil), and Delta (first detected in India), pose significant challenges to COVID-19 pandemic control [6,7]. These variants caused widespread concern since late 2020 and health authorities will continue to survey the appearance, spread and effects of emerging variants [8–10]. Most of the reported mutations focused on spike protein, as these could have a high impact in viral entry and also in antibody-mediated host immunity for the whole population [11,12] [13]. In addition, COVID-19 vaccines target the spike protein because it is the most immunogenic viral protein and hence the one that elicits the strongest immune response in the host [14–16]. As a result, outstanding questions relate to the duration of immunity and how novel variants impact the immunity developed by previous infections and vaccination.

So far, vaccination does not cover the entire population, excluding children under 12 years of age [5]. Although a large part of the population is fully immunized [3], children below this age may still contribute to the continuous spread of SARS-CoV-2 and its variants. Therefore, it is important to monitor viral spread and evolution in children, using the less invasive and easy-to-perform testing methodologies possible, at least until there is an answer to this question.

In this sense, the focus of this work was to contribute with tools and knowledge relevant to better management of the COVID-19 pandemic. In the second chapter, we developed spike-pseudotyped lentiviral particles to analyse mutations in spike protein able to reduce the neutralizing capacity of antibodies that arise upon natural infection and vaccination. Our results, published in PLOS Pathogens, show that there are mutations in the RBD able to reduce the neutralizing capacity of antibodies (E484K or S494P), but also synergetic combinations of mutations (S417N/N501Y [17]) displaying additive effects that relate to increased ability to bind the receptor ACE2 for viral entry rather than escape to antibody binding.

These mutations, present and also recurrent in several SARS-CoV-2 variants [7], or emergence of new ones [18], individually or in combination, require continuous monitoring. Their epidemiological surveillance is particularly important, relative to higher transmissibility and evasion of immunity [12,19–21]. In the future, it will be also important to analyse the effects of mutations in other parts of SARS-CoV-2 genome for their capacity to promote higher replication of the virus and immune escape, thus also impacting in severity of disease. These mutations could synergise with mutations in spike for severity of viral infection.

Furthermore, there are many questions outside of the scope of what we addressed in this thesis that are important to answer relative to SARS-CoV-2 pandemic. On one hand, there is the duration of immunity upon infection and vaccination with all the different vaccine platforms, as well as identification of genetic, environmental, biological markers for severity of the disease. On the other

hand, there is the issue of how people build immunity from childhood to protect against SARS-CoV-2, the issue of how SARS-CoV-2 infection interacts/associates with other viral and bacterial infections, as well as novel therapies and technologies to treat the disease and of course the origin, evolution, and spread to other species, namely domestic animals. As a final remark relative to this part, there is the necessity to define strategies to prepare and create awareness to mitigate the effects and better respond to the next viral challenge. In the third chapter, we addressed the need to develop and improve existing testing approaches, in this case, for monitoring children up to 10 years old, making them more straightforward, less invasive and involving less logistics. The gold standard SARS-CoV-2 detection method using nasopharyngeal swabs [22] can be highly invasive to children and babies. In addition, the capability of the same test was also tested in a protocol in which the RNA extraction steps were skipped to simplify the method. This is highly advantageous in low resource settings, resource-scarce countries, and even in developed countries. Greater speed in sample processing and diagnosis is essential for the control and management of potentially infected people.

Alternative methods available include both molecular tests (RT-LAMP, CRISPR-based technologies) [22,23] as well as antigenic tests using saliva, nasal samples, nasopharyngeal swabs and stool [24]. RT-LAMP is an isothermal nucleic acid amplification method that also has been evaluated as an alternative for detecting SARS-CoV-2 [22,25]. It is a colorimetric assay that amplifies nucleic acids at a constant temperature, eliminating the need for complex thermal cycles and laboratory equipment's. It simply requires a heating source, and because the test readout is a colour change, it can significantly reduce the time it takes to reach a diagnosis. However, this assay has some limitations, namely the proper design of primers, risk of sample contamination, robustness and sensitivity [25] and further studies are needed to optimize it to wide scale use, especially in low-income countries.

Assays based on CRISPR/Cas complex, a powerful genome editing tool have also been explored in the context of COVID-19 diagnostics [23,26]. Kits with high sensitivity (>95%) are currently available, such as the SARS-CoV-2 DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) reagent kit developed by Mammoth Biosciences, Inc. and also the specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) CRISPR SARS-CoV-2 kit developed by Sherlock Biosciences. Both kits are approved for the diagnosis of COVID-19 and detect viral RNA through reverse transcription and isothermal amplification using RT–LAMP [27]. A second step is then performed to transcribe the amplified cDNA and activate the collateral cleavage activity of a CRISPR/Cas complex (cas12 for DETECTR kit and cas13 for SHERLOCK kit) programmed to the target RNA sequence. These tests are less complex, faster and can give a diagnosis in up to 40 minutes but have some limitations as well. Sample processing requires several manual operations (includes a separate amplification step) increasing the risk of contamination [28].

Antigen tests are lateral flow immunoassays that target specific antigens of SARS-CoV-2 and are used to diagnose active infections. Due to their affordable price and short turnaround time (15-20 minutes) they have been used as a point of care (POC) test in COVID-19 diagnostics [24]. The result is read visually in a small portable device similar to pregnancy tests, allowing its use outside of laboratory settings. These advantages have allowed to scale up rapid antigen tests for the population, however, the sensitivity of these tests is lower than that of the reference RT-qPCR, which may produce more false-negative diagnostics. WHO recommends a minimum sensitivity and specificity of 80% and 97% for antigen tests in COVID-19 diagnostic [24]. Currently, there are hundreds of authorized tests based on different biological samples (saliva, nasal swabs, and nasopharyngeal swabs) and although less sensitive, when combined with molecular testing approaches could be extremely important managing the current pandemic.

Regarding project three, although the efficient detection of SARS-CoV-2 in the saliva is well documented [29–31], more studies are needed to understand better the viral load dynamics in children and their role in the active transmission of SARS-CoV-2 in the community and school settings. Studies that make it possible to assess the viral load of children at different time points and associate them with their ability to generate infectious SARS-CoV-2 particles will be helpful in this regard. Until a vaccine is available for this age group, it is crucial to keep infection rates under control, preferably with non-invasive methods such as the one proposed in this work, which can be applied in school contexts.

There is no perfect diagnostic test, however, the ideal test seeks to meet criteria such as the ASSURED criteria [32], created and defined by WHO in the context of infectious diseases for developing countries. Based on these criteria, tests should be affordable, sensitive, specific, user-friendly, rapid, equipment-free, and delivered to end-users. In developed countries, tests that meet these criteria can be crucial in managing outbreaks. All tests have their advantages and drawbacks, and they will continue to be evaluated and optimized, to better suit each country's context, resources, and epidemiological scenarios.

To summarize, the two projects described in this study enabled to generate knowledge and contribute to methodologies with significant potential in managing the current and future pandemic situations.

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