

REVIEW



## The importance of genomic analysis in cracking the coronavirus pandemic

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### ABSTRACT

**Introduction:** Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has pushed the scientific community to undertake intense research efforts. Understanding SARS-CoV-2 biology is necessary to discover therapeutic or preventive strategies capable of containing the pandemic. Knowledge of the structural characteristics of the virus genome and proteins is essential to find targets for therapies and immunological interventions.

**Areas covered:** This review covers different areas of expertise, genomic analysis of circulating strains, structural biology, viral mutations, molecular diagnostics, disease, and vaccines. In particular, the review is focused on the molecular approaches and modern clinical strategies used in these fields.

**Expert opinion:** Molecular approaches to SARS-CoV-2 pandemic have been critical to shorten time for new diagnostic, therapeutic and prevention strategies. In this perspective, the entire scientific community is moving in the same direction. Vaccines, together with the development of new drugs to treat the disease, represent the most important strategy to protect human from viral disease and prevent further spread. In this regard, new molecular technologies have been successfully implemented. The use of a novel strategy of communication is suggested for a better diffusion to the broader public of new data and results.

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## 1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) originated in Wuhan, China, in early December 2019, has rapidly spread worldwide, becoming one of the major global public health issues of the last centuries [1]. This virus belongs to the *Betacoronavirus* genus, and its genome is composed by 29,903 nucleotides [2]. Its genome organization is shared with other *Betacoronaviruses*, and it presents six functional open reading frames (ORFs) that are arranged in order from 5' to 3': replicase (ORF1a/ORF1), spike (S), envelope (E), membrane (M), and nucleocapsid (N) [3]. The virus likely emerged from several recombination events in bats and pangolins [4] and was subsequently introduced in the human population through zoonotic transmissions [5,6]. Later, it was recognized as the etiologic agent of Coronavirus Disease 2019 (COVID-19) [7].

Humanity is being seriously threatened by the SARS-CoV-2 pandemic, and the urgency to effectively tackle this devastating disease has pushed the scientific community to undertake intense research efforts.

Over the last year, SARS-CoV-2 has been rapidly moving across countries, causing an important pandemic that negatively influenced quality of life worldwide, and many different approaches have been proposed to actively fight the virus spreading as well as to face it. Molecular technologies have been largely applied from many perspectives, and this review summarizes these aspects. First, due to the introduction of whole-genome sequencing (WGS) methodology, viral genome sequences are available for deep phylogenetic and evolutionary analysis. This provides important insights into SARS-CoV-2 evolution driven by viral spreading and selective pressure. For this reason, it is important to identify mutations observed in SARS-CoV-2 and to determine whether they can be used to

provide an indication of viral fitness and adaptation. We should also consider that a number of factors are responsible for the viral genome mutagenic process, and that recombination events can contribute to the genomic variability, thus leading to the emergence of new virus variants [1].

Furthermore, understanding SARS-CoV-2 biology is necessary to discover therapeutic or preventive strategies capable of containing the pandemic. Within this framework, knowledge of the structural characteristics of the virus proteins is an essential prerequisite. Indeed, proteins are the effectors of the virus biological function and pathogenicity and are the natural targets for therapies and immunological interventions [7].

It is clear that the prompt diagnosis of SARS-CoV-2 is crucial to prevent contagion and provide rapid treatment of patients. For this purpose, during last year rapid diagnostic tests have been proposed based on RT-PCR technology as well as antigen detection immunoassay methods. These tests have been employed for diagnostic laboratory routine to reduce turn around time (TAT) of results and to provide rapid SARS-CoV-2 identification in the biological samples, mostly nasopharyngeal swab.

Given the shortage of therapeutic resources to counter the SARS-CoV-2 pandemic, clinical and pharmacological research is currently focused on exploring potential targets for the treatment of COVID-19. The ability of coronaviruses to rapidly evolve and adapt represents a significant potential obstacle and makes the development of effective and durable therapeutic strategies challenging [8,9]. Moreover, it is possible that mutational variants might modulate not only the spread of the disease but also the clinical presentation of COVID-19 [10–12]. Genomic RNA structures, expected to play crucial roles in several steps of the coronavirus replication cycle, including secondary structure maps of the full SARS-CoV-2 coronavirus genome and secondary structure-restrained 3D modeling, represent key resources for the identification of putative therapeutic targets [13].

Finally, vaccines are the best weapon to avoid and manage infectious diseases. In the fight against the spread of COVID-19, several vaccines have been approved for inoculation in the general population and many more remain in development.

### 1.1. Role of genomic analysis in SARS-CoV-2 infection

Over the course of the SARS-CoV-2 pandemic, due to whole-genome sequencing technologies, an unprecedented number of genomes have been generated, thus providing invaluable insights into the ongoing evolution and epidemiology of the virus. As of today, 21 March 2021, 830K complete or near-complete genome sequences are publicly available, and this large amount of data has been playing an important role in SARS-CoV-2 mitigation and control strategies.

On July 2020, Rambaut et al. [8,9] designed a *dynamic* nomenclature for SARS-CoV-2 lineage assignment in order to facilitate the real-time genomic epidemiology. By providing commonly agreed labels to refer to viruses circulating in different parts of the world, it was then possible to outline the links between outbreaks that share similar viral genomes. For this purpose, an algorithm named *Phylogenetic Assignment of Named Global Outbreak LINeages* [pangolin] [9], available at <https://github.com/hCoV-2019/pangolin>, was implemented.

According to this classification, two major lineages at the root of the phylogeny of the SARS-CoV-2 can be distinguished worldwide, namely lineages A and B [14–16]. After the worldwide spread, lineages A and B were further divided into sub-lineages and other two main lineages were detected, namely lineages C and D, that have been recently reassigned as alias of B lineage [17]. Furthermore, more than 1000 lineages have been identified worldwide (updated up to 21 January 2021), and since the start of the COVID-19 pandemic, several reports described unusual public health events possibly due to variants of SARS-CoV-2.

A variant of SARS-CoV-2 with D614G substitution in the gene encoding spike protein emerged in late January/early February 2020, becoming the dominant form of the virus circulating globally [17].

In August and September 2020, a SARS-CoV-2 variant linked to infection among farmed mink and subsequently transmitted to humans was identified in North Jutland, Denmark [18]. The variant presented a combination of mutations not previously observed, which raised concern regarding the potential role in reducing virus neutralization in humans, decreasing the extent and duration of immune protection following natural infection or vaccination. However, after extensive investigation and surveillance, it does not appear to have spread widely. Recent reports of virus variants from the UK, South Africa, and Brazil have raised interest in the impact of viral changes.

These three variants, identified as ‘variants of concern’ (VOCs) (B.1.1.7 or VOC202012/01, B.1.351 or 20 H/501Y.V2 and P.1) carrying several mutations in the receptor-binding domain (RBD) of the spike (S) protein, raise concerns about their potential to shift the dynamics and public health impact of the pandemic [19–22]. They appear potentially associated with (i) increased transmissibility, (ii) propensity for reinfection, (iii) escape from neutralizing antibodies, and (iv) increased affinity for the human ACE2 receptor [23–25].

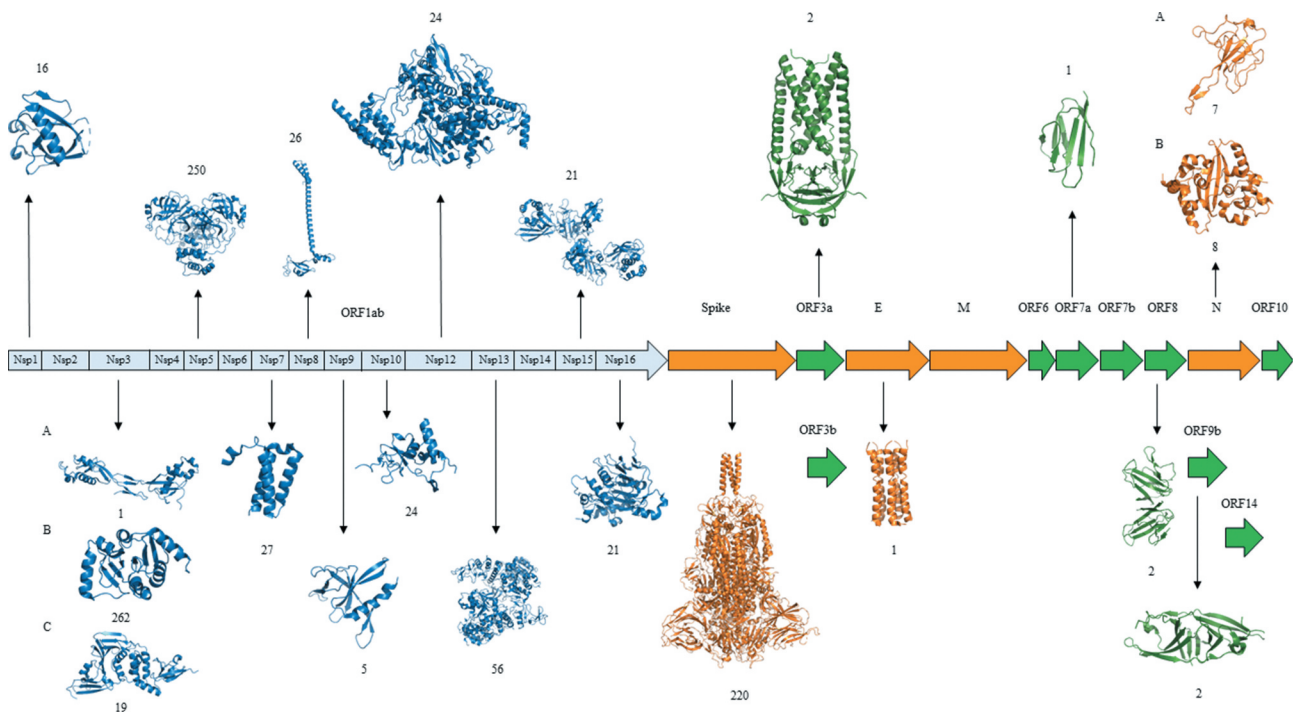
In December 2020, routine genomic surveillance in the UK reported a new and genetically distinct phylogenetic cluster of SARS-CoV-2 (variant VOC202012/01, lineage B.1.1.7) (Table 1) [26]. In October 2020, a separate SARS-CoV-2 cluster (variant 501Y.V2, lineage B.1.351), which carried a different constellation of genetic changes, was also detected by the Network for Genomic Surveillance in South Africa [20]. At the beginning of January, 45 countries reported the presence of B.1.1.7 and 13 countries had reported B.1.351/501Y.V2. B.1.1.7 and B.1.351 genome sequences were available for 28 and 8 countries, respectively [27] (Table 1).

A new variant in December 2020, named P.1 (descendant of B.1.1.28), has been detected in Manaus, Amazonas state, north Brazil [28]. Those lineages carry mutations, especially in the virus spike protein (among them N501Y and the E484K), that have raised concern on their potential impact on infectivity, immune escape and reinfection, and appear to have grown rapidly in relative frequency since their discovery. Early analyses emphasized the potential for rapid virus dissemination (Table 1). The discovery and rapid spread of these variants highlight the importance of real-time and open data for tracking the spread of SARS-CoV-2 and for informing future public health/immunization interventions and travel advice. Whole SARS-CoV-2 genome sequencing, or at least the sequencing of whole or partial S-gene, is important to confirm infection with a specific variant. In addition to those techniques,

Table 1. Real-time reverse transcription PCR assays authorized by the Italian Ministry of Health.

Assay	Specimen type	Gene target	Limit of detection (LOD)	Manufacturer
Bosphore Novel Coronavirus (2019-nCoV) Detection Kit	Nasopharyngeal swab, oropharyngeal swab, sputum, bronchoalveolar lavage	E, orf1ab	25 copies/reaction	Anatolia Genetik, Turkey
STANDARD M nCoV Real-Time Detection Kit	Nasopharyngeal swab and throat swab, sputum	E, ORF1ab	NA*	SD BIOSENSOR Inc, Korea
Allplex™ SARS-CoV-2 assay	Sputum nasopharyngeal swab, nasopharyngeal aspirate, bronchoalveolar lavage, throat swab	E, RdRp, N, S	50 copies/reaction	Seegene Inc, Korea
QUANTY COVID-19	Nasopharyngeal swab, oropharyngeal swab, sputum, serum	N	NA*	CLONIT SRL, Italy
GENEFINDER COVID-19 PLUS REALAMP KIT	Bronchoalveolar lavage fluid, throat swab, sputum	E, RdRp, N	10 copies/reaction	OSANG HEALTHCARE Co., Korea
Novel Coronavirus COVID-19 (2019 nCoV) Real Time Multiplex RT-PCR Kit	Nasopharyngeal swab, oropharyngeal swab, bronchoalveolar lavage, sputum, endotracheal aspirate	E, N, ORF1ab	$1 \times 10^3$ copies/ml	Liferiver, SHANGHAI ZI BIO-TECH CO., LTD, China
LabGun™ COVID-19 RT-PCR Kit	Nasopharyngeal swab, oropharyngeal swab, nasopharyngeal wash/aspirate, nasal aspirate, sputum	E, RdRp	20 copies/μl	LabGenomics Co., Ltd, Korea
REALQUALITY RQ-2019-nCoV CoVID-19 Detection Kit	Nasopharyngeal swab, bronchoalveolar lavage fluid, sputum	E, RdRp	NA*	AB ANALITICA s.r.l., Italy
<b>Rapid molecular tests (time to results)</b>	Nasopharyngeal swab, oropharyngeal swab, bronchoalveolar lavage	N, ORF1ab	500 copies/ml	OACP S.R.L., Italy
Xpert Xpress SARS-CoV-2 (45 min)	Nasopharyngeal, nasal, mid-turbinate swab	E, N2	250 copies/ml	Cepheid, Sunnyvale, CA, USA
QIAstat-Dx Respiratory SARS-CoV-2 Pane (60 min)	Nasopharyngeal swab	E, ORF1b, RdRp	500 copies/ml	Qiagen, Hilden, Germany
BIOFIRE® Respiratory panel 2.1 (45 min)	Nasopharyngeal swab	S, M	160 copies/ml	bioMérieux, Marcy l'Etoile, France
Simplexa™ COVID-19 Direct kit (60 min)	Nasal swab, nasopharyngeal swab, nasal wash/aspirate, and bronchoalveolar lavage	ORF1ab, S	500 copies/ml (NPS, NW/A); 242 copies/m I(NS): 1208 copies/ml (BAL)	DiaSorin Molecular LLC, Cypress, CA, USA
VitaPCRTM SARS-CoV-2 Assay (20 min)	Nasopharyngeal swab, oropharyngeal swab	N	2.73 copies/μl	Menarini Diagnostics, Florence, Italy
ID NOW COVID-19 assay (13 min)	Nasopharyngeal swab, throat swab	RdRp	125 genome equivalents/ml	Abbott Diagnostics, IL, USA

\*Not applicable



**Figure 1.** Genomic and proteomic organization of SARS-CoV-2. The proteins encoded by the SARS-CoV-2 genome consist of ORF1ab (that is proteolytically cleaved to form 16 nonstructural proteins, shown in sky-blue cartoon models), four structural proteins (shown in orange) and eight accessory proteins (shown in green). Number associated with each protein or protein domain represent the estimated number of PDB files. Letters indicate domains within the same protein whose structure has been solved independently.

alternative methods have been also developed, such as diagnostic screening PCR-based assays, that are currently recognized by the WHO. Among them, multiple real-time RT-PCR assays, targeting E and/or N and/or ORF-1 genes, have been used in combination with the S-gene target. This would allow the screening of the VOCs to be integrated in a single run with the normal routine [23]. Another recently developed diagnostic assay is based on the detection of the ORF1a gene (ORF1a  $\Delta$ 3675–3677) that exists in all three variants. Using ORF1a  $\Delta$ 3675–3677 as the primary target and spike  $\Delta$ 69–70 to differentiate the variants, an open-source PCR assay was also designed to detect SARS-CoV-2 variants of concern [24].

## 1.2. SARS-CoV-2 structural biology

The viral genome of SARS-CoV-2 encodes 16 nonstructural proteins (NSPs), four major structural proteins known as sSpike (S), Envelope (E), Membrane (M), Nucleocapsid (N) proteins, and several accessory proteins denoted as ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF10, and ORF14 [25].

The structural biology community has spent unprecedented efforts to rapidly gain knowledge on the proteins synthesized by the virus. Experimental (X-ray crystallography, NMR, cryo-electron microscopy) and theoretical (homology modeling, ab initio predictions) techniques have produced a vast, though not exhaustive, amount of data on SARS-CoV-2 proteins and their complexes with small molecules or with other protein components. As of January 2021, 921 files containing SARS-CoV-2 information are available with the PDB

([www.rcsb.org/pdb](http://www.rcsb.org/pdb)) repository. The number of PDB files containing experimental structures of SARS-CoV-2 proteins has been growing linearly (Figure 1). Besides PDB, other repositories organize and distribute structural data on SARS-CoV-2 proteins, e.g. PDBe-KB COVID-19 Data Portal ([www.ebi.ac.uk/pdbe/covid-19](http://www.ebi.ac.uk/pdbe/covid-19)) and NCBI SARS-CoV-2 Resources ([www.ncbi.nlm.nih.gov/sars-cov-2](http://www.ncbi.nlm.nih.gov/sars-cov-2)).

At the onset of the pandemic, theoretical methods played an important role in providing timely and valuable structural information [26]: for example, the I-Tasser [27] and SwissModel [28] servers have hosted a continuously updated set of predicted SARS-CoV-2 proteins. Homology modeling techniques generated structural information analyzed in several published papers [28–34]. Although experimental methods are actively providing structural knowledge, theoretical methods are still significantly contributing to the understanding of SARS-CoV-2 molecular properties [35,36] and to drug assessment or repurposing [36–38].

The first structure of a SARS-CoV-2 protein, the main protease NSP5 (denoted as 3CL<sup>Pro</sup> or M<sup>Pro</sup>), was deposited in January 2020 (PDB ID code 6LU7). This protein has been solved through X-ray crystallography experiments as a complex with the inhibitor N3 [39]. Up to January 2021, there were approximately 250 PDB files reporting structural data of the main protease complexes (Table 2). The M<sup>Pro</sup> is an obvious target for inhibitors acting as antiviral drugs, and many of the deposited structures are in fact complexes with a variety of inhibitors [40]. The coordinates of the complexes between the ACE2 receptor and the SARS-CoV-2 Spike receptor-binding domain were released (PDB ID code 6LZG)

**Table 2.** Proteins in the Sars-CoV-2 genome.

NSPs	Annotation	Sequence position within ORF1ab	Sample PDB structure <sup>a</sup>	No. of PDB files
NSP1	Suppresses host gene expression	1–180	7K3N: (1–180)	16
NSP2	Interacts with host factors PHB 1 and PHB2	181–818	none	0
NSP3	Papain-like proteinase	819–2763	7KAG: (1–111) 6WEY: (207–412) 6WX4: (746–1061)	282
NSP4	Required for viral replication	2764–3263	None	1*
NSP5	3CL-protease	3264–3569	6LU7: (1–306)	250
NSP6	Induces double-membrane vesicles in infected cells	3570–3859	None	0
NSP7	Cofactor for the RNA-dependent RNA polymerase	3860–3942	6YHU: A, C (1–83) 7BV2: C (1–83) 6XEZ: C (1–83) 6YYT: C (1–83)	27
NSP8	Cofactor for the RNA-dependent RNA polymerase	3943–4140	6YHU: B, D (1–198) 7BV2: B (1–198) 6XEZ: B (1–198) 6YYT: B, D (1–198)	26
NSP9	Functions in viral replication	4141–4253	6W9Q: (1–113)	5
NSP10	Stimulates the viral mRNA capping machinery.	4254–4392	6W4H: B (1–139)	24
NSP12	RNA-directed RNA polymerase	4393–5324	7BV2: A (1–932) 6XEZ: A (1–932) 6YYT: A (1–932)	24
NSP13	Helicase	5325–5925	6XEZ: E, F (1–601)	56
NSP14	Proofreading exoribonuclease/guanine-N7 methyltransferase	5926–6452	None	0
NSP15	Uridylate-specific endoribonuclease	6453–6798	6WXC: (1–346)	21
NSP16	2'-O-methyltransferase	6799–7096	6W4H: A (1–298)	21
<b>Accessory proteins</b>	<b>Annotation</b>	<b>Sequence length</b>	<b>Sample PDB structure</b>	<b>No. of PDB files</b>
ORF3a	Forms potassium ion channels	275	6XDC: (1–275)	2
ORF3b	Appears to block induction of IFN-I	22	None	0
ORF6	Virulence factor	61	None	0
ORF7a	Inhibits BST-2 glycosylation	121	6W37: (16–82)	1
ORF7b	Appears to be a viral attenuation factor	43	None	0
ORF8	May play a role in host-virus interaction	121	7JTL: (1–121)	2
ORF10	Hypothetical protein	38	None	0
ORF9b	Infects the host and promote virulence	97	6Z4U: (1–97)	2
ORF14	Infects the host and promote virulence	73	None	0
<b>Structural proteins</b>	<b>Annotation</b>	<b>Sequence length</b>	<b>Sample PDB structure</b>	<b>No. of PDB files</b>
Spike	Binds to the host receptors and initiate infection	1273	6XR8:(1–1273)	220
Envelope	Functions in virus morphogenesis and assembly	75	7K3G: (8–39)	1
Membrane	Functions in virus morphogenesis and assembly	222	None	0
Nucleocapsid	Packages the viral genome RNA	419	7CDZ: (47–174) 7CE0: (255–364)	21 (6*)

a) PDB code followed by the chain identifiers, in case of multichain assembly. Parentheses enclose the coverage, namely the sequence range within the protein covered by the structural coordinates.

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\*indicates fragment of the entire protein.

immediately after the main protease structure, in February 2020 [41,42]. Currently, there are about 220 PDB files containing the coordinates of the Spike protein solved with different techniques, in a variety of forms and complexes.

Figure 1 shows the map of the SARS-CoV-2 proteins with available structural data, along with the number of PDB files associated with each protein or protein domain. At present, M<sup>Pro</sup> and Spike are the most studied proteins, the latter being one of the most important targets of vaccines [43].

Nsp3 is also one of the most studied protein, as indicated by about 280 files deposited in the PDB. However, Nsp3 is a multidomain protein and the different domains have been studied with unequal focus. For example, the Papain-like and the Macrodomein domain are reported in 19 and about 250 PDB files, respectively. On the contrary, the ubiquitin-like and the RNA binding domains have one PDB file each.

In the following paragraph, other examples of solved non-structural SARS-CoV-2 proteins or protein domains are briefly described. The three-dimensional structure of Nsp1, a virulence factor inhibiting host gene expression, has been reported in 16 PDB files. Among these, the full-length crystal structure of the protein has been deposited with the PDB code 7K3N [44]. Nsp7 and Nsp8 participate in viral replication by forming a complex acting as a primase in viral RNA synthesis the structure of which is in the structure 6YHU [45]. This complex associates with Nsp12, the RNA-dependent RNA polymerase (RdRp), forming part of the replication and transcription assembly. The crystal structure of the SARS-CoV-2 complex composed of Nsp12 polymerase bound to Nsp7 and Nsp8, also called RdRp complex has been deposited under the PDB code 6YYT [46]. The RdRp complex is a target for antiviral agents. The crystal structure of RdRp complex in association with Remdesivir, an inhibitor that blocks synthesis

of RNA, is available with PDB code 7BV2 [47]. Recently, it has been reported the cryo-EM structure of SARS-CoV-2 Nsp12-Nsp7-Nsp8 in complex with two molecules of the SARS-CoV-2 Nsp13 helicase and an RNA template (PDB code: 6XEZ) [48].

Crystal structures of SARS-CoV-2 Nsp9, which may participate in viral replication as a ssRNA-binding protein, revealed a peptide-binding site that could be targeted by new antiviral drugs [PDB code: 6W9Q] [49]. Several crystallographic structures of SARS-CoV-2 Nsp10 have shown that it forms the 2'-O-methyltransferase complexes with Nsp16, thus causing its activation. The crystal structure of this complex associated with S-adenosylmethionine has been reported in the coordinate set denoted by the PDB code 6W4H [50]. Nsp15, the uridylate-specific endoribonuclease, has been determined as a complex with the repurposing drug Tipiracil bound to its active site (PDB code: 6WXC). This suggests that Nsp15 may be a target for antiviral molecules [51]. Among the structural proteins, SARS-CoV-2 N protein, that plays a role in the viral genomic RNA packaging, has been reported in 21 PDB files. In particular, the crystal structures of the N- and C-terminal domains are available (e.g. PDB code: 7CDZ, 7CE0) [52], while the coordinates of the linker portion are still missing.

Despite the fast accumulation of data, the structures of a few NSPs, including Nsp2, Nsp4, Nsp6, Nsp14, and several domains of Nsp3, ORF3b, ORF6, ORF7b, and ORF10 are still not available. Nevertheless, the structure of the Nsp4 and Nsp14 proteins was predicted using comparative modeling and structural templates from murine hepatitis virus and SARS-CoV, respectively [33]. The unsolved Mac2, Mac3, DPUP, and NAB domains of Nsp3 from SARS-CoV-2 have been modeled using the cognate crystal structures of proteins from SARS-CoV as templates [53,54].

Availability of structural information is also of utmost importance for understanding the mutation dynamics of the virus and the impact of the variations on SARS-CoV-2 biology and pathogenicity. For example, several surveillance activities are ongoing to map the Spike structure in order to define the position of the isolated variants and assess whether they have a potential impact on immunogenicity [55,56]. Likewise, data on the variants of single proteins have also been reported [27,57–60].

In conclusion, structural information on SARS-CoV-2 is accumulating at a fast pace. The wealth of data is now waiting for a careful and in-depth analysis to extract knowledge that will enable scientists to cope with this and future pandemics.

### 1.3. Viral mutations and their implications for the management of public health measures

While in most viruses the RNA polymerase lacks proofreading capability, there are nonetheless some exceptions like the *Nidovirales* order (which includes *Betacoronavirus*). In *Nidoviruses* a ternary complex composed by a polymerase (RNA-dependent RNA polymerase, RdRp) and two NSPs, is responsible for virus replication and transcription [61,62].

Analyses of sequenced genomes of SARS-CoV-2 are being actively examined to determine the extent of new mutations and how they affect viral/host relationship. Such variability is likely to have important implications for the control of the pandemic and to assess the emergence of viral strains with different characteristics, including increased or reduced

infectivity and lethality. Indeed, some studies indicated that specific mutations may correlate with increased transmissibility, while others showed that deletions in SARS-CoV-2 genomes are emerging. These changes may potentially reduce virus replication, similarly to what observed in the case of SARS-CoV [63–65]. These latter observations seem indeed to indicate that some variants of SARS-CoV-2 are trying to reach a point of equilibrium with their host, thus allowing spreading in the absence of high mortality rate, though this point of equilibrium has not been yet reached.

However, the contribution of specific mutations to these two parameters is difficult to determine, especially at the beginning of a pandemic event when the sequences would tend to be more homogenous and the virus is likely to be very aggressive. Besides, it is quite difficult to timely and correctly assess the exact number of infected subjects and calculate the correct mortality rate without a robust testing analysis in place to monitor the viral spread. To this regard, it is worth noting that in the initial phases of the pandemic, many patients were diagnosed with COVID-19 only after developing critical illness or even at the time of death. On the other hand, the vast majority of asymptomatic or paucisymptomatic patients were untested. This lack of precise data due to the initial shortage of testing capabilities leads to a miscalculation of the fatality rate [66], which in turn led to biases in determining the mortality curves. Over time, countries have been adopting better policies for diagnostic PCR testing and, as a result, the spread of the virus is better monitored and the data are more carefully determined. However, the process still needs improvement.

New mutation hotspots are now emerging in the spike portion of the genome, which is the one targeted by the vaccines currently in use. Indeed, closely monitoring changes in this region would ensure that the efficacy of the vaccines currently in use is not severely compromised and could allow us to promptly design new, improved versions. In addition, mutations in other parts of the viral genome need to be monitored and their biological significance addressed when the situation requires.

For these reasons, the current monitoring strategies need to incorporate a series of steps: (i) testing for rapid viral detection; (ii) collection on the territory and fast processing of samples to generate viral sequences, including whole-genome sequence; and (iii) quick analysis of viral sequences.

The concomitant execution of these steps would allow for the rapid recognition of new emerging clusters of infection, which in turn could help shape more focused interventions with the final goal of early containment and improved vaccination strategies.

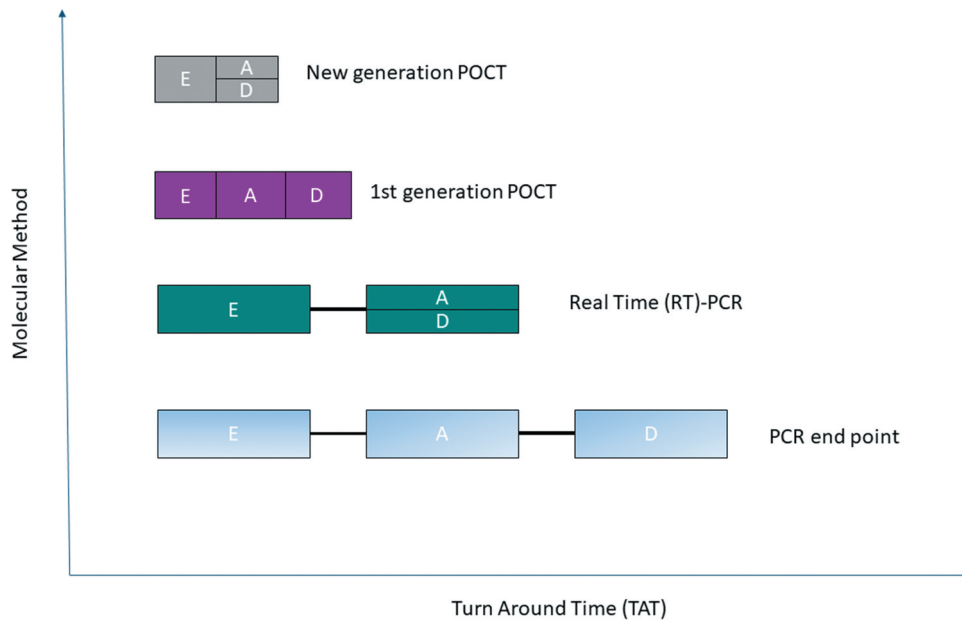
### 1.4. Molecular diagnostics

Nuclei acid testing (NAT) is currently the gold standard test for the molecular diagnosis of SARS-CoV-2 infection. NAT assays differ in terms of sensitivity, gene targets, and specimen type [67]. They are designed to include one specific target alone (e.g. different regions of the N gene, <https://www.fda.gov/media/134922/download>) or a combination of specific SARS-CoV-2 target genes (e.g. N, RdRp, ORF1ab, S). In addition, a region of the E gene shared by the

Table 3. List of validated molecular assays available on the market for the diagnosis of SARS-CoV-2 infection.

Assay	Specimen type	Gene target	Limit of detection (LOD)	Manufacturer
Bosphore Novel Coronavirus (2019-nCov) Detection Kit	Nasopharyngeal swab, oropharyngeal swab, sputum, bronchoalveolar lavage	E, orf1ab	25 copies/reaction	Anatolia Genetik, Turkey
STANDARD M nCoV Real-Time Detection Kit	Nasopharyngeal swab and throat swab, sputum	E, ORF1ab	NA*	SD BIOSENSOR Inc, Korea
Allplex™ SARS-CoV-2 assay	Sputum nasopharyngeal swab, nasopharyngeal aspirate, bronchoalveolar lavage, throat swab	E, RdRp, N, S	50 copies/reaction	Seegene Inc, Korea
QUANTITY COVID-19	Nasopharyngeal swab, oropharyngeal swab, sputum, serum	N	NA*	CLOMIT SRL, Italy
GENEFINDER COVID-19 PLUS REALAMP KIT	Bronchoalveolar lavage fluid, throat swab, sputum	E, RdRp, N	10 copies/reaction	OSANG HEALTHCARE Co., Korea
Novel Coronavirus COVID-19 (2019 nCoV) Real Time Multiplex RT PCR Kit	Nasopharyngeal swab, oropharyngeal swab, bronchoalveolar lavage, sputum, endotracheal aspirate	E, N, ORF1ab	1 × 10 <sup>3</sup> copies/ml	Liferiver, SHANGHAI ZI BIO-TECH CO., LTD, China
LabGun™ COVID-19 RT-PCR Kit	Nasopharyngeal swab, oropharyngeal swab, nasopharyngeal wash/aspirate, nasal aspirate, sputum	E, RdRp	20 copies/μl	LabGenomics Co., Ltd, Korea
REALQUALITY RQ-2019-nCoV COVID-19 Detection Kit	Nasopharyngeal swab, bronchoalveolar lavage fluid, sputum	E, RdRp	NA*	AB ANALITICA s.r.l., Italy
SARS-CoV-2 ddPCR Kit	Nasopharyngeal swab, oropharyngeal swab, bronchoalveolar lavage	N, ORF1ab	500 copies/ml	OACP S.R.L., Italy
	Nasopharyngeal wash/aspirate and nasal aspirate	N1 and N2	0.260 copies/μl to 0.351 copies/μl	Bio-Rad Laboratories, Inc, Italy
<b>Rapid molecular tests (time to results)</b>				
Xpert Xpress SARS-CoV-2 (45 min)	Nasopharyngeal, nasal, mid-turbinate swab	E, N2	250 copies/ml	Cepheid, Sunnyvale, CA, USA
QIAstat-Dx Respiratory SARS-CoV-2 Pane (60 min)	Nasopharyngeal swab	E, ORF1b, RdRp	500 copies/ml	Qiagen, Hilden, Germany
BIOFIRE® Respiratory panel 2.1 (45 min)	Nasopharyngeal swab	S, M	160 copies/ml	bioMérieux, Marcy l'Etoile, France
Simplexa™ COVID-19 Direct kit (60 min)	Nasal swab, nasopharyngeal swab, nasal wash/aspirate, and bronchoalveolar lavage	ORF1ab, S	500 copies/ml (NPS, NW/A); 242 copies/rm	DiaSorin Molecular LLC, Cypress, CA, USA
VitaPCR™ SARS-CoV-2 Assay (20 min)	Nasopharyngeal swab, oropharyngeal swab	N	I(NS): 1208 copies/ml (BAL)	Menarini Diagnostics, Florence, Italy
ID NOW COVID-19 assay (13 min)	Nasopharyngeal swab, throat swab	RdRp	2.73 copies/μl 125 genome equivalents/ml	Abbott Diagnostics, IL, USA

\*Not applicable



**Figure 2.** Turn around time for molecular testing during their evolution from end point PCR with by three different steps of nucleic acid extraction (e) amplification (a) and detection (d) through real-time (RT) PCR with simultaneous amplification and detection steps until point of care testing (POCT) testing more and more fast from first to last generation methods by integrated extraction, amplification and detection steps in a single cartridge system.

*Sarbecovirus* subgenus is amplified by some of the available validated commercial assays [68]. Although NAT is the reference method for the etiological diagnosis of COVID-19, false negative results may occur due to technical reasons, quality of the specimen, source of specimen [69], timing of specimen collection (too early or too late during infection), and low viral load. Droplet digital PCR (ddPCR) designed for absolute quantification of the target may perform better in detecting low viral load. ddPCR has been proposed for evaluating the efficacy of future anti-SARS-CoV-2 drugs through the determination of the viral load during the course of the disease [70]. Recently, the US FDA granted Emergency Use Authorization [EUA] for Bio-Rad's SARS-CoV-2 ddPCR Kit, which amplifies the N1 and N2 genes according to the CDC protocol. Other interesting assays have been developed that are worth mentioning. Among them, MGI Tech uses next-generation sequencing (NGS) to detect all pathogens in the specimen including SARS-CoV-2 or Innovita that uses isothermal amplification followed by chip detection [71]. A list of available molecular tests cleared by the Italian Ministry of Health is reported in Table 3.

#### 1.4.1. Future perspectives

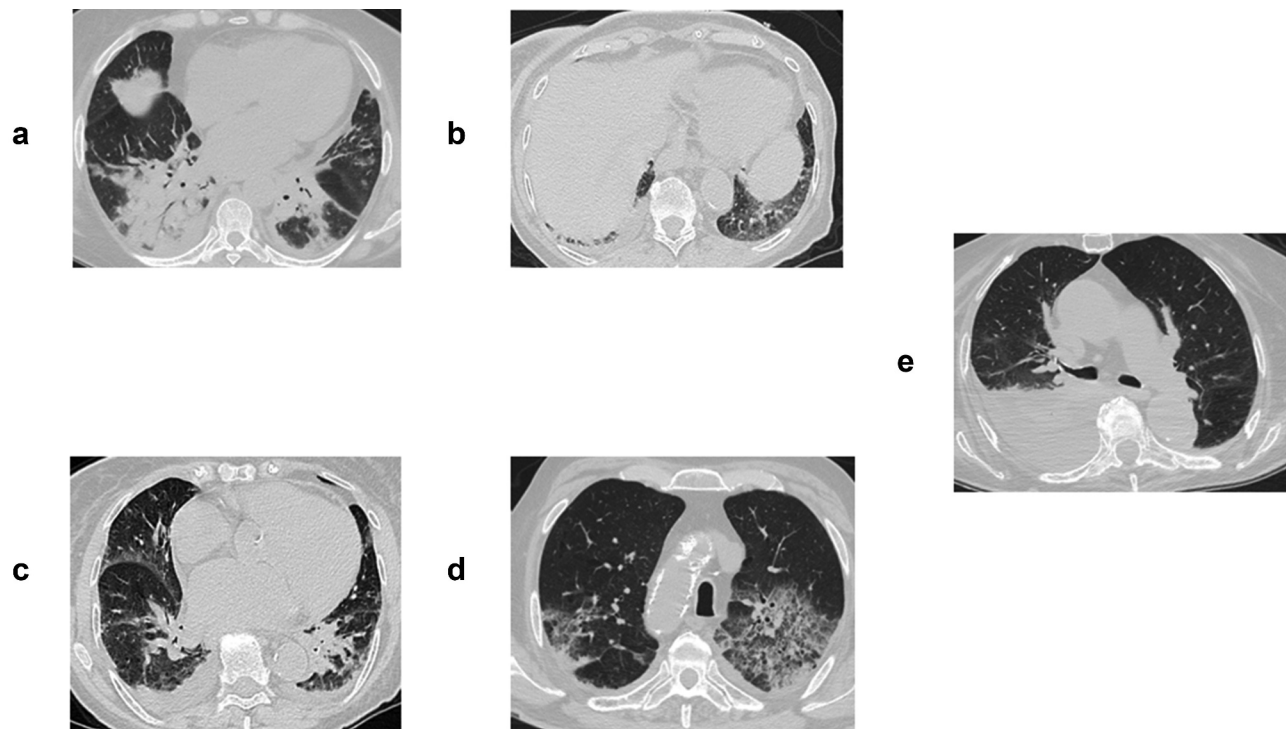
In recent years, faster and easy-to-use tests have been developed and a rapid evolution of this technology has been observed, as schematized in Figure 2. This fast approach includes rapid molecular testing as Cepheid's Xpert\_ Xpress SARS-CoV-2, DiaSorin's SimplexaTM COVID-19 Direct or ID NOWTM COVID-19 diagnostics. These tests have a fast turnaround time ranging from 1 h to about 13 min [72] (Table 3). These assays are generally single-cartridge-integrated nucleic acid extraction-amplification, easy to use that do not require a specific education on

molecular techniques. This kind of test, beyond being rapid, allows to manage emergency as in this pandemic period even in the absence of specific skilled staff. These tests, which offer fast and accurate detection of viral pathogens, are likely to have an immediate impact on rapid clinical diagnosis as well as on epidemiological decisions. They use a lysis buffer to inactivate the virus and provide the opportunity to be used as point of care testing (POCT), even when a biosafety cabinet is not available. Furthermore, POCT could help for triage of suspected cases of infection, especially in limited resource settings where it could guide quarantine restriction.

Future perspectives include testing kits that might be used at home for self-isolated patients with suspected SARS-CoV-2 infection. In case of positivity, patients could be monitored at home and further clinical decision could be taken based on the evolution of the clinical conditions [73]. Because of their intrinsic characteristics (fast, cheap, and easy to use), POC assays are amenable to large-scale use among the general population, particularly during the pandemic. Future researches are recommended and encouraged for improving the sensitivity and specificity of POC assays.

Recently, new SARS-CoV-2 variants have been described [71] and viral genome sequencing represents an important tool for fighting the spread of the virus. From this perspective, NGS represents a fundamental methodology which provides fast complete whole-genome sequence and that can be used by the scientific community for (i) molecular epidemiological studies, (ii) phylogenetic studies, (iii) development of antiviral drugs, (iv) vaccine development, and (v) design of diagnostic tests. Furthermore, NGS protocols have been revised to overcome some methodological limitations such as protocol length, reagents





**Figure 3.** Radiological features of COVID-19 disease: pneumonia with bacterial overinfection (a), ground-glass opacities of the lower lobes (b), bilateral infiltrates along with pericardial effusion (c), bilateral upper lobe ground-glass opacities, (d) and pneumonia with abundant pleural effusion (e).

availability, and sample degradation. The new protocols shortened library preparation from 12 to 3 hs, increased the success rate of viral genome sequencing, and provided a rapid bioinformatics workflow for genome assembly [74].

### 1.5. SARS-CoV-2 disease clinical characteristics, molecular approaches, and modern clinical strategies

COVID-19 accounts for over 100 million cases worldwide due to the infection with a mortality ranging from 2 to 10% [75]. A wide range of respiratory symptoms are described among which are dry cough, dyspnea, weakness, sore-throat, and fever [76,77].

The mortality was mainly observed in elderly patients affected by comorbidities [78]. One of the respiratory comorbidities that often occur in COVID-19 patients is chronic obstructive pulmonary disease (COPD).

COPD is a respiratory disease that is characterized by airflow limitation not fully reversible with a progressive decline in respiratory function [79]. Cigarette smoke is in turn the foremost cause of COPD development by inducing inflammation and bronchial remodeling [80].

Cross-sectional and longitudinal studies demonstrated that COPD worsens the prognosis of COVID-19 patients favoring the progression of the disease and determining a high risk of serious events [OR >6] such as lung failure, heart failure, and the need of mechanical ventilation [80].

Furthermore, COPD along with smoking habit affects overall survival in COVID-19 patients [81].

An increase in inflammatory markers is a common finding, including C-reactive protein (CRP), which is often associated with lymphopenia [82,83].

It is known that COPD and history of smoking could affect the immune response, leading to an altered blood cells number and notably a reduction of lymphocytes subtypes weakening the immune response and favoring bacterial overinfections [84].

Moreover, SARS-CoV-2 has a particular tropism for the lung, eliciting inflammation characterized by an increased level of inflammatory cells and causing mainly interstitial or lobar pneumonia [85].

This is typically characterized by bilateral, peripheral infiltrates, and multifocal ground-glass patchy opacities that could be found on computed tomography (CT) imaging associated with different levels of lung failure. In some cases, a superimposed bacterial infection may be found [86,87]. The extent of compromised parenchyma on CT correlates with the clinical severity.

The radiological manifestations are associated with the clinical symptoms development [88] and inflammatory alterations.

COVID-19 patients who developed pneumonia have higher serum level of cytokines such as TNF- $\alpha$ , IFN- $\gamma$ , IL-6 aside from CRP compared to healthy subjects. Within COVID-19 patients, serum IL-6 and IL-10 levels are significantly higher in severe

patients than in patients with mild clinical symptoms. As a result, higher levels of pro-inflammatory cytokines are associated with a more severe disease development [89].

Additionally, patients with inflammatory bronchopulmonary disease could also present an altered level of ferritin and D-dimer [90,91]. Some antiretroviral agents such as ritonavir and lopinavir could be effective by a strong binding to hydrogen residues of the virus [92].

Concerning the radiological manifestations, a wide variability of findings could be observed with different radiological and clinical outcome [93,94]. Chest radiography severity score is predictive of poor prognosis and mechanical ventilation risk.

There are several possible radiological and clinical features in order of frequency, namely ground glass opacities pleura thickening, interlobular septal thickening, pleural effusion, bronchiectasis, pericardial effusion, and lymphadenopathy (Figure 3).

A severe bilateral pneumonia often results in lung failure with two different outcomes: patients with mild hypoxia who need only oxygen therapy and patients with severe hypoxia and  $\text{PaO}_2/\text{FiO}_2$  rate  $<200$  who do not compensate hypoxia needing mechanical ventilation [95,96]. The latter is also applied in severe pneumonia patients with ARDS development.

A significant improvement in  $\text{PaO}_2/\text{FiO}_2$  ratio can be observed in patients with an acute respiratory distress syndrome (ARDS) evolution after mechanical noninvasive ventilation [97]. The improvement in  $\text{PaO}_2/\text{FiO}_2$  might be related to blood redistribution and recovery from hypoxic vasoconstriction. Noninvasive mechanical ventilation with CPAP modality could be effective in very sick hypoxic patients who are deemed to not benefit from invasive ventilation [98].

Patients with asthma and COVID-19 are more likely to be older, predominantly female, and smoke exposed, with a higher prevalence of comorbidities. The use of inhaled corticosteroids may exert a protective effect against severe COVID-19 symptoms [99].

Until now, a wide portion of therapeutic strategies for COVID-19 were based on previous experience in the treatment of SARS, MERS, or other related viral infections, exploiting the presence of possible common molecular targets and structural similarities between the different pathogens. Examples are

antiviral agents such as lopinavir/ritonavir and glycopeptides, which, however, have not yielded striking results [100–103].

Currently, a new promising area of research investigated by computational analysis has emerged, which takes advantage of genes networks analysis to gather information that could contribute to the understanding of the pathophysiology of COVID-19. Bioinformatic prediction models can thus lead to the rapid identification of existing drugs, which could be repurposed as treatments against COVID-19, by characterization of key genes involved in the host response to the pathogen and potentially able to interact with anti-SARS-CoV-2 molecules [104,105]. The molecular mapping of the viral sites responsible for interacting with the host and their potential variations and the whole-host genome sequencing analysis are the prerequisite for those types of approach. To this regard, a large clinical trial (CALYPSO trial, ClinicalTrials.gov Identifier: NCT04353401) involving a cohort of 5000 COVID-19-positive patients is currently ongoing to better understand the pathogenetic mechanisms of SARS-CoV-2 in the context of its interaction with the host genome through whole-genome sequencing analysis [106]. In fact, characterizing the role that human genetic diversity plays in the adaptive evolution of SARS-CoV-2 and the related variations in the viral genetic heritage can have a major impact on the understanding of therapeutic strategies to be adopted against COVID-19.

### 1.6. Platforms used in developing SARS-CoV-2 vaccine candidates

The WHO list of vaccines in development against SARS-CoV-2/COVID-19 is regularly updated [107]. At present, nine whole virus-inactivated candidate vaccines are in clinical trials. The first reported inactivated SARS-CoV-2 virus vaccine, called PiCoVacc, triggered an effective humoral immune response providing complete protection to SARS-CoV-2 infection in non-human primates [108]. However, the overall efficacy was of just over 50% in phase III clinical trial [109]. A second one, the BBIBP-CorV inactivated vaccine from Sinopharm, was reported to have an efficacy of 79.34% [110–112].

Protein-based vaccines against SARS-CoV-2 infection consist of (i) recombinant spike-protein-based, (ii) recombinant RBD-based, (iii) virus-like particle (VLP)-based, and 20 of them are in clinical phase. The NVX-CoV2373 vaccine from Novavax is based on full-length recombinant SARS-CoV-2 glycoprotein nanoparticle adjuvanted with Matrix M [113]. Preliminary

**Table 4.** Vaccine candidates in phase III clinical stage.

Platform	Vaccine type	Developers	Clinical stage	Ref.
Inactivated	PiCoVacc	Sinovac	III	1–5, 18,19
	BBIBP-CorV	Sinopharm/Wuhan Ins. Biol. Products		
	BBV152	Sinopharm/Beijing Ins. Biol. Products		
	QazCovid-in	Bharat Biotech Res. Ins. Biol. Saf. Problems, Rep of Kazakhstan		
Protein subunit Non-replicating viral vector	NVX-CoV2373	Novavax	III	6
	ChAdOx1-S – (AZD1222)	AstraZeneca/Uni. Oxford	III	7–9, 10, 11,12,20
	Convidecia/Ad5-nCoV	CanSino/Beijing Ins. Bio		
	Gam-COVID-Vac	Gamaleya/Health Min. Russian Federation		
DNA vaccines RNA vaccines	Ad26.COV2.S	Janssen	III	13–15, 16,17,21
	nCov vaccine	ZyduS Cadila		
	mRNA –1273	Moderna/NIAID		
	BNT162 (3LNP-mRNAs)	Pfizer		
	CVnCoV vaccine	CureVac AG		

evidence reported an efficacy rate of 89.3% in the UK phase III clinical trial, despite the massive presence of the B.1.1.7 variant. Conversely, the result was under 50% effective against 501Y.V2 variant, which was detected in South Africa.

Viral vector-based vaccines that may induce prior immunity to the vector failed during clinical trials. For this reason, either rare human or nonhuman-derived vectors were used to circumvent anti-vector immunity. Currently, there are 3 replication-competent and 10 replication-incompetent vector candidates in clinical phase, and of the latter candidates, the University of Oxford/AstraZeneca (ChAdOx1-S) [114–116], the Gamaleya Research Institute (Ad5/Ad26) [117], and the Janssen (Ad26.COVS.2.S) [118,119] are in phase III clinical trials. The ChAdOx1-S has an efficacy of 62–90% depending on dosage and has been authorized in the UK, European Union (EU), and other countries. The Ad5/Ad26, with an efficacy rate of 91.4%, is now being distributed in Russia and other countries.

Nucleic acid vaccines either RNA or DNA are delivered into human cells, where they will then be transcribed into viral proteins that will trigger an immune response. Eight DNA-based candidate vaccines, encoding different forms of the SARS-CoV-2 S protein, are in phase I/II/III clinical trials. Of the seven potential mRNA vaccines against SARS-CoV-2, three are in phase III clinical trials. Moderna mRNA-1273 and Pfizer BNT162 vaccines codify for the spike protein and have an efficacy of approximately 92–95% in protecting against COVID-19 [120–123]. New variant impacts on the effectiveness of COVID-19 vaccines are being studied. Both Moderna and Pfizer-BioNTech mRNA vaccines have been authorized in the USA, UK, EU, and other countries, and the monitoring and analysis of data continue [124–128]. Vaccine platforms for candidate vaccines are summarized in Table 4.

Safety supervising of COVID-19 vaccines at global level is ensured by the WHO. Vaccination can cause mild side effects and rare adverse events. WHO recommends monitoring for the effectiveness and adverse events of vaccines in elderly frail people and in individuals with comorbidities or immunocompromised [128]. To date, mortality linked to COVID-19 vaccine administration, which would indicate a safety problem with COVID-19 vaccines, was not detected ([https://www.who.int/news-room/q-a-detail/coronavirus-disease-\(covid-19\)-vaccines-safety](https://www.who.int/news-room/q-a-detail/coronavirus-disease-(covid-19)-vaccines-safety)).

The most recent data indicate that all the above-mentioned VOCs are susceptible to the inhibitory effect of three of the currently available vaccines (namely, BNT162b2, mRNA-1273, and AD26.COVS.2.S), although at different levels [120,129–132]. The fourth vaccine (ChAdOx1 nCoV-19) has been shown to be effective against the UK and Brazilian variants, but preliminary data indicate that it is not active against the South African variant B.1.351 [133].

Regarding cell-mediated immunity, it is known that convalescent patients have robust T cell immunity against SARS-CoV-2 [134], and recent preliminary data indicate that in subjects vaccinated with BNT162b2 and mRNA-1273 the same effect is observed against all the three VOCs [134,135]. Nonetheless, more data are needed to confirm and validate these results and to extend these observations to the other vaccines [135]. Finally, it should be pointed out that no data are available on

the efficacy of the vaccinated subjects T-cells activity. Data regarding the efficacy of other vaccines currently used against the VOCs and full duration of immunity are not available at the time of writing.

## 2. Conclusion

Molecular and evolutionary analyses have proven to be important and effective in informing public health in the context of this pandemic event. Given that this and other similar viruses are characterized by evolutionary and genetic changes accumulated in their genome, the use of new and improved phylodynamic techniques for the study of how epidemiological, immunological, and evolutionary processes act and potentially interact to shape viral phylogenies is extremely important and useful. In fact, phylogenetic trees constitute a crucial instrument to study virus evolution and molecular epidemiology, elucidating evolutionary relationships between sampled virus variants based on the temporal resolution in the genetic data, thus allowing to better understand their epidemiology. The integration of phylodynamic, epidemiological data and mathematical modeling in molecular evolution has been a fundamental point to better understand the pandemic events in terms of spread into different countries, mutations, and variants creation. Moreover, the phylodynamics concept has generated new opportunities to obtain a more detailed understanding of evolutionary histories. Thanks to the researchers' efforts and public funding efforts, more than 450,000 viral genomes are now available in public databases, in just about a year after the first viral genome was sequenced (gisaid.org). These sequences have allowed researchers to estimate the timing of SARS-CoV-2 spillover into humans, characterize the spread of the virus, and gauge virus adaptation to its new host.

SARS-CoV-2 virus determined a fundamental change in lifestyle and relationship worldwide, given its high contagiousity, the ability of spreading among individuals, the heterogenous clinical presentation, and the different clinical evolution from an individual to another. The virus pandemic surprised the world, pushing science to find new strategies to fight the virus and to save lives, with the ultimate goal now on the close horizon of allowing people to come back to a normal social life. On this basis, the application of new technologies allowed a better diagnostic as well as therapeutic approach for the control of this disease, which never before has been so determinant for many aspects of the modern life. Science moved quickly and the world began knowing molecular epidemiology based on whole-genome sequencing of the virus. SARS-CoV-2 structure biology was improved as well as molecular diagnostic by introducing RT-PCR-based kit on novel and improved molecular platforms for rapid diagnosis. An innovative vaccine based on mRNA was designed, produced in record time, and available worldwide. More and more targeted therapeutic strategies are increasingly being tested. SARS-CoV-2 pandemic radically changed public health approach, drastically marked human life, and will

represent a paradigm driving science and mankind behavior in the future.

### 3. Expert opinion

Molecular technology and novel molecular-based approaches to fight SARS-CoV-2 pandemic have demonstrated their fundamental importance. On this basis, viral evolution and adaptation strategies were able to provide ever new insights into viral biology, an extremely useful tool in understanding how to fight a new virus that endangers human lives and disrupts public health, thus creating a very important problem worldwide. In this landscape, everything needed to move at a faster speed, and the entire scientific community needed to coordinate at a different, improved level.

Vaccine represents the most important strategy to protect human from viral disease and prevent further spread, especially when the virus is highly contagious and easily transmits from one subject to another. New technologies and shortening time for vaccine production are necessary, as well as rapid approval process for their distribution, are needed. However, we must not compromise the required safety measures and studies necessary to establish their efficacy and safety. Also, the development of new drugs is necessary to treat the disease, as well as the implementation of molecular strategies, which showed to be a new frontier for successful results in the diagnostic field.

The communication of a complex and inter-systemic phenomenon such as a pandemic should be based on an inferential/probabilistic language transmitting the absence of a paradigm of certainty by strengthening the coherence paradigm. Furthermore, it may be useful to implement biostatistical models linked to nonlinear physics models with a predictive function, as in phylogenetic and evolutionary models. The different levels of communication should differ in terms of language and completeness of the transmitted content but must be consistent with each other. Consequently, the concrete and quantitative data should be presented in an inferential language, appropriately explaining that in some instances we may not have absolute certainty because, for example, there are false negatives/positives, or there may be delayed or missing data, human errors of transmission, etc. However, it is also very important that within this inferential approach the data transmitted daily should maintain as much as possible statistical significance, trying to keep constant biases.

By this new approach, a novel strategy to communicate results or new developments is suggested, in order to provide different levels of communication based on the desired target. To better inform the broadest share of the population, we propose to tailor the messages based on the following categories: (i) average culture (no specific scientific knowledge), (ii) culture modulated by incorrect/incomplete news and/or by COVID-19 denial realities, and (iii) political/professionals.

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