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Paul C. Guest *Editor*

Application of Omic Techniques to Identify New Biomarkers and Drug Targets for COVID-19

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Preface

Estimates indicate that the COVID-19 pandemic caused by the SARS-CoV-2 virus has now affected half of the world's population and caused more than 6.9 million deaths. Although the unprecedented worldwide vaccination effort has reduced the risk of serious disease outcomes, disparities in distribution and vaccine hesitancy have led to the rise of multiple waves of SARS-CoV-2 outbreaks. This has also led to the emergence of several SARS-CoV-2 variants of concern, some of which display enhanced infectivity and an ability to evade the existing vaccines and some therapeutics. This book describes how the application of techniques such as genomics, proteomics, metabolomics and artificial intelligence can be used to aid disease management during continuation of the current and future pandemics. Specifically, the book examines the most effective omic techniques for increasing our understanding of COVID-19 disease and for improved diagnostics, prognostics, patient stratification, treatment monitoring, genomic surveillance, and for facilitating the development of effective treatments and vaccines. It also describes deep learning approaches for more effective validation and translation of biomarker candidates into clinical, pharmaceutical company and genomic surveillance purposes. Given the worldwide interest in this topic, the authors in this series come from the six habitable continents, from countries including Australia, Bahrain, Brazil, Croatia, Cyprus, Germany, India, Iran, Italy, Mexico, South Africa, Turkey, the United Kingdom and the United States.

Campinas, São Paulo, Brazil — Paul C. Guest

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Background

Chapter 1
The COVID-19 Pandemic: SARS-CoV-2 Structure, Infection, Transmission, Symptomology, and Variants of Concern

Paul C. Guest, Prashant Kesharwani, Alexandra E. Butler, and Amirhossein Sahebkar

Abstract Since it was first detected in December 2019, the COVID-19 pandemic has spread across the world and affected virtually every country and territory. The pathogen driving this pandemic is SARS-CoV-2, a positive-sense single-stranded RNA virus which is primarily transmissible though the air and can cause mild to severe respiratory infections in humans. Within the first year of the pandemic, the situation worsened with the emergence of several SARS-CoV-2 variants. Some of these were observed to be more virulent with varying capacities to escape the existing vaccines and were, therefore, denoted as variants of concern. This chapter

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provides a general overview of the course of the COVID-19 pandemic up to April 2022 with a focus on the structure, infection, transmission, and symptomology of the SARS-CoV-2 virus. The main objectives were to investigate the effects of the variants of concern on the trajectory of the virus and to highlight a potential pathway for coping with the current and future pandemics.

Keywords COVID-19 · SARS-CoV-2 · Structure · Infection · Transmission · Symptomology · Variant of concern

1 Introduction

According to the Institute for Health Metrics and Evaluation Model, approximately 57% of the world population has had COVID-19 disease at least once, as of April 22, 2022 [1]. This constitutes a massive increase due to the most recent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) wave, driven mostly by the highly infectious Omicron variant. Similarly, a recent survey by the Office of National Statistics in the United Kingdom found that 72% of the population in England have been infected at least once, which is up from 30% before the Omicron wave [2]. Virtually every country or territory has been affected (Fig. 1.1), with the United States, India, and Brazil topping the list in both numbers of cases and deaths due to SARS-CoV-2 virus infections [3]. Most areas have seen multiple outbreaks propelled by the eruption of new variants of the virus with enhanced transmission and at least partial impacts on virulence and vaccine escape capabilities [4]. Although new treatments and vaccines have protected most patients from a more severe illness course, distribution of these medicines has not been equitable across the continents [5]. For example, for most of the countries in Africa, less than 20% of the population have received at least one dose of a World Health Organization-approved vaccine.

The first reported infection with the SARS-CoV-2 virus occurred in Wuhan, China, in late December 2019, which led to the derivation of the name COVID-19 (coronavirus 2019) for the disease caused by the virus [6, 7]. By March 11, 2020, COVID-19 was declared a pandemic by the World Health Organization [8] and has now plagued the world for more than two years. Potentially the largest contributing factor responsible for perpetuation of this pandemic has been the ongoing emergence of the SARS-COV-2 variants [4]. The rapid rise of the highly transmissible Omicron variant in November 2021 led to considerable alarm across the world with an unprecedented wave which peaked at almost four million cases per day in late January of 2022 [3]. Although this wave has mostly subsided (as of April 28, 2022), there are still fears that another surge involving a new variant is on the horizon. For these reasons, we are still under pressure to develop a sound infrastructure of new

Fig. 1.1 World map showing number of total COVID-19 cases as of April 2022 [https://www.bloomberg.com/graphics/2020-coronavirus-cases-world-map/]

methods for diagnosing, triaging, and tracking the current virus and to lay the groundwork to help us cope with the effects of future pandemics.

This chapter provides an overview of what we have learned about the COVID-19 pandemic up to April 2022, covering the structure, infection, transmission, and symptomology. The main objectives were to highlight the impact of the SARS-CoV-2 variants of concern on the pandemic and to describe the most effective strategies for helping us to manage the current and future outbreaks more effectively.

2 SARS-CoV-2 Structure

The COVID-19 outbreak is the third new acute infectious coronavirus disease to arise in the past two decades. It follows SARS-CoV in 2002–2004 [9, 10] and the Middle East respiratory syndrome (MERS)-CoV) from 2012 to the present (there are still occasional cases of this virus) [11, 12]. As with SARS-CoV and MERS-CoV, SARS-CoV-2 is a single-stranded, positive-sense RNA of the β-coronavirus genus in the *Coronaviridae* family [13, 14]. In electron microscopy images, these coronaviruses have a characteristic feature resembling a crown (from the Latin corona) due to the presence of the spike (S) protein projections (Fig. 1.2a) [15, 16]. The SARS-CoV-2 virus has a diameter of approximately 120 nm and is enveloped in a lipid bilayer. In addition to the prominent S protein, it features three additional major structural polypeptides, known as the envelope (E), membrane (M), and nucleocapsid (N) proteins (Fig. 1.2b) [17]. The SARS-CoV-2 genomic RNA is approximately 30 kb in length with 88% sequence homology to two recent bat coronaviruses (SL-CoVZC45 and SL-CoVZXC21) and approximately 79% and 50%

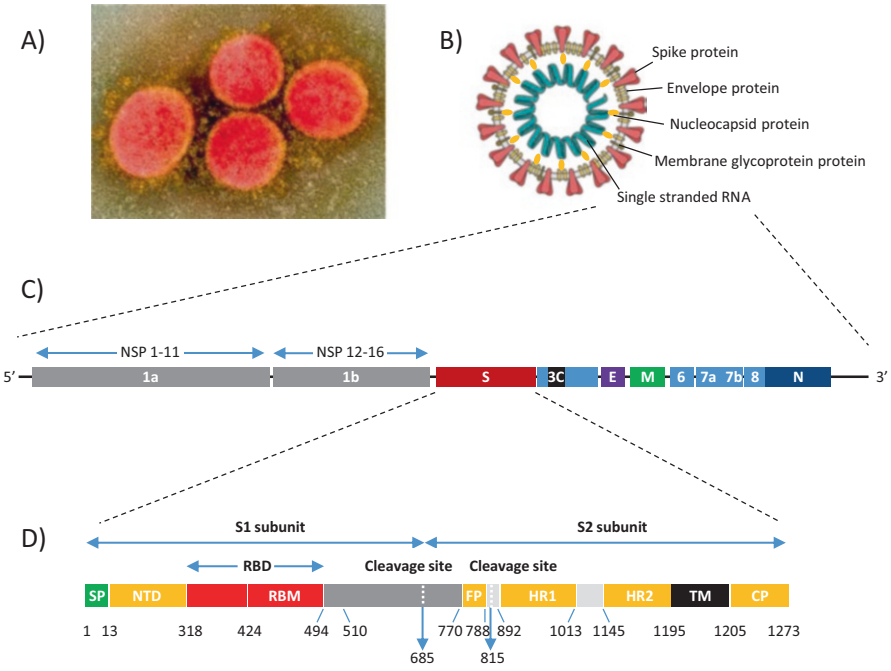

Fig. 1.2 Structure of SARS-CoV-2 virus. (**a**) Colorized electron micrograph image (free image obtained from the National Institute of Allergy and Infectious Diseases Rocky Mountain Laboratories (NIAID-RML) [https://www.niaid.nih.gov/news-events/novel-coronavirus-sarscov2-images]). (**b**) Diagram of virus structure showing the spike, envelope, nucleocapsid and membrane proteins, and the single-stranded virus. (**c**) Enlarged representation of SARS-CoV-2 single-stranded RNA showing the main encoded proteins (not to scale). S = spike, E = envelope, M = membrane, and N = nucleocapsid proteins. (**d**) Enlarged image of SARS-CoV-2 spike protein showing the main domains and S1-S2 cleavage site. SP = signal peptide, RBD = receptor binding domain, RBM = receptor-binding motif, FP = fusion peptide, HR1 = heptad repeat 1, HR2 = heptad repeat 2, TM = transmembrane domain, CP = C-terminal peptide

homology to SARS-CoV and MERS-CoV, respectively [18]. The SARS-CoV-2 genome includes two 5′ open reading frames (ORF1a and 1b), which are translated once inside a host cell to produce the pp1a and pp1b proteins. These undergo auto-proteolysis by the PLpro and 3CLpro proteases, which convert pp1a and pp1b into 16 non-structural proteins (nsps) (Fig. 1.2c) [19]. It also contains an RNA-dependent RNA polymerase which is essential for viral replication. The 3′ portion of the genome contains the ORFs encoding the S, E, M, and N proteins, as well as several accessory proteins.

The notable features in the S protein are the receptor binding domain (RBD) which provides the interaction with host cell receptors required for viral entry and a proteolytic cleavage site marked by an arginine-arginine-alanine-arginine (RRAR)

sequence at the junction of the S1 and S2 domains (Fig. 1.2d) [20]. The E protein is thought to be involved in SARS-CoV-2 pathogenesis, whereas the M protein is required in host-cell interactions and the N protein packs the viral RNA into a ribonucleoprotein complex. All four of these structural proteins play a role in various aspects of the virus lifecycle, including infection, assembly, budding, and release of new viral particles [21].

3 SARS-CoV-2 Infection of Host Cells

Transmission of SARS-CoV-2 occurs mostly through close contact with individuals who are already infected via expelled saliva or respiratory droplets [22]. This can by caused when the infected person coughs or sneezes or even through talking or singing. In these situations, droplets containing virus particles can enter the mouth, nose, or eyes of the new host and lead to infection. Once inside, entry of SARS-CoV-2 into tissues is initiated by fusion of the viral envelope with host cells and by binding of the S protein RBD to angiotensin-converting enzyme 2 (ACE2) receptors on these cells (Fig. 1.3) [17, 23–25]. This receptor is widely expressed in cells of multiple tissues such as the lungs, intestine, liver, heart, kidneys, skin, and brain, in line with the broad range of organ systems that this virus can affect [26, 27].

As part of the RBD-ACE2 receptor binding process, the S protein undergoes cleavage by host proteases to expose the internal fusion peptide (FP), which is an essential trigger for viral entry into cells [21]. This cleavage occurs on the carboxy terminal side of the arginine-arginine-alanine-arginine (RRAR) site located at the S1/S2 boundary (amino acids 682–685) and is carried out by the serine protease furin [28, 29]. The transmembrane serine protease 2 (TMPRSS2) and endosomal cathepsins B and L are also involved in proteolytic processing of the SARS-CoV-2 S protein at the lysine-arginine (KR) site (amino acids 814–815) to expose the FP (Fig. 1.3) [30, 31]. This drives the HR1 and HR2 domains in the S2 subunit to form a 6-helix bundle, allowing the fusion of the viral envelope and host cell membranes and release of the viral RNA into the host cell cytoplasm [23, 24].

Once inside the cell, the virus hijacks the transcriptional, translational, and secretory machinery to reproduce itself and release new viral particles for infection of other cells (Fig. 1.3) [32, 33]. In this process, the positive single-stranded RNA is translated into the 16 NSPs and the RNA-dependent RNA polymerase to initiate replication. This results in generation of the negative single-stranded RNA template for synthesis of new copies of the SARS-COV-2 genomic RNA. This is then trafficked through the endoplasmic reticulum and Golgi complex along with the newly translated structural proteins for final assembly in membrane-bound vesicles. These vesicles are then transported to the cell plasma membrane for fusion and release via exocytosis for infection of new cells [20, 34, 35]. Then the SARS-CoV-2 replication cycle repeats and the virus runs its course within the host.

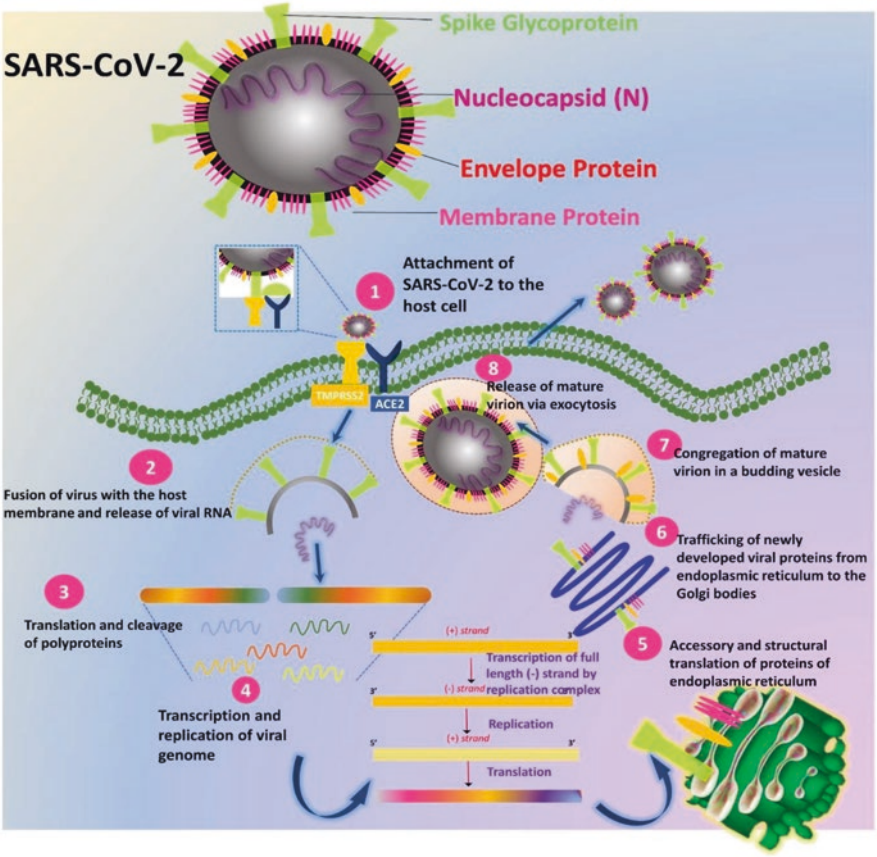

Fig. 1.3 Life cycle of the SARS-CoV-2 virus showing entry into host cells, replication, and assembly and release of new particles. SARS-CoV-2 binds to host cells by interaction of the viral S protein with host ACE2 receptors. The viral RNA enters the cytoplasm through proteolytic cleavage between the spike S1-S2 domains and within the S2 domain via the actions of endogenous proteases, furin, TMPRSS2, and cathepsins B and L. This results in a conformational change in the virus which permits fusion with the host cell membrane and release of the viral RNA into the cytoplasm. Once inside the cell, the virus utilizes the host cell transcriptional, translational, and secretory machinery to reproduce itself, assemble new viral particles, and release these outside the cell. After this, the virus can go on to infect other cells in an ongoing cycle [19, 33, 34]

4 Symptomology

SARS-CoV-2 infections can result in an extensive immune and inflammatory cytokine response by the host [36, 37]. In turn, this cytokine storm can cause an acute respiratory distress syndrome (ARDS), which can lead to severe tissue damage. At the clinical level, SARS-CoV-2 infections can generate a range of symptoms in infected individuals which typically present within 1–14 days of the initial infection

and vary from asymptomatic and mild cases to those with severe presentations [38–40]. The most common symptoms of COVID-19 infection include fever, cough, dyspnea, fatigue, and loss of taste and smell, with approximately one third of those infected having no symptoms [40–42]. Out of those who develop symptoms, approximately 80% have mild to moderate indications, 15% experience severe symptoms, and around 5% progress to a life-threatening condition [43]. Patients with mild to moderate symptoms are likely to recover within 1 to 2 weeks after initial presentation, while those with severe and life-threatening infections may deteriorate and progress to ARDS, acute cardiac and renal injury, multiple organ failure, and death [44, 45].

Common factors which have been linked to poor COVID-19 disease outcomes include age, gender, and the pre-existence of other diseases [46, 47]. A meta-analysis of 18 studies comprising 14,558 individuals found that chronic kidney disease had the highest risk of a severe COVID-19 disease course, followed by chronic obstructive pulmonary disease (COPD), cardiovascular disease, cancer, diabetes, and hypertension [48]. The same study showed that the risk of mortality was significantly higher in patients with pre-existing cerebrovascular, cardiovascular, and chronic kidney diseases, followed by cancer and COPD. Another meta-analysis of 20 studies consisting of a total of 28,355 hospitalized COVID-19 patients found that obesity was associated with a higher risk of severe illness and death outcomes when adjusted for age, gender, and other comorbidities [49]. A meta-analysis of 17 studies showed that being elderly (≥65 years-old) and male posed the greatest risk of a severe outcome or death from a COVID-19 infection, followed by the presence of comorbid diseases [50]. Another meta-analysis found that higher clinical frailty scores were associated with increased risk of death outcomes among elderly COVID-19 patients [51].

A recent meta-analysis of 10 studies incorporating a total of 1584 patients found significant differences in circulating white blood cell, neutrophil, lymphocyte, and platelet counts, as well as the levels of C-reactive protein, procalcitonin, ferritin, D-dimer, interleukin-6, and liver and heart damage biomarkers, in COVID-19 patients who died compared to those who survived their illness [52]. In addition, computed tomography (CT) chest scans of COVID-19 cases have demonstrated the presence of hazy ground-glass opacities with consolidative abnormalities in a bilateral distribution pattern [53, 54]. When combined, these latter two features have been associated with progression to severe disease [55, 56]. In support of this, autopsy reports of COVID-19 patients have demonstrated emboli and diffuse alveolar damage in the lungs as well as microthrombi in other organs such as the heart, kidney, and brain and other widespread effects such as hypercoagulation, hyperinflammation, and endothelial dysfunction [57–60]. In living patients, this can be seen through various systemic effects caused by the virus, such as hyperinflammation and autoimmunity [36, 61, 62], metabolic and hormonal dysfunction [63, 64], and thromboembolism and coagulopathies [65–67]. There have also been reports of direct effects on many organ systems including the brain [68, 69], heart [70], lungs [71], liver [72], kidneys [73, 74], and skin [75, 76]. This is thought to occur either

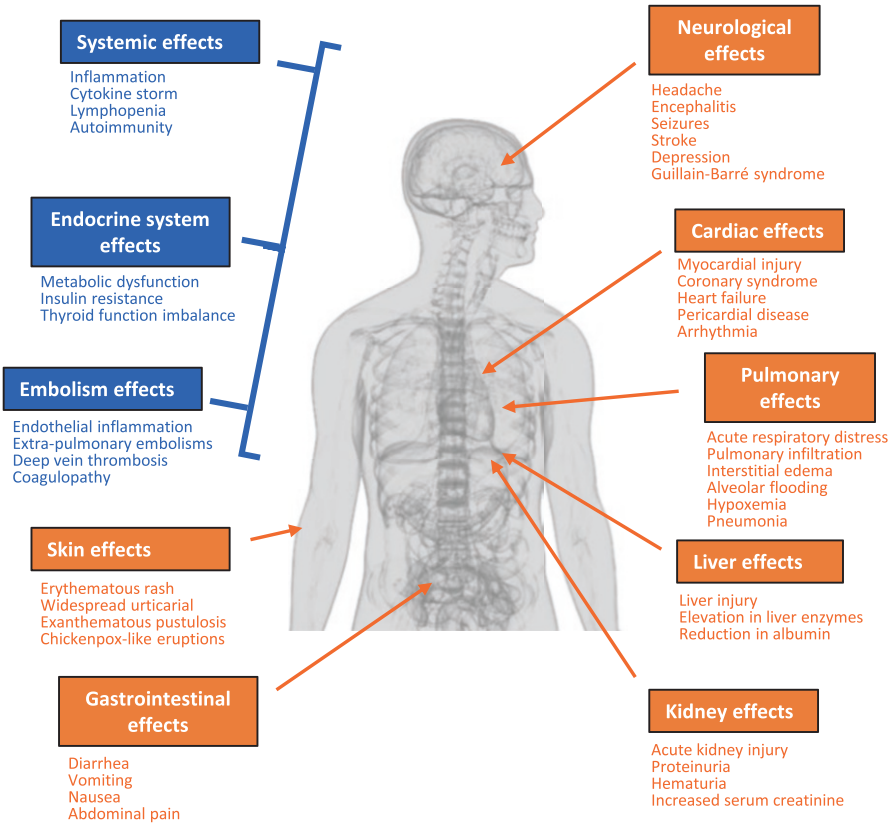

Fig. 1.4 Main effects of SARS-CoV-2 infection on the human body. Systemic effects are indicated in blue and direct effects on organs are given in orange font

via direct SARS-CoV-2 infection of these tissues [77] or though systemic effects of the hyperinflammation, autoimmune, and thromboembolic response (Fig. 1.4) [78].

5 Variants of Concern

Positive sense single-stranded RNA viruses such as SARS-CoV-2 are under continuous genetic pressure to alter their genomes via mutation or recombination in order to propagate themselves [79]. After infection, the SARS-COV-2 virus takes over the transcriptional and translational machinery in the host cell in order to make copies of itself. This involves use of the host ribosomes to translate the viral RNA into a single protein which is then altered by viral and host factors to generate the proteins necessary to allow replication. One of these proteins is an RNA-dependent RNA polymerase, which copies the viral RNA template to produce a double-stranded form which, in turn, acts as a template for production of new SARS-CoV-2 RNA strands.

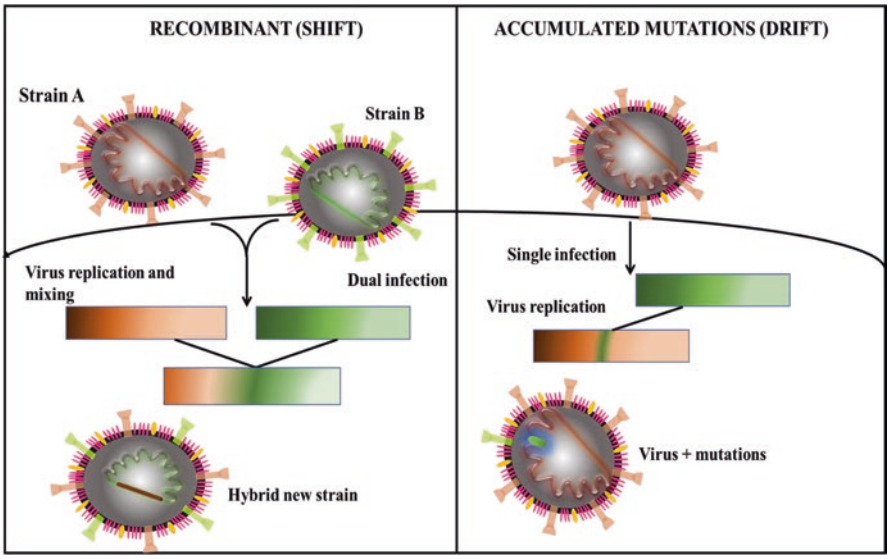

Fig. 1.5 The main routes by which RNA viruses such as SARS-CoV-2 can mutate and give rise to new variants. The pathway on the left shows recombination and that on the right shows amino acid replacement and deletions

Mutation of SARS-CoV-2 is caused by competitive mechanisms at the molecular, host, and population levels [80]. Molecular changes include errors in transcription and translation that can also be caused by recombination events (Fig. 1.5). Changes induced at the level of the host can be driven by the adaptive pressure of the immune response and by recombination events between the host and virus, or between two viruses infecting the host at the same time [81]. Population-induced changes occur because mutations leading to reproductive advantages of the virus are favored through the natural selection process.

Due to the nature of the process, RNA viruses have high mutation rates, at approximately one base per copy. Since mutations can be neutral, harmful, or favorable to the virus, only those that do not disrupt functioning can be propagated in a population. Changes that favor viral survival include those that increase infection and transmission rates, as well as those that allow the virus to escape the host immune system response. Below, we indicate the main SARS-CoV-2 variants of concern which have acquired these capacities and allowed the pandemic to persist.

5.1 Alpha

The emergence of SARS-CoV-2 variants of concern has led to new phases in the COVID-19 pandemic. The first of these variants was coded with the lineage B.1.1.7 and labeled as Alpha by the World Health Organization [82, 83] (Table 1.1 and Fig. 1.6).

Table 1.1 SARS-CoV-2 variants of concern

Lineage	Country first detected	Date first detected	Impact		
			Transmission	Severity	Immune escape
B.1.1.7 (Alpha)	United Kingdom	September 2020	↑	↑	No change
B.1.351 (Beta)	South Africa	September 2020	↑	↑	↑
P.1 (Gamma)	Brazil	December 2020	↑	↑	↑
B.1.617.2 (Delta)	India	December 2020	↑	↑	↑
B.1.1.529 (Omicron BA.1)	Botswana / South Africa	November 2021	↑	↓	↑
B.1.1.529 (Omicron BA.2)	South Africa	November 2021	↑	↓	↑

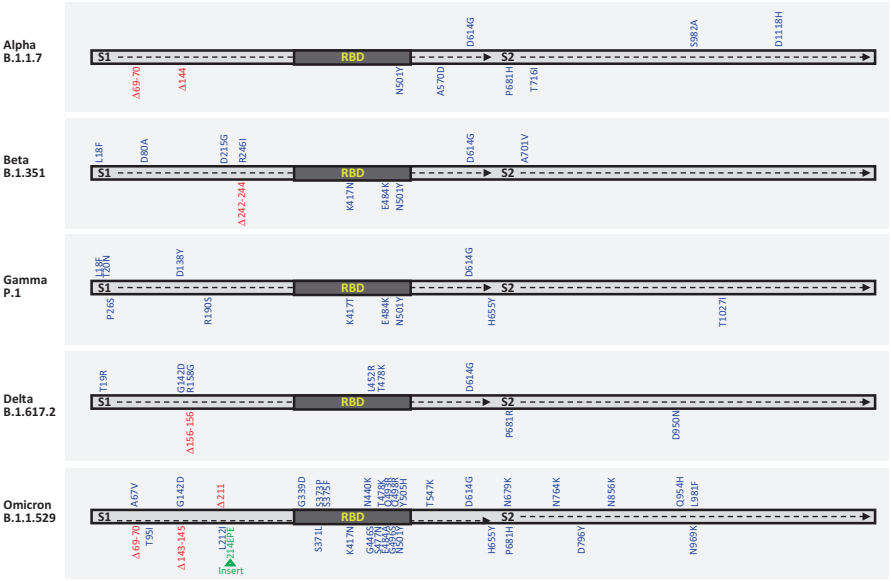

Fig. 1.6 Mutation sites in the spike gene of SARS-CoV-2 alpha (B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2) and Omicron (B.1.1.529, BA.1) variants of concern are shown. Mutations resulting in amino acid changes are given in blue text, those resulting in deletions are given in red text, and that indicating an insertion is given in green text (only in the case of the Omicron variant). Note that approximately one half of the mutations occur within the receptor binding domain (RBD) in the Omicron variant (only the BA.1 version of omicron is given). A = alanine, D = aspartate, E = glutamate, F = phenylalanine, G = glycine, H = histadine, I = isoleucine, K = lysine, L = leucine, N = asparagine, P = proline, R = arginine, S = serine, threonine, Q = glutamine, V = valine, and Y = tyrosine

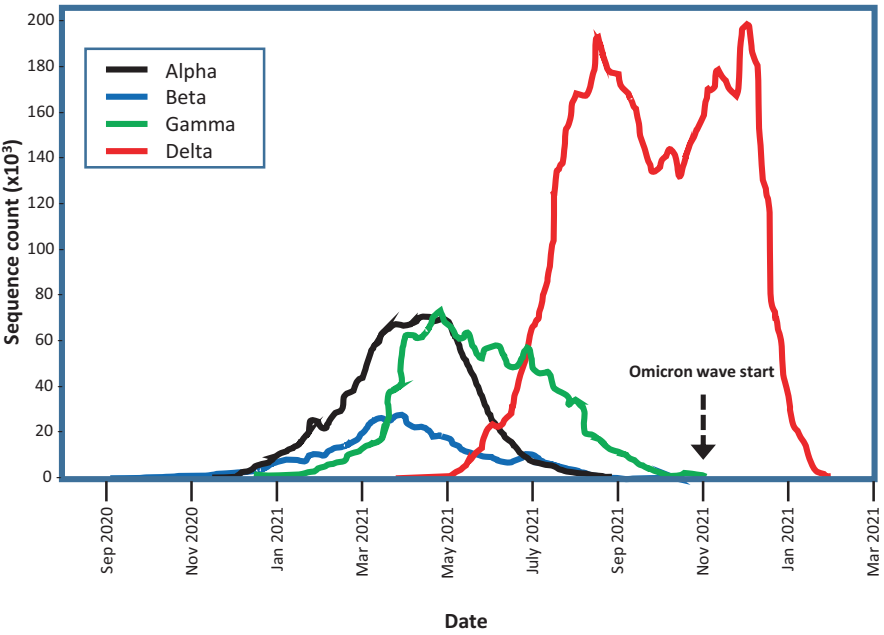

Fig. 1.7 Plot showing the time course and magnitude of the SARS-CoV-2 cases around the world for the Alpha (B.1.1.7; black), Beta (B.1.351; blue), Gamma (P.1; green) and Delta (B.1.617.2; red) variants. The initiation of the Omicron (B.1.1.529, BA.1) wave is indicated by the arrow

It first appeared in the United Kingdom in September 2020 [84] and spreaded rapidly to become the dominant variant there between December 2020 and May 2021 [85]. It has been detected in more than 160 countries and territories around the world but has now been largely superseded by other variants (Fig. 1.7) [86–88]. The SARS-CoV-2 Alpha variant contains a number of mutations in the S protein, including an asparagine to tyrosine substitution at amino acid position 501 (N501Y) (Fig. 1.6). This mutation is present in all of the variants of concern (apart from Delta) and is thought to alter the S protein RBD structural conformation, increasing its affinity by approximately twofold for the ACE2 receptor [89]. The N501Y mutation also appears to allow this variant to escape neutralization by disrupting binding to at least two anti-SARS-CoV-2 S protein monoclonal antibody binding sites [90]. Another mutation, deletion of the tyrosine residue at position 144 (Δ144), was seen to reduce neutralization by six monoclonal antibodies targeting this region of the S protein [91]. In addition, sera from individuals who had been administered the AstraZeneca, Pfizer, Moderna, and Novavax vaccines showed two- to threefold reductions in neutralization against the Alpha variant [92]. Other notable mutations in the Alpha variant S protein include the glutamate to lysine shift at position 484 (E484K) which may also reduce neutralization capabilities of the vaccines [91, 93]. There is also a deletion of the histadine (H) and valine (V) residues at positions 69

and 70 (Δ69–70), which is predicted to increase transmissibility and infectivity with a reproduction number estimated to be 50–100% higher than the initial SARS-CoV-2 strain [94, 95]. There is also evidence that the aspartate to glycine change at position 614 (D614G) increases infectivity of the Alpha strain compared to the original virus strain by enhancing ACE2 receptor binding and viral entry into host cells [96, 97]. Importantly, this mutation is present in all of the variants of concern (Fig. 1.6). Less is known about the other Alpha S protein mutations, which include alanine to aspartate at amino acid 570 (A570D), proline to histidine at 681 (P681H), threonine to isoleucine at 716 (T716I), serine to alanine at 982 (S982A), and aspartate to histidine at 1118 (D1118H). Apart from the P681H mutation which also appears in Omicron, none of these latter mutations appear in any of the other variants of concern.

5.2 Beta

The first cases of the Beta variant (B.1.351) emerged in September in South Africa and eventually spread to more than 120 countries and territories, albeit with fewer overall cases compared to the other variants of concern (Table 1.1, Figs. 1.6 and 1.7) [86–88]. This variant has 10 mutations in the S protein, including lysine to asparagine at 417 (K417N), glutamate to lysine at 484 (E484K), and the N501Y substitution within the RBD, which enhance the affinity the virus for the ACE2 receptor [98–100]. The same three mutations also occur in the Gamma variant [101]. Beta also contains five mutations in the N-terminal domain, which are leucine to phenylalanine at position 18 (L18F), aspartate to alanine at 80 (D80A), aspartate to glycine at 215 (D215G), and arginine to isoleucine at 246 (R246I), and there is a deletion of the lysine, serine, and phenylalanine residues at amino acids 241–243 (KSF; Δ241–243). There is also an alanine to valine substitution at position 701 (A701V) in the S2 domain. Finally, the D614G mutation found in all variants of concern is present in the subdomain 2 region. As with the Alpha variant, beta SARS-CoV-2 binds with higher affinity to the ACE2 receptor, allowing increased infectivity of host cells compared to the original SARS-CoV-2 strain [100, 102]. Also, the mutations in the N-terminal domain and RBD may aid the Beta variant to evade antibody neutralization. A study which used surface plasmon resonance (SPR) analysis found that multiple antibodies directed against these regions bound with lower affinity in the Beta variant compared to the original SARS-CoV-2 strain [103]. Wang et al. showed that the E484K mutation appears to be responsible for the complete block in neutralization capabilities of three RBD-directed antibodies (2–15, LY-CoV555 and C121), and the K417N substitution causes a reduction in neutralization activity of another antibody (910–30) [91]. However, another study found that the binding of two other RBD-directed antibodies (nAbs 1–57 and 2–7) to the Beta variant was unimpaired, suggesting that these may have therapeutic potential against this variant [104]. Finally, the neutralization capacity of sera from individuals vaccinated with the Pfizer and Moderna vaccines was reduced by more than tenfold against the

Beta variant compared to the original SARS-CoV-2 virus [91, 100]. This effect was attributed to the E484K substitution in the Beta SARS-CoV-2 S protein RBD domain [91]. In addition, another investigation found that entry of the Beta variant into host cells was blocked to a lesser extent when given sera from individuals that had been given the Pfizer vaccine [105]. However, an animal model study found that the AstraZeneca vaccine protected hamsters against infection by the Alpha and Beta variants [106]. From these mixed findings, it is apparent that considerable further studies are required to determine the effects of specific SARS-CoV-2 S protein mutations on efficacy of both therapeutic monoclonal antibodies and the various vaccines.

5.3 Gamma

The Gamma variant was first detected in samples from Manaus, Brazil, where it caused widespread infections in December 2020 (Table 1.1 and Fig. 1.6) [83, 84, 107]. This caused a sharp increase in COVID-19 infections in Brazil leaving it with the second highest death toll due to the virus after the United States [3]. By June 2021, the Gamma variant had spread to over 60 countries and territories and persisted to November 2021, beyond the span of the Alpha and Beta variants (Fig. 1.7) [84, 87, 88]. The Gamma variant has three mutations in common with the Beta variant (amino acids 417, 484, and 501) in the S protein RBD although one of these has a different substitution in Gamma (Fig. 1.6). While asparagine replaces lysine in the Beta variant (K417N), this is replaced with a threonine residue in the Gamma variant (K417T). As with the Beta variant, these substitutions have been predicted to increase the binding affinity of the S protein RBD to the ACE2 receptor, which is thought to be mainly driven by the N501Y mutation [98, 100, 104]. Other mutations found in the Gamma variant include five in the N-terminal domain: lysine is changed to phenylalanine at amino acid 18 (L18F), threonine is changed to asparagine at 20 (T20N), proline is replaced by serine at 26 (P26S), aspartate is changed to tyrosine at 138 (D138Y), and the arginine at 190 is replaced by serine (R190S). There is also the D614G substitution and three substitutions in the S2 subunit: aspartate to tyrosine at 655 (D655Y), threonine to isoleucine at 1027 (T1027I), and the valine at 1176 is replaced by phenylalanine (V1176F). The L18F N-terminal domain mutation in Gamma is also present in the Beta variant and has been attributed to a degree of immune escape from some of the vaccines [108, 109]. In addition, many RBD-targeted monoclonal antibodies have shown varying degrees of loss in their ability to neutralize the Gamma variant. For some of these antibodies, this loss has been attributed to the E484K mutation, whereas others appear to be diminished by the K417T and N501Y substitutions [100]. Also, three monoclonal antibodies (casirivimab, bamlanivimab, and etesevimab) were found to have little or no neutralizing capacity against the Gamma variant [100]. Surprisingly, neutralization of the Gamma variant by the Pfizer and AstraZeneca vaccines showed reductions of only two- to

threefold compared to the original strain, which was better than the loss in neutralizing capacity observed against the Beta variant [93].

5.4 Delta

The Delta variant was first detected in India in December 2020, and by July 2021, it had become the most prominent variant in Europe and accounted for the highest proportion of cases worldwide (Fig. 1.7) [88, 110]. Following this time, it became the most dominant SARS-CoV-2 strain and was reported in more than 170 countries and territories by November 2021 [81]. This variant has been shown to be more highly transmissible and twice as contagious compared to the previous variants [111, 112]. These effects are most likely due to mutations in the S protein, as detailed above for the other variants, which confer even stronger infectivity and transmission of the virus. Various versions of the Delta variant have emerged with 8–11 mutations in the S protein depending on the country or region of the outbreak [101, 112, 113]. Figure 1.6 shows the putative Delta B.1.617.2 parent form with nine mutations, as listed on the Stanford University Coronavirus Antiviral and Resistance Database [101]. Examination of the sequence shows that in the N-terminal domain there are four mutations: threonine to arginine at position 19 (T19R), glycine to aspartate at 142 (G142D), deletion of glutamate (E) and phenylalanine (F) at 156–157 (Δ156–157), and arginine to glycine at 158 (R158G). It is predicted that the deletion at 156–157 and the substitution at 158 may lead to an immune evasion phenotype as this sequence is incorporated in a key region in the N-terminal domain antigenic supersite recognized by SARS-CoV-2 S protein neutralizing antibodies [108]. The RBD contains two mutations which have not appeared in any of the other variants of concern: leucine to arginine at amino acid 452 (L452R) and threonine to lysine at 478 (T478K). As stated above for the other variants, mutations in the RBD can alter the affinity of the virus for the host ACE2 receptor and may also impede the binding of some neutralizing antibodies [114, 115]. Together, these properties can increase the transmissibility, infectivity, and pathogenicity of Delta strain over the other variants. The Delta variant contains three additional mutations in the C-terminal of the RBD which include the D614G substitution near the 3′ end of the S1 domain found in all of the variants and two in the S2 domain which are proline to arginine at 681 (P681R) and aspartate to asparagine at position 950 (D950N). The latter two mutations are unique to the Delta variant. The robust rise in cases due to the Delta variant is thought to be a result of the mutations in the N-terminal domain and RBD regions which have been shown to help this virus to at least partially evade the existing vaccines and to confer enhanced binding to the ACE2 receptor. This sets the scene for more efficient access of the virus into cells and for increased transmission due to the ensuing higher viral loads [111–113]. In terms of vaccine effectiveness, several investigations have now demonstrated that antibodies generated by the existing

vaccines, or from previous infections with other variants, show lower efficacy against the Delta variant [116, 117]. The number of Delta cases began to drop in December 2021 and appeared to be virtually non-existent by February 2022, when it was overtaken by another variant.

5.5 Omicron

The Omicron variant was first detected in Botswana and South Africa in November 2021 and spread at an unprecedented rate across South Africa and then around the globe to more than 190 countries and territories (Fig. 1.7) [118]. The Omicron wave peaked around January 20–21, 2022, at a rate of over 3.8 million cases per day [3]. This was approximately fourfold higher than the previous highest case rate observed towards the end of April 2021. Preliminary studies of the Omicron variant have demonstrated increased transmissibility and reduced protection by neutralizing antibodies, which are likely the main driving factors underlying the rapid spread of this variant and the increased numbers of reinfections [119, 120]. This has been attributed to the fact that this variant possesses the highest number of mutations compared to all of the other strains [101, 121]. This feature has also led to considerable fear, panic, and uncertainty across the globe, with concerns about how this affects infectivity and severity of COVID-19 disease, as well as the impact on existing treatments and vaccines [122]. Of the 34 mutations in the Omicron BA.1 subvariant, 15 amino acid substitutions occur in the RBD (Fig. 1.6) [101]. Within the RBD, the glutamine to arginine substitution at position 498 (Q498R) and the asparagine to tyrosine change at 501 (N501Y) have been shown to confer stronger affinity for the ACE2 receptor, which at least partly explains the high transmissibility of this variant [123–126]. As another possible explanation for the high transmission rate, Zhao et al. showed that Omicron may have a unique mechanism for host infection by gaining entry through the endocytotic pathway alone and without the need for TMPRSS2 cleavage [127]. Omicron is also predicted to escape immunity from antibodies generated by vaccinations or from previous infections, and considering the larger number of mutations, it appears that this effect is likely to be greater than that for any of the other SARS-CoV-2 strains [126, 128]. Using a computational model to predict antigenicity due to sequence changes in the S protein RBD of SARS-CoV-2 variants, Hu et al. identified a 17-fold decrease of Omicron in susceptibility to neutralization [129]. Further development of similar computational methods may offer a rapid means for prediction of antibody neutralization capacity of vaccines and monoclonal antibody therapeutics in future SARS-COV-2 variants of concern. Finally, it is now widely known that the Omicron variant appears to cause less severe symptoms compared to other SARS-CoV-2 variants and the percentage of cases resulting in COVID-19-related deaths is lower [130]. However, considering the high transmission rate and the recent emergence of new Omicron subvariants in South Africa [131, 132], serious concerns remain.

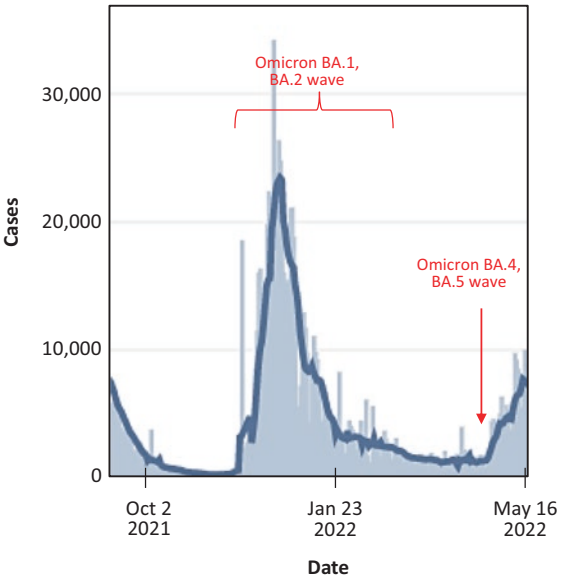

Fig. 1.8 COVID-19 case data for South Africa showing the previous Omicron BA.1 and BA.2 wave and the recent surge in BA.4 and BA.5 subvariants

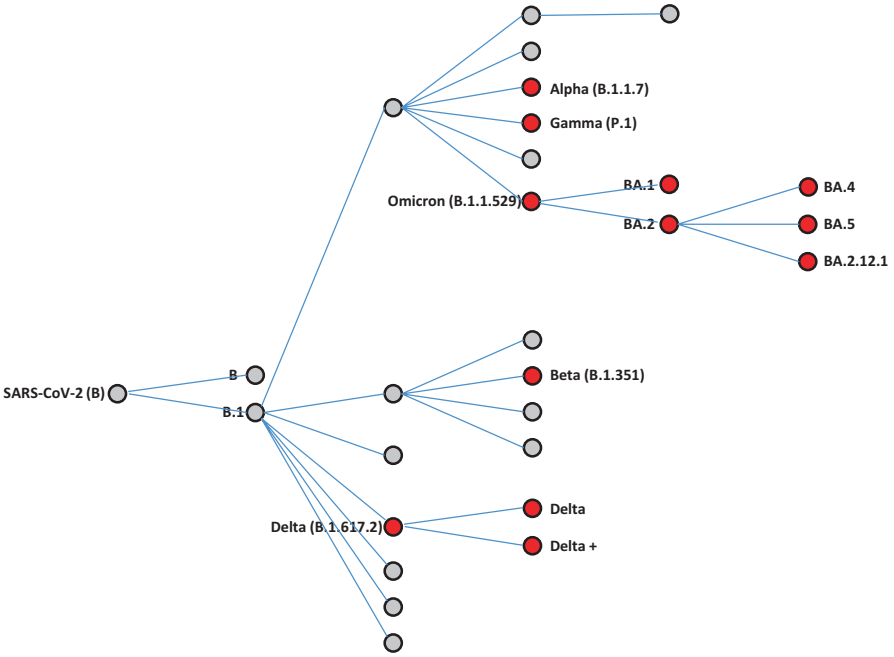

Fig. 1.9 Clade scheme showing descent of SARS-CoV-2 variants. (Modified from Nextstrain; https://nextstrain.org/sars-cov-2/ [136]). The variants of concern are indicated by red circles

6 Conclusions and Future Perspective

As of May 17, 2022, a new wave of the COVID-19 pandemic appears to be underway in some countries such as South Africa (Figs. 1.8 and 1.9) [3, 131]. The new surge in South Africa is being driven by two Omicron subvariants called BA.4 and BA.5 [131, 132]. Both appear to have evolved from the Omicron B.1.1.529.BA.2 strain which, with BA.1, accounted for a significant proportion of the cases in the previous SARS-CoV-2 wave. Although most parts of the world have apparently adjusted to living with COVID-19, appropriate measures should still be taken to prevent the spread of new and potentially more harmful variants. The most critical component which should be applied in this endeavor involves the use of genomic surveillance techniques to track any new strains of the virus and to enable predictions on virility so that appropriate steps can be taken to manage the outbreak. In line with this, the World Health Organization has advised that all nations should extend their research infrastructure to develop a science-based approach including vaccination to curb the spread of COVID-19 [133]. This includes ensuring equitable access to healthcare and vaccines in all countries. A study in the United Kingdom has shown that three doses of the SARS-CoV-2 vaccines provide 75%, 90%, and 95% protection against symptomatic illness, hospitalization, and death, respectively, from COVID-19 disease caused by the Omicron variant [134]. However, the newer Omicron BA.4 and BA.5 subvariants have now also been detected in the United Kingdom, and further work is required to understand their characteristics and determine vaccine efficacy [135]. This demonstrates the importance of detecting and tracking new variants to enable the appropriate healthcare steps to be taken at the individual, national, and global levels.

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Chapter 2
Long-Term Vaccination and Treatment Strategies for COVID-19 Disease and Future Coronavirus Pandemics

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Abstract The appearance of new severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants with increased infectivity and immune escape capabilities has allowed continuation of the COVID-19 pandemic for the foreseeable future. This review describes the worldwide efforts aimed at developing new vaccination and treatment strategies to keep pace with these variants as they emerge. In the case of vaccines and monoclonal antibody-based therapeutics, we describe the development of variant-specific, multivalent, and universal coronavirus directed approaches.

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Existing treatment approaches consist of repurposed medicines, such as antiviral compounds and anti-inflammatory agents, although efforts are underway to develop new ways of preventing or minimizing the effects of infection with the use of small molecules that disrupt binding the SARS-CoV-2 virus to host cells. Finally, we discuss the preclinical and clinical testing of natural products from medicinal herbs and spices, which have demonstrated anti-inflammatory and antiviral properties and therefore show potential as novel and safe COVID-19 treatment approaches.

Keywords Vaccination · COVID-19 · SARS-CoV-2 · Spike protein · Variant · Omicron

1 Introduction

As of June 23, 2022, 66.4% of the world population had received one or more doses of a World Health Organization (WHO)-approved COVID-19 vaccine, and over 12 billion doses have been administered in total [1]. However, the unequal distribution of vaccines has led to considerable moral outrage and could lead to epidemiological and economic disasters, as less than 20% of people in some low countries have received only one dose [2]. To compound the problem, the existing vaccines created to combat the original severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) strain which originated in Wuhan, China, may not work as effectively, if at all, against some of the newer SARS-CoV-2 versions, such as the Omicron subvariants [3]. Despite the devastating effects the COVID-19 pandemic has had on our world, the imbalance in vaccination has still not been corrected, and there is still a significant proportion of the population in many countries and territories that show vaccine hesitancy [4]. Thus, more studies are needed to understand and effectively correct this nonacceptance trend, which may threaten further efforts aimed at controlling the ongoing pandemic. Addressing the problem of how vaccines keep pace with new variants may be an even more difficult prospect. In terms of keeping pace with the emerging variants, it is still not clear whether the best strategy is to develop vaccines against each variant as these emerge in a continuous game of catch-up, or if the construction of vaccines targeting multiple variants simultaneously is the best approach [5].

In the meantime, effective therapeutics may be needed for those individuals who are not fully protected by vaccination, or those who are immunocompromised or have a high risk of experiencing a severe COVID-19 disease outcome [6]. Various monoclonal antibodies have been developed which target the SARS-CoV-2 spike protein, and some of these have demonstrated efficacy against the virus [7]. However, as with the vaccines, many of these are only partially effective or completely inactive against some of the variants [7]. The SARS-CoV-2 Omicron variant (lineage B.1.1.529) was detected in Botswana and South Africa in November 2021, and this spreads rapidly across South Africa and most of the world within 3 months

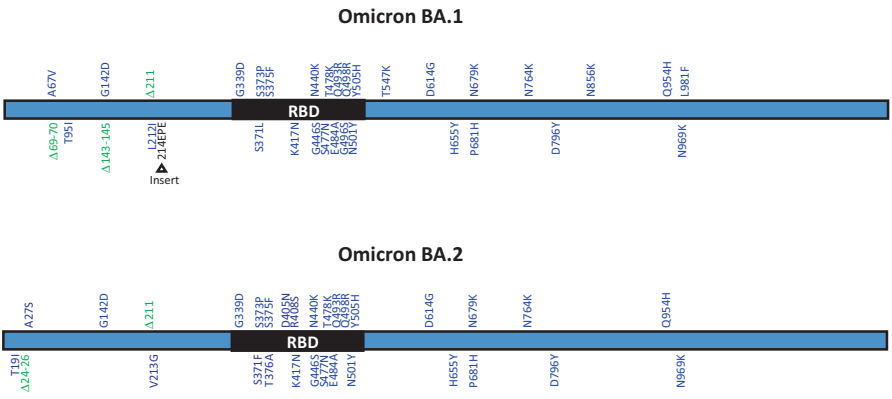

Fig. 2.1 Mutations of SARS-CoV-2 spike protein in the Omicron (B.1.1.529) BA.1 (top) and BA.2 (bottom) subvariants. The BA.1 subvariant contains 34 mutations and the BA.2 subvariant contains 31 mutations. Amino acid codes: A = alanine, D = aspartate, E = glutamate, F = phenylalanine, G = glycine, H = histadine, I = isoleucine, K = lysine, L = leucine, N = asparagine, P = proline, Q = glutamine, R = arginine, S = serine, T = threonine, V = valine, Y = tyrosine

[8]. This rapid spread is likely to be due to the increased transmissibility and strong ability of this variant to escape immune detection by neutralizing antibodies [9]. The property of increased transmission of this variant has been attributed to the enhanced capability of host infection via stronger interactions with the angiotensin-converting enzyme 2 (ACE2) receptor. The immune escape characteristic is a likely consequence of the higher number of mutations compared with other SARS-CoV-2 strains, rendering Omicron less recognizable to the existing vaccines and to convalescent sera from those had been infected by earlier strains (Fig. 2.1) [10, 11].

This review describes the effects that the continuous variation in the SARS-CoV-2 genome has had on the efficacy of existing vaccines and treatments. This has created an urgent need to fine tune and advance new vaccine and drug development strategies to cope with this protein virus and to prepare for the next pandemic.

2 Current COVID-19 Vaccines

The current vaccines approved by the WHO are indicated in Table 2.1. These are based on different strategies which can be classified as mRNA (Fig. 2.2a), non-replicating viral vector (Fig. 2.2b), inactivated (Fig. 2.2c), and recombinant protein nanoparticle (Fig. 2.2d) vaccines. The first of these to be approved by the WHO on Dec 21, 2020, was originally designated BNT162b1 and produced by Pfizer/BioNTech [12]. The International Non-proprietary Name (INN) is Tozinameran, and it is now sold under the tradename Comirnaty®. This was followed by Vaxveria (Oxford/AstraZeneca) [13], Covishield (Serum Institute of India) [14], Spikevax (Moderna) [15], Covilo (Sinopharm) [16], Ad26.COV2.S (Janssen) [17], and

Table 2.1 Current WHO-approved vaccines

Vaccine	Institution	Countries approved (No.)	Approval date	Description
Comirnaty	Pfizer/ BioNTech	146	Dec 31, 2020	mRNA encoding spike protein
Vaxzevria	Oxford/ AstraZeneca	140	Feb 15, 2021	Non-replicating viral vector
Covishield (Oxford/ AstraZeneca formulation)	Serum Institute of India	49	Feb 15, 2021	Non-replicating viral vector
Spikevax	Moderna	86	Apr 30, 2021	mRNA encoding spike protein
Covilo	Sinopharm (Beijing)	91	May 07, 2021	Inactivated
Ad26.COV2.S	Janssen	111	Mar 12, 2021	Non-replicating viral vector
CoronaVac	Sinovac	56	Jun 01, 2021	Inactivated
Covaxin	Bharat Biotech	14	Nov 03, 2021	Inactivated
COVOVAX (Novavax formulation)	Serum Institute of India	5	Dec 17, 2021	Recombinant spike protein nanoparticle
Nuvaxovid	Novavax	37	Dec 20, 2021	Recombinant spike protein nanoparticle with adjuvant
Convidecia	CanSino	10	May 19, 2022	Non-replicating viral vector

CoronaVac (Sinovac) [18] within a 6-month time span. After this, four more vaccines were developed which were approved within the next year (Covaxin; Bharat Biotech [19], COVOVAX; Serum Institute of India [20], Nuvaxovid; Novavax [21], and Convidecia; CanSino [22]). The rapid production of the above vaccines was unprecedented considering that it normally takes at least 10 years from discovery research of a new product through the preclinical, clinical, regulatory approval, manufacturing, and delivery stages [23–26]. However, this was driven by the deadly and disruptive nature of the pandemic and made possible by the unprecedented worldwide cooperation building on existing technologies and with new streamlined approaches to research, development, approval, global manufacturing, and distribution, without sacrificing testing and safety steps [27–32].

3 Treatments for COVID-19

The approved drugs for COVID-19 target different aspects of the SARS-CoV-2 infection cycle, for improving COVID-19 disease outcomes. These drugs include (1) monoclonal antibodies that interfere with binding of the receptor binding domain (RBD) of the SARS-CoV-2 spike protein to the ACE2 receptor (a critical step in viral entry into host cells) (Fig. 2.3a); (2) molecular compounds that minimize the

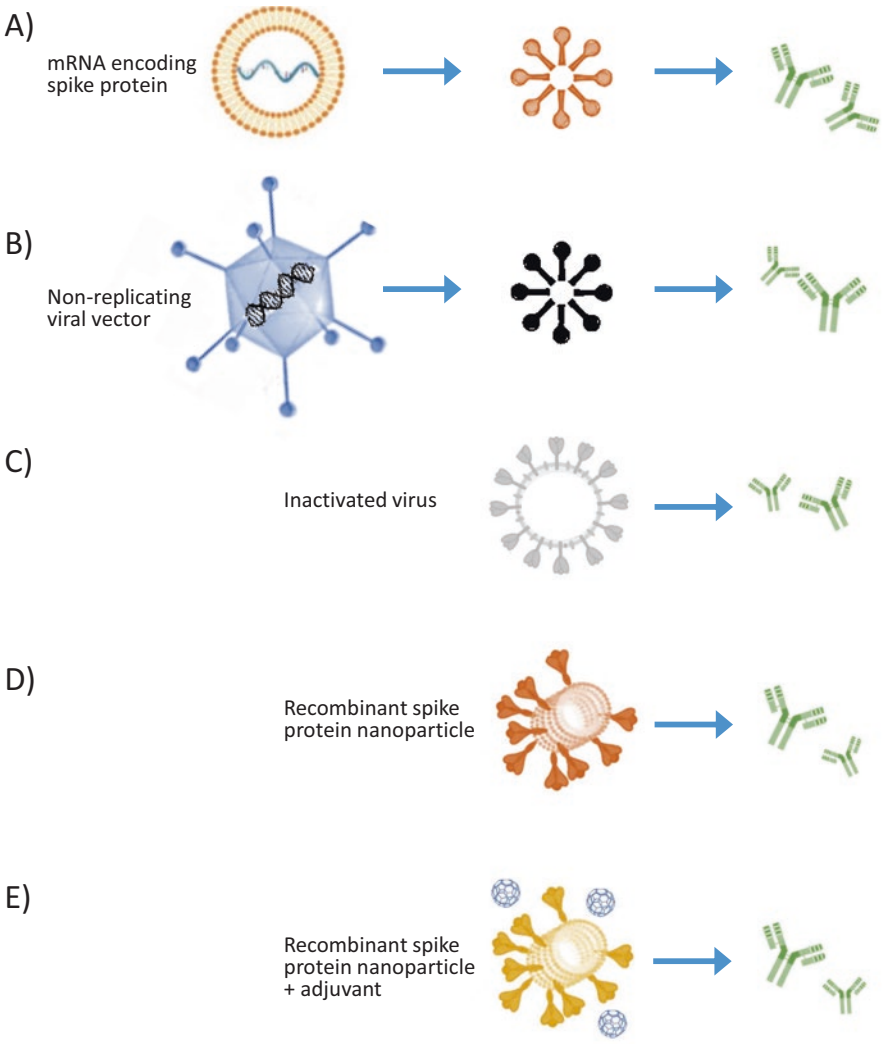

Fig. 2.2 Types of vaccines used as protection against COVID-19 disease. (**a**) mRNA-based vaccine (Comirnaty, SpikeVax). This type of vaccine consists of lipid nanoparticle-encapsulated mRNA molecules encoding a modified version of the SARS-CoV-2 spike protein. Once injected, this is translated by host immune cells to produce the modified spike protein molecules which stimulate an adaptive immune response. (**b**) Non-replicating viral vector (Vaxzevria, Covishield, Ad26.COV2.S, Convidecia). This vaccine type consists of a replication-deficient virus carrier containing the full-length DNA coding sequence of the SARS-CoV-2 spike protein which is transcribed into mRNA and then translated into proteins by the host cell to produce an immune response. (**c**) Inactivated vaccine (Covilo, CoronaVac, Covaxin). This type of vaccine contains the whole virus which has been inactivated either by deletion or chemical modification of the viral genetic material. (**d**) Recombinant spike protein nanoparticle (also known as a subunit vaccine and a virus-like particle vaccine; COVOVAX). These vaccines resemble virus particles to stimulate an immune response but contain no viral genetic material. (**e**) Recombinant spike protein nanoparticle containing adjuvant (Nuvaxovid). This type of vaccine is a virus-like particle containing an adjuvant to boost the host cell immune response

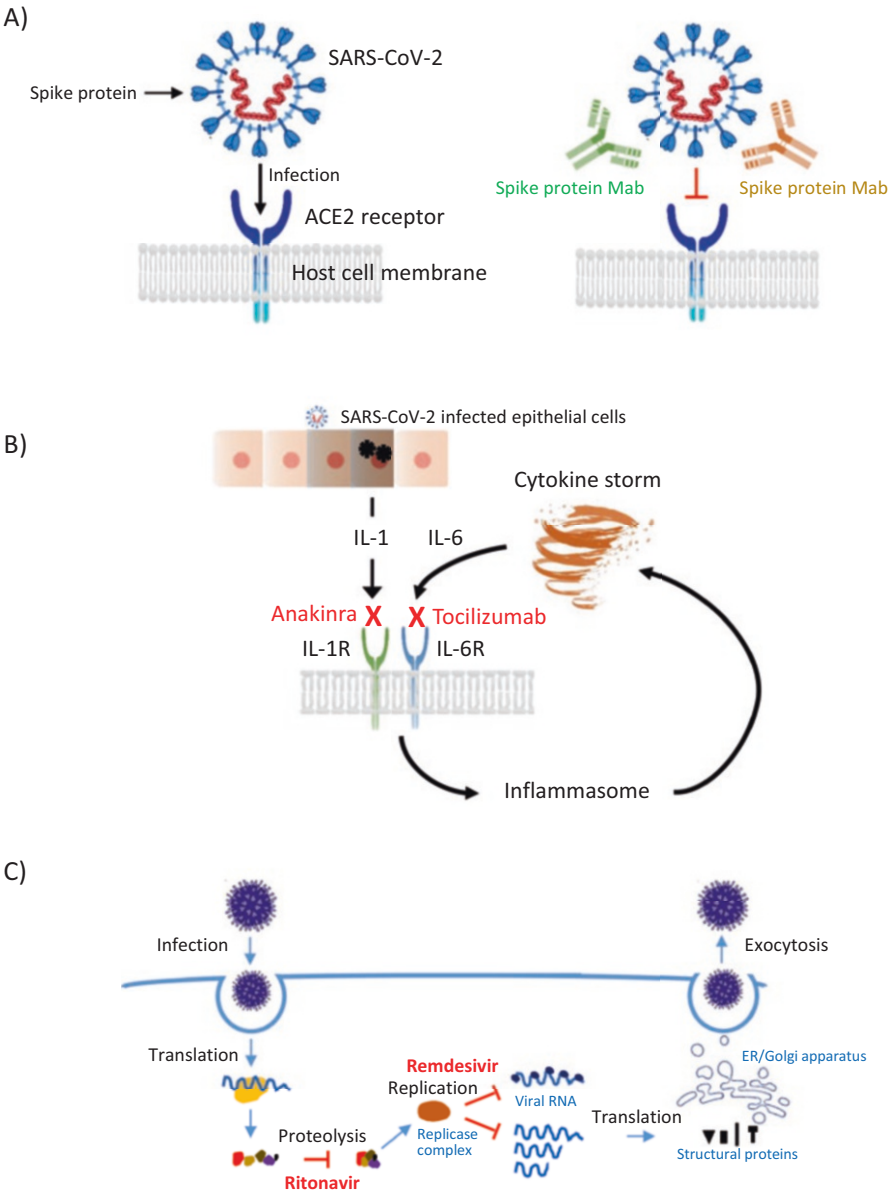

Fig. 2.3 Types of drugs used for treatment of COVID-19. (**a**) Monoclonal antibodies against spike protein (regdanvimab, casirivimab/imdevimab, sotrovimab, tixagevimab/cilgavimab). These antibody-based treatments disrupt binding of the SARS-CoV-2 RBD to the ACE2 receptor on host cells. (**b**) Anti-inflammatory drugs (tocilizumab, anakinra). These drugs block interaction of key cytokines with their receptor signaling cascades and thereby inhibit the hyperactivation of pro-inflammatory factors involved in the cytokine storm effect. (**c**) Antiviral (remdesivir, PF-07321332/ritonavir). These drugs inhibit key stages of the viral replication cycle

Table 2.2 List of COVID-19 treatments approved for use by the European Medicines Agency

Treatment	Institution	Authorization granted (date)	Mechanism
Veklury (Remdesivir)	Gilead Sciences	Jul 03, 2020	Anti-viral: Viral RNA polymerase inhibitor
Regkirona (Regdanvimab)	Celltrion	Nov 12, 2021	Monoclonal antibody: Targeting SARS-CoV-2 spike protein
Ronapreve (Casirivimab/ Imdevimab)	Regeneron pharmaceuticals	Nov 12, 2021	Monoclonal antibodies: Targeting SARS-CoV-2 spike protein
RoActemra (tocilizumab)	Hoffmann-La Roche	Dec 07, 2021	Anti-inflammatory: Monoclonal antibody targeting IL-6R
Kineret (Anakinra)	Swedish orphan Biovitrum	Dec 17, 2021	Anti-inflammatory: IL-1R antagonist
Xevudy (sotrovimab)	GlaxoSmithKline and Vir biotechnology, Inc.	Dec 17, 2021	Monoclonal antibody: Targeting SARS-CoV-2 spike protein
Paxlovid (PF-07321332/ritonavir)	Pfizer Inc.	Jan 28, 2022	Anti-viral: 3C-like protease inhibitor
Evusheld (tixagevimab/ cilgavimab)	AstraZeneca	Mar 25, 2022	Monoclonal antibodies: Targeting SARS-CoV-2 spike protein

damaging cytokine storm effects of viral infection (Fig. 2.3b); and (3) small molecules that prevent proteolytic activation of the SARS-CoV-2 non-structural proteins and replication of the viral RNA (Fig. 2.3c) (Table 2.2). The drugs which have been approved currently for use in either Europe, the United States, or by the World Health Organization (WHO), are indicated below in the order of approval date (earliest to most recent).

3.1 Remdesivir

Remdesivir was the first antiviral drug to be authorized by the WHO as a treatment for COVID-19. In the United States, the Food and Drug Administration (FDA) approved Remdesivir for emergency use for people greater than 12 years old and heavier than 40 kg (88 lbs) [33], and it has now been approved for temporary use in more than 50 countries [34]. It was first developed in 2016 as an antivirus drug called GS-5734 by Gilead Sciences for the treatment of Ebola virus [35]. Remdesivir is a nucleotide analogue that inhibits viral RNA synthesis by stalling RNA-dependent RNA polymerase complex activity (Fig. 2.3c) [36]. Clinical trials on the use of Remdesivir to improve clinical outcomes in COVID-19 patients have shown mixed results. A meta-analysis conducted by Angamo et al. found that treatment with Remdesivir led to an increase in clinical recovery rate by 21% and 29% on

days 7 and 14, respectively, and the need for supplemental oxygen or mechanical ventilation was reduced by 27% and 47%, respectively, compared to the placebo group [37]. The same study also found a 39% reduction in mortality on day 14 but with no significant difference in this outcome on day 28. One meta-analysis found that 10-day Remdesivir treatment was safe with some adverse effects in hospitalized COVID-19 patients, but there was no reduction in mortality compared to placebo [38]. A more recent meta-analysis of nine randomized controlled trials found no significant differences in mortality or use of mechanical ventilation between the Remdesivir and control groups [39]. However, the use of Remdesivir did significantly increase recovery ($p = 0.004$) and clinical improvement ($p = 0.020$) rates. Taken together, the results of these studies suggest that further work is required to determine if Remdesivir and related antiviral drugs are efficacious and safe for use in the treatment of COVID-19.

3.2 Anti-Spike Protein Monoclonal Antibodies

One of the most promising therapies in the treatment of COVID-19 disease is the use of monoclonal antibodies that target different epitopes of the spike protein RBD (Fig. 2.3a) (Table 2.2).

3.2.1 Regdanvimab

Regdanvimab (originally designated CTP59) was identified through screening a peripheral blood mononuclear cell library from a convalescent patient as a monoclonal antibody targeting the SARS-CoV-2 spike protein RBD of the viral spike protein [40]. A recent meta-analysis identified seven studies including 1350 patients in the Regdanvimab arm and 1983 patients in the control group, which showed that Regdanvimab treatment led to decreased mortality and need for supplemental oxygen and/or progression to severe disease outcomes [41]. However, this did not account for the effects of SARS-CoV-2 variants of concern on the outcomes. It was approved for use in COVID-19 patients with mild or moderate levels of illness by the European Medicines Agency in November 2021.

3.2.2 Casirivimab/Imdevimab Cocktail

Ronapreve (also known as REGN-COV2) is a neutralizing antibody cocktail consisting of Casirivimab and Imdevimab, which target distinct regions of the SARS-Cov-2 spike protein RBD [42]. Theoretically, the antibody cocktail approach may offer advantages over a single monoclonal antibody therapeutic by targeting multiple epitopes and thereby diminishing the chances of immune evasion by emerging SARS-CoV-2 variants. A study of 949 patients with mild-to-moderate COVID-19

who were admitted to hospital during the SARS-CoV-2 Delta wave (July 24 to September 30, 2021) in Fukushima Prefecture, Japan, found that those who received the Casirivimab/Imdevimab cocktail showed significantly lower deterioration of symptoms [43]. It was approved for use in COVID-19 patients with mild or moderate levels of illness by the European Medicines Agency on the same date as Regdanvimab (November 12, 2021).

3.2.3 Sotrovimab

Sotrovimab was identified initially by screening antibodies from a convalescent patient from the SARS-CoV-1 epidemic in 2003 [44]. This antibody recognizes a conserved epitope in both the SARS-CoV-1 and SARS-CoV-2 spike proteins outside the RBD. This property suggested that this epitope might forestall the mutational escape seen in different SARS-CoV-2 variants [45]. A meta-analysis on the efficacy of different SARS-CoV-2 monoclonal antibody therapies found that Sotrovimab ranked first by causing a significant decrease in the incidence of hospitalization compared to placebo, [46] and two studies showed that it retained the most activity in neutralizing the Omicron variant [47, 48]. Sotrovimab was approved for use by the European Medicines Agency on December 17, 2021 for the treatment of COVID-19 patients over 12 years old and weighing over 40 kg who do not require supplemental oxygen or who have a severe disease risk.

3.2.4 Tixagevimab/Cilgavimab Cocktail

A combination of two monoclonal antibodies, Tixagevimab (also known as AZD8895) and Cilgavimab (AZD1061), was isolated from patients who had recovered from COVID-19 disease [49]. As with the other monoclonal antibody cocktails listed above, Tixagevimab/Cilgavimab binds to non-overlapping epitopes on the spike protein RBD. A trial of 3460 participants who received one dose of this cocktail had a relative risk reduction of 82.8% compared to 1731 individuals who had received placebo [50]. It received approval for medical use for the treatment of COVID-19 in the European Union on March 15, 2022. However, as with the other monoclonal antibody therapeutics, this combination treatment showed a significant reduction in efficacy against the Omicron BA.1 and BA.2 SARS-CoV-2 variants [46, 48]. This calls to attention the need for new monoclonal antibody therapeutics which target the various Omicron subvariants more effectively.

3.3 Tocilizumab

As the levels of the proinflammatory cytokine interleukin-6 (IL-6) have been found to positively correlate with COVID-19, disease severity and death outcomes drugs which counteract IL-6 signaling might play a role in mitigating these effects [51, 52].

Tocilizumab is a monoclonal antibody that acts as an IL-6 receptor antagonist and has been approved for the treatment of rheumatoid arthritis, cytokine release syndrome, and other disorders marked by hyper-inflammation (Fig. 2.3b and Table 2.2) [53]. In a meta-analysis carried out by Maraolo et al., Tocilizumab was associated with higher survival in severe COVID-19 disease patients (odds ratio [OR]: 0.83, 95% confidence interval [CI]: 0.74–0.93), although a larger study size accounting for different dosage regimes will be required to confirm this [54]. Zhang et al. carried out a meta-analysis of 11 studies consisting of 3406 and 3173 patients assigned to the Tocilizumab and control groups, respectively [55]. They found that the Tocilizumab group had showed significant reductions in the following: 1) the 28–30-day mortality risk, 2) need for mechanical ventilation, 3) time-to-hospital discharge, 4) intensive care unit admission, 5) serious disease trajectory, and 6) serious adverse events, compared to the control group. However, another meta-analysis found that although Tocilizumab significantly increased the rate of hospital discharges in COVID-19 patients, it had no effect on all-cause mortality or risk of secondary infections [56].

Some studies have now been carried out to assess the combined use of Tocilizumab and corticosteroid treatment in COVID-19 patients, and these have generally showed positive effects. Lim et al. carried out a meta-analysis of 13 randomized controlled trials and 24 case-control studies to compare the efficacy of Tocilizumab with corticosteroid treatment on mortality outcomes in 18,702 COVID-19 patients [57]. This revealed significant reductions in mortality following Tocilizumab-dexamethasone (odds ratio [OR]: 0.71, 95% confidence interval [CI]: 0.55–0.92) and Tocilizumab-Methylprednisolone (OR: 0.52, 95% CI: 0.36–0.75) therapies. No reduction in mortality was observed for mono-treatment with Methylprednisolone, and none of the drugs significantly reduced the need for mechanical ventilation (OR: 0.72, 95%CI: 0.32–1.60). Hong et al. carried out a retrospective cohort study of 33 COVID-19 patients receiving dexamethasone alone and 33 receiving dexamethasone plus Tocilizumab [58]. This showed that the combination treatment led to a significant benefit in a 30-day clinical recovery and reduced the need for supplemental oxygen compared to the dexamethasone mono-treatment group. Furthermore, meta-analysis found that the risk of death for COVID-19 patients treated with a corticosteroid-Tocilizumab combination compared with Tocilizumab alone or placebo control was 26% and 52% lower, respectively [59]. Considering these promising results, these studies call to attention the need for further testing on the use of COVID-19 treatments targeting different aspects of inflammation and immune signaling pathways.

3.4 Anakinra

Considering that hyper-inflammation is a key factor in driving severe COVID-19 infections, elevated concentrations of pro-inflammatory biomarkers such as interleukin 1 (IL-1) have been identified in COVID-19 patients who experienced a severe

or critically ill outcome (Fig. 2.3b) [60]. Anakinra is a recombinant IL-1 receptor antagonist which has been approved for use in the European Union as an anti-inflammatory drug to reduce severity and mortality in COVID-19 patients (Table 2.2) [61]. A meta-analysis which assessed the effects of Anakinra treatment on key inflammatory biomarkers found that the serum levels of c-reactive protein (CRP), ferritin, and d-dimer were all reduced in the Anakinra compared to the standard care group [62]. Another meta-analysis found a significant reduction in mortality (OR = 0.34) and need for mechanical ventilation (OR = 0.68) in the Anakinra treatment arm compared with the standard care group [63]. However, the same study called to attention the need for further studies investigating the safety profile of this drug. These findings were confirmed by another meta-analysis, although this reported no difference in adverse events between the treatment and standard care groups [64].

3.5 Ritonavir

Ritonavir was originally developed as an inhibitor of the human immunodeficiency virus (HIV) protease [65, 66] and has been repurposed for similar use in COVID-19 patients via its ability to inhibit the SARS-CoV-2 3C-like protease enzyme (Fig. 2.3c and Table 2.2) [67]. Thus far, no meta-analyses have demonstrated the efficacy of this compound, either alone or in combination, in preventing serious disease in COVID-19 patients, with several reports of adverse effects [66, 68]. We suggest that further studies should be conducted to identify other more efficacious and safer antiviral drug candidates for COVID-19.

4 Effect of SARS-CoV-2 Variants on the Efficacy of Vaccines and Monoclonal Antibody Therapeutics

Although most of the developed vaccines worked well at preventing infections and serious illness courses with the original strain of the virus, most worked less efficaciously against the emerging SARS-CoV-2 variants. Planas et al. tested the sensitivity of Omicron compared to the Delta variant of the WHO-approved monoclonal antibody therapeutics using the S-Fuse assay [48]. All of these antibodies and antibody mixtures neutralized the Delta variant with IC_{50} concentrations ranging from 16 to 369 ng/mL (Table 2.3). However, the Tixagevimab/Cilgavimab combination (Evushield; AstarZeneca) and the Sotrovimab monotherapy (Xevudy; GlaxoSmithKline and Vir Biotechnology, Inc.) showed 85- and three-fold decreases in sensitivity, respectively, against Omicron compared to the Delta variant, and the Casirivimab/Imdevimab combination (Ronapreve; Regeneron) and Regdanvimab (Regkirona; Celltrion) had no detectable neutralizing activity towards the Omicron

Table 2.3 Sensitivity of omicron compared to delta variant to WHO-approved monoclonal antibody therapeutics. Data taken from Planas et al. [**Planas**]

	Delta variant (IC_{50} ng/mL)	Omicron variant (IC_{50} ng/mL)
Regdanvimab	92	9000+
Casirivimab/Imdevimab	98	9000+
Sotrovimab	369	1114
Tixagevimab/Cilgavimab	16	1355

variant. The same study also tested the potency of antibodies elicited by the Comirnaty (Pfizer/BioNTech) and Vaxzevria (AstraZeneca) vaccines to neutralize the Omicron variant relative to the original SARS-CoV-2 strain and the Delta variant [48]. For both vaccines, sera were sampled 5 months after a two-dose vaccination schedule. This showed that the neutralizing antibody activity in sera was 3.6-fold lower against the Delta variant compared to the original strain of the Comirnaty vaccine, with no neutralization activity detected against the Omicron variant at the highest concentration. Similarly, the levels of antibodies in sera from Vaxzevria-vaccinated individuals were 2.8-fold lower in the neutralizing the Delta variant compared to the original strain, and no activity was observed against the Omicron variant. Similar findings were reported by Zhang et al. [69], Cao et al. [70], and Carreño et al. [71]. This underscores the capacity of the Omicron variant to escape the existing therapeutic monoclonal antibody treatments and vaccines.

As a means of predicting the capability of SARS-CoV-2 variants to escape antibody neutralization, Hu et al. developed a computational model to estimate the effect of mutations in the spike protein RBD on antibody neutralization titers [72]. Their results were similar to the experimentally determined neutralization titers of the known variants of concern, and they predicted a 17.4-fold decrease in the susceptibility of Omicron to neutralization.

5 Identification of Monoclonal Antibodies and Development of New Vaccines to Overcome the Immune Escape Capabilities of SARS-CoV-2 Variants

5.1 Monoclonal Antibodies

Zakir et al. identified a broadly neutralizing monoclonal antibody (mAb 9G8) which potently neutralizes the SARS-CoV-2 wild-type, Alpha, and Delta variants [73]. However, this has not been tested with the Omicron variant. A similar result was obtained with mAb 2G1 with respect to neutralizing all SARS-CoV-2 strains, but without testing on the Omicron variant as above [74]. In two in vitro and in vivo studies, Wang et al. found that another monoclonal antibody (mAb 35B5) was capable of neutralizing the original SARS-CoV-2 virus and other variants of concern such as Delta [75] and Omicron [76]. By using cryo-electron microscopy, they

showed that this antibody targets a unique epitope outside the RBD, and this likely disrupts the conformational changes that allow SARS-CoV-2 binding to host ACE2 receptors [75, 76]. In a study of 30 healthy volunteers administering a mAb 35B5 nasal spray formulation, it was revealed that nasal mucosal samples collected within 24 h showed effective neutralization against pseudoviruses coated with SARS-CoV-2 spike protein variants including both Delta and Omicron [77]. However, full protection required daily inhalation of the spray, suggesting the need for further studies with optimized formulations to extend the duration of the antibody in the nasal mucosa.

Du et al. identified a monoclonal antibody (mAb 87G7) with potent in vitro neutralizing activity in vitro against all SARS-CoV-2 variants including the Omicron BA.1/BA.2 subvariants [78]. Using cryo-electron microscopy and site-directed mutagenesis, they showed that mAb 87G7 targets a conserved hydrophobic amino acid cluster in the ACE2 receptor binding site. Another study isolated two antibodies (EV053273 and EV053286) from convalescent patients after they had been infected with the wild-type version SARS-CoV-2 [79]. One of these antibodies (EV053273) had potent antiviral activity against wild-type SARS-CoV-2 and the Alpha and Delta variants, and the other (EV053286) had lower activity but neutralized all SARS-CoV-2 variants, including the Omicron BA.1 and BA.2 subvariants. They also found that a combination of these two antibodies blocked infection in vivo using a mouse model. In a similar study, Kovavech et al. identified a cocktail of two distinct monoclonal antibodies (AX290 and AX677) with high affinity to the SARS-CoV-2 spike protein RBD in all SARS-CoV-2 variants, including Omicron, and administration of this cocktail reduced viral burden and inflammation in the lungs of an infected mouse model in vivo [80]. Finally, another study developed monoclonal antibodies against Omicron and other SARS-CoV-2 variants elicited by vaccination with Convidecia [81]. One of these antibodies (ZWD12) showed potent neutralization against all strains of concern, including the Omicron variant.

5.2 SARS-CoV-2 Vaccines

5.2.1 Updated Vaccines

From the above findings, it is clear that the production of new vaccines against the current variant of concern is a pressing matter in gaining control over this pandemic. This includes the production of new vaccines specifically targeting the Omicron subvariants [82]. With this objective in mind, a recent study showed that the original Spikevax and Omicron-specific mRNA vaccines produced by Moderna elicited similar neutralizing responses to the Omicron BA.1 and BA.2 subvariants [83]. However, multiple countries and territories are now faced with outbreaks of Omicron BA.4 and BA.5, which may not be recognized by the time the above vaccines are rolled out. It is also possible that a new variant will branch out from a different part of the SARS-CoV-2 family tree. Thus, most scientists agree that constant updates to

the existing vaccines are essential. Other pharmaceutical companies are testing Omicron-specific vaccines. For example, Pfizer–BioNTech reported that their new Omicron BA.1-based vaccine produced neutralizing antibody responses against this subvariant that were 2–3 times higher than that seen with a booster dose of their original Comirnaty vaccine [84]. Another study tested adults who had been doubly vaccinated with Comirnaty and had never tested positive for COVID-19 and then received a booster vaccination with either 1) a third dose of Comirnaty; 2) a recombinant spike protein (MVD614) based on the original SARS-CoV-2 strain or 3) a recombinant spike protein (MVB.1.351) based on the Beta variant [85]. The results showed that boosting with the MVB.1.351 vaccine resulted in a higher neutralizing antibody response against the original virus as well as the Beta, Delta, and Omicron BA.1 strains, compared to boosting with either the Comirnaty or MVD614 vaccines.

5.2.2 Multivalent Vaccines

One approach that can be taken with vaccines is that of multivalent administrations that simultaneously neutralize multiple variants. This is not a new concept as it has been used for decades with influenza vaccines each year, such as the simultaneous targeting of different varieties of influenza A and B strains [86]. It follows that a similar approach could be used to spike RBD sequences from multiple SARS-CoV-2 variants of concern. In line with this objective, Moderna has now developed a bivalent vaccine called mRNA-1273.214, which targets the spike protein of the original SARS-CoV-2 virus as well as the highly mutated Omicron variant [87]. Initial reports from a small trial of 439 participants suggested that this vaccine met the clinical endpoints. The data showed that the mean titer was 2372 for the bivalent vaccine, compared to 1473 for the original Moderna mRNA-1273 vaccine [88–90]. The bivalent vaccine was also well tolerated with a similar side effect profile as the current vaccine. Moderna plans to submit the results of this analysis over the coming weeks to regulators.

6 Natural Products for Improved Management of COVID-19 Patients

Herb-derived natural products have long been used in the management of numerous human ailments since ancient times. With the aid of technical advances in instrumental and biological fields, numerous phytochemicals have been isolated and identified as active ingredients responsible for the pharmacological actions exerted by famous medicinal plants. With respect to COVID-19, several medicinal plants and phytochemicals have been suggested and explored as potential candidates for the treatment of the disease or alleviation of the symptoms [91–93]. In fact, herbal medicines have been among the first options to enter clinical phase testing for

COVID-19, owing to their availability and generally good safety and tolerability since most of the medicinal plants have a strong ethnobotanical background of use. From the mechanistic standpoint, phytochemicals might exert protective effects against COVID-19 through several mechanisms, including a direct impact on SARS-CoV-2 replication, and infectivity, regulation of ACE2 receptors and the renin-angiotensin system, anti-inflammatory action, and immunomodulatory properties [93, 94].

Among the phytochemicals, polyphenols have been the subject of a particular focus for their therapeutic potential in COVID-19 [91]. As a leading polyphenol, curcumin, the active ingredient of turmeric, has been the subject of several trials in patients at different stages of COVID-19 [95, 96]. A systematic review of clinical trials suggested the beneficial effects of different curcuminoid preparations, including nanoformulations and curcumin-piperine combinations, on symptom relief, hospitalization length, and mortality in patients suffering from COVID-19 [96]. The main mechanism suggested to explain the protective effects of curcumin in COVID-19 is the mitigation of inflammatory responses as well as the cytokine storm that is closely associated with end-stage adverse COVID-19 complications [95, 97, 98].

Another herbal product which has shown positive effects in clinical practice is the combination of glycyrrhizin and boswellic acids. Besides anti-inflammatory and immunomodulatory activities, both compounds have been reported to exert antiviral effects against SARS-CoV-2 [99, 100]. Glycyrrhizin has been proposed to inhibit the main protease (M^{pro}) of SARS-CoV-2, thereby interfering with viral replication [101]. Additionally, both glycyrrhizin and boswellic acids can interact with the functional spike protein of SARS-CoV-2 and reduce virus infectivity through mitigation of viral entry into the host cells [102, 103]. In a randomized, double-blind, and placebo-controlled trial, 50 hospitalized patients with moderate COVID-19 received either the combination of glycyrrhizin (60 mg twice daily) and boswellic acids (200 mg twice daily) or placebo for 14 days [104]. The findings revealed a significantly lower rate of mortality in the supplemented ($n = 0$) vs. placebo ($n = 5$) group. Moreover, there were significant improvements in terms of time to recovery, clinical status, serum CRP levels, and percentage of lymphocytes in the herbal combination group compared with the placebo group.

Chinese herbal medicine (CHM) is a comprehensive system of medicine with a strong ethnobotanical background dating to over 2000 years ago. Since the onset of the pandemic, CHM has been among the first therapeutic approaches tested for the management of COVID-19. Thus far, numerous herbs and formulae have been studied in patients with COVID-19, and several systematic review has been published [105–107]. However, the methodological limitations and risk of bias in several of the included trials precluded the possibility of reaching a definitive judgment on the efficacy and safety of CHM for the management of COVID-19. Recently, a systematic review and meta-analysis of 22 high-quality randomised controlled trials involving 1789 subjects assessed the value of adding CHM to Western medicine in controlling COVID-19 [108]. The results suggested the safety as well as the benefit of combining CHM with Western medicine in improving clinical, hematological,

and virological indices of COVID-19, particularly in those with mild-to-moderate symptoms [108]. Nevertheless, evidence from long-term and multicenter trials is still required to better clarify the role of CHM in the management of COVID-19.

7 Conclusions and Future Perspectives

The emergence of new highly infective SARS-CoV-2 variants such as Omicron has wreaked havoc around the world by allowing the persistence of a pandemic that has already resulted in considerable damage at the individual, societal, and financial levels. Although unprecedented achievements have been made in attempts to stop the spread of COVID-19 disease, the problem has continued due to the mutability of the virus, which renders it with new properties such as increased infectivity and the ability to evade our immune defenses. This review has described efforts aimed at developing new vaccination strategies to keep pace with new SARS-CoV-2 variants as they appear, including variant-specific and multivalent vaccine designs. This included the use of vaccines that target the spike protein of specific SARS-CoV-2 strains and multivalent approaches that are directed simultaneously against the original SARS-CoV-2 isolate as well as the Omicron variant. Another possibility is the targeting of other antigenic domains within the virus that lie outside the spike protein RBD, as this may allow the development of a universal coronavirus vaccine [109].

In addition to the developments in SARS-CoV-2 vaccination strategies, we described pharmaceutical approaches that are currently in use for the treatment of individuals who become ill or suffer from postviral sequelae. Most of the existing drugs consist of either repurposed medicines, such as antiviral compounds and anti-inflammatory agents, or monoclonal antibodies obtained from convalescent or vaccinated patients. In addition, other approaches are currently under development to help overcome the limitations of the current methods. In the case of antibody-based therapeutics, one potential strategy is the use of broad coronavirus-directed nanobodies isolated from dromedary camels, which are natural reservoirs of coronaviruses, as these molecules can recognize cavities in proteins that are inaccessible to larger conventional antibodies. With this in mind, Hong et al. constructed a phage display library from camels containing nanobodies capable of protecting transgenic mice-expressing human ACE2 receptors against challenge with the SARS-CoV-2 Beta and Delta variants [110]. In addition, several studies have been conducted which have attempted to identify small molecules that disrupt binding of the SARS-CoV-2 spike protein RBD to the ACE2 receptor. Mediouni et al. screened a library of 15,000 small molecules and identified a compound called calpeptin, which blocked the entry of some of the SARS'CoV-2 variants in whole cell infectivity assays [111]. Another study found that an engineered soluble ACE2 peptide had high binding affinity to the spike protein of the original SARS-CoV-2 isolate as well as to the Alpha, Beta, Gamma, and Delta variants [112]. The same study found that this peptide reduced disease severity and improved survival in a transgenic human

ACE2 mouse model infected with both the original SARS-CoV-2 strain and the Gamma variant. Due to the timing of the above studies, the effects of the SARS-CoV-2, RBD, and ACE2 inhibitors on the Omicron subvariants were not assessed. However, a recent study by Li et al. showed that an engineered ACE2 decoy protein had potent preventative and therapeutic efficacy against both Delta and Omicron in in vivo assays [113]. Finally, we described how several natural products are undergoing preclinical and clinical testing to determine their efficacy as preventative or therapeutic agents to prevent serious outcomes following SARS-CoV-2 infection. The advantage of these approaches is that the molecules concerned generally have good safety profiles and are predicted to work across all SARS-CoV-2 variants since they target the effects on the body and not the virus itself.

In conclusion, this review has described the importance of developing vaccines and treatment strategies that keep pace with the new SARS-CoV-2 variants as these emerge. In the case of vaccines and therapeutic antibodies, this could involve the production of broadly neutralizing or variant-specific products. For treatment approaches, considerable further work is required to identify the most efficacious approaches without the trade-off of poor safety profiles. Most of all, it will be important to lay the foundations for a procedural pipeline to cope with the likely appearance of new coronavirus variants.

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Part II
Neurological and Psychological Effects of the COVID-19 Pandemic

Chapter 3
Consequences of the Lockdown: Domestic Violence During the COVID-19 Pandemic

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Abstract

Background
The global pandemic of the coronavirus disease **2019** (COVID-**19**) has presented many unique challenges to health systems. The hidden impact of COVID-**19** and its associated lockdown have been an increased prevalence of domestic v**iolence.**

Objective
To increase our understanding of the connection between COVID-19 containment measures, domestic violence, and mental health in Germany, we conducted an online self-assessment survey of 98 domestic violence victims and 276 controls. All participants answered questions concerning domestic violence, emotional regula-

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tion skills, limitations due to and acceptance of containment measures, and quality of their contact experiences.Results

There was no significant effect of “gender” x “domestic violence.” Among victims of domestic violence, the number of women was considerably higher than the number of men. In addition, the factors “negative contact quality,” “emotional regulation,” and “resilience” differed significantly between the victims of domestic violence and the control group.Conclusions

The COVID-19 outbreak and associated containment and quarantine measures resulted in a “hidden pandemic” of domestic violence for which prevention programs and early victim assistance through the expansion of digital technologies are urgently needed. Prospective studies should expand empirical data to focus on the long-term psychological effects of domestic violence and biomarkers that can serve as warning signs of stress-related disorders.

Keywords COVID-19 · SARS-CoV-2 · Domestic violence · Mental health · Containment measures

1 Introduction

Coronavirus disease 2019 (COVID-19) is caused by acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. This infectious disease, which primarily affects the respiratory tract and broke out in Wuhan (Hubei Province, China) in 2019, spreads rapidly to various countries worldwide [2, 3]. On March 11, 2020, it was declared as a global pandemic [4]. To contain the spread of COVID-19, prevent increased morbidity, and avoid overburdening health systems, social containment measures were implemented [5–7]. These measures have included selective quarantines, stay-at-home orders, travel restrictions, and the closure of kindergartens, schools, and all nonessential services and businesses [6, 8]. Although these measures can be effective in containing the spread of disease, they also can lead to unintended, negative consequences [9]. Several new stressors, including physical and mental health risks as well as social and economic impacts, could result [1, 9]. There is evidence that quarantine, in particular, can lead to negative psychological outcomes such as posttraumatic stress symptoms, confusion, and anger [10].

Previous natural disasters and health crises have been associated with an increase in violence both inside and outside the home [11]. Similar to social isolation during previous epidemics and pandemics, the psychological effects of social isolation during the COVID-19 pandemic in particular may increase the risk and severity of domestic violence [9, 12–16]. Increases in domestic violence have been reported in the context of natural disasters, such as after the 2004 Indian Ocean earthquake and tsunami [17], Hurricane Katrina in the United States in 2005 [18], and the 2009 “Black Saturday” bushfires in Australia [13]. Following the 2004 tsunami in North Sumatra and the 2011 earthquake in Tōhoku, Japan, increased rates of violence

within couples persisted even a decade after these disasters [19, 20]. Women and girls also experienced more sexual violence, coercion, and exploitation during past epidemics such as those caused by the Ebola and Zika virus outbreaks [21, 22].

Domestic violence is a broad term that describes assault or abuse committed within a domestic setting by one person against another who are either in a current or former intimate relationship, cohabitation, or familial association [9, 23]. It is a global health problem that can lead to psychological trauma and accompanying mental, physical, and sexual health consequences for the victim and the entire family [24, 25]. In addition, domestic violence is a notable cause of mortality and morbidity among women [26]. The term domestic violence is interchangeably used with intimate partner violence or gender-based violence and also comprises elder abuse as well as child abuse [1, 27, 28]. A variety of behaviors fall within the scope of domestic violence [25]. These include physical (e.g., hitting, slapping), sexual (e.g., assault, rape), psychological (e.g., insult, manipulation), economic (e.g., prohibition from working, coercive control of finances), as well as social (e.g., social isolation, coercive control of messages) violence [29, 30].

Domestic violence can affect all types of age groups, ethnicities, relationship statuses, as well as socioeconomic levels [31]. It is typically experienced by women of all ages, and children and their mothers are particularly at risk of becoming victims of violence [9, 32]. In addition, domestic violence is the leading cause of homicide among women [32]. Despite this disproportionate distribution, men can also experience this type of violence. According to the Centers of Disease Control, one in four women and one in ten men report being victims of some form of intimate partner violence each year [33]. Prior to the COVID-19 pandemic, 35% of women worldwide were described as experiencing physical and/or sexual violence by an intimate partner during their lifetime [34]. In general, physical forms of violence are more severe against women than against men [35]. Individuals who have been a victim of intimate partner violence are at increased risk for various psychological (e.g., mood disorder, posttraumatic symptom disorder, substance abuse, suicidal behavior) and physical (e.g., cardiovascular disease, chronic pain, sleep disorders) health conditions [36]. This type of violence is a chronic and often persistent stressor, and some studies have even demonstrated the presence of hypothalamic-pituitary-adrenal (HPA) axis dysregulation involved in the stress response in victims of intimate partner violence [37–39].

Prolonged proximity to others, including family members or intimate partners, and external stressors can lead to an increased tension, feelings of isolation, loneliness, and worsening of existing mental health status [40]. In addition, individuals living in quarantine are described as more likely to experience anger and posttraumatic stress symptoms and have increased substance use, which may increase the risk for violent behavior, particularly in the home [41]. Furthermore, the risk of reabuse is known to increase when a person is unable to escape the abuser due to social isolation measures [24]. Therefore, the situation created by COVID-19, including the containment efforts, presents unique problems, particularly with regard to domestic violence. Social containment strategies have profound

implications for families experiencing domestic violence [42]. For children and adults living in these situations, the home is often where violence and abuse in various forms occurs [9, 32]. Contact with the abuser is a key factor in experiencing domestic violence [43]. It also increases the risk of health problems associated with domestic violence, such as chronic illness, gynecological morbidity, trauma-related injuries, and stress-related symptoms [44, 45]. Due to movement restrictions and the reduction of social contacts, the possibilities of benefiting from social and protective networks or escaping the violent situation are severely limited [43, 46]. In addition, access to public services and institutions that provide social support is disrupted [43, 47]. Moreover, in the exceptional circumstances of the COVID-19 pandemic, exposure to heightened external stressors may increase the risk for domestic violence [48]. These include situations such as unemployment and financial insecurity [14, 48, 49], fear for health [10, 50], and altered parenting responsibilities [48].

In the context of the current COVID-19 pandemic and associated lockdown, an increase in reports of domestic violence has been described worldwide [1, 9, 50–53]. Initial leads came from a police station in Jianli (Hubei Province, China) near the epicenter of the COVID-19 outbreak, where reports of domestic violence from February 2019 and February 2020 were compared. This revealed a tripling of domestic violence cases and estimated that 90% of these cases were related to COVID-19 [54–56]. In France, a 30% increase has been documented since the March 17, 2020 lockdown. Percentages are comparable for Argentina (25%), Cyprus (30%), and Singapore (33%) as evidenced by domestic violence counseling services [57]. In the United Kingdom, the number of deaths caused by domestic violence was found to have doubled between March 23 and April 21, 2020 ($n = 16$ deaths) compared with the average rate over the past 10 years [58]. In a study of maxillofacial surgery in the United Kingdom, Blackhall and colleagues reported cases of severe facial trauma ($n = 19$ cases) associated with domestic violence or self-harm [59].

Our aim was to examine the relationship between COVID-19, its associated containment measures, domestic violence, and mental health through an online survey. The specific objectives of this study were to determine (1) whether there are gender differences in domestic violence and (2) how victims of domestic violence differ from control individuals who did not experience domestic violence. Our study hypotheses are as follows:

1a) Women are more likely to be victims of domestic violence than men.
1b) Female victims report an increased frequency of domestic violence than male victims.
2a) Victims of domestic violence have more children attending kindergarten and school than the control group.
2b) Victims of domestic violence have more negative contact experiences compared to controls.
2c) Victims of domestic violence have lower emotional regulation skills compared to controls.

2d) Victims of domestic violence have more problems to endure and tolerate their feelings compared to controls (resilience).
2e) Victims of domestic violence report more restraints due to containment measures compared to controls.
2f) Victims of domestic violence show a lower willingness to implement containment measures (commitment) compared to controls.

Moreover, we conducted mediation analyses to see which factors can influence the above points on domestic violence.

2 Methods

2.1 Participants

The participants were recruited via local newspaper advertisements, social media, e-mail distribution lists for students and employees, newsletter for employees of Magdeburg University Hospital, information on the website of Otto-von-Guericke University Magdeburg, and distribution of flyers (including within the emergency department of Magdeburg University Hospital). All subjects gave written informed consent before enrollment in the study according to procedures approved by the institutional review board of the Medical Faculty (Otto-von-Guericke University Magdeburg) prior to study inclusion. Subjects received no financial compensation for their participation in the study. The study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

In total, 660 participants (n_{female} = 451, n_{male} = 172, $n_{diverse}$ = 5) aged 31.75 ± 12.26 years participated in the online survey study. Inclusion criteria were age of at least 18 years and participation between April 27, 2020, and June 8, 2020. One participant was excluded due to not giving sensible answers. Two participants were excluded because they indicated an age less than 18 years and a further 33 participants dropped out before the age question. Four were excluded because they indicated that they had not provided reliable responses. Furthermore, one participant was excluded because the DEG_TIME was >100 (negative points for extremely fast completion; the value is normalized so that values of more than 100 points indicate poor quality of the data) [60] and dwell time on 15 of 31 pages of the online survey fell below one-third of the mean time.

This resulted in a final sample of 619 participants. Within this sample, 98 reported at least one instance of domestic violence, while 276 reported not having been a victim of domestic violence. There was a high proportion of missing information (n = 245), because 140 participants dropped out of the survey before the domestic violence questions, 104 lived alone, and one did not answer all domestic violence questions.

2.2 Procedure

We conducted an anonymous online survey of mental health and well-being during the COVID-19 pandemic. The questionnaire was created using SoSci Survey [61]. The survey was compatible with desktop or laptop computers, tablets, and smartphones but was only available in German. The first page of the questionnaire contained information about the study, data protection, and points of contact in case of crisis. Before starting the survey, participants had to give their informed consent. The entire survey consisted of a variety of questions and psychological scales. At the end of the survey, participants were asked if they had provided sensible and reliable responses. The average time for survey completion was approximately 20 min. Only a subset of questions was selected to focus the statistical analyses, and these are explained in more detail in the following sections.

2.2.1 Demographic Information

Multiple-choice and open-ended questions were used to record gender (female/male/diverse), age in years, place of residence (country, state), education (level of education, professional qualification), profession, marital status, parenthood, and characteristics of the current household.

2.2.2 Domestic Violence

To assess the presence of domestic violence, participants were asked to indicate how often a person living in their household had perpetrated various types of violence against them in the past two weeks. Fifteen items required responses on a five-point Likert scale (1 = "never," 2 = "1 to 2 times," 3 = "3 to 5 times," 4 = "6 to 10 times," 5 = "more than 10 times"). These items included physical violence (e.g., "slapped you"), sexual violence (e.g., "had sexual intercourse with you by force"), psychological violence (e.g., "humiliated you"), economical violence (e.g., "forbade you to handle money") as well as social violence (e.g., "forbade you to have contact with your family"). A person was defined as a victim of domestic violence if at least one item had a value greater than 1. For further statistical analyses the variable "sum of domestic violence" was formed. This was the sum of all 15 items, reflecting the overall frequency/intensity of domestic violence.

2.2.3 Self-Report Measure for the Assessment of Emotion Regulation Skills

The ability of successful emotion regulation was assessed with the Self-Report Measure for the Assessment of Emotion Regulation Skills (SEK-27) [62]. This questionnaire consists of 27 items representing 9 different competencies in

dealing with problematic emotions during the past 14 days. Each item has to be answered on a five-point Likert scale (0 = "not at all," 1 = "infrequent," 2 = "sometimes," 3 = "frequent," 4 = "(almost) always"). For the present study, only six items from the "resilience" and "regulation" subscales were used for further statistical analyses.

2.2.4 Commitment Score

The following eight items were used to assess commitment to COVID-19 containment measures for the past 14 days: (1) "I comply to the measures"; (2) "I believe the measures are useful"; (3) "I believe the measures will be successful"; (4) "Complying with the measures is a challenge for me"; (5) "I believe the measures will have bad consequences for me"; (6) "I believe the measures will have bad consequences for my friends and/or relatives"; (7) "I believe the measures will have bad consequences for many people"; and (8) "I believe the measures can also be an opportunity for the future." Each item had to be scored on a five-point Likert scale from 0 = "not at all" to 4 = "very strong." To calculate the total score for all items (commitment score), ratings for items 4 to 7 were inverted.

2.2.5 Restrictions Due to Containment Measures

To assess the extent to which participants were personally affected by the COVID-19 mitigation measures, they were asked: "In terms of the past 14 days, what constraints and additional stresses are you experiencing as a result of the current situation?" Participants were instructed to select all that applied from a list of predefined constraints: "loss of earnings"; "child care"; "closing their own business/company"; "more work"; "home office"; "less work"; and "strenuous/stressful work." In addition, there was a blank space in which additional constraints could be entered. The score was calculated by counting the selected answers.

2.2.6 Contact Quality

To assess how participants described the quality of most of their face-to-face contacts, they were asked: "With regard to the past 14 days, how would you describe the quality of your current contacts?" Participants were instructed to rate the following seven items: "supportive," "friendly," "disruptive," "calming," "frightening," "stressful," and "upsetting." Each item had to be rated on a five-point Likert scale from 0 = "not at all" to 4 = "very strong." The score for "negative contact quality" was formed by taking the average of the inverted scores for the items "disruptive," "frightening," "stressful," and "upsetting."

2.3 Statistical Analyses

IBM SPSS Statistics Version 26 (Armonk, New York, United States) was used for descriptive inferential data analysis and hypothesis testing, and the PROCESS Version 3.5 [63] macro for SPSS was applied for mediation analyses. First, we tested for normal distribution ($p > 0.05$) using the Shapiro-Wilk test. To test for group differences, we performed parametric two-sample t-tests for normally distributed variables. Otherwise, nonparametric Mann-Whitney U tests were calculated. Chi-square tests were performed to test statistical independence. A p-value of less than 0.050 was considered statistically significant.

Mediation analyses were conducted using the PROCESS v3.5 macro for SPSS [63] which uses ordinary least squares regression, yielding unstandardized path coefficients for total, direct, and indirect effects. Bootstrapping with 5000 samples with heteroscedasticity-consistent inference (HC3) [64] was used to calculate confidence intervals and inferential statistics.

3 Results

3.1 Sociodemographic Data

The present sample includes 98 individuals who were victims of domestic violence as well as 276 controls who were not domestic violence victims during the first lockdown in Germany. Figure 3.1 gives an overview of the frequency of the different types of domestic violence. Psychological and economic violence were the most common forms in this present sample. Victims of domestic violence (median [Mdn] = 28.00, $Q1$ = 22.00, $Q3$ = 37.00) and controls (Mdn = 28.50, $Q1$ = 24.00, $Q3$ = 39.00) did not differ with respect to age (U = 12,370.00, $Z = -1.26$, $p = 0.209$). Furthermore, the two groups did not differ with respect to education, marital status, household structure, or lifestyle. Table 3.1 shows the detailed sample characteristics of domestic violence victims compared to control subjects.

3.2 Gender Differences Regarding Domestic Violence

A chi-square test was applied to examine 1a) the distribution of "gender" and "presence of domestic violence." Since the sample of diverse individuals was small (n = 2), we decided to exclude these two persons for the analyses of gender differences. The results showed no statistically significant association between gender and the presence of domestic violence ($\chi^2(2) = 0.39$, $p = 0.535$). Descriptively, 25.0% of the female and 28.3% of the male study participants reported being victims of domestic violence. Among victims of domestic violence, the proportion of

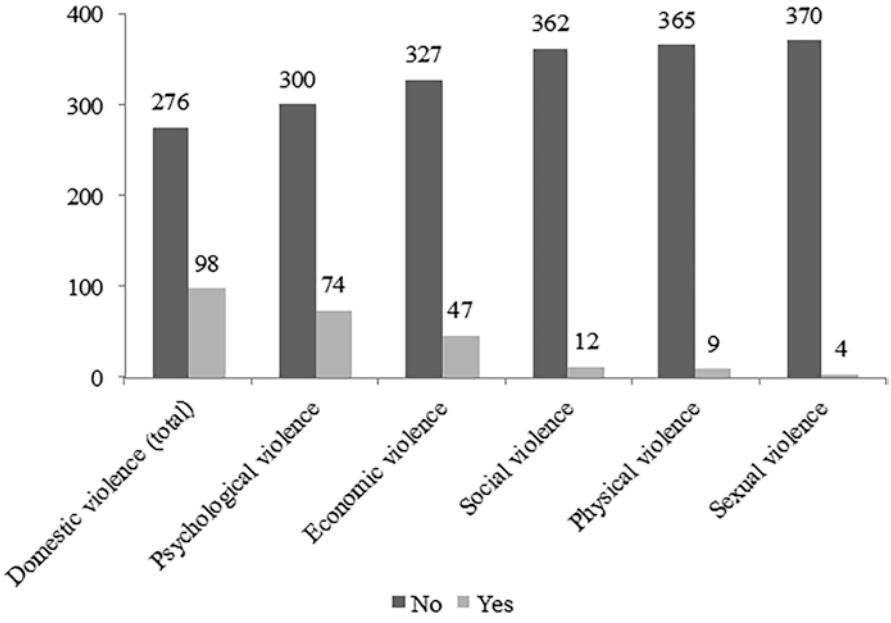

Fig. 3.1 Graphic representation of the frequency of different types of domestic violence (number of cases)

females ($n = 70$, 72.9%) was considerable higher than that of males ($n = 26$, 27.1%). Figure 3.2 provides an overview of the gender distribution in both groups.

To test whether 1b) female victims experienced domestic violence more frequently than male victims, a Mann-Whitney U test was calculated with the dependent variable "sum of domestic violence." Female (Mdn = 16.00, $Q1 = 16.00$, $Q3 = 17.00$) compared to male (Mdn = 17.00, $Q1 = 16.00$, $Q3 = 17.00$) victims did not differ significantly in terms of frequency ($U = 817.50$, $Z = -0.31$, $p = 0.757$).

3.3 Comparison of Domestic Violence Victims and Controls

To test whether 2a) victims of domestic violence had more kindergarten- or school-age children compared with controls, Mann-Whitney U tests were conducted with dependent variables "number of children in kindergarten" and "number of children in school." Victims of domestic violence (Mdn = 1.00, $Q1 = 0.00$, $Q3 = 1.00$) had more kindergarten-age children than controls (Mdn = 0.00, $Q1 = 0.00$, $Q3 = 1.00$; $U = 1380.00$, $Z = -2.04$, $p = 0.041$). Victims of domestic violence (Mdn = 0.00, $Q1 = 0.00$, $Q3 = 1.00$) did not differ from controls (Mdn = 0.00, $Q1 = 0.00$, $Q3 = 1.00$) in terms of school-age children ($U = 1552.50$, $Z = -1.03$, $p = 0.302$).

To test whether 2b) victims of domestic violence reported more negative contact experiences compared to controls, a Mann-Whitney U test was calculated with the

Table 3.1 Sample characteristics of domestic violence (DV) victims compared to controls

	Victims of DV (n = 98)	Controls (n = 276)	Statistics
Age [mean years]	Mdn = 28.00 ($Q1$ = 22.00, $Q3$ = 37.00)	Mdn = 28.50 ($Q1$ = 24.00, $Q3$ = 39.00)	U = 12,370.00, Z = −1.26, p = 0.209
Years of education [number] Low Middle High	n = 1 n = 113 n = 162	n = 1 n = 44 n = 53	U = 12,865.00, Z = −0.84, p = 0.404
Marital status [number] Single In relationship Married/registered partnership Divorced	n = 30 n = 39 n = 29	n = 82 n = 106 n = 81 n = 7	U = 13,121.00, Z = −0.46, p = 0.642
Household members [number]	Mdn = 2.00 ($Q1$ = 1.00, $Q3$ = 3.00)	Mdn = 2.00 ($Q1$ = 1.00, $Q3$ = 3.00)	U = 12,105.50, Z = −1.64, p = 0.102
Lifestyle [number] Rural community (<5.000) Small town (>5.000) Medium town (>20.000) Large city (>100.000)	n = 16 n = 10 n = 14 n = 58	n = 36 n = 23 n = 22 n = 195	U = 12,096.00, Z = −1.88, p = 0.061

Abbreviations: *DV* domestic violence, *Mdn* median, *n* number, *Q1* first quartile; *Q3* third quartile

dependent variable "negative contact quality." Contact quality was more negative for victims of domestic violence (Mdn = −2.00, $Q1$ = −2.50, $Q3$ = −1.50) than for controls (Mdn = −1.75, $Q1$ = −2.25, $Q3$ = −1.25; U = 9895.00, Z = −3.98, $p < 0.001$).

To test whether 2c) victims of domestic violence had lower emotional regulation competence, a Mann-Whitney U test was conducted with the dependent variable "SEK-27 subscale regulation." Emotional regulation competence was lower in victims of domestic violence (Mdn = 10.00, $Q1$ = 8.00, $Q3$ = 12.00) than in controls (Mdn = 11.00, $Q1$ = 9.00, $Q3$ = 12.00; U = 10,834.00, Z = −2.64, p = 0.008).

To test whether 2d) victims of domestic violence reported more difficulty coping with and tolerating their feelings than control subjects, a Mann-Whitney U test was calculated with the dependent variable "SEK-27 subscale resilience." Victims of domestic violence reported lower resilience scores (Mdn = 11.00, $Q1$ = 8.00, $Q3$ = 12.00) than control subjects (Mdn = 11.00, $Q1$ = 9.00, $Q3$ = 12.00; U = 11,397.50, Z = −2.01, p = 0.044).

To test whether 2e) victims of domestic violence reported more restraints due to containment measures compared to controls, a Mann-Whitney U test was conducted

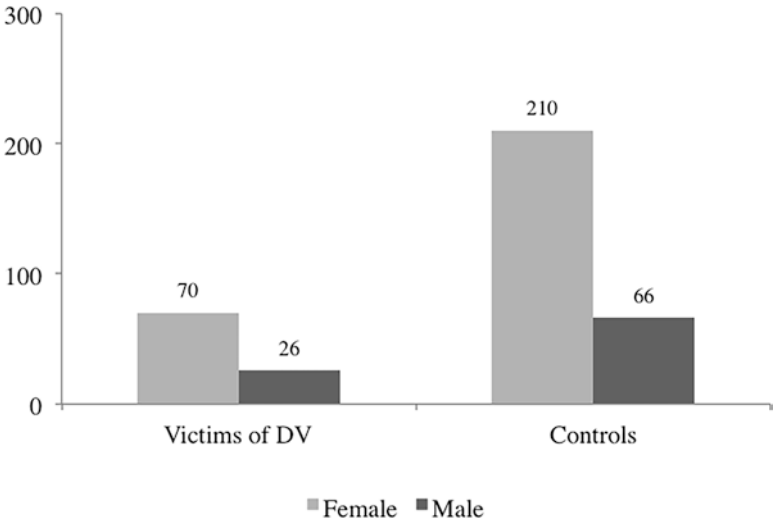

Fig. 3.2 Graphic representation of gender distribution among victims of domestic violence (DV) compared to controls (number of cases)

with dependent variable "number of restrictions." Victims of domestic violence (Mdn = 1.00, $Q1 = 1.00$, $Q3 = 2.00$) did not report significant more restraints than controls (Mdn = 1.00, $Q1 = 1.00$, $Q3 = 2.00$; $U = 12{,}496.50$, $Z = -1.21$, $p = 0.227$).

To test whether 2f) victims of domestic violence showed lower commitment for the containment measures compared to controls, a Mann-Whitney U test was calculated with dependent variable "commitment score." Victims of domestic violence (Mdn = 29.00, $Q1 = 26.00$, $Q3 = 32.00$) and controls (Mdn = 30.00, $Q1 = 27.00$, $Q3 = 33.00$) did not differ significantly in commitment ($U = 11{,}982.00$, $Z = -1.68$, $p = 0.093$).

3.4 Mediator Analyses

A simple mediation was performed to analyze whether negative contact quality predicted the presence of domestic violence and whether the direct path was mediated by the resilience score. An effect of negative contact quality on domestic violence was observed ($B = -0.20$, $p = 0.003$). After including the mediator into the model, negative contact quality significantly predicted the mediator ($B = 0.85$, $p < 0.001$), which in turn predicted the presence of domestic violence ($B = -0.04$, $p = 0.024$) (Fig. 3.3). We found that the association between negative contact quality and the presence of domestic violence was partially mediated by the resilience score.

Mediation was also performed to analyze whether negative contact quality predicted the presence of domestic violence and whether the direct path was mediated by emotional regulation competence. An effect of negative contact quality on

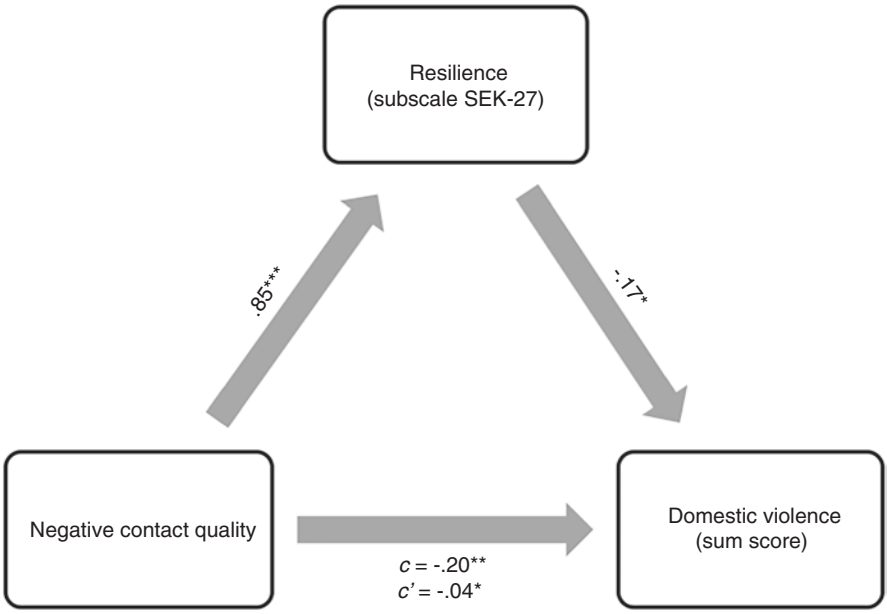

Fig. 3.3 Relationship between negative contact experiences and incidence of domestic violence, mediated in part by resilience score. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

domestic violence was observed ($B = -0.20$, $p = 0.003$). After entering the mediator into the model, negative contact quality significantly predicted the mediator ($B = 0.67$, $p < 0.001$), which in turn predicted the presence of domestic violence ($B = -0.05$, $p = 0.026$; Fig. 3.4). We found that the relationship between negative contact quality and the presence of domestic violence was partially mediated by emotional regulation competence.

4 Discussion

In the present study, we examined the impact of COVID-19-associated containment measures on mental health and domestic violence. In our statistical analyses, we examined differences in gender and between victims of domestic violence and non-victims, and we determined the most significant mediating factors in predicting domestic violence.

In terms of gender effects, we were able to show that, at a descriptive level, the number of female victims of domestic violence was significantly higher than that of male victims. This finding is consistent with previous studies reporting a disproportionate gender distribution in this parameter [9, 32]. The gender distribution of domestic violence victims was not significantly different. In our sample, 25% of the women and 28% of the men reported being victims of domestic violence. For women, this value is comparable to that reported by the Center of Disease Control

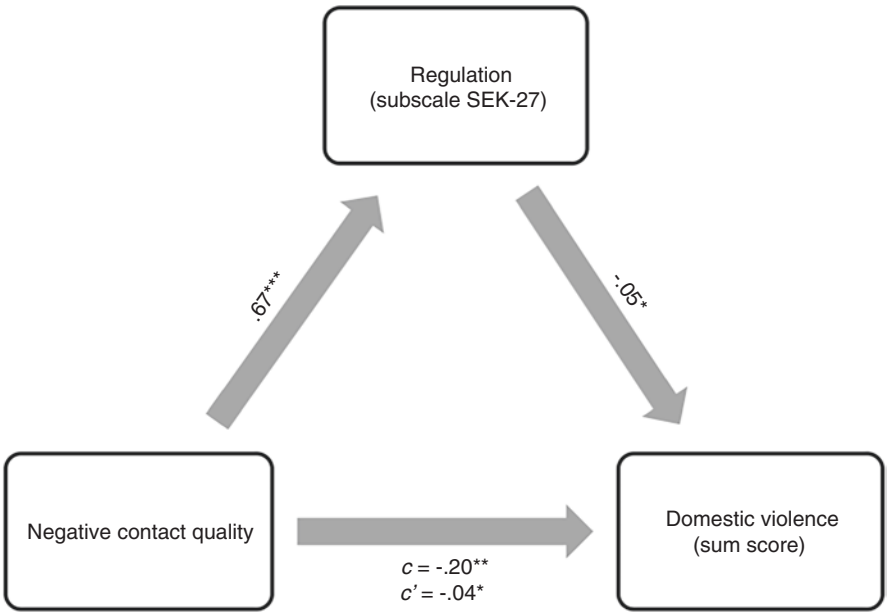

Fig. 3.4 Relationship between negative contact experiences and the incidence of domestic violence, partially mediated by emotional regulation competence. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

[33]. The value for men was higher than described in earlier studies [33]. No gender differences were found with respect to the incidence of domestic violence. This could be due to the relatively short reference period of the last 2 weeks.

In a second analysis step, the differences between the victims of domestic violence and the control group who did not experience domestic violence were examined. In terms of parenting, families where domestic violence occurred had significantly more children of kindergarten age than families in the control group. This could be explained by the closure of kindergartens as part of the containment efforts, which may have led to more stress at home and increased tension resulting from taking care of children and working from home at the same time [40]. This possibility is consistent with previous studies which reported that increased exposure to external stressors such as changes in parenting responsibilities can increase the risk for domestic violence [48]. Victims of domestic violence reported significantly more negative contact quality (more disturbing, frightening, stressful, and/or upsetting contact experiences) in the past 2 weeks, compared to control subjects. This result could be explained by the fact that quarantine can lead to negative psychological consequences and, in particular, to increased expression of anger [10, 41]. In addition, due to movement restrictions and social contact reduction measures, opportunities to benefit from protective, positive contact experiences were severely limited during lockdown [43, 46]. Rather than being supported by public services and institutions, victims of domestic violence were in constant contact with the perpetrator, which may have influenced their quality of contact [43, 47]. With

respect to the measures used to assess emotional regulation ability, victims of domestic violence reported more problems in the two subscales surveyed than did controls. Victims of domestic violence reported a lower ability to regulate emotions. They also reported more difficulties in coping with and tolerating their feelings.

No differences between victims and controls emerged in terms of constraints imposed by the containment measures or commitment to the measures. On the one hand, it could be that victims of domestic violence were similarly affected by the containment measures as the control subjects and therefore showed a comparable commitment to these. However, it is possible that both groups felt constrained by the interventions and were affected by the consequences, but other factors, such as negative contact characteristics and difficulties in emotion regulation, were more important determinants of one becoming a victim of domestic violence. It is also possible that the presence of domestic violence influences contact quality and this association is mediated by emotional regulation or resilience competencies. Following this interpretation, it is possible that in the presence of domestic violence, trust in social contacts diminishes, making the affected person more insecure, and further worsening the quality of contact.

Victims of domestic violence have been described as being at an increased risk for various mental health conditions [24, 25, 36]. It is possible that these difficulties in emotion regulation are associated in part with mental illness [65]. Nevertheless, the mechanisms by which domestic violence leads to mental illness are poorly understood. One underlying physiological mechanism that may contribute to stress-related disorders is the possibility of dysfunctions in the HPA axis, which produces the hormone cortisol [37, 66, 67]. The levels of cortisol rise as a natural response to acute stress, helping the organism to cope with homeostatic challenges by adjusting metabolic and cognitive functions and stimulating the "fight or flight" response [68, 69]. Most studies on this have demonstrated that there is a statistically significant relationship between cortisol levels and the experience of violence [69]. As a means of predicting or monitoring the stress response, measurements of salivary cortisol have been successfully used in epidemiological studies as a biological marker of HPA axis activity [70, 71], including females who have experienced domestic violence [72]. In addition, inflammation-related molecules such as C-reactive protein (CRP) have been used as an acute immune activation biomarker, providing a potential link between the experience of domestic violence and poor mental and/or physical health outcomes [73].

Some limitations must be considered when interpreting the present results. First, as we conducted a cross-sectional survey, no long-term data or pre-post comparisons were available. Therefore, it is not possible to draw a conclusion about any increase in the number of domestic violence cases due to the COVID-19 pandemic and related containment efforts. However, there are several studies that did report a substantial increase due to the pandemic, including a tripling effect described in Jianli (Hubei Province, China) [54–56] and a 30% increase recorded in France [57]. Second, considering the cross-sectional design, it was not possible to make conclusions on the direction of the relationship between the three factors: negative contact quality, emotional regulation, and domestic violence. A third limitation relates to

the fact that the start of the online survey occurred at a time when a gradual relaxation of restrictions had already begun in Germany. It would have been important to have also examined the impact of the measurements on mental health and domestic violence in March and during the first half of April, 2020. Fourth, there was a relative imbalance between the larger number of individuals who were not victims of domestic violence compared to the smaller number of domestic violence, which have affected the statistical analysis victims. In addition, all data were based on participant self-reports. However, we did use quality indicators, such as attributing minus points for extremely rapid completion and negative responses to the question about whether participants provided sensible and reliable responses. With regard to domestic violence, a caveat was that we did not have the opportunity to use a standardized questionnaire and therefore did not have normative data. In addition, for test economy reasons, we only collected information on victims of domestic violence and not on perpetrators, which would be of interest for further studies.

5 Conclusions and Future Perspectives

In conclusion, there is still much to be explored about the COVID-19 pandemic and the impact it has and will have on mental health, domestic violence, and our society in general. The psychological effects of the lockdowns are far-reaching and can be long-lasting [10]. The effects of the pandemic have also demonstrated that there is an urgent need for more empirical data on domestic violence in the (post)lockdown phases as well as on the long-term effects of domestic violence. It would be of interest to collect biological risk indicators such as salivary cortisol (e.g., diurnal cortisol slope, cortisol awakening response, mean cortisol concentration) and circulating CRP measurements to understand the pathophysiological mechanisms of violence-associated mental disorders and to inform researchers and practitioners about the possibility of using these as risk factors or for diagnosis, prognosis, and treatment [74]. These analytes and other stress-related biomarkers can be measured in parallel using multiplex immunoassay platforms to add further insights into the pathways affected [75–77]. Also, the assays could be translated to user-friendly lab-on-a-chip devices which would allow point-of-care testing [78–80]. In addition, there is a strong need for domestic violence prevention programs. Support networks for victims of domestic violence should be expanded in perspective, and the use of digital technologies, e.g., for remote detection of behavioral changes and tele-counseling [81, 82], should be pushed.

Ethics Approval and Consent Approval was obtained from the ethics committee of Otto von Guericke University Magdeburg. The procedures used in this study adhere to the tenets of the Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study.

Conflict of Interest The authors have no competing interests to declare that are relevant to the content of this article.

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Chapter 4
Psychological Distress Impact of Coronavirus Disease (COVID-19) Outbreak on Three Continents: A Systematic Review and Meta-analysis

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Abstract

Background: The dire state of coronavirus disease (COVID-19) outbreak has had a substantial psychological impact on society.

Methods: A systematic search was performed through Medline, PubMed, Embase, Scopus, and Web of Science, to investigate the impact of the COVID-19 pandemic on the psychological health of individuals in various countries. Subgroup analyses considered gender and classification of countries into three continents of America, Europe, and Asia. Only studies that used the COVID-19 Peritraumatic Distress Index (CPDI) questionnaire as a tool to assess mental distress were included

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in this meta-analysis. Heterogeneity among studies was assessed by I^2 statistic, and the random-effects model was utilized to obtain the pooled prevalence.

Results: This pooled analysis included a large data sample of 21 studies consisting of 94,414 participants. The pooled prevalence of the psychological distress during the time of COVID-19 pandemic by CPDI for the continent of Asia was 43% (34.6% mild-to-moderate and 8.4% severe) which was greater than that for Europe (35%; 30% mild-to-moderate and 5% severe) but lower than that for America (64.3%; 45.8% mild to moderate and 18.5% severe). In addition, the prevalence of psychological distress according to CPDI was higher in females (48%; 40% mild to moderate, 13% severe) compared with males (59%; 36% mild to moderate and 5% severe).

Conclusions: Our findings suggest that psychological distress in the Americas is a larger problem than in Asia and European continents. Females appear to be more vulnerable and may therefore require further attention in terms of preventive and management strategies. Implementation of both digital and molecular biomarkers is encouraged to increase objectivity and accuracy of assessing the dynamic changes in mental health in the current and future pandemics.

Keywords COVID-19 · SARS-COV-2 · Anxiety · Depression · Psychological distress · Posttraumatic stress syndrome

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1 Introduction

Coronavirus 2019 (COVID-19) was declared a pandemic after reaching more than 140 thousand cases by the World Health Organization (WHO) on March 11, 2020 [1]. The virus has since spread worldwide rapidly through several waves and emergence of numerous variants and reached more than 620 million cases with 6.5 million deaths by October 2022 [2]. Naturally, pandemics such as this coronavirus have a long-standing history of affecting physical and mental health in all demographic groups [3, 4].

To control and reduce the prevalence of the virus and save human lives, various strategies have been followed in the world, one of the most important being the different lockdown and quarantine approaches [5]. Over one-third of the global population has experienced periods of these steps, which has even been extended in some countries [6]. Due to these policies, the COVID-19 pandemic has had an unprecedented psychological effect on people from all walks of life [7]. While in quarantine, patients with confirmed or suspected COVID-19 disease can experience high levels of anxiety, depression, stress, fear, boredom, isolation, insecurity, posttraumatic stress (PTS) symptoms, confusion, and stigma, all of which are signs of psychological distress [8, 9].

Due to the rapid and evolving nature of this health emergency during the first year of the pandemic, a number of studies on associated emerging mental health problems have been published [10–16]. It is hoped that such analyses can help to prepare us from new outbreaks of the COVID-19 as well as in the likely event of future pandemics. For these reasons, we have updated these previous reports by conducting a systematic review and meta-analysis of studies published on a global prevalence of the psychological distress impact of COVID-19 pandemic in different countries. In addition, subgroup analyses were conducted to consider the effects of gender and regional distribution across three continents of North America, Europe, and Asia. In addition, we propose a route forward in preparedness for the future pandemics using a combination of psychological and molecular screening tools to aid in patient risk assessment.

2 Materials and Methods

2.1 Database and Search Strategy

We developed a protocol according to the PRISMA guidelines [17]. Published papers indexed in Medline-PubMed, Embase, Web of Science, and Scopus were searched using the following MeSH terms and keywords: “coronavirus diseases 19,” OR “SARS-CoV-2,” OR “COVID-19,” AND “psychological distress,” AND “prevalence”, AND “COVID-19 per traumatic Distress Index,” alone or combination. For preprint articles, we searched medRxiv and the Social Science Research Network (SSRN) COVID-19 Research Topic. References from selected articles were inspected to detect additional potential studies.

2.2 Eligibility Criteria

We selected studies that (a) reported psychological distress due to COVID-19, (b) used the COVID-19 Peritraumatic Distress Index (CPDI) questionnaire for the evaluation of psychological distress, (c) were published in English language, (d) were published between January 1, 2020 to January 1, 2021, and (e) were available as full texts. We excluded (a) interventional studies, (b) studies with incomplete or unclear methods/data, and (c) non-original or duplicate studies.

2.3 Introducing the CPDI

CPDI is an instrument for the evaluation of specific phobias and stress disorders due to COVID-19. This questionnaire was originally developed in Chinese [10] and then validated and used in many countries around the world. The 24-item CPDI questionnaire is designed in the form of 5-point Likert-type (0 "never," 1 "occasionally," 2 "sometimes," 3 "often," 4 "most of the time"). Items in the questionnaire inquire about the frequency of anxiety, depression, specific phobias, cognitive change, avoidance and compulsive behavior, physical symptoms, and loss of social functioning with a range from 0 to 100 and a higher final score indicating higher distress. A score $\leq$27 indicated normal distress, between 28 and 51 indicated mild-to-moderate distress, and a score $\geq$52 indicated severe distress [10].

2.4 Data Extraction

After obtaining full texts of relevant articles, two authors (SA and FRB) independently abstracted all studies using a pre-designed form. Inconsistencies between the two reviewers were adjudicated by a third independent reviewer (AVA). The data elements included the name of the first author (or authors if only two are listed), year of publication, place of study (country), population, sample size, study design, gender, age, number of individuals with normal, mild-to-moderate and severe psychological distress based on CPDI scores, and division of studies by country into three continents: The Americas (North and South America counted as one continent), Europe, and Asia which include Middle East countries.

2.5 Quality Assessment

Quality assessment of studies was conducted using the National Institutes of Health (NIH) tool [18]. This consists of 14-item questions and was used for observational cohort and cross-sectional studies. Items in the questionnaire inquired about the

research question (Q1), study population (Q2 and Q3), eligibility criteria (Q4), sample size justification (Q5), outcome measurement (Q6), timeframe sufficient (Q7), exposure of interest (Q8), exposure measures and assessment (Q9), repeated exposure assessment (Q10), outcome measures (Q11), blinding of outcome assessors (Q12), follow-up rate (Q13), and statistical analyses (Q14). The details of these questions are available at supplementary file as footnote of Supplemental Table S1. After evaluating all components of any given study and based on the number of "yes" responses, a rating of good (7–9), medium (4–6), or poor (≤3) was determined for each study [19]. Studies with a poor rating were excluded from the meta-analysis. Two reviewers (SA and FRB) assessed the quality of studies and disagreements between them was resolved with the final judgement offered by the senior investigator (AVA). The inter-rater agreement in ratings was also calculated, and the final rate of quality of included studies based on the number of "yes" according to inter-rater agreement is presented in Supplemental Table S1.

2.6 Statistical Analysis

We obtained the globally pooled prevalence for normal, mild-to-moderate, and severe psychological distress based on CPDI scores with confidence intervals (CI) for each study. Prevalence was calculated assuming binomial distribution. In addition, we calculated prevalence of normal, mild-to-moderate, and severe psychological distress for subgroups including gender (females versus males) and continent (Americas, Europe, and Asia). For analyses of pooled prevalence and CI, a random-effects model was used. Heterogeneity among studies was assessed using the I2 index, for which values >70% represented a high heterogeneity. When the data were homogeneous, a fixed-effects model was used, while a random-effects model was employed when the heterogeneity source was unknown. Publication bias was determined through visual inspection of a funnel plot. Additionally, to assess the bias, Egger's [20] and Begg's [21] tests were conducted. All analyses were performed using the STATA software (v16.0; College Station, TX, USA), and significant levels were set at $p < 0.05$.

3 Results

3.1 Search Outcomes

The search strategy yielded 2707 articles. After removal of duplicates ($n = 668$), a careful assessment of the title and abstracts resulted in the elimination of 1932 articles as they did not meet the inclusion criteria. Following examination of the reference lists of related articles, 8 studies were added, and 95 full text articles were assessed for eligibility. Of these, 74 full text articles were excluded because (1)

prevalence was reported as a mean instead of a proportion ($n = 29$), (2) prevalence was not reported ($n = 18$), (3) the methodology was unclear or of low quality ($n = 19$), and (4) it was a review article ($n = 8$). This left a final 21 articles that met our criteria and were included in the meta-analysis. These 21 studies comprised a total number of 94,414 participants, which included 5 studies with 5532 participants in the Americas [22–26], 6 with 27,269 participants in Europe [13, 26–32], and 10 with 61,613 participants in Asia [10–12, 33–39]. The PRISMA flowchart of study selections for the systematic review along with the reasons for exclusion is presented in Fig. 4.1.

3.2 Study Characteristics

The characteristics of studies included in the meta-analysis are presented in Table 4.1. The majority of studies were cross-sectional in nature and conducted during the period of the COVID-19 pandemic between January 2020 and January 2021. Out of the 21 studies, 10 were from Asia (including the Middle East) (1 from China, 1 from Iran, 1 from Saudi Arabia, 2 from India, 2 from Nepal, 1 from Egypt, 1 from Philippines, and 1 from Bangladesh), 6 from Europe (5 from Italy and 1 from Germany), and 5 from the Americas (1 from USA, 1 from Peru, and 3 from Brazil). All studies used the CPDI tool for assessment of psychological distress. Out of 21 studies, 15 were performed across the general population and 3 were on child welfare workers [22], students [24], and endodontists [36]. The study from Egypt estimated the prevalence of psychological distress based on CPDI among the general population and healthcare workers separately [39]. Two of the papers were designed as case-control studies on adults with autoimmune arthritis [27] or with cystic fibrosis [31], both compared to the general population. The mean age of study participants ranged from 32.0 to 57.7 years. The sample sizes in the studies varied from 45 to 52,730. Seventeen studies were of good quality and 4 were of medium quality based on use of the NIH tool.

3.3 Pooled Prevalence of Psychological Distress

Psychological distress was estimated using CPDI scores into normal, mild-to-moderate, and severe across categories as detailed in the methods section. We estimated the pooled prevalence of each category separately over the 21 studies with a sample size 94,414. The pooled prevalence percentages of the determined normal (Fig. 4.2), mild-to-moderate (Fig. 4.3a), and severe (Fig. 4.3b) psychological distress groups were 55% (95% CI: 47–63%, $I^2 = 98.97\%$, $p < 0.001$), 36% (30–41%, $I^2 = 97.42\%$, $p < 0.001$), and 10% (6–13%, $I^2 = 93.39\%$, $p < 0.001$), respectively.

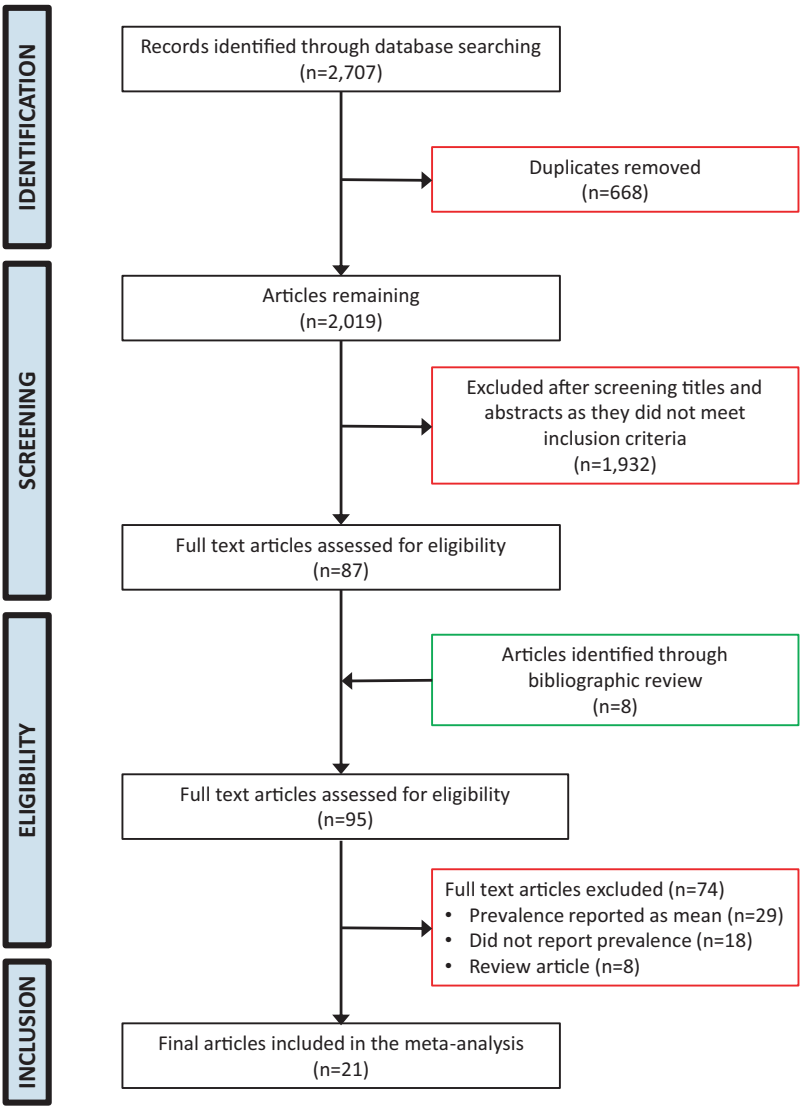

Fig. 4.1 PRISMA flowchart showing the selection of studies

3.4 Pooled Prevalence of Psychological Distress According to Continents

The normal, mild-to-moderate, and severe pooled prevalence percentages of psychological distress in the 5 studies from the Americas (sample size = 5532) were estimated at 35.7% (19.7–51.8%, I^2 = 96.99%, $p < 0.001$), 45.8% (39.8–51.8%, I^2 = 77.78%, $p < 0.001$), and 18.5% (8–28%, I^2 = 92.54%, $p < 0.001$), respectively.

Table 4.1 Characteristics of the included studies (a and b indicate different aspects of the same study)

First Author (Ref)	Country	Continent	Population	Size	Age (mean)	Female (%)	Prevalence of psychology distress based on CPDI tool (%)			Quality
							Normal	Mild-to-moderate	Severe	
Miller et al. [22]	USA	Americas	Child welfare workers	1996	41.4	90.4	53.6	40.5	5.9	Good
Abad et al. [23]	Brazil	Americas	General population	1844	36.2	79.8	28.7	47.5	23.8	Good
Hübner et al. [24]	Brazil	Americas	Student	654	NA	76.6	12.08	52.9	35.02	Good
Zhang et al. [25]	Brazil	Americas	General population	638	NA	57.7	29.15	52	18.8	Good
Krüger-Malpartida et al. [26]	Peru	Americas	General population	400	41.0	65.5	55.3	35.5	9.3	Good
Landi et al. [30]	Italy	Europe	General population	944	38.8	73.5	84.5	10.3	5.2	Good
Ciprandi et al. (a) [31]	Italy	Europe	Adults with cystic fibrosis	712	NA	59.3	54.5	40.2	5.3	Good
Ciprandi et al. (b) [31]	Italy	Europe	General population	3560	NA	59.3	49.9	43.9	6.2	Good
Liu and Heinzl [13]	Germany	Europe	General population	1007	42.0	74.4	75.9	20.6	3.6	Good
Diamanti et al. (a) [27]	Italy	Europe	General population	100	50.5	59.0	77	22	1	Good
Diamanti et al. (b) [27]	Italy	Europe	Adults with autoimmune arthritis	100	57.7	72.0	59	38	3	Good
Bonati et al. [28]	Italy	Europe	General population	20,518	NA	69.9	51.4	43.4	5.3	Good
Constantini and Mazzotti [32]	Italy	Europe	General population	328	46.5	58.2	74.7	20.4	4.88	Medium
Qiu et al. [10]	China	Asia	General population	52,730	NA	64.7	65.6	29.29	5.14	Good
Jahanshahi et al. [35]	Iran	Asia	General population	1058	NA	53.8	38.9	47	14.1	Good
Al-Hanawi et al. [11]	Saudi Arabia	Asia	General population & healthcare workers	3036	NA	49.9	59.9	39.2	7.2	Good
Ramasubramanian et al. [12]	India	Asia	General population	2317	NA	64.2	78.98	20.63	2.72	Good

Samson and Narayan Shah [37]	Nepal	Asia	General population	45	NA	37.7	82.22	17.82	0	Medium
Shrestha et al. [34]	Nepal	Asia	General population	410	34.8	35.1	88.5	11	0.5	Good
Marzo et al. [38]	Philippines	Asia	General population	407	32.0	56.8	52.1	39.5	8.4	Good
El-Abasiri et al. (a) [39]	Egypt	Asia	Healthcare workers	266	NA	40.2	33.1	51.9	15	Good
El-Abasiri et al. (b) [39]	Egypt	Asia	General population	257	NA	69.6	38.5	39.7	21.8	Good
Nair et al. [36]	India	Asia	Endodontists	586	NA	46.9	48.12	38.23	13.65	Good
Marzo et al. [33]	Bangladesh	Asia	General population	501	NA	65.6	46.11	44.3	9.5	Medium

NA Not available; All studies were cross-sectional

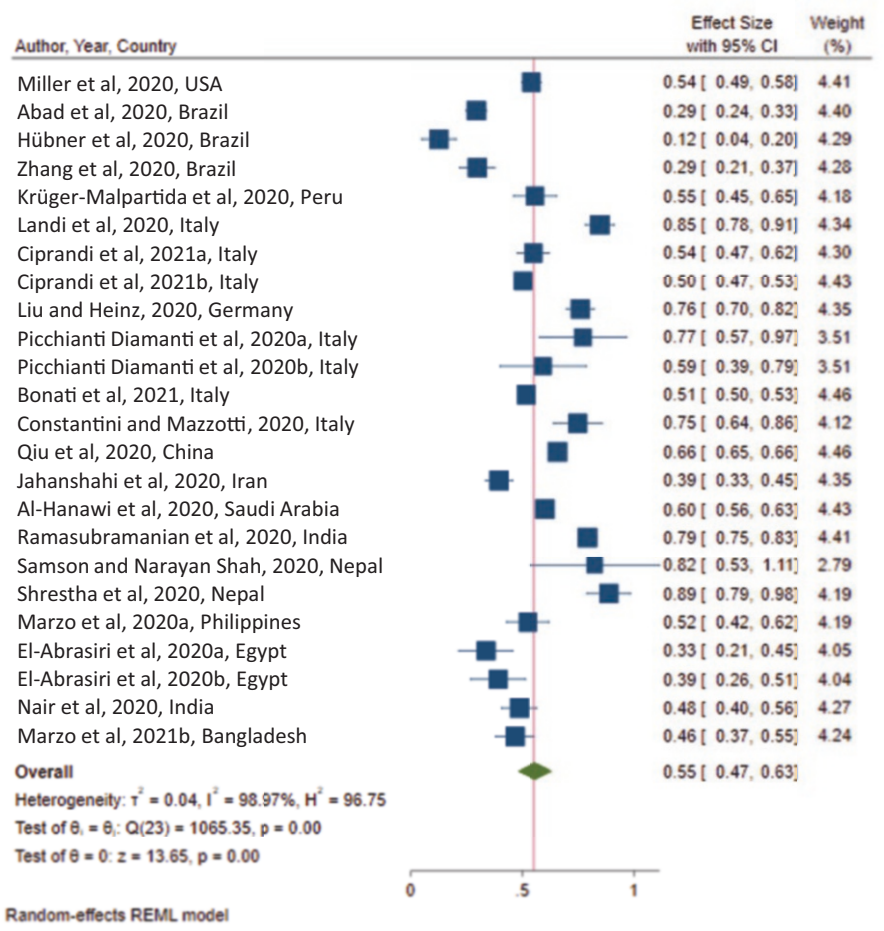

Fig. 4.2 Forest plot of CPDI-based pooled prevalence for normal psychological distress (note that years followed by a and b indicate different aspects of the same study). USA United States of America

For the European continent, the same pooled prevalence percentages of psychological distress across 8 studies (sample size = 27,269) were 65% (55.4–75.4%, I^2 = 96.67%, $p < 0.001$), 30% (20.5–39.7%, I^2 = 96.38%, $p < 0.001$), and 5% (4.1–6.5%, I^2 = 10%, $p < 0.001$), respectively. For the 11 studies from the Asian continent (sample size = 61,613), the normal, mild-to-moderate, and severe pooled prevalence percentages of psychological distress were 57% (46–67.8%, I^2 = 98.07%, $p < 0.001$), 34.6% (27.1–41.7%, I^2 = 95.42%, $p < 0.001$), and 8.4% (5.1–11.8%, I^2 = 72.61%, $p < 0.001$), respectively. Heterogeneity tests (I^2) indicated low heterogeneity in the prevalence of severe psychological distress in European countries. However, significant heterogeneity existed across the prevalence of all levels of psychological distress for Asia and the Americas as described above.

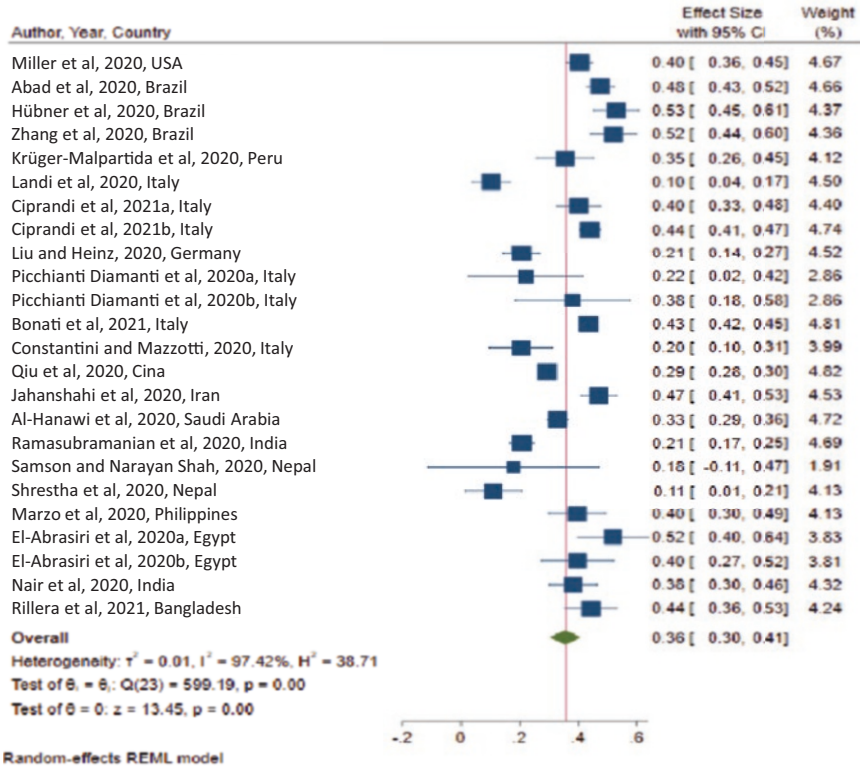

Fig. 4.3 Forest plot of CPDI-based pooled prevalence for (**a**) mild-to-moderate psychological distress and (**b**) severe psychological distress

3.5 Pooled Prevalence of Psychological Distress According to Gender

Prevalence data of CPDI-based psychological distress according to gender was available in seven studies (Table 4.2). The pooled prevalence of normal, mild-to-moderate, and severe psychological distress for females were 48% (34–63%, $I^2 = 97.52\%$, $p < 0.001$), 40% (31–48%, $I^2 = 91.17\%$, $p < 0.001$), and 13% (6–20%, $I^2 = 88.29\%$, $p < 0.001$), respectively. The pooled prevalence of normal, mild-to-moderate, and severe psychological distress for males were 59% (47–71%, $I^2 = 94.17\%$, $p < 0.001$), 34% (25–43%, $I^2 = 90.22\%$, $p < 0.001$), and 5% (2–9%, $I^2 = 26.36\%$, $p = 0.38$), respectively. A forest plot of the CPDI-based pooled prevalence for normal psychological distress in males and females is presented in Fig. 4.4 and for mild-to-moderate and severe psychological distress in Fig. 4.5a, b. I^2 tests indicated low heterogeneity in the prevalence of severe psychological distress among males and in the separate male and female analyses. However, the I^2 test indicated significant heterogeneity among the prevalence of psychological distress

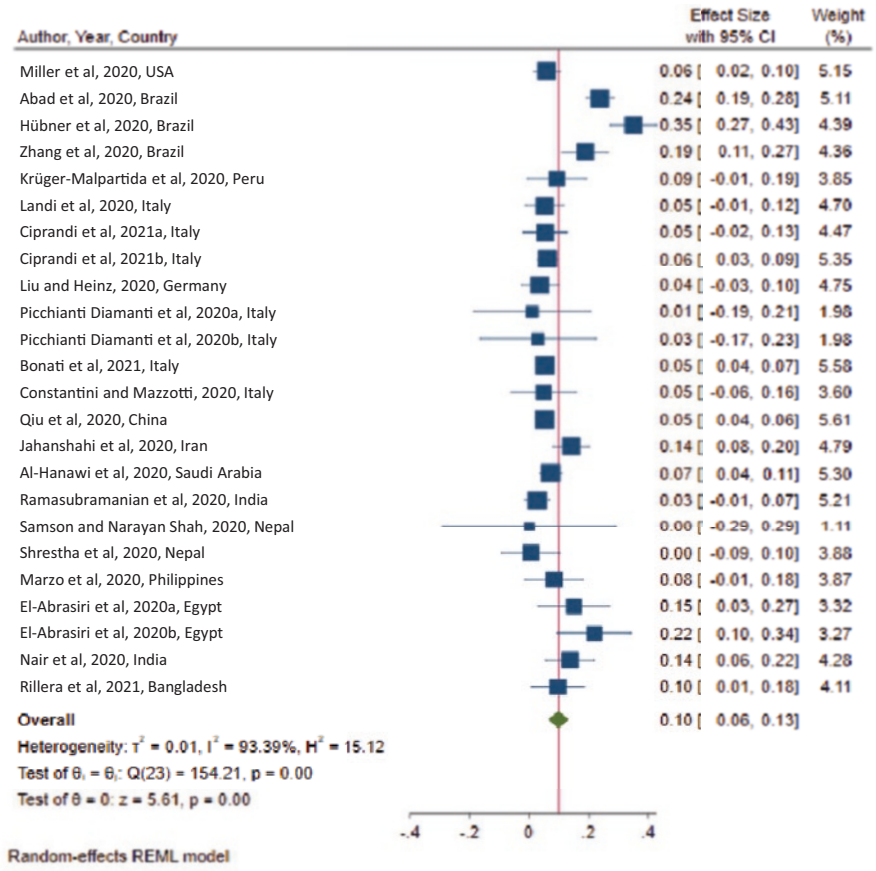

Fig. 4.3 (continued)

for females. Overall, the pooled prevalence of psychological distress was significantly higher in females than males (53% versus 39% $p < 0.001$).

3.6 Publication Bias

Egger's and Begg's tests were used to assess publication bias. As indicated by the *p*-values for the pooled prevalence of normal CPDI-based psychological distress (Egger: $p = 0.369$, Begg: $p = 0.551$) (Fig. 4.6a), mild-to-moderate psychological distress (Egger: $p = 0.439$, Begg: $p = 0.785$) (Fig. 4.6b), and severe psychological distress (Egger: $p = 0.995$, Begg: $p = 0.655$) (Fig. 4.6c), the funnel plots showed asymmetry and visual inspection confirmed the presence of publication bias.

Table 4.2 Prevalence of CPDI-based psychological distress by gender

Author	Country	Continent	Sample size	Prevalence of psychology distress based on CPDI for female (%)			Prevalence of psychology distress based on CPDI for male (%)		
				Normal	Mild-to-moderate	Severe	Normal	Mild-to-moderate	Severe
Abad et al. [23]	Brazil	America	1844	25.5	47.3	27.2	41.6	48.4	10
Bonati et al. [28]	Italy	Europe	20,518	45.02	48.51	6.46	65.95	31.54	2.5
Nair et al. [36]	India	Asia	586	41.81	41.81	16.36	53.69	35.05	11.25
Marzo et al. [38]	Philippines	Asia	407	57.2	33.3	9.5	45.5	47.7	6.8
El-Abasiri et al. [39]	Egypt	Asia	523	29.4	48.9	21.7	50.3	39	10.7
Shrestha et al. [34]	Nepal	Asia	410	86.8	12.5	0.7	89.4	10.2	0.4
Al-Hanawi et al. [11]	Saudi Arabia	Asia	3036	56.2	36.2	8.3	63.6	29.6	6.7

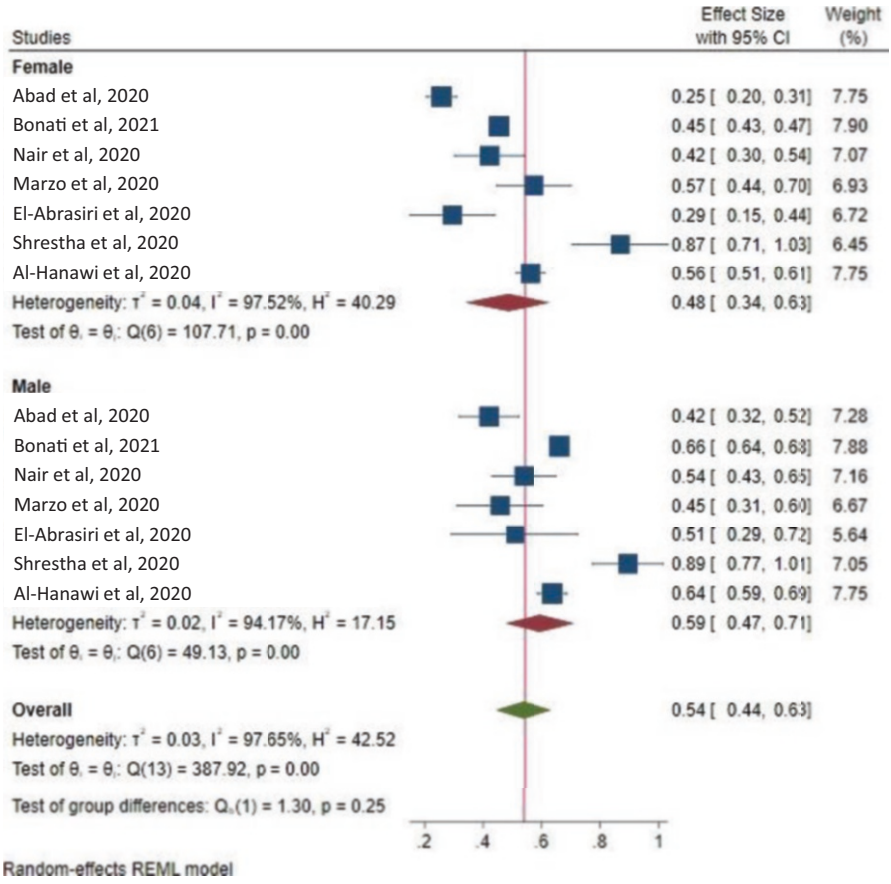

Fig. 4.4 Forest plot of CPDI-based pooled prevalence among females and males for normal psychological distress

4 Discussion

To our knowledge, this is the first study to report the prevalence of CPDI-based psychological distress impact of the COVID-19 pandemic in various countries, across the continents of the Americas, Europe, and Asia. We incorporated Middle East countries into the Asian continent as this region is officially classified as part of southwestern Asia [40]. We also included Egypt into the Asian continent as it officially recognized as part of the Middle East [41]. The analysis showed that the prevalence of the psychological distress in the mild-to-moderate and severe levels during the COVID-19 pandemic from January 2020 to January 2021 was highest for the Americas, followed by Asia and then Europe. In addition, the psychological distress in the mild-to-moderate and severe categories over this period was higher

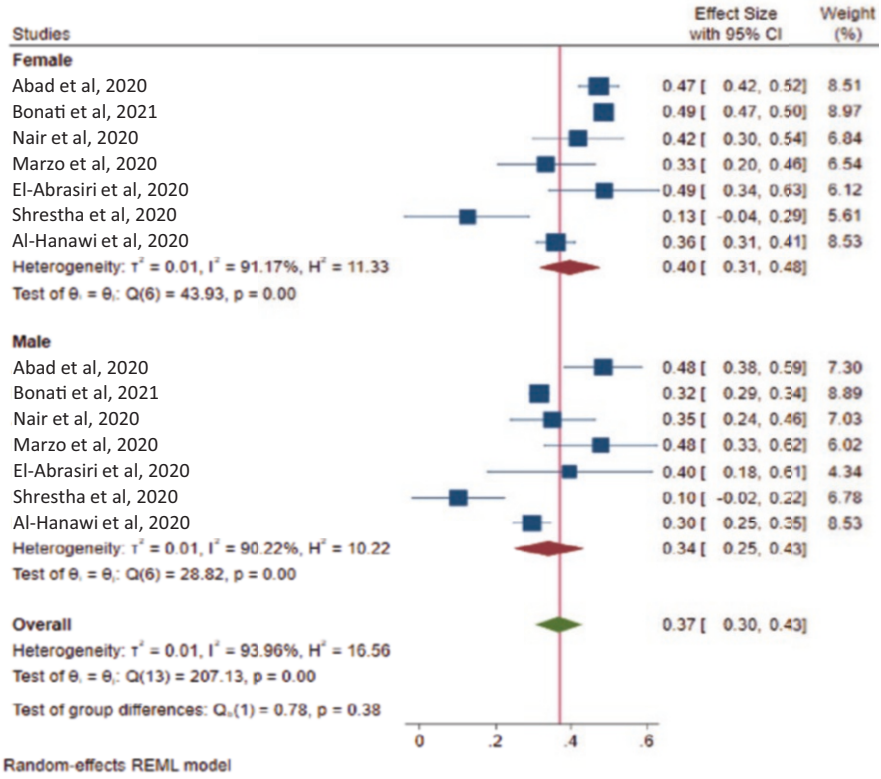

Fig. 4.5 Forest plot of CPDI-based pooled prevalence in females and males for (**a**) mild-to-moderate psychological distress and (**b**) severe psychological distress

for females compared to males. However, it should be noted that this finding was not analyzed across the separate continents.

The finding of greater psychological stress in females is consistent with the fact that women are generally more susceptible to depression and more likely to experience posttraumatic stress symptoms over time compared to males [42, 43]. In line with previous studies from Brazil [23] and Italy [28], women appear to show more psychological symptoms during quarantine in pandemics as compared to men. In this difficult situation, women can be faced with additional responsibilities, such as an increased role in family care, child support and teaching responsibilities due to school closures, as well existing gender inequalities and the potential for abuse from their partners [44]. In addition, the loss of daily routines, as well as social and physical contact with friends and family, can lead to isolation, boredom, or frustration [11, 34, 36, 38]. Moreover, previous studies have shown that fear of COVID-19 infection from family members and obsessive-compulsive disorders is higher in women [45, 46]. Taken together, these findings indicate that more careful attention

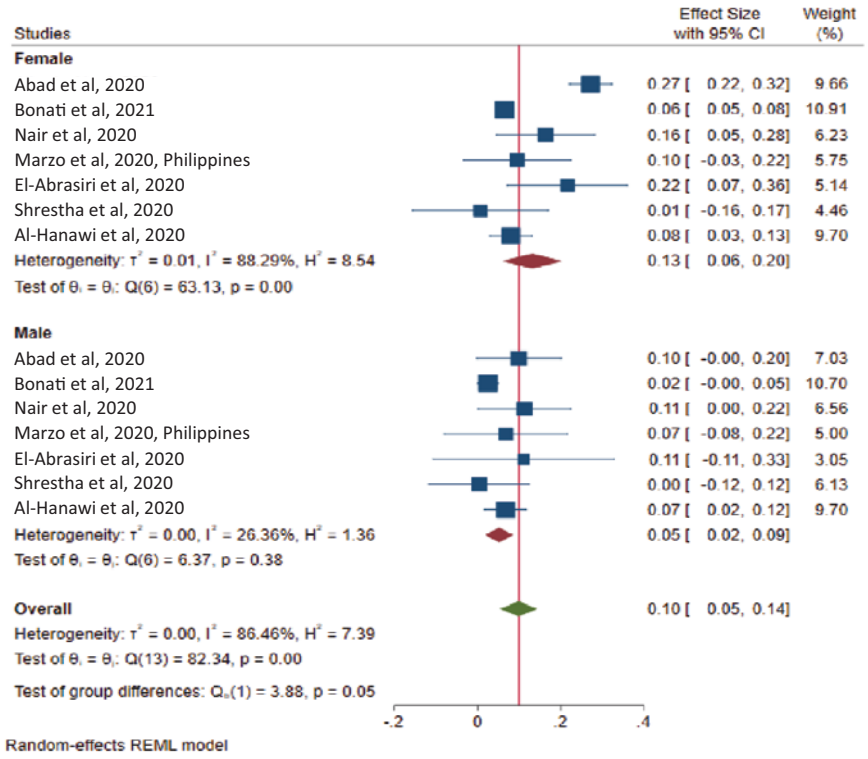

Fig. 4.5 (continued)

to risk identification and early intervention policies should be adopted for females during pandemics and other crises.

We found that the highest prevalence of psychological distress during the time of the COVID-19 lockdowns was highest in Brazil at 87.9% and lowest in Nepal (11.5%) for the combined mild-to-moderate and severe categories [24, 34]. The main difference in the prevalence of psychological distress between Brazil and Nepal is likely to be related to the more than 8.5 million persons in Brazil who had been infected by COVID-19 as of January 22, 2021 [2]. However, only 267 thousand cases of COVID-19 had been confirmed in Nepal over this same time period [2]. It is perhaps not surprising that the evidence shows a higher risk of mental distress in communities and countries with a higher prevalence of the disease [47], and this can also be related to the ensuing prolonged periods of quarantine and lockdown [3].

The finding that the pandemic-related psychological distress in the Americas was a larger problem than in Asia and Europe has not been reported previously. This is most likely driven by the high number of cases in both Brazil in South America and the USA in North America. In fact, the USA recorded the highest number of infections by January 22, 2021, at over 24 million cases [2]. Although COVID-19

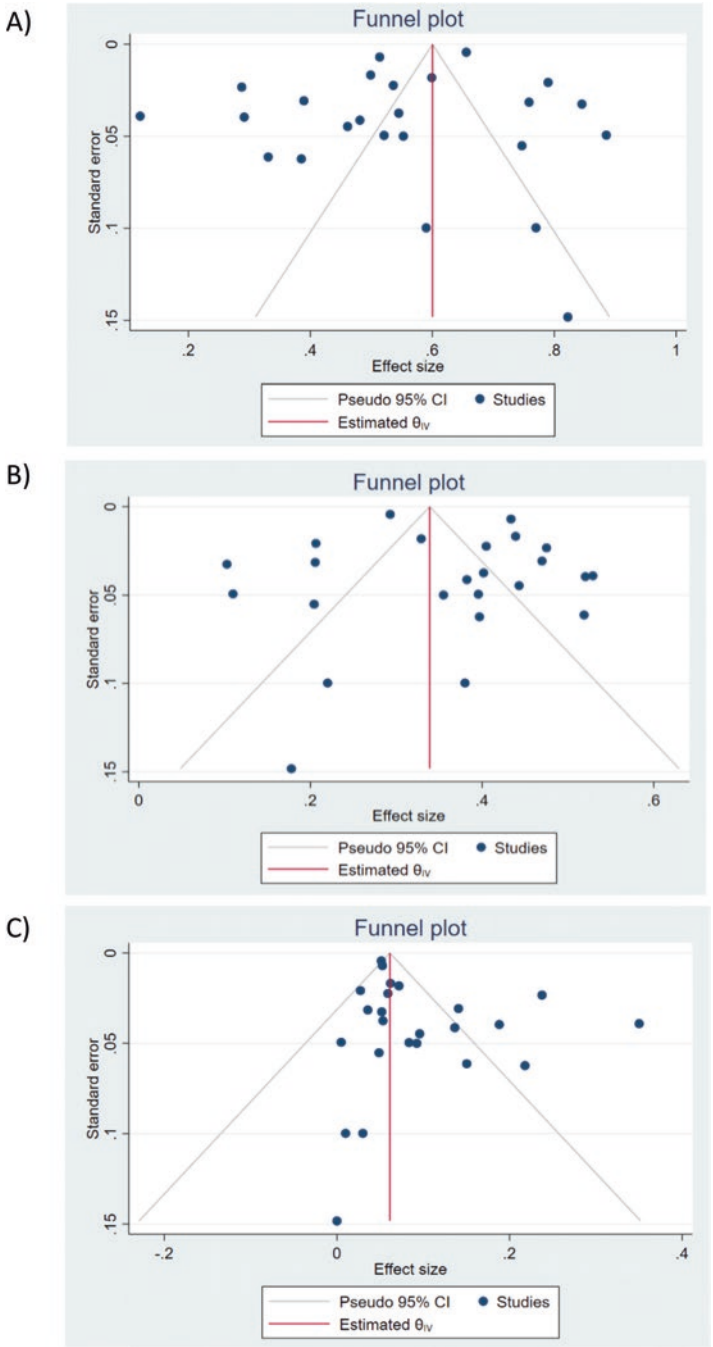

Fig. 4.6 Funnel plot showing publication bias on CPDI-based prevalence of psychological distress ranked as (**a**) normal, (**b**) mild-to-moderate, and (**c**) severe

infections were higher in Europe compared with Asia, a comparison of the two continents showed that the prevalence of psychological distress was significantly higher in Middle-East and Asian countries. Overall, this difference may be due to the higher testing rate in Europe and the potentially associated higher sense of security in the infected population. Moreover, European countries such as Germany and Italy had a lower COVID-19 case-fatality rate compared to China or Iran [2]. The results of previous studies on automobile accidents have suggest that self-reported fear is positively associated with mortality rate and differs across countries [48]. Other potential factors that may account for the observed variation in prevalence of COVID-19-related psychological distress across countries and continents include differences in restrictive measures, economic recessions, healthcare systems, biological, immunological, socio-demographics, and cultural differences [49–51].

There are some limitations to this study that should be considered in the interpretation of the data. First, all of the research in this meta-analysis were cross-sectional as they only gave a snapshot of the current situation with no longitudinal exploration. Second, there was lack of representation of studies in European countries other than Italy and Germany. Therefore, countries such as the UK where COVID-19 cases had reached over 3.5 million by the end of January, 2021 [2], were not represented. Third, it was not possible to assess gender differences between continents due to lack of data. For the same reasons, it was not possible to assess the prevalence of psychological distress for healthcare professionals compared to the general population. This is particularly important as many of these were on the front line exposed to high levels of physical and mental stress and had to cope with high levels of uncertainty, fear of contamination, and perceived lack of support [52–55]. Finally, the data provided by the studies included in this meta-analysis depended on the self-reported symptoms and signs via online survey. Thus, there is uncertainty related to actual mental status.

In future studies, the subjective nature of the online survey approach to assess psychological distress found be supported by more objective biomarker-based approaches. For example, the P1vital® PReDicT Test developed in Oxford, UK, provides an objective means of assessing a patient's mental state through a 15 min online test comprised of facial expression recognition tasks and a series of health-related questions [56]. The facial recognition aspect of the test works as people with a mental illness such as depression often show a negative bias by interpreting indistinct expressions as less happy compared to non-depressed controls [57]. In addition, there is now considerable evidence for the utility of easily accessible molecular biomarkers in assessing the mental state. For example, evening levels of salivary cortisol have been linked with anxiety, depression, and posttraumatic stress disorder [58]. The cortisol awakening response (CAR) which measures the increase in cortisol secretion 30 to 45 min after awakening has been used as a marker of hypothalamic-pituitary-adrenal (HPA) axis activation, which can occur in both physical and mental health conditions including psychological stress [59–61]. In these studies, cortisol can be measured by enzyme-linked immunoadsorbent assay (ELISA) [62]. In addition, salivary amylase enzymatic activity has been used to monitor the effects on workers in stressful or isolated environments [63]. There has been considerable

interest in the application of blood-based biomarkers such as cytokines, hormones, and growth factors in the study of mental disorders, which can be measured simultaneously using multiplex immunoassay platforms [64–66]. As examples, increased levels of C-reactive protein (CRP) and pro-inflammatory cytokines have been associated with depression following stroke [67], interleukins (IL)-1β, IL-5, and IL-6 have been detected in people with panic disorder [68], and the levels of brain-derived neurotrophic factor (BDNF) have been correlated with the disease progression of schizophrenia and depressive disorders [69]. Perhaps most critically, two studies also demonstrated the concept that mental illnesses could be detected several months or years before full manifestation with development of blood-based molecular biomarker algorithms for detection of individuals with a high risk of developing psychosis [70, 71]. In addition to assessing risk and current condition, all of the above digital and multiplex immunoassay approaches could be used to monitor any upsurge or recovery in mental status.

5 Conclusions and Future Perspectives

This meta-analysis suggested a high psychological impact due to the COVID-19 pandemic in many countries, with the highest levels detected in the Americas followed by Asian and European countries. In general, the distribution of the diverse spatiotemporal parameters of the pandemic may explain the heterogeneity in the degree of psychological distress among different geographical regions and countries. In addition, females appear to be more vulnerable to such distress and require further attention in terms of preventive and management strategies. However, the present study was limited by lack of a longitudinal analysis, poor representation of data from some countries in each continental group, and lack of data for assessment of gender differences on a per continent basis, and the reports of psychological stress levels were obtained by online survey and were therefore subjective in nature. Given these challenges, it will be important to incorporate the use of both digital and molecular biomarkers to increase the objectivity and accuracy of assessing the dynamic changes in mental health in the event of further disruptive waves of COVID-19 disease. There is also an urgent need for introduction of effective mental health interventions to assess and treat individuals in the highest risk groups for the best possible therapeutic outcomes.

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Chapter 5
A Molecular Biomarker-Based Triage Approach for Targeted Treatment of Post-COVID-19 Syndrome Patients with Persistent Neurological or Neuropsychiatric Symptoms

Paul C. Guest, Alexandra Neyazi, Rüdiger C. Braun-Dullaeus, Patrick Müller, Jens Schreiber, Aiden Haghikia, Veronika Vasilevska, and Johann Steiner

Abstract Approximately 30% of COVID-19 cases may experience chronic symptoms, known as post-COVID-19 syndrome (PCS). Common PCS symptoms can include fatigue, cognitive impairment, and persistent physical, neurological, and neuropsychiatric complaints. To improve healthcare and management of the current and future pandemics, we highlight the need for establishing interdisciplinary post-viral outpatient clinics comprised of specialists in fields such as psychiatry, psychotherapy, neurology, cardiology, pneumology, and immunology. In this way, PCS patients with a high health burden can receive modern diagnostics and targeted

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therapeutic recommendations. A key objective is to distinguish the “sick recovered” from the “healthy recovered.” Our hypothesis is that there is a PCS subgroup with autoimmune-mediated systemic and brain-vascular dysregulation, which may lead to circulatory disorders, fatigue, cognitive impairment, depression, and anxiety. This can be clarified using a combination of specific antibody diagnostics and precise clinical, psychological, and apparative testing.

Keywords COVID-19 · SARS-CoV-2 · Post-COVID-19 syndrome · PCS · Autoimmune · Autoantibodies · Neuropsychiatric complaints

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1 Introduction

Although there have been over 600 million confirmed cases of coronavirus 2019 (COVID-19) worldwide [1], estimates indicate that the actual proportion is considerably higher. From March 2020 to the appearance of the omicron variant (B.1.1.529) towards the end of 2021, a statistical analysis of 190 countries and territories indicated that approximately 3.4 billion people (almost 44% of the world population) had been infected at least once by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the pathogen responsible for COVID-19 disease [2]. A later figure was produced using the Institute for Health Metrics and Evaluation Model, which showed that the infection rate had increased to approximately 4.5 billion people (approximately 57% of the world population) by the end of January 2022 [3]. There has also been a high proportion of the population who were re-infected, particularly during the recent omicron waves [4–7]. This is likely to be due to the increased infectivity and enhanced ability of the omicron variant to evade the immune system.

Approximately one-third of COVID-19 cases may experience chronic symptoms, known as post-COVID-19 syndrome (PCS) [8, 9]. According to National Institute for Health and Care Excellence (NICE) definition, this syndrome is characterized as "signs and symptoms that develop during or after an infection consistent with COVID-19, continue for more than 12 weeks and are not explained by an alternative diagnosis" [10]. A similar clinical case definition was also put forward by the World Health Organization (WHO) [11]. However, the clinical characterization is not uniform, and the time criteria may be misleading as PCS may present with a variety of overlapping symptoms, which can fluctuate and have negative impact on many parts of the body. Common symptoms of PCS can include fatigue, cognitive impairment, as well as lasting physical and neurological or neuropsychiatric complaints [12, 13]. A meta-analysis of 68 studies comprising over 25,000 cases found that the percentage of people experiencing fatigue for 12 or more weeks after a COVID-19 diagnosis was 32% [14]. The same investigation also used a narrative synthesis of 43 studies encompassing more than 13,000 individuals, which found that 22% of these individuals exhibited cognitive impairment, as determined by a validated tool for performance-based cognitive function, clinical diagnostics, or self-report.

Although the precise cause of PCS is still not clear, many cases are associated with persistence of a proinflammatory state that may lead to an autoimmune response [15–17]. In the most severe cases of PCS, the latency in the effects on various organ systems resembles the course of post-infectious autoimmune diseases. As with other viral diseases, various auto-antibody-mediated syndromes such as N-methyl-D-aspartate receptor/contactin-associated protein-like 2 (NMDAR/Caspr2)-associated brain inflammation, Guillain-Barré syndrome, myasthenia, vasculitis, or postural tachycardia syndrome have been observed after SARS-COV-2 infections [18, 19].

In this paper, we review the mechanisms underlying PCS as it relates to a proinflammatory, autoimmune phenotype, and we describe potential treatment avenues

based on these observations. We believe that surveillance gained from clinical experience during rehabilitation of PCS patients might allow identification of subgroups with similar disease mechanisms, which could inform treatment options. Finally, we highlight the need for dedicated interdisciplinary post-viral outpatient clinics so that PCS patients with a high health burden can receive modern diagnostics and targeted therapeutics.

2 The SARS-CoV-2 Structure and Molecular Mimicry

The SARS-CoV-2 structure is shown in Fig. 5.1. The key features include an encapsulated positive-sense RNA genome consisting of approximately 30 kilobases, an enveloped structure containing a nucleocapsid (N) protein which stabilizes the genomic RNA, envelope (E) and membrane (M) proteins, and exterior projections of multiple spike (S) proteins that drive the attachment and infection process of host cells [20–22]. The first 70% of the genome encodes two macro polypeptides termed 1a and 1b. These undergo auto-proteolysis resulting in the production of the 16 non-structural proteins (NSPs) with various functions involved in the infection and replication processes [23, 24]. The remaining 30% of the genome encodes the major structural proteins S, E, M, and N, as well as the accessory proteins encoding by ORFs 3a, 6, 7a, 7b, 8, 9b, and 10 (Table 5.1) [25, 26].

As with many other environmental factors, viruses such as Epstein-Barr virus (EBV), cytomegalovirus (CMV), human immunodeficiency virus (HIV), and

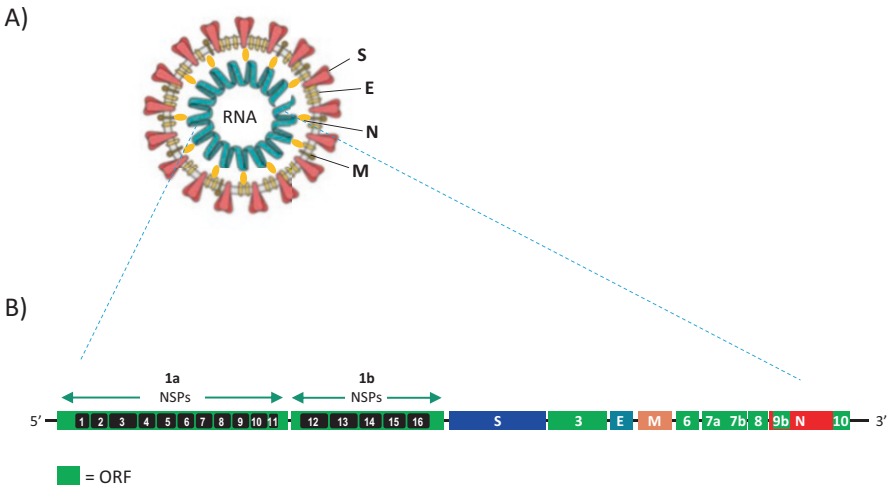

Fig. 5.1 (**a**) Schematic diagram of SARS-CoV-2 structure. (**b**) Structure of SARS-CoV-2 RNA showing open reading frames (ORFs), non-structural proteins (NSPs), spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins

Table 5.1 SARS-CoV-2 proteins and functions

Protein	Function
NSP1	Inhibits gene expression and degrades mRNA in host
NSP2	Disruption of cell cycle to alter host cell environment
NSP3	Papain-like protease in viral replication, forms NSP3,4,6 complex
NSP4	Probable membrane function, forms NSP3,4,6 complex
NSP5	3CL-like protease involved in proteolytic maturation of NSP proteins
NSP6	Forms NSP3,4,6 complex
NSP7	Forms NSP7,8 complex with NSP12 (RNA polymerase)
NSP8	Forms NSP8,12 complex (RNA polymease complex)
NSP9	Binds single stranded RNA in viral replication
NSP10	Interacts with NSP14 and stimulates methyltransferase
NSP11	Unknown
NSP12	RNA polymerase, forms NSP7,8,12 complex
NSP13	RNA helicase
NSP14	Exoribonuclease and N7-methyltransferase
NSP15	Endoribonuclease
NSP16	2′-O-methyltransferase in mRNA translation
S	Spike protein – binds virus to host cell
E	Envelope protein – creates ion channel in host cell
M	Membrane protein – viral assembly
N	Nucleocapsid protein – stabilizes viral RNA
Orf3a	Ion channel involved in NLRP3 inflammasome
Orf3b	
Orf6	Type I interferon antagonist involved in induced apoptosis
Orf7a	Transmembrane protein involved in induced apoptosis
Orf7b	
Orf8	
Orf9b	Type I interferon antagonist
Orf9c	
Orf10	

NSP non-structural protein, *S* spike, *E* envelope, *M* membrane, *N* nucleocapsid, *Orf* open reading frame

SARS-CoV-2 can contribute to production of an autoimmune response in the host [18]. Yapici-Eser et al. described how some of the neuropsychiatric and other symptoms of COVID-19 disease may be explained by SARS-CoV-2 protein mimicry of multiple host protein interactions, including those involved in neuronal functions. These can include targets such as G protein-coupled receptor (GPCR; e.g., β-adrenergic, serotonin and dopamine receptors) and ion channel receptor (e.g., NMDARs) signaling pathways (Fig. 5.2a) [27]. This means that the SARS-CoV-2 antigens share similarities with endogenous host antigens. Many of these SARS-CoV-2 proteins are also capable of mimicking interactions for synaptic, mitochondrial, and inflammatory functions (Table 5.2) [27]. Another computational study

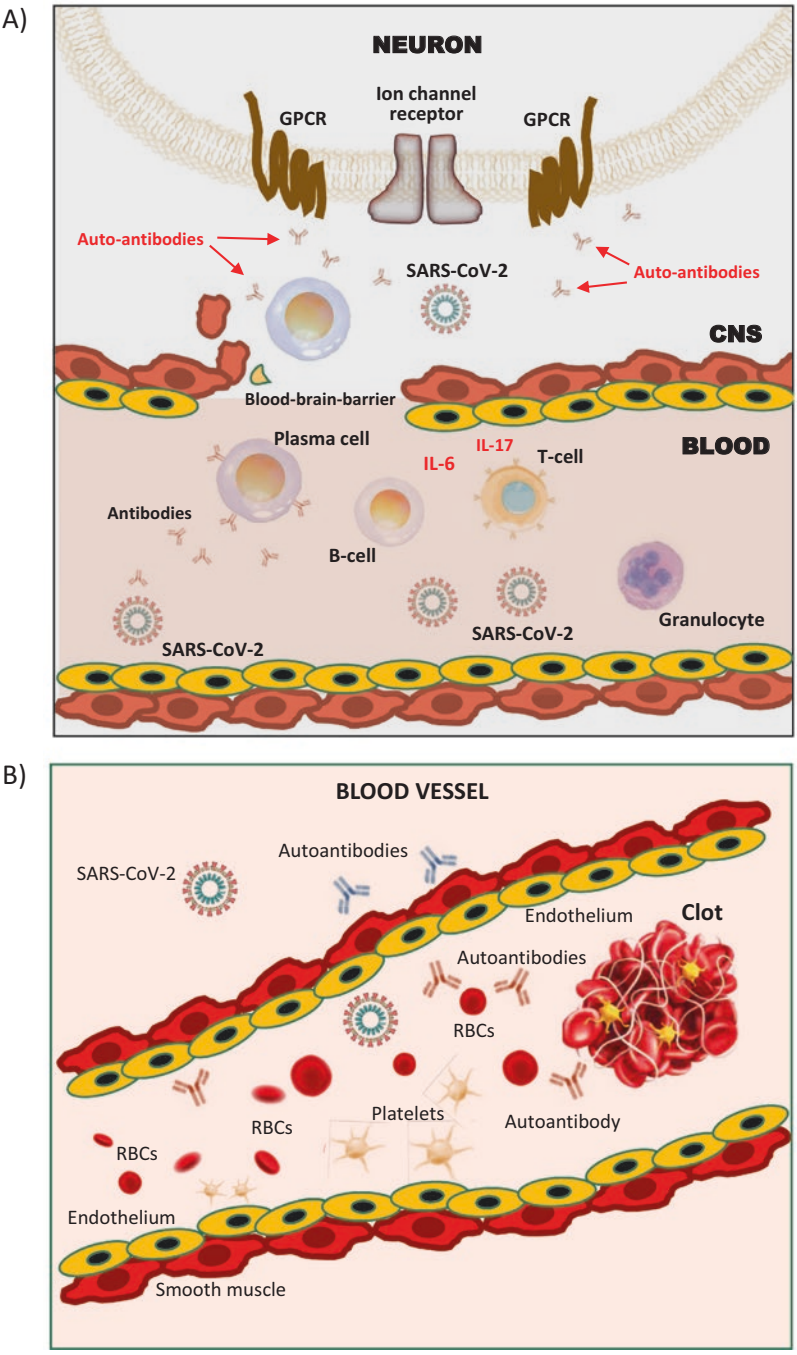

Fig. 5.2 (**a**) Possible pathophysiology of autoimmune response against host GPCR and ion channel receptors in the brain induced by SARS-CoV-2 infection. Viral particles in the bloodstream are recognized by T cells, leading to B-cell activation and sequential production of IgM and IgG antibodies

identified molecular mimicry hotspots in the S protein which shared antibody binding motifs with thrombopoietin, linked with blood coagulation, and tropomyosin, associated with cardiac health, and multiple other proteins involved in platelet activation and calcium regulation (Fig. 5.2b) [28]. In line with this, several studies have detected circulating autoantibodies in serum from COVID-19 patients with prothrombotic [29, 30] and hemolytic [31] activities, as well as those suspected of having damaging effects against the vascular endothelium [32] and smooth muscle [33, 34].

We recently proposed that mimicry of SARS-CoV-2 NSP8 and NSP9 with NMDAR NR1 and NR2A subunit epitopes may lead to autoimmune responses against these receptors in the brain as a potential cause of anti-NMDAR encephalitis [19]. This condition is an autoimmune disorder characterized by neurological and psychosis-like symptoms [35]. In our study, we identified eight SARS-CoV-2 cases with signs of anti-NMDAR encephalitis [19]. All of these patients had antibodies against the NMDAR in their cerebrospinal fluid (CSF) and showed a recent onset of deficits in working memory, mental status, or neuropsychiatric symptoms such as confusion, agitation, hallucination, or catatonia. Interestingly, all patients showed improvement after receiving steroid-based and immunoglobulin treatments. This suggested that there is considerable scope for effective treatments that can reduce PCS neurological symptoms.

There has now been a number of reports of neurological and neuropsychiatric conditions resulting from COVID-19 infections. One study showed that 39 out of 125 COVID-19 cases with such symptoms presented with altered mental status and 23 of these fit the definitions for either recent-onset psychosis, neurocognitive decline, or an affective disorder [36]. In a study on the effects of COVID-19 infection on brain pathology, Donaud et al. investigated brain changes in 401 individuals who were scanned by magnetic resonance imaging (MRI) before and after testing positive for a COVID-19 infection [37]. This revealed a significant reduction in gray matter thickness and tissue contrast in the orbitofrontal cortex and parahippocampal gyrus, as well as changes in biomarkers of tissue damage in olfactory regions. The researchers also found a reduction in global brain size in COVID-19 cases compared to controls, and PCS patients showed a cognitive decline between the two scans.

Fig. 5.2 (continued) against the SARS-CoV-2 NSPs, as well as the S, E, M, and N proteins. The SARS-CoV-2-mediated endothelitis and production of IL-17 by activated T cells disrupt the blood-brain barrier, allowing these antibodies to enter the CNS. The release of IL-6 alters glial cell activity, leading to neutrophil migration, inflammation, and further BBB damage. Antibodies produced against the SARS-CoV-2 proteins produced by plasma cells in the central nervous system can cross-react as auto-antibodies with the brain receptors indicated in Table 5.2, leading to neurological and neuropsychiatric manifestations. BBB: blood-brain barrier; CNS: central nervous system; NSP: non-structural proteins. (**b**) Possible pathophysiological autoimmune response following SARS-CoV-2 infection against smooth muscle, endothelial proteins, phospholipids, membrane receptors and components of inflammatory pathways via mimicry of viral proteins, leading to thrombus formation in blood vessels in the brain and disruption of blood supply. RBC = red blood cell

Table 5.2 SARS-CoV-2 proteins which may act as molecular mimics of host protein interactions linked to neuropsychiatric diseases

Code	Name	Interacting SARS-COV-2 protein
AA2AR	Adenosine receptor A2a	NSP5, NSP7, N
ACES	Acetylcholinesterase	S
ACHA2	Neuronal acetylcholine receptor alpha-2	NSP5
ACHA4	Neuronal acetylcholine receptor alpha-4	NSP5, NSP7, S
ACHB2	Neuronal acetylcholine receptor beta-2	NSP7, S
AL1A3	Aldehyde dehydrogenase 1A3	NSP10
AL4A1	D-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	NSP5
AL7A1	Alpha-aminoadipic semialdehyde dehydrogenase	NSP5
ARHG1	Rho guanine nucleotide exchange factor 1	NSP8, N
CAC1C	Alpha-1C	NSP5, NSP7, NSP10, S
CAC1D	Voltage-dependent L-type calcium channel alpha-1D	NSP8
CALM1	Calmodulin-1	NSP5, NSP7, NSP8, NSP9, NSP10, S
CALM2	Calmodulin-2	NSP3, NSP7, NSP8, NSP9
CBP	CREB-binding protein	NSP7, NSP8
CDK5	Cyclin-dependent-like kinase 5	NSP10, S
CHLE	Cholinesterase	NSP5
CNGA3	Cyclic nucleotide-gated cation channel alpha-3	NSP10, S
CREB1	Cyclic AMP-responsive element-binding protein 1	NSP5, NSP7, NSP9, S
DCHS	Histidine decarboxylase	NSP5
DOPO	Dopamine beta-hydroxylase	NSP10
DRD2	D2 dopamine receptor	NSP7, NSP8
EP300	Histone acetyltransferase p300	NSP7, NSP8, NSP10, NSP16, S
GABR1	Gamma-aminobutyric acid B receptor 1	NSP3, NSP5, NSP7, S
GABR2	Gamma-aminobutyric acid B receptor 2	NSP3, NSP9, S
GBB1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta-1	NSP8, S
GBG2	Guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-2	NSP8
GBRA1	Gamma-aminobutyric acid receptor alpha-1	S
GBRB2	Gamma-aminobutyric acid receptor beta-2	NSP5, S
GBRB3	Gamma-aminobutyric acid receptor beta-3	NSP5, NSP7, S
GBRG2	Gamma-aminobutyric acid receptor gamma-2	NSP5
GCR	Glucocorticoid receptor	NSP7, S
GLRA1	Glycine receptor subunit alpha-1	S
GLRA3	Glycine receptor subunit alpha-3	NSP5, NSP7, S
GNAI1	Guanine nucleotide-binding protein G(i) subunit alpha-1	NSP7
GNAI3	Guanine nucleotide-binding protein G(k) alpha	NSP7
GPSM2	G-protein-signaling modulator 2	NSP8, S
GRB2	Growth factor receptor-bound protein 2	NSP5
GRIA2	Glutamate receptor 2	NSP7
GRM1	Metabotropic glutamate receptor 1	NSP5, NSP12

(continued)

Table 5.2 (continued)

Code	Name	Interacting SARS-COV-2 protein
GRM2	Metabotropic glutamate receptor 2	NSP16
GRM5	Metabotropic glutamate receptor 5	NSP7
GRM8	Metabotropic glutamate receptor 8	NSP7, NSP8, NSP12, S
GRP1	RAS guanyl-releasing protein 1	S, N
GSK3B	Glycogen synthase kinase-3 beta	NSP10, NSP15
HVCN1	Voltage-gated hydrogen channel 1	S
KAP1	cAMP-dependent protein kinase I-beta regulatory subunit	NSP8
KAP2	cAMP-dependent protein kinase II alpha regulatory subunit	S
KCC2A	Calcium/calmodulin-dependent protein kinase II alpha	N
KCC2D	Calcium/calmodulin-dependent protein kinase II delta	NSP3, NSP5, NSP7, NSP8, NSP16
KCJ11	ATP-sensitive inward rectifier potassium channel 11	NSP7, NSP8
KCNH1	Potassium voltage-gated channel subfamily H1	NSP8
KCNN4	Intermediate conductance calcium-activated potassium channel protein 4	NSP9, NSP12, S
KCNQ1	Potassium voltage-gated channel subfamily KQT1	NSP7, NSP8, S
KCNQ2	Potassium voltage-gated channel subfamily KQT2	NSP8
KCNQ4	Potassium voltage-gated channel subfamily KQT4	NSP8
KPCG	Protein kinase C gamma type	NSP5
MCR	Mineralocorticoid receptor	NSP8
MTOR	Serine/threonine-protein kinase mTOR	NSP7, NSP8, S
NMDE1	Glutamate receptor ionotropic, NMDA 2A	NSP9
NMDZ1	Glutamate receptor ionotropic, NMDA 1	NSP8
NNMT	Nicotinamide N-methyltransferase	S
PENK	Proenkephalin-A	NSP5, NSP12
PHKG2	Phosphorylase b kinase gamma catalytic chain, liver/testis isoform	NSP5, NSP16
PLCE1	1-Phosphatidylinositol 4,5-bisphosphate phosphodiesterase epsilon-1	NSP5
PLCG1	1-Phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1	NSP5, NSP7, NSP8
PLCG2	1-Phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2	NSP5, NSP8, NSP16
PYGL	Glycogen phosphorylase, liver form	NSP5
RAP1A	Ras-related protein Rap-1A	NSP7
RAP1B	Ras-related protein Rap-1b	NSP3, NSP7
RGS16	Regulator of G-protein signaling 16	NSP7, S
RHOA	Transforming protein RhoA	NSP3, NSP8, NSP10, S
RPGP1	Rap1 GTPase-activating protein 1	NSP5
SCN5A	Sodium channel protein 5 alpha	NSP5, NSP7, NSP9, NSP12
SYUA	Alpha-synuclein	NSP5, NSP7
TPH2	Tryptophan 5-hydroxylase 2	S
TRPM4	Transient receptor potential cation channel M 4	NSP7, S
TY3H	Tyrosine 3-monooxygenase	NSP7, NSP9
VDAC1	Voltage-dependent anion-selective channel protein 1	NSP7, NSP8, S

3 Autoantibodies in PCS

In order to increase our understanding of neuropsychiatric conditions in PCS, analyses of autoimmune disorders of vascular regulation and the autonomic nervous system may be required. Although autoantibodies against GPCRs and ion channel receptors have been detected in COVID-19 disease, these have not been systematically studied in PCS. The presence of antibodies against α- and β-adrenergic, M1, M2, M3, M4, and M5 muscarinic acetylcholine, angiotensin II, and endothelin-A receptors could explain many of the symptoms such as peripheral and cerebral blood flow disturbances, cardiac arrhythmias, consequent chronic fatigue, as well as cognitive, depressive, and anxiety disorders [18, 38, 39]. To characterize such PCS cases, differential diagnosis at the clinical level is crucial to differentiate these from non-COVID-19 related mental disorders, intensive care unit (ICU) complications, reduced general conditions, or cardiac, respiratory, or renal insufficiencies.

Wallukat et al. investigated the association of neurological or cardiac symptoms with the presence of functionally active autoantibodies against GPCRs, following the acute phase of COVID-19 infection in 31 patients [40]. They found that 29 of the patients showed a spectrum of neurological symptoms such as fatigue, alopecia, and attention deficits, and 17 patients showed a combination of neurological and cardiovascular symptoms. Screening in rat neonatal cardiomyocytes revealed the presence of two to seven different GPCR autoantibodies, some of which either increased (angiotensin II type 1 receptor, α1-adrenoceptor, β2-adrenoceptor, nociceptin-like opioid receptor) or decreased (muscarinic M2-receptor, MAS-receptor, endothelin type A receptor) the heart rate. In each case, the antibodies targeted the extracellular domains of the receptors.

A recent study investigated the association of autoantibodies against GPCRs with impaired retinal microcirculation in PCS [41]. All 42 PCS patients showed seropositivity for different autoantibodies against GPCRs, while none of the controls ($n = 6$) did. Furthermore, a decrease in retinal vessel density was associated with autoantibodies targeting the adrenergic β2, MAS, angiotensin-II-type-1, and α1 adrenergic receptors. This suggests the possibility that techniques such as optical coherence tomography (OCT) may be useful clinical tools to search for such vascular dysregulations in the retina of PCS patients [42]. Furthermore, analyses of the blood vessels of the retina and optic nerve using OCT may lead to useful insights into the vascularization of the brain since many neurological diseases have early retinal manifestations [43, 44].

4 Autoantibody Screening and Treatment Options for PCS

Our investigation of anti-NMDAR encephalitis patients described above demonstrated the importance of early detection using antibody diagnostic screening in severe cases of COVID-19 infection [19]. We also suggest the use of

electroencephalography (EEG) and CSF testing for detection of autoimmune encephalitis. Confirmed positive cases could be treated with immunotherapeutics to prevent severe neurological impairments. However, this will first require testing in large randomized trials to show that these therapies help in PCS. There are available screening panels for PCS patients to test for the presence of autoantibodies. This includes assays from CellTrend (Berlin, Germany) which test for antibodies against the M1, M2, and M5 muscarinic acetylcholine receptors, the α1- and α2- adrenergic receptors, as well as the angiotensin-II-receptor-1(AT1R) and the endothelin A receptor [45]. In addition, EUROIMMUN (Lübeck, Germany) offers tests for autoantibodies against other neurological/neuropsychiatric-related markers such as the NMDAR as well as for components of myelin and the α-amino-3-hydroxy-5--methyl-4-isoxazolepropionic acid (AMPA) and gamma-aminobutyric acid ($GABA_B$) receptors [46].

A case report from the eye clinic of the University Hospital of Friedrich-Alexander-Universität (FAU) gave cause for optimism that there may soon be an effective therapeutic intervention for PCS [47]. This study showed that a 59-year-old man who had been suffering from PCS was discharged symptom-free after treatment with the active substance BC 007. This compound acts to bind autoantibodies against GPCRs, including the α1-, β1-, and β2-adrenergic receptors, as well as the endothelin-A receptor, which have been implicated cardiomyopathies [48, 49]. The treatment led to an improvement in this patient in symptoms such as concentration and sense of smell, as well as blood flow in the eyes. Since this time, two further patients treated with this compound have shown improvements in their PCS symptoms [50]. Other potential treatments which have shown successful outcomes in autoimmune conditions include intravenous immunoglobulin infusion, which provides passive immune protection against multiple pathogens [51, 52] and extracorporeal apheresis [53].

5 The Case for More Studies on PCS

To improve outcomes in patients with PCS, we propose that there is an urgent need for establishment of interdisciplinary outpatient clinics dedicated to this purpose. This platform will also enable carrying out research to determine the frequency of autonomic and vascular dysregulation mediated by autoantibodies in patients with post-COVID syndrome compared to those without. For example, we propose such a clinic should perform accurate neuropsychiatric and autonomic phenotyping to increase our understanding of autoimmunity associated with the clinical presentation and complaint patterns.

Table 5.3 (a) General aims of the interdisciplinary outpatient clinic dedicated to improving outcomes in patients with PCS. (b) Scientific and technical aims

Aim	(a) *General objectives*
1	Improvement of health care for PCS patients with neuropsychiatric and neurological impairments
2	Identification of a PCS subgroup with GPCR or ion channel receptor autoantibodies and correlation with autonomic dysfunction and neuropsychiatric/neurological symptoms
3	Development of a staged diagnostic and treatment scheme for autoimmune-mediated PCS
Aim	(b) *Scientific and technical objectives*
1	Establishing an interdisciplinary collaboration of different clinical specialities since COVID-19 can affect many organ systems
2	Neuropsychiatric and vascular phenotyping of patients with/without GPCR or ion channel receptor autoantibodies
3	Identification of risk profiles and resilience factors (e.g., stress, autoimmune or mental health history, predisposition) by comparing patients with and without PCS
4	Establishment of a clinical diagnostic scheme guided by "alarm symptoms" to identify inflammatory PCS subtypes with vascular dysregulation
5	Determination of the most appropriate treatment options based on symptoms and autoantibody screening results

5.1 The Need for Dedicated PCS Outpatient Clinics: Using Saxony-Anhalt as an Example

As of April 6, 2022, more than 625 thousand COVID-19 infections were detected in Saxony-Anhalt, Germany, out of approximately 2.2 million inhabitants (around 30% of the population) [54]. From this number, it is expected that 30% of the infected group will experience late and long-term health effects, based on data from the REACT-2 study in England [55]. As with many other regions in Germany and other countries, rehabilitation clinics in Saxony-Anhalt have been stretched to their capacity, and there is only one PCS outpatient clinic at Klinikum Bergmannstrost Halle [56]. Furthermore, only a small proportion of rehabilitation clinics with PCS experience and university research hospitals have shown effective interactions. The specific aims of a proposed clinic are indicated in Table 5.3. Using this interdisciplinary approach, we aim to test the hypothesis that there is a PCS subgroup with antibody-mediated vascular dysregulation that differs from other PCS cases and healthy recovered patients.

5.2 Proposed Methodology for Interdisciplinary PCS Outpatient Clinic

In our case, patient recruitment will occur via university hospital and rehabilitation clinics at Bad Salzelmen and Bad Suderode, Germany. Recruitment of controls will occur via the Internet. As shown in Fig. 5.3, the following information will be

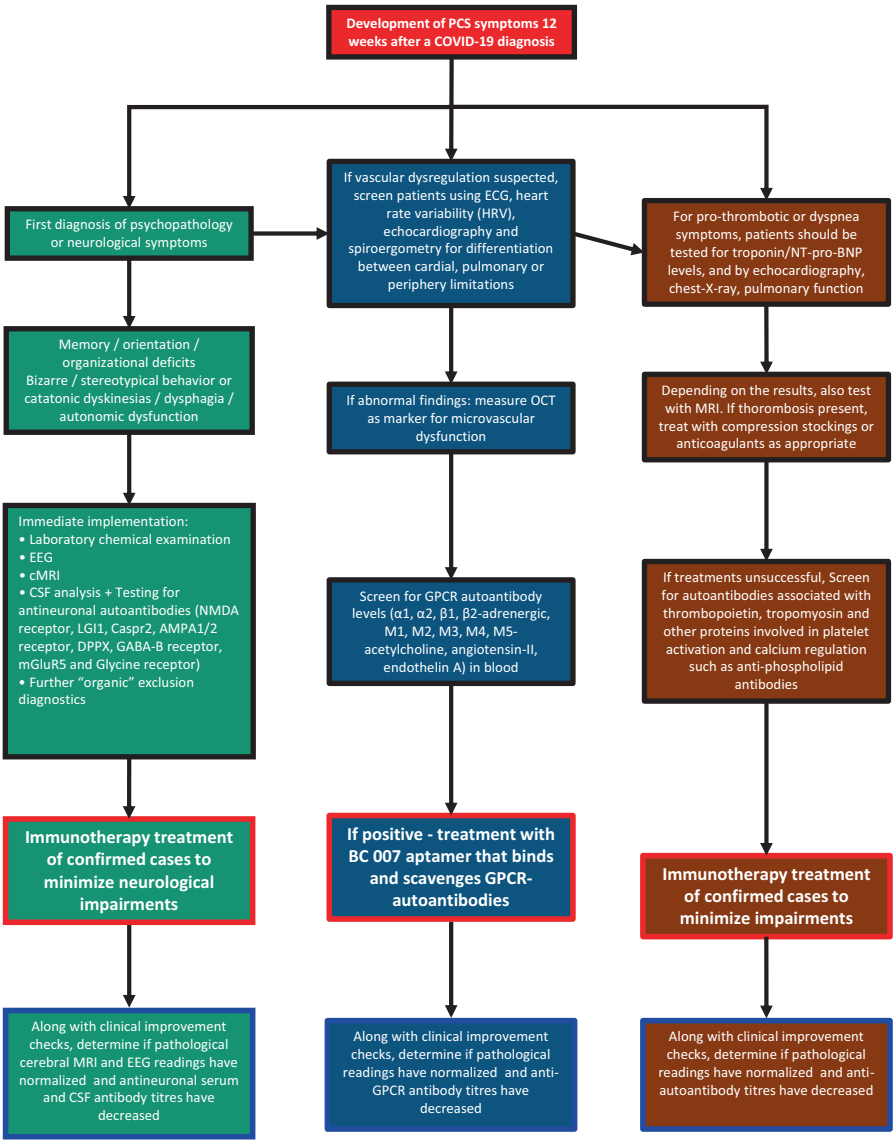

Fig. 5.3 Diagnostic algorithm to test for an autoimmune origin of neurological/neuropsychiatric symptoms. Since some symptoms cannot be excluded by negative findings from EEG, MRI, or CSF profile, screening should be carried out for the presence of autoantibodies to aid in stratification of the most appropriate treatment options on a case-by-case basis. Those who test positive for the presence of neuronal (left), vascular (middle), thrombotic (right), or other relevant autoantibodies can be treated with immunotherapies and other drugs as appropriate. EEG: electroencephalography; MRI: magnetic resonance imaging; CSF: cerebrospinal fluid; NMDA: N-methyl-D-aspartate, CASPR2: contactin-associated protein 2, AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, LGI1: leucine-rich glioma-inactivated protein 1, DPPX: dipeptidyl aminopeptidase-like protein 6, GABAB: γ-aminobutyric acid B; OCT: optical coherence tomographya

obtained for all patients who had been infected with COVID-19 to guide the most appropriate treatment options:

1. Medical history, psychiatric, physical neurological-internal examination
2. Psychological and cognitive testing
    - Current well-being/cognition: Hospital Anxiety and Depression Scale (HADS), Fatigue Scale (FS), Symptom Checklist-90-Revised (SCL-90), mini-mental state examination, Consortium to Establish a Registry for Alzheimer's Disease (CERAD), Brief Neuropsychological Cognitive Examination (BNCE)
    - Risk/stress factors: Childhood Trauma Questionnaire (CTQ), prolonged standing strain index (PSSI)
3. Routine laboratory blood analysis
4. Autoantibody screening:
    - Screening for circulating neuronal antibodies: NMDA receptor, LGI1 (leucine-rich glioma inactivated 1), Caspr2, AMPA1/2 (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid 1/2) receptor, DPPX (dipeptidyl-peptidase-like protein-6), GABA-B receptor, mGluR5 (metabotropic glutamate receptor 5) and GlyR (glycine receptor)
    - Determination of circulating GPCR-antibodies: α1, α2, β1, β2-adrenergic, M1, M2, M3, M4, M5-acetylcholine, angiotensin-II and endothelin A
    - Screening for antibody-associated brain inflammation: Lumbar puncture/CSF analysis (lymphocytic pleocytosis: cell count >5/μL, CSF-specific oligoclonal bands or blood–CSF barrier impairment) and EEG (epileptic or slow-wave activity, possibly with temporal focus, "extreme delta brush") have the highest sensitivity. Magnetic resonance imaging (MRI) is abnormal in only about 50% of patients with definite autoimmune encephalitis
5. Cardiovascular and pulmonary diagnostics
    - ECG, heart rate variability (HRV), and echocardiography
    - In case of exertional dyspnea, chest pain, and exercise-induced tachycardia, apply spiroergometry and exercise stress test
    - In case of abnormal findings, test vascular stiffness using pulse wave velocity and microvascular changes, OCT ocular fundus to assess cerebrovascular regulation, autonomic nervous system (orthostasis test with tilt table if necessary) and sleep diagnostics (Pittsburgh Sleep Quality Questionaire [PSQI]), use of wearables devices
6. Review of findings, differential diagnostic assessment, and therapeutic recommendation by interdisciplinary team if necessary
    - Application of machine and deep learning for selection of discriminating variables for PCS endophenotypes with GPCR-antibodies and vascular dysregulation

- Data analysis to determine variance, correlation, factor analyses, logistic regression, cluster analyses for group comparisons regarding GPCR and other autoantibodies, their correlation with clinical-apparative findings, and identification of PCS subtypes

7. Development of a diagnostic and treatment scheme based on clinical experience and data

Patients found to have new onset neurological or neuropsychiatric symptoms persisting for 12 or more weeks following a COVID-19 diagnosis will be tested as above and screened for the presence of antineuronal antibodies (Fig. 5.3). Those found to be positive for neuronal or vascular-related autoantibodies can be treated as appropriate with immunotherapies and anti-inflammatory compounds to minimize neurological damage, given positive results from clinical trials.

5.3 Methodologies for Autoimmune-Associated Neuronal, Vascular, or Thrombotic Dysregulation

For patients with confirmed autoimmune encephalitis and neuropsychiatric symptoms (Fig. 5.3), the following therapeutic procedure can be followed as described previously [19, 57, 58]. Firstly, assessment and screening should be performed as described above. If anti-neuronal antibodies are detected, immunosuppression can be attempted using corticosteroid therapy (1 g methyl-prednisolone/day for 5 days), intravenous human immunoglobulin administration (0.4 g/kg/day for 5 days), or immunoadsorption or plasmapheresis for rapid removal of pathogenic autoantibodies. If there is no improvement, treatment can be extended with rituximab administration (2 × 1000 mg i.v. or s.c. 2–4 week intervals). In refractory cases, combination treatment can be performed with cyclophosphamide (750 mg/m^2 body surface area every 4 weeks) and mycophenolate mofetil or methotrexate. Bortezomib may be applied (1–6 cycles of 1.3 mg/m^2 body surface area, 21 days/cycle) to eliminate plasma cells in the case of patients who require artificial ventilation and do not respond adequately to the above treatments. Normalization can be assessed by clinical improvement in symptoms, and pathological cardiac MRI and EEG findings can be used to monitor treatment response. Finally, antineuronal serum and CSF antibody titres should be measured after a few weeks of treatment to determine if these have normalized.

In case of neuropsychiatric symptoms associated with clinical or apparative warning signs for autoimmune-triggered vascular dysregulation [59] or other conditions such as postural orthostatic tachycardia syndrome (POTS) [60], patients should be screened for heart rate variability (HRV), using electrocardiography (ECG), echocardiography, and spiroergometry for differentiation between cardiac, pulmonary or peripheral limitations (Fig. 5.3). If abnormalities are detected, OCT can be measured as marker for microvascular dysfunction. Screening should then be performed for GPCR autoantibody levels (α1, α2, β1, β2-adrenergic, M1, M2, M3,

M4, M5-acetylcholine, angiotensin-II, endothelin A). Given the presence of autoantibodies, treatment can be performed with BC 007 to scavenge GPCR-autoantibodies with and other immunotherapies as described above.

For neuropsychiatric manifestations associated with autoimmune-associated pro-thrombotic syndromes or dyspnea [61, 62], it is recommended that patients are tested for troponin/NT-pro-BNP levels and by echocardiography, chest-X-ray, pulmonary function, and, depending on the results, MRI (Fig. 5.3). Screening should then be carried out for anti-phospholipid antibodies and other antibodies associated with thrombopoietin, tropomyosin, platelet activation, and calcium regulation. If autoantibodies are detected, immunotherapies can be performed as described above.

6 Conclusions and Future Perspectives

Given the high proportion of COVID-19 cases that result in PCS, urgent steps are required to identify those patients most at risk and to develop routine screening procedures at the clinical and molecular levels. This will enable identification of the underlying causes to facilitate the most appropriate therapeutic treatments. The consensus now appears to indicate that a high proportion of PCS cases result from inappropriate hyper-inflammatory and autoimmune states resulting from SARS-CoV-2 infection. In order to improve care of PCS patients, we aim to open an interdisciplinary PCS outpatient clinic encompassing clinical and technical teams from the fields of psychiatry, psychotherapy, neurology, immunology, cardiology, angiology, and pneumology at Otto von Guericke-University in Magdeburg, Germany. This will enable patients with a high health burden to receive modern diagnostic and competent therapeutic recommendations. The main aim is to test our hypothesis that there is a PCS subgroup with autoimmune-mediated systemic and brain-vascular dysregulation, which may lead to conditions such as circulatory disorders, fatigue, cognitive impairment, depression and anxiety. This will be assessed using a combination of specific autoantibody screening diagnostics and precise clinical, psychological, and apparative testing. This system could be used as a model for identifying those individuals most at risk of developing PCS for prevention, or for treatment-focussed clinical trials, and for planning education and rehabilitation services in the event of a continuing COVID-19 pandemic and/or the emergence of future coronavirus outbreaks.

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Part III
Omic Techniques for COVID-19 Diagnostics

Chapter 6
Genetic Associations with Coronavirus Susceptibility and Disease Severity

Fatima Barmania, Juanita Mellet, Megan A. Holborn, and Michael S. Pepper

Abstract The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is responsible for the coronavirus disease 2019 (COVID-19) global public health emergency, and the disease it causes is highly variable in its clinical presentation. Host genetic factors are increasingly recognised as a determinant of infection susceptibility and disease severity. Several initiatives and groups have been established to analyse and review host genetic epidemiology associated with COVID-19 outcomes. Here, we review the genetic loci associated with COVID-19 susceptibility and severity focusing on the common variants identified in genome-wide association studies.

Keywords SARS-CoV-2 · COVID-19 · Variants · Host genetics · GWAS · Susceptibility · Severity · Candidate gene

1 Introduction

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a highly transmissible pathogenic virus which emerged in 2019 as the cause of coronavirus disease (COVID-19) [1, 2]. One of the most striking features of the virus is the heterogeneity in clinical presentation of symptoms ranging from asymptomatic or mild illness to severe forms of the disease and death. The severity of symptoms and the mortality rate increase in individuals who have known risk factors such as advanced age, male gender, and comorbidities such as diabetes, hypertension, and cancer [3]. However, these factors do not fully account for the variation seen in outcomes.

Infectious diseases may present with a range of clinical presentations indicative of variable and complex pathogen-host interactions. Variability in risk of infection

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or severity of disease symptoms has been attributed to several factors including differences in host genetics. Several common infectious diseases, including human immunodeficiency virus (HIV)-1, malaria, and tuberculosis, present with susceptibility or clinical presentation differences across individuals which have been attributed to host genetic factors [4]. For example, genetic variants affecting the amino acid sequence of the host CD4-positive immune cell C–C motif chemokine receptor 5 (CCR5) can reduce the expression, intracellular signalling, or ligand-binding capacity of CCR5. The consequential reduction in host cell CCR5 expression or functionality interferes with HIV-CCR5 interaction, reducing the rate of R5-tropic viral entry and infection [5, 6]. Like other infectious diseases, an association between COVID-19 susceptibility or severity and host genetic factors has been demonstrated [7]. Host genetic variation in genes involved in COVID-19 pathogenesis, including those involved in viral entry, replication, or the host immune response, may explain some of the heterogeneity in COVID-19 cases. Studies are currently underway to identify genetic factors that may underlie host predisposition to COVID-19 and severity of disease.

SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) host cell receptor, found predominantly on the surface of cells in the lower respiratory tract, for viral entry. The viral glycoprotein used for entry consists of two subunits which require cleavage by host cellular proteases. The host enzymes transmembrane protease serine 2 (TMPRSS2), lysosomal cathepsins, and furin are utilized by the virus for cleavage so that membrane fusion can occur. The viral RNA genome is released into the host cell cytoplasm by endocytosis where the virus uses host cell machinery to replicate [8].

The innate immune system is essential for detecting and restricting SARS-CoV-2 and for activating the adaptive immune response. The innate immune system recognizes SARS-CoV-2 using pattern recognition receptors (PRRs) including toll like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I) receptors (RLRs), and C-type lectin receptors (CLRs) [9]. These PRRs recognize pathogen-associated molecular patterns (PAMPs) leading to the production of antiviral molecules, such as inflammatory chemokines and cytokines, and interferons (IFNs). In turn, this results in dendritic cell (DC) maturation, activation of natural killer (NK) cells, and macrophage phagocytosis of viral antigens. DCs and macrophages are antigen-presenting cells (APCs) which activate naïve and memory T cells [10].

The adaptive immune response is usually slower compared to the innate immune response. This is because expansion of naïve cells into effector B and T cells is required for viral control [11]. SARS-CoV-2-specific antibodies and CD4+ and CD8+ T cells are produced in response to viral infection and have various roles. CD4+ T-cell responses are more prominent in SARS-CoV-2 than CD8+ T-cell responses. Circulating and memory T follicular helper (Tfh) cells are produced in response to SARS-CoV-2 infection [12], and the number of Tfh cells has been directly associated with less severe COVID-19 [13]. CD4+ T cells likewise assist CD8+ T cells in their effector function during viral infection. CD8+ T cells are involved in clearing viral infections through their ability to destroy infected cells using potent cytotoxic effector functions. In SARS-CoV-2, the presence of CD8+ T cells has been linked to better outcomes.

B cells are involved in the production of virus-specific antibodies against the SARS-CoV-2 nucleocapsid and spike proteins, with the majority of neutralizing antibodies targeting the spike protein receptor binding domain [14]. Heterogeneity in the presentation of COVID-19 might occur due to a reduced type I IFN response during initial infection [15, 16] and elevated pro-inflammatory cytokine secretion [17]. SARS-CoV-2-specific T-cell responses in convalescent COVID-19 patients have been shown to be associated with milder COVID-19 [18, 19]. This suggests that the adaptive T-cell response to SARS-CoV-2 is essential for viral control and resolution. In some cases, increased neutralizing antibody titers have been found to correlate directly with COVID-19 severity [14, 20].

The pathogenesis of severe COVID-19 outcomes is likely to be the result of abnormal host responses or an over- or under-reaction of the immune system in some patients. Although viral proteins are known to influence immune responses, individuals with variations in essential genes may have varied responses. This could lead to uncontrolled immune responses later in infection or reduced immune responses during early infection.

2 Global Response to COVID-19 Host Genetics

Shortly after the first cases of COVID-19 pneumonia was reported, and even before the World Health Organization declared a pandemic, the global research community began to collaborate and investigate the genetic architecture of COVID-19 host disease [21]. Since then, a plethora of articles have been published relating to the discovery of both common and rare variants in host genes involved in COVID-19 outcomes. This global collaboration has created an environment that fosters open science and immediate sharing of meta-data, analysis pipelines, and resources which allowed for an ultra-rapid dissemination of multiple human genetic determinants of disease severity early in the pandemic (Fig. 6.1).

Large-scale whole exome sequencing (WES), whole genome sequencing (WGS), and genome-wide association studies (GWAS) were the focus of several of these large consortia including but not limited to the COVID-19 host genetic initiative (HGI), GenOMICC and ISARIC groups, and the Severe COVID-19 GWAS group, as well as commercial genomic service providers such as 23andMe and AncestryDNA and independent academic working groups. These studies identified population-specific common genetic variation data for loci enriched in susceptible or severe COVID-19 individuals which have contributed important information regarding the biological pathways involved in disease pathogenesis. The discovery of rare genetic variants is limited with GWAS; therefore, groups such as the COVID-19 Human Genetic Effort (HGE) focused on targeted gene approaches of known or novel monogenic disorders that are associated with a subset of individuals with extreme COVID-19 phenotypes/outcomes.

Many of the variants or genes identified can be mapped to distinct pathophysiological aspects of COVID-19 disease which include viral entry, viral replication,

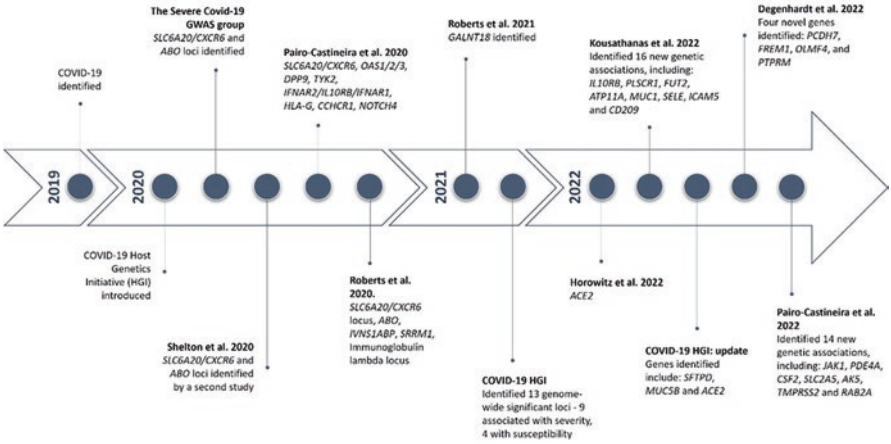

Fig. 6.1 Brief timeline of the main genome wide association study (GWAS) data releases

and the immunological response to SARS-CoV-2. For the purposes of this review, we will focus on the more robust GWAS associations and results from the larger consortia.

3 Genetic Findings

Efforts to understand the relationship between host genetics and susceptibility to SARS-CoV-2 infection and COVID-19 outcomes have identified key loci and genes, some of which are discussed below (Fig. 6.2). These loci have been replicated in one or more studies, and in some cases functional, computational, and statistical approaches have been used to elucidate the likely causal gene/variant.

3.1 Chromosome 1

An intronic variant (rs67579710) at locus 1q22 was found in the thrombospondin 3 (*THSB3*) gene, which was inversely associated with COVID-19 hospitalization [7]. This locus was replicated by the GenOMICC group which identified three independent single nucleotide polymorphisms (SNPs) in this region [22]. The first variant was mapped to the ephrin A4 (*EFNA4*) gene and the second to an intronic variant in *THSB3*, which was near a significant expression quantitative trait locus (eQTL) from genotype tissue expression (GTEx) v8 [23] for mucin 1 (*MUC1*). The third signal was associated with a variant in the intron of tripartite motif containing 46 (*TRIM46*), which is also a splicing quantitative trait locus (sQTL) and an eQTL for *MUC1*. Fine mapping, colocalization, and transcriptome-wide association studies

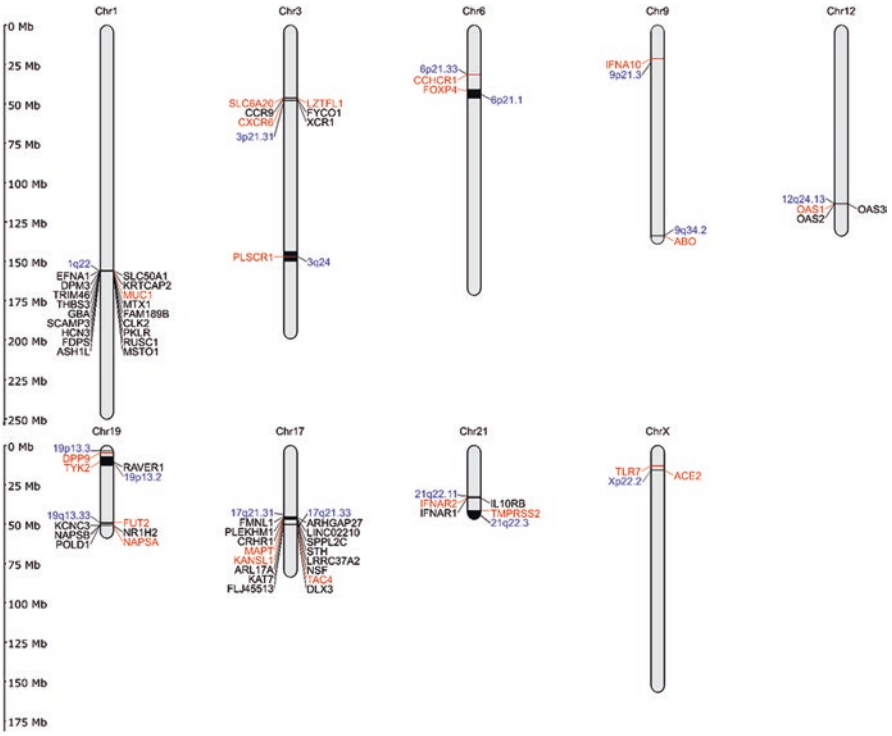

Fig. 6.2 Loci/genes identified through GWAS results from large-scale COVID-19 host genetics studies. Loci are shown in blue. Potential causal genes are depicted in red, while genes in linkage disequilibrium (LD) with the lead variants are depicted in black

(TWAS) found that these variants increase expression of *MUC1* suggesting that this gene is a mediator for the locus discovered. Mucin proteins are abundant in mucus that lubricates the lining of the airways, digestive tract, and various tissues and organs. These proteins also play essential roles in intracellular signaling, protection of the host from pathogens, and regulation of inflammatory responses to infection. The MUC1 protein can influence signaling of activated T cells and plays a significant role in apoptosis. Studies done on *MUC1*-deficient mice show that they are unable to phagocytose pneumococci pathogens [24], which demonstrates the importance of this protein in the innate immune response.

3.2 Chromosome 3

The 3p21.31 locus was one of the two genetic loci discovered by the severe COVID-19 GWAS group in the earliest GWAS of critically ill COVID-19 patients with respiratory failure [25]. This locus exceeds 50 kb in size and is inherited from

Neanderthals with the lead risk variant having a high frequency in the south Asian and European population groups while being absent in the African population [26]. Since its initial discovery, the 3p21.31 locus remains the most consistent and strongest association across multiple studies associating with both susceptibility and severity outcomes in several independent GWAS cohorts [27–30]. This locus consists of multiple GWAS signals [30–32] that are independent from one another in both 1KGP CEU and AncestryDNA population groups [31].

This locus encompasses six genes which include solute carrier family 6 member 20 (*SLC6A20*), leucine zipper transcription factor like 1 (*LZTFL1*), *CCR9*, FYVE and coiled-coil domain containing 1 (*FYCO1*), C-X-C motif chemokine receptor 6 (*CXCR6*), and X-C motif chemokine receptor 1 (*XCR1*). Considering that this locus is part of a large genomic segment, identifying the causal variants and genes to understand the biological mechanism responsible for the association signals has been challenging. The lead severity variant (rs35081325/rs10490770) is closest to the *LZTFL1* gene, while the lead susceptibility variant (rs73062389/rs2271616), 54 kb distant, is closest to the *SLC6A20* gene [30]. COVID-19 HGI identified *CXCR6* as the causal gene for the severity signal using the Variant2Gene algorithm [30], while the GenOMICC group identified *SLC6A20* as the causal gene for the susceptibility signal using Bayesian fine mapping techniques [22]. The third signal in this locus, tagged by lead variant rs2531743 near the *SLC6A20* gene, was identified as a susceptibility signal in COVID-19 positive versus negative cases and may provide a protective effect [32].

The 3p21.31 locus contains the chemokine receptor genes *CXCR6*, *CCR9*, and *XCR1*. *CXCR6* is involved in chemokine signaling [33] and recruitment of CD8-memory T cells to the respiratory tract to combat respiratory pathogens [34]. *CXCR6* and *CCR9* have a protective effect in the lung with TWAS indicating lower levels of CXCR6 and CD8+ T cells in severe COVID-19 patients [35].

The *LZTFL1* gene has also been implicated as the candidate effector gene for this risk locus. Using multi-omic analyses and machine learning, a gain-of-function variant in an enhancer region of *LZTFL1* was identified [36]. The variant upregulates the expression of *LZTFL1* in the lung and in turn delays a lung-specific viral response pathway that can inhibit infection by downregulating known viral host entry receptors in the respiratory tract [37].

Genome and epigenome editing techniques using CRISPR/Cas9-mediated genomic deletion identified both *CCR9* and *SLC6A20* as potential target genes [38]. Further evidence for *SLC6A20* association is supported by the sodium transporter being known to directly interact with *ACE2*, the SARS-CoV-2 entry receptor [39].

Given the current discrepancies involving this locus, additional studies are needed to determine the true causal variants and genes responsible for these associations.

Kousathanas and co-workers identified a variant (rs343320) in the phospholipid scramblase 1 (*PLSCR1*) gene at locus 3q24.2 that is associated with critical COVID-19 cases [22]. Bayesian fine mapping identified a missense variant that can affect the gene with Combined Annotation Dependent Deletion (CADD) scores indicating the variant as deleterious [40]. Structural protein predictions revealed

that the missense mutation results in the disruption of a crucial nuclear localization signal required for the antiviral interferon effect [41]. Studies have also shown the PLSCR1 protein is involved in gene regulation and IFN-induced immune responses [41]. *PLSCR1* has previously been shown to control replication of RNA viruses such as influenza A [42].

3.3 Chromosome 6

The 6p21.1 locus with lead variant rs1886814, found within the transcription factor forkhead box P4 (*FOXP4*), was identified in the COVID-19 HGI meta-analysis in hospitalized cases [30]. FOXP4 presents an attractive biological target as it plays a role in controlling epithelial cell fate during lung development and regeneration [43] and is required for normal T-cell recall responses [44]. The *FOXP4* variant modified gene expression in the lung as reported by lung-specific *cis*-eQTL from GTEx [23] and the lung eQTL consortium [45], through the use of knockdown experiments which resulted in inhibition of lung epithelial regeneration [43]. The lead variant identified correlated with lead variants for lung adenocarcinoma [46] and subclinical interstitial lung disease [47]. The leading cause of mortality in severe hospitalized COVID-19 patients is respiratory failure [48]. Therefore, the involvement of *FOXP4* in lung regeneration and other lung-associated diseases suggests this gene is a likely candidate for severe COVID-19 outcomes.

The 6p21.33 locus was first identified in critical COVID-19 cases in the GenOMICC study, and the finding was replicated using HGI and 23andMe datasets [27]. The coiled-coil alpha-helical rod protein 1 (*CCHCR1*) gene is implicated as the causal gene at this locus as it is the closest gene with the highest Variant2Gene score and has coding variants in linkage disequilibrium (LD) with the lead variant identified [30]. Additional fine mapping and eQTL analysis have identified a functional variant in the *CCHCR1* gene that further supports its role as the causal gene [32]. The *CCHCR1* gene encodes a P-body protein involved in cytoskeletal remodeling and messenger RNA (mRNA) turnover [49, 50]. However, the exact mechanism of action of *CCHCR1* in COVID-19 is unclear. The locus is in a region encompassing nine genes which include human leukocyte antigen (HLA)-B and HLA-C. Further study in this region is needed to identify the causal gene.

3.4 Chromosome 9

The 9q34 locus was one of the first loci associated with COVID-19 outcomes and represents the *ABO* gene. The *ABO* gene was first discovered in 1901 and has three allelic forms, A, B, and O [51]. This gene is responsible for the presence of antigens on red blood cells which determine blood type. The *ABO* gene has been linked to several infectious and non-infectious diseases [52] and was initially thought to be

linked to severe COVID-19 disease [25]. It now represents one of the strongest signals associated with susceptibility to COVID-19, with data suggesting that individuals with blood group O have protection while blood group A is associated with susceptibility to SARS-CoV-2 infection [29, 30]. This was further supported by observational studies that explored ABO blood groups and clinical characteristics of COVID-19 cases [53, 54].

The mechanism of *ABO* involvement in COVID-19 outcomes is still not fully understood. However, some studies have indicated that anti-immunoglobulin G (IgG) antibodies may exert a protective effect [55]. Other studies have suggested a role for cluster of differentiation (CD)209, a protein that directly interacts with the SARS-CoV-2 spike protein. CD209 is found at higher levels in cases with the variant that confers blood group O [56]. COVID-19 transmission rates were also found to be lower in individuals receiving transfusion with incompatible ABO blood groups, which suggests that anti-ABO antibodies might contribute to viral neutralization [57].

A lead variant (rs28368148) in the IFN alpha 10 (*IFNA10*) gene at locus 9p21.3 was identified in critical COVID-19 cases [22, 58]. The IFNA10 ligand, part of the type I IFN family, has a direct role in the immune response against pathogens. Identification of a potential role for the variant was replicated in hospitalized patients in a combined GWAS meta-analysis of four different cohorts [22]. Bayesian fine mapping identified a missense variant that can affect the IFNA10 ligand, with CADD scores indicating that the variant was deleterious [40]. Structural prediction of the variant shows that the amino acid change affected an evolutionarily conserved region which likely destabilizes the protein and causes functional effects [22].

3.5 Chromosome 12

A protective haplotype of approximately 75 kb encompassing the oligoadenylate synthetase (*OAS*)1, 2, and 3 genes, derived from Neanderthal ancestry [59], was identified as a COVID-19 risk locus (12q24.13) in association studies of individuals of mostly European ancestry [27, 30]. The OAS genes encode enzymes involved in antiviral effects such as activation of ribonuclease L which is responsible for degrading intracellular double-stranded (ds) RNA [60]. The candidate causal variant, rs10774671, is found within the splice acceptor site on exon seven of the *OAS1* gene. The protective G allele results in a longer OAS1 enzyme which is approximately 60% more active [61]. The allele results in the expression of a prenylated C-terminal form of OAS1 which facilitates targeting of the protein to intracellular sites containing viral dsRNA [62]. Therefore, the expression of prenylated *OAS1* has been associated with protection from severe COVID-19. Mendelian randomization studies have also indicated that increased circulating levels of OAS1 are associated with a reduced risk of severe COVID-19 and hospitalization [63]. Due to the candidate variant being in strong LD ($r^2 > 0.8$) with more than 130 variants in the OAS1-3 region, Huffman and co-workers used trans-ancestry fine mapping in

20,779 hospitalized cases, which demonstrated that the splice variant was the likely causal variant [61], and implicated *OAS1* as the causal gene in locus 12q24.13 affecting COVID-19 severity.

3.6 Chromosome 16

A missense variant (rs117169628) at locus 16q24.3 in the *SLC22A1* gene has been identified and replicated in severe cases requiring hospitalization [22, 58, 64]. This gene belongs to the solute carrier protein family that facilitates cell membrane transport and is co-regulated with numerous surfactant proteins [65]. The importance of the SLC6A20 solute carrier in COVID-19 was reviewed previously [66]. The solute carrier family consists of integral cell membrane proteins involved in many essential processes, and its members have been shown to be dysregulated in various human diseases.

3.7 Chromosome 17

Two loci have been identified on chromosome 17, namely, 17q21.31, and 17q21.33.

The COVID-19 HGI paper published in 2021 identified a lead variant (rs1819040) in KAT8 regulatory NSL complex subunit 1 (*KANSL1*) at the 17q21.31 locus that associated protectively against COVID-19 hospitalization [30]. The locus was replicated with lead variants rs2532300 [22] and rs8080583 [58], which likewise demonstrated a protective association in critical cases. Degenhardt and co-workers found no association with their lead variant (rs8065800) and severe COVID-19 disease [67]. The 17q21.31 locus is known to have structural variants and has previously been linked to a mega-base inversion polymorphism [68]. Bayesian fine mapping has shown that there are approximately 1530 variants in this region that are proxies for the inversion polymorphism [69]. It is likely that all variants indicated in this region in the aforementioned studies involve this inversion. An in-depth characterization was performed on this region, with functional analysis of the variants showing associations with possible COVID-19 pathology traits associated with lung function, blood, and immune cells [67]. Furthermore, the inversion locus strongly colocalized with eQTLs and sQTLs in genes such as *KANSL1*, microtubule-associated protein tau (*MAPT*), formin like 1 (*FMNL1*), and corticotropin-releasing hormone receptor 1 (*CRHR1*), which were identified as the best candidates. *KANSL1* is expressed in lung tissue-resident alveolar macrophages, and *FMNL1* is expressed in immune cells of the nasal and bronchial tissue [67]. Both genes showed a significantly higher expression in various lung cell types in patients who died from acute COVID-19 disease [70]. The mechanism of action of these genes in COVID-19 disease outcome has yet to be determined.

The 17q21.33 locus was identified in critically ill COVID-19 cases [30] with the lead variant localized 15.5 kb upstream of tachykinin precursor 4 (*TAC4*) in a regulatory region [22]. *TAC4* is involved in regulation of B-cell lymphopoiesis [71]. It can also activate kinase pathways that enhance B-cell proliferation and antibody production [72], and it can promote DC survival [73]. The causal gene has not yet been identified although other genes such as lysine acetyltransferase (*KAT7*), distal-less homeobox 3 (*DLX3*), xylosyltransferase 2 (*XYLT2*), and *FLJ45513* have been proposed through gene annotation methods [64].

3.8 Chromosome 19

An association signal for severe COVID-19 at locus 19p13.2 was identified by the GenOMICC group with lead variant rs11085727 located close to tyrosine kinase 2 (*TYK2*) [27]. The locus has also been replicated for severe or critical illness by various other groups [22, 28, 32] including the HGI GWAS meta-analysis study which identified lead variant rs74956615 also within the *TYK2* region [30]. *TYK2* codes for a kinase that activates IFN-stimulated genes such as the OAS antiviral genes discussed earlier [74]. This kinase is also required for the secretion and release of various interleukins and T-helper cell immune responses [75] and is therefore involved in a fine balance of the cytokine response. Individuals with loss of *TYK2* function present with immunodeficiencies [76, 77], but those with low expression of *TYK2* can be protected from various autoimmune disorders [78]. Other studies have shown that low expression of *TYK2* can make individuals more susceptible to various infections due to dysregulated immune signaling [79, 80]. The rs74956615 lead variant is in strong LD with a *TYK2* missense variant that is known to reduce *TYK2* function [81, 82]. The GenOMICC group showed that individuals with severe COVID-19 had higher *TYK2* expression [27]. Therefore, there may be more than one variant in this region affecting COVID-19 outcome.

A lead variant (rs2109069) at locus 19p13.3 was identified in severe COVID-19 cases within the dipeptidyl peptidase 9 (*DPP9*) gene [27, 30, 32]. *DPP9* is a member of the serine protease family with substrates including CXCL10, CXCL11, and CXCL12, which have been induced *in vitro* by SARS-CoV-2 [83, 84]. DPP9 is also an inflammasome regulator and can downregulate the inflammasome sensor NLR family pyrin domain containing 1 (NLRP1) protein [85]. The lead variant is in strong LD with an eQTL variant in lung tissue that leads to decreased DPP9 levels [86, 87]. A functional loss of *DPP9* is associated with increased expression of interleukin (IL)-1β and IL-18 which have both been associated with severe COVID-19 disease [88].

An intronic lead variant (rs368565) at locus 19q13.33 was recently identified in severe COVID-19 cases in the fucosyltransferase (*FUT2*) gene [22, 67]. Although the mechanism of COVID-19 disease association is unclear, genetic variation in the *FUT2* gene determines secretion of ABO antigens into body fluids [89], and as previously described, the ABO blood group locus is strongly associated with COVID-19

susceptibility. Genetic variation in the *FUT2* gene has also been associated with resistance to norovirus [90] and progression of HIV-1 infection [91].

A lead variant rs1405655, also at locus 19q13.33, was found to be associated with COVID-19 disease severity [64, 67]. In-depth characterization of the locus was performed with Bayesian fine mapping, and this led to identification of several credible genes including napsin A aspartic peptidase (*NAPSA*), nuclear receptor subfamily 1 group H member 2 (*NR1H2*), and potassium voltage-gated channel subfamily C member 3 (*KCNC3*) [67]. The *NAPSA* gene is a likely candidate as it encodes a protease which may play a role in proteolytic processing of pulmonary surfactant B in lung tissue [92]. Gene expression analysis found high levels of the *NAPSA* gene in lung parenchymal tissue, and its expression has been associated with lung adenocarcinomas [93]. Studies have also shown that the NAPSA protein is significantly increased in type I alveolar cells of COVID-19 patients compared to healthy control subjects [70].

3.9 Chromosome 21

The rs2236757 lead variant in locus 21q22.1 was first identified in critically ill COVID-19 intensive care unit patients [27], and this finding has been replicated in numerous studies [28, 30, 32]. The variant was found within the IFN alpha and beta receptor subunit 2 (*IFNAR2*) gene, which is a cytokine receptor component in the antiviral type I IFN pathway, a key pathway often dysregulated in severe SARS-CoV-2 cases [15, 94]. Both the IL-10 receptor subunit beta (*IL10RB*) and *IFNAR1* genes are near the identified signals. Two missense variants in high LD with rs2236757 were identified in the *IFNAR2* gene which could explain the observed associations found in severe COVID-19 cases [32]. The COVID-19 HGE group observed 13 genes involved in the type I IFN pathway with loss of function mutations associated with critical COVID-19 disease [94]. One of the genes identified was *IFNAR2* presenting with a novel loss of function variation.

TMPRSS2 is the host enzyme used by the SARS-CoV-2 virus for membrane fusion and viral entry into the host cell [95]. Russo and co-workers postulated that genetic variants in the *TMPRSS2* gene may make an individual susceptible to SARS-CoV-2 infection [96] and explored this hypothesis using an in-depth genetic analysis of chromosome 21 by exploiting data from 7970 hospitalized COVID-19 cases from the COVID-19 HGI data release [97]. They identified five SNPs within the *TMPRSS2* gene, the minor alleles of which correlate with a reduced risk of developing severe COVID-19. One of these SNPs, rs12329760, is a coding variant and eQTL for *TMPRSS2* and has been associated with COVID-19 susceptibility by multiple independent study groups [98]. The missense variant results in the destabilization of the protein structure which inhibits viral binding [99]. Recent GWAS identified variants in the *TMPRSS2* gene, one with a protective effect (rs2298661) [31] and the other (rs915823) associated with critical COVID-19 cases [58].

3.10 Chromosome X

ACE2 is the host receptor used by SARS-CoV-2 for viral entry [100]. Recently, a novel rare variant in the *ACE2* gene was identified by the Regeneron group [32]. The variant, rs190509934, located 60 bp upstream of the *ACE2* gene, provides evidence that *ACE2* expression levels can affect COVID-19 outcomes. The variant results in a 37% reduction in *ACE2* expression which reduced the risk of SARS-CoV-2 infection by up to 40%. Although the variant is well imputed and has no significant difference in effect size across studies or ancestries, it has a stronger association with SARS-CoV-2 infection in males likely due to its location on chromosome X. Further investigation of the association between the variant and severity indicated that carriers of the variant have a lower risk of severe outcomes compared to individuals who do not carry the variant. Thus, the variant in *ACE2* can confer protection against SARS-CoV-2 infection but also possibly modify disease severity. Candidate-based gene studies have also found an association between *ACE2* variants and COVID-19 outcome [98, 101, 102].

The COVID-19 HGE group postulated that some patients with life-threatening COVID-19 may have monogenic inborn errors of immunity to SARS-CoV-2, specifically in genes found in the IFN I pathway [94]. The study identified an enrichment of predicted loss of function variants in eight candidate gene loci from the IFN I pathway in 3.5% of critical COVID-19 patients. TLRs play an essential role in the initiation of the innate immune response as discussed earlier and are especially important in the production of pro-inflammatory cytokines and type I and II IFNs. TLR7 has been shown to recognize single-stranded RNA viruses such as SARS-CoV-2 [103].

A candidate gene-based WES study performed on four young male individuals with critical COVID-19 disease identified loss of function variants in the X-linked *TLR7* gene [104]. Functional analysis illustrated a downregulation of type I IFN from primary immune cells and a decrease in mRNA expression of various type I IFN pathway genes. This prompted a nested case-control study to compare critical COVID-19 male patients with asymptomatic controls [105]. The results of the study indicated that approximately 2% of severely infected male patients have loss of function variants in *TLR7* that causes decreased *TLR7* gene expression and a defective IFN I and II response. Similar results were found in young male patients with extreme COVID-19 phenotypes by other independent study groups [106, 107]. The Regeneron group performed an exome-wide association study in 586,157 individuals including 20,952 with COVID-19 to discover associations between rare variants and COVID-19 outcomes. They found no significant associations with rare protein-coding variants and COVID-19 disease [108]. However, a recent study using HGI results which included 5085 severe cases and 571,737 controls found that the *TLR7* gene was an important determinant of severe COVID-19, and despite its location on chromosome X, the finding was significant across the sexes [109].

4 Potentially Relevant Loci/Genes

This chapter has thus far discussed the host genetic findings associated with COVID-19 outcomes mainly from larger study groups, focusing on associations that have been replicated in more than one study or dataset (Fig. 6.2). There are additional loci identified which have not been replicated but are worth mentioning since the genes they encompass may influence COVID-19 pathology. One such signal is the 5q31.1 locus which was identified in critical COVID-19 cases with fine mapping, indicating an intronic variant affecting expression of the acyl-CoA synthetase long chain family member 6 (*ACSL6*) gene as well as a missense variant in the colony stimulating factor 2 (*CSF2*) gene [22]. The *CSF2* gene encodes granulocyte-macrophage colony stimulating factor (GM-CSF) which has been found to be significantly upregulated in critical COVID-19 patients [88].

A locus (10q22.3) identified in the recently published COVID-19 HGI paper was discovered in hospitalized cases. The lead SNP implicates a missense variant in the gene for surfactant protein D (*SFTPD*), which encodes the surfactant D protein (SP-D) [64]. The SP-D is involved in pathogen clearance by enhancing uptake by phagocytes and thereby maintaining healthy lung function. It regulates both innate and adaptive immunity, specifically DCs, macrophages, neutrophils, and T cells [110]. SP-D knockout mice have increased inflammation and susceptibility to infection [110] with studies showing that recombinant protein fragments can bind to the SARS-CoV-2 spike protein and potentially inhibit binding to the ACE2 receptor, thereby reducing viral infection [111]. Additionally, the gene was associated with reduced lung function and increased COVID-19 severity.

A promoter variant in the mucin 5B (*MUC5B*) gene was identified at locus 11p15.5 as a protective variant in hospitalized patients with severe COVID-19 [64]. This variant is known to increase *MUC5B* expression in the lung and has been strongly associated with increased risk of idiopathic pulmonary fibrosis (IPF) [112], while improving survival rates of these patients by up to twofold [113]. IPF is a non-infectious lung disease that usually occurs in older individuals and has been associated with progressive lung scarring [114]. *MUC5B* deficiency in mice can lead to chronic infection and inflammation due to decreased mucociliary clearance which may exacerbate lung fibrosis [115]. A retrospective candidate gene-based case-control study illustrated that this variant confers protection against hospitalization from COVID-19 disease [116]. Since *MUC5B* is essential for mucociliary clearance and infection control, the increased expression of the protein may provide a mechanism of protection in airway infections such as COVID-19 [115].

HLA plays an important role in antigen binding and recognition and in eliciting an efficient immune response, with different HLA variants known to affect infectious disease outcomes [117]. The GenOMICC group identified variants in *HLA-G* associated with critical illness [27], and this finding was replicated in the HGI data release 5 although a high degree of heterogeneity was observed [30]. HGI data release 6 identified variants in *HLA-DPB1* associated with susceptibility to infection. Apart from GWAS, smaller HLA-based target studies with high HLA

resolution have identified variants associated with COVID-19 outcome [118]. Nguyen and co-workers assessed 145 known HLA types in silico against SARS-CoV-2 proteins and found that *HLA-A*25:01*, *B*46:01*, and *C*01:02* bind fewer SARS-CoV-2 viral peptides and are thus more likely to have reduced viral antigen presentation, while *HLA-A*02:02*, *B*15:03*, and *C*12:03* have higher binding affinities [119]. The HLA region is highly complex and has significant variation among different ancestries and so was not included in the genes discussed above in which findings were replicated in different studies.

Other GWAS lead variants of possible significance in COVID-19 disease outcomes include BAF chromatin remodeling complex subunit (*BCL11A*) involved in leukocyte differentiation, member RAS oncogene family (*RAB2A*) involved in viral replication, and Janus kinase I (*JAK1*) which is a kinase required for the type I IFN pathway [22, 58]. The latest GenOMMIC study was also the first to identify coagulation and platelet activation genes, coagulation factor 8 (*F8*), and platelet-derived growth factor receptor like (*PDGFRL*), with possible causal roles in critical COVID-19 outcomes [58].

5 Global Approach to COVID-19 Host Genetics

Most COVID-19 genetic studies have been performed in the European population group. Some population groups, such as those of African descent, have been understudied [120]. It is imperative to include other population groups when analyzing the impact of genetic variation on COVID-19 outcomes, since this may be population-specific [121]. For example, risk allele frequencies of genetic variants involved in several complex diseases, including cancer, stroke, and type 2 diabetes, have been shown to vary greatly between European and non-European population groups, potentially influencing the prevalence and incidence of these diseases [122]. Additionally, some monogenic diseases, such as cystic fibrosis, are caused by different variants in the same gene, which differ in prevalence between population groups [123].

The 3p21.31 risk locus, the most consistent association identified for COVID-19 outcomes, is based on a 50 kb genomic segment inherited from Neanderthals, carried by around 50% of people in south Asia, 16% of people in Europe, but is absent in African and east Asian individuals [26].

The *OAS* gene haplotype on chromosome 12, associated with a ~22% reduction in relative risk of becoming severely ill with COVID-19, is also inherited from Neanderthals. This haplotype is present at substantial frequencies in all regions of the world outside of Africa [59].

Novel associations with other genes have been found in diverse population groups. The Japan COVID-19 task force identified a population-specific risk variant in the dedicator of cytokinesis 2 (*DOCK2*) gene, which is involved in chemokine signaling, type I IFN production, and lymphocyte migration. This variant is either absent or present at a low concentration in other population groups [124].

It has also been observed that *ACE2* and *TMPRSS2* expression varies across populations. East Asians presented with the highest level of *TMPRSS2* expression, while Africans had the lowest [125]. The recently discovered *ACE2* variant (rs190509934) that confers protection against SARS-CoV-2 infection is also ten times more common in the south Asian population compared to Europeans [64].

Three independent signals were found for the locus on chromosome 1 implicating the *MUC1* gene, one of which was only discovered in multi-ancestry analyses [22]. Similarly, COVID-19 HGI meta-analysis only identified a severity-associated locus at *FOXP4* by including multiple ancestries. The variant (rs1886814) is found at a low frequency in the European population (<3%) but at high frequencies in east Asians (32%) [30].

Clearly, further investigations of understudied population groups are likely to provide a more comprehensive insight into the clinical heterogeneity of COVID-19. For instance, the increased genetic variation and consequential increased presence of rare variants in the African population may help in the discovery of rare variants influencing COVID-19 susceptibility and outcomes.

6 Conclusion

The rapid dissemination of findings and collaborative efforts that have accompanied the COVID-19 pandemic have increased our understanding of disease etiology and provided routes for management of COVID-19. Mounting an efficient early innate immune response to COVID-19 disease has been highlighted as crucial to COVID-19 outcomes. In particular, the IFN-related genes (*IFNAR2*, *TYK2*, *IL10RB*, *PLSCR1*, *IFNA10*, and *JAK1*) make up a significant number of COVID-19-host genetic associations identified. IFN [126], TYK2, and JAK1 therapies for COVID-19 [127, 128] are already underway in clinical trials. Genes involved in inflammation resulting in lung injury (*DPP9*, *TYK2*, *TLR7*) as well as those that have previously been linked to lung diseases (*DPP9*, *FOXP4*, *SFTPD*, and *MUC5B*) such as interstitial lung disease, lung fibrosis, and lung carcinoma, have also been associated with severe COVID-19 outcomes.

Although important insight into disease pathogenesis has been obtained, there is an overrepresentation of cases with severe outcomes in many of the studies. This type of approach has resulted in clarifying susceptibility or severity associations such as in the case of the *ABO* gene locus and has aided in isolating the third independent signal on the 3p21.31 locus associated with a protective effect. However, few studies [31, 32] have focused on genetic analysis of individuals with asymptomatic or milder phenotypes. Additionally, most of the GWAS studies have used control cohorts which include population controls or people who have not been screened for COVID-19. This has led to misclassification of the COVID-19 genetic outcomes such as the ABO locus which was originally associated with severity. More recent studies have used COVID-19-tested controls which have shown more robust COVID-19 gene associations.

Genes involved in the immune response to the virus represent the majority of identified genetic determinants of COVID-19 outcomes. Host viral entry (*ACE2*) and replication (*RAB2A*) genes have also been identified.

In conclusion, this chapter describes the association between COVID-19 disease outcomes and host genetic variation and describes molecular mechanisms underlying an inherited predisposition to SARS-CoV-2 infection and COVID-19 severity. Further studies are needed to identify the exact causal genes and to understand the functional mechanisms underlying many of the associations described.

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Chapter 7
COVID Diagnostics: From Molecules to Omics

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Abstract The identification and genetic sequencing of a novel coronavirus was key to the diagnosis and management of the global pandemic. An understanding of the SARS-CoV-2 structure and mechanism of injury is vital to explaining the disease course and the pathophysiology of the signs and symptoms observed. This particularly as the presentation, disease course, and severity are noted to be highly variable. The role of the spike protein and angiotensin-converting enzyme 2 (ACE-2) receptor in immune response and viral entry provides great insight into current and future diagnostics and therapeutics. This article reviews the traditional diagnostic methods, which include molecular testing methods, antigen testing, and antibody testing. The gold standard for diagnosis of COVID-19 is reverse transcriptase polymerase chain reaction (RT-PCR). There have been multiple improvements to these principles to help optimize the sensitivity, specificity, and user friendliness of the method. In addition, advancements in gene sequencing and identification have been integral to identifying variants and managing outbreaks. Serological and immunological testing have made significant contributions to the management of the COVID-19 pandemic, each with its unique benefits and limitations. A growing role of the laboratory is in triaging patients to determine which patients will most benefit from hospitalization and specialized care. This is imperative for rationalizing resources during outbreaks. As we learn to live with the pandemic, novel testing methods include the use of multiomic technologies and the greater utility of point of care.

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1 Introduction

In December 2019, Chinese health authorities identified an outbreak of pneumonia of unknown origin with high mortality, which raised intense concern not only in China but also internationally as well. In attempts to control the spread of the disease, Chinese authorities isolated infected people and monitored close contacts. They characterized the clinical presentation and sought to develop diagnostic and treatment modalities. By January 2020, they isolated a novel coronavirus, and the genetic sequencing of this virus [1] enabled the development of molecular tests specific for the virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

The disease spreads rapidly, and the World Health Organization (WHO) declared it a global pandemic in March of 2020, with more than 100,000 cases and 4000 deaths reported worldwide at that time [2]. In Africa, the first case was reported in February 2020, and by March of the same year, cases were reported from across the continent. To date, over 650 million cases have been reported globally, with over 6 million deaths.

2 Transmission and Pathogenesis

SARS-CoV-2 belongs to a group of viruses of the *Betacoronavirus* genus, which includes SARS Co-V and Middle East respiratory syndrome coronavirus (MERS-CoV). SARS-CoV-2 shares 75–80% of its viral genome with SARS-CoV [3]. It is an enveloped, single-stranded RNA virus and has four structural proteins: the spike, nucleocapsid, membrane, and envelope proteins. These proteins play a key role in the pathogenesis of disease. The spike protein is used for viral entry via the angiotensin-converting enzyme 2 (ACE2) receptor and causes membrane fusion which is important for viral entry into cells [4–6]. The spike protein is also the primary target of neutralizing antibodies and the focus of vaccine development.

The major route of transmission is from infected patients via respiratory droplets and possibly contact with fomites and when aerosols are generated during medical procedures like endotracheal intubation [7, 8]. Transmission can occur in asymptomatic people and during the early incubation phase [9]. Viral load in the upper respiratory tract appears to peak around the time of symptom development, with viral shedding starting 2–3 days before the onset of symptoms [10]. Presymptomatic transmission is thought to be a major route for the spread of infection, with

modeling studies estimating transmission rates ranging from 48% to 62% [11]. While perinatal transmission from mother to babies can occur, this is rare [12].

Disease presentation is varied, with many patients remaining asymptomatic or having mild disease and quick recovery. The most common symptoms are flu-like with a sore throat, fever, cough, muscle pains, and headache [13]. In severe cases, patients may go on to develop a pneumonia and then acute respiratory distress syndrome (ARDS) [14].

As severe COVID-19 presents with multisystem involvement, the role of the laboratory is key in not only the diagnosis of the disease but also in detecting system involvement and in monitoring the disease [15–20] (Table 7.1). Reported cases of COVID-19 infection and death appear to be far less in Africa compared to the rest of the world [21]. This may be due to a number of factors such as the relatively young population and perhaps unexplored protective genetic factors [22]. It may also be a result of underreporting as testing capacity is less in South Africa than many other areas in the world. Large swathes of the population live in rural areas where communities may have limited access to healthcare facilities. Risk factors for COVID-19 include older age, obesity, diabetes, hypertension, and existing kidney disease. There is some evidence that human immunodeficiency virus (HIV) and tuberculosis, both of which are major causes of death in sub-Saharan Africa, increase risk for morbidity and mortality from COVID [23, 24]. With limited healthcare resources, it is important to look at rapid diagnostic tests for COVID-19 as well as for complications of the disease. This review highlights traditional diagnostic and point of care tests for COVID-19 and related diagnostics, as well as the potential role of "Omics" in the laboratory management of this disease.

3 COVID-19 Diagnosis

3.1 Traditional Diagnostic Methods

SARS-Co-V-2 diagnosis is based on the clinical suspicion, laboratory investigations, and imaging modalities. Laboratory testing for the diagnosis of SARS-CoV2 is based on the identification of viral nucleic acid, antigen, or host-antibody responses. Table 7.2 summarizes these tests and their clinical utility.

Molecular tests allow for viral RNA detection by using nucleic acid amplification and detection techniques [25]. Among these and widely used globally is real-time reverse transcriptase polymerase chain reaction (RT-PCR). The Wuhan scientists isolated the virus from a bronchoalveolar lavage specimens and used a combination of molecular techniques including Sanger, Illumina, and nanopore sequencing to establish the complete genome.

The principle of molecular testing is that different genome regions are used to develop primers and probes for the PCR tests. Targeted regions of the viral genome include the RNA polymerase region, spike, nucleocapsid, and envelope proteins

Table 7.1 System involvement and their laboratory tests

System involved	Severe disease presentation	Pathophysiology	Diagnostic test
Pulmonary	Severe hypoxemia Acute respiratory distress syndrome (ARDS) Respiratory failure and death (if untreated)	Endothelial barrier disruption and impaired oxygen diffusion capacity are characteristic features of COVID-19 in the respiratory system. Early stage of SARS-CoV-2 infection targets the nasal and bronchial epithelial cells and pneumocytes. Later, SARS-CoV-2 infects pulmonary capillary endothelial cells, triggering an inflammatory response. There may be activation of the coagulation cascade and disseminated intravascular coagulation	X-rays: ground glass opacities Blood gas: decrease pO2 [15]
Liver	Generally mild disease	Cause may be multifactorial: direct viral cytotoxicity, immune mediated, vascular changes due to coagulopathy, congestion following right sided heart failure, drug induced	Elevated bilirubin and liver enzymes [16]
Cardiac	Cardiomyopathy, heart failure Myocardial injury		ECG changes Elevated cardiac troponins Natriuretic peptides Elevated cardiac enzymes [17]
Kidney	Acute kidney injury Renal failure	Direct cytopathic effect Inflammatory mediated Complement activation Disseminated intravascular coagulation Rhabdomyolysis Organ cross talk e.g., hepatorenal syndrome Volume depletion	Elevated urea and creatinine Proteinuria, or albuminuria Abnormal blood electrolytes [18]
Vascular	Large vessels emboli Disseminated intravascular coagulation	Activation of renin angiotensin system cytokine storm	Elevated D dimers Low platelets Prolonged APTT and INR [19]
Neurological	Meningoencephalitis Seizures Cerebrovascular accidents Guillain-Barre syndrome Coma	Direct infection of neurons via ACE2 receptor Endothelial damage and hypercoagulation Immune mediated cytokine storm	CSF positive for SARS-CoV2 [20]

Table 7.2 Traditional diagnostic tests and their clinical utility

	RT-PCR	Antigen detection	Antibody detection
Specificity	Highly specific in *acute SARS-CoV-2	Specific RT-PCR is required in negative results	Variable - dependent on kit Indicates current or past infection
Technical requirements: Equipment Personnel Site	Requires expensive equipment and reagents		
	Highly skilled technicians	Minimal technical skills required	Minimal technical skills required
	Centralized laboratory testing	Can be done within hospital/ at point of care	Can be done within hospital/at point of care
Turnaround time	Extended	Short	Short
Advantages	Sensitive – Early diagnosis Specific	Scalable Can be automated Specific	Scalable Can be automated
Disadvantages	Non-automated Long Turnaround Times	Cross reactivity with related coronaviruses Poorer Sensitivity Variable performance depending on kit	Cross reactivity with related coronaviruses Variable performance depending on kit
Sample type	Naso/Oro pharyngeal Swab	Naso/Oro pharyngeal Swab Blood	Blood

[26]. This method is considered the gold standard. Its high sensitivity and specificity make it a good choice during the early phase of diagnosis when the viral load is low, with the diagnostic window preceding the onset of symptoms [25]. It does, however, require technical expertise and sophisticated equipment, requiring a laboratory environment to process the samples. The quality of the sample is imperative, and factors including sample type, collection, transportation, and storage can affect test performance [27]. These constraints result in a longer turnaround time which impacts service delivery and patient outcomes especially during periods of high demands like during an upward trend in infections, often termed a "wave."

There have been many advancements on the principles of RT-PCR which have significantly improved the utility of this test in diagnosis. These include techniques like the use of isothermal detection, next-generation sequencing (NGS), clustered regularly interspaced short palindromic repeats (CRISPR), and digital PCR. Isothermal amplification and detection techniques accumulate nucleic acids at a constant temperature, unlike traditional PCR which requires cyclic temperature changes. When combined with simpler readout methods and microfluidics, this has resulted in portable, accessible, and easy to use devices [26]. One such example is loop-mediated isothermal amplification (LAMP), which has been utilized widely in COVID-19 diagnostics. Advantages of this method include a greater yield than RT-PCR, it eliminates the need for sophisticated equipment, and it is cost-effective, easy to use, and accurate [2]. Studies evaluating its utility compared to the gold standard of RT-PCR have demonstrated excellent sensitivity of up to 97% [25].

NGS has been critical in the evolution of COVID-19 diagnostics. It allows for the description of the entire SARS-CoV-2 genome and therefore has been used to detect changes to the genome and identify emerging molecular variants. Its utility is confined to surveillance and epidemiology due the cost and technical requirements, but it has been key in managing the pandemic worldwide. Improvements in the NGS methods include amplicon-based metagenomics sequencing [26].

Clustered regularly interspaced palindromic repeats (CRISPR) technology is based on genome editing systems normally found in bacteria. It uses the collateral cleavage activity of endonucleases for viral nucleic acid detection [26]. Advantages of CRISPR over routine PCR-based methods include speed, sensitivity, specificity, and user-friendliness. The Sherlock CRISPR SARS-CoV-2 kit is the first CRISPR-based test to be used in patient testing that is US Food and Drug Administration (FDA)-approved [27].

Digital PCR is an improvement on routine PCR in which smaller volumes of sample are used. Droplet digital PCR uses the principles of micro-partitioning and ultra-dilutions. Each PCR reaction is conducted in multiple discrete replicate droplets and then detected by fluorescence [26].

Serological and immunology tests also have an important role in the COVID-19 healthcare response [28]. This can be by viral antigen detection or the patient's response to infection via antibody detection [29].

Tests that detect viral antigens can be utilized for diagnosis. Although these are less sensitive than the molecular testing, they have the potential to provide results quicker and cheaper and are, therefore, useful in settings where an urgent result is needed [30]. Antigen testing allows rapid identification of possible cases to help curb transmission. This includes fit for traveling and resumption of school or work, identifying patients who pose a risk of spreading infection, and in cases where laboratories are unable to keep up with the demand of molecular testing [25].

Antibody testing can be considered to provide indirect evidence of viral exposure at least within the past 1–2 weeks, and antibodies can persist up to 6 months [28, 30]. These rely on the detection of antibodies (IgG, IgA, IgM, and/or total antibodies), which may be specific for the receptor binding domain, nucleocapsid protein, spike protein, or both nucleocapsid and spike proteins of the SARS-CoV-2 virus. IgA levels increase early (within 1 week of symptoms) but usually decline rapidly within a few weeks. IgM levels also increase rapidly but decrease early on in the disease course. IgG levels can peak within 1–2 weeks but are valuable in that they can remain increased for up to 6 months [28]. Different assays detect any one or a combination of these antibodies, so it is vital to understand the characteristics of the test being used to aid interpretation of the findings.

While these antibody tests are inadequate for diagnosis, they can be useful in epidemiologic studies, surveillance, and vaccine development, as well as being useful for screening healthcare workers [27]. They allow for the evaluation of seroprevalence, which indicates if our control and containment measures have been effective [28]. The stability of human antibodies is thought to be superior to viral RNA, especially when considering pre-analytical issues like sample type, collection, transport, and storage [25]. This makes serological testing a good alternative in certain circumstances.

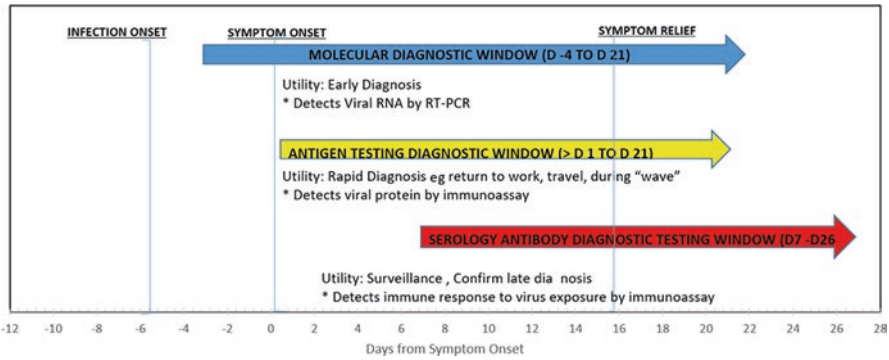

Fig. 7.1 Traditional tests: Diagnostic window and utility

Many methodologies have been approved by the WHO, ranging from manual assays to highly automated assays. These include enzyme-linked immunosorbent assays (ELISA), Western blot, immunofluorescence assays (IFAs), chemiluminescence assays (CLIAs), and protein microarrays [25, 26]. A benefit of antigen and antibody testing is the scalable nature of the testing which allows laboratories to meet the demands for testing during the different stages of a pandemic.

A limitation of the serological assays is related to the potential for cross-reactivity between antigens of SARS-CoV-2 and related coronaviruses like MERS-CoV [26]. The specificity has been reported to be from 96 to 100%. The lag between infection onset and the finding of a positive test limits its utility for early diagnostics. Studies to date have demonstrated that 5% of symptomatic and up to 40% of asymptomatic PCR-positive patients can remain seronegative [27]. It also remains to be determined what the effect of widespread vaccination drives will be on interpretation of the serology tests.

The clinical utility of these tests varies with time from infection, and this is summarized in Fig. 7.1.

4 COVID-19 Risk Biomarkers

The management of SARS-Cov-2-infected patients entails using biomarkers to aid in the diagnosis, prognostication, stratification, and therapeutic intervention, as well as monitoring and assessment of long-term COVID-19 sequelae. The mortality and severe morbidity associated with infection by this virus have been associated with many risk factors. A systematic review by Dessie et al. reported that chronic noncommunicable diseases, age, demographic variables, and lifestyle behavior were significant risk factors for severe disease and mortality [31]. Despite the earlier assertion that COVID-19 brings about a respiratory disease, the mortality has been linked to multiorgan dysfunction, which is secondary to viral infection and the immune response. Thus, early detection of organ dysfunction can help to mitigate

disease severity and aid in choices of therapeutic interventions for systemic SARS-CoV-2 disease.

The pathophysiology for the multiorgan failure with the SARS-Cov-2 virus is variable and is organ- and system-specific [32–35] (Table 7.3). Most organ failures are secondary to the overt immune response and direct infection of the cells by the virus. For instance, COVID-19-associated coagulopathy is associated with endothelial damage secondary to the inflammatory response. Some studies have indicated that the magnitude of the humoral response is proportional to disease severity.

The use of biomarker-based tests can aid in identifying organ involvement and can be used for risk stratification. The use of certain biomarkers has not been consistent across all studies. Hyperferritenima is marked by high levels of ferritin, a positive acute-phase protein associated with inflammatory disease, multiorgan dysfunction, and overt infections. Nonetheless, the evidence for its use in prognosticating patients has been inconsistent. Williams et al. reported that serum ferritin did not predict mortality in sepsis, although some studies looking at COVID-19 demonstrated that ferritin was a good prognostic marker [36, 37]. Most biomarkers follow different patterns depending on the phase/time since seroconversion. For instance, full blood count markers such as white cell, neutrophil, and platelet counts reach a nadir by day 8–9 of illness and subsequently improve. Therefore, these markers can be used in the first week of life to predict prognosis, and after 14 days, the increase in these markers can be used to assess recovery.

4.1 Risk Stratification and Prognostication of COVID-19 Patients

The limited hospital and critical care beds in resource-restricted African countries necessitated using prediction models to ensure timely intervention and deployment of true distributive justice. Disease stratification and prognostication are based on clinical presentation, medical history, bedside investigations (vitals and electrocardiography), radiological findings, and biochemical evidence of impending organ failure. Symptoms such as cyanosis, shortness of breath and altered mental status, and signs like SpO2 <94%, respiratory rate >30/min, systolic blood pressure <90 mm Hg, or other signs of shock or complications are associated with high risk. High-risk and severe disease patients require urgent hospitalization, and critical care is needed in extreme cases. Many institutions, including the WHO, have provided algorithms to ensure quick and efficient patient triaging during a crisis. A scoring system is mandatory to assist resource allocation in a resource-limited setting.

In South Africa, the sequential organ failure assessment (SOFA) score was adopted in many high care and intensive care units. This scoring system determines the level of organ dysfunction and mortality risk in ICU patients. The score was first reported by Vincent et al. and has 0 to 4 points assigned to each of 6 organ systems based on several analytes and the Glasgow coma scale [38]. Thus, the SOFA score

Table 7.3 Pathophysiology of multiorgan failure associated with the SARS-Cov-2 virus

Organ/system	Pathogenesis	Biomarkers
Respiratory	Bronchopneumonia and acute respiratory distress syndrome are common pulmonary presentations. The ventilation and perfusion abnormalities are due to the following Direct viral infection of the bronchial epithelial cells and the alveolar type I and type II pneumocytes Inflammatory response Activation of coagulation and formation of microthrombi Vascular permeability due to lack of ACE 2 receptors and inflammatory response Atelectasis, Pulmonary oedema and fibrosis	Blood gas: pO_2, pCO_2, bicarbonate Neuron specific enolase Lactate dehydrogenase Metabolomics markers: peroxisome proliferator-activated receptors PPAR, D-arginine, D-ornithine, TRP, alpha-linoleic [32]
Hematological	COVID-associated lymphocytopenia is secondary to the direct infection of cells by the virus via the ACE receptors, resulting in cell death. The cytokine storm is also linked to cell apoptosis. Cytokine storm-induced atrophy of lymphoid organs and reduced lymphocyte proliferation due to lactic acidosis	High Neutrophil: lymphocyte ratio Peak platelet/lymphocyte ratio Thrombocytopenia Lymphopenia Neutrophilia [33]
Coagulation	The patients are prone to venous thromboembolic events (VTE) and disseminated intravascular coagulopathy (DIC). Endothelial dysfunction is secondary to the virus binding to the ACE 2 receptor and the release of inflammatory mediators, which result in increased blood viscosity.	Marked prolongation of PT and aPTT elevated d-dimer Elevated fibrinogen [34]
Inflammation	The virus triggers host and innate immunity responses upon entry into the host cells. Neutrophils are recruited, and these release cytokines. The cytokine response leads to a wide spectrum of systems dysfunctions	Elevated CRP Elevated IL-6 Neutrophilia Elevated ESR (erythrocyte sedimentation rate) Elevated serum ferritin Elevated PCT Omics: microRNA
Cardiac	Cardiac dysfunction is attributed to the direct viral invasion of cardiomyocytes, secondary to VTE, and the immune-mediated response. The cardiac complication noted are namely myocarditis, cardiac failure, cardiomyopathy, acute myocardial infarction, arrhythmias and cardiac arrest	Elevation in cardiac Troponin I and T Elevation on NT ProBNP /or BNP [35]
Musculoskeletal	Direct viral infection of cells and immune response affect the myocytes. Severe myositis	Creatine-kinase (CK) Myoglobin

(continued)

Table 7.3 (continued)

Organ/system	Pathogenesis	Biomarkers
Hepatic	Direct virus infection of hepatocytes, endothelial damage secondary to cytokine storm, tissue hypoxia, and VTE result in hepatobiliary dysfunction. The decreased synthetic function increased capillary permeability and increased turnover of albumin, resulting in hypoalbuminemia,	Elevated transaminases: AST and ALT Hypoalbumin Elevated lactate dehydrogenase Elevated bilirubin
Renal	Kidney damage is mainly an acute kidney injury that may lead to chronic nephropathy. The pathophysiology of kidney dysfunction in SARS-CoV-2 is due to direct nephron infections, endothelial vasculitis, VTE and hypoxia of the kidney cells	Creatinine Neutrophil gelatinase-associated lipocalin (NGAL), Cystatin C, Kidney injury molecule-1 (KIM-1), Urine protein creatinine clearance
Electrolytes	The reduction in aldosterone activity is secondary to drugs such as chloroquine. Hypokalemia is secondary to GIT losses, increased angiotensin II and kidney disease. Hyponatremia is due to SIADH secondary to cytokines noted in pneumonia and ARDS Hypocalcaemia is attributed to lower intracellular calcium, as two Ca^{2+} ions bind to the SARS-COV-2 fusion peptide	Hyponatremia/hypernatremia Hypokalemia Hypocalcaemia

ranges from 0 to 24 points, and higher scores indicate worse organ function. However, this scoring system was deemed ineffective during the early phase of pandemic, as COVID-19 was hypothesized to be a single-organ dysfunction disease.

In developing countries, the need to decide the level of care is critical due to the limited availability of critical care units. The scoring tools used to evaluate the patients and decide the patient therapeutic plans require a number of biochemical and hematological analytes. These can be measured on point of care devises thus allowing for rapid triage of patients.

4.2 Cytokine Testing

The SARS-CoV-2 infection triggers both innate and adaptive immune responses, with a characteristically excessive pro-inflammatory response of the innate immune system [39]. Adding on to this, the dysregulated host response of the adaptive immune system can lead to tissue damage. As a result, a massive amount of cytokines and chemokines are released, mainly interleukins 2 and 6 (IL-2 and IL-6) as well as tumor necrosis factor (TNF-α). This cytokine storm is a hallmark of severe SARS-CoV-2 infection, and the cytokines released can cause endothelial damage, hypercoagulability, alveolar damage, and multiorgan failure [40].

Table 7.4 Clinical utility of IL-6

Clinical utility	IL-6 levels
Assessment of severity	Increased
Response to therapy	Decreases
Predicting outcome	Variable
Multisystem inflammatory syndrome in children	Increased
Cytokine storm	Increased

IL-6 is a circulating multifunctional 26 kDa protein consisting of 26 amino acids. It has a pro-inflammatory function and may be acutely elevated in COVID-19 patients. IL-6 stimulates production of acute phase proteins, acts as a maturing agent for B lymphocytes, stimulates the synthesis of immunoglobulins, induces proliferation of T cells, and activates natural killer cells. In COVID-19, IL-6 levels follow a temporal course with a peak between 7–14 days post-infection [41]. The levels of IL-6 with other cytokines may remain elevated for 4 weeks post-infection in severe cases [42]. IL-6 levels can be used for prognostication, with higher IL-6 baseline results correlating with severe, bilateral interstitial involvement, in keeping with other acute inflammatory markers [43]. IL-6 may also be useful in monitoring therapeutic response [44] (Table 7.4).

Another cytokine of clinical importance in COVID-19 is TNF-α. Active TNF-α is a pro-inflammatory homotrimer of 17 kDa polypeptides with a total molecular weight of 52 kDa. It is produced by activated macrophages, monocytes, T lymphocytes, and natural killer cells. TNF-α mediates and regulates development of the immune system, proliferation, cell survival signaling, and metabolic processes, as well as apoptosis [45]. Elevated serum TNF-α was found in patients with severe COVID-19 and in those admitted to the ICU and with poor clinical outcomes [46]. Together with measurements of IL-6, TNF-α was shown to be predictive of COVID-19 disease severity and mortality. The role of TNF-α in disease pathogenesis has also highlighted a potential role for anti-TNF-α therapeutics. This therapy aims to reverse TNF-induced immunopathology to improve the prognosis of COVID-19 patients. Therefore, measurements of TNF-α have a potential role in monitoring disease severity and prognosticating in COVID-19 patients [47]. TNF-α can be measured using flow cytometry, ELISA, and chemiluminescence as well as by microfluidic methods.

IL-10 is produced by regulatory T cells and T helper 1 cells for immunoregulation and as part the inflammatory response. IL-10 may be pro-inflammatory and immune-activating in COVID-19 pathogenesis. Studies have shown that patients with elevated IL-6 also have higher circulating levels of IL-10 and TNF-α. This relationship was observed in COVID-19 patients with severe disease and positively correlated with mortality. Therefore, IL-10 has been identified as a disease severity and mortality biomarker in COVID 19. Similar to TNF-α, IL-10 is a potential target for therapeutic intervention to reduce mortality in SARS-CoV-2 infections [47].

The use of point of care testing (POCT) detection of cytokines has become imperative in the context of COVID-19 as it offers rapid assessment of disease severity. The POCT cytokine measurement allows for early diagnosis and monitoring of the cytokine storm in particular. Cytokines in the context of COVID-19 have been measured mainly in serum or plasma in clinical practice [48]. However, they can also be measured in matrices such as whole blood, interstitial fluid, and cerebrospinal fluid. Methodologies that are in use for cytokine detection currently are immunoassays, including electrochemiluminescent multiplex immunoassays and enzyme-linked immunosorbent assays (ELISA). Immunoassays are often automated in a central laboratory and thus not suitable for near patient testing. Also, the interpretation of results is challenging due to differences in method standardization and potential errors due to the presence of cytokine binding proteins and variable cytokine forms [49].

Commonly available POCT designs have been used in cytokine measurements. These include colorimetric lateral flow assays, fluorescence lateral flow assays, electrochemical impedance spectrometry spectroscopy, and field effect transistors [49]. Improvements on POCT devices have been made possible by the use of biosensors for the detection of cytokines. These use biochemical reactions and bioelectronic technologies for quick and reliable detection of pathogens [26]. Biosensors also allow for improved analytical sensitivity, analysis time, and smaller sample volume and offer multiplex detection [49].

5 The Use of Multiomics in Understanding SARS-CoV-2 Infection

Multiomic technologies have been used to describe the viral genotype and the pathogenesis of SARS-CoV-2. For example, NGS allowed the original identification of SARS-CoV-2 as well as the origins of the virus [50, 51]. It was then possible to develop RT-PCR tests for diagnostic use. This was subsequently followed by parallel detection of SARS-CoV-2 and other viruses that cause respiratory tract infections [52, 53].

The areas of research included in the omics field include proteomics, transcriptomics, genomics, metabolomics, lipidomics, and epigenomics, which allow parallel and comprehensive analyses of proteins, RNA, genes, metabolites, lipids, and methylated DNA or modified histone proteins in chromosomes, respectively.

Genomics has enabled understanding and diagnosis of SARS-CoV-2 from first identification to current identification of mutant strains. The African continent was not left out in these developments, as there is now genomic-based surveillance for COVID-19 informing diagnostic tests and vaccines. This surveillance was based on genome sequencing of the SARS-CoV-2 virus and has enabled Kenya and South Africa to delineate imported cases involving community transmission. These findings were also crucial to direct public health policies and containment responses to the COVID-19 pandemic in the early stages [54].

Table 7.5 Postulated transcriptome-based immune profiling implications

Method	Findings	Profiling implication
Viral transcriptome analysis	41 sites of RNA 5 – methyl cytosine modification	Instability of viral RNA's and immune escape [55]
Single cell transcriptomes	Upregulated Squamous epithelial cells ANXA1, S100A8 and S100A9 with upregulated Neutrophil and Macrophages FPR1 and TLR4	Clarify immune characteristics and mechanisms resulting in the cytokine storm [56]
Sequencing non coding RNA and mRNA	miR-146a-5p; miR-21-5p; miR-142-3p; miR-15b-5p were related to the severity of COVID 19	Heterogeneity of COVID 19 and classifying COVID 19 severity [57]
Plasma multiomics	Dysfunctional $S100^{high}$ $HLA\text{-}DR^{low}$ monocyte subpopulation is related to COVID 19 severity	Differentiation between levels of severity in COVID 19 [58]

Transcriptomics has been enabled by the progression in sequencing technology. The genomic transcriptome analysis of the SARS-CoV-2 has elucidated gene expression information of the virus and an understanding interaction of the virus with the host. Importantly, it has allowed for immune profiling as illustrated in Table 7.5 [55–58] and for understanding the pathogenesis as illustrated in Table 7.6 [59–62].

Metabolomics which studies small molecules with a relative molecular weight of less than 1000 Da has also been applied in the study of COVID-19. Through quantitative analysis of metabolites, their mechanistic relationship with physiological and pathological changes has been explored. Techniques used include ultraperformance liquid chromatography/tandem mass spectrometry and multiomic approaches, such as combined metabolomic and lipidomic profiling. One study found that a plasma lipid monosialodihexosyl ganglioside (GM3) was inversely associated with CD4+ T cell count in COVID-19 patients [63]. The study suggested that GM3-rich exosomes may be involved in the pathogenesis of COVID-19 by affecting microenvironmental homoeostasis. This study also identified an association between GM3-enriched exosomes and COVID-19 severity. Such findings can inform development of diagnostic assays to detect small changes in GM3 with the potential value for diagnosing and classifying COVID-19 patients. In the future, it is anticipated that omics platforms will inform practice through diagnostics, prognostication, surveillance, and clinical decision making, which are all relevant to improving COVID-19 disease outcomes.

6 Conclusions and Future Perspectives

The complexity and heterogeneity of COVID-19 infection is challenging for diagnostic sciences. However, there has been rapid progress from identification of the virus and diagnosis based on RT-PCR, through point of care tests and management

Table 7.6 Pathogenic mechanisms generated by multiomic studies

Method	Findings	Profiling implication	Reference
Multiorgan proteomic profile; Autopsies analysis of 5336 protein molecules	Upregulated cathepsin L1 in the lungs, dysregulation of factors related to hypoxia, angiogenesis, coagulation and fibrosis in multiple organs	Differentially expressed proteins may be candidate biomarkers for diagnosis and prognosis of severe COVID -19 cases	[59]
Model based on machine learning: Prioritization of optimal biomarker Combinations for COVID-19 (POC-19)	1. Four protein biomarkers were identified as classifiers include orosomucoid-1/alpha-1-acid glycoprotein-1 (ORM1/AGP1), Alpha-1-acid glycoprotein 2 (ORM2), fetuin-B (FETUB), and cholesteryl ester transfer protein (CETP) as classifies and 2. Outcome markers identified were zinc-a2-glycoprotein 1 (AZGP1), ORM2, and complement factor I (CFI) alone or in combination, 3. Markers predicting recovery include combination of serine proteinase inhibitor A3/a1- antichymotrypsin (SERPINA3/ACT), lymphocyte cytosolic protein 1/L-plastin (LCP1/LPL), and peptidase inhibitor 16 (PI16)	COVID 19 patient classification, disease progression prediction and prediction of recovery. Investigation in a large cohort is required	[60]
Time resolved proteomics using Flow chromatography and mass spectrometry, SWATH-MS quantitative and deep-neural network methods	Dynamic changes in markers reflecting progression of disease: immuno-inflammatory mediators CD44 and B2 M, complement cascade components CFD and CFHRs, coagulation components HRG and PLG, apolipoprotein APOA2, APOC3 and angiotensin (AGT), as well as the organ dysfunction indicators NT-proBNP and troponin	Prediction model of disease progression, and oxygen therapy intervention, Identify early infected individuals and direct risk stratification	[61]
Ultra-high throughput proteomic assay using short-gradient highflow liquid chromatography (LC)	27 proteins identified that are closely associated with IL-6-mediated proinflammatory signaling	Valuable biomarkers of disease severity	[62]

of risk factors for severe disease to the use of multiomics. The application of many of these tests in resource poor countries remains suboptimal. Current point of care COVID-19 tests may not perform well early in the course of infection. Improved and affordable diagnostics are needed in resource constrained countries. Some tests such as IL-6 are used to predict disease severity and response to treatment and are

currently not widely available in Africa. The dream would be to have a widely available and easily accessible point of care for multi-array diagnosis followed by tests for risk stratification. The principles established from the COVID-19 pandemic should guide the future of pandemic diagnostics.

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Chapter 8
Assessing Biomarkers in Viral Infection

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Abstract Current biomarkers to assess the risk of complications of both acute and chronic viral infection are suboptimal. Prevalent viral infections like human immunodeficiency virus (HIV), hepatitis B and C virus, herpes viruses, and, more recently, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) may be associated with significant sequelae including the risk of cardiovascular disease, other end-organ diseases, and malignancies. This review considers some biomarkers which have been investigated in diagnosis and prognosis of key viral infections including inflammatory cytokines, markers of endothelial dysfunction and activation and coagulation, and the role that more conventional diagnostic markers, such as C-reactive protein and procalcitonin, can play in predicting these secondary complications, as markers of severity and to distinguish viral and bacterial infection. Although many of these are still only available in the research setting, these markers show promise for incorporation in diagnostic algorithms which may assist to predict adverse outcomes and to guide therapy.

Keywords Biomarker · Viral infection · C-reactive peptide · Procalcitonin · Inflammation · Coagulation · SARS-CoV-2

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1 Introduction

Chronic viral infections are associated with immune system activation and inflammation which may be responsible for a number of non-infectious disease complications. These can include the development of autoimmune manifestations including cytopenias, malignancy, and cardiovascular disease (CVD) [1, 2]. Recently, there has been increasing interest in predicting adverse outcomes from these infections resulting in the identification of biomarkers which may indicate the development of chronicity and assist with treatment decisions. With the most recent severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) pandemic, infection in some patients was prolonged resulting in the development of syndromes including long-COVID-19 (also known as post-COVID-19) and multisystem inflammatory disorder of childhood (MISC-C) [3, 4]. Inflammatory markers including interleukin-6 (IL-6), and more conventional markers like C-reactive protein (CRP) and procalcitonin (PCT) [5, 6], were offered as a component of the laboratory management of these patients although the interpretation of the results was not always straightforward. CRP and PCT are used routinely in severely ill patients, but a number of other inflammatory biomarkers, including endothelial markers and other cytokines, are not offered routinely. In some cases, inflammatory biomarkers have not been fully evaluated as prognostic markers although they are available as routine tests. D-dimers (or additional fibrin-degradation products) are a measure of fibrinolysis and are increased with bleeding and clotting [7], but this test has more recently been utilized to assess prognosis in patients with SARS-CoV-2 infections, independently of overt underlying coagulopathy or thrombosis [8]. The timing of sample collection, assay type, and the number of repeat analyses are poorly standardized, and this may reduce the utility of these markers in the clinical setting [6, 9]. Diagnostic and management guidelines have been issued by scientific bodies although these do not fully cover all clinical scenarios [10–12].

This review will focus on some chronic viral test cases including human immunodeficiency virus (HIV) infection, hepatitis B and C virus (HBV and HCV) infection, selected human herpes viruses, Kaposi-sarcoma herpesvirus (KSHV), and Epstein-Barr virus (EBV), as well as SARS-CoV-2.

2 Inflammatory Cytokines in Viral Infections

Cytokines are small protein molecules which are released by both immune effector cells and non-immune cells and which act to regulate immune function [13, 14]. A comprehensive discussion of all cytokines is outside the scope of this review, but recently, 3 cytokines, interleukin-6 (IL-6), IL-1, and tumor necrosis factor alpha (TNF-α) have been an area of focus in viral disease. These pleiotropic cytokines are the chief regulators of multiple inflammatory pathways [13, 15–17].

IL-6, TNF-α, and IL-1α are secreted by multiple cells including non-immune cells like epithelial and endothelial cells and some leukocytes [15, 18, 19]. IL-1β

production is more restricted to leukocytes (primarily myeloid cells) [15]. Production of these cytokines is upregulated in response to innate immune system activation through the binding of pathogen-associated molecular patterns (PAMPs) to highly conserved pattern-recognition receptors (PRRs) [13]. An important mediator of secretion of IL-1β specifically is the inflammasome, a complex of proteins containing PRRs, which recognize specific microbial patterns including the nucleotide oligomerization domain, leucine-rich repeat receptors (NLRs). The nitrogen permease regulator-like 3 (NLRP3) inflammasome activates caspase 1 which cleaves pro-IL1 into active components, IL-18 and IL-1β [20, 21]. TNF-α production is upregulated in response to IL-1β and toll-like receptor (TLR) activation through upregulation of TNF-α gene transcription. TNF-α is converted to a soluble form by the metalloproteinase TNF-α converting enzyme (TACE) [15]. Levels of IL-6, the principal member of the IL-6 family of cytokines, are low in healthy individuals but rise rapidly with inflammation [17]. IL-6 gene transcription is upregulated by nuclear factor kappa B (NFκB), nuclear factor IL-6 (NF-IL-6), and activation protein-1 among other pro-inflammatory signaling pathways, typically in response to PAMPs or danger-associated molecular patterns (DAMPs) [18]. Further secretion is stimulated by the action of the IL-6 amplifier which also positively influences secretion of other pro-inflammatory cytokines [18]. Elevated cytokine levels in chronic viral infections are attributed to a number of stimuli. In HIV infection, chronic activation has been linked to ongoing low-grade viral replication, presence of opportunistic infections, and microbial translocation [22]. Both EBV and KSHV promote inflammatory gene transcription, and KSHV produces viral cytokine homologs including viral IL-6 [23].

IL-1β, TNF-α, and IL-6 are crucial to pro-inflammatory responses [15, 18, 19]. All three are associated with monocyte and neutrophil recruitment and activation, dendritic cell maturation, increased endothelial permeability, fever, and pain. In response to these cytokines, there is release of acute phase proteins and hepcidin from the liver [24]. IL-1β, IL-6, and TNF-α (sometimes also classed as sT-helper 1 cytokines) promote a pro-inflammatory T-cell response and inhibit regulatory T-cell differentiation [25]. IL-6 specifically stimulates Th17 T-cell differentiation, in conjugation with transforming growth factor beta (TGF-β). It also has a non-redundant function in plasma cell differentiation and antibody secretion. IL-6 hypersecretion is also associated with increased platelet production and bone remodeling [17]. IL-1β favors Th17 differentiation in response to increased IL-6 levels by suppressing suppressor of cytokine signaling 3 (SOCS3) [15]. The IL-1 receptors are common entry sites for microorganisms, and expression and activity are therefore tightly regulated by mechanisms involving decoy receptors and proteolytic degradation [15]. As pro-inflammatory cytokines, IL-1β, TNF-α, and IL-6 promote an important antiviral and antibacterial response. However, under chronic infection and inflammation conditions, cytokine levels remain elevated, and this can become pathogenic [13, 16]. Therefore, these cytokines can have both beneficial and detrimental effects in viral infections [5, 6, 13, 26–61] (Table 8.1).

Table 8.1 Secretion and effects of inflammatory cytokines in selected viral infections

	Interleukin 1β (IL-1 β)	Interleukin-6 (IL-6)	Tumor necrosis factor-α (TNF- α)
Hepatitis B (HB) virus [13, 26–29]	Downregulation of secretion by HBe Antigen(Ag) and upregulation by HBcAg; increased levels associated with viral replication and disease complications including cirrhosis and HCC	Elevated levels inhibit viral entry and transcription; ongoing hypersecretion predicts mortality in acute on chronic liver failure and contributes to development of HCC through activation of the STAT3 pathway	Inhibition is associated with HBV reactivation; increased production also associated with liver fibrosis, hepatocyte apoptosis and pyroptosis
Human immunodeficiency virus (HIV) [30–32]	Augmentation of NLRP3 and IL-1B gene expression culminating in activation of the inflammasome in dendritic and related monocyte lineage cells with IL-1β hypersecretion	Elevated levels associated with lower CD4+ T-cell count and higher HIV viral load; strongly predictive of all-cause mortality and specifically HIV-associated CVD and non-AIDS defining malignancies	Increased secretion primarily by macrophages through action of viral proteins nef, tat and gp120; causes bystander immune cell apoptosis; elevated levels associated with increased mortality and disease progression
Hepatitis C (HC) virus [28, 33]	Upregulated in response to hypoxia during chronic inflammation; activates production of membrane metalloproteinase 9 with subsequent fibrosis; also linked to HCC and stimulation of an epithelial-mesenchymal transition	IL-6 polymorphisms linked to poorer outcomes with chronic HCV infection; may stimulate tumorigenesis through action on JAK-STAT pathway	Inhibition not conclusively linked to reactivation; putative role in hepatic fibrosis and hepatocyte pyroptosis
Epstein-Barr Virus (EBV) [32, 34–41]	Upregulated in response to viral proteins including LMP-1 although other viral proteins may inhibit secretion of IL-1 and downregulate its cognate receptors; increases are associated with pyroptosis but also with increased development of nasopharyngeal carcinoma and angiopathy in chronic infection; associated with development of chronic EBV disease and with HLH	Elevation predicts mortality in primary effusion lymphoma; biomarker for development of HL; independently associated with mortality in HL; Viral IL-6 associated with B-cell immortalization and hyperproliferation; prognostic marker and possible therapeutic target in EBV-associated HLH	High levels associated with elevation of early lytic proteins, including LMP-1, resulting in B-cell proliferation; elevated levels independently associated with EBV associated chronic fatigue syndrome and HLH

(continued)

Table 8.1 (continued)

	Interleukin 1β (IL-1 β)	Interleukin-6 (IL-6)	Tumor necrosis factor-α (TNF- α)
Kaposi-sarcoma herpesvirus (KSHV) [42–46]	IL-1α and/or IL1-β increased in response to vOX_2 glycoprotein b and other viral proteins; stimulates angiogenesis and abnormal cell proliferation and upregulates PD-1L to effect tumor cell escape; increased levels associated with tumorigenesis in KS, primary effusion lymphoma and multicentric Castleman's disease	Increased levels predictive of development of KSHV-associated malignancies including primary effusion lymphoma, KS and multicentric Castleman's disease; upregulates growth factors including Vascular Endothelial Growth Factor; high levels associated with KSHV-associated cytokine syndrome	Upregulated levels in response to KSHV glycoprotein b although other factors may inhibit secretion; elevated levels associated with viral reactivation, KS and B-cell lymphomagenesis; elevated levels may also be associated with decreased viral load
SARS-CoV-2 [5, 6, 47–61]	Levels of IL-1β, IL-6 and TNF-α are all raised in SARS-CoV-2 disease and have been predictive of severity, mortality and disease complications including neurological disease, severe viral pneumonia and development of lung fibrosis, multisystem inflammatory disorder of children, SARS-CoV-2 associated HSH and long COVID-19 syndrome; SARS-CoV2 cytokine release syndrome has been targeted with immunotherapies		

HCC hepatocellular carcinoma (HCC); *STAT3* signal transducer and activator of transcription 3; *NLPR3* nitrogen permease regulator-like 3; *CVD* cardiovascular disease; *nef* negative factor; *tat* transactivator of transcription; *GP* glycoprotein; *JAK-STAT* Janus kinase-signal transducer and activator of transcription; *LMP-1* latent membrane protein 1; *HLH* hemophagocytic lymphohistiocytosis; *PD-L1* programmed cell death Ligand-1; *KS* Kaposi sarcoma

3 Coagulation as a Biomarker of Viral Infection

Coagulation is a component of an innate immune response, and a procoagulant state is a feature of dysregulated inflammation [62]. Cardiovascular events including venous thromboembolic disease, myocardial infarction, cerebrovascular accidents, and thrombotic microangiopathies are a cause of virus-related morbidity and mortality [62]. Biomarkers may assess endothelial cell activation or clot formation or breakdown [7, 63]. Classically, disseminated intravascular coagulation (DIC) is a complication of severe sepsis and has been associated both with primary viral infection as a trigger and also with secondary conditions specifically cancer and bacterial or viral superinfection [64].

Both humoral and cellular effectors of coagulation have prognostic value in severe viral disease [14, 65, 66]. Thrombocytopenia is a key feature of ongoing microvascular thrombosis and chronic inflammation which can result in dysmegakaryopoiesis [67]. In addition, immune-mediated platelet destruction is associated with multiple viral diseases including hepatitis C [33], HIV [68], SARS-CoV-2

[69], and the herpes viruses [70]. On the other hand, platelet sequestration is associated with hypersplenism, which may complicate liver disease or may be a direct result of infection [71]. Increased platelet numbers may also be present specifically in response to elevated IL-6 [18]. Platelet activation is increased by multiple inflammatory mediators including the lipid mediators of inflammation contributing to pathological thrombosis [65].

Leukocytes can also contribute to infection-related thrombosis by interacting with both platelets and the endothelial surface. In HIV, there is upregulation of leukocyte expression of tissue factor which can activate factor VII stimulating the coagulation cascade [72]. Both platelets and monocytes upregulate expression of adhesion markers like P-selectin and its cognate ligand, P-selectin glycoprotein ligand [73]. Measurement of these markers, by immunophenotyping, can be an important adjunct in assessing risk and has been shown to correlate with CVD development and with other markers of viral severity [62]. Neutrophils, under inflammatory conditions, release neutrophil extravasation traps which also contribute to immunothrombosis by activating platelets and physically blocking the vascular lumen [74].

Chronic inflammation activates endothelial cells to a procoagulant and proinflammatory phenotype [62]. Endothelial dysfunction, a state of dysregulated contractility and endothelial cell activation, contributes to the development of CVD. Surrogate markers of endothelial dysfunction include the release of endothelial cell adhesion markers like intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and the procoagulant factors, factor VIII, and von Willebrand factor [62]. These factors can be pathogenic in thrombosis and have predictive value in critically ill patients.

Independent from CVD risk, coagulation system activation can predict severity in other complications of viral infection. For example, increased levels of ICAM-1 were found to be predictive of development of hepatocellular carcinoma (HCC) in chronic HBV and HCV infection [27], as well as decompensating cirrhosis [75]. Elevated levels of D-dimers are a strong predictor of mortality in HIV and specifically for CVD-related complications [76–78], and more recently, D-dimers have been used to prognosticate in severe SARS-CoV-2 infection [79]. Importantly, D-dimers show high negative predictive value in patients with suspected venous thromboembolic disease, and longitudinal measurement may indicate treatment adherence and clinical improvement [7].

4 Traditional Biomarkers of Severe Viral Disease

It can be difficult to distinguish bacterial from viral infections especially in the lower respiratory tract. Untreated bacterial infections can result in serious complications, while the use of antibiotics in inflammation or viral infections leads to the development of antibiotic resistance, increased costs, and possible unwanted side effects [80]. The most accurate way to diagnose these infections is by culture in the

case of bacterial infections, or serology for antibodies or antigens, or molecular tests. Culture results and ancillary test results are generally not available immediately, and there is a need for alternative approaches. Both CRP and PCT concentrations have been used to initiate and monitor the antibiotic use for lower respiratory tract infections [81].

These biomarkers also are elevated in people with inflammation resulting from causes other than infections such as trauma, autoimmune diseases, and metabolic disease [82]. Early studies during the COVID-19 pandemic suggested that these may be used as markers of disease severity.

4.1 CRP

CRP is an acute inflammatory protein discovered in 1930 by Tillet and Francis, while investigating the effects of sera of patients with pneumococcal pneumonia [83]. CRP binds to polysaccharides on microorganisms and activates C1q of the classical complement pathway [84]. CRP is synthesized primarily in hepatocytes, but is also produced in adipocytes, endothelial cells, lymphocytes, macrophages, and smooth muscle cells [85–87]. CRP is found in two forms: a pentameric form which can then dissociate to form monomers. These two forms of CRP play distinct roles in the inflammatory process [88]. Monomeric CRP is involved in the innate immune system by activation of the complement cascade and stimulation of both angiogenesis and thrombosis, whereas pentameric CRP is mostly released to the circulation after an inflammatory stimulus and recognizes phosphocholine on bacterial cells and damaged host cells [89].

CRP triggers C1q activation in the complement pathway leading to the opsonization of pathogens. It can also stimulate cell-mediated pathways via complement activation and by binding Fc receptors of IgG [90]. CRP increases within 4–6 h, in response to injury, infection, and inflammation, and peaks at about 36 h. In general inflammation, CRP levels can rise beyond 10 mg/L [89]. Lower concentrations of CRP, in the range of 0.01 to <10 mg/L (high sensitivity CRP or hsCRP), are associated with low grades of systemic inflammation. Low grade systemic inflammation is associated with elevated hsCRP levels, and use of this biomarker to detect atherosclerotic vascular disease has been intensely investigated through observational studies and clinical trials over the past two decades. On the basis of evidence that has accrued, hsCRP measurement has been integrated into the Reynolds risk scoring system to predict cardiovascular risk [91]. It is used at concentrations of <1 mg/L, 1–3 mg/L, and >3 mg/L to classify individuals as low, intermediate, or high risk for CVD, respectively [24].

Sequential CRP levels are a sensitive and specific biomarker to improve the differential diagnosis between acute bacterial and viral infections, although this may be less accurate in severe viral disease cases and with prolonged inflammation [92]. CRP is raised in patients with severe SARS-CoV-2 [93, 94] and can predict mortality [49, 95] especially in patients aged 60 years and older [96]. CRP levels show a

downward trend in survivors and tend to increase prior to death in non-survivors [97]. CRP kinetics in SARS-CoV-2-infected patients admitted to intensive care units were similar to those seen in bacterial sepsis with an initial rise followed by a decline during recovery, although levels are typically higher in patients with bacterial sepsis compared to patients with severe COVID-19 disease [98]. Mortality in patients with SARS-CoV-2 is higher in patients with comorbidities such as type II diabetes mellitus and preexisting CVD [99]. SARS-CoV-2 infection itself can cause cardiovascular damage and impaired glucose control. While biomarkers such as high sensitivity Troponin and pro brain natriuretic peptide (proBNP) are better markers of CVD, CRP is also elevated signifying the underlying inflammatory process [100]. CRP measurement can be an important ancillary test in these patients as it may directly damage cardiac tissue by activating complement, reducing nitric oxide (NO) release and CRP-mediated inhibition of angiogenesis, and stimulating endothelial cell apoptosis [101].

Elevated CRP levels have been associated with poorer outcomes in other viral infections such as SARS-related pneumonia, Middle East respiratory syndrome (MERS) infection, and H7N9 influenza. High levels of CRP were consistently seen with severe disease outcomes in H1N1 influenza patients [102–105]. Elevated CRP is also predictive of mortality in HIV particularly from CVD, and the levels of this biomarker are further elevated in patients with co-infection with other viruses like HCV [106]. The IL-6 expressed by KSHV also stimulates CRP secretion, and high CRP levels are a feature of a cytokine storm in a number of different viral diseases [14]. Taken together, these findings indicate that CRP is elevated in several viral infections and, therefore, cannot be used to differentiate between them.

4.2 PCT

PCT is a glycoprotein precursor of calcitonin released by the thyroid parafollicular cells. In healthy subjects, calcitonin is released, but in the presence of an inflammatory stimulus, particularly bacterial endotoxin or pro-inflammatory cytokines, there is increased calcitonin gene expression, and PCT mRNA is synthesized. This leads to release of PCT from all parenchymal tissues. PCT is a useful biomarker to differentiate between bacterial and viral infections as a concentration $\geq$0.5 μg/L is suggestive of a possible bacterial infection [107]. PCT may be used in the early diagnosis of bacterial pneumonias and to guide initiation of antibiotic therapy [108].

Although relatively specific for bacterial infections, serum PCT levels also correlate with disease severity and thus cannot reliably distinguish between bacterial and nonbacterial infections in the setting of critical illness, particularly in cases of severe influenza and SARS-CoV-2 infection [6, 52]. However, the value of PCT as a prognostic marker in SARS-CoV-2 is unclear. Meta-analyses have shown that those patients with severe disease had higher PCT levels compared to those with

non-severe disease [6, 109], although this was inconsistent with some studies failing to find a significant difference [51]. The reasons for these discrepancies may be attributed to variable cut-offs, patient ages, or other factors impacting PCT release. PCT release is inhibited by interferon (INF)-γ, for example, and levels of this cytokine may differ in different patient populations or with different administered therapies. Since INF-γ is a key antiviral cytokine, this could explain the differences in PCT level in viral and bacterial infection [110]. However, all three pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) stimulate parenchymal PCT production. PCT levels are typically normal in uncomplicated viral infections [111] but may rise with severe complications including, for example, the development of hemophagocytic lymphohistiocytosis (HLH) [9] or the development of secondary bacterial infection in patients with severe viral disease including H1N1 influenza [112]. In general, however, PCT appears to be a more specific marker of bacterial sepsis than CRP, albeit with some limitations. This has prompted a search for more specific markers or combinations of markers that can be used reliably to differentiate bacterial and viral infections.

One potential biomarker for distinguishing between bacterial and viral infections is myxovirus resistance protein A (MxA), an IFN-inducible protein with antiviral activity. MxA has been investigated for use as a biomarker because of its rapid induction in acute, symptomatic viral infections and low levels in bacterial infections and in healthy individuals [113–115]. Clinical studies, mostly involving children, suggest that MxA is selectively increased in viral infections and have the potential to rapidly distinguish viral and bacterial disease [116, 117]. It has been used in the emergency department setting to distinguish SARS-CoV-2 from bacterial and non-infectious causes of respiratory disease [118].

5 Conclusions and Future Perspectives

Viral infections cause significant morbidity and mortality. Host- and virus-specific factors can determine patient outcomes in both acute and chronic infection although these outcomes cannot always be predicted in clinical settings with the current biomarkers available, as demonstrated during the COVID-19 pandemic. In this review, we considered some of the biomarkers that are used in the clinical setting and in research to monitor viral infections. These biomarkers may predict the development of end-organ diseases including CVD and malignancies and contribute to acute viral immune escape or control, or they may indicate severe complications including HLH and cytokine release syndromes. Combinations of these markers can also help to distinguish between bacterial and viral infection which is critical for effective antimicrobial stewardship. Into the future, standardization of biomarker panels, validation of new markers, and appropriate age-specific, disease-specific reference ranges will assist to make these biomarkers more clinically relevant.

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Chapter 9
Proteomic Investigation of COVID-19 Severity During the Tsunamic Second Wave in Mumbai

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Abstract Maharashtra was severely affected during the noxious second wave of COVID-19, with the highest number of cases recorded across India. The emergence of new symptoms and dysregulation of multiple organs resulted in high disease severity during the second wave which led to increased difficulties in understanding

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the molecular mechanisms behind the disease pathology. Exploring the underlying factors can help to relieve the burden on the medical communities to some extent by prioritizing the patients and, at the same time, opening avenues for improved treatments. In the current study, we have performed a mass-spectrometry-based proteomic analysis to investigate the disease pathology using nasopharyngeal swab samples collected from the COVID-19 patients in the Mumbai region of Maharashtra over the period of March–June 2021, the peak of the second wave. A total of 59 patients, including 32 non-severe and 27 severe cases, were considered for this proteomic study. We identified 23 differentially regulated proteins in severe patients as a host response to infection. In addition to the previously identified innate mechanisms of neutrophil and platelet degranulation, this study revealed significant alterations of anti-microbial peptide pathways in severe conditions, illustrating its role in the severity of the infectious strain of COVID-19 during the second wave. Furthermore, myeloperoxidase, cathepsin G, and profilin-1 were identified as potential therapeutic targets of the FDA-approved drugs dabrafenib, ZINC4097343, and ritonavir. This study has enlightened the role of the anti-microbial peptide pathway associated with the second wave in India and proposed its importance in potential therapeutics for COVID-19.

Keywords Proteomics · COVID-19 · Host response · Severity markers · Nasopharyngeal swab · MRM · Second wave

1 Introduction

Fatality in the second wave of COVID-19 driven mainly by the delta SARS-CoV-2 variant was far higher than in the first, especially in India. As of December 31, 2021, there were a total of 34,799,691 confirmed COVID-19 cases and 480,290 deaths in India, of which >45% were from Maharashtra, making it the maximally affected state in India [1]. Owing to the dense population, and several other factors such as the economic pursuits and the substantially populated slum area, Mumbai added to the vast spread of COVID-19 [2]. Population groups with previous comorbidities like diabetes, cardiac diseases, and microbial co-infections emerged as a major challenge chiefly during the second wave of COVID-19 and were associated with higher mortality, severity, increased duration of hospital stays, and the need for mechanical ventilation [3, 4].

Most research has focused on developing vaccines against the SARS-CoV-2 virus to control this pandemic [5]. Although vaccination provided a statistical advantage toward risk compensation of the disease, several variants of SARS-CoV-2 became capable of escaping the neutralizing action of the immune response [6, 7]. These variants spread to various countries worldwide, including some regions of European nations, the United States, and India, where they became dominant [8]. In addition to the immune-escape capacity, the enhanced transmission and pathogenicity of these variants worsened the situation in the disastrous second wave of the

pandemic [9]. In India, this resulted in a massive surge in the total number of cases and deaths due to depleted supplies in essential treatments [10].

COVID-19 disease has a wide range of disease effects ranging in outcomes from mild to severe. Moreover, many COVID-19 cases are reported to be asymptomatic [11]. Besides some of the common symptoms of COVID-19 like fever, cough, general weakness, and body pain, the loss of smell and taste can also occur [12–14]. Patients suffering from severe COVID-19 disease mainly experience a substantial decrease in oxygen saturation levels with a significant increase in clinical parameters like interleukin-6 (IL-6), C-reactive protein (CRP), procalcitonin, and D-dimer levels [15]. However, the transition from non-severe to severe phase of disease in COVID-19 patients can be promoted by underlying medical conditions, which also act as a risk factor of a severe disease course [16, 17]. In many cases, the host immune response can damage the infected alveolar cells causing acute respiratory distress syndrome (ARDS).

With the burden of the COVID-19 pandemic crumbling the healthcare systems in countries like India, efficient patient triage and appropriate resource allocation at all levels are required to mitigate morbidity and mortality. Therefore, identifying potential risk factors that predict the disease course may be useful for healthcare professionals [18]. Studying the complex molecular and immune response events in infected hosts is an important step in increasing our understanding of pathobiology of SARS-CoV-2. Nasopharyngeal (NP) swabs have become the sample of choice as the nasopharynx is the primary site of viral entry and is essential for diagnosis. Moreover, it shows association with disease transmission and other risk factors of COVID-19. For these reasons, many research groups have used mass-spectrometry-based proteomics during the initial peak of the COVID-19 pandemic to study the effects of SARS-CoV-2 infection in NP samples [19–24].

The use of high-throughput "omic" technologies can help in understanding the pathobiology of SARS-CoV-2 infection in human [25]. The host response to this virus has been investigated in various clinical specimens such as serum [26–29], plasma [30–33], saliva [34, 35], and NP swabs [19, 36, 37]. However, only a few studies have analyzed the host proteome alterations in nasopharyngeal swabs using mass-spectrometric-based proteomic approaches [17, 37]. These studies have shown that SARS-CoV-2 infection induces alterations in the host proteome, particularly in biological processes involving the innate immune response (IFN signaling, neutrophil degranulation, and complement activation) and viral replication (exocytosis, endoplasmic reticulum/Golgi transport, and translation of viral proteins). However, none of these studies considered the disease severity as a variable while classifying the participants. Therefore, studies focusing on the severity of the disease could provide insights into the pathogenesis of COVID-19.

Therefore, to elucidate the pathobiology of the underlying disease severity of the second wave, we analyzed the NP swab samples collected for routine RT-PCR testing to perform proteomic analysis. Using mass-spectrometry-based label-free quantification methods we have evaluated the NP proteome alterations. In addition, validation of the significantly dysregulated proteins was performed using the targeted multiple reaction monitoring (MRM). Finally, *in silico* pathway analysis was

performed to investigate the role of the putative severity markers identified in the current study, and we have tried to predict possible drug molecules which target the affected pathways using a molecular docking approach.

2 Materials and Methods

2.1 Sample Collection and Processing

The nasopharyngeal swab samples used in this study were acquired from Kasturba Hospital for Infectious diseases, Mumbai, with approval from the Institute Ethics Committee, Indian Institute of Technology Bombay, and Institutional Review Board, Kasturba Hospital for Infectious Diseases. The samples were collected during the second wave of the COVID-19 pandemic between March 26 and June 16, 2021. Patients below 18 years of age and pregnant women were excluded from the study. The samples were classified into severe and non-severe groups based on symptoms like respiratory distress, low SpO_2 levels, and the need for ventilation. Patients classified as severe showed significant differences in duration of hospital stay, death outcomes, and some blood laboratory factors (Table 9.1 and Table 9.S1). Of 59 patients selected for this study, 32 were categorized as non-severe and 27 as severe (PXD041609). A subset of these samples has been used for another study, raw data for which is available at PXD029300 [70].

2.2 Mass Spectrometry Settings and Data Analysis

The collection, inactivation, and preparation of samples for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was performed as described in our previous study [19]. Desalted peptide digests (1 µg) were injected into the LC column. A gradient of 120 min with a constant flow rate of 300 nL/min was used to separate peptides on the nano-LC column. Solvent A consisted of 0.1% formic acid (FA) in water and solvent B consisted of 80% acetonitrile and 0.1% FA in water and the gradient was followed as given in Table 9.2 [38]. Mass spectrometric data were acquired using an Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Fisher Scientific) in data-dependent mode. The following data acquisition parameters were used: mass range, 375–1700 m/z; mass resolution, 60,000; dynamic exclusion, 40 s; and mass tolerance, 10 ppm. The MS/MS acquisition was performed using higher energy collision dissociation with a fixed collision energy of 30%, 15,000 resolution, and a maximum injection time of 30 ms.

The acquired data were analyzed using MaxQuant (v 2.0.0) [38] against the Human Swiss-Prot Database (downloaded on August 8, 2021) which identified a total of 1981 proteins. For MaxQuant search, the raw files were processed using

Table 9.1 Clinical status for the patients in the cohort selected for the study

	COVID-19 positive		
	Non-severe	Severe	*p*-Value
Patient information			
Number of participants	33	27[a]	NA
Age (years)	46.5 (33–55)	54 (45–60)	0.0175
Gender			
Males	23 (69.69)	13 (48.14)	n.s.
Females	10 (30.30)	14 (51.85)	
Patient outcome			
Discharged	30 (90.90)	17 (62.96)	0.011
Dead	1 (3.03)	10 (37.03)	
Duration of hospital stay	8 (6–9)	13 (7.5–15.5)	0.0142
Ventilation status			
Ventilation required	7 (19.44)	27 (100)	–
NRBM (>6 L O_2 supplementation)	0	18	–
BiPAP	0	8	–
Symptoms on admission			
Fever	29 (80.55)	16 (61.53)	–
Cough	21 (58.33)	16 (61.53)	–
Sore throat	4 (11.11)	0	–
General weakness	14 (36.11)	12 (46.15)	–
Breathlessness	10 (27.77)	19 (73.07)	–
Respiratory symptoms	2 (36.11)	20 (74.07)	–
Comorbidities			
Diabetes	3 (9.09)	6 (22.22)	–
Hypertension	5 (15.15)	4 (14.81)	–
Others	1 (3.03)	2 (7.40)	–
N/A	28 (77.78)	13 (48.14)	–
Hematological parameters			
Hemoglobin (g/dL)	12.6 (11.25–13.9)	11.1 (9.3–12.9)	0.0014
Polymorphs (40–75%)	73 (65.25–81)	81 (75–86)	0.0093
Lymphocytes (20–40%)	27 (19–34.75)	19 (14–25)	0.0152
Platelets (1.5–4.5 lakhs/μL)	2 (1.6–2.5)	2.1 (1.7–3)	n.s.
Biochemical parameters			
SGOT (0–40 U/L)	34 (30–45.5)	42 (32–56.75)	n.s.
SGPT (5–34 U/L)	24 (16–35.5)	22 (14–36)	n.s.
$AlkPO_4$ (15–112 IU/L)	53 (45–64)	54 (45.75–62.25)	n.s.
Total bilirubin (0.1–1.2 mg%)	0.7 (0.6–0.7)	0.7 (0.6–0.8)	n.s.
D. bilirubin (0–0.3 mg%)	0.2	0.2	n.s.
Total protein (6–8.4 g%)	7 (6.5–7.3)	6.45 (6.17–6.95)	n.s.
Albumin (3.2–5 g%)	4.4 (4–4.6)	3.85 (3.55–4.12)	0.0239
Globulin (2–2.5 g%)	2.9 (2.5–3)	2.8 (2.4–2.9)	n.s.
Sodium (133–146 m.Eq/L)	138 (135–140)	137 (135–140)	n.s.

(continued)

Table 9.1 (continued)

	COVID-19 positive		
	Non-severe	Severe	*p*-Value
Potassium (3.8–5.6 m.Eq/L)	3.45 (3–4.05)	3.75 (3–4.65)	n.s.
Blood urea nitrogen (6–21 mg%)	14 (10–15)	18 (14–22)	n.s.
Creatinine (1–2 mg%)	1.1 (1.1–1.4)	1.2 (1–1.4)	n.s.
D-dimer (<500 ng FEU/mL)	0.81 (0.66–1.06)	5 (1.27–8.59)	0.0079
CRP (<5 mg/L)	20 (11–79.7)	32 (11.6–71.3)	n.s.
Ferritin (22–322 ng/mL)	392.5 (252.35–930.775)	580 (251.75–1456.82)	n.s.
IL-6 (0–7.0 pg/mL)	9.7 (5.75–41.8)	33.4 (7.35–87.3)	n.s.

All the data are represented as a number, median (interquartile range), or number (percentage)
Abbreviations: *NRBM* non-rebreather mask, *BiPAP* bilevel positive airway pressure ventilation, *SGOT* serum glutamic-oxaloacetic transaminase, *SGPT* serum glutamate pyruvate transaminase, *AlkPO4* alkaline phosphatase, *CRP* C-reactive protein, *n.s.* not significant
[a]Clinical details for one sample were not available

Table 9.2 Gradient used for chromatographic separation

Time (min)	Duration (min)	Flow (nL/min)	%B	%A
00:00	00:00	300	0	100
05:00	05:00	300	5	95
80:00	75:00	300	30	70
110:00	30:00	300	60	40
115:00	05:00	300	90	10
120:00	05:00	300	90	10

label-free quantification (LFQ) parameters, setting the label type as standard with a multiplicity of 1, the instrument as Orbitrap Fusion, enzyme as trypsin, and 2 as the maximum missed cleavages. The algorithm used for this analysis was match between runs (MBR). Carbamidomethylation of cysteine was set as fixed modification and oxidation of methionine as the variable modification. The false discovery rate (FDR) was set at 0.01 for protein and peptide levels to ensure high reliability of the protein detection. Decoy mode was enabled, and type of identified peptides was set to unique + razor (Table 9.S2).

2.3 Statistical Analyses

The LFQ intensities of the identified proteins from MaxQuant were taken forward for analysis. The missing values were estimated using the *k*-nearest neighbors (kNN) imputation algorithm with a filtering threshold of 30%. The dataset was then median-normalized and log-transformed. Outlier detection was done using

correlation analysis, and outlying samples were removed before statistical analysis. Identification of significantly expressed proteins was based on *t*-test with a *p*-value of <0.05. The entire analysis was performed using the online tool, MetaboAnalyst 5.0 [39]. To plot the heatmap for the top differentially expressed proteins, the distance measure parameter was set to Euclidean and the clustering algorithm to Ward clustering.

2.4 Multiple Reaction Monitoring Validation

Some of the differentially regulated proteins identified from the LFQ analysis were validated using the MRM approach for preliminary analysis. MRM experiments were performed using the triple quadrupole instrument TSQ Altis (Thermo Fisher Scientific). The transition list was prepared using SRMAtlas [40] and Skyline [41]. The most observed peptides and list of their transitions were downloaded from SRMAtlas separately for each targeted protein and these were combined in Skyline. To prepare the combined transition list, the peptide length was kept in the range of 8–16 amino acids. The list included only y ions with precursor charges +2 and +3 and product ion charges +1 and +2. Proteins with less than three peptides or peptides with few transitions were discarded. Samples (750 ng) were injected and run in the Orbitrap Fusion mass spectrometer, thus generating a combined list to acquire the MRM data.

The acquired MRM data were analyzed in Skyline using stringent guidelines. A Prosit-generated library was used to check the reliability of the detected peaks. Only those peptides with dotP values strictly above 0.5 were considered for the analysis [42]. A standard BSA sample was prepared and desalted in a procedure similar to that followed for the samples and peptides were reconstituted in the same buffer. This sample (300 ng) was run daily before the sample sequence to check for variations in instrument response. Similarly, 500 ng of peptides from MCF7 cell lysates was run once per day. All relevant transition lists are given in Table 9.S3.

2.5 In Silico Pathway Analysis

Integrative gene ontology analysis was performed using STRING v11.5. All significant proteins were used as the input for pathway analysis. Initially, enriched protein–protein interaction (PPI) networks were obtained with a *p*-value of 5.66e-15 with a total of 21 nodes. The Reactome pathways were used for mapping significant biological pathways with the input proteins. Networks with an FDR less than 0.05 were considered for the analysis and validation.

2.6 *In Silico Molecular Docking*

The upregulated proteins identified from the proteomics study were taken forward for the *in silico* molecular docking analysis. The structure for each target protein was obtained from the Protein Data Bank (PDB) [43] and the AlphaFold protein structure database (https://alphafold.ebi.ac.uk/). A library of 95 potential drug candidates was prepared, of which 49 were FDA approved, 29 were in clinical trials, and 17 were in pre-clinical trials (Table 9.S4). The 3D structure of each drug was downloaded from the PubChem [44] and ZINC15 [45] databases. Control inhibitors were identified for each target protein from the available literature to set the binding affinity threshold and identify the active sites of the proteins. Blind docking approach was used by keeping the grid box large enough to fit the whole protein in the box. The *in silico* molecular docking experiment was performed using Autodock Vina 1.1.2 in the PyRx software (https://pyrx.sourceforge.io/) [46], setting the exhaustiveness value to 50. The output files generated after molecular docking analysis were split into separate poses based on binding energy. The pose having the lowest binding energy was selected and taken forward for the post-docking analysis. We used PyMOL (version 2.4) and Discovery Studio Visualizer Software (version 4.0) to visualize the docked structures and identify the drug-binding pockets. Additionally, the protein–ligand interaction profiler (PLIP) server was used to obtain the types of binding interactions between drugs and the proteins [47].

2.7 *Data Availability*

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD029300 and PXD041609.

3 Results

3.1 *Analysis of Significantly Altered Proteins in COVID-19 Severe Samples*

Figure 9.1 is a schematic of the sample collection, processing, and various analysis tools involved in the study. Initial analysis using partial least squares-discriminant analysis (PLS-DA) showed that sample ID 82 was an outlier that did not cluster into either of the severe or non-severe groups and was therefore removed from the study. Figure 9.2a shows the 3D score plot for the 58 samples segregated into severe (n = 27) and non-severe (green = 32) groups. A total of 1981 proteins were identified after MaxQuant analysis. These proteins were then subjected to statistical

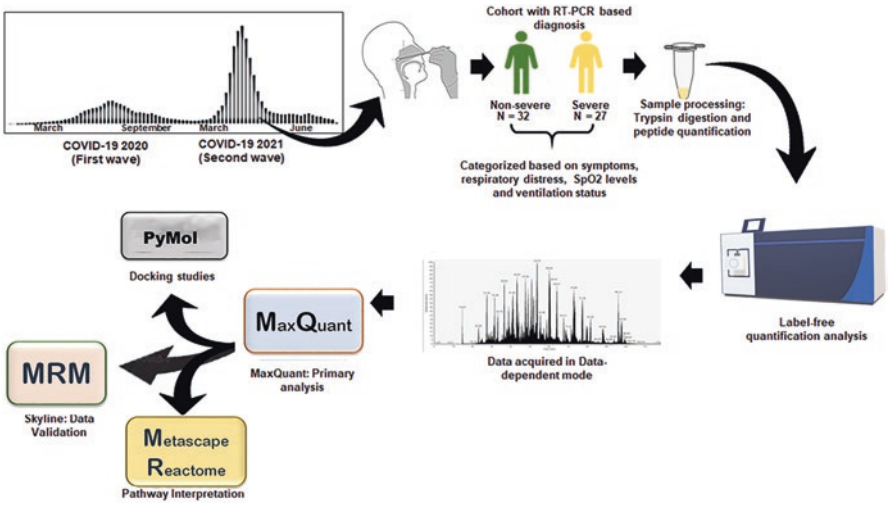

Fig. 9.1 Roadmap to the sample preparation for mass spectrometric analysis of NP swab samples. Collection of the COVID-19 swab samples was done between March and June 2021 from the Kasturba Hospital for Infectious Diseases, Mumbai. The RT-PCR-positive samples were classified based on their severity. We processed the heat-inactivated samples in the lab and performed mass spectrometry-based analysis. The list of proteins after MaxQuant analysis was validated using a Skyline MRM-based approach. Pathway prediction and docking studies were then performed for the significant proteins

analysis using the online software MetaboAnalyst. We found that 23 proteins were differentially regulated in severe compared to the non-severe patients (Table 9.S5). From the differentially expressed proteins, 19 proteins including lactotransferrin, neutrophil gelatinase-associated lipocalin, cathepsin G, and neutrophil defensin 3 were found to be upregulated, whereas 4 proteins like cystatin, WAP four-disulfide core domain protein 2, glucose-6-phosphate isomerase, and filamin-A were down-regulated. A heat map was generated for eight representative proteins (Fig. 9.2b) that are involved in the pathways reported here in the study. Segregation of the proteins into two groups can be seen in the heat map. Figure 9.2c shows the fold change in six of the proteins, of which five of them are upregulated and one down-regulated (TSPAN14).

3.2 Anti-microbial Peptide Pathway as a Host Response to COVID-19 Infection

All the 23 proteins showing differential expression patterns were used for pathway analysis using the Reactome software. We also used Metascape to increase confidence in the identified pathways. The pathways altered in this study include anti-microbial peptide, neutrophil degranulation, and platelet degranulation pathway,

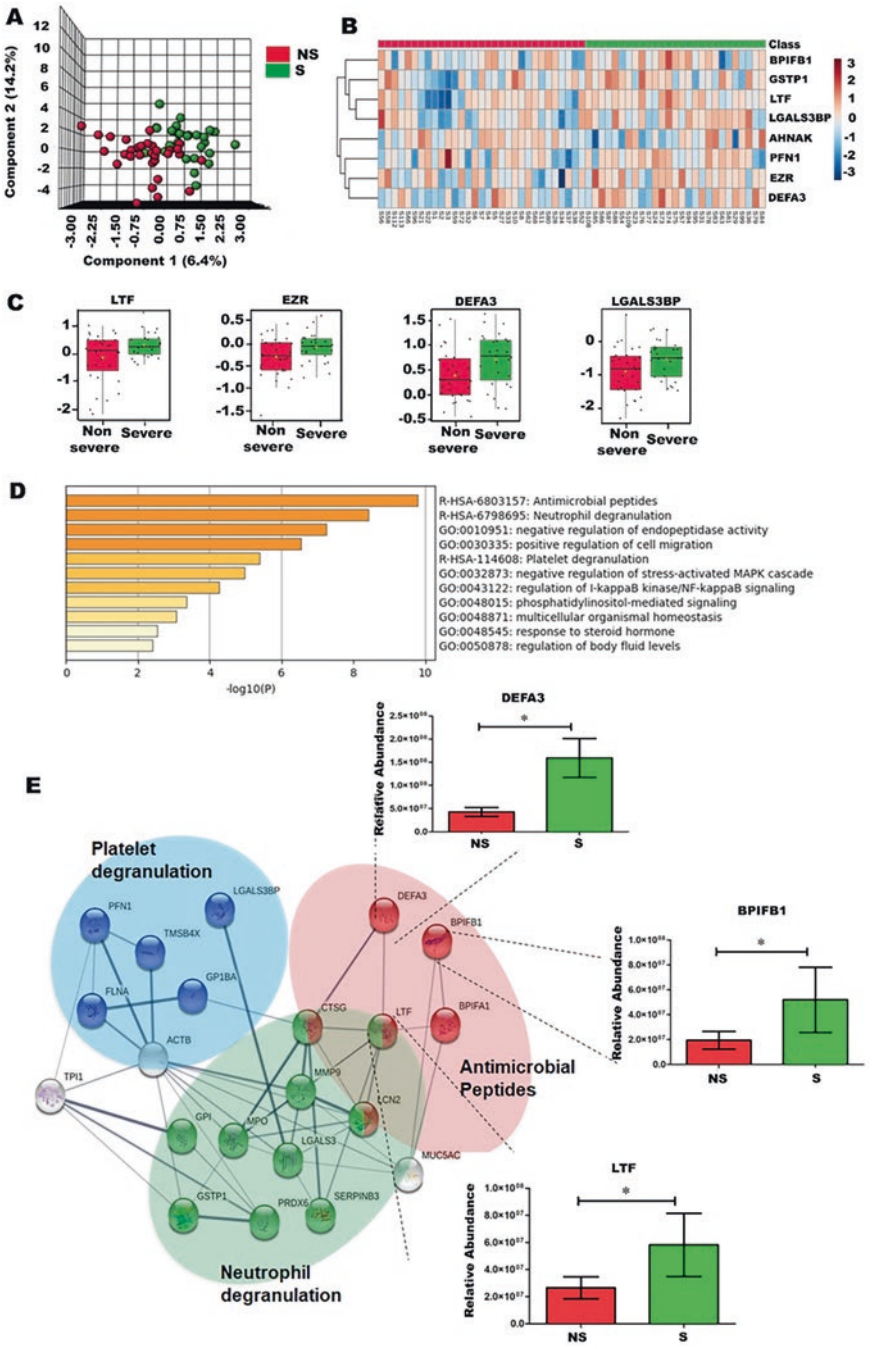

Fig. 9.2 Data analysis for the altered proteins between the two groups. (**a**) 3D scores plot showing segregation of 27 severe (green) and 31 non-severe (red) samples. Some of the samples were clustered between the severe and non-severe transition stage. (**b**) Heat map of eight representative

which are components of the complex immune system (Fig. 9.2d, e). Our analysis found that six proteins (lactotransferrin, neutrophil gelatinase-associated lipocalin (NGAL), cathepsin G, BPI fold-containing family B member 1, neutrophil defensin 3, and BPI fold-containing family A member 1) were mapped to anti-microbial peptide as the highest scoring pathway (FDR = 0.000012) (Table 9.S6). The proteins peroxiredoxin-6, glutathione S-transferase P, serpin B3, lactotransferrin, myeloperoxidase, mucin-5AC, BPI fold-containing family B member 1, BPI fold-containing family A member 1, neutrophil defensin 3, and glucose-6-phosphate isomerase were mapped to innate immune system (FDR = 0.000507) and neutrophil degranulation (FDR = 0.000507) as the next most significant pathways. The table in Fig. 9.S1a summarizes the STRING analysis indicating the proteins associated with each pathway. The protein–protein interaction between each pathway is indicated in Fig. 9.S1b.

3.3 Validation of Severity Markers by MRM Analysis

For the targeted study, 10 severe and 10 non-severe samples were used. From our preliminary MRM analysis, one peptide of MUC5AC and two peptides of LTF showed upregulation in severe cases of COVID-19 compared to the cumulative group-wise peak areas (Fig. 9.3). The boxplots in Fig. 9.3 clearly depict that the peptides from the COVID-19 severe samples showed a minimum of 1.2-fold change compared with the non-severe samples. However, for a thorough validation of the proteins that were differentially regulated in our discovery dataset, the MRM analysis needs further optimization.

The uniformity in the response of the BSA sample helped to ensure the proper functioning of the instrument (Fig. 9.S2). A similar consistency in the day-wise response of MCF7 samples confirmed the consistency of the MRM setup (Fig. 9.S3).

3.4 Virtual Screening of Drugs for Selected Proteins

Three of the upregulated proteins (myeloperoxidase, cathepsin G, and profilin-1) were considered for the *in silico* molecular docking studies with the library of 95 drugs (Table 9.S4). Based on the literature, a small molecule inhibitor for each

Fig. 9.2 (continued) proteins showing differential expression in both groups. (**c**) Box plots depicting the fold change for the six proteins shown in the heat map with the significance level of $p < 0.05$. (**d**) Data obtained from the Metascape analysis representing pathways such as antimicrobial peptides, neutrophil degranulation, platelet degranulation, stress response, and innate immune system. (**e**) Representative image depicting the enriched pathways and interlinkage of proteins involved in these. A graphical representation showing differential expression of BPIFB1, DEFA3, and LTF is also displayed

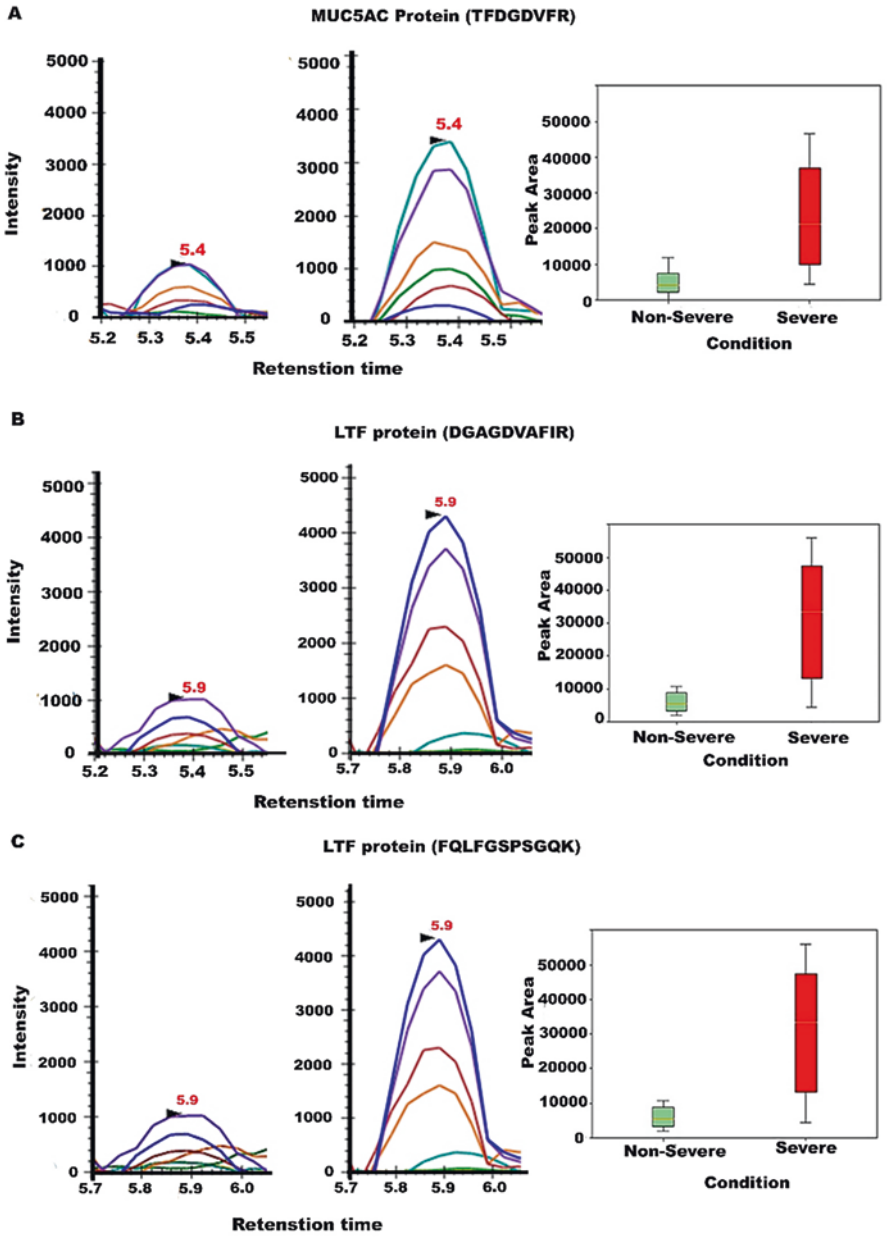

Fig. 9.3 Analysis of differential expression of peptides using the MRM approach. The figure shows the MRM peak and box plot representing the variation in the group-wise level of peptide (**a**) TFDGDVFR from MUC5AC protein, (**b**) DGAGDVAFIR from LTF protein, and (**c**) FQLFGSPSGQK from LTF protein

protein was identified and used as a positive control. Nabumetone with a binding affinity of −7.7 kcal/mol, [2-[3-[(1-benzoylpiperidin-4-yl)-methylcarbamoyl]naphthalen-2-yl]-1-naphthalen-1-yl-2 oxoethyl]phosphonic acid with the binding energy of −8.4 kcal/mol and Pfn1-IN-C1 with a binding affinity of −7 kcal/mol were used as control inhibitors for myeloperoxidase (P05164), cathepsin G (P08311), and profilin-1 (P07737) respectively. Positive control drugs were used to set a cut-off for the docking score and identify the active sites in each protein. A summarized table is included in the supplementary representing the protein, control inhibitor, and pathway involved (Table 9.S7). The pictorial representation of the control inhibitor and the shortlisted drug with the representative protein is given in Fig. 9.S4. Drugs with more negative binding energy than the control inhibitor were included in the study, and we found that their binding pocket was similar to the control drug. Only ten drugs passed these criteria (Table 9.S8). Of these, three drugs (dabrafenib, ritonavir, and itraconazole) were FDA approved (Fig. 9.4), one drug (CPI-0610) was in a clinical trial, and two drugs (4E2RCat and GB110) were in pre-clinical trials. Myeloperoxidase was inhibited by all six drugs, while only CPI-0610 inhibited

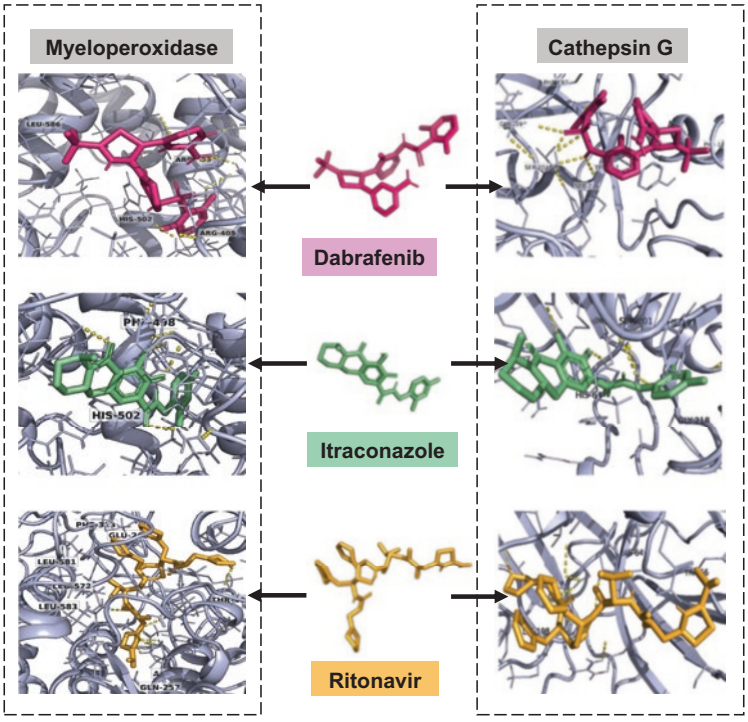

Fig. 9.4 *In silico* molecular docking studies. Three FDA-approved drugs, dabrafenib, itraconazole and ritonavir, were screened from customized drug library and were docked against the target proteins; myeloperoxidase and cathepsin G

profilin-1. For cathepsin G, all of the selected drugs except CPI-0610 acted as inhibitors.

4 Discussion

During the second wave of the COVID-19 pandemic, SARS-CoV-2 emerged as a pestilence with increased misdiagnosis, inaccurate treatment, and an incorrect disease prognosis surging as crucial challenges. In the present study, a comprehensive mass-spectrometry-based proteomic analysis using nasopharyngeal swab samples was performed to understand the variations in the host response among severe and non-severe patients with COVID-19. Investigating the host response to the pathogen is crucial to monitor the disease severity and identify newer target molecules for drug discovery or therapeutic use. Similar studies on host response were reported by Samprathi et al., who reviewed different classes of biomarkers and their importance at the clinical level [48].

C-reactive protein (CRP), lactate dehydrogenase, interleukin-6, and ferritin are some of the severity markers that have been reported in various studies of COVID-19 [49]. Most of the available studies on host severity marker proteins using swab samples considered positive and negative patients from the first wave [50]. Reports using second wave samples have been scarce. Here, we collected samples during the peak of second wave, between March and June 2021 to explore differences between severe and non-severe PCR-confirmed COVID-19 patients. From Fig. 9.2a, the scores plot showed that most of the samples fell into two groups. From the many possible factors, the emergence of different viral strains during the second wave most likely led to the increase in COVID-19 disease severity during this period. Recently, we have observed the emergence of new variants such as omicron, which has been shown be less severe with a lower impact than earlier variants that prevailed in the first and second wave of COVID-19 [51]. During the second wave, along with multiple variants, double mutant and triple mutant strains of SARS-CoV-2 including B.1.617.2 (delta) and B.1.618 emerged as the main variants in India, and the delta version remained the most dominant in the Maharashtra region [9]. However, genetic studies were not conducted in the present analysis so we could not classify the patients according to which SARS-CoV-2 strain they had been infected with. Moreover, lower vaccination rates in India during this period further accelerated the pace of disease transmission. According to reports, only 2% of total population of India had received both vaccine doses and approximately 8% had been singly vaccinated [52, 53].

In addition to SARS-CoV-2 variant type, other factors like age, gender, and comorbidities have been associated with disease severity [54, 55]. Although we found that patients categorized as severe were significantly older than those in the non-severe group, chi-square tests revealed that gender and severity were not

significantly associated. However, this could be due to low sample number and might therefore require testing using a larger cohort. Lactotransferrin, neutrophil defensin 3, galectin 3-binding protein, and neutrophil gelatinase-associated lipocalin (NGAL) were some of the significant proteins identified in the current study. In addition, we detected severity-associated changes in other proteins like peroxiredoxin-6, glutathione S-transferase, and angiotensin that were reported previously from our lab [19].

Using *in silico* pathway analysis tools, we discovered that these proteins were most significantly associated with anti-microbial peptides, immune system, and neutrophil degranulation pathways, which are components of the immune system. On mapping, proteins such as lactotransferrin, peroxiredoxin 6, myeloperoxidase, glutathione S-transferase P, and serpin B3 were found to be involved in neutrophil degranulation and immune system pathways. On the other hand, the proteins filamin-A, profilin-1, galactin-3 binding protein, and thymosin beta-4 were associated with platelet degranulation. It is now understood that SARS-CoV-2 disrupts innate immune mechanisms including platelet and neutrophil degranulation in severe cases [19, 56].

Potentially the most novel finding of the current study was our detection of the anti-microbial peptide pathway in severe cases during the second wave of SARS-CoV-2 infections. Anti-microbial peptides are bioactive molecules released as an essential component of the innate immune response. They are amphiphilic molecules with a short sequence of peptides ranging from 10 to 100 amino acids. The anti-microbial peptide database (APD) focuses on naturally occurring anti-microbial peptides isolated from organisms, including humans. This shows that many of these have an antiviral effect against enveloped RNA and DNA viruses. Most studies on anti-microbial peptides and COVID-19 have focused on the spike (S) protein that mediates viral entry into the cells. Given our present findings, we suggest that the anti-microbial peptide pathway may also help to reduce viral transmission and the resulting infection severity [57]. In line with this idea, we identified host proteins such as lactotransferrin, neutrophil gelatinase-associated lipocalin (NGAL), cathepsin G, neutrophil defensin 3, BPI fold-containing family B member 1, and BPI fold-containing family A member 1 that were mapped to this pathway. Among these proteins, lactotransferrin shows significant antimicrobial activity. Reports have demonstrated the action of this protein in blocking the spike protein in host cells, indicating an inhibitory effect in the viral attachment stage. Moreover, lactotransferrin increases host immunity and reduces viral replication after viral invasion of cells. Because of these properties, we suggest that lactotransferrin is a potential antiviral agent against COVID-19 infection [58].

Cathepsin G is another protein from the antimicrobial peptide family which showed markedly increased levels in severe COVID-19 patients. Cathepsin G is a serine protease that eliminates intracellular pathogens by breaking down tissues at inflammatory sites. It stimulates cytokine production and recruits the immune cells to the site of tissue damage. It has also been reported to be involved in the

pathogenesis of acute respiratory distress syndrome (ARDS) in severe cases of COVID-19. It is interesting in this regard that Korkmaz and colleagues suggested that the inhibition of cathepsin might help in controlling ARDS and lung injury due to cytokine storm effects [59]. Of particular relevance to this suggestion, we found that ZINC4097343 can be used to inhibit cathepsin G as a potential target in the treatment of severe COVID-19 patients.

Neutrophil defensin 3 belongs to a class of antimicrobial peptide family secreted by neutrophils in pathogenic responses. Interestingly, neutrophilia and elevated levels of defensin have been associated previously with disease severity in COVID-19 [60]. Studies conducted by Xu et al. showed that neutrophil peptides and defensin exhibited significant antiviral activity against expressed SARS-CoV-2 spike proteins [61]. Our analysis found that the defensin levels were upregulated in severe compared to the non-severe samples. Schulte-Schrepping et al. also reported a higher expression of defensin in severe patients which supports our analysis and the case for this protein as a COVID-19 severity marker [62]. This may also be the case for BPI fold-containing family A member 1 and BPI fold-containing family B member 1, as we found that both proteins were associated with disease severity in the present study. Both of these proteins are expressed by the innate immune system in response to the bacterial infection in the mouth, nasal cavities, and lungs. Studies have found higher expression of both proteins associated with COVID-19 disease severity. Upregulation of the BPIF gene has also been reported in chronic obstructive pulmonary disease (COPD) and patients with a history of this disease are prone to a more severe disease course during COVID-19 infections [63, 64].

Finally, we selected myeloperoxidase, cathepsin G, and profilin-1 for *in silico* docking studies due to their potential roles in severe COVID-19 disease and because a molecular inhibitor had been reported in the literature for each of these. In disease conditions, myeloperoxidase can induce vasoconstriction [65] and hypoxia and cathepsin causes ARDS [66]. In addition, profilin is involved in viral replication and inhibiting this protein can block viral maturation [67]. Both myeloperoxidase and cathepsin G showed inhibitory effects against the FDA-approved drugs dabrafenib, itraconazole, and ritonavir. Dabrafenib is a kinase inhibitor used to treat melanoma, non-small cell lung cancer, and thyroid cancer [68]. Similarly, ritonavir is an HIV protease inhibitor used in combination with other antivirals in the treatment of HIV infection [69]. Our *in silico* molecular docking study showed that ritonavir can be used to target myeloperoxidase and cathepsin G. ZINC4097343 is another FDA-approved drug which showed inhibition activity against myeloperoxidase and cathepsin G. Additionally, CPI-0610 drug can also be explored to target COVID-19. This drug is in clinical trials and has shown inhibitory effects against myeloperoxidase and profilin-1.

To summarize, this is the first study to identify significant alterations in proteins associated with the anti-microbial peptide pathway in severe COVID-19 infections as part of the disastrous second wave in India. Based on our preliminary analysis, we predict that proteins associated with this pathway such as myeloperoxidase, cathepsin G, and profilin-1 represent potential therapeutic targets of dabrafenib,

ZINC4097343, and ritonavir, respectively. Nonetheless, further validation studies are required.

4.1 Limitations

For the current study, cohort size and lack of complete medical history were the main limiting factors. Although we have the clinical information of the patients, various parameters like medical history and comorbidities were not accounted for in the clinical records. Furthermore, the effects of gender and age were not considered in the present analysis. Thus, this study needs to be validated using a larger well-characterized cohort with consideration of demographic factors, as well as comorbidities and medications.

4.2 Conclusions

The findings of this study suggest that changes in the expression of proteins associated with antimicrobial pathways played a prominent role during the second wave of COVID-19 infection. The altered expression of these proteins in severe patients could be explored further as potential biomarkers to predict severity of the infection. Validation of the findings of this study in additional well-characterized clinical cohorts will benefit the scientific community trying to decipher the dynamic mechanism of the SARS-CoV-2 infection with time and guide clinicians to better manage patients for more favorable clinical outcomes.

Author Contributions The study was designed by S.S., S.R., and D.N. including hypothesis and experimental sketch. Collection of clinical specimens was performed by V.P. and A.S. Sample preparation for mass spectrometry was done by S.R., D.N., and K.S. Data analysis was done by D.N., S.R., M.G., A.G., and H.D. In data visualization D.N., S.R., K.S., D.B., and A.B. were involved. Drug docking studies were conducted by A.V., V.D., and A.B. Data uploading on PRIDE was done by A.B. Review and writing part of the manuscript was done by S.S., S.R., D.N., K.S, A.S., V.P., M.G., A.V., and D.B.

Competing Interests Statement and Notes The authors declare no competing financial interest. All the proteomics data obtained from the swab samples are deposited on the ProteomeXchange Consortium via the PRIDE partner repository with data set identifiers PXD029300 and PXD041609.

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Chapter 10
NMR-Metabolomics in COVID-19 Research

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Abstract COVID-19 stands for Corona Virus Disease 2019, which starts as a viral infection that provokes illness with different symptoms and severity. The infected individuals can be asymptomatic or present with mild, moderate, severe, and critical illness with acute respiratory distress syndrome (ARDS), acute cardiac injury, and multiorgan failure. When the virus enters the cells, it replicates and provokes responses. Most diseased individuals resolve the problems in a short time but unfortunately, some may die, and almost 3 years after the first reported cases, COVID-19 still kills thousands per day worldwide. One of the problems in not curing the viral infection is that the virus passes by undetected in cells. This can be caused by the lack of pathogen-associated molecular patterns (PAMPs) that start an orchestrated immune response, such as activation of type 1 interferons (IFNs), inflammatory cytokines, chemokines, and antiviral defenses. Before all of these events can happen, the virus uses the infected cells and numerous small molecules as sources of energy and building blocks for newly synthesized viral nanoparticles that travel to and infect other host cells. Therefore, studying the cell metabolome and metabolomic changes in biofluids might give insights into the state of the viral infection, viral loads, and defense response. NMR-metabolomics can help in solving the real-time host interactions by monitoring concentration changes in metabolites. This chapter addresses the state of the art of COVIDomics by NMR analyses and presents exemplified biomolecules identified in different world regions and gravities of illness as potential biomarkers.

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Keywords COVID-19 · SARS-CoV-2 · Thrombosis · Biomarkers · Metabolomics · NMR spectroscopy · COVIDomics

1 Introduction

Coronaviruses belong to a group of infectious viruses, which infect animals and humans, causing respiratory illness [1, 2]. In December 2019, a new zoonotic coronavirus was reported in Wuhan City, China [2, 3]. This was designated as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causal agent of coronavirus disease 2019 (COVID-19), which is highly transmissible, as seen by its rapid spread all over the world. It was estimated that 17.1 million to 19.6 million people died in 2020 and 2021 due to COVID-19 complications [4]. Even now, the virus is causing infections and deaths although vaccination has mitigated the COVID-19 pandemic situation. There is still the risk of the emergence of new coronavirus variants because low-income and middle-income countries (LMICs) are only partially vaccinated, due to limited access to vaccines or the lack of awareness of the importance of vaccination [5].

The clinical features of COVID-19 are classified into cases with different severity such as asymptomatic, mild disease (fever, fatigue, myalgia, dry cough, sore throat, and headache), moderate (fever greater than 37.8 °C and symptoms of pneumonia), severe (dyspnea and hospitalization in intensive care unit), and critical cases (acute respiratory distress syndrome [ARDS], acute cardiac injury, and multiorgan failure) (Fig. 10.1) [2, 6]. The gravity of the symptoms is linked to age, comorbidities (diabetes, cardiovascular disease, hypertension, and others), genetic factors, unhealthy eating habits, and lifestyle factors, such as the lack of physical exercise [2, 7].

In this sense, it is important to diagnose COVID-19 during the asymptomatic stage or when the first mild symptoms appear because the infected individuals with SARS-CoV-2 in this disease stage are a source of infection [8].

Diverse strategies have been tested and/or used for the discovery of COVID-19 biomarkers to aid in diagnosis [9–11], with mass spectrometry and NMR spectroscopy being the most commonly used techniques in COVID-19 metabolomics research [12]. NMR-based metabolomics is an approach that has allowed the identification of biomarkers in different sample types and biological mixtures [13]. Furthermore, NMR spectroscopy is a suitable analytical platform for disease studies, because it is a highly reproducible technique, which can be used in studies that require large-scale and longitudinal research [12, 14].

In addition to the identification of biomarkers for COVID-19 disease severity, NMR spectroscopy has also been used for metabolite quantification [15], evaluation of vaccine effects [16, 17], studies of viral cell shielding [18], identification of the

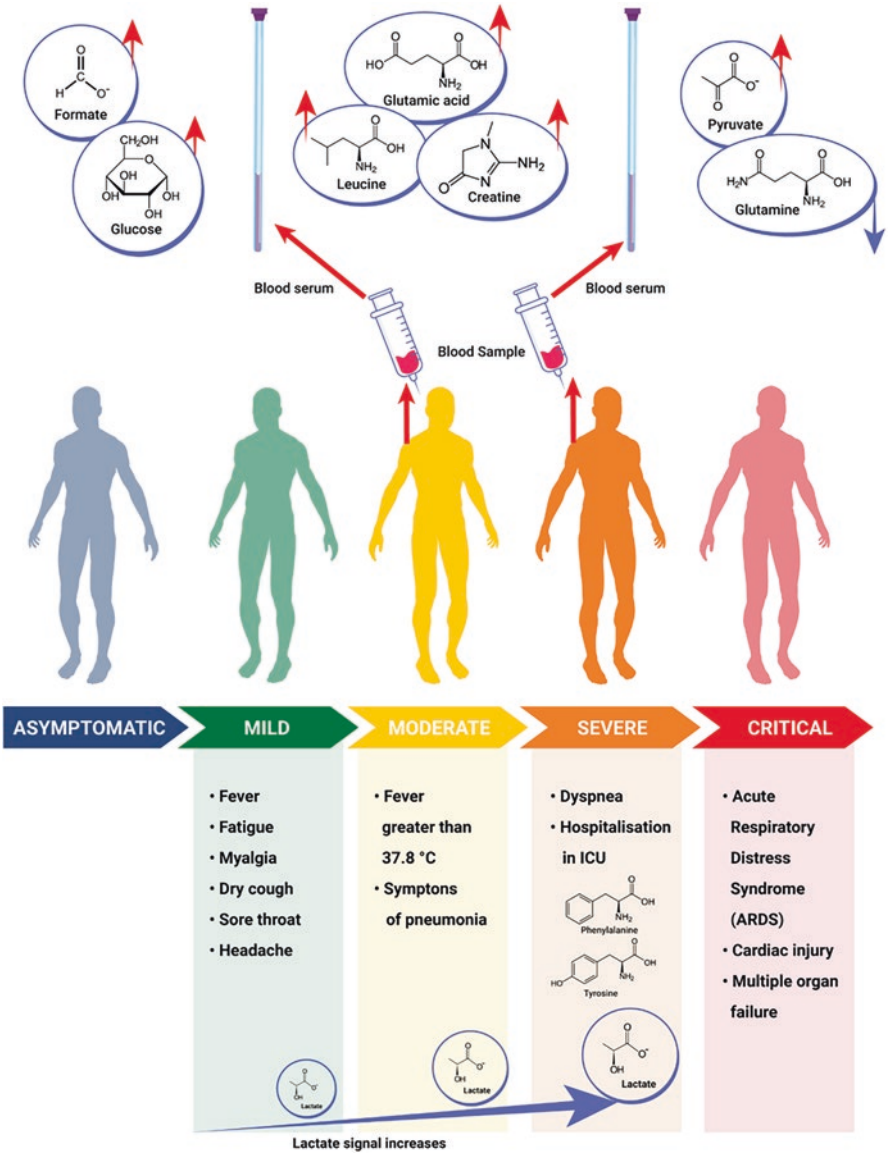

Fig. 10.1 Five stages of COVID-19. The asymptomatic profile does not have biomarkers due to difficulties in recruiting people to collect blood for testing at this stage

etiological causes of ARDS [19], and monitoring the mental health of patients affected by COVID-19 [20]. In this chapter, we discuss about the COVID-19 severity biomarkers detected by NMR spectroscopy approaches and those found in cardiovascular and thrombosis risks resulting from the disease.

2 Biomarkers of COVID-19 Severity

Since the escalation of the first COVID-19 cases, the scientific community has performed a collective effort to ameliorate the pandemic situation. Omic-based searches have acquired, interpreted, and integrated different data about this concern, which has been named "COVIDomics." Blood plasma and serum have been mostly used as biological samples in metabolomics studies [21]. Table 10.1 and Fig. 10.1 summarize the metabolites detected by ^{1}H-NMR spectroscopy as biomarkers of different COVID-19 stages.

There are few studies which have used saliva as an NMR sample, which could indicate the nasopharyngeal state of COVID-19 patients [12]. However, there are some limitations to the use of these approaches, such as the low concentration of metabolites (99% water and 1% inorganic and organic compounds), diary fluctuations of metabolite concentrations, and the possible influence of oral injuries. In these cases, it would be necessary to perform metabolomics analyses in an NMR

Table 10.1 Biomarkers of COVID-19 severity detected using ^{1}H-NMR spectroscopy

Metabolites	Samples (concentration in relation to disease stage)	Peak Assignments	References
Creatine	Plasma (increase in moderate-severe)	3.04 (s), 3.94 (s)	[15, 23]
Formate	Blood and urine (higher levels in moderate)	8.45 (s)	[15, 24, 25]
Glucose	Plasma (higher levels in moderate)	3.23 (m), 3.40 (m), 3.46 (m), 3.52 (dd), 3.78 (m), 3.82 (m), 3.89 (dd), 4.64 (d), 5.23 (d)	[15, 23, 26]
Glutamate	Plasma (higher levels in moderate)	2.04 (m), 2.13 (m), 3.35 (m), 3.75 (m)	[15, 27]
Glutamine	Blood (decrease in severe)	2.12 (m), 2.15 (m), 2.44 (m), 2.48 (m), 3.77 (dd)	[23, 24, 26]
Lactate	Blood (increase in accordance to the infection process)	1.33 (d; J = 7.0 Hz), 4.12 (q; J = 7.0 Hz)	[15, 24, 28]
Leucine	Plasma (decrease in mild stage and a slight increase in moderate-severe)	0.96 (d; J = 6.2 Hz), 0.97 (d; J = 6.1 Hz), 1.68 (m), 1.72 (m), 1.75 (m)	[15, 23]
Phenylalanine	Blood (increase in accordance to the infection process)	3.13 (m), 3.28 (m), 7.34 (d; J = 7.5 Hz), 7.38 (t; J = 7.4 Hz), 7.44 (t)	[15, 23, 24, 28, 29]
Pyruvate	Blood (higher levels in severe)	2.38 (s)	[15, 24]
Tyrosine	Blood (increase in accordance to the infection process)	3.05 (dd), 3.20 (dd), 3.93 (dd), 6.91 (d; J = 8.5 Hz), 7.20 (d; J = 8.5 Hz)	[15, 23, 28]

spectrometer with pulsed and magnetic field gradients to improve sensitivity and accuracy [12, 22].

Glutamine deficiency has been reported in severe COVID-19 patients [23, 26, 30]. The acute inflammatory process featured in COVID-19 requires energy from an increase in the concentration of some metabolites of the tricarboxylic acid cycle (TCA), such as glucose and glutamine [31]. When the endogenous synthesis of glutamine is not sufficient to supply all the needs of the infected body, the plasma glutamine levels are reduced [23]. The use of glutamine supplementation has been suggested for COVID-19 patients [30], since glutamine performs important roles in energy metabolism, the immune response, and cytokine production [23, 26].

Like glutamine, glutamate also takes part in energy metabolism, being the first step in glutathione biosynthesis. Furthermore, glutamate and glutamine act in nucleotide biosynthesis as nitrogen donors [26, 30]. In COVID-19, the unregulated glutamate levels in patients with hypoxia may cause neurological abnormalities since glutamate is also an important neurotransmitter [32]. Reductions in glutamine and increased glutamate in blood plasma are associated with health risks [30].

Elevated creatine levels have been associated with muscular energy metabolism in severe COVID-19 patients [15, 23]. Creatine-kinase converts creatine to the energy storage form of phosphocreatine. The high creatine concentration in infected cells is possibly related to the energy consumption changes during viral replication [15], as well as in COVID-19 patients, who spend significant time in the hospital without utilizing muscular energy [23].

Unregulated leucine, isoleucine, and valine concentrations in blood plasma are harmful to the organism, because these compounds may cause inflammation and neurological impact, and promote oxidative stress [23]. Other unregulated amino acid concentrations observed in severe COVID-19 groups include phenylalanine and tyrosine [33]. Barberis et al. observed perturbations in phenylalanine metabolism, arachidonic acid metabolism, and tricarboxylic acid cycle (TCA) in COVID-19 patients [33]. Since this report, the increase in phenylalanine and tyrosine concentrations in blood samples has been reported in a various researches on this issue (Table 10.1). Correia et al. quantified these amino acids and observed that although tyrosine concentration increased with COVID-19 severity, tyrosine levels remained slightly lower than those of phenylalanine during the infection process, which may point to a disturbance in the immune system [15].

The increase in pyruvate levels in blood samples from COVID-19 patients may be related to breathing difficulties felt by these individuals, which decreases the oxygenation rate essential in biochemical processes. In aerobic conditions, pyruvate is converted to lactate and eliminated from the TCA [15]. However, in hypoxic conditions, pyruvate is regulated by anoxic respiration, which increases the NADH concentration. Under these conditions, pyruvate dehydrogenase is allosterically hampered in performing pyruvate oxidation [27].

Pyruvate is not commonly used as a biomarker because it is sensitive to preanalytical procedures [34]. Due to this problem, other methods to monitor COVID-19 have been performed through measurements of lactate or the lactate-to-pyruvate conversion rate, which has been reported as a biomarker of severe respiratory

dysfunctions [26]. Hyperlactatemia is featured in sepsis and some damages caused by COVID-19 such as end-organ injury, systemic dysfunctions, ischemia, and thrombosis [35].

Elevated formate levels in plasma and urine samples have been associated with indicators of environmental exposure to contaminants or as biomarkers of impaired one-carbon metabolism [25, 36]. However, in COVID-19 cases, patients with moderate and severe disease may be in isolation or hospitalized. Therefore, they are not likely to be exposed to environmental contaminants. In these cases, formate changes may be related to sarcopenia or kidney damage [25].

It is worth mentioning that acetylated glycoproteins and phospholipids have been reported as changed in COVID-19 and might be considered important inflammatory biomarkers that can be measured in clinics by applying low-field NMR [37]. For instance, *N*-acetyl signals from glycosylated serum proteins were found to be elevated and phospholipids showed an inverse relationship in COVID-19 patients. Therefore, the phospholipid:acetylated glycoprotein ratio has been suggested as a biomarker for inflammation assessment [26, 37, 38].

Finally, it can be assumed that NMR-monitored metabolic changes during COVID-19 are driven by immune system regulation of key metabolic enzymes by cytokines, the energy consumption of cytokine-secreting cells, or by the effect of immune cells on other tissues [39]. In line with this, altered lipid metabolism has been observed in COVID-19, including effects on cholesterol and cholesterol esters, sphingolipids, and saturated fatty acids (FAs). Furthermore, an increase in the triglyceride levels of lipoproteins with different densities, such as very-low (VLDLs), low- (LDLs), and high-density lipoproteins (HDLs), and the fatty acids (FAs) saturation state have been associated with increased disease severity.

3 Principal Risks Caused by COVID-19

There are many consequences reported in convalescent COVID-19 patients, some of which have been linked to thromboembolic episodes. Therefore, it is crucial to understand in which way COVID and post-COVID syndrome metabolomic and lipidomic alterations are linked to thrombosis. Although NMR studies of blood metabolite alterations in thrombosis have not been thoroughly explored in the literature [40, 41], some works have reported effects on lipids, free fatty acids (FFAs), acylcarnitines, trimethylamine *N*-oxide, and their involvement in thromboinflammation and platelet dysfunctions [42]. It is also important to note that low-density lipoproteins (LDLs) and their oxidation products (oxLDLs) take part in prothrombotic responses. Another important class of metabolites linked to thrombosis is sterol and derivatives, such as cholesterol and cholesterol esters. Their involvement in thrombosis is expected, as well as other thrombotic risks, such as hypercholesterolemia, the enhanced inflammatory potential of lipid-laden platelets, and changes in circulatory lipids and atherogenic chemokines. Also, imbalances in the ratio of branched-chain amino acids (BCAAs) to alanine, as seen in thrombosis [41], as

well as alterations in lipids, acetoacetate, pyruvate, glucose, 3-hydroxybutyrate, lactate, creatine, and phenylalanine [41, 43], might be linked to pathological conditions in thrombosis and have been observed in COVID-19 (Fig. 10.2). Thus, these represent potential biomarker candidates for prediction of thrombotic risk in moderate and severe patients. It is worth mentioning that, of the 20 biomarkers reported for thrombosis, most of them are also increased in COVID-19 patients. In addition, the potential role of platelets beyond thrombosis and hemostasis is coming under increasing attention. It is known that platelets mediate inflammation through

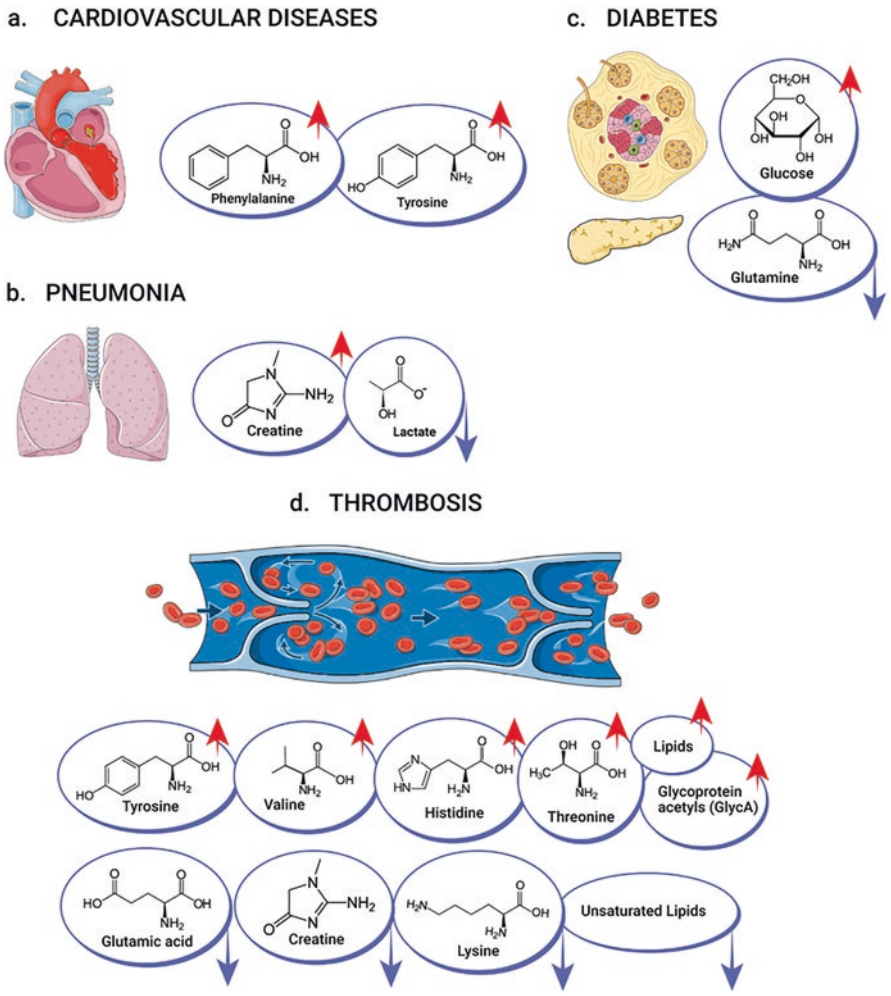

Fig. 10.2 Principal risks associated with COVID-19 disease: (**a**) cardiovascular diseases, (**b**) pneumonia, (**c**) diabetes, and (**d**) thrombosis. The associated metabolites are also indicated by either increased (up, red arrows) or decreased (down, blue arrows) concentrations. Heart, lung, and vein were adapted from link smart.servier.com (free medical images)

interactions with immune cells or cytokine/chemokine secretion [44] and patients affected by COVID-19 have been reported to have micro-thromboses due to altered immune function [45]. Furthermore, pro- and anti-inflammatory lipid mediators may be generated by activated platelets during the inflammation course [42].

Other important roles of platelets in the immune response have been recognized, such as the capacity to guide immune cells to the infected area and in recognizing and neutralizing pathogens. Platelets also play an important role in the coagulation cascade and in resolving bleeding problems during vascular injuries, but their inappropriate activation can cause thrombosis. Many proteins that interact with platelets, such as fibrin and collagen, are also recognized as key players in the clotting process.

Platelets are blood cells that circulate freely till the moment the blood vessel suffers an injury. Then, the platelets organize themselves and initiate clot formation by forming spider-net-like 3D structures with actin and fibrin, which are adhesion matrix proteins with motifs that recognize and stick to the platelets. As stated earlier, platelets also take part in immune responses. For example, CD8+ T-cells which take part in the adaptive immune response are orchestrated by platelets against viral hepatitis [46]. Considering these dual roles, an infection can cause secondary thrombocytosis, a condition marked by increased platelets and thrombosis formation.

During the process of clot formation, platelets participate in the regulation, recruitment, and functions of innate immune cells. As part of this, the platelets release the following mediators: chemokine ligand 1 (CXCL 1), CXCL 3, 5, and 7, and beta-chemokines ligand 5 (CCL 5) and 7, among others that trigger receptors expressed on myeloid cells. There are two families of cytokines which can be distinguished based on the first cysteine residue. The first is the family called CC chemokines, also known as beta-chemokines, and the second is called CXC chemokines, alpha-chemokines, which have an amino acid between the first two cysteines. CC chemokines mainly stimulate monocytes, as well as basophils, eosinophils, T-lymphocytes, and natural killer (NK) cells. On the other hand, CXC chemokines, which primarily stimulate neutrophil chemotaxis, contain a glutamate-leucine-arginine (ELR) sequence at the N-terminus that is essential for receptor binding. These mediators support leukocyte accumulation and promote their microbicidal activity. Furthermore, platelets interact with innate immune cells affecting effector functions such as the formation of extracellular neutrophil trap (NETosis) and cell migration. During the formation of neutrophils and other white blood cells, such cells expel their chromatin content, which participates in the formation of the extracellular fiber network, capturing and eliminating pathogens. So, NETs act in different biological activities through activation and promotion of recruitment of platelets as well as their feedback mechanisms, thus causing a thrombosis [46, 47].

Although NETose is generally associated with responses against extracellular pathogens, there is increasing evidence that NETose also occurs in viral infections [48]. During the COVID-19 pandemic, several reviews cited the formation of NETs as a major defense mechanism [49]. This was especially marked in the cases of ARDS, being easy to identify due to its high presence in plasma [49, 50]; Autopsy of COVID-19 victims also showed a high number of neutrophils [51].

In addition to immunothrombosis, COVID-19 can cause hyperinflammation and hypercoagulopathy [47]. One of the causes of hyperinflammation is ARDS, which is triggered by primary micro-thrombi in the pulmonary vessels, evolving to systemic microangiopathy and can lead to a variety of damaging scenarios in the COVID-19 patient such as the encompassing cardiomyopathy, multiple organ dysfunction syndromes (MODS), hepatic and renal failure, mesenteric ischemia, and neurological dysfunctions (Fig. 10.2) [47]. Another cause of hyperinflammation commonly seen in COVID-19 is the NLRP3 inflammasome activation, which can result in various cardiovascular disorders [52], characterized by increasing concentrations of plasminogen activator inhibitor I (PAI-1), free fatty acids (FFAs), leptin, interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) [53].

The NLRP3 inflammasome (NOD-, LRR-, and pyrin domain-containing protein 3) is a sensor, with an adapter (ASC or PYCARD) and an effector (caspase 1). For example, the protein (NLRP3) that plays an important function is a three-domain ATPase, which also has a purine domain on its amino-terminal, and a carboxy-terminal rich in leucine and arginine residues (LRR domain). Its central domain is an ATPase that is crucial for self-association and function. The final LRR domain is considered important for self-inhibition by reducing the ATPase function and stopping the auto-oligomerization. NLRP3 oligomers recruit ASC using homotypic interactions of the pyrin domain (PYD–PYD interactions) and act through nucleation in the formation of helical ASC filaments, which also occur by PYD–PYD interactions. Then, the multiple ASC filaments coalesce into a macromolecular focus, named the ASC spot. Further, processing between the carboxy-terminal caspase recruitment domain (CARD) and p20 releases p20–p10 from the ASC. The protease activity of the p20-p10 heterotetramer is lost in the cell, due to its instability. Recently, NIMA-related kinase 7 (NEK7), an enzyme that plays a role during the mitosis process, has been reported as an essential enzyme for the NLRP3 inflammasome activation. The assembly of NEK7 leads to caspase 1-dependent release of antibodies (IL-1β and IL-18) and gasdermin D-mediated pyroptosis. Activation of NLRP3 results in several biochemical responses, most of which are not mutually exclusive, including ion efflux (K^+ and Cl^-), calcium ion flux, metabolic changes, and cellular consequences such as mitochondrial dysfunction, lysosomal disruption, and trans-Golgi disassembly [54].

As explained briefly, blood clotting is a complex process involving an orchestrated and precise action of many proteins such as clotting factors, chemokines, receptors, binding motifs, signaling molecules, and the participation of platelets. Nevertheless, if provoked by inflammation and unchecked by normal feedback mechanisms, enhanced clotting can occur, which can cause severe damage to health and even death, as in embolisms, stroke, or other grave thrombotic events. It appears that in COVID and post-COVID micro-thrombosis, the associated hyperinflammation and altered blood metabolites can drive such undesired and uncontrollable clotting events, leading to thrombosis and potentially fatal outcomes.

4 Conclusions and Perspectives

COVIDomics by NMR has shown significant potential in mapping serum and plasma samples, and some blood metabolites are now being considered as potentially disease-relevant prognostic biomarkers. We have cited the main roles and explored the potential of the most common metabolites described in the recent literature. Among these, the lipoproteins, some lipids, lactate, glucose, aromatic amino acids, and creatine draw the most attention and concerns when disease gravity is discussed. Interestingly, most of the discovered metabolites showed higher concentrations of biofluids in the disease state, which might point to the changing viscosity of the blood and increased ionic force. This suggests that the resulting concentrated blood may lead to an increased risk for coagulation abnormalities and blood clotting dysregulation as shown by the micro-thrombotic events and thromboses in COVID-19.

Alongside mapping blood alterations provoked by COVID-19, there are many efforts to link the disease hallmarks with factors such as the risk of hospitalization and negative outcomes. As NMR-metabolomics is expensive due to equipment costs, and due to practical considerations such as the lack of trained professionals in clinics, the new wave in exploring NMR-metabolomics in COVID-19 research is to design pulse-sequences for low-field and portable NMR types of equipment that trained professionals without a strong background in NMR can use with ease and obtain similar results as with the high-field equipment. The progress in this regard is not only relevant for COVID-19 risks but also for clinical monitoring of other diseases that are still challenged with imprecise or doubtful diagnoses or with difficulties in predicting outcomes or treatment effects.

Another important issue is the critical factor of obtaining reliable samples for NMR analysis and overcoming any bioanalytical drawbacks among the different sample handling and storage steps [49]. This is especially so when the samples in question have a viral and potentially life-threatening origin. Even so, recent procedures have been put in place to overcome these issues [55].

Last, but not least, NMR-metabolomics might shed the light on disease mechanisms and immune response, track early markers of disease severity, and bring insights into treatment effects, among others, therefore enabling us to estimate the recovery or the need for hospitalization. Many benefits of metabolomics by NMR in revealing COVID-19 early markers might positively affect the present and future pandemics, making this exciting research field one of the most important diagnostics and prognostics tools.

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Chapter 11
Potential Biomarkers of Mitochondrial Dysfunction Associated with COVID-19 Infection

Nadia Turton, Lauren Millichap, and Iain P. Hargreaves

Abstract Mitochondria play crucial roles in modulating immune responses, and viruses can in turn moderate mitochondrial functioning. Therefore, it is not judicious to assume that clinical outcome experienced in patients with COVID-19 or long COVID may be influenced by mitochondrial dysfunction in this infection. Also, patients who are predisposed to mitochondrial respiratory chain (MRC) disorders may be more susceptible to worsened clinical outcome associated with COVID-19 infection and long COVID. MRC disorders and dysfunction require a multidisciplinary approach for their diagnosis of which blood and urinary metabolite analysis may be utilized, including the measurement of lactate, organic acid and amino acid levels. More recently, hormone-like cytokines including fibroblast growth factor-21 (FGF-21) have also been used to assess possible evidence of MRC dysfunction. In view of their association with MRC dysfunction, assessing evidence of oxidative stress parameters including GSH and coenzyme Q10 (CoQ10) status may also provide useful biomarkers for diagnosis of MRC dysfunction. To date, the most reliable biomarker available for assessing MRC dysfunction is the spectrophotometric determination of MRC enzyme activities in skeletal muscle or tissue from the disease-presenting organ. Moreover, the combined use of these biomarkers in a multiplexed targeted metabolic profiling strategy may further improve the diagnostic yield of the individual tests for assessing evidence of mitochondrial dysfunction in patients pre- and post-COVID-19 infection.

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1 Introduction

The mitochondrial electron transport chain (ETC) is composed of four enzyme complexes: complex I (NADH: ubiquinone reductase; EC 1.6.5.3); complex II (succinate:ubiquinone reductase; EC 1.3.5.1); complex III (ubiquinol: cytochrome *c* reductase; EC 1.10.2.2) and complex IV (cytochrome *c* oxidase; EC 1.9.3.1) [1, 2]. It is located in the inner mitochondrial membrane and contains two electron carriers, coenzyme Q10 (CoQ10) and cytochrome *c*. The synthesis of ATP by oxidative phosphorylation is operated by the ETC together with complex V (ATP synthase) (Fig. 11.1). Mitochondria play crucial roles in modulating immune responses, and viruses can in turn moderate mitochondrial functioning [3]. Thus, clinical outcome experienced in patients with COVID-19 disease or those suffering from long COVID syndromes may be influenced by mitochondrial dysfunction [4]. Moreover, patients who are predisposed to mitochondrial respiratory chain (MRC) disorders may be more susceptible to worsened clinical outcomes [5]. The most common group of

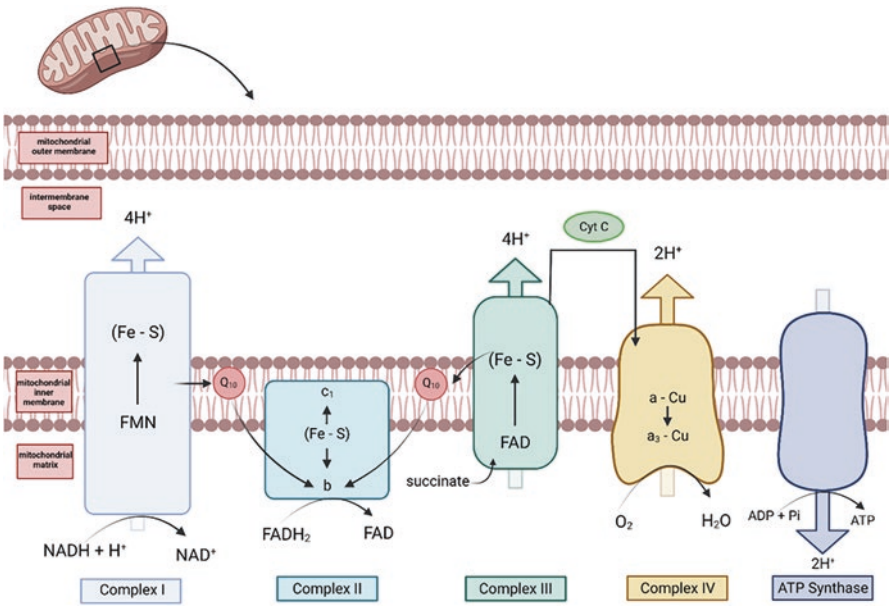

Fig. 11.1 Diagram showing the role of coenzyme Q10 (CoQ10) in the mitochondrial electron transport chain (ETC). (Created using Biorender.com)

metabolic diseases which affect 1 out of 5000 individuals is MRC disorders [6]. The incidence of this disorder in the United Kingdom has been estimated to be as high as 1 in 4300 [7]. MRC disorders may be associated with a decrease in the levels of the lipid-soluble antioxidant CoQ10 in patients [8]. This lipophilic molecule exists in either the reduced, ubiquinol form (CoQ10H2) or an oxidized, ubiquinone form (CoQ10) [8]. Its primary role is in the mitochondrial ETC, where it accepts electrons derived from complex I (NADH ubiquinone reductase; EC 1.6.5.3) and complex II (succinate ubiquinone reductase; EC 1.3.5.1) and transfers these to complex III (ubiquinol cytochrome *c* reductase; EC 1.10.2.2) [9]. In view of the role of COQ10 in the MRC as well as its function in mitigating oxidative stress and inflammation, it cannot be assumed that a deficiency of this coenzyme could be useful as a specific biomarker of disease susceptibility in COVID-19-related disorders. Thus, assessment of this molecule in combination with other biomarkers of oxidative stress such as reduced glutathione (GSH) could add a degree of specificity to this prospect and provide further insights into the cause of MRC dysfunction in COVID-19 patients [8, 10]. Alternatively, other means of assessing MRC dysfunction may be utilized in patients with COVID-19 infections. These may include metabolite analysis in urine and blood samples, such as measurement of plasma pyruvate, lactate and amino acids, as well as urine organic acid profile analyses [11]. In view of this, urinary/blood samples may be utilised in multiplex approaches to assess parameters of MRC dysfunction via the use of both high-performance liquid chromatography (HPLC) and mass spectrometry (MS).

As potential components of these multiplex approaches, hormone-like cytokines, fibroblast growth factor-21 (FGF-21) and growth differentiation factor-15 (GDF-15) may have some utility as biomarkers [11, 12]. However, the most reliable biochemical assessment for the determination of MRC dysfunction in patients is the spectrophotometric measurement of the MRC complex activities in a skeletal muscle biopsy or in other tissues where the disease is presenting itself [11]. Although these biochemical methods have certain limitations, a global approach which assesses the measured biomarkers synergistically may increase the diagnostic accuracy. Thus, the present review highlights biochemical methods used in identification of biomarkers of MRC dysfunction. Although these are presented in isolation, this is done with a view to combining these in multiplex approaches for use in screening and diagnosis of COVID-19 disease and as a guide to targeted therapeutics (Fig. 11.2).

2 Assessment of Mitochondrial Respiratory Function

Assessing MRC enzyme activities is critical for the investigation of mitochondrial function in a range of disorders. To date, the most reliable biochemical method for analysing evidence of MRC dysfunction in patients, which may be applicable in COVID-19 disease or long COVID patients, is spectrophotometric assay of MRC enzyme activities [6, 11, 13].

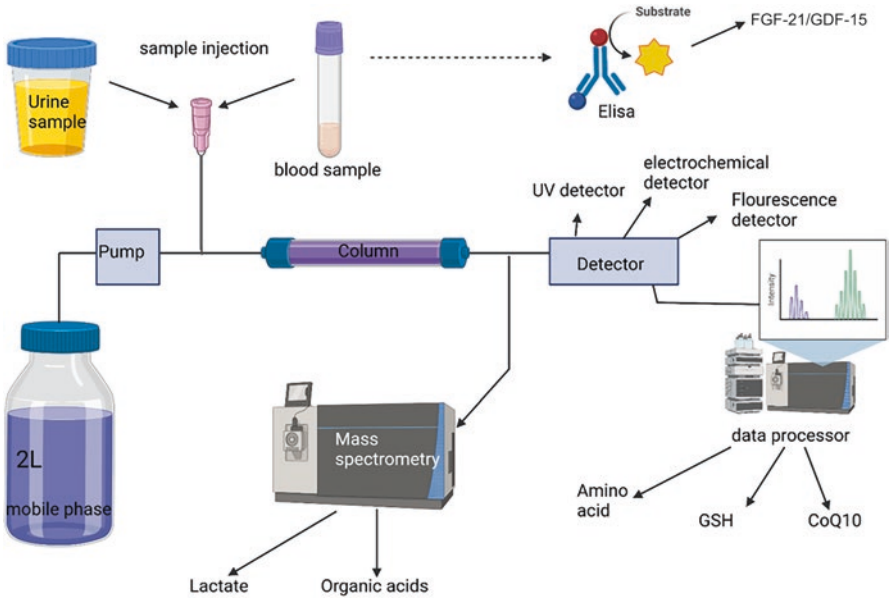

Fig. 11.2 Theoretical multiplex approach to assessing parameters of MRC dysfunction and oxidative stress in COVID patients using two possible matrixes, urine and blood samples. (Created using biorender.com)

To gain sufficient access to the substrates required for determination of MRC enzyme activities, cycles of freeze–thaw can be used to compromise the inner mitochondrial membrane [14]. Then, the spectrophotometric assay can provide information on the maximal activities of the exposed MRC enzymes. However, this assay is not performed under physiological conditions since the substrate concentrations, pH and other assay conditions are optimized to allow for maximal activities to be measured [6, 14]. Nonetheless, these assays still hold benefits in being easily performed and reproducible. The activities of complexes I, II, III and IV can be determined using spectrophotometric assays and are usually expressed as normalized values with respect to the total protein concentration of the sample [15]. Alternatively, activity can be expressed as a ratio to citrate synthase (CS; EC 1.1.1.27) activity for normalization to mitochondrial content [15–17]. This latter approach allows for age effects to be taken into account and removes the requirement for age-specific reference intervals [17]. In addition, measurement of the electron carrier CoQ10 can be performed by determining the activities of complexes II–III (succinate:cytochrome *c* reductase; EC. 1.3.5.1 + 1.10.2.2) and I–III (NADH:cytochrome *c* reductase; EC 1.6.5.3 + 1.10.2.2) as these require endogenous CoQ10 for the transfer of electrons [16]. In-house quality controls are used frequently to test for reliability of such spectrophotometric assays as these are generally not commercially available [10]. These can be obtained from frozen skeletal muscle tissue with no prior evidence of an MRC disorder. At present, there are no external quality assurance schemes available for MRC enzyme activity assessment. Thus, specialist centres are unable to

compare their results to agree on appropriate diagnostic criteria of MRC enzyme deficiencies in patients [10]. Also, misdiagnoses can occur as a result of poor storage/handling of skeletal muscle samples as this can result in a significant decrease in the levels of mitochondrial (mt)DNA and a simultaneous loss in MRC enzyme activity in the tissue. Thus, it is important to follow strict sample handling procedures for obtaining the most accurate results [6, 10, 13].

3 Pyruvate and Lactate

MRC disorders can also be detected by identifying lactate levels in cerebrospinal fluid (CSF), urine and blood serum [18]. Under normal conditions, pyruvate is converted via aerobic respiration into acetyl-CoA through the activity of pyruvate dehydrogenase. Acetyl-CoA can then enter the Krebs cycle for complete oxidation to CO_2 and H_2O, driving ATP production [19]. However, under conditions of impaired pyruvate utilization, it is reduced to lactate by the enzyme lactate dehydrogenase, which, in turn, leads to an elevation in blood lactate levels [10]. Thus, whole blood serum or plasma samples that contain lactate levels greater than 2.1 mM are indicative of possible mitochondrial dysfunction. Methods used in this measurement include a flow injection MS approach and liquid chromatography-tandem MS (LC-MS/MS) technique [20].

Although elevated lactate levels in patient blood may indicate MRC dysfunction, it should also be considered that this can be caused by a deficiency of vitamin B1 (thiamine), which is an essential cofactor of pyruvate dehydrogenase activity [21]. Also, increased lactate levels can result from cardiac dysfunction or by improper or prolonged use of tourniquets upon blood collection [6, 21]. Conversely, elevations in blood and CSF lactate levels may not always display in patients with MRC dysfunction. Based on these factors, the possibility that patients with lactate levels that lie within the reference range have an underlying MRC disorder cannot be ruled out [10]. To further complicate matters, elevations in CSF lactate levels may be a consequence of inflammation, infections, seizures or malignancies of the central nervous system (CNS) and not exclusive to MRC disorders [22]. In view of this, the use of a secondary marker of MRC dysfunction such as the NADH:NAD ratio (an indication of the lactate:pyruvate ratio) may be used in conjunction with measurement of lactate levels to add some degree of specificity to the findings [13, 23]. However, it should be noted that blood and/or CSF pyruvate levels may also be elevated in patients with pyruvate dehydrogenase, biotinidase or pyruvate carboxylase deficiencies, all of which can contribute to possible defects in pyruvate metabolism [6, 21, 23]. Another potential indicator of an accumulation of pyruvate in MRC disorders is an elevation in blood alanine concentration (see later). However, due to diagnostic limitations of these biochemical markers, they may not be suitable as stand-alone tests to provide diagnostic evidence of an underlying MRC defect, although they can be a useful indicator of possible perturbations in oxidative phosphorylation. Thus, further biochemical, genetic and histological investigations are required to provide additional diagnostic tools [6, 13, 23, 24].

4 Amino Acids

Elevated levels of amino acids such as alanine may be found via analysis of plasma and/or CSF from MRC disorder patients. The enzyme alanine aminotransferase catalyses the transamination of pyruvate to produce alanine [25]. Thus, an increase in the levels of alanine could reflect an accumulation of cellular pyruvate and provide evidence of MRC dysfunction. Amino acids are usually analysed in plasma by ion exchange chromatography, although Schwarz et al. [26] demonstrated a reverse-phase HPLC method for this purpose. Although this can be performed in a relatively short time, regular washes are required to maintain column integrity.

It should be noted that elevations in alanine could also be caused by a pyruvate dehydrogenase deficiency [27]. Abnormal alanine levels have been defined by the mitochondrial disease criteria (MDC) scoring system as a minor criterion of mitochondrial dysfunction [28]. The Nijmegen protocol determines the likelihood of mitochondrial dysfunction in patients if plasma alanine levels are greater than 450 mM [28]. However, increased serum alanine concentrations have also been identified in conditions such as sepsis, hyperinsulinaemia and chronic thiamine deficiency [29].

Upon assessment of hyperalaninaemia in patients, samples should be obtained from fasting patients to avoid dietary influences on amino acid levels [13, 27]. Parikh et al. [30] reported that the exact specificity and sensitivity of alanine elevations in patients with primary mitochondrial dysfunction still require elucidation. A retrospective cohort study suggested that individuals who present with MRC dysfunction along with elevations in alanine may also have increased levels of branch chain amino acids (BCAA: leucine, isoleucine, valine) as well as an increase in the BCAA:glutamate ratio [24]. Thus, it can be assumed that in MRC disorder, an accumulation of BCAAs and alanine could reflect a rise in glutamate-linked transamination reactions. In turn, this could be linked to an impairment of NAD^+-dependent keto-acid oxidation.

The accumulation of BCAAs such as leucine can inhibit the function of MRC enzymes including α-ketoglutarate dehydrogenase and pyruvate dehydrogenase [24, 28]. Recently, the BCAA homeostasis has been recognized for its importance in adequate brain functioning, and alterations in these amino acids directly correlate with severe neurological disease outcomes [28]. Presently, the assessment of urinary amino acid status has proved useful in the identification of renal tubulopathy in patients with MRC dysfunction, with aminoaciduria occasionally reported [29]. Similarly, Shatla et al. [31] added further support to the measurement of plasma and urinary amino acids in the diagnosis of mitochondrial disease in children. In light of this, amino acid profiles analysed in patient samples could be informative in the diagnosis of MRC dysfunction/disorder with a view to determining clinical outcomes in patients with COVID-19.

5 Organic Acid Profiles

Organic acids occur as intermediates and by-products of intracellular metabolic pathways such as glycolysis, the citric acid cycle, ketone metabolism and fatty acid oxidation. In addition, they can be used as markers of detoxification and are present in urine, CSF and plasma [13, 32]. Analysis of urinary organic acids is usually performed using gas chromatography-MS (GC-MS) [33]. However, due to this technique being time consuming, LC-MS/MS techniques have been developed to allow for faster and more accurate quantitative organic acid analysis [34]. Urine is generally the choice of biological fluid in these studies due to its accessibility [13]. However, in patients with suspected disorders of neurotransmitter metabolism, assessment of organic acids in CSF may be more specific.

Patients who are reported to be clinically normal may still contain organic acids in their urine, although their diagnostic utility may be limited by the differing concentration ranges for each of the acids present [32]. Alban et al. [35] reported that in patients with MRC enzyme deficiencies, 82% had an abnormal organic acid profiles. For example, urinary 3-methylglutaconic acid (an intermediate in the mitochondrial metabolism of leucine) levels were found to be elevated in patients with MRC complex IV and V deficiencies and urine lactate levels were elevated in patients with isolated MRC complex I and II defects. Increased levels of dicarboxylic acids are another common finding in these patients [13, 36]. This effect may occur as a result of impaired fatty acid ω-oxidation, a possible consequence of defective MRC functioning.

There are some limitations associated with the analysis of urinary organic acids. For example, during a period of clinical stability in patients with MRC dysfunction, such analyses may not be evidence of a metabolic abnormality [37]. Furthermore, patients who are experiencing dehydration or poor perfusion at the time of sample collection may show an abnormal urine organic profile [13, 37]. It should also be noted that TCA cycle intermediates may be present in the urine of patients with renal immaturity. Thus, in patients less than 1 year of age, the urine organic acid profile should be interpreted with caution [13].

6 Coenzyme Q10

The measurement of CoQ10 may be a useful biomarker of MRC disorder following COVID-19 infection as several clinical studies have demonstrated an association between depleted CoQ10 levels and increased susceptibility to COVID-19 infection [38]. A clinical study carried out by Sumbalova et al. [39] found that infection by the SARS-CoV-2 virus reduced endogenous biosynthesis of CoQ10, therefore partially blocking electron transfer in the MRC and resulting in mitochondrial

impairments and increased oxidative stress in patients [39]. Older age groups tend to have lower CoQ10 levels, and the severity of SARS-CoV-2 infection and risk of mortality are known to increase with age. In patients greater than 80 years of age, the largest depletion of CoQ10 was found in the lungs (51.7%) and heart (42.9%) [40]. CoQ10 can also be found within lysosomal membranes, and these organelles play an important role in the immune system responses [41]. CoQ10 serves as both an electron and a proton carrier within the tentative lysosomal respiratory chain, as well as having an important role in maintaining acidity of the lysosome. Therefore, a CoQ10 deficiency in COVID-19 patients may impair both MRC and lysosomal functions.

The CoQ10 content of a patient can be determined via biochemical assessment of plasma, white blood cells, skin fibroblasts or skeletal muscle tissue biopsies [42]. Clinically, the assessment of CoQ10 deficiency is generally based on the plasma CoQ10 content, and CoQ10 supplementation therapy is monitored to assess bioavailability and absorption [16]. However, plasma CoQ10 status can be influenced by dietary supply [43]. Additionally, the status of plasma CoQ10 is dependent on circulating lipoproteins, which are the major carriers of CoQ10 within the circulation. Approximately 58% of the total plasma CoQ10 content is associated with low-density lipoproteins (LDL) fraction [16]. In order to improve the diagnostic value of plasma CoQ10 and to exclude any influence of an age-induced CoQ10 decrease, plasma CoQ10 levels can be expressed as a ratio to the total plasma cholesterol or LDL cholesterol content.

HPLC with ultraviolet (HPLC-UV) or electrochemical detection (HPLC-ED) is the most common laboratory analytical method used to determine tissue CoQ10 status, although the lack of commercially available nonphysiological internal standards makes it difficult to assess this in tissue samples [16]. HPLC-ED has a higher sensitivity in comparison to HPLC-UV for diagnosis of deficiencies, in addition to having the ability to measure both oxidized and reduced forms of CoQ10 simultaneously [42]. However, the simultaneous measurement of both forms of CoQ10 may not be suitable for diagnostic purposes, as this requires complex preanalytical sample management techniques [16]. Alternatively, the determination of total tissue CoQ10 status can provide an accurate determination of a CoQ10 deficiency. Other methods used to provide evidence of potential CoQ10 deficiency include LC-MS/MS and spectrophotometric assessment of MRC complexes II–III and complex I–III activities [10, 16].

7 Reduced Glutathione

Several studies have reported redox homeostasis impairments associated with COVID-19 infection, leading to increased free radical-mediated inflammation and potentially causing depletion of endogenous reduced glutathione (GSH) pools [44]. GSH is a ubiquitous antioxidant molecule and exists in both the reduced GSH and oxidized GSSG states. Glutathione is located within the cytosol of cells at 1–10 mM

levels and exists predominately in the mitochondria in the reduced state (at 10–14 mM levels) where it protects the MRC against reactive oxygen species (ROS)-induced damage [45].

In a study carried out by Kumar et al. [46], COVID-19 infection was found to be associated with severe intracellular GSH deficiency, in addition to increased oxidative stress. However, it is not known whether oxidative stress and GSH deficiency can increase the risk of hospitalization and exacerbate recovery in these patients. The severity of COVID-19 is dramatically increased in GSH-deficient patients, which could be due to weakened antioxidant defence systems and aggravated by COVID-19 infection [44]. As a consequence, glutathione may be an important therapeutic target for COVID-19 patients in order to prevent exacerbated inflammation that could lead to further complications, including multiorgan failure [44]. The study by Kumar et al. [46] found that GlyNAC (glycine and *N*-acetylcysteine) supplementation may be beneficial in reducing COVID-19 complications, as it has been shown to be effective in ameliorating GSH deficiency, inflammation and oxidative stress-induced damage in diabetes and older patients [46].

The measurement of glutathione is based on the GSH:GSSG ratio [47–49]. Decreased levels of cellular GSH have been associated with MRC dysfunction [50]. Several analytical techniques have been implemented for determination of GSH and GSSG concentrations, including the most commonly used methods of HPLC-UV, HPLC-ED and HPLC with fluorescence detection (HPLC-FD), as well as MS-based approaches [45, 47]. HPLC-ED may be the most advantageous of these for the detection of GSH:GSSG ratio in patient blood serum samples due to its simplicity, high sensitivity and low cost [47]. Although LC-MS/MS has also been used to measure GSH and GSSG concentrations in human plasma samples [51], care should be taken with sample handling in this method as oxidation of GSH to GSSG can occur, resulting in a biased GSH:GSSG ratio [52].

The biological samples that can be used to determine patient GSH and GSSG levels include whole blood, plasma, erythrocytes and urine. However, it should be noted that plasma samples contain a relatively low GSH and GSSG concentration in comparison to the other biological samples [10, 51, 53]. On the other hand, skeletal muscle seems to be the most reliable sample source to determine patient GSH and GSSG status, as this tissue contains the largest GSH pool at a concentration of approximately 5 mM [54].

8 FGF-21/GDF-15

Several studies have demonstrated that fibroblast growth factor-21 (FGF-21) and growth differentiation factor-15 (GDF-15) are useful biomarkers in the study of multiple diseases, including primary mitochondrial disorders [55]. Therefore, FGF-21 and GDF-15 may be promising biomarkers to assess evidence of mitochondrial dysfunction in patients with COVID-19, in addition to predicting disease severity and mortality in this patient population [55–57].

FGF-21 is increased in response to mitochondrial stress and inflammatory stimuli, and several studies have analysed the levels of this growth factor in patients infected with COVID-19 compared to healthy controls [56, 58]. FGF-21 is a hormone-like cytokine predominately expressed in metabolically active tissues, such as liver, skeletal muscle, adipose tissue and pancreas [59]. It acts as an essential regulator of energy homeostasis, in addition to being a potent endocrine regulator of glucose and lipid metabolism [59]. In healthy humans, FGF-21 is found in the serum at a range of 5 pg/mL to 5 ng/mL [60]. Several studies have identified increased levels of FGF-21 as a result of mitochondrial dysfunction in patients with suspected primary mitochondrial disorders, and significantly higher plasma levels of this growth factor have also been observed in patients with COVID-19, suggesting that disease severity and mortality may correlate with increased FGF-21 levels [61, 62]. FGF-21 has also been used as a biomarker for muscle-presenting and primary mitochondrial disorders, with mitochondrial translation and mtDNA maintenance defects [55]. However, FGF-21 is also elevated in several nonmitochondrial disorders, including obesity, diabetes mellitus and renal and liver diseases. A study by Ajaz et al. [56] also found elevated levels of FGF-21 released in response to mitochondrial stress and damage in peripheral blood mononuclear cells (PMBCs) of COVID-19 patients, which also correlated with increased disease severity and mortality [56, 63]. This finding supports the concept that the SARS-CoV-2 virus can hijack the host mitochondria and compromise mitochondrial respiration in the viral replication process [56].

GDF-15, belonging to the transforming growth factor-β (TGFβ) protein superfamily, has an essential role as a metabolic regulator and is used as biomarker of cellular stress and injury [64]. GDF-15 is expressed in several tissues, including skeletal muscle, heart, lung, liver and kidney and, in mouse models, it was shown to protect several organs from adverse outcomes caused by viral infection [65–68]. This growth factor can also be useful as a biomarker in combination with FGF-21 to detect muscle-presenting mitochondrial diseases [55]. This was demonstrated in a study carried out by Montero et al., in which elevated serum GDF-15 was found to be induced by mitochondrial dysfunction in children with mitochondrial disease [69]. A study carried out on patients with severe COVID-19 found that these patients also had elevated levels of GDF-15 in comparison to healthy controls. These findings suggest that measurement of GDF-15 levels could be useful as a biomarker to identify patients with suspected mitochondrial disorders who are potentially at risk of a more severe COVID-19 disease course [70]. However, increased plasma GDF-15 concentrations are also associated with older age and physical inactivity as well as some conditions involving secondary mitochondrial dysfunction [55, 64].

The levels of FGF-21 and GDF-15 are commonly measured by enzyme-linked immunosorbent assay (ELISA) and both are noninvasive and easily obtainable serum biomarkers [71]. It has been suggested by Varhaug et al. [71] that the combined measurement of these biomarkers would be beneficial as a diagnostic tool and in clinical follow-up studies of primary mitochondrial disorders. However, it is important to take into consideration that GDF-15 is not as stable as FGF-21, which could affect the results of such analyses [61, 72].

9 Conclusion

In view of the potential involvement of mitochondrial dysfunction as an indicator of both disease susceptibility and severity following COVID-19 infection, the assessment of this parameter could provide important information to guide patient management and treatment. Although numerous biochemical assays exist to determine evidence of mitochondrial dysfunction, the 'gold standard' test is still considered to be spectrophotometric assay of MRC enzyme activities in skeletal muscle. However, there has been recent interest in identifying accurate and accessible biomarkers of mitochondrial dysfunction in order to alleviate the need for invasive muscle biopsies. Although there are some limitations, assessment of lactate, FGF-21, GDF-15 and CoQ10 levels as well as organic acid and amino acid profiles in patients have demonstrated some degree of clinical utility. Moreover, the combined use of these biomarkers in a multiplexed targeted metabolic profiling strategy may further improve the diagnostic sensitivity and specificity for detection of mitochondrial dysfunction in patients pre- and post-COVID-19 infection. Finally, the inclusion of GSH determinations in this multiplex approach would also provide important information on the antioxidant status of patients, which may guide therapeutic interventions.

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Chapter 12
Red Cell Distribution Width as a Prognostic Indicator for Mortality and ICU Admission in Patients with COVID-19

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Abstract

Background

COVID-19 disease caused by the SARS-CoV-2 virus can lead to an acute respiratory illness with a high hospitalization and mortality risk. Therefore, prognostic indicators are essential for early interventions. As a component of complete blood counts, the coefficient of variation (CV) of red blood cell distribution width (RDW) reflects cellular volume variations. It has been shown that RDW is associated with increased mortality risk in a wide range of diseases. This study aimed to determine the relationship between RDW and mortality risk in COVID-19 patients.

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Methods
This retrospective study was performed on 592 patients admitted to hospital between February 2020 and December 2020. Patients were divided into low and high RDW groups and the relationship between RDW and mortality, intubation, admission to intensive care unit (ICU), and need for oxygen therapy was investigated.Results

The mortality rate in the low RDW group was 9.4%, while that in the high group was 20% ($p < 0.001$). Also, ICU admission in the low group was 8%, whereas this was 10% in the high RDW group ($p = 0.040$). The results of the Kaplan–Meyer curve showed that the survival rate was higher in the low group compared to the high RDW group. Cox results in the crude model showed that higher RDW values were directly related to increased mortality, although this was not significant after adjustment for other covariates.

Conclusion
The results of our study reveal that high RDW is associated with increased hospitalization and risk of death and that RDW may be a reliable indicator of COVID-19 prognosis.

Keywords COVID-19 · Red cell distribution width · RDW · Mortality · Intensive care unit

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1 Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection which causes COVID-19 disease can induce a hyperinflammatory condition that may lead to acute respiratory syndrome (ARDS) in the host [1]. Therefore, early diagnosis and treatment plays an important role in determining patient outcomes and preventing life-threatening complications. To meet these objectives, the use of biomarkers, especially laboratory biomarkers for assessing the prognosis of patients, has a vital role in the management of this disease [2]. The coefficient of variation of red blood cell distribution width (RDW) is a hematologic parameter routinely measured in blood cell count analysis. RDW readings show the size heterogeneity of circulating red blood cells (RBCs), otherwise known as the degree of anisocytosis [3]. Changes in erythropoiesis can cause heterogeneity of RBC size as an indicator of certain pathological conditions [4]. For example, RDW values are known to be higher in malnutrition, tuberculosis, hemolytic anemia, myelodysplastic syndrome, cardiovascular disease, pneumonia, sepsis, influenza, viral hepatitis, and cancer [5, 6]. Increasing levels of RDW can also be indicative of an imbalance in RBC production in the bone marrow or a high turnover rate of these cells. Importantly, high RDW values have also been associated with an increased risk of mortality [7–9].

In a prospective study of 240,477 healthy individuals, participants were followed for nine years to investigate the prognostic role of RDW [10]. This showed that the levels of RDW in cardiovascular diseases and cancer (especially colorectal cancer and leukemia) were increased, and this was associated with an increased risk of mortality in patients. Recent studies have reported the prognostic role of RDW in COVID-19 patients (for a meta-analysis on this topic see [8]). Various mechanisms have been suggested for an elevation in RDW in COVID-19 patients. One of these is the potential increase in the levels of inflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α [11]. These cytokines increase hepcidin (as a negative regulator of iron) and decrease the release of stored iron, leading to an impairment of iron metabolism [12]. In addition, CD147, also known as the OK blood group antigen, is expressed on erythrocyte lineage cells and is known to act as a novel receptor for SARS-CoV-2 binding [13]. It has also been suggested that this binding on RBCs can lead to viral invasion of these cells and their subsequent destruction, an outcome predicted to affect RDW values.

Here, we have investigated the relationship between RDW levels and the outcomes of COVID-19-hospitalized patients. Our main focus was on intubation rate, need for oxygen therapy, intensive care unit (ICU) admission, and length of hospital stay.

2 Materials and Methods

2.1 Study Design

This retrospective single-center study was conducted at Shahid Mostafa Khomeini Hospital in Tabas, Iran. The study population consisted of COVID-19 patients with a positive polymerase chain reaction (PCR) test hospitalized in Shahid Mostafa Khomeini Hospital between February 2020 and December 2020. People under 18 years or with hematologic malignancies were excluded. Finally, 592 patients were included in the study.

This study was approved by the ethics committee of Birjand University of Medical Sciences, Iran (IR.BUMS.REC.1400.006). Patient electronic medical records were reviewed, and demographic characteristics, clinical signs, comorbidities, laboratory test results [complete blood count (CBC) [14], blood urea nitrogen (BUN), creatinine (Cr), and C-reactive protein (CRP)], PO_2 (partial pressure of oxygen), computerized tomography (CT) scan results, hospitalization duration time, and body temperature were determined. CBCs were analyzed by Sysmex analyzer model Kx-21. The normal range of the coefficient of variation of RDW in our laboratory was 11.5–14.5. Patients were divided into two groups: those with RDW less than 14.5 (RDW < 14.5) and those with RDW above 14.5 (RDW > 14.5).

The relationship between RDW and mortality, intubation, admission to intensive care unit, and the need for oxygen therapy was assessed in both groups.

2.2 Statistical Analysis

The Stata software (version 14) was used for data analysis. Descriptive data were presented as mean, standard deviation (SD), frequency, and frequency percentage. The Kolmogorov–Smirnov test evaluated the normality of continuous variables, and the Schoenfeld residuals test was performed to check the proportional hazards (PH) assumption in the simple and Cox multiple models. To determine the difference between the means of variable data between the two groups (RDW > 14.5 vs. RDW < 14.5), an independent *t*-test was used for continuous variables such as PO_2 status, temperature, white blood cells (WBC), platelet (PLT), neutrophil, and lymphocyte counts. For nonparametric variables, comparisons between groups were performed with the Mann–Whitney *U* test. Fisher's exact test and chi-square test were used for categorical variables.

A Kaplan–Meyer curve was drawn to show the survival time of patients in the RDW > 14.5 and RDW < 14.5 groups, and a log-rank test was performed to check the difference in survival time between the two groups. In addition, a Cox simple regression model was used to determine the factors related to survival time in patients with COVID-19, and a multivariable Cox regression model was performed for variables that were found to have significant effects in the simple Cox regression

model. Finally, hazard ratio (HR) and 95% confidence interval (CI) were reported for each variable related to patient survival time in the two simple models and the Cox multivariable model. *P*-values less than 0.05 were considered as significant.

3 Results

The present study was performed on 592 patients with COVID-19 disease. Of these, 73.3% ($n = 434$) were classed in the RDW < 14.5 set, and the remaining participants were in the RDW > 14.5 group. The mean age of all subjects was 60.4 ± 21.5 years, 56.7 ± 21.0 years in the RDW < 14.5 group and 70.5 ± 19.7 years in the RDW > 14.5 group. The independent *t*-test showed that the difference in mean age between the two groups was statistically significant (Table 12.1). The chi-square test results showed that RDW level was not statistically related to gender.

In addition, we found that age was directly related to higher RDW values ($p = 0.001$). Also, higher RDW levels were related to higher ICU admission rates (RDW < 14.5: 8%, RDW > 14.5: 10%, $p = 0.04$). Furthermore, the prevalence of cancer and cardiovascular disease was higher in the group with RDW > 14.5 than in the RDW < 14.5 group.

The mortality rate in the RDW < 14.5 group was 9.4% while that in the RDW > 14.5 group was higher at 20.2% ($p = 0.001$). PO_2 was also significantly associated with RDW as 78% of the patients with a PO_2 greater than 93% were in the RDW < 14.5 group, compared to only 22% of the RDW > 14.5 patient group ($p = 0.004$). The group with RDW > 14.5 also had lower hemoglobin and higher BUN levels than the group with RDW < 14.5 ($p = 0.001$).

The results of the Kaplan–Meyer curve showed that the survival rate was higher in the group with RDW < 14.5 than in the RDW > 14.5 group. In addition, the Cox results showed that the rate of intubation was directly related to mortality. Finally, Cox analyses of the crude and adjusted models showed that higher RDW values were directly related to the increase in death, although this relationship was not significant after adjustment for age, fever, cough, cardiovascular disease, oxygen therapy status, temperature, intubation, PO_2, WBC count, and BUN (Fig. 12.1).

4 Discussion

The results of this study showed that elevated RDW was associated with higher ICU admission rates and an increased risk of death. This suggests that RDW can be considered as a negative prognostic indicator of clinical conditions of COVID-19 patient clinical conditions. In line with our study, a retrospective study of 1198 COVID-19 patients found that having an RDW > 14.5 was associated with an increased risk of death at all ages [15]. Also, the Cox model used in this previous study showed that, after adjusting for age, lymphocyte count, and D-dimer levels in

Table 12.1 Characteristics of patients in RDW < 14.5 and RDW > 14.5 groups

Variable	Level variable	Total (n = 592)	RDW ≤ 14.5 (n = 434, 73.3%)	RDW > 14.5 (n = 158, 26.7%)	Test result	p
Age (mean ± SD)		60.39 ± 21.52	56.72 ± 21.00	70.49 ± 19.65	3.92	**0.001**
Sex	Female (n, %)	280(47.3)	202(72.1)	78(27.9)	0.37	0.543
	Male (n, %)	312(52.7)	232(74.4)	80(25.6)		
Name of the ward	Isolate	244(78.7)	204(83.6)	40(16.4)	6.15	**0.046**
	ICU (n, %)	51(16.5)	35(68.6)	16(31.4)		
	Other (n, %)	15(4.8)	12(80.0)	3(20.0)		
Sign and symptoms						
Fever	No (n, %)	298(50.3)	203(68.1)	95(31.9)	8.26	**0.004**
	Yes (n, %)	294(49.7)	231(78.6)	63(21.4)		
Cough	No (n, %)	376(63.5)	267(71.0)	109(29.0)	2.79	0.095
	Yes (n, %)	216(36.5)	167(77.3)	49(22.7)		
Muscular pain	No (n, %)	526(88.9)	380(72.2)	146(27.8)	2.79	0.097
	Yes (n, %)	66(11.1)	54(81.8)	12(18.2)		
ARDS	No (n, %)	388(65.5)	285(73.5)	103(26.5)	0.01	0.914
	Yes (n, %)	204(34.5)	149(73.0)	55(27.0)		
Consciousness	No (n, %)	559(94.4)	420(75.1)	139(24.9)	17.04	**0.001**
	Yes (n, %)	33(5.6)	14(42.9)	19(57.6)		
Olfactory	No (n, %)	588(99.3)	430(73.1)	158(26.9)	1.47	0.578
	Yes (n, %)	4(0.7)	4(100.0)	00(00.0)		
Taste	No (n, %)	588(99.3)	430(73.1)	158(26.9)	1.47	0.578
	Yes (n, %)	4(0.7)	4(100.0)	00(00.0)		
Convulsions	No (n, %)	588(99.3)	431(73.3)	157(26.7)	0.01	0.999
	Yes (n, %)	4(0.7)	3(75.0)	1(25.5)		
Stomach ache	No (n, %)	564(96.7)	411(72.9)	153(27.1)	1.21	0.429
	Yes (n, %)	19(3.3)	16(84.2)	3(15.8)		
Nausea	No (n, %)	503(86.3)	365(72.6)	138(27.4)	0.86	0.354
	Yes (n, %)	80(13.7)	62(77.5)	18(22.5)		
Vomit	No (n, %)	528(90.6)	383(72.5)	145(27.5)	1.41	0.234
	Yes (n, %)	55(9.4)	44(80.0)	11(20.0)		
Diarrhea	No (n, %)	533(91.4)	391(73.4)	142(26.6)	0.04	0.836
	Yes (n, %)	50(8.6)	36(72.0)	14(28.0)		
Anorexia	No (n, %)	532(91.3)	385(72.4)	147(27.6)	2.37	0.124
	Yes (n, %)	51(8.7)	42(82.4)	9(17.6)		
Headache	No (n, %)	546(93.7)	398(546)	148(27.1)	0.53	0.466
	Yes (n, %)	37(6.3)	29(78.4)	8(21.66)		
Vertigo	No (n, %)	569(97.6)	415(72.9)	154(27.1)	1.14	0.373
	Yes (n, %)	14(2.4)	12(85.7)	2(14.3)		

(continued)

Table 12.1 (continued)

Variable	Level variable	Total (n = 592)	RDW ≤ 14.5 (n = 434, 73.3%)	RDW > 14.5 (n = 158, 26.7%)	Test result	p
Paralysis	No (n, %)	582(99.8)	427(73.4)	155(26.6)	2.74	0.268
	Yes (n, %)	1(0.2)	00(00.0)	1(100.0)		
Plegia	No (n, %)	582(99.8)	427(73.4)	155(26.6)	2.74	0.268
	Yes (n, %)	1(0.2)	00(00.0)	1(100.0)		
Chest pain	No (n, %)	558(95.7)	108(73.1)	150(26.9)	0.10	0.999
	Yes (n, %)	25(4.3)	19(76.0)	6(24.0)		
Inflammation	No (n, %)	582(99.8)	427(73.4)	155(26.6)	2.74	0.098
	Yes (n, %)	1(0.2)	00(00.0)	1(100.0)		
Comorbidities						
Cancer	No (n, %)	585(98.8)	432(73.8)	153(26.2)	7.25	**0.007**
	Yes (n, %)	7(1.2)	2(28.6)	5(71.4)		
Liver disease	No (n, %)	589(99.5)	432(73.3)	157(26.7)	0.07	0.999
	Yes (n, %)	3(0.5)	2(66.7)	1(33.3)		
Diabetes	No (n, %)	510(86.1)	376(73.7)	134(26.3)	0.32	0.569
	Yes (n, %)	82(13.9)	58(70.7)	24(29.3)		
Cardiovascular diseases	No (n, %)	446(75.3)	347(77.8)	99(22.2)	18.65	**0.001**
	Yes (n, %)	146(24.7)	87(59.6)	59(40.4)		
Renal diseases	No (n, %)	580(98.0)	428(73.8)	152(26.2)	3.40	0.065
	Yes (n, %)	12(2.0)	6(50.0)	6(50.0)		
Asthma	No (n, %)	574(97.1)	422(73.5)	152(26.5)	0.66	0.418
	Yes (n, %)	17(2.9)	11(64.7)	6(35.3)		
Neurologic diseases	No (n, %)	583(98.5)	428(73.4)	155(26.6)	0.21	0.706
	Yes (n, %)	9(1.5)	6(66.7)	3(3.3)		
Hypertension	No (n, %)	437(73.8)	328(75.1)	109(24.9)	2.60	0.107
	Yes (n, %)	155(26.2)	106(68.4)	49(31.6)		
Oxygen therapy status	No (n, %)	99(63.9)	77(77.8)	22(22.2)	0.02	0.887
	Yes (n, %)	56(36.1)	43(76.8)	13(23.2)		
Death	No (n, %)	518(87.6)	393(75.9)	125(24.1)	12.73	**0.001**
	Yes (n, %)	73(12.4)	41(56.2)	32(43.8)		
Other parameters						
Intubation	No (n, %)	286(92.3)	233(81.5)	53(18.5)	0.60	0.438
	Yes (n, %)	24(7.7)	18(75.0)	6(25.0)		
PO_2	<93%	297(50.2)	204(68.7)	93(31.3)	6.51	**0.011**
	>93%	295(19.8)	230(78.0)	65(22.0)		
Number of breaths	<18	30(6.8)	24(80.0)	6(20.0)	7.53	0.057
	18–22	99(22.6)	76(76.8)	23(23.2)		
	22–28	23(5.3)	18(78.3)	5(21.7)		
	>28	286(65.3)	186(65.0)	100(35.0)		
CT scan	No (n, %)	151(49.5)	126(83.4)	25(16.6)	1.49	0.222
	Yes (n, %)	154(50.5)	120(77.9)	34(22.1)		

(continued)

Table 12.1 (continued)

Variable	Level variable	Total (n = 592)	RDW ≤ 14.5 (n = 434, 73.3%)	RDW > 14.5 (n = 158, 26.7%)	Test result	p
Smoking	No (n, %)	586(99.0)	430(73.4)	156(26.6)	0.14	0.660
	Yes (n, %)	6(1.0)	4(66.7)	2(33.3)		
Opium intake	No (n, %)	551(93.1)	409(74.2)	142(25.8)	3.43	0.064
	Yes (n, %)	41(6.9)	25(61.0)	16(39.0)		
Blood group	O (n, %)	60(35.9)	44(73.3)	16(26.7)	1.40	0.704
	A (n, %)	46(27.5)	31(67.4)	15(32.6)		
	B (n, %)	42(25.1)	33(78.6)	9(21.4)		
	AB (n, %)	19(11.5)	14(73.7)	5(26.3)		
Hospitalization time	mean ± SD	5.53 ± 9.22	5.29 ± 8.55	6.19 ± 10.85	0.65	0.294
PO_2	mean ± SD	91.27 ± 7.21	92.03 ± 6.08	89.54 ± 9.05	11.22	**0.004**
Temperature	mean ± SD	37.08 ± 0.69	37.12 ± 0.69	36.96 ± 0.69	0.48	0.240
Laboratory measures						
WBC (× 10^9/L)	mean ± SD	8.48 ± 6.42	6.90 ± 0.033	9.61 ± 4.71	0.15	**0.010**
HB (g/dL)	mean ± SD	12.79 ± 6.42	13.22 ± 5.74	11.65 ± 2.86	0.07	**0.001**
PLT (× 10^9/L)	mean ± SD	201.60 ± 107.78	198.63 ± 101.59	209.69 ± 123.07	4.37	0.313
Neut (%)	mean ± SD	74.21 ± 13.99	73.33 ± 13.48	76.64 ± 15.11	1.11	**0.011**
Lymph (%)	mean ± SD	18.97 ± 12.38	19.43 ± 11.01	17.71 ± 15.48	2.95	0.133
BUN (μg/dL)	mean ± SD	17.77 ± 14.48	15.81 ± 8.95	23.16 ± 23.03	44.83	**0.001**
MPV (FL)	mean ± SD	9.89 ± 4.38	10.16 ± 4.81	9.16 ± 2.78	1.27	**0.014**
Cr (μg/dL)	mean ± SD	1.32 ± 1.47	1.23 ± 1.11	1.58 ± 2.17	13.16	0.059
CRP (μg/dL)	mean ± SD	1.14 ± 0.87	1.12 ± 0.89	1.18 ± 0.79	3.32	0.476

* p-Values calculated by t-test continuous variables and Fisher's (exact) test or χ^2 test for the categorical variables; $p < 0.05$ considered significant

patients with RDW > 14.5, the mortality rate was higher. In our study, the results of the Cox model in the crude model showed that increased RDW values were associated with an increased risk of death. However, this association was not significant after adjusting for other covariates including age, which is an important risk factor for severe COVID-19 and ensuing hard outcomes.

We found that patients with higher RDW had higher rates of ICU admission. In line with our analysis, a study conducted in Ankara on 127 COVID-19 patients showed that patients with higher RDW values had higher ICU admissions [16]. In a study on 294 COVID-19 patients in Brooklyn, Ramachandran et al. examined the association with mortality, septic shock, and the need for mechanical ventilation [17]. The results showed elevated RDW was associated with increased mortality and septic shock. However, they found no association between increased RDW and increased need for ventilation, which is in line with the findings of our study.

The pathological mechanisms underlying RDW increase in COVID-19 are unknown. However, previous studies have shown that elevated RDW in COVID-19 is associated with increased inflammatory cytokines such as IL-1 and TNF-α, which

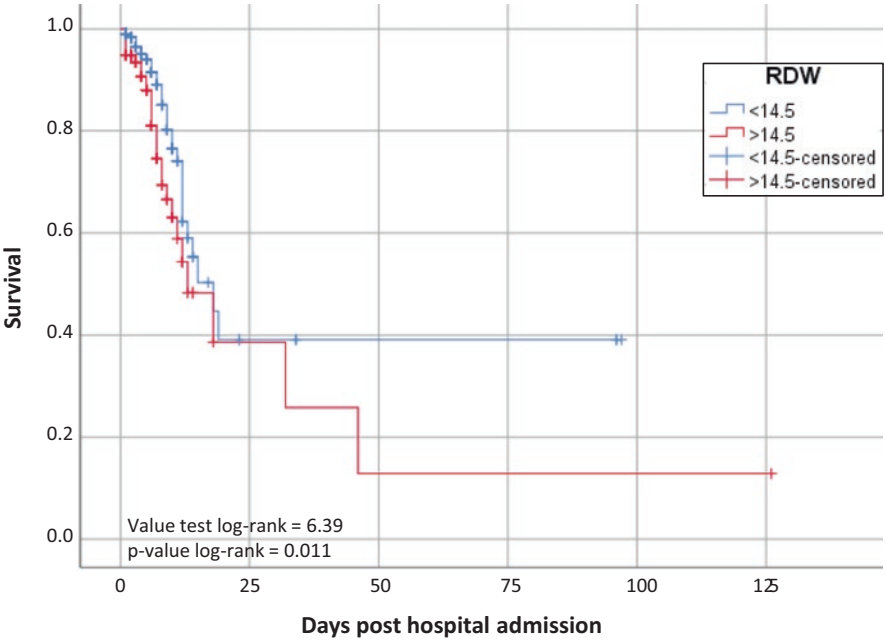

Fig. 12.1 Cox results on the survival rate of patients in the two groups (RDW < 14.5 and RDW > 14.5) after admission to ICU. The survival rate was higher in the group with RDW < 14.5

can disrupt iron metabolism and cause anemia and increased RBC apoptosis [18]. Also, an increased RDW reflects an imbalance between hematopoiesis and survival of RBCs, and a delay in removing old RBCs from the peripheral blood [18]. Kaufman et al. also reported that elevated RDW was associated with higher CRP and BUN levels along with increased mortality risk [19]. This is in line with our findings as we also found that BUN was higher in the group with higher RDW. High BUN levels are used an indicator of kidney dysfunction which can also be manifested in patients with SARS-CoV-2 infections [20].

One of the limitations of our study is this retrospective design, which limits our access to information. Another limitation of our study is that it was performed in only one center. Lastly, our analysis only considered the effects of a single biomarker (RDW values) on ICU admission and death outcomes in COVID-19 patients admitted to hospital. Future studies should attempt to incorporate additional markers with RDW values such as BUN, CRP, IL-1, IL-6, and TNFα using a multiplex algorithm. These analyte values can be obtained using cytokine arrays or multiplex immunoassay panels [21, 22]. Also, in addition to the current study of inpatients here, future investigations should analyze the effects on outpatients. Thus, further studies are required in multiple centers and on larger population groups.

In conclusion, the results of our study showed that increased RDW is associated with an increase in hospitalization in ICU and an increased risk of death, and can be used as a nonspecific, inexpensive, and accessible indicator to determine the

prognosis of COVID-19 patients. Thus, future studies should be carried out to validate and optimize the performance of this biomarker and associated algorithms in prediction of COVID-19 disease outcomes. This will help to stratify patients according to the most appropriate therapeutic options.

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Conflict of Interest The authors declare that they have no conflict of interest.

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Chapter 13
Predicting the COVID-19 Patients Status Using Chest CT Scan Findings: A Risk Assessment Model Based on Decision Tree Analysis

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Abstract

Background

The role of chest computed tomography (CT) to diagnose coronavirus disease 2019 (COVID-19) is still an open field to be explored. The aim of this study was to apply the decision tree (DT) model to predict critical or non-critical status of patients infected with COVID-19 based on available information on non-contrast CT scans.

Methods

This retrospective study was performed on patients with COVID-19 who underwent chest CT scans. Medical records of 1078 patients with COVID-19 were evaluated. The classification and regression tree (CART) of decision tree model and *k*-fold

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cross-validation were used to predict the status of patients using sensitivity, specificity, and area under the curve (AUC) assessments.

Results
The subjects comprised of 169 critical cases and 909 non-critical cases. The bilateral distribution and multifocal lung involvement were 165 (97.6%) and 766 (84.3%) in critical patients, respectively. According to the DT model, total opacity score, age, lesion types, and gender were statistically significant predictors for critical outcomes. Moreover, the results showed that the accuracy, sensitivity and specificity of the DT model were 93.3%, 72.8%, and 97.1%, respectively.

Conclusions
The presented algorithm demonstrates the factors affecting health conditions in COVID-19 disease patients. This model has the potential characteristics for clinical applications and can identify high-risk subpopulations that need specific prevention. Further developments including integration of blood biomarkers are underway to increase the performance of the model.

Keywords Chest CT scan without contrast · Coronavirus disease · COVID-19 · Disease outcome · Decision tree

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1 Introduction

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which causes coronavirus 2019 (COVID-19) disease appears to have emerged at the Huanan Seafood Market in Wuhan, China [1, 2]. On March 11, 2020, this disease was declared as a pandemic by the World Health Organization (WHO) [3]. As of October 18, 2022, COVID-19 has affected virtually all countries and territories of the world, through successive outbreaks of SARS-CoV-2 variants of differing virulence [4]. To date, more than 630 million confirmed COVID-19 cases and 6.5 million deaths have been reported in the world [5]. The first reported COVID-19 case in Iran was identified in Qom on February 19, 2020 [6]. Since that time the number of Iranian cases has risen to over 7.5 million with more than 144 thousand deaths [5].

COVID-19 can lead to respiratory infection, liver disease, gastrointestinal and neurological disorders [7, 8]. In addition, the virus can cause respiratory conditions such as pneumonia, pulmonary edema, and acute respiratory distress syndrome (ARDS) [9]. For this reason, imaging tools such as non-contrast chest computed tomography (CT) scanning have been applied as an unambiguous tool in diagnosis quantification and follow-up of patients with COVID-19 [10]. The lungs of patients with COVID-19 symptoms show visual hallmarks, such as ground-glass opacities (GGOs) and areas of increased lung density called consolidation [10]. Furthermore, patients with more severe forms of the disease have shown more extensive effects with increasing time from onset of symptoms such as linear opacities, a crazy-paving pattern, reverse halo signs, pleural effusion, intralesional traction bronchiectasis, and lymphadenopathy [11, 12].

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Classification and regression tree (CART) decision tree (DT) analysis is a data mining technique used for establishing classification in systems based on multiple covariates or for developing prediction algorithms for a target variable [13]. The analysis has been widely applied in medicine and public health. Moreover, the DT model is a strong statistical method for classifying, predicting, interpreting, and processing data. The algorithm can be considered as nonparametric and can efficiently manage large, complex datasets without imposing a complex parametric structure. Furthermore, both heavily skewed data and missing values are easily managed without the need for data transformation. Numerous factors have been shown to influence the conditions of COVID-19 patients such as specific signs on high-resolution computed tomography (HRCT), lesion type, presence of diffuse opacity, age, and gender. The computer-based model can be graphically represented as a tree structure that makes the interpretation easy and useful in clinical approaches. In addition, the algorithm has numerous merits including the capability of splitting sequential data into the best predictive groups [14].

The aim of the current retrospective study, with such a large sample size population, was to apply the CART decision tree model to predict critical/non-critical status of patients with COVID-19 based on chest CT findings. We also attempted to identify independent risk factors in the patients. Additionally, receiver operating characteristic (ROC) analysis was applied to assess the ability of DT model for the prediction of critical and non-critical status.

2 Methods

2.1 Study Design and Patients

This was a retrospective study in which we collected both demographic characteristics and radiologic information of 1078 patients with COVID-19, who were referred to Baqiyatallah Hospital, Tehran, Iran, during the first wave of the pandemic, from March to April 2020. The inclusion criteria were (1) positive results on a reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay of a specimen obtained on a nasopharyngeal swab; (2) having related symptoms (like fever, dry cough, shortness of breath, and aches); and (3) willingness of the patients to participate in the study. The exclusion criteria were (1) logistical impediments to data collection; (2) incomplete data; and (3) revoking of consent [15]. According to patient clinical outcomes, the individuals were divided into two groups as critical and non-critical. Patients admitted to the routine ward of the hospital and then discharged ($n = 909$) were considered as non-critical patients. The critical group included those who died ($n = 104$) or who were admitted to the intensive care unit (ICU) ($n = 65$). This study was approved by the Ethics Committee of Baqiyatallah University of Medical Sciences, Tehran, Iran, with code IR.BMSU.REC.1399.024 and the patients were enrolled after giving written informed consent.

2.2 CT Protocol and Evaluation of Chest CT

The images of non-contrast chest CT scans were acquired using a 16-row detector CT scanner (General Electric GE, Optima, USA), with patients in a supine position and at full inspiration. The detailed parameters for CT acquisition based on a low-dose thoracic CT scan protocol were as follows: tube voltage 100 kVp, 120 mA, slice thickness of 2.5 mm, reconstruction interval of 1.25 mm, pitch 1.75, speed 35 mm/rot, detector configuration 16 × 1.25, computed tomography dose index 3.5 mGy. The findings of CT scans were evaluated by two blinded radiologists who were in agreement with the results of images. The inter-rater coefficient agreement between the two radiologists was $r = 0.98$; $p < 0.0001$. If the radiologists disagreed about the COVID-19 diagnosis, a third party joined the discussion and this was continued until agreement was achieved. According to Fleischner Society Nomenclature recommendations [16, 17], the images of initial chest CT scan were assessed for some features of patients, including GGO (Fig. 13.1) pericardial effusion, crazy-paving pattern (Fig. 13.2), consolidation (Fig. 13.3), pleural effusion,

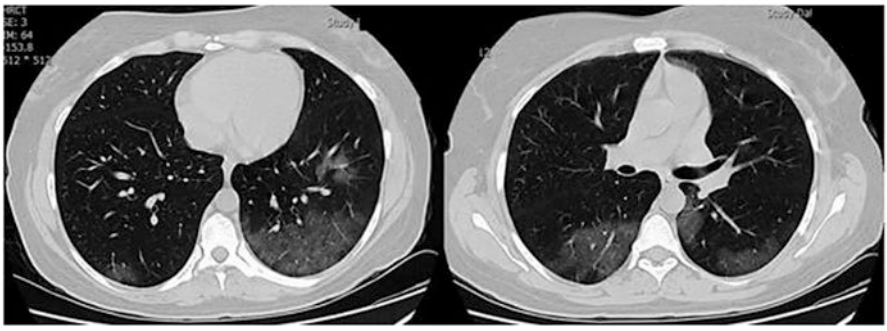

Fig. 13.1 Two axial chest CT scans without contrast show bilateral and multifocal patchy subpleural ground-glass opacities (GGOs) in a patient with COVID-19 pneumonitis

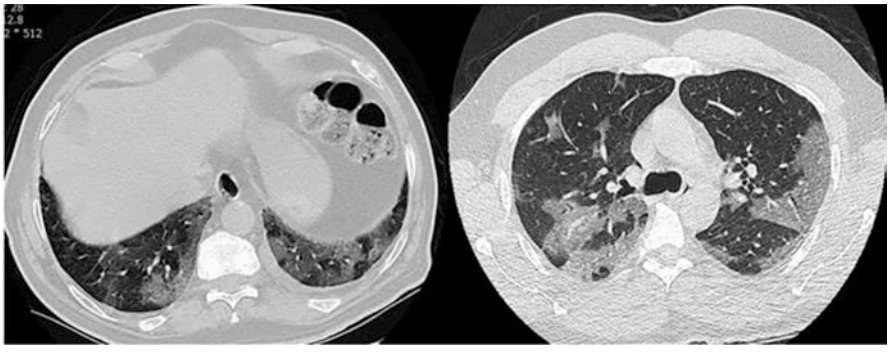

Fig. 13.2 Two axial chest CT scans without contrast show multifocal subpleural patchy ground-glass opacities (GGOs) with interlobular septal thickening (crazy-paving) in lower lobes of both lungs in a patient with COVID-19 pneumonitis

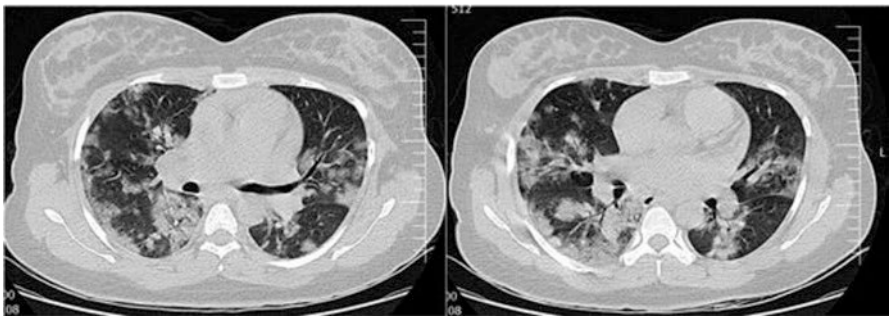

Fig. 13.3 Two axial chest CT scans without contrast showing bilateral and multifocal patchy consolidation in a patient with COVID-19 pneumonitis

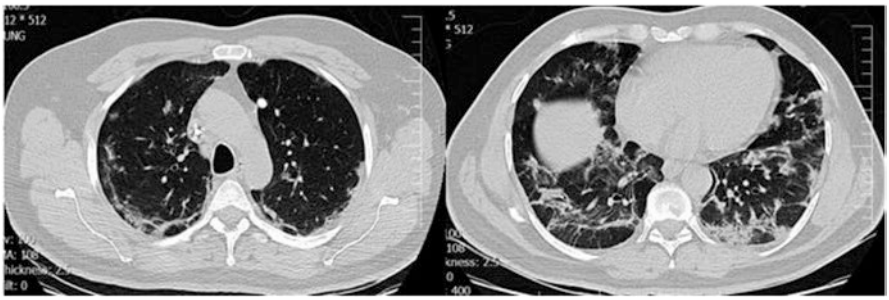

Fig. 13.4 Two axial chest CT scans without contrast show bilateral and multifocal linear opacities with architectural distortion in a patient with COVID-19 pneumonitis

reversed halo sign, linear opacities (Fig. 13.4), intralesional traction bronchiectasis, and lymph node enlargement [16]. Afterward, scores of thin-section CT involvement were assigned based on the abnormal areas involved as a way of measuring the extent of lesions [18]. A score, ranging from 0 to 5, was given to each lobe as follows: 0 (no involvement); 1 (<5% involvement); 2 (25% involvement); 3 (26–49% involvement); 4 (50–75% involvement); and 5 (>75% involvement). A score from 0 to 5 was assigned to each lobe, with a total possible score from 0 to 25.

2.3 Statistical Analysis

The results were described as mean ± SD in continuous variables. In addition, frequency and percentage of categorical variables were reported. The chi-square test was used to evaluate the association between categorical variables and the Mann–Whitney *U* and independent *t* tests were performed to compare means between number of involved lobes and age in the two groups. In addition, the CART method

was used to build a risk assessment model to predict critical/non-critical patient conditions using both demographical and clinical factors, including age, gender, lesion type, specific signs, presence of diffuse opacity, underlying disease, number of involved lobes, and total opacity score. Afterward, the *k*-fold cross-validation method was used to validate the model. The value of *K* was considered equal to 10 and the set of *N* (1078) patients was split into *k* mutually exclusive subsets of size *N*/*k*. Afterward, *k*–1 subsets were used as a training set to fit a model, which was used to predict the left-out validation subset. Next, this process was repeated *k* times, each time excluding a different validation subset and then an estimate of the model performance was calculated from the predicted values. Therefore, each patient was included in a validation set once and *k*–1 times in the training sets. Lower *k* values typically led to estimates of prediction error biased upward and higher *k* values minimized bias but increased variance [19, 20]. In the DT analysis, each fork was split into a predictor variable and each end node contained a prediction for the outcome variable. Additionally, ROC analysis was performed to assess the ability of DT model for prediction of critical and non-critical condition. The level of significance for statistical tests was 0.05. The R-4.0.0 software (dtree package) was used for statistical analysis.

3 Results

The study population consisted of 1078 confirmed patients with COVID-19 who underwent CT scans including 169 critical and 909 non-critical subjects. The baseline characteristics and chest CT features according to critical and non-critical status are given in Table 13.1. The age of participants in the critical group was significantly higher than those in the non-critical group (61.24 ± 13.48 vs. 51.47 ± 14.02, $p < 0.001$). The frequency of the involved lobe number in the non-critical group was higher than that in the critical group, except for the number of lymph nodes less than 1, which was significantly different between the groups ($p < 0.001$). The results showed that there was a significant relationship between gender, lesion distribution, lesion type, specific HRCT signs, presence of diffuse opacity, and underlying disease ($p < 0.001$).

The DT derived from CART analysis is shown in Fig. 13.5. This had a depth of three levels from the root node and three intermediate nodes, including six terminal nodes. Each node represented the probability of being critical/non-critical for the corresponding branches. This shows that in order to predict patient status, the total opacity score should be bifurcated at a score of 7.5. If the value was more than 7.5, the lesion type was checked in the next step. If this value was less than 7.5, age was bifurcated at 62.5 (years). Then, comparisons with the presented variables continued at each node split to reach a branch, to predict either the critical or non-critical status of the patient. The number and percentage of cases that we obtained using this model are presented at the end of each branch.

Table 13.1 Baseline characteristics and chest CT features in patients with COVID-19 based on critical and non-critical status

Parameter	Critical patients (n = 169)	Non-critical patients (n = 909)	Total patients (n = 1078)	p-Value
Age (years), mean±SD	61.24 ± 13.48	51.47 ± 14.02	53 ± 14.37	<0.001[a]
Total opacity score, mean±SD	13.71 ± 6.26	4.86 ± 3.52	6.24 ± 5.19	<0.001[a]
Male gender, n (%)	123 (72.8)	614 (67.5)	737 (68.4)	0.179[b]
Lesions distribution, n (%)				<0.001[b]
Bilateral + multifocal	165 (97.6)	766 (84.3)	931 (86.4)	
Others	4 (2.4)	143 (15.7)	147 (13.6)	
Lesions type, n (%)				
GGO*	13 (7.7)	401 (44.1)	414 (38.4)	<0.001[b]
GGO + crazy paving	19 (11.2)	114 (12.5)	133 (12.3)	0.637
Consolidation	12 (7.1)	30 (3.3)	42 (3.9)	0.019
GGO + Consolidation	125 (74)	364 (40)	489 (45.4)	<0.001
Specific signs of HRCT#, n (%)				
None	78 (46.2)	617 (67.9)	695 (64.5)	
Liner opacity	24 (14.2)	150 (16.5)	174 (16.1)	0.455[b]
Reversed halo sign	6 (3.6)	43 (4.7)	49 (4.5)	0.499
Pleural effusion	34 (20.1)	21 (2.3)	55 (5.1)	<0.001
Intralesional traction bronchiectasis	17 (10.1)	44 (4.8)	61 (5.7)	0.007
Lymphadenopathy	10 (5.9)	34 (3.7)	44 (4.1)	0.189
Presence of diffuse opacity, n (%)				
Yes	118 (69.8)	63 (6.9)	181 (16.8)	<0.001[b]
No	51 (30.2)	846 (93.1)	897 (83.2)	
Number of involved lobes, n (%)				<0.001[c]
0	51 (30.2)	846 (93.1)	897 (83.2)	
1	1 (0.6)	5 (0.6)	6 (0.6)	
2	33 (19.5)	10 (1.8)	49 (4.5)	
3	35 (20.7)	15 (1.7)	50 (4.6)	
4	30 (17.8)	13 (1.4)	43 (4)	
5	19 (11.2)	14 (1.5)	33 (3.1)	
Underlying disease, n (%)				
None	159 (94.1)	882 (97)	1041 (96.6)	
Pulmonary	1 (0.6)	6 (0.7)	7 (0.6)	0.919[b]
Cardiac	8 (4.7)	20 (2.2)	28 (2.6)	0.057
Kidney	1 (0.6)	1 (0.1)	2 (0.2)	0.289

*GGO ground-glass opacities, #$HRCT$ high-resolution computed tomography
[a]Independent t test
[b]Chi-square test
[c]Mann–Whitney U test

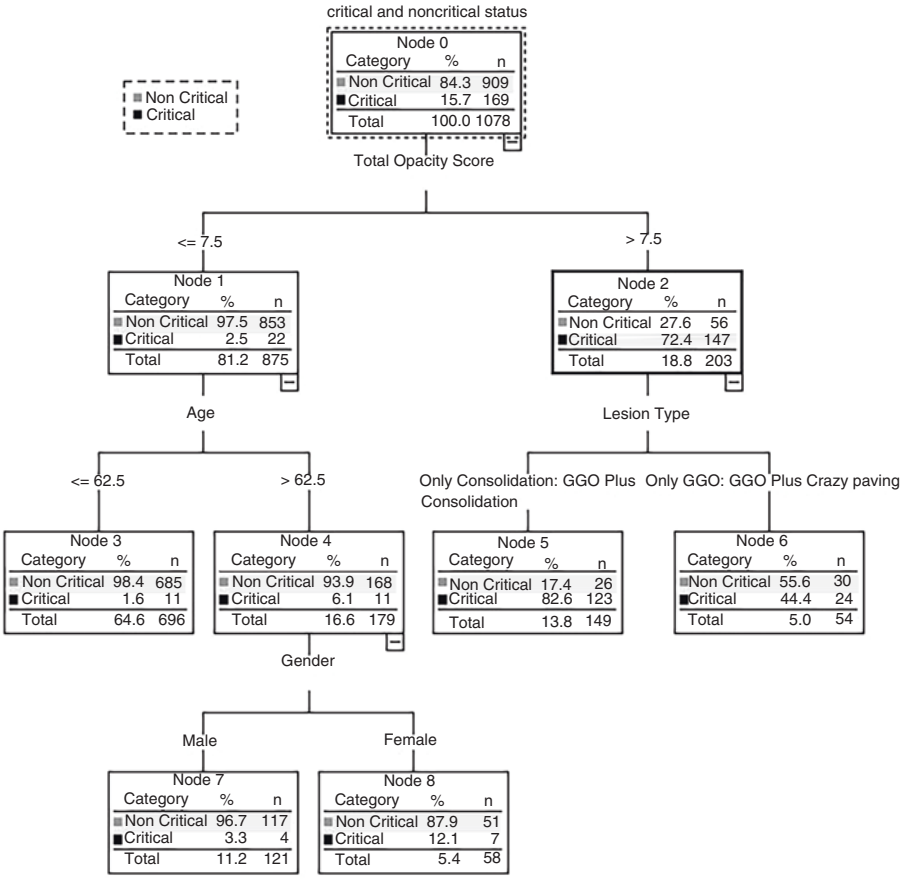

Fig. 13.5 Decision tree predicting the risk for critical or non-critical situation of patients with COVID-19

The use of DT model showed that 72.8% with a critical condition (sensitivity) and 98% of patients with a non-critical status (specificity) were correctly predicted. Also the accuracy index which showed the percentage of true prediction of the patient conditions was 93.3 (accuracy). The risk estimate showed that the proportion of cases that were incorrectly classified was 0.068 (standard error = 0.008).

Based on Fig. 13.6, the ROC analysis of the DT showed excellent performance in predicting the status of patients with COVID-19. The area under the ROC curve (AUC) of the CT-derived opacity score was 0.93 (95% confidence interval 0.91–0.96; $p < 0.001$).

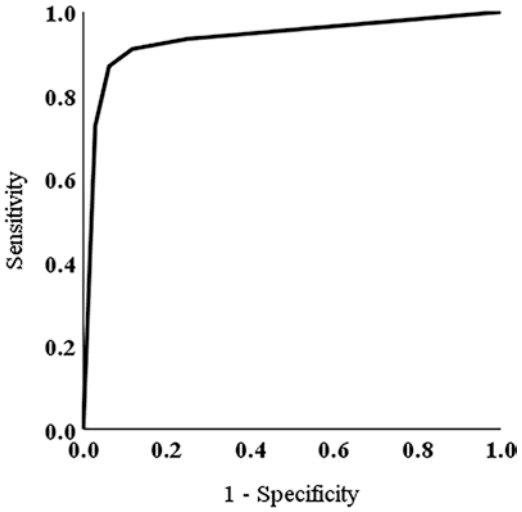

Fig. 13.6 ROC curve for DT, AUC = 93%

4 Discussion

This report describes a means of predicting COVID-19 disease status, which fits with the concept that early diagnosis can aid in patient assessment for enabling the appropriate therapeutic intervention, if needed [21]. Here, we have provided a quantitative means of assessing chest CT imaging as an indicator of signs related to disease advancement, including increase in GGOs, interstitial septal thickening, and consolidative opacities [22].

We found that linear opacities, pure GGOs, mixed GGOs with consolidation, and mixed GGOs with crazy-paving pattern were the most frequent types of lesions with bilateral and multifocal distributions. The total opacity score, number of lung lobes involved, and presence of diffuse opacity were regarded as noticeable variables by data mining. In the DT model, we considered that if the variable scored lower than 7.5, the next essential variable will be age. Using the total opacity score with a score greater than 7.5, along with lesion type as GGOs plus consolidation, we found that the occurrence of the critical condition would give a score of 82.6. It is worth mentioning that when the total opacity score is less than 7.5 and the age of the patient is less than 62.5, the predicted percentage of patients with a non-critical status would be 98.4.

In our study, the difference in age between the two groups was statistically significant consistent with reports that age is one of the most significant risk factors for severe COVID-19 disease outcomes [23–25]. Similar to other chest CT studies, we observed bilateral lung involvement in most of the patients and a reversed halo sign in a small number of patients in both groups [26, 27].

In both groups of this study, the common types of lesions were mixed GGOs with consolidation, mixed GGOs with crazy-paving pattern, liner opacities, and pure GGOs. The frequency of pure consolidation and mixed GGOs with

consolidation lesions showed a significant difference between the groups, being more common in critical patients than in non-critical patients. This implies that the virus has diffused into the respiratory epithelium where it can cause necrotizing bronchitis and diffuse alveolar damage in the critical patients [28]. Also, critical patients showed more intralesional traction bronchiectasis and pleural effusion lesions than the non-critical patients. These extra pulmonary lesions indicate the occurrence of severe inflammation in critical group and are consistent with the findings of other chest CT studies of COVID-19 disease patients [29, 30].

According to our DT model, the total opacity score was the main feature for distinguishing the critical from the non-critical group, with an accuracy of 93.3%. Our findings are consistent with previous studies regarding sensitivity and specificity scores derived from CT imaging of lung lesions of COVID-19 patients [31–33]. However, it is clear that there is considerable scope for further progress in this area in forthcoming studies. One possibility is to incorporate machine learning techniques to extract the most important features for CT image-based classifications, as described in two recent studies [34–36]. As more data become accessible, the procedure described here could be easily repeated to acquire more exact models. We also suggest that further improvements in the predictive performance could be achieved through incorporation of laboratory data into the model. For example, molecular biomarkers could be used to allow determination of the pneumonia-related markers associated with CT features [37–40].

4.1 Strengths and Limitations

The strength of this retrospective study was the large sample size, which enabled a sufficiently powered statistical comparison. Potentially, one of the most important strengths was the use of data derived from chest CT imaging. This is the gold standard method for unambiguous determination of interstitial pneumonia, a distinctive feature of respiratory virus infection [41]. In addition, this method can serve as an additional screening tool to add confidence to a diagnosis, particularly with regard to disease staging [42]. It is also easily implemented and can be particularly valuable in the early stages of a viral outbreak, when molecular diagnostic tools have not been optimized (as seen in the early stages of the current pandemic).

One limitation of this study was that the time of chest CT examination and the onset symptoms were not simultaneous. This made it difficult to summarize the features of a CT scan that could be associated with specific symptoms during the course of the disease. Another limitation was the dependence of this study on the CT and demographic data. The incorporation of data from laboratory biomarker measurements could add further value to the model. For example, point-of-care array devices which provide readouts of circulating molecules associated with the cytokine storm effect could be incorporated into the DT model to increase robustness and performance values [43].

4.2 Conclusion

In summary the results showed that chest CT imaging features were helpful in identifying pulmonary parenchymal abnormalities in patients suspected of having COVID-19 disease. We used the total opacity score as the main feature of the CT results in predicting which patients will develop a critical or non-critical status. The main results of the study showed that 98% of patients with non-critical condition and 72.8% of patients with critical situation were correctly diagnosed. We conclude that the established DT model had high sensitivity and specificity and aided in the identification of risk factors in COVID-19 patients associated with different severity outcomes. We suggest that the use of machine learning approaches with incorporation of molecular and laboratory-based biomarkers will help to improve the performance of the model. Such approaches will help us to manage the current and future pandemics caused by respiratory viruses more effectively.

Availability of Data and Materials

Data are available by contacting the corresponding authors with a reasonable request.

Acknowledgments An early version of this manuscript was submitted as a preprint and is available at https://www.researchsquare.com/article/rs-56387/v3. The present version contains updated information.

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Part IV
Tracking SARS-CoV-2 Variants

Chapter 14
Inferring Recombination Events in SARS-CoV-2 Variants In Silico

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Abstract Over the last 34 months, at least 10 severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) distinct variants have evolved. Among these, some were more infectious while others were not. These variants may serve as candidates for identification of the signature sequences linked to infectivity and viral transgres-

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sions. Based on our previous hijacking and transgression hypothesis, we aimed to investigate whether SARS-CoV-2 sequences associated with infectivity and trespassing of long noncoding RNAs (lncRNAs) provide a possible recombination mechanism to drive the formation of new variants. This work involved a sequence and structure-based approach to screen SARS-CoV-2 variants in silico, taking into account effects of glycosylation and links to known lncRNAs. Taken together, the findings suggest that transgressions involving lncRNAs may be linked with changes in SARS-CoV-2–host interactions driven by glycosylation events.

Keywords SARS-CoV-2 · COVID-19 · Spike protein · Variant · Glycosylation · lncRNA

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1 Introduction

Since the emergence of the unique coronavirus 2019 (COVID-19) which first appeared in Wuhan, China, the mechanism of how the severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) spike protein mediates viral binding and how post-translational modifications affect this is beginning to be understood [1, 2]. Glycosylation is a significant post-translational event that can influence protein structure and functional characteristics either directly or indirectly. Structural studies have shown that the spike protein and spike-angiotensin converting enzyme 2 (ACE2) complexes exhibit several glycosylations, which may have a substantial impact on the ability of the virus to infect and induce an immune response in the host [1]. The SARS-CoV-2 spike protein glycans are sometimes referred to as a "glycan shield" because they sterically obscure the underlying polypeptide epitopes from detection from potentially neutralizing antibodies [2–4]. In addition, the spike protein receptor binding domain (RBD) glycans play a critical role in binding proteins involved in COVID-19 pathogenesis, including the ACE2 receptor and transmembrane protease, serine 2 (TMPRSS2), to host glycoproteins [5–6]. Because viral glycoproteins are exposed on the virus surface, they are the primary targets of host antibodies [7]. In turn, all antibodies are glycoproteins and the attached glycans can have a major impact on their function in the immune response [8]. Therefore, understanding how the spike protein is glycosylated has crucial implications for studies on SARS-CoV-2 pathobiology and vaccine development.

Glycosylation is the enzyme-catalyzed addition of a sugar molecule/oligosaccharide to a macromolecule such as a protein. Nitrogen (N)-linked glycosylation takes place co-translationally on asparagine residues at a specific sequence on the nascent protein known as a sequon, which consists of asparagine-X-threonine/

serine/cysteine (AsnXThr/Ser/Cys), where X cannot be proline (Pro) [9]. Oxygen (O)-linked glycosylation occurs post-translationally on the side chain of Ser or Thr residues during transport of the nascent proteins through the Golgi compartment of cells [9]. *N*-glycans contain a common pentacore which consists of two *N*-acetylglucosamine (GlcNAc) and three mannose residues which can be extended by various monosaccharide units via the action of various glycosyl-transferases, and these units can be modified by glycosidases. Since the process depends on multiple factors such as cell type and metabolic state, the resultant glycan structures are often heterogeneous in nature. If the pentacore is extended only with mannose, the structure is known as high mannose. *N*-glycans of the second type are complex sugars where the two antennae of the pentacore are extended by different sugars including GlcNAc, galactose, fucose, and sialic acid residues. If one antenna is extended with mannose and other with various monosaccharides the sugar structure is known as hybrid. Based on linkages and composition, the structure can be further divided to many subtypes creating high complexity [10].

Host glycoproteins on cells such as those of the immune system play a major role in the pathogenic and immunogenic activity during infections. SARS-CoV-2 infection induces changes in the pattern of host antibody glycosylations with significant variations in the levels of IgG galactosylation and fucosylation [11]. The increase in fucosylation can lead to increased production of proinflammatory cytokines which can lead to the damaging cytokine storm effect in patients [12–17]. In addition to these effects, spike protein glycan variations can modify binding of viruses to host receptors and alter the severity of the pathogenesis and immune responses [10, 18]. Importantly, the composition of N-glycosylation modifications on viruses and host cell receptors has been reported to have a significant impact on virus-receptor identification, binding, and cellular penetration [19, 20]. For example, seven glycosylation sites on the SARS-CoV-1 spike protein from the 2002–2004 epidemic were shown to be necessary for dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC/L-SIGN)-mediated infection [21]. The extracellular domain of the human ACE2 receptor contains seven N-glycosylations (Asn53, Asn90, Asn103, Asn322, Asn432, Asn546, and Asn690) and several O-glycosylations, which are likely to impact viral entry into host cells [22, 23]. The glycosylations at N90 and N322 appear to be important in binding to SARS-CoV-2 spike protein RBD [21, 24]. Also, molecular dynamic simulations have shown that the glycan linked to the ACE2 Asn90 position interferes with virus binding, explaining reports of heightened susceptibility to infection when glycosylation at this site is removed [25]. With hyper sialylation and oligomannose-type modification of ACE2 glycans, the binding affinity between ACE2 and SARS-CoV-2 spike decreases modestly [26]. Acting in concert with the ACE2 receptor, the TMPRSS2 protease involved in SARS-CoV-2 entry into host cells is glycosylated at amino acids Asn213 and Asn249 [6]. However, the structural impact of this has not been investigated extensively. In terms of glycosylated structures, SARS-CoV-2 is reported to bind specifically to heparan sulfate and sialic acid residues on host cells [27, 28]. As many immune cells such as macrophages, dendritic cells, T cells and B cells, and

immune system proteins are glycosylated, it is likely that SARS-CoV-2 may have interactions with these in the ensuing pathogenic and immunogenic processes.

Although most studies on the host response to viral infections have focused on genes that encode proteins, it is now emerging that noncoding RNA molecules are also involved [29]. Early studies in this field found that changes in the expression of long noncoding RNAs (lncRNAs) can alter the innate immune response during viral infections [30, 31]. A more recent study found changes in the expression of multiple lncRNAs during SARS-CoV-2 infection of human bronchial epithelial cells [32]. Another investigation found that dysregulated lncRNAs in SARS-CoV-2 infection are involved in multiple aspects of the infection process including viral proliferation, the host immune response, and disease outcome [33]. There is also evidence that rearrangements or polymorphisms in lncRNAs may drive disease-causing mutations, as shown in cancer research [34, 35].

In this study, we have carried out in silico analyses to determine (1) if any new lncRNAs are known in transgression pathways induced by SARS-CoV-2 infection, (2) whether or not lncRNAs encoded or transgressed by the virus could provide clues into the mechanisms of how the SARS-CoV-2 variants have emerged, and (3) if changes in RBD N-glycosylation status are associated with the altered binding affinity of different SARS-CoV-2 variants. For the latter, we used in silico docking complex analyses to calculate the effect of binding energies between host glycan and spike protein variants. This involved comparison of the binding energies of the Omicron (7WPB) and Delta variants (7TEW) with that of the Wuhan strain (6LZG), with respect to the three commonly found host glycan structures A2F, 6G1, and Man 5.

2 Methods

2.1 Datasets

SARS-CoV-2 and selected nucleotide sequences were retrieved from an NCBI database search [36]. We filtered the several thousands of results using Boolean expressions AND, OR, and NOT and retrieved RefSeq accession numbers of relevant annotated sequences. Following this, we performed NCBI BLAST search in which SARS-CoV-2 reference sequences were compared to the SARS-CoV-2 genome using different databases (nucleotide collection [nr/nt], sequence read archives, refseq representative genome, Protein Data Bank, refseq genome database, whole-genome shotgun contigs, refseq select RNA sequences, expressed sequence tag). The results obtained were tabulated with information on similarity and dissimilarity between the query and subject (Fig. 14.1a). We chose accessions based on characteristics such as e-value, mismatches, and % identity, and selected hits were used for downstream analysis (Table 14.1 and Supplementary Table 14. ST1). We then used Protein Data Bank (PDB) to screen candidate SARS-CoV-2

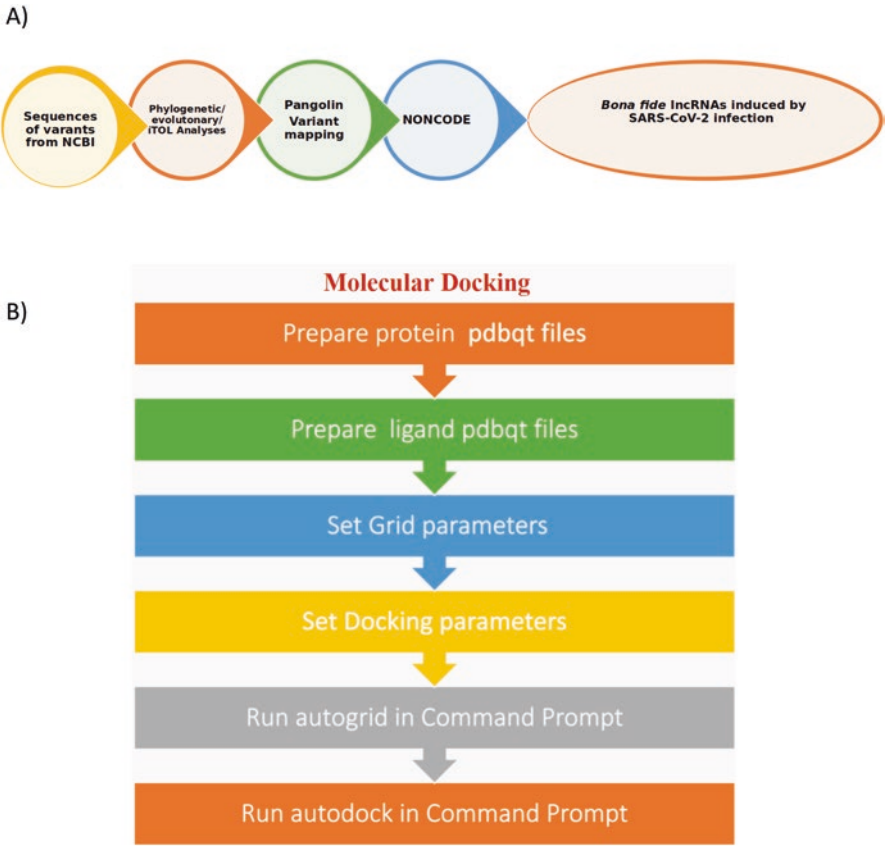


Fig. 14.1 Pictorial methodology of the tools used for the analysis. (**a**) Sequences of the SARS-CoV-2 variants were downloaded from NCBI and subjected to phylogenetic/evolutionary analyses before reconfirming their lineages with Pangolin. As a final check to understand the matching lncRNAs, we used the NONCODE.org database to analyze these by BLAST. (**b**) Overview of the molecular docking analysis

spike protein sequences from the Delta (pdb id: 7TEW) and Omicron (pdb id: 7WPB) variants against the spike sequences of the Alpha, Beta, and Gamma variants as reference (pdb id: 6LZG) (Fig. 14.1b).

2.2 Structural Interpretation and Docking

MolView was used to visualize small molecules of 2D and 3D structures. We inserted the Simplified Molecular Input Line Entry System (SMILE) of the molecule to obtain 2D and 3D structures and downloaded these in spatial data file (SDF)

Table 14.1 List of spike proteins associated with lncRNAs

Accession number	lncRNA	Query length	Putative variant
MZ558096.1	NONHSAT156862.1	21,705–21,726	Deltacron
MZ558096.1	NONHSAT079728.2	21,793–21,814	Deltacron
MZ427312.1	NONHSAT156862.1	21,701–21,722	Gamma
MZ427312.1	NONHSAT079728.2	21,789–21,810	Gamma
MZ433432.1	NONHSAT156862.1	21,713–21,734	Beta
MZ433432.1	NONHSAT079728.2	21,801–21,822	Beta
MZ 297238.1	NONHSAT247026.1	12,481–12,504	Beta
OK189649.1	NONHSAT247026.1	12,494–12,517	Delta
ON017450.1	NONHSAT156862.1	21,701–21,722	Zeta
ON017450.1	NONHSAT079728.2	21,789–21,810	Zeta
MZ780476.1	NONHSAT156862.1	21,720–21,741	Beta
MZ780476.1	NONHSAT079728.2	21,808–21,829	Beta
ON017446.1	NONHSAT247026.1	12,461–12,484	Zeta

format [37]. AutoDock containing Molecular Graphics Laboratory (MGL) tools and Autodock4 was used for in silico docking, evaluating the binding energy (ΔG) and binding inhibition constant (K_i). We used MGL tools to set the parameters of ligand and protein by minimizing the energies and converted the files to Protein Data Bank, Partial Charge (Q), and Atom Type (T) (pdbqt) files for both ligands and proteins. The grid parameters were set for each protein with respect to each ligand separately by considering the *X*, *Y*, and *Z* coordinates, and the Grid Parameter Files (GPFs) were generated. Furthermore, the docking parameters for each protein with respect to each ligand were set considering Lamarckian and generic algorithms and the files were saved as dock parameter files (DPFs). Autogrid in Autodock commands were run using command prompt (Fig. 14.1). Chimera was used for visualization (https://www.cgl.ucsf.edu/chimera/download.html) in the analysis of spike protein from three different SARS-CoV-2 strains (6LZG: original Wuhan strain, 7TEW: Delta variant, and 7WPB: Omicron variant) (Fig. 14.2).

2.3 Selection of Ligands

The ligands were chosen based on binding patterns of host glycans to the spike protein RBD with steric hindrance checked for each [28]. We used the glycan structures from PubChem to obtain 2D and 3D structures [38]. These structures were downloaded in SDF format and then converted into PDB format (Table 14.2). The three ligands used were A2F *N*-glycan, 6 G1-glycan, and mannose.

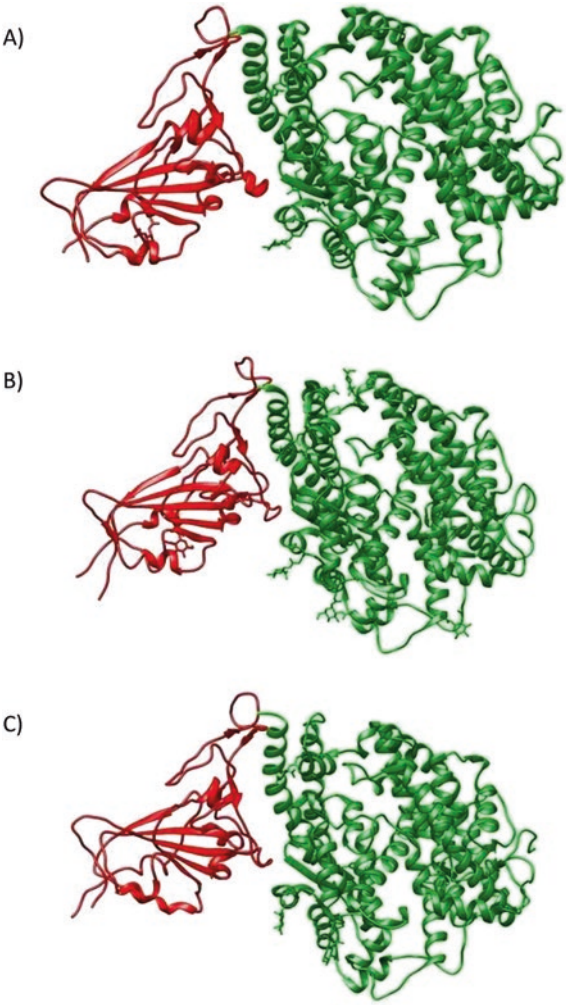
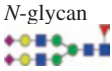
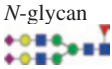
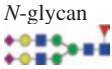


Fig. 14.2 Representation of SARS-CoV-2 spike protein 3D structures from (**a**) the March 2020 original strain (6LZG) and (**b**) the Delta (7TEW) and (**c**) Omicron (7WPB) variants

2.4 Phylogenetic and Pangolin Analyses

Clustal Omega was used to align multiple sequences [39]. The sequences from best hits selected from BLAST searching were converted into FASTA files which were uploaded in Clustal Omega to obtain the alignment results [40]. From this, we obtained guides and phylogenetic trees showing the evolution of the different strains. The guide tree data from the Clustal Omega analysis was uploaded to the Interactive Tree of Life (iTOL) online tool to obtain a circular phylogenetic tree [41]. We also used Pangolin (Phylogenetic Assignment of Named Global Outbreak Lineages) to assign lineages to genome sequences of SARS CoV-2 [42].

Table 14.2 Docking results showing the binding energy and affinity of the ligands for the indicated amino acids

Protein	PBD ID	ligand	ΔG	K_i	AA residues
SARS-CoV-2 spike protein (reference sequence)	6LZG	A2F *N*-glycan 	–9.11 kcal/mol	210.60 nM	ASP364, CYS336, NAG601, GLY339, LEU441, ASN440
		6 G1-glycan 	–7.19 kcal/mol	5.37 µM	ASN370, SER371, LEU368, PHE374, NAG601
		Man-5 	–4.89 kcal/mol	261.35 µM	NAG601, PHE342
SARS-CoV-2 Delta spike protein	7TEW	A2F *N*-glycan 	–7.79 kcal/mol	1.94 µM	NAG706, SER317, VAL316, NAG704, GLU312, LYS313
		6 G1-glycan 	–6.97 kcal/mol	7.75 µM	NAG704, VAL316, LYS313
		Man-5 	–3.62 kcal/mol	2.22 µM	NAG706, SER545, SER317, LYS313, ILE421
SARS-CoV-2 Omicron spike protein	7WPB	A2F *N*-glycan 	–7.79 kcal/mol	1.94 µM	NAG902, ASN546, SER317, LYS313
		6 G1-glycan 	–6.37 kcal/mol	21.24 µM	NAG902, VAL316, LYS313
		Man-5 	–4.29 kcal/mol	711.08 µM	PHE374, TYR362, ILE431, LEU365, VAL364, PHE339

◆ = sialic acid, ● = galactose, ■ = GlcNAc, ● = mannose, ▲ = core fucose
Asp aspartate, *Cys* cysteine, *Gly* glycine, *Leu* leucine, *Asn* asparagine, *Ser* serine, *Phe* phenylalanine, *Val* valine, *Lys* lysine, *Ile* isoleucine, *Tyr* tyrosone, *NAG N*-acetylglucosamine

2.5 *LncRNA Analysis*

We used the Noncode RNA database [43] to enable retrieval of data and to compare lncRNA sequences with SARS-CoV-2 and host protein sequences using BLAST. The query sequence was given in FASTA format and the database used was NONCODE V6 animal. From the obtained hits, the ones with e-values less than zero, we chose human lncRNAs and sought to check the expression profile data to know where the particular lncRNA is expressed in human. Similarly, BLAST was performed for every other accession selected from NCBI earlier. Finally, data wrapper was used to design charts ranging from simple bars and lines to arrow, range, and scatter plots, which can be done using steps [44].

3 Results and Discussion

3.1 Sequence Similarities and Dissimilarities for All SARS-CoV-2 Variants

The SARS CoV-2 nucleotide sequences downloaded from the NCBI database were BLAST-searched against the RefSeq database and the resulting candidate hits are listed in Supplementary Table 14.ST2. The cut off for the e-value was set at <0 which indicates that the sequence is an exact match to the query. Thus lower e-values are indicative of better hits with respect to % identity and query coverage. The % identity for the 27 assemblies ranged between 89.5% and 100%. The assembled sequences were then assigned to specific SARS-CoV-2 variant sequences. For this, we employed a similar strategy as above and the best hits were tabulated in Supplementary Table 14.ST3. While a large number of sequences were mapped to Beta and Zeta, some were mapped to Gamma and a lower number to the Delta and Deltacron [45] variants.

3.2 Pangolin Outbreak Lineages of 38 Different Variants

The Pangolin tree yielded seeded guide alignments, and hidden Markov model (HMM) profile–profile techniques were used to generate alignments between three or more sequences. We considered the accession numbers of 38 different variants of the virus, with the rest of the information downloaded in FASTA format for multiple sequence alignment (Fig. 14.3a). The tree showed distinct clades with many variants sub-claded together. The ones which were sub-claded were assumed to belong to the same variants which we confirmed. We also checked assignment conflicts, ambiguity, and lineages showing the metadata files of the given accession number of viruses including the type of variants, dates, and regions where these variants were also depicted. From this, we finally considered 11 sequences as belonging to the Gamma, Delta, and Beta variants (Fig. 14.3b).

3.3 Noncoding RNA Sequences Known to Be Trespassed

We searched the NONCODE database to identify lncRNA sequences within the complete genome sequences of the selected viral accessions in FASTA format. From the resulting table, we considered human lncRNA sequences that showed 100% sequence identity (Fig. 14.4). Binary values of 0 and 1 were ascribed to a lncRNA sequence if absent or present, respectively, in specific viral accession numbers and entries were summed row- and column-wise to obtain the final lists of matching lncRNAs. This showed that MW562722.1 had the lowest sum of 17

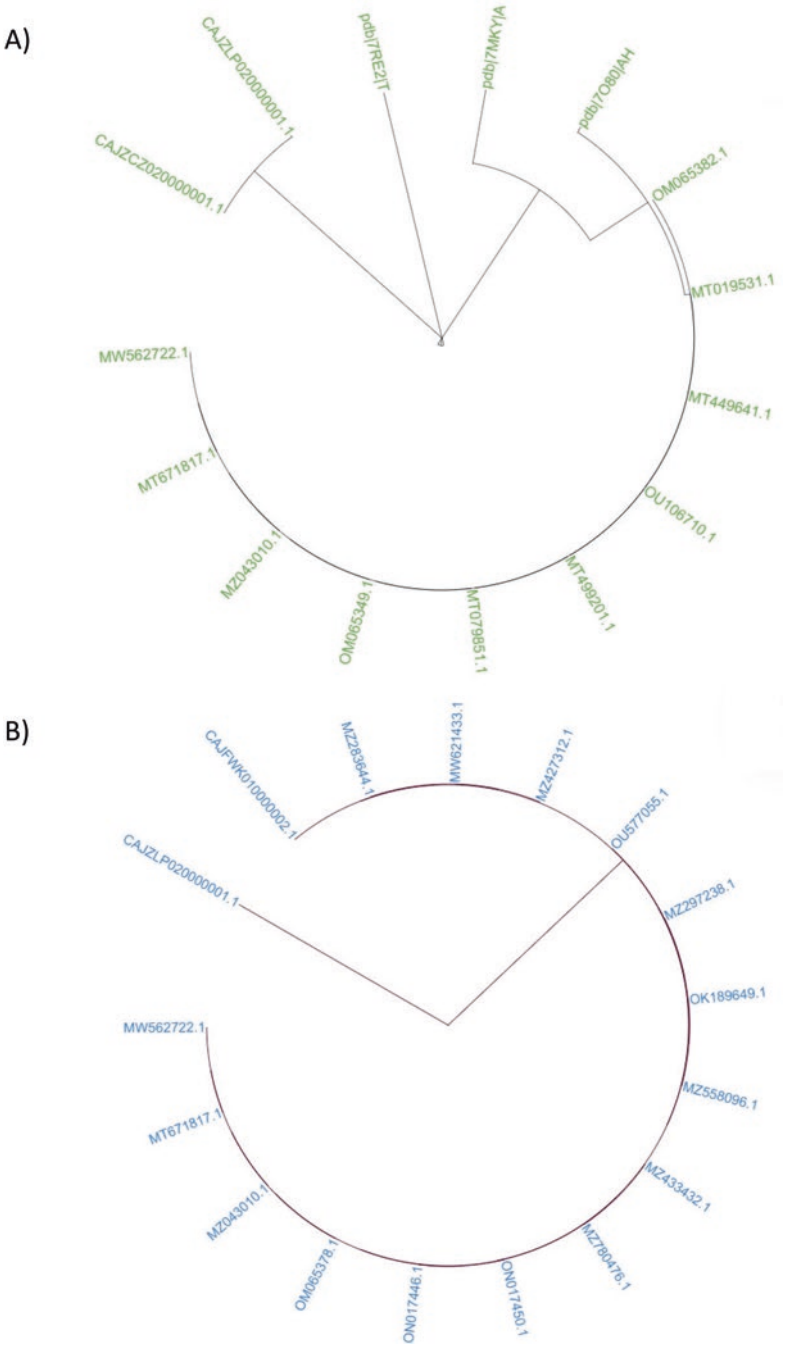


Fig. 14.3 Circular phylogenetic trees of (**a**) all similar SARS-CoV-2 sequences and (**b**) dissimilar sequences emerging from different SARS-CoV-2 variants

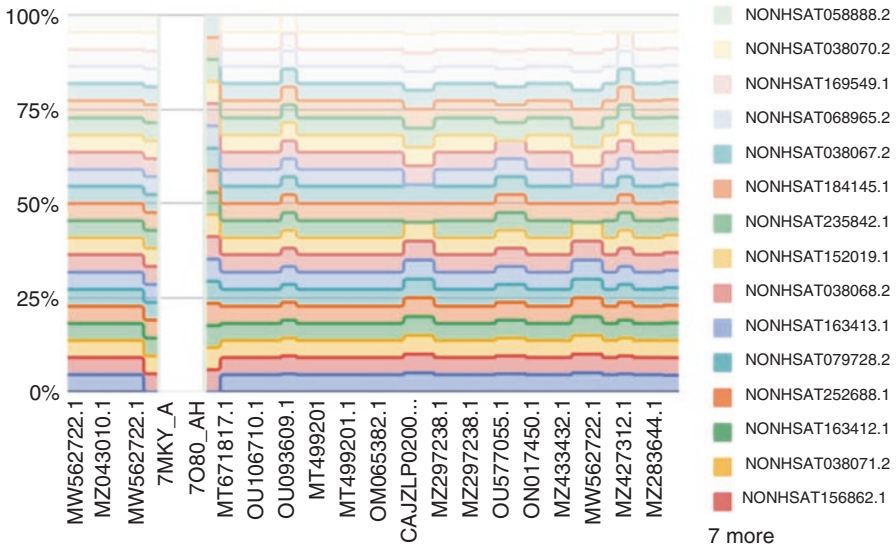


Fig. 14.4 Visualization of lncRNAs (NONHSAT codes) versus viral accessions. The Y-axis shows percentage of lncRNAs present for each viral accession (X-axis). This analysis revealed no lncRNAs in two viral accessions: 7MKY_A (SARS-CoV-2 chain A, RNA 66-MER) and 7O80_AH (SARS-CoV-2 chain AH, mRNA). Note that the figure shows only 15 of the 22 lncRNAs

lncRNA sequences (NONHSAT252687.1, NONHSAT209697.1, NONHSAT209698.1, NONHSAT169548.1, NON HSAT155452.1, NONHSAT209695.1, NONHSAT156862.1, NONHSAT038071.2, NON HSAT163412.1, NONHSAT252688.1, NONHSAT079728.2, NONHSAT163413.1, NONHSAT038068.2, NONHSAT152019.1, NONHSAT235842.1, NONHSAT184145.1, NON HSAT038067.2). We also found strains with a sum of 22 as the highest number of lncRNA sequences. Some of the lncRNA sequences such as NONHSAT163412.1 and NONHSAT163413.1 were repeated in more than 30 viral sequences. We also identified the loci of the genes which code for the spike protein by examining sequences of the identified strains using the Pangolin tool. We then selected these stains and retrieved the sequences from the NCBI database and searched for those with 100% identity with lncRNA sequences. This resulted in the identification of seven viral accession numbers which had similarities to some lncRNAs (Table 14.1). Finally, the hypothesized transgression hypothesis is depicted in Fig. 14.5.

3.4 Molecular Interaction Studies

Our in silico molecular docking approach investigated the binding of the SARS-CoV-2 spike protein variants to specific carbohydrate groups. It revealed that the original Wuhan SARS-CoV-2 spike protein (6LZG) bound to A2F *N*-glycan with a

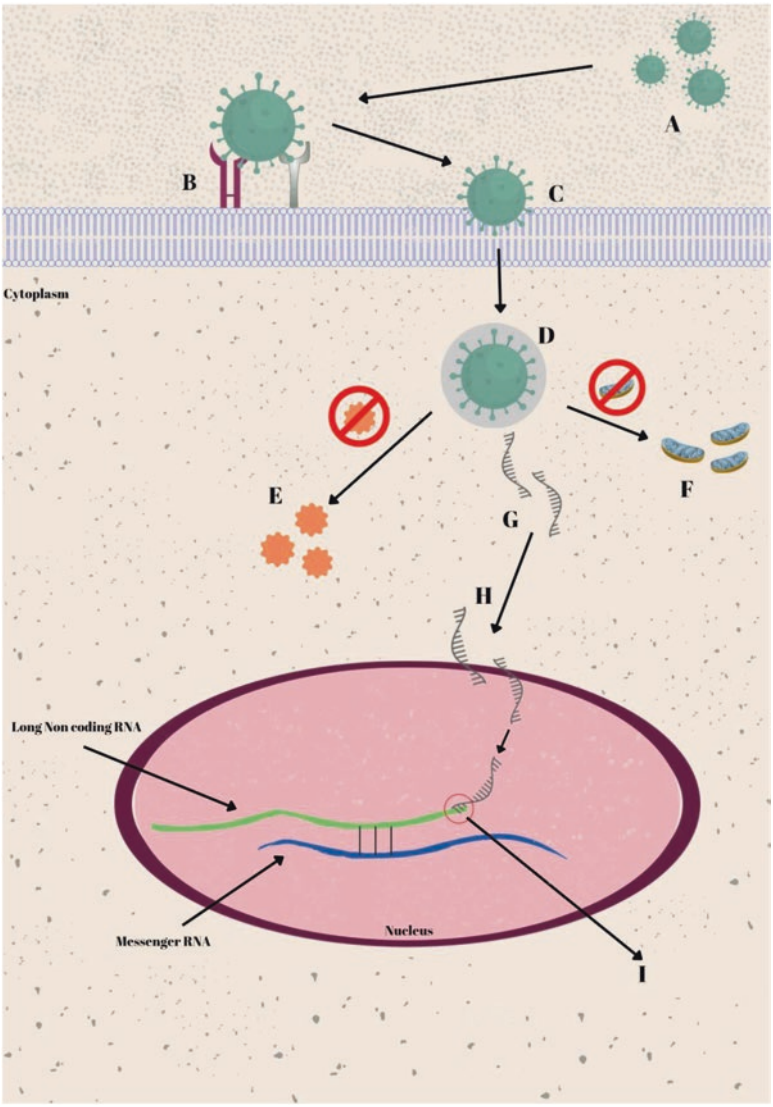


Fig. 14.5 Hypothesis of how lncRNAs transgress: (A) SARS-CoV-2 particles enter the body; (B) the spike protein binds to the host ACE2 receptor, followed by cleavage by TMPRSS2 protease which activates the fusion process; (C) the virus fuses with host cell membrane; (D) the virus enters the cell by endocytosis; (E) the virus destroys or deactivates interferons and interleukins responsible for innate immunity; (F) the virus hijacks mitochondria; (G) the virus undergoes uncoating and release of RNA; (H) the viral RNA enters the nuclei; and (I) the viral RNA transgresses specific lncRNAs of the host cell and takes the neighboring genes under its control

low free energy (ΔG) of −9.11 kcal/mol and high affinity (K_i) of 210.60 nM, at the indicated RBD amino acids (Fig. 14.6 and Table 14.2). 6 G1-glycan bound to the 6LZG spike protein with a ΔG of −7.19 kcal/mol and K_i of 5.37 μM as indicated. Mannose was also bound with a ΔG of −4.89 kcal/mol and a low affinity (261.35 μM).

The same analysis of the SARS-CoV-2 Delta spike protein (7TEW) revealed binding to A2F *N*-glycan with a ΔG of −7.79 kcal/mol and a K_i of 1.94 μM. 6 G1-glycan bound with a ΔG of −6.97 kcal/mol and K_i of 7.75 μM, and mannose bound to the Delta variant with ΔG equal to −3.62 kcal/mol and high binding affinity of 2.22 μM (Fig. 14.7 and Table 14.2).

Finally, SARS-CoV-2 Omicron spike protein (7WPB) bound to A2F *N*-glycan with a ΔG of −7.79 kcal/mol and K_i of 1.94 μM, 6G1-glycan was bound with a ΔG of −6.37 kcal/mol and a K_i of 21.24 μM, and mannose was bound at −4.29 kcal/mol with a low affinity of 711.08 μM (Fig. 14.8).

We next differentiated the number of H-bonds formed between the host glycan and the spike RBD with the reference spike protein (6LZG). This showed that A2F *N*-glycan formed six H-bonds, 6 G1-glycan formed five H-bonds, and mannose had two H-bonds. The Delta spike protein (7TEW) interacts with A2F *N*-glycan forming six H-bonds, while interaction with 6 G1-glycan gave three H-bonds and mannose had five H-bonds. Lastly, the Omicron spike protein (7WPB) interacted with A2F

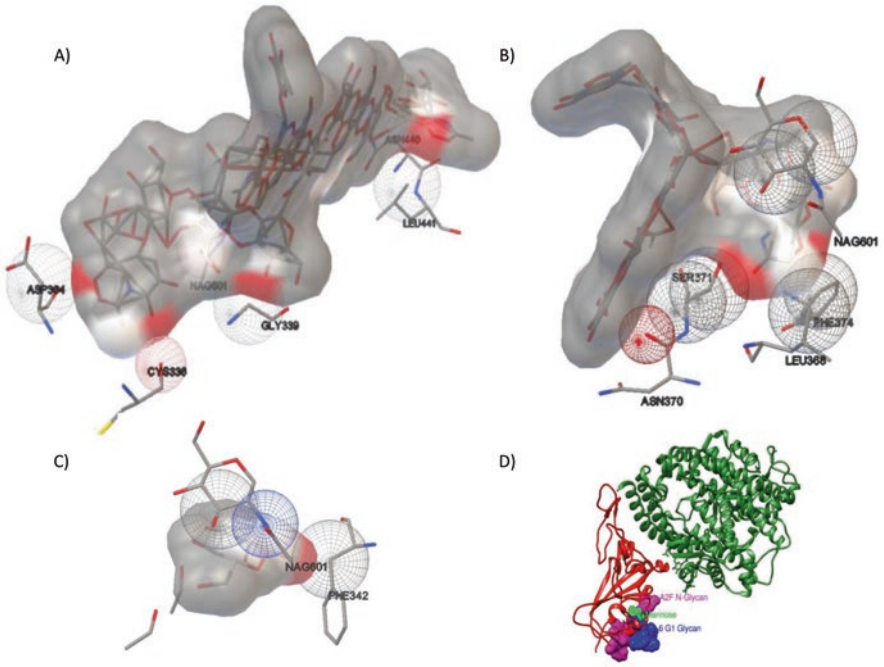


Fig. 14.6 2D representation of (**a**) A2F *N*-glycan, (**b**) 6 G1 glycan, and (**c**) mannose structures of 6LZG SARS-CoV-2, and (**d**) 3D representation of 6LZG SARS-CoV-2 with glycan complexes

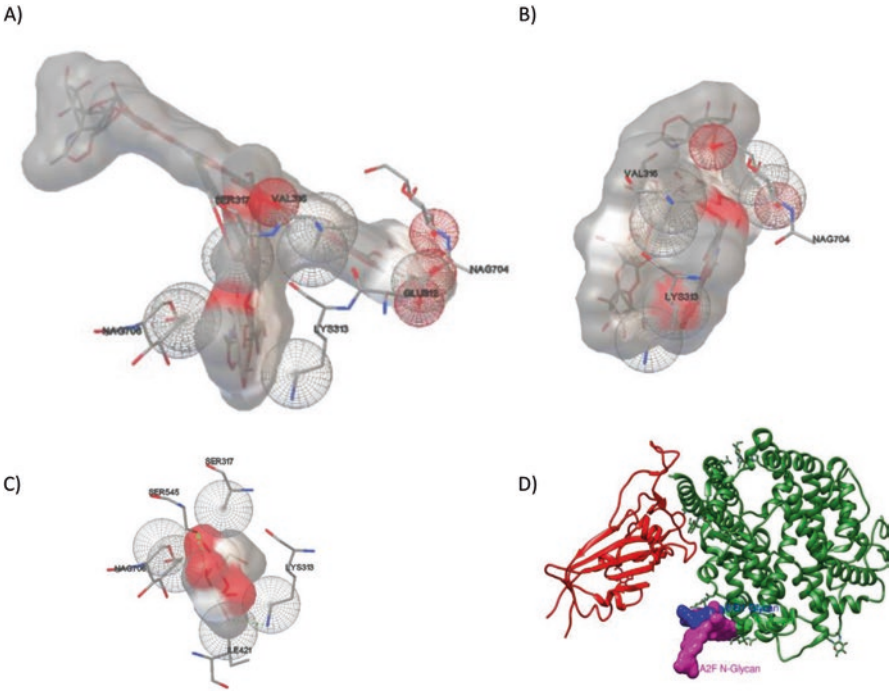


Fig. 14.7 2D representation of (**a**) A2F *N*-glycan, (**b**) 6 G1 glycan, and (**c**) mannose structures of 7TEW SARS-CoV-2, and (**d**) 3D representation of 7TEW SARS-CoV-2 with glycan complexes

N-glycan forming four H-bonds, while interaction with 6 G1-glycan gave three H-bonds and mannose had six H-bonds.

In summary, the analysis revealed that A2F *N*-glycan had the lowest binding affinity for the Delta and Omicron spike protein RBD sites. However, A2F *N*-glycan and 6 G1-glycan are bound with a lower free energy at RBD sites for all spike proteins compared to mannose. 6 G1-glycan had marginally lower affinity for the Omicron spike RBD (21.24 μM) compared to the Wuhan strain and the Delta variant. In contrast, mannose is bound to the Delta variant with markedly higher affinity (2.22 μM) compared to the original strain (K_i = 261.35 μM) and the Omicron variant (K_i = 711.08 μM).

4 Conclusions

The sequence similarity and dissimilarity approaches helped us to increase our understanding of how the SARS-CoV-2 variants achieve different binding, infectivity, and transmission properties in host cells. In the first part of the study, we identified key lncRNAs that could play a role in these transgression effects, and in the

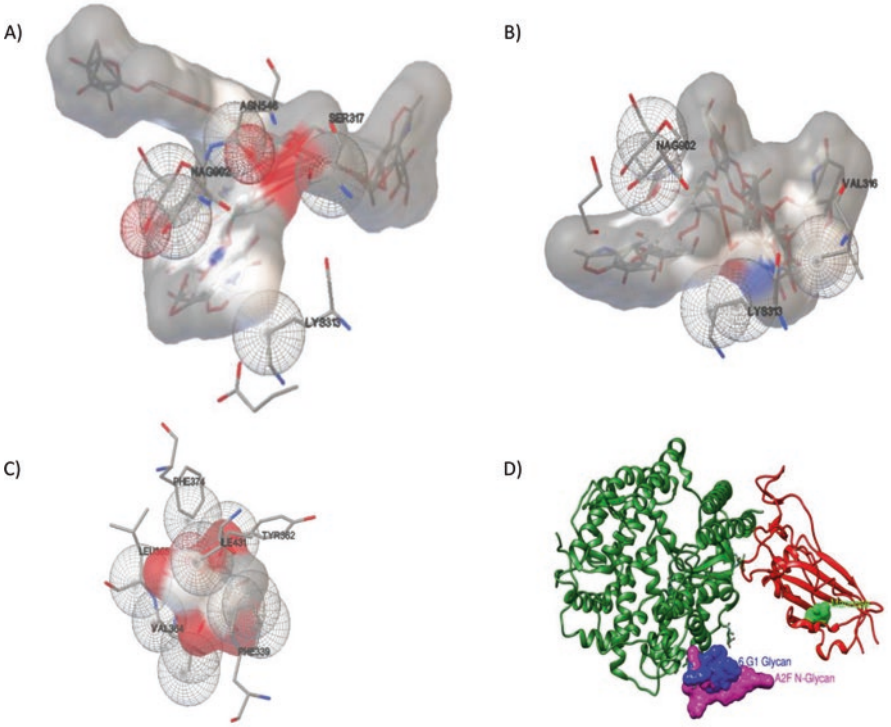


Fig. 14.8 2D representation of (**a**) A2F *N*-glycan, (**b**) 6 G1 glycan, and (**c**) mannose structures of 7WPB SARS-CoV-2, and (**d**) 3D representation of 7WPB SARS-CoV-2with glycan complexes

second part, we focused on the sequence differences in spike proteins from the Delta and Omicron variants with regard to glycan binding in the host. Taken together, the findings revealed that the sequence differences in the variants of concern can affect glycosylation of the SARS-CoV-2 spike and host proteins which, in turn, can impact on the various transgression pathways. For example, such changes could increase infectivity by enhancing interactions with the ACE2 receptor or block the effect of neutralizing antibodies by disrupting their binding to the virus. In the current study, we examined the effects on three *N*-glycan structures (A2F, 6-G1, and high mannose) which differed with respect to fucosylation and terminal sugar composition, and showed differential binding with the SARS-CoV-2 Delta and Omicron spike RBDs compared to that of the original Wuhan strain. We suggest that the methods described in this study could be used to predict the virulence and transmissibility of new SARS-CoV-2 variants as these emerge. This would enable implementation of appropriate response measures and help to prepare us for the next pandemic.

Acknowledgments The authors gratefully acknowledge Revered Amma, Mata Amritanandamayi, Chancellor of Amrita Vishwa Vidyapeetham.

Author Contributions NN visualized and performed the major structural docking analysis; AM, HH, and AJ along with AR, MJ, PA, SR, and AAN wrote the first draft. All other authors contributed with lateral texts. PS, AA, and RS mentored the project. PS proofread the manuscript before all authors agreed to it.

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Chapter 15
Amplicon-Based Nanopore Sequencing of Patients Infected by the SARS-CoV-2 Omicron (B.1.1.529) Variant in India

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Somesh Kumar, Avinash Lomash and Sunil K. Polipalli contributed equally with all other contributors.

Abstract We report the sequencing of SARS-CoV-2 Omicron variants from 75 patients, using nanopore long-read sequencing chemistry. These data show a range of mutations in spike glycoprotein that are both unique and common to other populations.

Keywords SARS-CoV-2 · COVID-19 · Omicron · Mutation · Sequencing

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1 Introduction

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a betacoronaviridae family member, and has been a primary and urgent concern worldwide [1–3]. As of March 4, 2022, over 107 countries had reported infections due to Omicron variants, since the reporting of first case on November 29, 2021 [4]. India saw the first few Omicron cases originating in the state of Karnataka on December 1, 2021 [5], with Delhi reporting a case later from a Tanzania returnee [6]. In this study, we sought to sequence all COVID-19 samples including Omicron variants that were reported in our tertiary care to gain further insights into the mutations occurring in this SARS-CoV-2 variant.

2 Methods

Nasopharyngeal swab samples were collected from 75 patients with a travel history of Africa/Middle East. Here, we randomly analysed samples from 10 representative patients who presented with mild symptoms (fever, cold, cough, sore throat and mild weakness) within 3 days of onset of infection and prior to hospitalization. The samples were used as an input for the ARTIC network "Midnight" protocol (Fig. 15.1) for PCR tiling of SARS-CoV-2, including sequencing with Oxford Nanopore Technologies (ONT) long-read whole-genome sequencing (Rapid Barcoding Kit 96/SQL-RBK-110-96) [7, 8].

3 Results and Discussion

ONT sequencing yielded an average of 25 million reads from all 10 samples, spanning 96.28% of the SARS-CoV-2 genome (20× coverage depth) (Table 15.1). To check the transmissibility associated with the number of mutations in the spike glycoprotein associated with receptor-binding domain (RBD), we compared the 44 common mutations from our samples with the recently emerging mutations of Omicron. Our preliminary analysis indicated that the Omicron variant subcladed with the dominant Delta variant and might have evolved rapidly from multiple mutations (Tables 15.2a, 15.2b, 15.3 and 15.4). A neighbourhood joining tree was constructed using Clustal Omega with the sequences sorted vertically, thereby drawing a circular and unrooted tree (Fig. 15.2a) [9]. We observed that the Indian Omicron variants were clustered together with a root emerging from OL815455, the variant that was first detected from Botswana. The iTOL containing the 75 sequenced samples and Wuhan reference yielded distinct clades in both unrooted and rooted circular tree (data not shown) and the four samples that were claded separately suggested that these were among the first suspected Omicron cases in India (Fig. 15.2a)

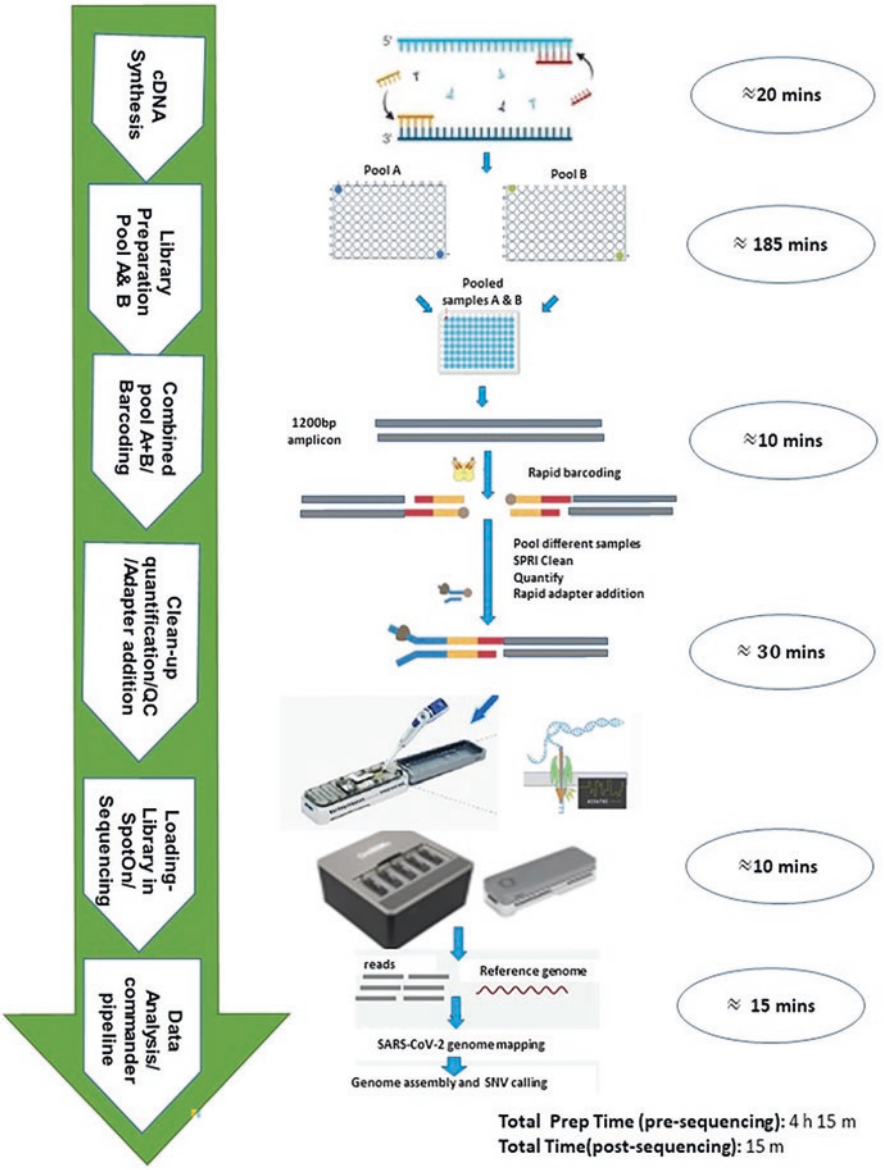

Fig. 15.1 Midnight workflow for preparation of SARS-CoV-2 whole-genome sequencing. This method was similar to the ARTIC amplicon sequencing protocol for MinION for SARS-CoV-2 v3 (LoCost) by Josh Quick and the method used in Freed et al. [8]

[10–12]. We obtained p.Thr614Ile, p.Thr1822Ile, p.Thr6098Ile and p.Asp155Tyr from LNHD9, p.Ala701Val and p.Val1887Ile from LNHD8 and p.Gly667Ser from LNHD1. However, our preliminary observations indicated that none of these are known to confer detrimental properties to the spike (e.g. changes in transmissibility,

Table 15.1 List of the 10 samples with coverage, CT values, clinical symptoms and age/sex

S. No.	GenBank	Size (bp)	GISAID	Age/Sex	Lab ID	CT VALUE	Clinical Symptoms	Coverage 20×
1	ON063250.1	29,746	EPI_ISL_7877026	35Y/M	LNHD4	E gene-25, rdrp gene-26	Fever, cough or mild weakness	99.94
2	ON063249.1	29,768	EPI_ISL_7877093	39Y/F	LNHD5	E gene-19, rdrp gene-22	Fever, cough, cold or mild weakness	98.39
3	ON063248.1	29,742	EPI_ISL_7877115	6Y/M	LNHD6	E gene-27, rdrp gene-27,	Not available	91.31
4	ON063247.1	29,779	EPI_ISL_7877191	40Y/M	LNHD7	E gene-20, rdrp gene-23	Fever, cough, cold, mild weakness	97.16
5	ON063246.1	29,751	EPI_ISL_7877201	18Y/M	LNHD8	E gene-24, rdrp gene-26	Fever, cough, mild weakness	97.27
6	ON063245.1	29,739	EPI_ISL_7877202	57Y/M	LNHD9	E gene-18, rdrp gene-20	Fever, cough, cold, sore throat, mild weakness	97.22
7	ON063244.1	29,780	EPI_ISL_7877203	19Y/F	LNHD10	E gene-30, rdrp gene-30	Not available	97.23
8	ON063243.1	29,737	EPI_ISL_7877297	23Y/M	LNHD11	E gene-21, rdrp gene-23	Fever, cough, cold, mild weakness	97.25
9	ON063242.1	29,739	EPI_ISL_7889640	31Y/M	LNHD12	E gene-20, rdrp gene-22	Fever, cough, cold, mild weakness	94.3
10	ON063241.1	29,743	EPI_ISL_7889641	42Y/M	LNHD13	E gene-17, rdrp gene-19	Fever, cough, cold, sore throat, mild weakness	93.2

S. No. sample number, *bp* base pairs, *CT* cycle threshold

severity or immune evasion). Mutations in the spike proteins (Fig. 15.2b(i–iii)) of SARS-CoV-2 variants of concern have also been compared to the parental SARS-CoV-2 isolate B.1 suggesting that the amino acid substitutions are already found in altered positions but with distinct substitutions (Supplementary Tables 15.1 and 15.2).

Table 15.2a Genome Coverage and mutations in the cohort

Sample	Query	Length (nucleotides)	Length (amino acids)	Total mutations (number)	Unique mutations (number)	Reference
1	hCoV-19/ India/un-LNHD4/2021|EPI_ISL_7877026	29,751	9710	41	0	hCoV19/Wuhan/ WIV04/2019
2	hCoV-19/ India/un-LNHD5/2021|EPI_ISL_7877093	29,779	9710	45	0	hCoV19/Wuhan/ WIV04/2019
3	hCoV-19/ India/un-LNHD6/2021|EPI_ISL_7877115	29,742	9710	43	0	hCoV19/Wuhan/ WIV04/2019
4	hCoV-19/ India/un-LNHD7/2021|EPI_ISL_7877191	29,768	9710	45	0	hCoV19/Wuhan/ WIV04/2019
5	hCoV-19/ India/un-LNHD8/2021|EPI_ISL_7877201	29,746	9710	45	0	hCoV19/Wuhan/ WIV04/2019
6	hCoV-19/ India/un-LNHD9/2021|EPI_ISL_7877202	29,771	9710	49	0	hCoV19/Wuhan/ WIV04/2019
7	hCoV-19/India/ un-LNHD10/2021|EPI_ISL_7877203	29,738	9710	43	0	hCoV19/Wuhan/ WIV04/2019
8	hCoV-19/India/ un-LNHD11/2021|EPI_ISL_7877297	29,752	9710	47	1	hCoV19/Wuhan/ WIV04/2019
9	hCoV-19/India/ un-LNHD12/2021|EPI_ISL_7889640	29,745	9710	44	0	hCoV19/Wuhan/ WIV04/2019
10	hCoV-19/India/ un-LNHD13/2021|EPI_ISL_7889641	29,764	9710	41	0	hCoV19/Wuhan/ WIV04/2019

Table 15.2b Multiple mutations identified in the study cohort

1	NSP3_K38R,NSP3_A1892T,NSP4_T492I,NSP5_P132H,NSP6_I189V,NSP12_P323L,NSP14_I42V,Spike_N679K,Spike_Q493R,Spike_G339D,Spike_G446S,Spike_P681H,Spike_D614G,Spike_N969K,Spike_N764K,Spike_T478K,Spike_H655Y,Spike_G496S,Spike_N856K,Spike_N440K,Spike_A67V,Spike_S371L,Spike_Q498R,Spike_K417N,Spike_T547K,Spike_L981F,Spike_S375F,Spike_Q954H,Spike_S477N,Spike_N501Y,Spike_T95I,Spike_Y505H,Spike_D796Y,Spike_S373P,Spike_E484A,E_T9I,M_A63T,M_D3G,M_Q19E,N_G204R,N_R203K
2	NSP3_S1265N,NSP3_L1266I,NSP3_K38R,NSP3_A1892T,NSP4_T492I,NSP5_P132H,NSP6_I189V,NSP12_P323L,NSP14_I42V,Spike_N679K,Spike_Q493R,Spike_G339D,Spike_G446S,Spike_P681H,Spike_D614G,Spike_N969K,Spike_R346K,Spike_N764K,Spike_T478K,Spike_H655Y,Spike_G496S,Spike_N856K,Spike_N440K,Spike_A67V,Spike_S371L,Spike_Q498R,Spike_K417N,Spike_T547K,Spike_L981F,Spike_S375F,Spike_Q954H,Spike_S477N,Spike_N501Y,Spike_T95I,Spike_Y505H,Spike_D796Y,Spike_S373P,Spike_E484A,E_T9I,M_A63T,M_D3G,M_Q19E,N_G204R,N_P13L,N_R203K
3	NSP3_K38R,NSP3_A1892T,NSP4_T492I,NSP5_P132H,NSP6_G107S,NSP6_I189V,NSP12_P323L,NSP14_I42V,Spike_N679K,Spike_Q493R,Spike_G339D,Spike_G446S,Spike_P681H,Spike_D614G,Spike_N969K,Spike_R346K,Spike_N764K,Spike_T478K,Spike_H655Y,Spike_G496S,Spike_N856K,Spike_N440K,Spike_S371L,Spike_Q498R,Spike_K417N,Spike_T547K,Spike_L981F,Spike_S375F,Spike_Q954H,Spike_S477N,Spike_N501Y,Spike_T95I,Spike_Y505H,Spike_D796Y,Spike_S373P,Spike_E484A,E_T9I,M_A63T,M_D3G,M_Q19E,N_G204R,N_D63G,N_R203K
4	NSP3_S1265N,NSP3_L1266I,NSP3_K38R,NSP3_A1892T,NSP4_T492I,NSP5_P132H,NSP6_G107S,NSP6_I189V,NSP12_P323L,NSP14_I42V,Spike_N679K,Spike_Q493R,Spike_G339D,Spike_G446S,Spike_P681H,Spike_D614G,Spike_N969K,Spike_R346K,Spike_N764K,Spike_T478K,Spike_H655Y,Spike_G496S,Spike_N856K,Spike_N440K,Spike_A67V,Spike_S371L,Spike_Q498R,Spike_K417N,Spike_T547K,Spike_L981F,Spike_S375F,Spike_Q954H,Spike_S477N,Spike_N501Y,Spike_T95I,Spike_Y505H,Spike_D796Y,Spike_S373P,Spike_E484A,E_T9I,M_A63T,M_D3G,M_Q19E,N_G204R,N_R203K
5	NSP3_S1265N,NSP3_K38R,NSP3_A1892T,NSP3_V1069I,NSP4_T492I,NSP5_P132H,NSP6_I189V,NSP12_P323L,NSP14_I42V,Spike_N679K,Spike_Q493R,Spike_G339D,Spike_G446S,Spike_P681H,Spike_D614G,Spike_N969K,Spike_N764K,Spike_T478K,Spike_H655Y,Spike_G496S,Spike_N856K,Spike_N440K,Spike_A67V,Spike_S371L,Spike_Q498R,Spike_K417N,Spike_T547K,Spike_L981F,Spike_V70I,Spike_S375F,Spike_Q954H,Spike_S477N,Spike_N501Y,Spike_T95I,Spike_A701V,Spike_Y505H,Spike_D796Y,Spike_S373P,Spike_E484A,E_T9I,M_A63T,M_D3G,M_Q19E,N_G204R,N_R203K

(continued)

Table 15.2b (continued)

6	NSP2_T434I,NSP3_S1265N,NSP3_L1266I,NSP3_K38R,NSP3_T1004I,NSP3_A1892T,NSP4_T492I,NSP5_P132H,NSP6_I189V,NSP12_P323L,NSP14_T173I,NSP14_I42V,Spike_N679K,Spike_Q493R,Spike_G339D,Spike_G446S,Spike_P681H,Spike_D614G,Spike_N969K,Spike_N764K,Spike_T478K,Spike_H655Y,Spike_G496S,Spike_N856K,Spike_N440K,Spike_A67V,Spike_S371L,Spike_Q498R,Spike_K417N,Spike_T547K,Spike_L981F,Spike_S375F,Spike_Q954H,Spike_S477N,Spike_N501Y,Spike_T95I,Spike_Y505H,Spike_D796Y,Spike_Y145H,Spike_S373P,Spike_E484A,NS3_D155Y,E_T9I,M_A63T,M_D3G,M_Q19E,N_G204R,N_P13L,N_R203K
7	NSP3_K38R,NSP3_A1892T,NSP4_T492I,NSP5_P132H,NSP6_I189V,NSP12_P323L,NSP14_I42V,Spike_N679K,Spike_Q493R,Spike_G339D,Spike_G446S,Spike_P681H,Spike_D614G,Spike_N969K,Spike_N764K,Spike_T478K,Spike_H655Y,Spike_G496S,Spike_N856K,Spike_N440K,Spike_A67V,Spike_S371L,Spike_Q498R,Spike_K417N,Spike_T547K,Spike_L981F,Spike_S375F,Spike_Q954H,Spike_S477N,Spike_N501Y,Spike_T95I,Spike_Y505H,Spike_D796Y,Spike_S373P,Spike_E484A,E_T9I,M_A63T,M_D3G,M_Q19E,N_G204R,N_D63G,N_R203K,N_Q9L
9	NSP2_R46K,NSP3_K38R,NSP3_A1892T,NSP4_T492I,NSP5_P132H,NSP6_I189V,NSP12_P323L,NSP14_I42V,Spike_N679K,Spike_Q493R,Spike_G339D,Spike_N211I,Spike_G446S,Spike_P681H,Spike_D614G,Spike_N969K,Spike_N764K,Spike_T478K,Spike_H655Y,Spike_G496S,Spike_N440K,Spike_A67V,Spike_S371L,Spike_Q498R,Spike_K417N,Spike_T547K,Spike_S375F,Spike_S477N,Spike_N501Y,Spike_T95I,Spike_Y505H,Spike_D796Y,Spike_G142V,Spike_S373P,Spike_L212I,Spike_E484A,NS3_L41F,E_T9I,M_A63T,M_D3G,M_Q19E,N_G204R,N_D63G,N_R203K
10	NSP3_S1265N,NSP3_L1266I,NSP3_K38R,NSP3_A1892T,NSP4_T492I,NSP5_P132H,NSP6_I189V,NSP12_P323L,NSP14_I42V,Spike_N679K,Spike_Q493R,Spike_G339D,Spike_G446S,Spike_P681H,Spike_D614G,Spike_N969K,Spike_N764K,Spike_T478K,Spike_H655Y,Spike_G496S,Spike_N856K,Spike_N440K,Spike_A67V,Spike_S371L,Spike_Q498R,Spike_K417N,Spike_T547K,Spike_L981F,Spike_S375F,Spike_Q954H,Spike_S477N,Spike_N501Y,Spike_T95I,Spike_Y505H,Spike_D796Y,Spike_S373P,Spike_E484A,NS3_L106F,N_G204R,N_D343G,N_R203K

Table 15.3 Amino acid substitutions in the spike region observed in the study cohort

Spike mutation	Occurrences
K417N	9
T478K	9
S477N	9
E484A	9
G339D	9
N440K	9
G496S	9
Q493R	9
T547K	9
G446S	9
S375F	9
D614G	9
S373P	9
N764K	9
N679K	9
S371L	9
Y505H	9
Q498R	9
P681H	9
T95I	9
H655Y	9
N501Y	9
D796Y	9
N969K	9
A67V	8
Q954H	8
N856K	8
L981F	8
R346K	4
S373P	1
A67V	1
N764K	1
N679K	1
Q954H	1
K417N	1
S371L	1
T478K	1
S477N	1
Y505H	1
N856K	1

(continued)

Table 15.3 (continued)

Spike mutation	Occurrences
E484A	1
L981F	1
G339D	1
Q498R	1
P681H	1
N440K	1
H655Y	1
G496S	1
T95I	1
Q493R	1
T547K	1
G446S	1
S375F	1
D796Y	1
D614G	1
N501Y	1
N969K	1
N211I	1
Y145H	1
A701V	1
G142V	1
V70I	1
L212I	1

The limitation of our study is that although the adopted ARTIC sequencing protocol allowed the confirmation of SARS-CoV-2 infections, we did not carry out analyses to determine the probable structural impact of mutations on binding of antibodies produced by existing vaccines or previous SARS-CoV-2 infections, as described by Kannan et al. [13].

In conclusion, our study has demonstrated the utility of nanopore sequencing for SARS-CoV-2 genomes from clinical specimens. We firmly hope that prompt diagnosis and rapid whole-genome analysis would allow a decisive response to the SARS-CoV-2 outbreak that will bring disease control and prevention efforts.

Table 15.4 Common mutations ($n = 44$) seen across the Indian cohort

p.Ala67Val
p.Thr95Ile
p.Gly339Asp
p.Ser371Pro
p.Ser371Phe
p.Ser373Pro
p.Ser375Phe
p.Lys417Asn
p.Asn440Lys
p.Gly446Ser
p.Ser477Asn
p.Thr478Lys
p.Glu484Ala
p.Gln493Arg
p.Gly496Ser
p.Gln498Arg
p.Asn501Tyr
p.Tyr505His
p.Thr547Lys
p.Asp614Gly
p.His655Tyr
p.Asn679Lys
p.Pro681His
p.Asn764Lys
p.Asp796Tyr
p.Asn856Lys
p.Gln954His
p.Asn969Lys
p.Leu981Phe
p.Thr9Ile
p.Asp3Gly
p.Gln19Glu
p.Ala63Thr
p.Arg203Lys
p.Gly204Arg
p.Gly645Ser
p.Lys856Arg
p.Ala2710Thr
p.Thr3255Ile
p.Pro3395His
p.Ile3758Val
p.Ala4409Thr
p.Pro4715Leu
p.Ile5967Val

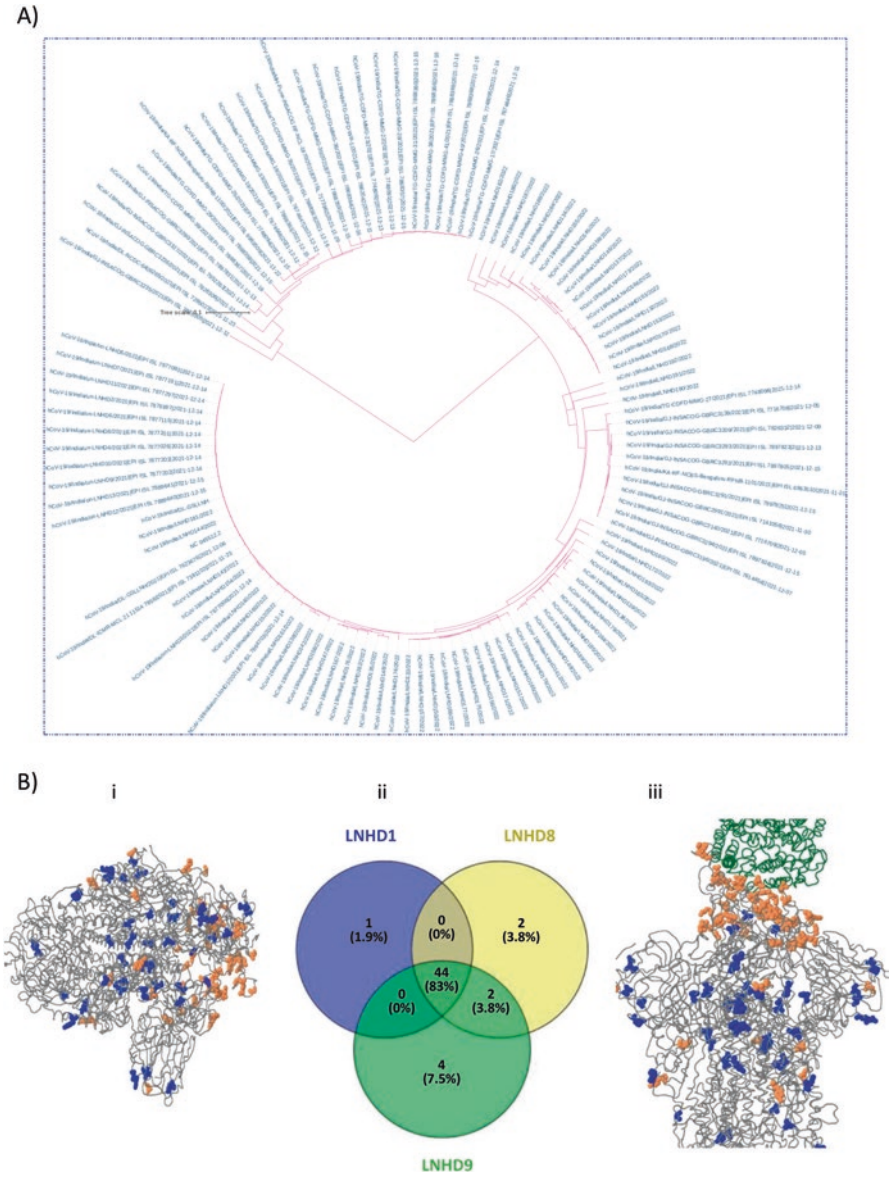

Fig. 15.2 (**a**) Circular phylogenetic tree of all 75 samples from India claded with the Wuhan reference genome. The unrooted tree shows a clear dissection of Wuhan from other lineages. All LNHD accessions are labelled. In the Indian sub-population, spike mutations ($n = 35$) were seen with the nearest residue if in loop/termini region (A67V, V70I(69), T95I, G142V, Y145H(143), N211I, L212I, G339D, R346K, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H T547K, D614G, H655Y, N679K(674), P681H(674), A701V, N764K, D796Y, N856K, Q954H, N969K and L981F). (**b**) (i) Spike glycoprotein (PDB: 6acc, EM 3.6 Angstrom) with RBD in down conformation. (ii) Multi-Venn diagram of three samples LNHD1, LNHD8 and LNHD9 showing unique and common mutations to all the LNHD series. (iii) Spike glycoprotein (PDB: 6acj, EM 4.2 Angstrom) in complex with host cell receptor ACE2 (green ribbon). (Also see links to Supplementary Tables 15.1 and 15.2)

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Ethics Statement Informed consent was judiciously taken before the sample was sequenced. The Institutional Ethics Committee of Maulana Azad Medical College, Delhi, India, had given approval for the study (F.1/IEC/MAMC/85/03/2021).

Data Availability All Omicron variant samples have been uploaded to NCBI-GenBank with accession IDs ON063241–ON063253 (https://www.ncbi.nlm.nih.gov/nuccore/?term=ON063241:ON063253[accn]) and ON060006–ON060067 (https://www.ncbi.nlm.nih.gov/nuccore/?term=ON060006:ON060067[accn]). The same have also been submitted to GISAID.org with hCoV-19/India/un-LNHD**XX**/2021 series

EPI_ISL_7864703
EPI_ISL_7876997
EPI_ISL_7877026
EPI_ISL_7877006
EPI_ISL_7877093
EPI_ISL_7877115
EPI_ISL_7877191
EPI_ISL_7877201
EPI_ISL_7877202
EPI_ISL_7877203
EPI_ISL_7877297
EPI_ISL_7889640

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Chapter 16
Perspectives on Rapid Antigen Tests for Downstream Validation and Development of Theranostics

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Abstract Point-of-care SARS-CoV-2 rapid antigen tests have proven to be useful over the years and have become more apparent to the public eye during COVID-19 pandemic due to their ease of use, rapid processing and result times, and low cost. Here, we have assessed the effectiveness and accuracy of rapid antigen tests in comparison to the standard real-time polymerase chain reaction analyses of the same samples.

Keywords COVID-19 · SARS-CoV-2 · Diagnosis · Polymerase chain reaction · Rapid antigen test · Sensitivity · Specificity

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1 Introduction

The SARS-CoV-2 virus has been at the center of the current COVID-19 pandemic. To gain information about the uncertain pathogenic aspects of SARS-CoV-2 researchers have looked into the immunopathogenic responses by rigorous investigation of the four major structural proteins: membrane, envelope, nucleocapsid, and spike. Efforts to visualize the high-resolution structure of the SARS-CoV-2 Exo-non-structural protein (nsp)-10 complex may lead to development of anti-coronavirus medications or approaches to lower viral virulence [1]. However, the rapid mutations leading to the emergence of new SARS-CoV-2 variants necessitate the development of efficient methods for detecting genetically diverse viral strains. Although the standard real-time polymerase chain reaction (PCR) detection assay can take into account the inherent polymorphism of the virus caused by genetic drift and recombination, mismatch-tolerant molecular beacons have specifically targeted detection of other emerging and rapidly mutating pathogens [2]. As SARS-CoV-2 infections burgeon, one intriguing feature of viruses transgressing the host sequences was beginning to be understood which could be detected using the diagnostic framework [3]. However, real-time PCR platforms have some weaknesses including potentially inadequate procedures for sample collection, contamination, manual errors, or use of inadequately validated assays, as well as the potential difficulties in screening subjects under antiretroviral therapies, testing subjects outside the diagnostic window, or problems arising due to mutation or recombination of the viral pathogen. Some practical indications for reducing the risk of diagnostic errors can thus be identified, including improving diagnostic accuracy; interpreting results based on epidemiological, clinical, and radiological data of the subjects; screening for upper or lower respiratory infections in patients with negative PCR test results; and improving management and storage of samples [4]. On the other hand, rapid antigen tests have steadily grown in numbers and since the spurt in Omicron cases, massive numbers of these tests have been deployed in India.

Several approaches to measure antiviral activities based on the SARS-CoV-2 spike protein have been developed to measure quantitatively the neutralizing activities of both human monoclonal antibodies and antibodies present in convalescent plasma, as well as those produced by the vaccines. These assays have also proved useful for serological immunity evaluations [5]. Current approaches for

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SARS-CoV-2 RNA detection are based on nucleic acid signal amplification which relies primarily on biological enzyme functions, which may impart the need for strict transit and storage conditions, high costs, and global supply constraints.

To circumvent these potential issues, a simple isothermal signal amplification method can be used for quick whole SARS-CoV-2 RNA genome detection via a non-enzymatic approach. This method has been termed non-enzymatic isothermal strand displacement and amplification (NISDA) and can detect down to 10 copies of RNA. In addition to having an assay time of less than 30 min, the NISDA assay is inexpensive, highly robust at room temperature, isothermal (42 °C), and non-invasive. This assay also requires no RNA reverse transcription, is simple to use, and is good for broad-based testing [6]. In addition, a number of commercial immunoassays are available for detecting SARS-CoV-2 antigens and antibodies. However, as many of these were developed with early strains of the virus in mind, some may have a diminished or total loss of capacity to detect sequences of the circulating variants or the antibodies raised against these [7].

To meet the demands of detected newly emerging variants in rapid time, a high-throughput next-generation sequencing-based approach was developed for screening over 100,000 samples per day. The amplification of SARS-CoV-2 and control amplicons by two-barcoded amplification, downstream library preparation for Illumina sequencing, and bioinformatics analysis can be done using the REcombinase Mediated BaRcoding and AmplificatioN Diagnostic Tool (REMBRANDT) as described by Palmieri et al. [8]. This can also be adapted to analysis of any pathogenic or non-pathogenic genome. On the other hand, point-of-care SARS-CoV-2 antigen tests have also proven to be useful over the years and more so during COVID-19 pandemic due to their ease of use, rapid processing and result times, and low cost. Here, we have assessed the effectiveness and accuracy of rapid antigen tests by comparison against real-time PCR analysis of the same samples.

2 Methods

The work received ethics clearance from Institutional Ethics Committee, Kurnool Medical College, Kurnool, India, with prior informed consent from all subjects. Symptomatic adults with suspected COVID-19 (n = 315) and non-infected controls (n = 525) from Pulivendula, India, were recruited for the current study. Two throat swabs were collected, one for real-time RT-PCR and another for the rapid antigen test. The study was intended to evaluate the efficacy of in-house-developed rapid antigen test (Genomix Biotech; Hyderabad, Telangana, India) against real-time PCR (Huwel Lifesciences Pvt. Ltd.; Hyderabad, Telangana, India). The oral swabs collected for the rapid antigen test were dipped in 400 µl viral lysis medium supplied with the rapid antigen test cassette and mixed several times by inversion. Three to four drops of the lysate was added to the sample window of the cassette and the results read within 10 min. The diagnostic sensitivity and specificity were calculated.

3 Results and Discussion

Analysis of samples from the 840 subjects yielded a diagnostic sensitivity of 96.51% and specificity of 100% (Fig. 16.1). To mitigate the diagnostic challenge of false positives, a viable option would have been to carry out RT-PCR screening for variants of concern, such as the omicron sub-variants (B.1.1.529 BA.1, BA.2, BA.3, BA.4, BA.5, and descendent lineages) [9]. From our analysis, we highlight the findings in Tables 16.1 and 16.2 to show the challenges and provide evidence which could be implemented for rapid diagnostics. We argue that comparative analysis of real-time RT-PCR and rapid antigen test results is a good prelude to assess cost effectiveness and to bring point-of-care diagnostics void of false positives to the forefront.

Finally, we suggest the following future aims to enable rapid point-of-care diagnostics:

- Identify potential signature sequences in the form of motifs and signals that would ideally serve as epitope targets.
- Characterize the hypothetical open reading frames (ORFs) which could possibly be associated with pathophysiological mechanisms governing co-morbidities.
- The aforementioned approaches could help design aptamers as small molecules which could be tested against the targeted SARS-CoV-2 signature sequences to enhance the translational value.

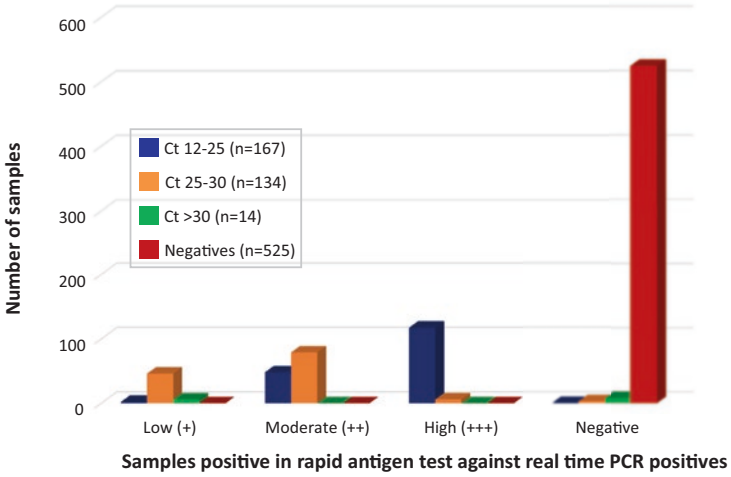

Fig. 16.1 Comparative analysis between the rapid antigen test and real-time PCR for screening SARS-CoV-2. In samples with Ct values between 12 and 25, rapid antigen test showed a greater number of high positives (+++). In samples with Ct values between 25 and 30, a higher number of samples showed moderate (++) and low positivity (+) with a small number of negatives. In samples with Ct values >30, only low positives and negatives were observed. All samples negative for SARS-CoV-2 PCR were also negative in rapid antigen tests

Table 16.1 Comparative analysis of real-time RT-PCR and rapid antigen test results from new nasopharyngeal swab samples. Samples with low, medium, and high Ct values in real-time RT-PCR showed a good, optimal, and weak or negative band intensities, respectively, in rapid antigen tests. Weak intensities could result from low viral load in the sample, which cannot be picked up by the rapid antigen test. Although rapid antigen tests are less sensitive in comparison to real-time RT-PCR tests, they might be more useful due to their cost effectiveness and ease of use at resource-limited and point-of-care areas

S. No.	Sample ID	Result	Ct values		RAT result
			E GENE	N GENE	
1	5381211	**Positive**	**19.26**	**21.17**	+++
2	5382982	**Positive**	**21.17**	**22.26**	+++
3	5383574	**Positive**	**24.18**	**23.33**	++
4	5384435	**Positive**	**22.19**	**21.99**	+++
5	5385806	**Positive**	**24.11**	**23.19**	++
6	5437967	**Positive**	**25.77**	**27.78**	+
7	5438748	**Positive**	**27.97**	**28.56**	+
8	5441956	**Positive**	**21.23**	**21.67**	+++
9	5442315	**Positive**	**26.13**	**22.86**	++
10	5442883	**Positive**	**26.71**	**26.82**	+++
11	5444575	**Positive**	**24.92**	**25.42**	++
12	5444606	**Positive**	**24.16**	**26.12**	++
13	5446555	**Positive**	**27.17**	**26.91**	+
14	5724719	**Positive**	**27.25**	**28.79**	+
15	5726576	**Positive**	**25.18**	**25.89**	++
16	5731303	**Positive**	**22.93**	**22.89**	++
17	5744680	**Positive**	**20.67**	**22.16**	++
18	5760227	**Positive**	**19.26**	**19.77**	+++
19	5760861	**Positive**	**28.78**	**28.70**	+
20	5761999	**Positive**	**28.20**	**27.86**	+
21	5763217	**Positive**	**28.20**	**29.32**	+
22	5828129	**Positive**	**21.89**	**25.43**	++
23	5836531	**Positive**	**24.52**	**24.70**	++
24	5837275	**Positive**	**21.10**	**21.77**	+++
25	5838721	**Positive**	**28.83**	**29.86**	+
26	5842656	**Positive**	**26.32**	**26.72**	+
27	5844450	**Positive**	**15.21**	**15.87**	+++
28	5844596	**Positive**	**24.53**	**24.48**	++
29	5845607	**Positive**	**24.27**	**24.08**	++
30	5846975	**Positive**	**19.50**	**20.54**	+++
31	5865404	**Positive**	**23.13**	**24.15**	+++
32	5869121	**Positive**	**21.72**	**21.68**	+++
33	5870272	**Positive**	**20.09**	**20.58**	+++
34	5872016	**Positive**	**29.28**	**28.88**	+
35	5875119	**Positive**	**20.34**	**20.42**	+++
36	5876281	**Positive**	**23.91**	**23.72**	++

(continued)

Table 16.1 (continued)

S. No.	Sample ID	Result	Ct values		RAT result
			E GENE	N GENE	
37	5877193	**Positive**	**28.56**	**28.17**	+
38	5877358	**Positive**	**18.63**	**19.42**	+++
39	5877667	**Positive**	**18.45**	**18.92**	+++
40	5887458	**Positive**	**15.72**	**16.12**	+++
41	5891815	**Positive**	**20.74**	**21.34**	+++
42	5987537	**Positive**	**23.51**	**25.91**	+++
43	5988318	**Positive**	**20.26**	**23.14**	+++
44	5988587	**Positive**	**24.53**	**27.21**	++
45	6007118	**Positive**	**16.94**	**20.52**	+++
46	6019072	**Positive**	**21.88**	**24.66**	+++
47	6021370	**Positive**	**21.14**	**24.11**	++
48	6024546	**Positive**	**22.24**	**25.56**	++
49	6025784	**Positive**	**19.67**	**22.33**	+++
50	6029109	**Positive**	**15.05**	**18.07**	+++
51	6029902	**Positive**	**21.24**	**24.94**	++
52	6036893	**Positive**	**16.43**	**19.12**	+++
53	6039577	**Positive**	**20.97**	**23.88**	+++
54	6040490	**Positive**	**19.73**	**23.6**	+++
55	6042457	**Positive**	**16.59**	**20.1**	+++
56	6043676	**Positive**	**21.49**	**24.09**	+++
57	6051584	**Positive**	**21.77**	**24.79**	+++
58	6057042	**Positive**	**21.75**	**24.83**	+++
59	6057240	**Positive**	**23.86**	**28.14**	++
60	6060860	**Positive**	**14.32**	**17.88**	+++
61	7663395	**Positive**	**26.34**	**26.52**	+
62	7729469	**Positive**	**26.08**	**26.53**	+
63	7738247	**Positive**	**25.27**	**25.40**	+
64	7739045	**Positive**	**23.27**	**23.67**	+++
65	7743953	**Positive**	**16.37**	**16.58**	+++
66	7745524	**Positive**	**28.52**	**24.03**	+
67	7747599	**Positive**	**29.47**	**29.39**	+
68	7749637	**Positive**	**29.28**	**29.09**	+
69	7751104	**Positive**	**27.19**	**27.96**	++
70	7752249	**Positive**	**21.30**	**21.93**	+++
71	7753732	**Positive**	**27.42**	**27.50**	+
72	7754504	**Positive**	**28.49**	**24.01**	++
73	7754833	**Positive**	**23.33**	**23.36**	++
74	7756437	**Positive**	**20.41**	**20.94**	+++
75	7757469	**Positive**	**26.79**	**26.98**	++
76	7758603	**Positive**	**24.66**	**24.17**	++
77	7760136	**Positive**	**26.33**	**26.18**	++

(continued)

Table 16.1 (continued)

S. No.	Sample ID	Result	Ct values		RAT result
			E GENE	N GENE	
78	7766869	**Positive**	**29.42**	**29.71**	+
79	7768257	**Positive**	**23.78**	**23..02**	++
80	7768477	**Positive**	**28.28**	**29.90**	+
81	7774337	**Positive**	**28.02**	**28.13**	+
82	7774880	**Positive**	**27.70**	**27.78**	++
83	7776175	**Positive**	**23.67**	**25.14**	++
84	7777731	**Positive**	**25.69**	**25.11**	++
85	7778540	**Positive**	**24.30**	**24.82**	++
86	7778750	**Positive**	**29.47**	**29.30**	++
87	7784811	**Positive**	**28.75**	**28.45**	+
88	7784877	**Positive**	**21.81**	**21.99**	+++
89	7785275	**Positive**	**26.01**	**26.64**	++
90	7785929	**Positive**	**29.23**	**29.50**	+++
91	7803763	**Positive**	**21.68**	**21.53**	+++
92	7804936	**Positive**	**26.87**	**26.47**	++
93	7806385	**Positive**	**28.46**	**28.02**	+
94	7806795	**Positive**	**27.52**	**27.04**	+
95	7807991	**Positive**	**21.31**	**21.85**	+++
96	7824231	**Positive**	**28.16**	**28.23**	+
97	7835492	**Positive**	**23.01**	**23.74**	++
98	7835531	**Positive**	**26.48**	**24.53**	++
99	8521806	**Positive**	**28.02**	**25.52**	+
100	8523159	**Positive**	**25.13**	**22.69**	++
101	8523476	**Positive**	**17.31**	**17.69**	+++
102	8523704	**Positive**	**27.40**	**26.01**	++
103	8530394	**Positive**	**25.30**	**23.17**	++
104	8539235	**Positive**	**27.96**	**30.57**	+
105	8545675	**Positive**	**25.54**	**19.03**	+++
106	8552462	**Positive**	**17.99**	**13.03**	+++
107	8554090	**Positive**	**22.36**	**24.38**	+++
108	8554353	**Positive**	**24.22**	**22.22**	++
109	8560562	**Positive**	**30.07**	**24.58**	+
110	8565635	**Positive**	**25.42**	**23.16**	++
111	8577854	**Positive**	**17.49**	**15.62**	+++
112	8580477	**Positive**	**29.97**	**28.34**	+
113	8581088	**Positive**	**26.18**	**24.52**	++
114	8581434	**Positive**	**27.23**	**25.04**	++
115	8582610	**Positive**	**26.77**	**25.07**	++
116	8583677	**Positive**	**15.91**	**13.48**	+++
117	8584269	**Positive**	**28.64**	**27.22**	+
118	8584498	**Positive**	**13.58**	**12.25**	+++

(continued)

Table 16.1 (continued)

S. No.	Sample ID	Result	Ct values		RAT result
			E GENE	N GENE	
119	10485651	**Positive**	**29.02**	**26.66**	+
120	10487405	**Positive**	**27.65**	**25.38**	++
121	10548216	**Positive**	**31.25**	**31.11**	–
122	10549790	**Postive**	**28.47**	**26.62**	++
123	10580349	**Positive**	**32.52**	**31.90**	–
124	10580800	**Positive**	**30.52**	**28.22**	+
125	10581455	**Positive**	**21.88**	**20.93**	+++
126	10590245	**Positive**	**32.07**	**29.54**	+
127	10602284	**Positive**	**32.63**	**32.14**	–
128	10603038	**Positive**	**32.57**	**30.98**	–
129	10606931	**Positive**	**29.63**	**28.02**	+
130	10607027	**Positive**	**24.06**	**20.70**	+++
131	10607059	**Positive**	**18.30**	**17.10**	+++
132	10608582	**Positive**	**26.81**	**25.63**	++
133	10609327	**Positive**	**32.98**	**31.66**	–
134	10612153	**Positive**	**22.36**	**21.63**	+++
135	10646258	**Positive**	**32.98**	**31.09**	–
136	10656115	**Positive**	**32.36**	**30.60**	+
137	10663001	**Positive**	**32.37**	**31.20**	–
138	10693247	**Positive**	**23.13**	**22.42**	+++
139	12730122	**Positive**	**18.63**	**21.92**	+++
140	12788156	**Positive**	**24.99**	**20.23**	++
141	12842650	**Positive**	**28.80**	**23.24**	++
142	12855334	**Positive**	**23.51**	**17.19**	+++
143	12860364	**Positive**	**26.92**	**26.72**	++
144	12874600	**Positive**	**29.03**	**22.96**	++
145	13909807	**Positive**	**25.64**	**27.07**	++
146	14685805	**Positave**	**24.73**	**22.71**	++
147	14719026	**Positave**	**32.19**	**26.40**	+
148	26468585	**Positive**	**25.72**	**22.00**	++
149	26476621	**Positive**	**26.06**	**20.89**	++
150	26500764	**Positive**	**20.71**	**23.53**	+++
151	26501031	**Positive**	**26.17**	**24.42**	++
152	26505227	**Positive**	**22.24**	**20.42**	+++
153	26523895	**Positive**	**12.50**	**12.31**	+++
154	26535815	**Positive**	**25.56**	**23.24**	++
155	26569776	**Positive**	**24.42**	**23.12**	++
156	26585025	**Positive**	**26.47**	**24.30**	++
157	26589252	**Positive**	**25.23**	**22.62**	++
158	26613155	**Positive**	**24.49**	**22.07**	+++
159	26623616	**Positive**	**24.18**	**22.00**	++

(continued)

Table 16.1 (continued)

S. No.	Sample ID	Result	Ct values		RAT result
			E GENE	N GENE	
160	26623791	**Positive**	**26.62**	**24.87**	++
161	26655577	**Positive**	**20.42**	**19.18**	+++
162	26714058	**Positive**	**22.16**	**28.10**	++
163	26749316	**Positive**	**26.92**	**24.74**	++
164	26750979	**Positive**	**15.95**	**15.01**	+++
165	26754829	**Positive**	**18.01**	**20.65**	+++
166	26762606	**Positive**	**26.25**	**24.90**	++
167	26762607	**Positive**	**27.25**	**23.85**	++
168	26784484	**Positive**	**25.15**	**23.54**	++
169	26798681	**Positive**	**20.85**	**18.36**	+++
170	26798930	**Positive**	**32.43**	**28.77**	+
171	26798930	**Positive**	**32.43**	**28.77**	+
172	26799072	**Positive**	**24.68**	**22.74**	++
173	26835814	**Positive**	**21.63**	**18.61**	+++
174	26836505	**Positive**	**23.99**	**23.11**	+++
175	26862439	**Positive**	**23.40**	**20.57**	+++
176	26862440	**Positive**	**23.40**	**20.57**	+++
177	26999387	**Positive**	**21.15**	**17.27**	+++
178	27014428	**Positive**	**14.80**	**12.32**	+++
179	27028210	**Positive**	**22.25**	**20.81**	+++
180	27029240	**Positive**	**20.89**	**19.77**	+++
181	27042961	**Positive**	**16.45**	**15.08**	+++
182	27047432	**Positive**	**23.35**	**21.81**	++
183	27049052	**Positive**	**22.03**	**20.58**	+++
184	27049923	**Positive**	**17.92**	**16.71**	+++
185	27167761	**Positive**	**18.68**	**16.43**	+++
186	27168185	**Positive**	**21.50**	**20.25**	+++
187	27243163	**Positive**	**22.85**	**11.13**	+++
188	27308549	**Positive**	**28.95**	**26.46**	+++
189	27324816	**Positive**	**24.65**	**18.36**	+++
190	27598753	**Positive**	**25.35**	**24.84**	++
191	27619887	**Positive**	**29.56**	**24.17**	+
192	27676099	**Positive**	**27.77**	**24.10**	++
193	27818140	**Positive**	**23.17**	**24.78**	+++
194	28042421	**Positive**	**27.76**	**28.08**	+++
195	28075589	**Positive**	**20.56**	**13.23**	+++
196	28078579	**Positive**	**27.02**	**21.50**	++
197	28078657	**Positive**	**21.66**	**16.69**	+++
198	28078778	**Positive**	**22.34**	**20.10**	+++
199	28094220	**Positive**	**25.88**	**27.38**	++
200	28112368	**Positive**	**24.48**	**25.09**	+++

(continued)

Table 16.1 (continued)

S. No.	Sample ID	Result	Ct values		RAT result
			E GENE	N GENE	
201	28137767	**Positive**	**27.59**	**28.62**	++
202	28151326	**Positive**	**23.20**	**18.10**	+++
203	28152347	**Positive**	**21.74**	**22.65**	+++
204	28159889	**Positive**	**27.25**	**30.49**	+
205	28160441	**Positive**	**20.61**	**23.41**	+++
206	28160668	**Positive**	**25.67**	**28.66**	++
207	28217105	**Positive**	**18.38**	**23.78**	++
208	28217328	**Positive**	**24.29**	**19.34**	+++
209	28233054	**Positive**	**26.60**	**26.40**	++
210	28248751	**Positive**	**22.95**	**18.59**	+++
211	28257370	**Positive**	**25.54**	**22.60**	++
212	28261726	**Positive**	**24.52**	**23.13**	++
213	28262504	**Positive**	**23.13**	**18.74**	+++
214	28264527	**Positive**	**28.08**	**22.60**	+
215	28276601	**Positive**	**26.87**	**21.75**	+
216	28279738	**Positive**	**18.39**	**22.90**	++
217	28281860	**Positive**	**22.17**	**18.61**	+++
218	28293262	**Positive**	**26.59**	**23.17**	++
219	28298748	**Positive**	**20.14**	**14.89**	+++
220	28899021	**Positive**	**25.27**	**21.50**	++
221	28940879	**Positive**	**29.12**	**25.27**	++
222	28985439	**Positive**	**21.21**	**19.68**	+++
223	28985443	**Positive**	**27.80**	**24.84**	++
224	29004949	**Positive**	**29.56**	**27.21**	+
225	29029431	**Positive**	**20.46**	**18.64**	+++
226	29081047	**Positive**	**24.60**	**20.60**	+++
227	29085339	**Positive**	**28.15**	**25.31**	++
228	29158568	**Positive**	**24.1**	**21.87**	+
229	29187334	**Positive**	**26.58**	**24.01**	++
230	29197328	**Positive**	**25.15**	**22.58**	++
231	29228294	**Positive**	**13.18**	**12.88**	+++
232	29230884	**Positive**	**26.22**	**23.52**	++
233	29231543	**Positive**	**15.54**	**12.77**	+++
234	29237707	**Positive**	**23.76**	**20.52**	+++
235	29246034	**Positive**	**26.42**	**26.66**	++
236	29256107	**Positive**	**17.70**	**16.26**	+++
237	29266016	**Positive**	**23.04**	**19.80**	++
238	29272402	**Positive**	**13.21**	**11.61**	+++
239	29342609	**Positive**	**23.48**	**20.33**	+++
240	29358290	**Positive**	**22.00**	**18.04**	+++
241	29398311	**Positive**	**23.71**	**18.58**	+++

(continued)

Table 16.1 (continued)

S. No.	Sample ID	Result	Ct values		RAT result
			E GENE	N GENE	
242	29437396	**Positive**	**25.23**	**22.62**	++
243	29481857	**Positive**	**25.84**	**21.42**	++
244	29484250	**Positive**	**23.74**	**19.03**	++
245	29488969	**Positive**	**24.16**	**21.35**	++
246	29528402	**Positive**	**28.06**	**27.26**	++
247	29551530	**Positive**	**16.28**	**14.15**	+++
248	29553020	**Positive**	**25.65**	**22.71**	++
249	29556904	**Positive**	**26.29**	**23.22**	++
250	29578862	**Positive**	**26.98**	**25.16**	++
251	29673768	**Positive**	**25.52**	**20.03**	++
252	29691520	**Positive**	**28.04**	**23.60**	+
253	29722346	**Positive**	**27.49**	**23.03**	++
254	29730156	**Positive**	**27.98**	**22.23**	++
255	29734697	**Positive**	**23.04**	**18.44**	++
256	29745074	**Positive**	**24.30**	**19.37**	++
257	29752364	**Positive**	**25.01**	**22.34**	++
258	29765216	**Positive**	**35.52**	**29.47**	–
259	30073332	**Positive**	**22.57**	**20.49**	+++
260	30089593	**Positive**	**25.90**	**23.01**	++
261	30197905	**Positive**	**25.63**	**24.46**	++
262	30278151	**Positive**	**25.59**	**24.91**	++
263	30335237	**Positive**	**27.27**	**27.42**	+
264	PS-90553018-2	**Positive**	**21.63**	**23.22**	+++
265	PS-90553018-3	**Positive**	**20.78**	**23.51**	+++
266	PS-90554836-2	**Positive**	**21.73**	**22.89**	+++
267	PS-90556367-3	**Positive**	**22.25**	**22.24**	+
268	PS-90556720-3	**Positive**	**28.86**	**29.17**	–
269	PS-90556802-4	**Positive**	**20.01**	**21.61**	+++
270	PS-90557105-2	**Positive**	**26.18**	**27.18**	++
271	PS-90557105-5	**Positive**	**27.18**	**23.19**	++
272	PS-90558818-2	**Positive**	**24.19**	**25.17**	++
273	PS-90560724-5	**Positive**	**23.57**	**24.45**	+++
274	PS-90562303-4	**Positive**	**26.04**	**28.56**	++
275	PS-90562496-2	**Positive**	**28.38**	**19.22**	+
276	PS-90562683-4	**Positive**	**26.18**	**27.63**	+
277	PS-90563044-1	**Positive**	**19.2**	**19.32**	+++
278	PS-90563136-2	**Positive**	**28.34**	**27.11**	+
279	PS-90563518-3	**Positive**	**26.18**	**28.47**	++
280	PS-90563518-4	**Positive**	**25.33**	**26.27**	++
281	PS-90563609-1	**Positive**	**24.12**	**26.18**	++
282	PS-90563609-2	**Positive**	**25.19**	**24.29**	+++

(continued)

Table 16.1 (continued)

S. No.	Sample ID	Result	Ct values		RAT result
			E GENE	N GENE	
283	PS-90563746-3	**Positive**	**18.11**	**19.72**	+++
284	PS-90564636-1	**Positive**	**29.14**	**28.13**	+
285	PS-90564636-4	**Positive**	**24.17**	**25.62**	++
286	PS-90564636-5	**Positive**	**26.17**	**26.81**	++
287	PS-90564743-2	**Positive**	**23.95**	**24.75**	+++
288	PS-90564743-3	**Positive**	**21.56**	**26.66**	++
289	PS-90564743-5	**Positive**	**18.51**	**18.17**	+++
290	PS-90565001-4	**Positive**	**29.14**	**31.97**	–
291	PS-90565027-3	**Positive**	**29.18**	**27.62**	+
292	PS-90565070-1	**Positive**	**22.93**	**23.04**	++
293	PS-90565345-3	**Positive**	**28.53**	**29.89**	+
294	PS-90565368-1	**Positive**	**26.18**	**27.99**	++
295	PS-90565368-5	**Positive**	**28.51**	**18.71**	++
296	PS-90565449-5	**Positive**	**24.06**	**24.89**	++
297	PS-90565672-1	**Positive**	**30.16**	**32.37**	–
298	PS-90565672-2	**Positive**	**22.54**	**23.36**	+++
299	PS-90565963-4	**Positive**	**22.65**	**23.12**	+++
300	PS-90566677-4	**Positive**	**18.92**	**17.69**	+++
301	PS-90566808-3	**Positive**	**21.09**	**21.61**	+++
302	PS-90566945-1	**Positive**	**22.66**	**23.51**	++
303	PS-90567175-1	**Positive**	**19.01**	**20.98**	+++
304	PS-90567175-3	**Positive**	**22.38**	**23.49**	+++
305	PS-90567347-3	**Positive**	**24.12**	**25.17**	++
306	PS-90567504-1	**Positive**	**25.62**	**24.13**	++
307	PS-90567504-3	**Positive**	**26.18**	**26.19**	++
308	PS-90567504-4	**Positive**	**27.18**	**26.72**	++
309	PS-90567813-1	**Positive**	**20.01**	**20.71**	+++
310	PS-90567922-1	**Positive**	**26.18**	**27.19**	++
311	PS-90567923-1	**Positive**	**22.72**	**23.14**	+++
312	PS-90567923-2	**Positive**	**26.27**	**27.98**	+
313	PS-90567963-3	**Positive**	**24.13**	**25.12**	++
314	PS-90568098-4	**Positive**	**20.19**	**21.11**	+++
315	PS-90568098-5	**Positive**	**25.63**	**23.17**	++

Table 16.2 Negative results both by real-time RT-PCR and rapid antigen tests from new nasopharyngeal swab samples. All data show that rapid antigen tests can detect negatives on par with real-time RT-PCR with no false positives

S. No.	Sample ID	Result
1	5348137	**Negative**
2	5348330	**Negative**
3	5350502	**Negative**
4	5351301	**Negative**
5	5351829	**Negative**
6	5352411	**Negative**
7	5352701	**Negative**
8	5354626	**Negative**
9	5354979	**Negative**
10	5364597	**Negative**
11	5386331	**Negative**
12	5387247	**Negative**
13	5388227	**Negative**
14	5388766	**Negative**
15	5389811	**Negative**
16	5405835	**Negative**
17	5406864	**Negative**
18	5406889	**Negative**
19	5407799	**Negative**
20	5408907	**Negative**
21	5409689	**Negative**
22	5410150	**Negative**
23	5411700	**Negative**
24	5412350	**Negative**
25	5413981	**Negative**
26	5415018	**Negative**
27	5415239	**Negative**
28	5479160	**Negative**
29	5479786	**Negative**
30	5481835	**Negative**
31	5482348	**Negative**
32	5483062	**Negative**
33	5483350	**Negative**
34	5483707	**Negative**
35	5485061	**Negative**
36	5485550	**Negative**
37	5488436	**Negative**
38	5488801	**Negative**
39	5489655	**Negative**

(continued)

Table 16.2 (continued)

S. No.	Sample ID	Result
40	5490013	**Negative**
41	5490368	**Negative**
42	5490726	**Negative**
43	5491094	**Negative**
44	5500021	**Negative**
45	5502143	**Negative**
46	5502745	**Negative**
47	5510071	**Negative**
48	5511628	**Negative**
49	5514011	**Negative**
50	5515486	**Negative**
51	5517179	**Negative**
52	5750027	**Negative**
53	5752839	**Negative**
54	5756075	**Negative**
55	5757820	**Negative**
56	5770447	**Negative**
57	5797585	**Negative**
58	5797797	**Negative**
59	5797981	**Negative**
60	5798228	**Negative**
61	5798331	**Negative**
62	5798692	**Negative**
63	5798784	**Negative**
64	5798998	**Negative**
65	5809266	**Negative**
66	5809329	**Negative**
67	5813631	**Negative**
68	5814955	**Negative**
69	5829807	**Negative**
70	5834756	**Negative**
71	5835581	**Negative**
72	5836225	**Negative**
73	5836841	**Negative**
74	5839668	**Negative**
75	5847311	**Negative**
76	5850627	**Negative**
77	5850957	**Negative**
78	5863154	**Negative**
79	5867115	**Negative**
80	5867638	**Negative**
81	5869593	**Negative**

(continued)

Table 16.2 (continued)

S. No.	Sample ID	Result
82	5871059	**Negative**
83	5871513	**Negative**
84	5871524	**Negative**
85	5872751	**Negative**
86	5873342	**Negative**
87	5873846	**Negative**
88	5874548	**Negative**
89	5878850	**Negative**
90	5879733	**Negative**
91	5883989	**Negative**
92	5890561	**Negative**
93	5892497	**Negative**
94	5978060	**Negative**
95	5978779	**Negative**
96	5979611	**Negative**
97	5982391	**Negative**
98	6015247	**Negative**
99	6015931	**Negative**
100	6016810	**Negative**
101	6020373	**Negative**
102	6027039	**Negative**
103	6028555	**Negative**
104	6035052	**Negative**
105	6035761	**Negative**
106	6036967	**Negative**
107	6037230	**Negative**
108	6038405	**Negative**
109	6039142	**Negative**
110	6039218	**Negative**
111	6039390	**Negative**
112	6039737	**Negative**
113	6039792	**Negative**
114	6039846	**Negative**
115	6040373	**Negative**
116	6040797	**Negative**
117	6041801	**Negative**
118	6042628	**Negative**
119	6056101	**Negative**
120	6056439	**Negative**
121	7740155	**Negative**
122	7740787	**Negative**
123	7741603	**Negative**

(continued)

Table 16.2 (continued)

S. No.	Sample ID	Result
124	7744065	**Negative**
125	7745131	**Negative**
126	7754048	**Negative**
127	7759728	**Negative**
128	7760005	**Negative**
129	7760375	**Negative**
130	7760617	**Negative**
131	7761049	**Negative**
132	7762677	**Negative**
133	7763217	**Negative**
134	7764119	**Negative**
135	7767984	**Negative**
136	7768127	**Negative**
137	7768392	**Negative**
138	7768578	**Negative**
139	7768729	**Negative**
140	7768742	**Negative**
141	7773170	**Negative**
142	7773451	**Negative**
143	7773663	**Negative**
144	7773839	**Negative**
145	7774092	**Negative**
146	7774302	**Negative**
147	7774706	**Negative**
148	7775643	**Negative**
149	7776122	**Negative**
150	7776134	**Negative**
151	7776325	**Negative**
152	7776450	**Negative**
153	7776584	**Negative**
154	7776614	**Negative**
155	7776681	**Negative**
156	7776817	**Negative**
157	7776826	**Negative**
158	7776918	**Negative**
159	7776964	**Negative**
160	7777190	**Negative**
161	7777332	**Negative**
162	7777855	**Negative**
163	7778015	**Negative**
164	7778104	**Negative**
165	7778865	**Negative**

(continued)

Table 16.2 (continued)

S. No.	Sample ID	Result
166	7778962	**Negative**
167	7779052	**Negative**
168	7779219	**Negative**
169	7779414	**Negative**
170	7779863	**Negative**
171	7785855	**Negative**
172	7786445	**Negative**
173	8529605	**Negative**
174	8529844	**Negative**
175	8531148	**Negative**
176	8545694	**Negative**
177	8547154	**Negative**
178	8550675	**Negative**
179	8553015	**Negative**
180	8553017	**Negative**
181	8553072	**Negative**
182	8553437	**Negative**
183	8554614	**Negative**
184	8560427	**Negative**
185	8561169	**Negative**
186	8561305	**Negative**
187	8574485	**Negative**
188	10044349	**Negative**
189	10045506	**Negative**
190	10048456	**Negative**
191	10051249	**Negative**
192	10051352	**Negative**
193	10051975	**Negative**
194	10052372	**Negative**
195	10053106	**Negative**
196	10053123	**Negative**
197	10053297	**Negative**
198	10053356	**Negative**
199	10053594	**Negative**
200	10053742	**Negative**
201	10484365	**Negative**
202	10484597	**Negative**
203	10484746	**Negative**
204	10484925	**Negative**
205	10485737	**Negative**
206	10486634	**Negative**
207	10486817	**Negative**

(continued)

Table 16.2 (continued)

S. No.	Sample ID	Result
208	10487331	**Negative**
209	10516958	**Negative**
210	10525060	**Negative**
211	10544199	**Negative**
212	10548343	**Negative**
213	10551978	**Negative**
214	10557415	**Negative**
215	10559285	**Negative**
216	10591101	**Negative**
217	10601022	**Negative**
218	10607637	**Negative**
219	10608457	**Negative**
220	10619200	**Negative**
221	10620503	**Negative**
222	10622824	**Negative**
223	12706487	**Negative**
224	12707697	**Negative**
225	12708171	**Negative**
226	12708910	**Negative**
227	12709742	**Negative**
228	12710350	**Negative**
229	12729863	**Negative**
230	12730013	**Negative**
231	12730201	**Negative**
232	12731774	**Negative**
233	12731916	**Negative**
234	12739410	**Negative**
235	12753670	**Negative**
236	12758113	**Negative**
237	12759091	**Negative**
238	12771257	**Negative**
239	12773309	**Negative**
240	12774446	**Negative**
241	12785010	**Negative**
242	12786841	**Negative**
243	12828059	**Negative**
244	12837397	**Negative**
245	12838472	**Negative**
246	12840709	**Negative**
247	12842294	**Negative**
248	12842732	**Negative**
249	12843406	**Negative**

(continued)

Table 16.2 (continued)

S. No.	Sample ID	Result
250	12843858	**Negative**
251	12853721	**Negative**
252	12854045	**Negative**
253	12854504	**Negative**
254	12855078	**Negative**
255	12855514	**Negative**
256	12855547	**Negative**
257	12856574	**Negative**
258	12859637	**Negative**
259	12873469	**Negative**
260	12873667	**Negative**
261	12873837	**Negative**
262	12874018	**Negative**
263	12874338	**Negative**
264	13934543	**Negative**
265	13934576	**Negative**
266	13934881	**Negative**
267	13935273	**Negative**
268	13935306	**Negative**
269	13938159	**Negative**
270	13942206	**Negative**
271	13946769	**Negative**
272	13948177	**Negative**
273	13950319	**Negative**
274	13954349	**Negative**
275	13955416	**Negative**
276	13956035	**Negative**
277	13966975	**Negative**
278	13967039	**Negative**
279	13967180	**Negative**
280	13967307	**Negative**
281	13967793	**Negative**
282	13968005	**Negative**
283	13968213	**Negative**
284	13968581	**Negative**
285	13969422	**Negative**
286	13971218	**Negative**
287	13971679	**Negative**
288	13971764	**Negative**
289	13972104	**Negative**
290	13972268	**Negative**
291	13972390	**Negative**

(continued)

Table 16.2 (continued)

S. No.	Sample ID	Result
292	13972916	**Negative**
293	13976396	**Negative**
294	13976548	**Negative**
295	13976674	**Negative**
296	13977605	**Negative**
297	13978320	**Negative**
298	13978587	**Negative**
299	13978758	**Negative**
300	13979582	**Negative**
301	13979837	**Negative**
302	13979970	**Negative**
303	13980154	**Negative**
304	13980364	**Negative**
305	13980975	**Negative**
306	14316576	**Negative**
307	14316802	**Negative**
308	14317032	**Negative**
309	14317118	**Negative**
310	14317668	**Negative**
311	14317958	**Negative**
312	14317981	**Negative**
313	14318037	**Negative**
314	14330769	**Negative**
315	14331113	**Negative**
316	14331526	**Negative**
317	14331816	**Negative**
318	14332666	**Negative**
319	14332717	**Negative**
320	14333676	**Negative**
321	14333903	**Negative**
322	14335175	**Negative**
323	14336154	**Negative**
324	14336682	**Negative**
325	14337514	**Negative**
326	14343064	**Negative**
327	14343680	**Negative**
328	14349080	**Negative**
329	14349246	**Negative**
330	14351183	**Negative**
331	14351532	**Negative**
332	14354530	**Negative**
333	14355876	**Negative**

(continued)

Table 16.2 (continued)

S. No.	Sample ID	Result
334	14356847	**Negative**
335	14366461	**Negative**
336	14366674	**Negative**
337	14369376	**Negative**
338	14375158	**Negative**
339	14375571	**Negative**
340	14626831	**Negative**
341	14647075	**Negative**
342	14664465	**Negative**
343	14670777	**Negative**
344	14671449	**Negative**
345	14699130	**Negative**
346	14699589	**Negative**
347	14701844	**Negative**
348	14703732	**Negative**
349	14705671	**Negative**
350	14708638	**Negative**
351	14709106	**Negative**
352	14712479	**Negative**
353	14717241	**Negative**
354	14718748	**Negative**
355	14719253	**Negative**
356	14720402	**Negative**
357	14720952	**Negative**
358	14722339	**Negative**
359	14723093	**Negative**
360	14736095	**Negative**
361	14736937	**Negative**
362	14737189	**Negative**
363	14737378	**Negative**
364	14738545	**Negative**
365	14739151	**Negative**
366	26482113	**Negative**
367	26488363	**Negative**
368	26491428	**Negative**
369	26492140	**Negative**
370	26492486	**Negative**
371	26493780	**Negative**
372	26494843	**Negative**
373	26501650	**Negative**
374	26502339	**Negative**
375	26505892	**Negative**

(continued)

Table 16.2 (continued)

S. No.	Sample ID	Result
376	26506525	**Negative**
377	26511388	**Negative**
378	26512125	**Negative**
379	26512601	**Negative**
380	26512991	**Negative**
381	26513440	**Negative**
382	26513804	**Negative**
383	26514335	**Negative**
384	26514648	**Negative**
385	26529470	**Negative**
386	26530799	**Negative**
387	26533883	**Negative**
388	26534852	**Negative**
389	26535005	**Negative**
390	26535567	**Negative**
391	26535619	**Negative**
392	26596453	**Negative**
393	26599446	**Negative**
394	26600066	**Negative**
395	26600987	**Negative**
396	26601749	**Negative**
397	26603321	**Negative**
398	26611246	**Negative**
399	26611822	**Negative**
400	26611880	**Negative**
401	26613019	**Negative**
402	26613410	**Negative**
403	26613758	**Negative**
404	26614104	**Negative**
405	26614450	**Negative**
406	26614750	**Negative**
407	26742778	**Negative**
408	26802560	**Negative**
409	26802749	**Negative**
410	26809619	**Negative**
411	26809824	**Negative**
412	26810000	**Negative**
413	26810139	**Negative**
414	26810670	**Negative**
415	26810906	**Negative**
416	26811096	**Negative**
417	26811278	**Negative**

(continued)

Table 16.2 (continued)

S. No.	Sample ID	Result
418	26839344	**Negative**
419	26840068	**Negative**
420	26872183	**Negative**
421	26873223	**Negative**
422	26875238	**Negative**
423	26876153	**Negative**
424	26881786	**Negative**
425	26892127	**Negative**
426	29181631	**Negative**
427	29203668	**Negative**
428	29203800	**Negative**
429	29204664	**Negative**
430	29205120	**Negative**
431	29205238	**Negative**
432	29231744	**Negative**
433	29232811	**Negative**
434	29233252	**Negative**
435	29233621	**Negative**
436	29234183	**Negative**
437	29234844	**Negative**
438	29241316	**Negative**
439	29245191	**Negative**
440	29245677	**Negative**
441	29246048	**Negative**
442	29246362	**Negative**
443	29249510	**Negative**
444	29252637	**Negative**
445	29259077	**Negative**
446	29259933	**Negative**
447	29262004	**Negative**
448	29304162	**Negative**
449	29306136	**Negative**
450	29306628	**Negative**
451	29306991	**Negative**
452	29307782	**Negative**
453	29308157	**Negative**
454	29308610	**Negative**
455	29336811	**Negative**
456	29339697	**Negative**
457	29346768	**Negative**
458	29348294	**Negative**
459	29349603	**Negative**

(continued)

Table 16.2 (continued)

S. No.	Sample ID	Result
460	29350066	**Negative**
461	29351099	**Negative**
462	29351686	**Negative**
463	29352322	**Negative**
464	29352999	**Negative**
465	29355182	**Negative**
466	29355772	**Negative**
467	29356058	**Negative**
468	29357596	**Negative**
469	29357926	**Negative**
470	29359047	**Negative**
471	29359384	**Negative**
472	29359909	**Negative**
473	29360529	**Negative**
474	29360990	**Negative**
475	29362380	**Negative**
476	29362679	**Negative**
477	29363415	**Negative**
478	29364246	**Negative**
479	29366858	**Negative**
480	29370559	**Negative**
481	29372167	**Negative**
482	29372668	**Negative**
483	29375922	**Negative**
484	29379234	**Negative**
485	29383008	**Negative**
486	29388993	**Negative**
487	29391531	**Negative**
488	29392662	**Negative**
489	29393034	**Negative**
490	29411460	**Negative**
491	29420066	**Negative**
492	29422716	**Negative**
493	29422846	**Negative**
494	29423073	**Negative**
495	29436236	**Negative**
496	29437112	**Negative**
497	29440579	**Negative**
498	29442351	**Negative**
499	29450602	**Negative**
500	29452032	**Negative**
501	29452935	**Negative**

(continued)

Table 16.2 (continued)

S. No.	Sample ID	Result
502	29453799	**Negative**
503	29454024	**Negative**
504	29454496	**Negative**
505	29455913	**Negative**
506	29467399	**Negative**
507	29468033	**Negative**
508	29469120	**Negative**
509	29470148	**Negative**
510	29471761	**Negative**
511	29476507	**Negative**
512	29478033	**Negative**
513	29478459	**Negative**
514	29478816	**Negative**
515	29478873	**Negative**
516	29479379	**Negative**
517	29481955	**Negative**
518	29484175	**Negative**
519	29489579	**Negative**
520	29489795	**Negative**
521	29490000	**Negative**
522	29490181	**Negative**
523	29493565	**Negative**
524	29493831	**Negative**
525	29493950	**Negative**

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Chapter 17
Machine Learning and COVID-19: Lessons from SARS-CoV-2

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Abstract Currently, methods in machine learning have opened a significant number of applications to construct classifiers with capacities to recognize, identify, and interpret patterns hidden in massive amounts of data. This technology has been used to solve a variety of social and health issues against coronavirus disease 2019 (COVID-19). In this chapter, we present some supervised and unsupervised machine learning techniques that have contributed in three aspects to supplying information to health authorities and diminishing the deadly effects of the current worldwide outbreak on the population. First is the identification and construction of powerful classifiers capable of predicting severe, moderate, or asymptomatic responses in COVID-19 patients starting from clinical or high-throughput technologies. Second is the identification of groups of patients with similar physiological responses to

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improve the triage classification and inform treatments. The final aspect is the combination of machine learning methods and schemes from systems biology to link associative studies with mechanistic frameworks. This chapter aims to discuss some practical applications in the use of machine learning techniques to handle data coming from social behavior and high-throughput technologies, associated with COVID-19 evolution.

Keywords COVID-19 · SARS-CoV-2 · Machine Learning · scRNASeq · Metabolome · Systems biology

1 Introduction

In December 2019, a new coronavirus emerged in the city of Wuhan in China and caused an increase in infections in the respiratory tract [1]. The virus was quickly identified and named the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes the novel coronavirus disease 2019 (COVID-19) [2, 3]. Most individuals infected with the virus will develop mild symptoms, although some (especially the elderly) may develop severe symptoms leading to hospitalization and, in some cases, death [4]. The most common symptoms are fever, cough, and shortness of breath among others [5, 6]. Individuals get infected through contact with droplets or aerosol particles expelled by carriers of the SARS-CoV-2 virus [7]. Once the virus enters the human body, it will target multi-ciliated cells in the trachea or sustentacular cells in nasal mucosa, by binding to the receptor angiotensin-converting enzyme 2 (ACE2). After anchoring to the host cell, the virus injects genomic information to replicate itself and secrete new virus particles [8–11]. The swift development and application of vaccines against COVID-19 have had a great impact on reducing deaths [12]. However, the uneven accessibility and distribution of vaccines around the world, compounded with the loss of effectiveness in the face of new variants, have made it difficult to reduce new waves of contagion [13–15]. At the time of writing this chapter (November 2022), COVID-19 is still present in more than 200 countries, causing more than 610 million infections and 6.52 million deaths.

The mechanistic explanation of how the disease overcomes the immune system and uses it to its advantage to reproduce inside the human body and how this influences the spread of the disease is still a challenge to solve. To this end, there have been unprecedented efforts in the construction of multiple mathematical models to understand not only the spread of the virus (SEIAR-type differential equations [16, 17]) but also to piece together the puzzle of how the virus responds to and affects our immune system [18–21]. Even though the SARS-CoV-2 virus has been studied extensively, we still need to develop effective tools to characterize the spread and to enhance surveillance and diagnosis of COVID-19 to help in guiding data-informed

decisions by the policymakers in public health. In this scenario, the interconnection of different scientific disciplines, such as biology, medicine, and computer science, can contribute to the identification of biomarkers for COVID-19 and the clarification of the molecular mechanisms impacted by the disease.

Artificial intelligence (AI) and, in particular, machine learning, a branch of AI, are fields of computer science devoted to the development and implementation of algorithms that allow us to learn, find patterns, and make predictions from large data sets [22, 23]. Decision-making in machine learning can be of two types. The first type uses a supervised approach to develop predictive algorithms using regression or classification methods. In contrast, the unsupervised strategy allows the computer to explore large amounts of data without classification in order to find some kind of pattern (Fig. 17.1). On the other hand, systems biology integrates computational models at the gene-scale level and high-throughput data coming from different technologies to compose a theoretical framework capable of building testable hypotheses of the regulatory mechanism in living organisms.

This chapter is divided into three sections. In the first section, we present some of the main public databases that currently hold data related to public health policies, epidemiology, social behavior, and genetic information of SARS-CoV-2. The second section is devoted to presenting some applications of machine learning techniques to identify biomarkers in different types of high-throughput data. As shown in Fig. 17.1, this chapter is mainly focused on the genome, transcriptome, metabolome, and microbiome measurements. In addition, given their relevance, we have included a section that exemplifies the impact that neural networks have on image analysis, and in the proper identification of parameters in epidemiological models. Finally, the last section is devoted to discussing some advances in the integrative description between machine learning and systems biology when studying the immunological response in the host. We expect that this chapter serves the reader as a guide to how machine learning should be implemented in clinical areas to identify biomarkers in COVID-19 patients in combination with other areas such as systems biology to unwind their molecular mechanisms. All these activities are in close relationship with ethical purposes and are guided by the principle that machine learning should benefit health in our current society, with or without being driven by a pandemic urgency.

2 Databases

Since the outbreak started in December 2019, there have been remarkable efforts to integrate epidemiological and genetic information to evaluate the social impact that SARS-CoV-2 has had on a worldwide scale. The pandemic has promoted the integration of databases that contain epidemiological, social, and health-driven policy factors, as well as serving as repositories of biological data. As a consequence of the unprecedented availability of information, there has been an explosion of machine learning models to try to understand the pandemic both from the social and

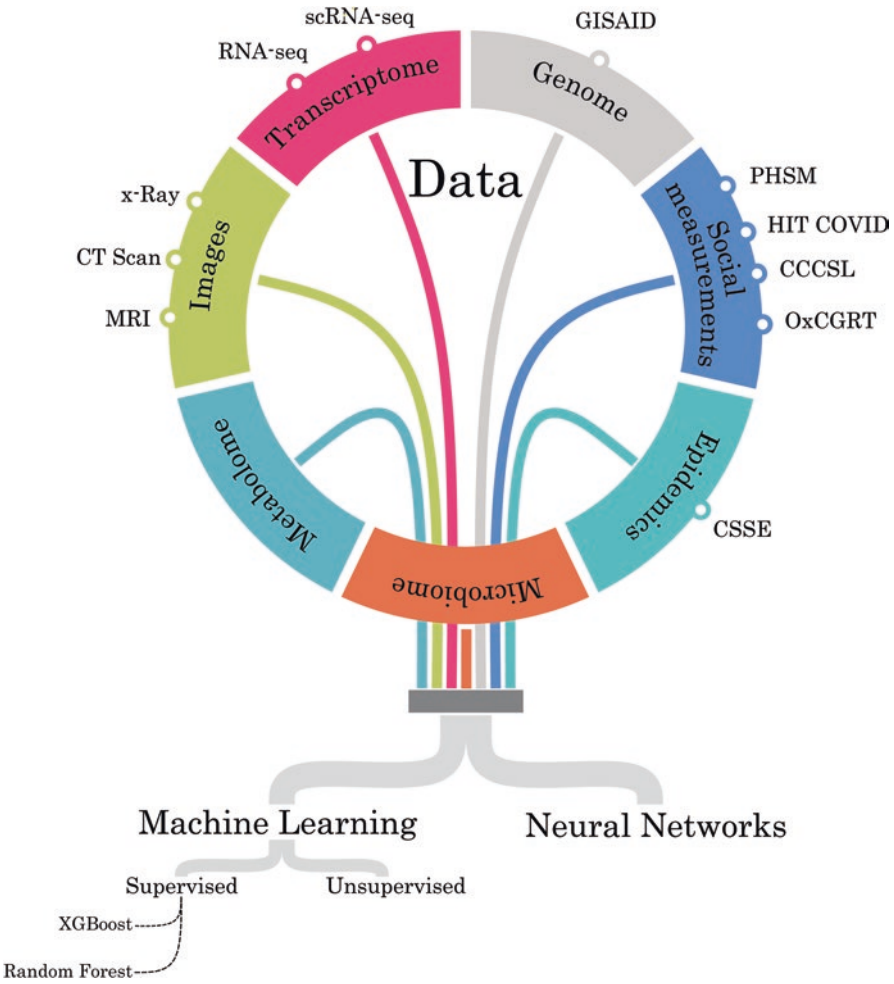

Fig. 17.1 The impact of machine learning (ML) and deep learning on studies related to health. As shown in the figure, ML and neural networks methods (supervised and unsupervised) have been extensively applied in high-throughput data to identify biomarkers associated with mild, moderate, or severe responses in COVID-19 patients. Magnetic Resonance Imaging (MRI), Computed Tomography Scan (CT Scan), Single-Cell RNAseq (scRNA-seq), Global Initiative on Sharing All Influenza Data (GISAID), Public Health and Social Measurements (PHSMs), Health Intervention Tracking for COVID-19 (HIT-COVID), Complexity Science Hub COVID-19 Control Strategies List (CCCSL), Oxford COVID-19 Government Response Tracker (OxCGRT), and COVID-19 Dashboard by the Center for Systems Science and Engineering (CSSE)

biological standpoint. Notably, these databases are a valuable source of information to monitor the progression of the disease and eventually identify strategies to reduce the spread. For instance, the Public Health and Social Measurements (PHSMs) data set was implemented through a collaborative effort between the World Health Organization (WHO) and the London School of Hygiene and Tropical Medicine

(https://www.who.int/emergencies/diseases/novel-coronavirus-2019/phsm). This database is useful for tracking the impact of a variety of illnesses around the world, including the COVID-19 breakout, and evaluating whether or not governmental decisions stopped or slowed the damaging effects on health. Furthermore, the Health Intervention Tracking for COVID-19 (HIT-COVID) project is another remarkable high-quality database that provides important public health and social parameters useful for evaluating how the disease propagates and evolves [24]. HIT-COVID is a curated and standardized global database that catalogs COVID-19-related data through PHSMs, better known as non-pharmaceutical interventions (NPIs), at different geographical positions. Thus, by tracking variables such as restriction and travel movements, social and physical distancing, surveillance and response measures, one can conclude how decisions taken by health authorities and policymakers impact the transmission of the disease. Interestingly, data from HIT-COVID can also be used to analyze historical trends in disease transmission, which will help further governments and health systems to make informed decisions when dealing with diseases and their control [24].

Continuing with the goal of learning, optimizing, and analyzing the effectiveness of the different strategies that governments have implemented against COVID-19, the Complexity Science Hub COVID-19 Control Strategies List (CCCSL) data set represents a source of consultation on the impact of NPIs from 56 countries [25]. Notably, with this publicly available data set and machine learning methods, anyone can evaluate the effectiveness of the control policies taken during the COVID-19 epidemic.

Additional projects that aim for similar goals are the Our World in Data COVID-19 vaccination [26], The Oxford COVID-19 Government Response Tracker (OxCGRT) [27], and the dashboard hosted by Johns Hopkins University [28]. The Our World in Data COVID-19 vaccination source gathers information monitoring the application of first and second doses of the vaccines in various countries. This database stores variables such as the time intervals that countries implemented between the application of the first and second dose, accessibility, time of starting the vaccination program, and aspects related to the organization and distribution of the vaccine within the population. Despite the relevance of the data, its purpose is not to evaluate the effectiveness of vaccines in controlling the pandemic, but rather to store the information of the first and the second dose for further analyses, to show the inequality in access to the vaccine, and to promote the idea that everyone should be vaccinated.

Continuing in the same line, the OxCGRT is a database that analyses the different policies that some governments are implementing in controlling the pandemic. This data set has helped researchers and public decision-makers to analyze the effects of the pandemic in the economy and on social behaviors [27]. In parallel, an online interactive dashboard, hosted by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins University, was created to visualize the cases of infected, recovered, and deceased patients from COVID-19 in real time on a worldwide level. With daily updates, this database provides a snapshot of the pandemic in most parts of the world, which is invaluable information for assessing the

current situation and for predicting future caseloads. Despite the challenge of integrating daily COVID-19 reports from tens of worldwide sources, this dashboard has been a success and operates with continuous improvements [28]. Complementary to these global strategies for scanning public health and social parameters, the genomic surveillance of SARS-CoV-2 is positioned at the frontline of the battle to halt the spread of COVID-19. One remarkable database that falls in this category is the Global Initiative on Sharing All Influenza Data (GISAID), which contains detailed genome sequences of the SARS-CoV-2 variants, and clinical/epidemiological data of affected patients around the world [29]. Overall, these and other databases contribute in at least two aspects directed at reducing the pandemic effects on the entire population. First, these databases supply health authorities and policymakers with important information to implement and compare strategies to contain the pandemic, depending on local factors such as culture, economy, and geographical context [25]. Second, genomic repositories in combination with public health surveillance data provide information that can be integrated with mathematical and computational models [25, 30]. With these aims, the proper organization of the data is the first step toward controlling the pandemic. The next step would be the analysis of the data to identify patterns of behavior and the parameters that have higher probabilities of influencing the propagation of the virus. In order to achieve this goal, machine learning has supplied different strategies (Fig. 17.1).

In addition to epidemiologic and genomic information of the different variants of SARS-CoV-2, high-throughput technologies have contributed substantially to applying machine learning methods for classifying and predicting the clinical result of patients with COVID-19. In the next sections, we discuss some machine learning applications where high-throughput technologies have been used to identify biomarkers and explore potential explanations of how SARS-CoV-2 affects human health.

3 High-Throughput Technologies in COVID-19

High-throughput technologies have opened a window to explore cellular activity at different biological levels. The massive amount of data generated from these technologies (commonly known as “omics” sciences) supports the understanding of our body as a complex system, which is integrated by a variety of components interacting in a non-linear manner. The pandemic has exacerbated the need for application of these technologies in identifying potential biomarkers associated with mild, severe, or critical response on COVID-19 patients. In combination with frameworks in systems biology, these data sets can provide a great opportunity to formulate mechanistic explanations of how SARS-CoV-2 virus could alter the normal metabolic and immunological mechanisms in the human body. With this in mind, the next section is devoted to discussion of some applications of machine learning in three omic areas applied to understanding COVID-19 disease: viral genomics, metabolomics, and microbiome profiles in the host. The main objective of the next

sections is to show the relevance and power of machine learning in the identification of biomarkers that can differentiate the distinct severity levels of COVID-19 patients, a central aim in the design of preventive strategies in this pandemic time.

3.1 Genome of SARS-CoV-2

Next-generation sequencing (NGS) technologies have been used to monitor the mutation rate of the virus in a variety of countries. Consequently, there have been remarkable efforts to integrate the information and track the different variants of SARS-CoV-2 on a worldwide scale. The GISAID database contains a detailed temporal track of most of the variants around the world [29]. This database has allowed us to apply machine learning methods to predict the fitness of SARS-CoV-2 variants taking into account the mutation in the genome and the growth rate in the pandemic. For instance, PyRo is a hierarchical Bayesian multinomial logistic regression model that has been applied to predict and emit warnings of mutations that may result in new SARS-Cov-2 waves [31]. PyRo has the ambition not only to be a program focused on dealing with SARS-CoV-2 but also other viral phenotypes. The task has some difficulties starting with the struggle to collect and organize large amounts of information. Although the goal is ambitious, it is a program that is certainly important for improvement of the health system.

3.2 Single-Cell RNASeq (sc-RNASeq)

After the characterization of SARS-CoV-2 genome, scientists started to evaluate the viral infection from different perspectives. At the transcriptional level, the efforts were focused on identifying alterations occurring inside infected cells and to classify the severity in patients according to their molecular characteristics. Single-cell RNA sequencing (sc-RNASeq) has provided transcriptional information of thousands of individual cells at the same time. In particular, sc-RNASeq analyses of bronchoalveolar samples in patients with COVID-19 allowed the description of the immunological alterations induced by the SARS-CoV-2 virus. Moreover, COVID-19 sc-RNASeq data are highly dimensional. Therefore, the use of dimensionality reduction algorithms is often applied to visualize and extract information from these complex data sets [32–35]. In addition, machine learning has been used to annotate cellular types over several COVID-19 data sets, verifying the consistency and validity of results on small cohorts to be extended as general findings [36].

A natural research question surveyed by the community is the study of the molecular changes and factors that promote/restrict patient prognoses. Algorithms based on cluster-based minimum spanning trees and deep learning have been used to study the altered transcriptional programs and the trajectories in the transition from healthy to moderately severe patients and from healthy to severe patients [37].

In terms of physiological classification, illness severity is catalogued according to the clinical manifestations. However, the symptoms alone do not provide enough information to understand the pathophysiology. Therefore, molecular characterization is necessary to elucidate the systemic condition of the patients and inform clinical management in a patient-specific manner. For instance, a classification model built using the XGBoost gradient boosting algorithm was capable of classifying moderate and severe patients with an accuracy greater than 95% in the data subset and at 98% in a new data set [38]. These types of models showed transcriptional signatures that can be used to improve treatments. Finally, it should be mentioned that SARS-CoV-2-infected tissues can be characterized through spatial transcriptomics [39]. In this manner, infected organs are assessed considering both the structure and the spatial disposition of the tissue providing a new layer of information that can help unravel the biology of the disease. Despite advancements in the field, more studies that exploit single-cell transcriptomic data with machine and deep learning algorithms are still only emerging. There is no doubt that as more studies become available, as well as more methods to interpret these data sets, we will gain a better understanding of COVID-19 mechanisms.

3.3 Metabolomics and Machine Learning

Metabolites are the direct sub-products of the reactions, processes, and functions occurring inside cells and tissues. Metabolomic studies are possible through measurement techniques such as liquid or gas chromatography coupled with mass spectrometry (LC/GC-MS) or nuclear magnetic resonance (NMR) spectroscopy. The signals obtained in this manner are then interpreted with the aid of machine learning algorithms. These algorithms can be applied in all steps of the data analysis workflow, including peak calling, normalization, metabolite identification, and interpretation via pinpointing the activated metabolic pathways [40]. Metabolomic studies are in many cases performed in unison with proteomics since they are measured using similar technologies and their combination gives a broader picture of the situation inside cells. In the specific case of emerging diseases such as COVID-19, being able to identify the activation or disruption of signaling pathways in affected individuals can provide valuable insights into the disease physiology.

The applications of machine learning and metabolomics for COVID-19 include diagnosis, clinical sample analysis, and prognosis. Early on in the pandemic, there was a great need to accurately diagnose large amounts of patients and, therefore, breath analysis tests garnered interest [41, 42]. Although some metabolites were identified, these studies suffered from small sample sizes, and diagnosis of COVID-19 in this manner did not reach the clinical stage [41]. To date, no method has replaced reverse transcription polymerase chain reaction (RT-PCR) or the less sensitive rapid antigen tests, but detection of SARS-CoV-2 from nasal-swabs metabolomics in combination with support vector machines (SVMs) has shown promising results [41, 43]. Prediction of disease severity has been attempted via

metabolite analysis from breath samples [44], nasal swabs [45], plasma [46–48], and serum [49–51]. Although several proteins and metabolites have been identified that correlate with severe COVID-19 such as cytokine storm-related molecules, Il-6, and other inflammation markers, these have proven highly variable depending on the response of the host, and none have been sufficiently specific to be used in a clinical setting.

Most of the above metabolomic studies were performed through a combination of both targeted and untargeted approaches in conjunction with machine learning algorithms for model construction. Partial least squares discriminant analysis (PLS-DA) and related techniques are the most popular methods utilized in metabolomic analyses in general, although classical machine learning algorithms such as random forests, XGboost, elastic nets, Bayesian and logistic regression, and SVMs have also been applied. The past 4 years have observed a boom in the development of deep learning methods for all steps of the metabolomics workflow [52]. Direct applications of deep learning to COVID-19 have been, for the most part, focused on the classification of chest X-rays or CT scans for diagnosis [53]. One of the few exceptions is a study that integrated proteomic, lipidomic, and metabolomic data sets for COVID-19 prognosis [54]. In this study, a total of 1463 proteins, 902 lipids, and 1018 metabolites were measured from 455 patients who had visited one of three Mayo Clinic sites in the United States. These patients were assigned one of the three severity classes recognized by the WHO: outpatient, severe, or critical. The integrated list of molecular features was fed to AutoGluon tabular prediction algorithm for automated machine learning analysis [55]. The resulting model outperformed IL-6 and cytokine storm biomarker signatures, both considered as good predictors of adverse outcomes in COVID-19. The new signature consisted of 53 proteins, 12 lipids, and 37 metabolites, including both known and novel COVID-19 biomarkers. As expected, this signature also contained IL-6 and several cytokines from the cytokine panel as well as several markers of immune response that have been previously associated with COVID-19. The model also identified proteins related to inflammation and apoptosis pathways that have not been previously reported in the context of COVID-19. This application of deep learning in omic data sets showed that higher specificity can be achieved, although the caveat is that large signatures such as the one identified by AutoGluon of 102 analytes are harder to implement in clinical settings where only a few biomarkers can be tested [56]. Despite the drawbacks, and considering that the mechanisms behind mild and severe COVID-19 remain as one of the most baffling and important questions from the pandemic, the use of robust methods that can help us unravel hidden patterns behind COVID-19 progression is of utmost importance.

3.4 Microbiome and Machine Learning

The human microbiota comprises the microorganisms (bacteria, archaea, fungi, and viruses) that reside in the human body. Almost 90 trillion microorganisms colonize the skin and mucosal epithelia in the body [57]. In addition, each person has a

unique microbiota profile according to anatomical body site, with many beneficial functions in the host metabolism, such as modulation of the immune response, maintenance of the epithelial barrier, and defence against potential pathogens [58]. Accordingly, the gut and respiratory microbiota are essential to induce and maintain an adequate immune response against respiratory infections, including those caused by the SARS-CoV-2 virus. Therefore, an alteration in the microbiota structure and function is associated with the progression of COVID-19 patients [59]. Therefore, uncovering the changes in the microbiota composition from severe COVID-19 patients might help to set new biomarkers and develop microbiota-based therapies. However, microbiota data analysis is complex and can suffer from many problems, such as high dimensionality, sparsity, and inherent data [60]. At this point, machine learning can serve as an appealing strategy to overcome these challenges and identify bacteria associated with the clinical manifestation of the disease. In this way, machine learning models in combination with conventional statistical analyses can contribute to the identification of patterns of microbial communities in distinguishing patients with COVID-19 from healthy individuals. Currently, the most commonly machine learning supervised methods used in microbiome studies are SVM, random forest, XGBoost, and artificial neural networks, with variable predictive accuracy [61].

Generally, there are two principal approaches for microbiome characterization using high-throughput technologies: amplicon sequencing of the 16S rRNA gene and whole-metagenome shotgun (WMS) gene sequencing [62]. Amplicon sequencing uses a marker gene (commonly 16S rRNA) to count the microbes and achieve taxonomic classification using bioinformatics tools. In contrast, WMS surveys all accessible DNA present in the sample to characterize the microbiome profile with better resolution at the strain or species levels than those based on 16S marker genes. In this way, WMS studies allow us to infer the functional profile in the microbial community [63]. Both 16S rRNA and WMS have been widely used to characterize the microbiome profile and provide meaningful information to machine learning models for predicting human diseases and uncovering their potential host microbial signatures [64].

Supervised machine learning algorithms have been successful in solving regression and classification tasks. Specifically, in COVID-19 patients, this method was shown high potential in the use of gut microbiota as a diagnostic tool to detect the disease [65]. In this study, Ke et al. characterized the gut microbiome profile inferring the metagenome-assembled genomes (MAGs) from WMS sequencing data of faecal samples in two different data sets. The first data set had 50 patients with COVID-19 and 15 non-COVID-19 subjects as controls. The second data set had 195 patients with COVID-19 and 78 non-COVID-19 controls. Remarkably, using the MAGs of gut microbiota profiles as input, a random forest algorithm was used to accurately classify COVID-19 patients versus healthy controls with receiver operating characteristic area under the curve (ROC-AUC) scores of about 0.98 and 0.92 (using a fivefold CV technique) in the two independent cohorts, respectively. Through this machine learning interpretation analysis, this study was also able to obtain some important genera to predict COVID-19 disease status. Among the most

important elements to classify the patients, they reported genera such as *Blautia* and *Faecalibacterium prausnitzii* [65]. Interestingly, these taxa are well-known short fatty chain acids (SCFAs) producers. In agreement with this finding, other articles described a fall in microbiome SCFA producers in severe COVID-19 patients [66]. Production of SCFAs, such as butyrate, propionate, and acetate, has positive effects on human metabolism as well as anti-inflammatory properties. Also, butyrate is essential to maintain a healthy epithelial barrier and prevent the translocation of toxins (e.g., lipopolysaccharides [LPS]) and opportunistic pathogens. These toxins and microbes are potential signals that can trigger the immune system and induce a pro-inflammatory response. Surprisingly, inadequate production of SCFAs is correlated with IL-6 serum concentrations and may play a role in the progression to severe COVID-19 outcomes [66].

Nonsupervised clustering techniques have also been helpful in the stratification of individuals according to their microbiome profiles. Specifically, different enterotypes can be identified in group-specific populations using the gut microbiome. For example, a study that analyzed the gut microbiome profile from 953 patients and ten countries (UK, Canada, Japan, Mexico, and others) used an unsupervised cluster algorithm called LIGER, which showed that the profiles in the entire data set could be classified into five unique enterotypes (denoted from 1 to 5) [67]. Notably, the authors found that these enterotypes were associated significantly with an increase in the COVID-19-related mortality rate in ascending order from groups 1 to 5. Moreover, some genera, such as *Collinsella*, were proposed as essential taxa in this study because this genus was the most highly correlated with mortality rate, as determined using a generalized linear model (GML). Interestingly, the genus *Collinsella* showed a steady decline in abundance levels from enterotype groups 1 to 5 and was, therefore, inversely correlated with mortality rate. From a metabolic point of view, there is evidence that *Collinsella* produces deoxycholic acid (DCA), a metabolite of secondary bile acids, and also SCFAs. Recently, DCA was shown to have COVID-19-relevant functions, such as suppression of proinflammatory cytokines (e.g., IL-1, IL-6, and TNF-alpha), as well as antiapoptotic and antioxidant effects, and in promotion of clearance of alveolar liquids from the lungs [67]. Despite these findings, the mechanisms by which the microbiome can alter the immune and metabolic systems in the host are still under investigation [38].

4 Neural Networks on COVID-19

4.1 Neural Networks in ODE

Throughout the pandemic, the use of computational methods to forecast the spread of the virus has been of paramount importance. Notably, these methods have been effective in predicting the trajectory of the pandemic based on the enormous amount of data collected by each country regarding the number of individuals who were

infected, died, or recuperated [28], as well as the number and type of vaccines applied in each country [26]. In order to carry out these prospective projections, three types of mathematical/computational tools have been used: machine learning, compartmental models (susceptible-infected-recover [SIR]), and hybrids that mix both machine learning and SIR schemes. There are a large number of mathematical models that use machine learning methods trained with infection, death, and recovery data. Among these, the most popular for the study of COVID-19 data are SVM, random forests, and autoregressive integrated moving average (ARIMA). SVMs and random forests are tools to solve classification [68–72] and regression problems, a necessary first to make a forecast. For example, Parbat et al. estimated the total number of expected cases and deaths based on the pattern obtained in a time series data through an SVM analysis [73]. Along the same line, Yadav et al. forecasted with high efficiency the spread of the virus and presented an approximation of the mitigation strategies to acknowledge their importance at the beginning of the pandemic [74]. Two complementary studies compared multiple machine learning methods to evaluate which one was better for predicting short-term forecasts in Brazil and USA [75, 76]. Both studies concluded that the SVM approach had the highest efficiency in predicting future COVID-19 cases in these countries. However, when a random forest analysis was combined with an estimation algorithm (e.g., Kalman Filter), the hybrid model showed a higher efficiency in forecasting COVID-19 cases [77]. In another application using a random forest technique, instead of using time series data, the authors obtained a prediction based on the test positivity rate and effective reproduction number estimated from the USA population [78]. In this model, they concluded that the random forest analysis maintained good performance in forecasting the behavior of the pandemic.

ARIMA is the most frequently used machine learning strategy to forecast regarding COVID-19 cases, despite its limitations in not capturing non-linearities in time series data. This approach has been applied to the Johns Hopkins epidemiological data [79] and in countries like Kuwait, Spain, India, France, Italy, among others [80–84]. In all of these studies, the application of the ARIMA method resulted in a good performance for short-term predictions, a valuable result that can be used to make public health-informed decisions. SIR models have been heavily used in predicting case, death, and hospitalization spikes; however, their main limitation is that they are highly dependent on the accuracy of their assumptions. Since these models are beyond the scope of this chapter, we recommend the following extensive reviews on this subject [85, 86]. The most interesting models are the hybrids, which combine the long-term forecasting and theoretical capabilities of compartmental models with methods capable of predicting and elucidating patterns in the data. This type of model has been applied to anticipate the future behaviour of the pandemic in countries like China, USA, Switzerland, Bangladesh, among others [87–91]. Most of the hybrid models discussed here used a modified SIR approach and multiple neural networks for their forecasts.

During the pandemic, most countries focused their attention on a number of key coping practices such as never exceeding hospital bed capacity, estimating the real-time world toll of positive infections of COVID-19, and quarantining individuals who

were infected. One study developed a modification of a SIR approach to incorporate risk parameters based on where the individuals work: from home (low contact) and onsite (high contact) [89]. The model was trained using hospitalized data, and this led to the prediction of the number of intensive care unit (ICU) beds that would eventually be needed based on a 7-day forecast. Additionally, they assessed the importance of how augmentative reopening policies would affect hospitalization and death rates [89]. A hybrid neural-susceptible-exposed-infectious-removed (SEIR) network model mixed with a feedforward neural (FFL) network and trained with data from Switzerland was shown to have a better fit compared to only-SEIR and only-FFL in predicting the increased need of ICU beds [92]. By incorporating machine learning methods with SEIR, it is possible to consider covariates such as the proportion of positive particular SARS-CoV-2 variant and use of NPIs, which can affect the pattern of hospitalization [92]. The latter study used NPIs as a covariate, although this did not predict the number of individuals that needed to be quarantined due to testing positive for the SARS-CoV-2 virus. In another study, a hybrid model was derived and trained with recurrent neural networks [88]. This resulted in a quantifiable number of individuals that needed to be quarantined to prevent a higher spread of the virus. A similar hybrid approach demonstrated the importance of quarantine policy strategies to prevent the spread in European countries and the USA [93]. To improve the model, they considered the effects of the socioeconomic landscape and the relationship with mortality as a covariate [93]. Another study used the ARIMA method to obtain the parameters to visualize the dynamics of a modified SIR mathematical model in making a two-month forecast to guide preparedness of hospital resources, such as the availability of ICU beds [94]. Another important variable that none of the previous models considered is that humans are active and tend to mobilize to one place and another. Rahmadani et al. built a hybrid model capable of taking into account the mobility in comparison to results obtained from deep neural network and long-short-term memory network (LSTM) approaches [95]. Both machine learning methods had the ability to accurately parametrize the SIR-type model, compared with the common statistical methods (e.g., the sum of errors squared). By including mobilization in the model, they described the shape of the pattern of transmission between geographical regions, which may give insights into how this virus became a pandemic. A hybrid model that used LSTM and trained with data from Wuhan demonstrated the importance of disease heterogeneity, in that not all individuals who test positive for the virus are affected in the same way [91]. By taking infection rate as a variable, they demonstrated that infections act more as a pulse than as a daily infection rate. This meant that the number of susceptible individuals decreases at different rates instead of at a daily constant rate.

4.2 Deep Learning Applied in Image Classification

Despite being the most accurate molecular test and the one with the capability to detect patients not experiencing symptoms, the use of RT-PCR complicates the diagnosis of COVID-19 due to the need for specialized lab equipment and trained

professionals. Another drawback is that COVID-19 is rapidly spread and RT-PCR analysis is a time-consuming process. Other techniques used to determine the severity of COVID-19 is the use lung imaging from X-ray and computerized tomography (CT) scans [96]. In addition, magnetic resonance imaging (MRI) is a radiation-free approach that can help evaluate COVID-19 status in children and pregnant women [97]. In these imaging approaches, CT is the method of choice because the resulting images can convey a detailed information regarding the infected area [98]. One limitation of using medical images is the need for specialized knowledge of the disease by the medical staff. Medical imaging along with deep learning algorithms are valuable tools that yield faster and more accurate diagnoses. In these approaches, patient classification is based on pattern recognition using deep learning models. A widely used approach for this is the convolutional neural network (CNN), which consists of an input layer, interconnected hidden layers, and an output layer. These approaches use few pre-processing steps and the network filter parameters are optimized through automated learning. Nevertheless, CNNs are prone to overfitting due to each node being connected to all the nodes in the next layer.

In terms of the model implementation, the data are separated into three subsets: training, validation, and testing. The training data set is the biggest partition and is used to train the model to perform the classification. The validation subset helps to monitor model performance and fine-tune the hyper-parameters. Lastly, the testing subset is used to evaluate model accuracy. Considering that COVID-19 is a recent pandemic, the available data sets are still insufficient to train models [99]. To deal with the lack of inadequately sized data sets, two main approaches can be used. First, data augmentation can be performed by virtually increasing the number of images by making slight changes such as rotation, noise addition, flipping, and mixing. As a result, the accuracy of such models has been shown to increase above 95% [98, 100–104]. Second, transfer learning can be applied here, which essentially consists of the use of pre-trained models on a sufficient amount of data for a specific task, for then used it on a new problem. Successful integration of transferred models to COVID-19 include ResNet50 [105], MobileNet [106], and Inception [107], to name a few (a more detailed review can be found in [108]). Although several implementations have high accuracy, Ahsan et al. [109] evaluated the performance of pre-trained models and obtained 100% accuracy for VGG-16 and MobileNet V2 in identifying COVID-19 patients using X-ray images. In addition, multilayer perceptron neural network obtained an accuracy within the Kaggle data set of 99.26% and 99.7% using the chest X-ray COVID-19 GitHub images [110]. As for CT images, the CovTANet hybrid neural network has been used for COVID-19 severity at an accuracy of 95.8% [111]. Despite the need to increase the number of images to train the models, these works demonstrate a plausible implementation strategy for AI patient classification to improve medical care.

5 Towards Construction of Mechanistic Explanations: When Systems Biology Meets Machine Learning

As described in the previous sections, AI and machine learning methods are technologies that can have a positive impact on health programs, such as the pandemic caused by the SARS-CoV-2 virus. However, machine learning per se is not enough to elucidate molecular mechanisms that explain the observed associations in the data. To understand the patterns hidden in the data, and move towards more comprehensible and understandable theories, other sciences such as systems biology are needed. In this last section, we focus our attention on presenting and discussing the two specific cases of the immune system and the metabolic activity of the microbiota. The combination of systems biology and machine learning can serve as a powerful strategy not only to identify relevant variables in prospective studies of diseases but also to build hypotheses around those associations. It is anticipated that this synergistic approach will contribute to developing the basis of precision medicine for the translation of tangible benefits to the population.

5.1 Regulatory Mechanisms of Immunological Cells

Our immune system is the primary defence once the physical barriers such as the skin are overcome by pathogens. Briefly, the immune system is divided into two types of response: innate (rapid response) and adaptive (slow response). Once the SARS-CoV-2 virus enters the host, the innate response can recognize the virus because of the existence of pathogen associated molecular patterns (PAMPs). The PAMPs will eventually activate transcription factors leading to the secretion of type 1 interferons (IFN-1s), which are the first line of defence against a viral infection. However, the virus uses its assortment of proteins to inhibit the secretion of IFN-1 and avoid being eliminated by the innate system and forcing activation of the adaptive response. The combination of the innate, and adaptive immune responses, and proliferation of the virus leads to a dysregulation and a continuous secretion of pro-inflammatory cytokines, which manifests as the cytokine storm. To fully understand the pathogenesis of this virus, we need to analyze data on the immune response to the virus in the lungs and circulating blood. To accomplish this, we need machine learning methods to characterize this complex response mechanism.

Lie et al. [112] used two methods of reduction on single-cell data focused on six types of immune cells: B cells, CD4+ T and CD8+ T cells, dendritic cells (DCs), natural killer cells (NKCs) and monocytes. The output of the reduction was a reference list to train and compare random forest and decision tree (DT) classification machine learning methods. All of the genes found by the authors were associated with enhancing a dysregulated immune response and a reduction in viral elimination. The cells that comprise the adaptive response (B cells, CD4+ and CD8+ T cells) have the ability to reduce the clearance of SARS-CoV-2 the IFN-1 pathway.

However, the virus can create a microenvironment that depletes the necessary T cells in the lungs. While some cells participating in the innate response (DCs, NKCs, and monocytes) are associated with enhanced secretion of pro-inflammatory cytokines implicated in the cytokine storm effect and chemokine secretion to recruit more monocytes/macrophages in the lungs. In addition, Chen et al. used the same protocol using a reduction followed by a classification method for analysis of the same types of immune cells [113]. In severe COVID-19 cases, B cell discriminative genes are implicated in the maturation and survival in an inflamed lung. Consequently, they are unable to function correctly. Meanwhile, CD4+ T cells are associated with the secretion of pro-inflammatory cytokines because the virus has the ability to infect this cell type. Cells that form part of the innate immune system and their discriminative genes are implicated in the secretion of cytokines and the regulation of the immune response towards a hyper-inflammatory lung.

The previous articles discussed in this section were focused on trying to describe the genes associated with a specific response from six types of immune cells with emphasis placed on the extensive inflammation response. The following articles use machine learning methods on single-cell data to visualize the immune profiles based on the severity of the disease. One study used a random forest classifier to identify transcriptional signatures and their association with disease severity and death [114]. This study evaluated immune cell types like monocytes, DC T and B cells, and trained the machine learning methods with transcriptome cell-specific data. They found that monocytes were the cell type with the highest predictive power, and the identified immune genes were implicated in the regulation of IL-6 pathway. Specifically, individuals who survived had a positive regulation of IL-6 at the beginning of the infection. The genes of the B cells and DC were associated with survival, activation, antigen-presentation capacity, and interferon response. Meanwhile, T cell immune profiles were associated with fatal outcomes, meaning that when COVID-19 was severe, it was associated with a depletion of T cells.

In another study, the same random forest classifier was used to separate immune cell behavior as well as the difference in cytokine expression [115]. IL-6 and IL-10 are cytokines associated with disease severity and implicated in enhancing the cytokine storm. IL-10 has the capacity to regulate T cell behavior by inducing T cell exhaustion, a trademark of COVID-19, as well as the ability to activate regulatory T cells to evade an immune antiviral response. The levels of IFN-γ and IL-2 were found to be high in severe COVID-19 cases, and this combination has been implicated in the activation of T cells in the pro-inflammatory response related to the cytokine storm. High levels of IL-2 are associated with B cell activation and thereby blocking a proper humoral response. Accordingly, the expression of cytokines in severe COVID-19 cases is of high importance because these molecules have the ability to modify the behavior of the immune system, creating a feedback between cytokines and immune cells to favour viral growth. At the same time, Mueller et al. characterized blood profiles to define three types of immune-related phenotypes based on cytokine expression [116]. These were a balanced response (BRI), excessive inflammation (EXI) and low antibody (LAI) immunotypes [116]. EXI and LAI were associated with hospitalized patients who worsened with time. EXI has been

implicated with an uncontrolled secretion of cytokines associated with the cytokine storm, and LAI has been linked with a delay in the adaptive immune system and IFN-1 response. Therefore, both of these immunotypes support viral replication. Patients with BRI did not worsen and survived, because of a controlled immune response, timed to eliminate viral replication.

Another study applied RefMap, a machine learning method that allows prioritization of genes by integrating genome-wide association studies (GWAS) and identified the involvement of NKs in the severity of COVID-19 [117]. To be exact, the lack of maturation of these cells affects the immune response, therefore resulting in the development of more severe symptoms. One limitation of this work is that they only used data from European patients. Another study, which used a random forest approach to classify metabolic changes in plasma in association with disease severity, found that five metabolites predicted the survival of COVID-19 patients who were hospitalized [118]. To train the random forest, they used data not only from plasma, but also from specific immune cells.

Most of the studies that mixed single-cell data and machine learning have been aimed at developing a classification based on gene expression changes in lungs infected with SARS-CoV-2. One study used a machine learning method called a slingshot algorithm and then a deep learning method called DrivAER [37]. This study only described the transcriptional behavior of macrophages and T cells because these were the most abundant cell types in their sample. They found that T cells were present when an individual shifted from healthy to moderate (H to M) and macrophages from healthy to severe (H to S). This suggested that T cells from the H to M group were involved in eliminating the virus, whereas those in the H to S group may be associated with the depletion of T cells. Macrophages from H to S samples resembled a pro-inflammatory and recruiting behavior, correlated with the enhancement of the cytokine storm.

5.2 Microbiome and COVID-19

As described in Sect. 3.4, the microbiome has been a new target to identify possible bacteria that can differentiate patients with severe or moderate responses of COVID-19. The advent of genome-scale metabolic reconstructions has made it possible to simulate the metabolic activities of the microbiome and link these with different metabolic diseases such as type 2 diabetes or cancer. Most of these strategies start with genome-scale metabolic reconstruction of the bacteria identified with 16S or metagenome measurement of a patient and define the dietary macronutrients. After this, the metabolic activity of the community is simulated through constraint-based modeling, which includes algorithms such as flux balance analysis (FBA), flux variability analysis (FVA), or metabolic control theory. To this end, a special case is the microbial community (MICOM) software, which has the capability to simultaneously calculate the flux in the metabolic reconstructions for almost 840 bacteria constrained by a specific diet in the patient [119]. Despite the high

number of variables, computational modeling of metabolic activity in gut microbiota has been successfully applied in some cases. For instance, in colon cancer, the model has been able to suggest that hydrogen sulfide is a metabolite with relevant abundance to identify normal from cancer tissues in 106 patients, a result that was in agreement with target metabolome assessment [120]. In addition, given that the MICOM contains the metabolic detail of the bacteria, by tracking the metabolic activity associated with the cancer region, the authors were able to identify those bacteria that produce this and are therefore potentially associated with the malignant phenotype. Notably, MICOM was able to identify *Clostridium perfringens* as a main producer of hydrogen sulphide. Su et al. described a machine-learning multiclass microbiome-based model using faecal samples from over two thousand individuals with distinct diseases, including COVID-19 syndrome [121]. Their trained model resulted in a sensitivity of 0.95 and specificity of 0.92 for the identification of post-COVID-19 syndrome. Based on such studies, we suggest that the combined application of methods in machine learning and systems biology can be used to create testable hypotheses that contribute to our understanding of how complex diseases such as COVID-19 can affect the host at a systemic level.

6 Conclusions and Future Perspectives

Machine learning is a disruptive technology that can be used to replace old ways of deciphering complex systems. In the pandemic situation that we live in, open databases that include social parameters for evaluating both the spread of the COVID-19 and the efficacy of the implemented policies are a necessity to improve preventive strategies around the world. In this light, the advent of high-throughput data and systems biology approaches has opened a window for exploring the mechanism of how SARS-CoV-2 affects the metabolism and immunological systems in the host. Together, both fields of research are useful to design optimal strategies to contain the outbreak, understand the effect on the human body, and eventually contribute to the design of effective treatments for the wellness of the human population. In this chapter, we have described some applications of omics and machine learning both at the population and clinical levels with this end in mind. Our list of references is not exhaustive, and we hope that further advances in machine learning and systems biology can be used to help us to manage the current and future pandemics more effectively. There are still some challenges to be addressed, not only in the technical sense but also at the social and ethical levels. One of the most prominent challenges is the need for a clear regulation of rights in the data sets and a prompt distribution of the benefits that this technology can generate.

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Part V
Omic Approaches in the Development of New Treatments and Vaccines for COVID-19

Chapter 18
The Relationship Between Psoriasis, COVID-19 Infection and Vaccination During Treatment of Patients

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Abstract Since the outbreak of the COVID-19 pandemic in December 2019, scientists worldwide have been looking for a way to control this global threat. One of the most successful and practical solutions has been the development and worldwide distribution of the COVID-19 vaccines. However, in a small percentage of cases, vaccination can lead to de novo development or exacerbation of immune or inflammatory conditions such as psoriasis. Due to the immunomodulatory nature of this disease, people affected by psoriasis and other related skin conditions have been encouraged to receive COVID-19 vaccines, which are immunomodulatory by nature. As such, dermatological reactions are possible in these patients, and cases of onset, exacerbation or change in the type of psoriasis have been observed in patients

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administered with COVID-19 vaccines. Considering the rarity and minor nature of some of these cutaneous reactions to COVID-19 vaccination, there is a general consensus that the benefits of vaccination outweigh the potential risks of experiencing such side effects. Nevertheless, healthcare workers who administer vaccines should be made aware of the potential risks and advise recipients accordingly. Furthermore, we suggest careful monitoring for potentially deleterious autoimmune and hyperinflammatory responses using point-of-care biomarker monitoring.

Keywords COVID-19 · SARS-CoV-2 · Infection · Vaccination · Psoriasis

1 Introduction

The pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first reported in December 2019. This is the third coronavirus transmitted from zoonotic species to humans after the H1N1 influenza outbreak in 2016 [1]. COVID-19 is associated chiefly with self-limiting upper respiratory tract infections. However, a small but significant proportion of patients develop acute respiratory distress syndrome (ARDS), which cannot be treated effectively and may increase risk of death [2]. In cases of severe COVID-19, the host immune system appears to respond excessively, producing a damaging hyperinflammatory response known as a cytokine storm [3].

Potentially the most effective way of halting the spread of viral infections is through the development and deployment of approved vaccines. As of October 18, 2022, more than 68% of the world population have been administered at least one

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dose of an approved COVID-19 vaccine [4]. However, the rate of vaccination has declined [4], and there is still a significant proportion of populations around the world that show vaccine hesitancy [5]. One factor that may affect public confidence in vaccine uptake is the potential of long- and short-term adverse effects [6–8]. Although uncommon, some cases of new-onset psoriasis and exacerbation of existing ones have been reported around the world, following a COVID-19 vaccination [9–14]. However, it should be noted that both new psoriasis cases and exacerbation of existing ones were reported even before rollout of the COVID-19 vaccines [15–18]. This suggests the involvement of a common underlying mechanism in psoriasis, COVID-19 infection and vaccination. The most likely link is through immune and inflammatory pathway modulation as all of these work as a result of effects on these systems, including through activation of autoimmune mechanisms [19–23].

In this study, we have evaluated studies on the effect of various COVID-19 vaccines as a potential causative factor in the de novo appearance or exacerbation of psoriasis in the individuals who received these. We also make recommendations on how to deal with this potential issue while at the same time maintaining an effective vaccination approach.

2 Methods

Data were collected from papers published in PubMed, Scopus, Google Scholar and Cochrane library for Clinical Studies. This required that the papers were published in English up to January, 2022. Search terms included “psoriasis” OR “dermatological reactions” AND “COVID-19” OR “COVID-19 vaccines” AND “adverse reaction” OR “side effects” AND “immunological response.”

3 Psoriasis and Correlation with COVID-19

Psoriasis is an inflammatory skin disease that affects 0.09–11.43% of people in different countries around the world [24]. Psoriasis patients may be prescribed systemic immunomodulatory or immunosuppressive treatments depending on location and severity of the lesions or if they are resistant to topical treatments [25]. However, these therapies have been associated with the increased risk of infections, and there has been considerable controversy regarding the potential of increased susceptibility of psoriasis patients on such treatments to COVID-19 infections and/or a more serious disease course [26–31]. In this section, we review some of the relevant studies addressing this controversy.

Kara Polat et al. found no difference in the incidence, length of hospital stay, intensive care unit (ICU) admittance or death outcomes in psoriasis patients on immunosuppressive or biologic treatments with COVID-19 infections compared to those who had not been treated with these compounds [32]. None of the other tested potential risk factors that were assessed had an influence on COVID-19 disease trajectory apart from the presence of diabetes. However, there was an exacerbation of psoriasis with COVID-19 infections.

A retrospective multicentre study in Italy of 5206 patients with chronic plaque psoriasis on biological therapies found no deaths from COVID-19 and four hospitalizations for COVID-related interstitial pneumonia, which did not differ from the general population [33]. However, the authors acknowledged limitations due to the lack of standardization of the control group.

Carugno et al. evaluated 159 psoriasis patients during the first 45 days of the COVID-19 pandemic in the Lombardy region of Italy for SARS-CoV-2 infections [34]. They found no serious cases of COVID-19 and no difference in patients who continued or did not continue their psoriasis treatment.

A case–control study performed in 2020 by Damiani et al. of 1193 psoriasis patients in Lombardy receiving small molecules or biological drugs found that 22 of these tested positive for COVID-19, with 5 of these being hospitalized and none admitted to the ICU or who died [35]. In comparison to the general population, the researchers found that patients were at higher risk to test positive for COVID-19 and hospitalized. These findings suggested that treatment with biologic or immunosuppressive therapeutics may increase the risk of contracting mild forms of COVID-19 disease.

Other studies showed de novo or exacerbation of psoriasis in COVID-19 cases who were not on immunosuppressive therapies. A case study of a 38-year-old man with a single psoriatic plaque but who had received no treatment for this condition was diagnosed with COVID-19 infection after a nasopharyngeal test [36]. Six days after the onset of COVID-19 symptoms, several psoriatic lesions formed on his knee with no improvement after 22 days. After this, treatment with topical betamethasone cream led to significant clinical improvement after 2 weeks. Another case report of a 25-year-old male diagnosed with a COVID-19 infection developed multiple psoriatic lesions 15 days later [37]. As above, treatment with topical betamethasone led to recovery.

Taken together, these studies provide no evidence that biologic or immunosuppressive treatments increase the risk of COVID-19 infection or severity of disease course. However, they do suggest that COVID-19 disease can lead to de novo eruptions or exacerbations of existing psoriatic lesions. This was supported by a study covering the first (February 15, 2020 to June 30, 2020) and second (October 1, 2020 to January 31, 2021) waves of the pandemic in France [38]. This investigation found that COVID-19 patients who had received systemic treatments for psoriasis did not show an increased risk of in-hospital mortality due to COVID-19 infection.

4 Efficacy and Safety of COVID-19 Vaccines in Patients with Psoriasis

Wack et al. reviewed the evidence related to COVID-19 vaccine safety and efficacy in patients with immune-mediated inflammatory diseases [39]. They found no evidence to support the point that these patients are at a higher risk of harmful side effects from a COVID-19 vaccination compared to healthy controls. However, they could not determine if patients on biologics or immunosuppresants produce a sufficient immune response to the vaccine, as this may depend on the specific indication and therapeutic employed.

A study conducted by Geisen et al. showed that SARS-CoV-2 mRNA vaccines produce antibodies with neutralizing activity in healthy controls as well as in patients who were on immunosuppressant therapies for chronic inflammatory conditions [40]. However, the immunoglobulin G (IgG) titres were significantly lower in the immunosuppressant-treated patients compared to controls. It should be noted that vaccination did not lead to significant side effects or disease flare-ups in the immunosuppressed group.

Along the same lines, another study found that patients with immune-mediated inflammatory diseases who received the Pfizer-BioNTech mRNA vaccine produced slower antibody responses compared to the control group, and a higher proportion of these patients showed no detectable response [41]. Furthermore, those patients with immune-mediated inflammatory diseases who had not been treated showed a similar diminished response, suggesting that this effect may be linked to the disease rather than to a treatment effect.

Skroza et al. evaluated the safety of COVID-19 vaccination in psioriasis patients who had received biological or immunosuppressive treatment for at least 24 weeks [42]. The study found that all patients showed a similar reduction in their psoriasis area severity index scores, and this did not differ between vaccinated and non-vaccinated individuals. In addition, no adverse effects were detected in either group.

In another study, Damiani et al. evaluated four psoriatic cases who took biological or immunomodulatory medications and received two doses of the Pfizer-BioNTech vaccine [43]. This showed that none of the patients showed changes in cutaneous manifestations or a psoriasis flare up. Furthermore, all patients showed an effective response to the vaccine.

In order to promote optimal treatment of patients with psoriasis during the pandemic, the National Psoriasis Foundation COVID-19 Task Force guideline has proposed that patients with psoriasis should receive their COVID-19 vaccine in the shortest possible time while continuing with their biological or immunomodulatory treatments drugs [44]. However, this proposal stipulates that the ultimate judgement should be made by the treating clinician and the patient due to variability of psoriatic diseases and the medications used to treat them.

5 Psoriasis After COVID-19 Vaccination

5.1 COVID-19 Vaccination Leading to De Novo Psoriasis

A number of studies have reported on cases of individuals who developed different forms of psoriasis for the first time after receiving a COVID-19 vaccine (see Table 18.1). This includes de novo psoriasis cases following the first [46, 46] or second [46] dose of Oxford-AstraZeneca vaccine. In addition, there have been reports of new psoriasis eruptions following the first [47, 48] or second [49] dose of the Pfizer-BioNTech vaccine. Although the mechanism for these spontaneous eruptions is not clear, it is possible that it is linked to dysregulation of immune system due to the virus or vaccine components, as proposed by Gunes et all for other vaccines such as influenza, BCG and tetanus-diphtheria vaccines [50]. In addition, mRNA vaccines such as Pfizer-BioNTech vaccine can lead to increased levels of interleukin 6 (IL-6) and Th17 cell activation, which are known to be involved in the pathological mechanism of psoriasis [51, 52]. Even though these cases of de novo medical professionals are still advised to pay close attention to side effects and take appropriate measures in the treatment of the clinical condition on a case-by-case basis.

5.2 COVID-19 Vaccinations Which Exacerbates Psoriasis

5.2.1 Pfizer-BioNTech mRNA Vaccine

A number of studies have reported on exacerbations or flare-ups of psoriasis that may be linked to vaccination with the Pfizer-BioNTech mRNA vaccine: Durmaz et al. described three different cases where psoriasis was exacerbated after the first, second and third doses of the Pfizer-BioNTech vaccine [53]. A case study also reported exacerbation of existing psoriasis in a 40-year-old man after vaccination with the first dose of the same vaccine [54]. Two cases of underlying dermatitis were reported to be exacerbated upon receipt of the third dose of Pfizer-BioNTech vaccine [55]. In a recent study, Michkowska et al. reported a case of a 65-year-old male with a history of hepatocellular carcinoma previously treated with nivolumab and poorly controlled psoriasis that was exacerbated one week after he received the first dose of the Pfizer-BioNTech vaccine [56]. Another case study reported on a man who developed psoriatic lesions on the lower legs 5 days after a second dose of this vaccine [52]. Finally, one study reported on a 51-year-old man whose existing psoriatic lesions enlarged after receipt of his first dose of the Pfizer-BioNTech vaccine [57]. The same report described the case of a second man with a complaint of skin rash that started on his buttocks 1 month after the second dose of inactivated CoronaVac vaccine [57].

Table 18.1 Cases of psoriasis after COVID-19 vaccination

Case	Vaccine type	Vaccination dose	Psoriasis type before/after vaccination	Description of lesions	Result	Ref
66-year-old female	Oxford/AstraZeneca	1st dose	_/Generalized pustular psoriasis	1. Erythematous pustular rashes to the trunk and proximal part of th limbs	Development of psoriasis	[45]
65-year-old male		2nd dose	_/Psoriasis	1. Erythematous papules and plaques on the trunk and extremities	Development of psoriasis	[46]
56-year-old female	Covishield	1st and 2nd dose	Psoriasis/Psoriasis	_	Exacerbation of psoriasis	[46]
23-year-old female	Pfizer/BioNTech	1st dose	_/Guttate psoriasis	1. Erythematous papules and plaques on the dorsum of her hand, then on the elbows, arms, legs and trunk	Development of psoriasis	[47]
79-year-old female	Pfizer/BioNTech	1st and 2nd dose	_/Guttate psoriasis	1. Disseminated erythematous papules and scaly plaques on the arms and thighs	Developoing of psoriasis	[48]
76-year-old female	Pfizer/BioNTech	2nd dose	_/Nail psoriasis	1. Red spots and dilated capillaries in proximal nailfold 2. subungual parakeratosis and entrapment of neutrophils	Development of psoriasis	[49]
46-year-old male	Pfizer/BioNTech	2nd dose	Plaque psoriasis/Plaque psoriasis	Silver scaling and inflamed psoriatic plaques on legs, extremities and trunk	Exacerbation of psoriasis	[52]
64-year-old male	Pfizer/BioNTech	3rd dose	Psoriasis vulgaris/Psoriasis vulgaris	1. Erythematous, silver-coloured scaly plaques on the bilateral dorsum of the hand, elbow, leg extensor surfaces and intergluteal area	Exacerbation of psoriasis	[53]
64-year-old male	Pfizer/BioNTech	2nd dose	Palmoplantar psoriasis/ Palmoplantar psoriasis	1.Silvery-scaly plaques in the hypothenar region of palms 2. Hyperkeratotic plaques and fissures in the plantar area	Exacerbation of psoriasis	[53]

(continued)

Table 18.1 (continued)

Case	Vaccine type	Vaccination dose	Psoriasis type before/after vaccination	Description of lesions	Result	Ref
25-year-old female	Pfizer/BioNTech	1st dose	Pustular psoriasis/Pustular psoriasis	1.Non-follicular pustules and localized scaling on erythematous plaques in the anterior-posterior of the trunk, arms and proximal thighs	Exacerbation of psoriasis	[53]
40-year-old male	Pfizer/BioNTech	1st dose	Acute generalized pustular psoriasis/Acute generalized pustular psoriasis	Erythematous patches and plaques on abdomen, arms, legs and buttocks Psoriasiform dermatitis with intraepidermal neutrophilic pustules	Exacerbation of psoriasis	[54]
71-year-old male	Pfizer/BioNTech	2nd and 3rd dose	_/Vesicular and discoid eczema	Discoid plaques and multiple deep-seated vesicles on the palms and trunk Eczematous and weeping discoid plaques on the limbs and chest	Exacerbation of eczema	[55]
80-year-old female	Pfizer/BioNTech	3rd dose	Quiescent psoriasis/Guttate psoriasis	Scaly erythematous plaques and papules on lateral side of lower right limb	Exacerbation of psoriasis	[55]
65-year-old male	Pfizer/BioNTech	1st dose	Hepatocellular carcinoma/psoriasis	Erythematous scaly plaques on chest, abdomen, back and extremities	Exacerbation of psoriasis	[56]
51-year-old male	Pfizer/BioNTech	1st and 2nd dose	Plaque psoriasis/Plaque psoriasis	Confluent, erythematous, scaly, thick plaques on knees, upper extremities, buttocks, trunk, thighs and legs	Exacerbation of psoriasis	[57]
52-year-old male	CoronaVac	2nd dose	Plaque psoriasis/Plaque psoriasis	Erythematous and scaly plaques on extremities, neck and trunk	Exacerbation of psoriasis	[57]
34-year-old female	Oxford/AstraZeneca	1st dose	Psoriasis vulgaris/Psoriasis vulgaris	Papules and plaques on trunk and extremities	Exacerbation of psoriasis	[58]
12 patients	Pfizer/BioNTech Oxford/AstraZeneca	1st or 2nd dose	Plaque psoriasis or pustular psoriasis/Plaque psoriasis or pustular psoriasis	_	Exacerbation of psoriasis	[59]

89-year-old male	Moderna	2nd dose	_/psoriasis	1. lesions on the scalp, torso, arms, legs 2. 60% body surface area	Developing of psoriasis	[60]
6 patients	Moderna (5 cases) Pfizer/BioNTech (1 case)	1st and 2nd dose (1 case) 2nd dose (5 cases)	_	_	Exacerbation of psoriasis	[60]
14 patients	Pfizer/BioNTech Oxford/AstraZeneca Moderna	1st or 2nd dose	13 cases plaque psoriasis and 1 case guttate psoriasis/plaque psoriasis	Generalized papulosquamous rash	Exacerbation of psoriasis	[61]
11 patients	Pfizer/BioNTech Oxford/AstraZeneca Moderna	Mainly 2nd dose	10 cases plaque and 1 case guttate psoriasis/plaque or guttate psoriasis	_	Exacerbation of psoriasis	[62]
51 patients	Moderna Oxford/AstraZeneca	_	Psoriasis/psoriatic arthritis or guttate psoriasis	Erythematous scaly patches Increase in PASI index	Exacerbation of psoriasis/changing type of psoriasis	[63]
72-year-old male	CoronaVac	1st dose	Plaque psoriasis/generalized pustular psoriasis	Ddesquamation, diffuse erythema and coalescing pustules all over the body	Changing type of psoriasis	[64]
57-year-old male	Pfizer/BioNTech	_	Plaque psoriasis/palmo plantar psoriasis	_	Changing type of psoriasis	[65]
63-year-old female	Pfizer/BioNTech	_	Plaque psoriasis/Pustular plantar psoriasis	_	Changing type of psoriasis	[65]
83-year-old female	Pfizer/BioNTech	2nd dose	Palmoplantar psoriasis/ Psoriatic arthritis	Stiffness, swelling and desquamation of palmar skin of hands Edema on the back of left hand and wrist joint.	Changing type of psoriasis	[66]
58 patients	Moderna Janssen Pfizer/BioNTech Oxford/AstraZeneca	1st or 2nd dose	_	Erythematous plaques, silvery scale Epidermal acanthosis, confluent parakeratosis with trapped neutrophils and focal spongiform pustules Reduced thickness of the granular layer in the trunk, extremities, head, neck and face	Exacerbation of psoriasis	[82]

5.2.2 Oxford/AstraZeneca Vaccine

There have also been reports of exacerbated psoriasis conditions linked to vaccination with the AstraZeneca vaccine. For example, Fang et al. reported a case of a 34-year-old woman with a history of psoriasis who was being treated successfully with biologic and immunosuppressant drugs [58]. One week after being injected with the first dose of the AstraZeneca vaccine, an erythematous scaly plaque was seen around the injection site and psoriasis plaques developed on her trunk and extremities. Another case presented by Nagrani et al. described a 56-year-old woman with a history of psoriasis who showed a flare-up of psoriatic lesions after receiving her first dose of the Covishield version of the AstraZeneca vaccine [46].

5.2.3 Studies Carried Out at Single Centres

In a retrospective study, Koumaki et al. identified 12 patients at a single centre who showed an exacerbation in their psoriasis condition after receiving either the Pfizer-BioNTech or AstraZeneca vaccine [59]. Likewise, Wei et al. carried out a retrospective analysis at a single centre in New York to investigate cases of new-onset or exacerbation of existing psoriasis after COVID-19 vaccination [60]. They identified 7 patients who showed new onset or psoriasis flare-ups of pre-existing psoriasis after receiving either the Modena or Pfizer-BioNTech mRNA vaccines. Sotiriou et al. reported 14 cases of psoriasis flares from a single centre after patients were vaccinated with either of the Pfizer, Moderna or AstraZeneca vaccines [61]. Similarly, Megna et al. reported on 11 cases of psoriasis exacerbation over a 6-month period in early 2021 following vaccination with Pfizer-BioNTech, Moderna or Oxford-AstraZeneca vaccines [62].

In a larger study, Huang et al. recruited 32 volunteers with psoriasis who had never been immunized and 51 psoriasis patients who had been vaccinated who had been vaccinated with either the Moderna or AstraZeneca vaccine [63]. They observed 15 cases of exacerbations that occurred within 9 days of vaccination compared to two cases in the non-vaccinated control group. Taken together these results suggest that there is some risk of flare-ups or exacerbations of pre-existing psoriasis conditions following the administration of many of the COVID-19 vaccines.

5.3 COVID-19 Vaccination Changing Type of Psoriasis

Onsun et al. reported on a case involving a 72-year-old male patient with a history of plaque psoriasis using a topical treatment for his condition [64]. Four days after receiving the first dose of the CoronaVac vaccine, he manifested a number of alterations in his condition including desquamation, diffuse erythema and coalescing pustules. Another study showed that two cases of mild plaque-type psoriasis appeared to develop into the pustular palmoplantar psoriasis form one month after

administration of the Pfizer-BioNTech vaccine [65]. Finally, Quattrini et al. reported the case of an 83-year-old female with a history of palmoplantar psoriasis. Two days after being administered her second dose of the Pfizer-BioNTech vaccine, she presented to the hospital with symptoms of stiffness, swelling and desquamation of palmar skin of both hands along with oedema on the back of the left hand and wrist [66].

6 Conclusions and Future Perspectives

The current vaccines currently approved by the WHO consist of four different types [67] which can be classified as:

1. mRNA (spike protein)
   (a) Comirnaty (Pfizer/BioNTech)
   (b) Spikevax (Moderna)
2. Viral vector (spike protein)
   (a) Vaxzevria (Oxford/AstraZeneca)
   (b) Covishield (Oxford/AstraZeneca)
   (c) Jcovden (Janssen),
   (d) Convidecia (CanSino)
3. Inactivated virus
   (a) Covilo (Sinopharm)
   (b) CoronaVac (Sinovac)
   (c) Covaxin (Bharat Biotech)
4. Recombinant spike protein
   (a) COVOVAX (Novavax)
   (b) Nuvaxovid (Novavax)

In addition, there are adapted bivalent versions of authorized COVID-19 vaccines from Pfizer/BioNTech and Moderna Biotech using the mRNA spike protein strategy for broader protection against the variants [68]:

1. Pfizer/BioNTech
   (a) Comirnaty bivalent – Original + Omicron BA.1 spike protein (Authorized: September 1, 2022)
   (b) Comirnaty bivalent – Original + Omicron BA.4-5 (Authorized: September 9, 2022)
2. Moderna Biotech
   (a) Spikevax bivalent Original/Omicron BA.1 (Authorized: September 1, 2022)
   (b) Spikevax bivalent Original/Omicron BA.4-5 (Under evaluation: from September 26, 2022)

Like all medications, vaccines can cause side effects such as psoriasis [50, 69]. Since the COVID-19 vaccines work in different ways, this is likely to occur via some overlapping and some distinct mechanisms. However, this review revealed that most of the above types of COVID-19 vaccines were associated with psoriatic side effects. Also, given that psoriasis cases were reported in response to SARS-CoV-2 infections before the COVID-19 vaccines were rolled out [15–18], a likely common mechanism is through perturbations in immune and/or inflammatory pathways, including potential autoimmune responses [19–23]. This suggests that individuals with pre-existing psoriasis or other autoimmune-related conditions should be advised and then monitored for worsening of their conditions after a COVID-19 infection or vaccination. In cases where a de novo eruption or exacerbation does occur, treatment with some biologics, immunosuppressive agents and anti-inflammatory drugs can be helpful [21, 70, 71]. However, some of these could also lead to a worsening of the condition, which suggests that techniques for monitoring potential autoimmune and pro-inflammatory effects should be applied.

Four psoriasis-associated autoantigens have been identified as cathelicidin LL-37, melanocyte A disintegrin-like and metalloprotease domain containing thrombospondin type 1 motif-like 5 (ADAMTSL5), phospholipase A2 group IVD (PLA2G4D) and keratin 17, and autoreactive T cells against these have been found in some psoriasis patients [72]. Another study reported on the discovery of autoantibodies against LL-37 and ADAMTSL5 associated with both psoriasis and psoriatic arthritis, suggesting a potential role of these autoantibodies in disease pathogenesis [73]. We suggest the use of screening panels for monitoring the levels of these and other autoantibodies, using platforms such as those developed by the German companies CellTrend [74] and EUROIMMUN [75]. Other technologies such as multiplex immunoassay [76] and cytokine arrays [77] could be used to detect inflammation-related changes for disease detection and monitoring. For more rapid analyses in a doctor's office or clinic, lab-on-a-chip devices incorporating rapid and sensitive tests for some of these biomarkers could be employed for point-of-care-testing [78–80].

At this stage, no specific emphasis can be given on the cause of psoriasis onset or exacerbation based on the type of COVID-19 vaccine. The matter is further complicated by the fact that some cases were apparently caused in people who did not have a history of psoriasis, and some existing psoriasis cases had received biological or immunosuppressant drug therapies, while others were in remission. In addition, where cases emerged or were exacerbated, these varied in their degree of severity or chronicity. Also, the low severity of the disease in some cases was so low that receiving an emollient was sufficient for the symptom relief. Furthermore, 0.1–0.5% of the European population have reported any adverse responses associated with a COVID-19 vaccination [81].

Considering that cutaneous reactions to COVID-19 vaccination are rare and, when they do occur, they are mostly minor and self-limiting, there is a general consensus that the benefits of vaccination outweigh the potential risks of experiencing such side effects [63, 82–86]. This is especially true since bivalent vaccines are now available which are capable of neutralizing the highly infectious omicron variant,

maximizing the benefit-to-risk ratio. Nevertheless, healthcare workers administering the vaccines must be made aware of these potential risks and advise the recipients accordingly. To add an extra layer of safety, careful monitoring for potentially deleterious autoimmune and hyperinflammatory responses can be employed. These can include screening for the presence of autoantibodies and inflammation-related molecules for both risk assessment and for monitoring patient responses to either COVID-19 infection, COVID-19 vaccination or biologic and anti-inflammatory treatments.

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Chapter 19
Immunogenicity of Inactivated SARS-CoV-2 Vaccine (BBIBP-CorV; Sinopharm) and Short-Term Clinical Outcomes in Vaccinated Solid Organ Transplant Recipients: A Prospective Cohort Study

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Abstract

Background

Immunocompromised patients have lower seroconversion rate in response to COVID-19 vaccination. The aim of this study is to evaluate the humoral immune response with short-term clinical outcomes in solid organ transplant recipients vaccinated with SARS-CoV-2 vaccine (BBIBP-CorV; Sinopharm).

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Methods
This prospective cohort was conducted from March to December 2021 in Abu Ali Sina hospital, Iran. All transplant recipients, older than 18 years were recruited. The patients received two doses of Sinopharm vaccine 4 weeks apart. Immunogenicity was evaluated through assessment of antibodies against the receptor-binding domain (RBD) of SARS-CoV-2 after the first and second dose of vaccine. The patients were followed up for 6 months after vaccination.

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Results
Out of 921 transplant patients, 115 (12.5%) and 239 (26%) had acceptable anti S-RBD immunoglobulin G (IgG) levels after the first and second dose, respectively. Eighty patients (8.68%) got infected with COVID-19 which led to 45 (4.9%) of patients being hospitalized. None of the patients died during follow-up period. Twenty-four (10.9%) liver transplant recipients developed liver enzyme elevation, and increased serum creatinine was observed in 86 (13.5%) kidney transplant patients. Two patients experienced biopsy-proven rejection without any graft loss.

Conclusion
Our study revealed that humoral response rate of solid organ transplant recipients to Sinopharm vaccine was low.

Keywords Liver transplant · Kidney transplant · COVID-19 · Vaccination · Humoral response · Sinopharm

1 Introduction

To date, coronavirus disease 2019 (COVID-19) is still considered as a serious global health problem. By September 5, 2022, more than 604 million infected patients with COVID-19 and 6,493,867 deaths have been identified based on World Health Organization (WHO) statistics [1]. Some studies have reported the mortality rate of COVID-19 in solid organ transplant (SOT) recipients to be approximately 20% [2, 3]. The most effective approach for COVID-19 prevention and reduction of its burden on health systems is rapid and widespread vaccination. Currently, several different vaccine platforms against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; the cause of COVID-19 disease) are available worldwide, some of which are authorized for emergency use. Since SOT recipients have been excluded from vaccine trials, there is insufficient information regarding safety and efficacy of vaccination in this population [4]. SOT recipients receive immunosuppressive therapy and are at risk for lower immunogenicity than the non-transplant population [5]. Most of studies in this context have focused on messenger ribonucleic acid (mRNA)-based vaccines, which mainly indicate low immune responses of SOT recipients against these types of vaccines [5, 6]. However, only eight studies thus far have evaluated the immunogenicity of inactivated anti-SARS-CoV-2 vaccines in SOT patients, and these have had mixed results [7–14].

Sinopharm COVID-19 vaccine or BBIBP-CorV is an inactivated vaccine produced by Beijing Bio-Institute of Biological Products (BBIBP) and authorized for emergency use by the WHO. Its efficacy against symptomatic COVID-19 and hospitalization rate has been reported to be 79%. According to the Strategic Advisory Group of Experts on Immunization, the Sinopharm vaccine should be administered over a two-dose schedule, given 3–4 weeks apart [15]. The trials have proved the

efficacy of this vaccine. The most reported adverse reactions were injection site pain and fever which were mild and safe limiting, with no serious adverse reactions [16, 17]. So far, only one large-scale study has been published on the use of this vaccine in transplant recipients [8].

Here, we present an evaluation of the humoral response, clinical outcomes, and adverse effects of this vaccine in a large population of SOT recipients.

2 Material and Methods

2.1 Study Design and Participants

This prospective observational cohort study was conducted from March to December 2021 on SOT patients whose date of surgery exceeded 6 months. The patients received two doses of COVID-19 vaccine BIBP developed by China National Biotec Group (CNBG), Sinopharm, 4 weeks apart in Shiraz Transplant Center, Abu Ali Sina Hospital, Shiraz, Iran, as the largest SOT center in Asia. The study was approved by the regional board of Shiraz University of Medical Sciences, Iran (#IR.SUMS.REC.1400.447).

The inclusion criteria were as follows: age over 18 years; having been transplanted more than 6 months prior to recruitment; and eligibility to receive COVID-19 vaccination according to relevant guidelines [18]. Exclusion criteria were patients with a laboratory-confirmed diagnosis of SARS-CoV-2 infection either by polymerase chain reaction (PCR) or serology; acute transplant rejection at the time of vaccination; inability to complete study-related procedures; and pregnancy or lactation.

2.2 Immunogenicity Assay

Blood samples were obtained from all participants before the first dose, 4 weeks after the first dose and 4 weeks after the second dose. Samples were tested by antibody-capture enzyme-linked immunosorbent assay (ELISA) [19] which detects immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies against the receptor-binding domain (RBD) of SARS-CoV-2 using commercial kits (Chemobind®, Iran) and an ELISA reader (Awareness Technologies Stat Fax 2100 Microplate Reader; Westport, CT, USA). The commercial anti-RBD IgM kit used in this study had 100% specificity (95% CI: 99.0–100) and 91.8% sensitivity (95% CI 94.9–99.9), while both specificity and sensitivity of the anti-RBD IgG kit were 100% (95% CI: 97.4–99.9). The levels of IgG and IgM antibodies were measured according to the manufacturer's instructions and ELISA index values above 1.1 were considered as a positive response.

2.3 Patients Follow-Up

The demographic and clinical information of all patients was collected in a predesigned form. All patients were monitored daily during the first week after each vaccination dose and then monthly up to 6 months after second dose by trained healthcare providers in Shiraz Transplant Center by telephone or in person. They were evaluated for any sign of vaccine adverse reactions or contracting COVID-19 and its complications. The patients were visited face to face by the transplant team and infectious disease specialist if needed.

2.4 Statistical Methods

In this study, continuous data were expressed as the mean ± standard deviation (SD) or median (IQR) and categorical data were given as frequency and percentage. In order to compare the responder and non-responder groups, student's t-test was used for continuous data and categorical data were analyzed using Chi-squared or Fisher's exact test. Univariate and multiple logistic regression analyses were performed to assess the potential predictors of non-responsiveness to the vaccination, using variables which were significant at the level of 0.2 ($P \leq 0.2$) in univariate analyses. Data were analyzed using the SPSS 16 package (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 Participant Characteristics

Out of 921 transplant recipients who had received two doses of vaccine, 35.9% were females and 64.1% males (Table 19.1). The mean age of participants was 47.81 ± 13.42 (18–80) years. Overall, 665 and 221 patients had received kidney or liver transplants, respectively. The number of simultaneous pancreas kidney (SPK) and heart transplant recipients were 28 and 7, respectively. The most common comorbidities found were hypertension (44.4%), diabetes mellitus (28.2%), and dyslipidemia (14.8%).

The major underlying causes leading to end-stage renal disease (ESRD) in kidney transplant recipients were hypertension (54.5%), diabetes (24.8%), autosomal-dominant polycystic kidney disease (ADPKD; 15.2%), and systemic lupus erythematosus (SLE; 5.5%). The most important indications for liver transplantation were cryptogenic (23.2%), primary sclerosing cholangitis (PSC; 20.8%), hepatitis B (17.4%), Wilson disease (6.8%), and autoimmune hepatitis [20] (16.4%).

Table 19.1 The solid organ transplant recipient demographic data of those who received first and second dose of Sinopharm COVID-19 vaccine ($N = 921$)

Variables	Responder $N = 239$	Non responder $N = 682$	Total	p-value
Age, *n* (%)				
<30 years old	74 (10.9%)	20 (8.4%)	94 (10.3%)	0.44
30–50 years old	298 (44%)	102 (42.9%)	400 (43.7%)	
>50 years old	306 (45.1%)	116 (48.7%)	422 (46.1%)	
Sex, *n* (%)				0.86
Male	152 (63.6%)	438 (64.2%)	590 (64.1%)	
Female	87 (36.4%)	244 (35.8%)	331 (35.9%)	
Comorbid disease, *n* (%)				
Diabetes mellitus	69(28.9%)	191(28%)	260 (28.2%)	0.79
Hypertension	117 (49%)	292 (42.8%)	409 (44.4%)	0.13
Dyslipidemia	43 (18%)	93 (13.6%)	136 (14.8%)	0.19
Type of transplantation, *n* (%)				0.42
Liver	48 (20.1%)	173 (25.4%)	221 (24.0%)	
Kidney	182 (76.2%)	483 (70.8%)	665 (72.2%)	
Simultaneous pancreas-kidney	7 (2.9%)	21 (3.1%)	28 (3%)	
Heart	2 (0.8%)	5 (0.7%)	7 (0.8%)	
Type of donor, *n* (%)				0.09
Living	50 (21.4%)	93 (14.5%)	143 (16.3%)	
Deceased donor	184 (78.6%)	549 (85.5%)	733 (83.7%)	
Time passed from transplantation, *n* (%)				0.07
6 months–1 year	8 (3.4%)	50 (7.4%)	58 (6.4%)	
1–3 years	48 (20.3%)	144 (21.4%)	192 (21.1%)	
More than 3 years	180 (76.3%)	479 (71.2%)	659 (72.5%)	
Immunosuppressive medications, *n* (%)				
Anti-metabolites	225 (94.5%)	640 (93.8%)	865 (94%)	0.69
Calcineurin inhibitors	201 (84.8%)	593 (87%)	794 (86.4%)	0.40
Corticosteroids	182 (76.5%)	493 (72.3%)	675 (73.4%)	0.27
Mammalian target of rapamycin inhibitors	30 (12.6%)	79 (11.6%)	109 (11.8%)	0.67
Tacrolimus level, ng/ml, mean ± SD	5.96 ± 2.21	6.16 ± 3.86	6.14 ± 3.55	0.58
Everolimus level, ng/ml, mean ± SD	5.88 ± 1.83	5.48 ± 3.2	5.57 ± 2.89	0.79
Alanine transaminase, U/L, mean ± SD	37.10 ± 15.00	39.92 ± 17.23	39.18 ± 16.71	0.42
Aspartate aminotransferase, U/L, mean ± SD	41.00 ± 14.02	45.61 ± 12.00	44.41 ± 12.70	0.38
Serum creatinine, mg/dL, mean ± SD	2.76 ± 1.90	2.97 ± 1.00	2.81 ± 1.71	0.61
Glomerular filtration rate, mL/min/1.73 m2, mean ± SD	81.20 ± 22.18	73.98 ± 25.00	79.32 ± 23.14	0.93

(continued)

Table 19.1 (continued)

Variables	Responder $N = 239$	Non responder $N = 682$	Total	p-value
Underlying liver disease, *n* (%)				0.87
Primary sclerosing cholangitis	12 (25.5%)	31 (19.4%)	43 (20.8%)	
Wilson disease	1 (2.1%)	13 (8.1%)	14 (6.8%)	
Hepatitis B	9 (19.1%)	27 (16.9%)	36 (17.4%)	
Cryptogenic	12 (25.5%)	36 (22.5%)	48 (23.2%)	
Non-alcoholic steatohepatitis	4 (8.5%)	15 (9.4%)	19 (9.2%)	
Autoimmune hepatitis	7 (14.9%)	27 (16.9%)	34 (16.4%)	
Budd–Chiari syndrome	1 (2.1%)	7 (4.4%)	8 (3.9%)	
Alcoholic	1 (2.1%)	4 (2.5%)	5 (2.4%)	
Underlying kidney disease, *n* (%)				0.11
Diabetes mellitus	21 (21.2%)	64 (26.2%)	85 (24.8%)	
Hypertension	53 (53.5%)	134 (54.9%)	187 (54.45%)	
Autosomal-dominant polycystic kidney disease	15 (15.2%)	37 (15.2%)	52 (15.2%)	
Systemic lupus erythematosus	10 (10.1%)	9 (3.7%)	19 (5.5%)	
History of rejection 1 year before transplantation, *n* (%)				0.34
Yes	16 (6.7%)	60 (8.8%)	76 (8.3%)	
No	223 (93.3%)	621 (91.2%)	844 (91.7%)	
History of re-transplantation, *n* (%)				0.51
Yes	10 (4.2%)	36 (5.3%)	46 (5%)	
No	228 (95.8%)	644 (94.7%)	872 (95%)	
History of positive COVID-19 PCR before vaccination, *n* (%)				0.33
Yes	28 (11.7%)	65 (9.5%)	93 (10.1%)	
No	211 (88.3%)	617 (90.5%)	828 (89.9%)	
Time of COVID-19 with positive PCR before vaccination, *n* (%)				0.09
1–3 months ago	2 (7.1%)	15 (23.1%)	17 (18.3%)	
3–6 months ago	10 (35.7%)	26 (40%)	36 (38.7%)	
6–12 months ago	16 (57.1%)	24 (36.9%)	40 (43%)	
History of negative PCR but symptomatic COVID-19, *n* (%)				0.15
Yes	24 (10%)	49 (7.2%)	73 (7.9%)	
No	215 (90%)	633 (92.8%)	848 (92.1%)	
Time of symptomatic COVID-19 with negative PCR, *n* (%)				0.07
1–3 months ago	2 (9.5%)	9 (20.9%)	11 (17.2%)	
3–6 months ago	6 (28.6%)	20 (46.5%)	26 (40.6%)	
6–12 months ago	13 (61.9%)	14 (32.6%)	27 (42.2%)	

(continued)

Table 19.1 (continued)

Variables	Responder $N = 239$	Non responder $N = 682$	Total	p-value
History of admission due to COVID-19 before vaccination, *n* (%)				0.10
Yes	16 (6.7%)	28 (4.1%)	44 (4.8%)	
No	233 (93.3%)	654 (95.9%)	877 (95.2%)	
Time of admission due to COVID-19 before vaccination, *n* (%)				0.94
1–3 months ago	2 (15.4%)	5 (19.2%)	7 (17.9%)	
3–6 months ago	4 (30.8%)	10 (38.5%)	14 (35.9%)	
More than 6 months ago	7 (53.8%)	11 (42.3%)	18 (46.2%)	

Among participants, 72.5% had undergone transplantation more than 3 years previously and 6.4% had received transplants 6–12 months prior to study enrollment.

In total, 60.8% of patients were taking calcineurin inhibitors (CNIs), corticosteroids, and antimetabolites at the time of the first and second doses of the vaccine. Also, 3.8% and 17.9% of patients were receiving a combination of mTOR inhibitors, corticosteroids and antimetabolites, and CNIs combined with antimetabolites, respectively.

3.2 SARS-CoV-2 Vaccination Immunogenicity

The median (IQR) plasma level of anti S-RBD IgM and IgG before vaccination was 0.08 [0.06, 0.15] and 0.31 [0.13, 0.57], respectively. Out of the 921 SOT recipients, 115 (12.5%) and 239 (26%) patients had acceptable anti S-RBD IgG levels (>1.1) 4 weeks after the first and second dose, respectively. After omitting cases who had shown a positive PCR test for COVID-19 within 6 months prior to vaccination, 104 (12.6%) and 211 (25.5%) patients had acceptable anti RBD levels 4 weeks after the first and second dose.

3.3 Clinical Outcomes 6 Months Post-Vaccination

A total of 80 patients (8.68%) got infected with COVID-19 after vaccination, eight (0.9%) of those who were infected between the first and second (8.08 ± 2.21 days) dose and 72 (7.8%) were infected 133.90 ± 54.94 days after the second dose. Also, among the COVID-19 infected patients ($n = 80$), 13 and 24 had acceptable anti-RBD IgG levels between the first and second dose and after the second dose, respectively. Forty-five (4.9%) patients were admitted to hospital due to COVID-19 after

Table 19.2 Univariate and multivariate analysis regarding qualitative and quantitative variables between seroconversion and non-seroconversion to Sinopharm COVID vaccine

	Univariate analysis		Multivariate analysis	
Variables	OR (CI)	*P*-value	OR (CI)	*P*-Value
Age				
<30 years old	1.4 (0.81,2.4)	0.20	0.82(0.23, 2.94)	0.77
30–50 years old	1.1(0.81,1.5)	0.51	0.76 (0.44,1.3)	0.32
>50 years old	Ref	–		
Sex				
Male	1.02(0.75,1.39)	0.86		
Female	Ref.	–		
Type of transplantation				
Liver	1.24(0.54,2.84)	0.59		
Kidney	0.91(0.42,1.99)	0.83		
Others	Ref.	–		
Comorbid disease				
Diabetes mellitus	0.95(0.69,1.32)	0.79		
Hypertension	0.78(0.58,1.04)	0.14	0.63 (0.35,1.16)	0.14
Dyslipidemia	0.72(0.48,1.06)	0.17	0.83 (0.46,1.16)	0.52
Time passed from transplantation				
6 months–1 year	2.34(1.09,5.05)	0.02	5.75(1.29, 25.48)	0.02
1–3 years	1.12(0.78,1.63)	0.52	1.22(0.67,2.21)	0.51
More than 3 years	Ref.		Ref.	
Immunosuppressive medications				
Anti-metabolites	0.88(0.46,1.67)	0.69		
Calcineurin inhibitors	1.19(0.78,1.81)	0.49		
Corticosteroids	0.8(0.56,1.13)	0.20	0.69(0.33,1.42)	0.32
Mammalian target of rapamycin inhibitors	0.9(0.58,1.42)	0.67		
Tacrolimus level	1.01(0.95,1.08)	0.58		
Certicane level	0.95(0.67,1.34)	0.78		
Underlying liver disease				
Primary sclerosing cholangitis / Autoimmune hepatitis	0.71(0.31,1.62)	0.42		
Cryptogenic/Non-alcoholic steatohepatitis	0.75(0.32,1.74)	0.51		
Others (Wilson/Budd–Chiari/Alcoholic)	Ref.	–		
Underlying kidney disease				
Diabetes mellitus	1.65(0.82,3.31)	0.15	1.65 (0.78,3.5)	0.18
Hypertension	1.37(0.76,2.45)	0.28	1.62 (0.83,3.12)	0.15
Others (Autosomal-dominant polycystic kidney disease, Systemic lupus erythematosus)	Ref.	–	Ref.	

(continued)

Table 19.2 (continued)

Variables	Univariate analysis		Multivariate analysis	
	OR (CI)	*P*-value	OR (CI)	*P*-Value
History of rejection 1 year before transplantation				
Yes	1.34(0.76,2.38)	0.35		
No	Ref.	–		
History of positive PCR before vaccination				
Yes	0.79(0.49,1.27)	0.33		
No	Ref.	–		
History of negative PCR but symptomatic COVID-19				
Yes	0.69(0.41,1.15)	0.16	1.04 (0.37, 2.87)	0.93
No	Ref.	–	Ref.	
History of admission due to COVID-19 before vaccination				
Yes	0.59 (0.31,1.12)	0.11	0.71 (0.23,2.13)	0.54
No	Ref.	–	Ref.	

receiving the second dose of vaccine. None of the patients died during the 6-month follow-up period.

The univariate analyses showed that hypertension, dyslipidemia, the time from transplantation, receiving corticosteroid, underlying kidney diseases, history of symptomatic COVID-19 with negative PCR, and history of hospital admission before vaccination due to COVID-19 were considered as risk factors for non-responsiveness to the vaccination (Table 19.2). However, the multivariate analysis demonstrated that time from transplantation was the only significant risk factor for non-responsiveness (OR = 5.75, 95% CI = 1.29–25.48; $p = 0.02$). This showed that the odds of non-responsiveness to the vaccination in patients who had undergone transplantation 6–12 months before vaccination compared to people who were transplanted >3 years before vaccination was 5.75.

3.4 Adverse Events

Figure 19.1 shows the rate of adverse events (AEs) after the first and second dose of the vaccine. Fatigue, injection site pain, and fever were the most frequent AEs found in patients. Five and three patients visited the hospital emergency room due to AEs (allergic reactions, hypotension, and severe headache) after the first and second dose of vaccine, respectively.

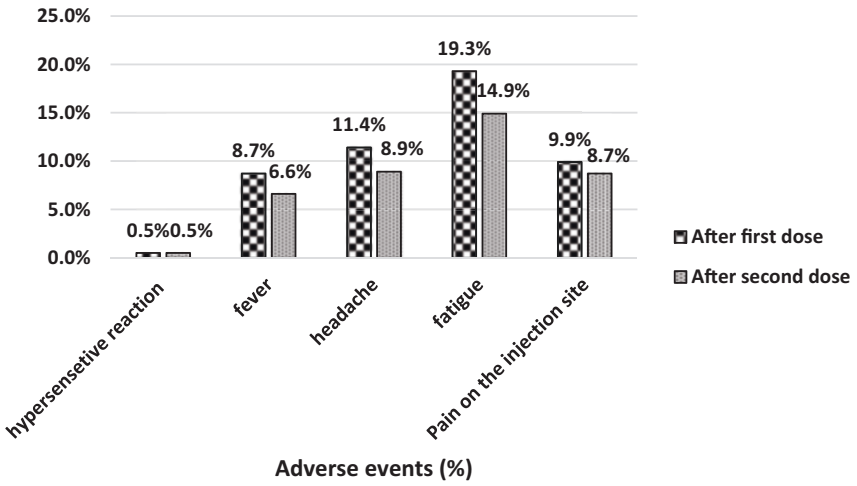

Fig. 19.1 Adverse events following first and second dose of Sinopharm COVID-19 vaccine among solid organ transplant recipients ($N = 921$)

Among the liver transplant recipients (n = 221), 24 (10.9%) developed liver enzyme elevation (17 cases after first dose and 7 patients after second dose). Also, elevated serum creatinine was observed in 86 (13.5%) (44 cases after the first and 42 after the second dose) of the kidney transplant recipients. In all of these patients, other reasons for serum creatinine or liver enzymes elevation were evaluated and ruled out. Two liver transplant recipients who experienced liver enzyme elevation (more than fivefold over the upper limit of normal) needed hospital admission and received corticosteroids. However, none of the above kidney transplant recipients needed hospitalization, hemodialysis, or continuous renal replacement therapy (CRRT).

Among the vaccine recipients, two patients developed antibody-mediated rejection confirmed by biopsy, one of whom was a kidney transplant recipient (8 days post-second dose) and the other patient had received a liver transplant (11 days after the second dose). Both of these patients were admitted, evaluated regarding the cause of rejection and received methylprednisolone. Biopsies after treatment in both patients showed normal histopathology, neither of them experienced graft loss and both were discharged 12 and 9 days after admission.

4 Discussion

Immunosuppressed patients, including SOT recipients, have a weaker humoral and cellular immune response compared to normal population regarding vaccination. In this study, the humoral response rate to Sinopharm COVID-19 vaccine and short-time clinical outcomes were evaluated. Nearly 13% and 25.5% of patients had

acceptable anti-spike protein RBD IgG levels after the first and second dose of vaccine, respectively. The trials on Sinopharm vaccine immunogenicity in the general population demonstrated a seroconversion rate after receiving two doses of vaccine to be more than 90% [21, 22]. In the case of SOT recipients, a number of vaccine types have been tested. Boyarsky et al. evaluated the immunogenicity of the SARS-CoV-2 mRNA vaccines in SOT recipients and observed that only 15% and 54% of patients had acceptable antibody level after the first and second dose, respectively [23]. Also, it has been reported that the antibody response to the Janssen viral vector-based COVID-19 vaccine was 16% [5]. Another study showed that 24% and 34.8% of heart transplant patients vaccinated with ChAdOx1 (AZD1222), another viral vector vaccine, developed a detectable antibody response after the first and second dose [24]. In one study on kidney transplant recipients vaccinated with inactivated Sinopharm-CoronaVac vaccine (BBIBP-CorV), it was demonstrated that only 9% of the transplant recipients had an acceptable antibody level, while the antibody level was acceptable in 100% of participants in the control group [25]. These differences in seroconversion rates across the various vaccine types may have been caused by the type of vaccine platform, number of participants, and factors affecting seroconversion in transplant recipients, such as type of immunosuppressive regimens, time passed since transplantation, and underlying diseases. However, the lower rates of seroconversion in transplant recipients compared to the normal population have been a common finding across these studies.

The univariate analysis revealed that age, diabetes, hypertension, a recent transplant operation, history of hospitalization due to COVID-19 before vaccination, and kidney transplantation secondary to diabetes or hypertension were risk factors for low immunogenicity response. However, logistic regression demonstrated that the only significant predictor of low immunogenicity response was vaccination within 6 months to 1 year following transplantation. Advanced age is one of the well-established risk factors for lower antibody titers in transplant and non-transplant patients receiving influenza, hepatitis B, and pneumococcal vaccines [26]. Also, many studies have identified advanced age as one of the strongest risk factors for a weak response to COVID-19 vaccines [27–30]. Diabetes and hypertension were among risk factors for poor response to vaccination in our patients, especially among kidney transplant recipients. Such metabolic disorders are common among SOT patients, mainly due to treatment with immunosuppressants such as CNIs and corticosteroids. For example, Mazzola et al. found that diabetes was a risk factor for lack of response to vaccination among kidney transplant recipients [31], and similar results were reported with seasonal influenza vaccination in diabetic patients in some countries [32, 33]. It seems that low antibody response is secondary to diabetes-induced immune dysfunction. Furthermore, two studies evaluating risk factors for attenuated response to mRNA COVID-19 vaccines found that the presence of hypertension was for a contributor to poor seroconversion due to its negative effect on immune function [34, 35].

Transplant recipients who have recently undergone transplantation are expected to have lower seroconversion rate to COVID-19 vaccination due to their need for treatment with higher doses of immunosuppressive medications, particularly

antimetabolites [30, 36, 37]. Marta et al. found that the unfavorable effect of mycophenolate mofetil (MMF) on seroconversion was dose-dependent and MMF dose modification prior to vaccination can improve the immune system response to COVID-19 vaccination [38]. Also, our study revealed that receiving corticosteroids can have a negative effect on seroconversion. The COViNEPH Project, which evaluated different aspects of COVID-19 infection in nephrology including effective factors on humoral immune response to COVID-19 vaccination, found that seroconversion rate was 66.7% in patients who did not receive corticosteroids in their maintenance immunosuppressive regimen [37]. Also, a similar result was observed in immunocompromised hematologic cancer patients receiving prednisolone [39]. Although some studies have demonstrated that COVID-19 infection prior to vaccination leads to increased immunogenicity of COVID-19 vaccines in transplant and non-transplant patients [31, 40], this association was not observed in our study. Moreover, our results showed that transplant patients with a history of COVID-19 and COVID-19-related hospitalization had a lower response rate to vaccination. It is possible that a high percentage of patients with prior COVID-19 infections in our study became infected or were hospitalized due to COVID-19 more than 6 months prior to vaccination. Previous studies have shown that IgG levels against the SARS-CoV-2 spike protein decrease with time [41, 42]. In addition, Yalcin et al. demonstrated that the patients who had been infected more than 6 months prior to COVID-19 vaccination had the lowest antibody titers and antibody responses were highest in patients who had been infected 3–6 months before vaccination [40]. Another possible explanation involves the potential negative effect of corticosteroids on response to vaccination [43]. Administration of high doses of corticosteroids to our transplant recipients suffering from moderate to severe COVID-19 infection could possibly have caused low response rates to vaccination in spite of prior COVID-19 infection [44].

Our results showed that nearly 1% of patients became infected with COVID-19 after their first vaccination and just under 8% were infected within 3 months after receiving the second dose. Among the infected patients, only 5% were hospitalized due to COVID-19 during the time period of 6 months after vaccination and no COVID-19-related mortality occurred. Previous studies revealed that getting COVID-19 is possible after vaccination in transplant recipients due to their lower rates of seroconversion [31, 45]. A multicenter study showed that COVID-19 related hospitalization, critical COVID-19, and subsequent mortality were more prevalent in transplant recipients compared to normal population groups (7% vs. 2%), which indicates the importance of the third dose of vaccine in transplant recipients [46]. In support of this, a recently published meta-analysis found that transplant recipients who were seronegative after two doses of COVID-19 vaccines turned seropositive after receiving the third dose [47].

Our findings are in line with other studies which showed that fatigue, injection site pain, and fever were the most frequent AEs of Sinopharm vaccination [16, 48]. In our study, liver enzyme elevation occurred in 11% of patients, two of them required medical intervention. Similar findings and new onset or activation of autoimmune hepatitis have also been reported following administration of mRNA

(Pfizer-BioNTech; Moderna Biotech) and viral vector-based (OxfordAstraZeneca) vaccines [49–51]. Hepatic artery thromboembolism has also been reported which resulted in death in some cases [52]. A rise in serum creatinine in kidney transplant patients following vaccination has been observed in our research and in other studies [53, 54]. Also, some investigations have reported cases of acute kidney injury and minimal change disease following COVID-19 vaccination [55]. A possible explanation of this is that interferon-γ (INF-γ), tumor necrosis factor-α (TNF-α), and interleukin-2 (IL-2) produced as a result of T-cell responses to foreign mRNA could lead to podocytopathies and B-cell production of disease-specific antibodies in susceptible patients and to aggravation of subclinical or quiescent glomerular diseases. Also, SARS-CoV-2 infection itself can cause activation of diverse autoimmune and alloimmune renal diseases by a similar pathogenesis [56].

Two of the participants in our study experienced organ rejection after the second dose of vaccine. However, those grafts were recovered after administration of methyl prednisolone to both patients. Some studies have reported organ rejection after receiving COVID-19 vaccines although rejection prognoses were generally good [57–59]. However, one case of steroid-resistant acute cellular rejection was reported in a liver transplant recipient vaccinated with a COVID-19 mRNA vaccine [60]. Although the possibility of rejection following vaccination exists in SOT recipients, the association with vaccination has not been proved in large studies or trials. Some studies have mentioned that nonspecific immune activation (adjuvant effect) or induction of cross-reactive immunity coincident with vaccinations is responsible for cellular or humoral antidonor alloresponses and consequently rejection [61, 62]. Although the majority of organ rejections after vaccination have occurred following administration of mRNA vaccines, it cannot be concluded that a definite correlation exists between vaccine platform and organ rejection. This finding may be a result of higher percentage of SOT recipients vaccinated with mRNA vaccines.

4.1 Limitations

Although our study is one of the largest studies conducted on inactivated COVID-19 vaccination in SOT recipients, it should be interpreted cautiously due to some limitations. First, only the inactivated Sinopharm BBIBP-CorV vaccine was investigated. This obviated comparisons of the results with other vaccines. Second, no control group was included in this study and, therefore, a comparison between transplant and non-transplant patients was not possible. Furthermore, due to the 6-month follow-up period, data regarding a third vaccine dose was not available. Finally, this study focused on humoral immune response, while evaluation of cellular immune responses can provide more comprehensive information regarding vaccination efficacy.

4.2 Conclusion

The results of our study are consistent with those of previous investigations which showed that the humoral response rate to the Sinopharm vaccine was low in SOT recipients. The short time interval between transplantation and vaccination may cause low seroconversion rates in SOT recipients, due to the high dosages of immunosuppressive medications used during this period. It is recommended that a third dose of a different vaccine type or use of adjuvants may be employed in SOT recipients who have been previously vaccinated with two doses of inactivated vaccine. For example, a third vaccine dose using one of the new bivalent versions of the spike protein mRNA vaccines from Pfizer/BioNTech and Moderna Biotech [63] to giver better protection against the SARS-CoV-2 Omicron sub-variants.

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Disclosure The authors of this manuscript have no conflicts of interest to disclose.

Declarations

Ethics Approval and Consent to Participate

The study was approved by the regional board of Shiraz University of Medical Sciences, Iran (#IR.SUMS.REC.1400.447). Informed consent from each study participant was also obtained before data collection.

Consent for Publication

Not applicable.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Competing Interests

The authors declare that we do not have any conflict of interest.

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Chapter 20
Spices and Biomarkers of COVID-19: A Mechanistic and Therapeutic Perspective

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Abstract In the face of the COVID-19 pandemic, many people around the world have increased their healthy behaviors to prevent transmission of the virus and potentially improve their immune systems. Therefore, the role of diet and food compounds such as spices with bioactive and antiviral properties may be important in

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these efforts. In this chapter, we review the efficacy of spices such as turmeric (curcumin), cinnamon, ginger, black pepper, saffron, capsaicin, and cumin by investigating the effects of these compounds of COVID-19 disease severity biomarkers.

Keywords Spices · Curcumin · Ginger · Cinnamon · Turmeric · COVID-19

1 Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus which has caused and perpetuated the COVID-19 pandemic [1, 2]. The results of a study conducted in early 2021 showed that the disease originated from a single strain of the virus, which infected wild bats and appeared to spread to humans via an intermediate host [3]. The latency period of the disease varies between 1 and 14 days, and most people experience symptoms such as fever, cough, headache, and fatigue within the first 7 days of exposure to the virus [4]. Among the people who show significant symptoms, about 81% have mild to moderate symptoms, 14% have severe symptoms such as shortness of breath and hypoxia, and 5% may show life-threatening symptoms such as respiratory failure or multi-organ dysfunction [5]. In the case of the Omicron variant which erupted around the world at the end of 2021 and the beginning of 2022, the severity of symptoms appeared less than in the other variants of concern [6]. Recently, new strains of SARS-CoV-2, including XBB and BF7, have been identified. The BF7 strain is increasing in the United States and XBB has appeared in Singapore, Bangladesh, and India. Some experts believe that by identifying these sub-strains, the virus may turn into SARS-COVID type 1 or 3. According to forecasts, there is a possibility of spreading new strains, such as BQ1, BQ1.1, BQ1.3, and XBB, which mutate quickly, especially in the cold season and wintertime. These strains have high infectivity but low mortality. One of the characteristics of these sub-strains is that despite low level symptoms, cardiovascular, digestive, bone or muscle complications, and even diabetes may occur after infection. Although these new strains of the virus have become more similar to the common cold in terms of symptoms and mortality, long-term and different complications may occur [7].

2 Immunopathology

Although this virus has the highest affinity for angiotensin-converting enzyme 2 (ACE2)-expressing epithelial cells in the lungs, people with COVID-19 infection have systemic inflammation. This can lead to vasodilation and allow infiltration of lymphocytes and inflamed monocytes into the lungs and heart [8]. In addition, clinical laboratory findings indicate increased levels of interleukin (IL)-2, IL-7, IL-6, interferon-gamma-induced protein 10 (IP-10), tumor necrosis factor alpha (TNF-α)

and cytokine release syndrome (CRS), which represent a type of underlying immune system pathology in COVID-19 infections [9]. Preventive actions to diminish the chance of infection include observing healthcare principles and other preventive methods such as getting vaccinated, wearing a mask, ventilating indoor spaces, managing the duration of exposure to infected people, and washing hands [10].

3 Herbal Remedies

Although many medications have been found to improve COVID-19 symptoms, people in all countries cannot access them. As most COVID-19 patients experience a mild form of the disease, supportive care in these cases includes medications such as non-steroidal anti-inflammatory drugs (NSAIDs) to relieve symptoms, adequate fluid intake, rest, observance of personal hygiene, and a healthy diet. Some severe cases may be caused by excessive systemic inflammation known as a cytokine storm [11].

Plants with medicinal properties have always been effective treatments against many disorders, including infectious diseases. According to a study conducted between 1940 and 2014, about half of the micromolecules approved by the United States Food and Drug Administration (FDA), originated from natural products or their derivatives [12]. This is not surprising as foods such as spices contain many bioactive substances, including phenolic compounds, flavonoids, tannins, sulfur-containing compounds, and alkaloids [13, 14]. However, since there are still no completely effective treatments for COVID-19 to date, India's Ministry of Ayush has released guidelines on promoting traditional Ayurveda methods for self-care, including the use of seasonings such as cumin, turmeric, garlic, and ginger in cooking [15].

4 Spices

Spices are obtained from dried parts of a plant and used to add taste, flavor, and color to foods and preserve them. In addition to their application as a condiment of foods, several health benefits have been traditionally linked to spices. Spices can be considered an inexpensive and available therapy to combat various diseases, including diabetes, neurological conditions, renal disorders, prostate diseases, osteoarthritis, rheumatoid arthritis, asthma, and cancer [16–20]. As inflammation plays a significant role in the pathogenesis of all of these diseases, the major effects of spices on these diseases have been attributed to the anti-inflammatory and antioxidant effects of the active ingredients. Furthermore, considering the cytokine storm that can occur in severe COVID-19 cases, spices might have added beneficial effects. The potential effects of some of these spices against COVID-19 disease are reviewed below.

*4.1 Turmeric (*Curcuma longa *L.)*

Turmeric belongs to the ginger plant group (Zingiberaceae), which has been used in traditional medicines for thousands of years. This plant grows naturally in India but is now grown and used worldwide, including in Southeast Asia [16]. Turmeric rhizomes contain several metabolites as major bioactive substances, such as sesquiterpenes, curcuminoids, steroids, and polyphenols [21]. Curcumin, a polyphenolic product and the main bioactive compound in turmeric rhizomes, is also known as diferuloylmethane [22]. Several mechanisms have been proposed for curcumin's protective action against many diseases due to its biological properties, including inhibition of inflammatory mediators and cytokines, preventing the creation of reactive oxygen species (ROS) in macrophages, and regulating the pro-inflammatory cytokine secretion and adhesion molecules [23, 24]. In line with this, this spice has been found to have multiple pharmacological effects, including antioxidant, anticancer, anti-diabetic, lipid-lowering, antiviral, antiseptic, and anti-pneumonia properties [25–43].

Recently, curcumin–piperine co-supplementation was shown to cause a significant reduction in weakness and prevent muscle wasting by inhibiting NF-κB in outpatients with COVID-19 [44, 45]. Despite several unique properties of curcumin, its low absorption and bio-availability have challenged its applicability in different diseases. Recently, new formulations of curcumin, such as phospholipid-modified curcumin and nano-curcumin, or combinations with other herbs, such as piperine, were introduced to circumvent this limitation [34]. As shown in Table 20.1, several clinical trials were undertaken using nano-curcumin or curcumin piperine in COVID-19 patients. Nano-curcumin was used at a dosage of 80 to 160 mg/day for 14 to 21 days [25, 46–49]. In almost all of these studies, nano-curcumin had beneficial effects on clinical outcomes and COVID-19-related biomarkers, such as inflammatory molecules. Compared to the control group, nano-curcumin significantly reduced inflammatory biomarkers such as IL-17, interferon-gamma (IFN-γ), T cell helper (Th)-1 and Th-17 responses and clinical symptoms, including weakness, tiredness, cough, chills, myalgia, olfactory and taste disturbances, duration of fever, and recovery time. In addition, it increased anti-inflammatory cytokines and T-cell regulatory (Treg) responses, transforming growth factor-beta (TGF-β), lymphocyte counts, and oxygen saturation (SpO_2) levels [25, 46–49]. Similarly, curcumin piperine had beneficial effects on diverse symptoms of patients with COVID-19, such as reduction in weakness and tiredness, fever, cough, sore throat, dyspnea, deterioration, duration of hospitalization, and mortality rate. Likewise, maintenance of oxygen saturation levels above 94%, reduction of the need for mechanical ventilation and lower D-dimer levels were observed in the curcumin piperine group compared with controls [44, 50]. Several potential mechanisms have been attributed to curcumin as a natural agent for the treatment of COVID-19 (Fig. 20.1). First, curcumin may directly inhibit viral adhesion and entry via blocking the binding of the SARS-CoV-2 spike protein to ACE2 receptors on host cell membranes. Second, viral RNA transcription and replication may also be disrupted by curcumin. In this action,

Table 20.1 Randomized clinical trials regarding effects of curcumin and ginger against COVID-19

First author (publication year), Reference No.	Sample number	COVID-19 Patients	Age/mean age	Intervention	Control/ placebo	Duration (days)	Main results↑↓↔
Askari, 2022, [44]	46	Outpatients	18–65 years	1000 mg curcumin + 10 mg piperine/ day	Placebo capsule	14	Clinical symptoms: ↓weakness and tiredness Biochemical items: Complete blood count, liver enzymes, blood glucose levels, lipid parameters, kidney function ↔Inflammatory indices: CRP ↔
Pawar, 2021 [50]	140	Inpatients	18–85 years	1050 mg Curcumin with 5 mg bioperine/day	Placebo capsule	14	Clinical symptoms: fever, cough, sore throat, breathlessness, deterioration, duration of hospitalization, mortality rate ↓ Clinical manifestation: SpO2 levels ↑ Mechanical ventilation ↓ D-Dimer levels↓ Neutrophil/lymphocyte ratio↔

(continued)

Table 20.1 (continued)

First author (publication year), Reference No.	Sample number	COVID-19 Patients	Age/mean age	Intervention	Control/ placebo	Duration (days)	Main results↑↓↔
Hassaniazad, 2021 [47]	40	Inpatients	18–75 years	160 mg of nano-curcumin/day	Placebo capsule	14	Inflammatory indices: Expression TBX1 gene of Th1 responses ↓ Expression FOXP3 gene of regulatory T cells↑ Expression RORC gene of Th17 response ↓ Serum level of IL-17 and IFN-γ ↓ Serum level of TGF-β and IL-4↑ Up regulation of FOXP3 and GATA3 genes ↑
Valizadeh, 2020 [25]	80	Inpatients	19–69 years	160 mg of nano-curcumin/day	Placebo capsule	14	Inflammatory indices: Expression level of IL-1β, IL-6↓ Expression level of IL-18 and TNF-α↔ Serum levels of IL-1β, IL-6, IL-18 and TNF-α↓ Clinical manifestation: Mortality rate↓ Fever, cough and dyspnea ↓

Ahmadi, 2021 [46]	60	Outpatient	18–65 years	160 mg nano-curcumin/day	Placebo capsule	14 days	Clinical symptoms: Cough, chills, myalgia, olfactory and taste disturbances ↓ Fever, headache, sore throat, weakness, dyspnea, GI disturbances, dermatological disturbances ↔ Inflammatory indices: Serum level of CRP ↔ Biochemical Indices: Lymphocyte count↑
Tahmasebi, 2021 [49]	120	Inpatients	21–73 years	160 mg nano-curcumin/day	Placebo capsule	21	Inflammatory indices: Serum level of IL-35, IL-10, TGF-β↑ FoxP3, IL-35, IL-10,TGF-β expression levels ↑ Mortality rate↓
Saber-Moghaddam, 2021 [48]	41	Inpatients	18–75 years	160 mg nano-curcumin/day	Placebo capsule	14	Clinical symptoms: Fever, chills, tachypnea, myalgia, cough↓ Recovery time ↓ SpO2 levels↑ Biochemical Indices: Lymphocytes count↑ Serum level of ALT, AST, CRP↔

(continued)

Table 20.1 (continued)

First author (publication year), Reference No.	Sample number	COVID-19 Patients	Age/mean age	Intervention	Control/ placebo	Duration (days)	Main results↑↓↔
Hellou, 2021 [116]	50	Inpatients	38–66 years	1 ml artemiC (12 mg artemisinin, 40 mg curcumin, 30 mg frankincense and 120 mg vitamin C)/day	Placebo capsule	15	Clinical symptoms: Duration of fever ↓ Biochemical Indices: SpO2 levels↑
Li, 2022 [67]	109	Inpatients	Mean age: 52.7 years	First group:1500 mg ginger/ daySecond group: 3000 mg ginger/ day	Third group as control	14	Clinical symptoms: SpO2 levels↑ Consciousness frequency of patients↑

Abbreviations: ↑ significant increase in the parameter in comparison to control group, ↓ significant decrease in the parameter in comparison to control group, ↔ no significant change. *M* male, *F* female, *Spo2* oxygen saturation profiles, *Th1* T hehper1, *Th17* T helper 17, *IL-17* interleukin 17, *IFN-γ* interferon γ, *TGF-β* tumor necrosis factor-β, *IL-4* interleukin 4

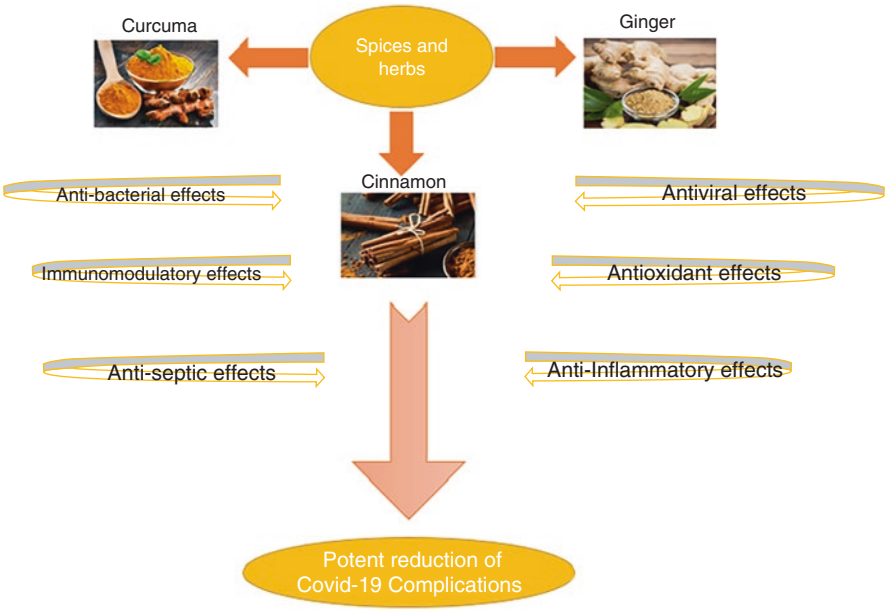

Fig. 20.1 Potential mechanisms of turmeric (curcumin), cinnamon, and ginger against COIVD-19 infection

curcumin suppresses the formation of the replicas–transcriptase complex by binding to the main protease of SARS-CoV-2. Next, the host antiviral response can be increased by curcumin by induction of IFN-stimulated genes at the mRNA level [51, 52]. In addition, curcumin can prevent secondary bacterial infections in COVID-19 patients [37, 53, 54].

4.1.1 Safety of Curcumin

Curcumin is regarded as a safe phytochemical as clinical studies have found that up to 12 g of curcumin per day did not result in any serious side effects [55]. Furthermore, a study by Srivastava showed that the consumption of curcumin at a dose of 2.5 to 8 g per day for 3 months was not associated with any toxic effects [56]. Curcumin has also been shown to be safe and well tolerated in the pediatric population [57].

*4.2 Ginger (*Zingiber officinale*)*

Ginger is one of the important medicinal plants that naturally exist in different countries. It belongs to the ginger family (*Zingiber officinale*) and is a well-known herbal remedy in the traditional Unani system of medicine [58]. Ginger is a rich

source of bioactive molecules such as phenolic compounds, alkaloids, and steroids. Moreover, ginger contains sub-compounds such as 4-gingerol, 6-gingerol, 8-gingerol, 10-gingerol and 6-shugaol, and also 14-shogaolsm. Various studies have shown the anti-vomiting, anti-fever, anti-pain, anti-arthritic, and anti-inflammatory effects of ginger (Table 20.2). Recently some studies have shown the antiviral activity and anti-influenza effects of ginger and its bioactive compounds [59, 60]. The antiviral activity of lyophilized extract from *Zingiber officinale* on hepatitis C virus has been investigated at different concentrations from 5 to 200 μg/mL. The results showed that the 100 μg/mL dose effectively inhibited the amplification of viral RNA segments and prevented virus replication [61]. The potential of several ginger bioactive compounds, namely, gingeranone A, geraniol, zingiberene, zingibernol, and zingerone, as anti-SARSCoV-2 compounds has also been investigated. In a molecular binding study, researchers found that the bioactive compounds of ginger inhibited the binding of the SARS-CoV-2 spike protein to the ACE2 receptor and acted as an inhibitor for the main protease protein (MPro) [62, 63]. Mpro is responsible for processing the poly-proteins pp1a and pp1ab during viral replication [64]. Mpro plays a central role in mediating the replication and transcription of SARS-CoV-2 mRNA. Based on molecular binding modeling, two potential candidates from ginger (zingiberenol and zingiberol) act as Mpro receptor inhibitors against the virus [65]. Also, in a study by Jeena et al. on a ginger essential oil, it was shown that this substance has antioxidant effects and increases blood levels of antioxidant enzymes such as catalase, superoxide dismutase, glutathione, and glutathione reductase [66]. Furthermore, a recent study showed that the consumption of ginger in COVID-19 patients resulted in increased SpO_2 levels and consciousness frequency of patients [67].

4.2.1 Safety of Ginger

According to survey results, 71.8% of the people of India were consuming the kadha (traditional Indian drink containing cinnamon, basil, ginger, black pepper, and raisins) prescribed by the Ministry of Ayush. About 52.4% of them used these compounds once daily, and 24.1% used them twice daily. In addition, 68.8% of people used ginger, cloves, dill, black pepper, and tulsi in their kadha. Most of these people (86.1%) did not report any side effects after consuming kadha, while 13.9%, especially the elderly, experienced side effects such as heartburn, constipation, diarrhea, mouth ulcers, and hypertension. Therefore, according to Ayurveda, consuming these spices in large quantities might have some complications (Table 20.1) [68].

*4.3 Cinnamon (*Cinnamomum cassia*)*

Cinnamomum cassia is an aromatic plant belonging to the Lauraceae family. It has been a popular spice in Chinese, Indian, Iranian, and Greek medicine since ancient times. This plant is extracted from the bark of young branches and used as a daily

Table 20.2 Potential mechanisms of turmeric (curcumin), cinnamon, and ginger against COIVD-19 infection

First author (publication year) Reference No.	Plant parts, extracts, and compounds	Possible mechanisms
Zhang, 2019 [117]	Curcumin	1.Reduction of IL-1β, IL-6, TNF-α, NF-κB activation, 2.Reduction of stressed-induced P2X7R/NLRP3 inflammasome axis activation
Peng, 2021 [118]	Curcumin	1. Regulating Janus kinase/signal transducer and activator of transcription (JAK/STAT) inflammatory signaling way 2. Inhibition of the accumulation of NLRP3 inflammasome, or inhibition the NF-κB pathway
Zhang, 2019 [119]	Curcumin	1.Reduction of NO, IL-1β, IL-6, iNOS levels 2. Increased level of IL-4, IL-10, Arg-1 promoted microglial polarization to the M2 phenotype
Li, 2019 [120]	Curcumin	Reduction in IL-1β, TNF-α, NLRP3, caspase 1
Zhang, 2018 [121]	Curcumin	Reduction of TLR4, IL-1β, TNF-α, VCAM-1, ICAM-1, NF-κB
Atabaki, 2020 [122]	Curcumin	Reduction of CRP, CD4+ and CD8+ T cells, Th17 cells and B cell frequency
Dai, 2018 [123]	Curcumin	Inhibition of virus uptake, proliferation, and particle production
Ahkam, 2020 [62]	Ginger	Inhibition of the spike protein combination to ACE2 receptor or inhibition of main protease
Zhuanga, 2009 [124]	Procyanidins and butanol extract of ginger	Disruption of the clathrin-dependent endocytosis pathway
Jeena, 2013 [66]	Ginger essential oil	1. Increased blood level of antioxidant enzymes including catalase, super oxide dismutase, glutathione, glutathione reductase 2. Increased level of superoxide dismutase, glutathione peroxidase and glutathione-s-transferase in liver
Rabie, 2022 [65]	Ginger compounds (zingiberenol and zingiberol)	Inhibition of main protease activity
Al-Sanea, 2021 [125]	Strawberry and methanolic extract of ginger	Neohesperidin is of particular interest as a potential dual inhibitory compound with its binding potential to human AAK1 protein and SARS-CoV-2 NSP16 protein
Zareie, 2021 [73]	Eugenol Extracted oil of cinnamon	Disturbance of ERK, (p38MAPK) and IKK/NF-kB signaling pathways
Raina, 2015 [126]	Aqueous extracts and methanolic extracts of cinnamon	Inhibition of NO, PGE2, LTB4, and MMP production

(continued)

Table 20.2 (continued)

First author (publication year) Reference No.	Plant parts, extracts, and compounds	Possible mechanisms
Gunawardena, 2015 [127]	Water extract/ cinnzeylanine	1. Blocking LPS + IFN-γ induced NO, and TNF-α production 2. Strong activity related to inhibition of TNF-α production
Rathi, 2013 [128]	Polyphenol fraction of cinnamon	1.Reduction of Serum TNF-α density 2. Inhibition of cytokine (IL-2, IL-4, and IFN-γ) release 3. Inhibition of prostaglandin
Vetal, 2013 [129]	Type-A procyanidin polyphenols	1. Reduction of serum CRP level 2. Reduction of serum turbidity
Hagenlocher, 2015 [130]	Cinnamaldehyde	1. Inhibition of degranulation and mRNA expression 2. Reduction of mediator release 3. Reduction of cytokine expression 4. Reduction of pro-inflammatory mast cell mediators release and expression
Han, 2017 [131]	Essential oil blends of cinnamon	1. Dramatic impacts on levels of protein biomarkers involved in inflammation, immune modulation, and tissue remodeling 2. Effects on signaling pathways such as mitotic roles of the polo-like kinase canonical pathway

Abbreviations: *ACE2* angiotensin-converting enzyme 2, *ERK* extracellular signal-regulated kinase, *NO* nitric oxide, *PGE2* prostaglandin E2, *LTB4* leukotriene B4, *LPS* lipopolysaccharide, *IFN-γ* interferon γ, *TNF-α* tumor necrosis factor-α, *TGF-β* transforming growth factor-β, *IL* interleukin 4, *CRP* C-reactive protein

seasoning worldwide. The main uses of cinnamon include the treatment of flatulence, diarrhea, toothache, fever, leucorrhoea, and headache [69]. Additionally, reports indicate the effectiveness of regular consumption of cinnamon in preventing throat infections. Previous studies have shown that cinnamon contains 21 bioactive molecules, including two well-known compounds, cinnamaldehyde (60.41%) and eugenol (3.19%), which have antibacterial effects. In addition, antimicrobial, antiviral, antifungal, antioxidant, anti-hypertensive, anti-diabetic, anti-tumor, and immune-modulating effects of cinnamon have been reported in recent studies [70–74]. According to one study, a higher dose of cinnamon (100 mg/kg) strongly enhanced serum phagocytic index, immunoglobulin levels, and antibody titers. A lower dose (10 mg/kg) only improved serum immunoglobulin levels [75]. The higher dose promoted cellular and humoral immunity, while the lower dose only affected humoral immunity [75, 76]. Cinnamon, like other herbs, has shown immunomodulatory, antiseptic, and antiviral properties, which can be a complementary treatment in inhibiting inflammation-related diseases, such as COVID-19 [77]. In addition to cinnamaldehyde and eugenol, other important bioactive substances of cinnamon include trans-cinnamaldehyde, cinnamic acid, p-cymene, and essential

oils [78, 79]. The promising activity of eugenol in the treatment of influenza A and Ebola virus, and reports of the antimicrobial, antifungal, and anti-inflammatory properties of this substance are available. Evidence suggests that eugenol inhibits autophagy and replication of influenza A virus by interfering with extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38-MAPK), and inhibitor of nuclear factor-κB (IκB) kinase (IKK) signaling pathways [80–82]. It has also been demonstrated that the active substances in cinnamon suppress expression of cyco-oxygenase-2 (COX-2) and the inducible nitric oxide synthase (iNOS) pathways and therefore diminish the production of inflammatory cytokines such as IL6, IL-1β, and TNF-α [83, 84]. These properties explain the anti-inflammatory and analgesic effects of cinnamon in inflammatory illnesses such as rheumatoid arthritis, diabetes, heart disease, cancer, and neurological disorders such as Alzheimer disease [85, 86]. This suggests that cinnamon may be able to suppress inflammation and disrupt COVID-19 disease complications. Cinnamon extract (CE) and cinnamaldehyde are used as anti-allergic agents by reducing the release and expression of specific mediators of mast cells [87]. CE, p-cymene, and cinnamaldehyde have been shown to potentiate mature monocyte-derived dendritic cells (DCs) and, subsequently, allergen-specific immune responses in the co-generation of human DC-T cells in vitro. Furthermore, these treatments were shown to reduce expression of mast cell-specific proteases, total IgE production, and histamine levels. These results could be due to the suppression of the production of nitric oxide (NO), TNF-α, IL-1β, and IL-6, and blocking MAPK and nuclear factor κB (NF-κB) pathways [88, 89]. Another study showed that nine cinnamon phytochemicals likely have suppressive effects against the SARS-CoV-2 MPro enzyme. Using the available strategies, these naturally derived plant compounds may create a potential reliable drug [90].

4.3.1 Safety of Cinnamon

The FDA stated that cinnamon is well tolerated in amounts commonly found in food. Additionally, the cinnamon extract is secure and exempt from toxicity data requirements by the US Environmental Protection Agency (EPA) [91].

4.4 Other Spices

4.4.1 Black Pepper

Black pepper is another spice with several potential health advantages. Previously, ethanol fractions of *Piper nigrum* have been used in mouse models with ovalbumin-induced asthma, in which IL-1β, IL-4, IL-6, IL-17A, and TNF-α were found to be reduced, and IL-10 and INF-γ increased by the extracts [92]. Inflammatory cell

infiltration and the state of fibrosis were also decreased. In addition, other studies have indicated that piperine was effective in blocking bacterial sepsis mediated by prevention of pyroptosis through reduced levels of IL-1β and AMP-activated protein kinase (AMPK) [93]. Piperine also attenuated acute pancreatitis by diminishing the levels of IL-1β, IL-6, and TNF-α [94].

4.4.2 Saffron

Previous studies have shown that saffron can have favorable effects against a wide range of human diseases, including metabolic syndrome, diabetes, psychological conditions, cancer, neurological disorders, gastrointestinal disorders, and cardiovascular diseases [95–108]. These effects could be mediated through its actions as an immuno-modulatory, antioxidant, anti-inflammatory, anticonvulsant, anti-mutagenic, antidepressant, anti-carcinogenic, and anti-diabetic agent. As an example, crocin, a major component of saffron, was shown to be effective against bacterial lipopolysaccharide (LPS)-induced sepsis and cardiotoxicity in H9c2 cells. In line with this, the biomarkers TNF-α, prostaglandin E2 (PGE2), IL-1β, and IL-6 were significantly downregulated, and NO, COX-2, and iNOS mRNA expression was significantly reduced by this treatment [109]. Also, the efficacy of saffron, particularly crocin and picrocrocin, against infection by herpes simplex virus 1 (HSV-1) and human immunodeficiency virus 1 (HIV-1) has been documented. Crocin and picrocrocin inhibited virus entry and replication [110, 111]. Moreover, the efficacy of saffron in asthmatic patients has been shown [112].

4.4.3 Capsaicin

Capsaicin is one the most important constituents of capsicum, which is useful against diabetes, asthma, cancer, and other diseases [113]. Inflammatory biomarkers such as IL-6, IL-1β, and TNF-α were found to be reduced in response to capsaicin [114]. In addition, this molecule reduced inflammation of the salivary glands by reducing mRNA and protein expression of TNF-α and IL-6 in the human salivary gland (HSG) cell line [115].

4.4.4 Cumin

Cumin is another common spice widely used in food preparation and which has anti-oxidant and anti-inflammatory properties. Cumin has been applied to treat some diseases such as cancer, diabetes, hyperlipidemia, among others [92]. Cumin contains phenols and flavonoids which give it antibacterial, antifungal, and antiviral properties, which may be useful against COVID-19 disease [92].

5 Conclusion and Future Perspectives

In this study, we reviewed the potential mechanisms of spices, including turmeric (curcumin), ginger, and cinnamon, as well as some other spices, such as black pepper, saffron, capsaicin, and cumin, with emphasis on their bioactive properties against different aspects of the SARS-CoV-2 life cycle. The beneficial effects of curcumin on several biomarkers and clinical symptoms of patients with COVID-19 have been shown in multiple clinical studies. However, these studies have been limited such that a definitive conclusion cannot be reached. Also, clinical trials on the actions of ginger, cinnamon, black pepper, saffron, capsaicin, and cumin are scarce. Considering the results of preclinical studies, it is clear that these spices contain a diverse array of bioactive compounds known to decrease oxidative stress and inflammation, modulate the immune system, and prevent viral, bacterial, and fungal infections in COVID-19 patients. In the future, more clinical studies consisting of biomarker-stratified patients and employing biomarker readouts of therapeutic or toxicity-related responses are urgently needed. This will help us to prepare for the next pandemic, which may be on the horizon sooner than we think.

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Chapter 21
Antiviral Mechanisms of Curcumin and Its Derivatives in Prevention and Treatment of COVID-19: A Review

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Abstract The COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has now plagued the world for almost 3 years. Although vaccines are now available, the severity of the pandemic and the current dearth of approved effective medications have prompted the need for novel treatment approaches. Curcumin, as a food nutraceutical with anti-inflammatory and antioxidant effects, is now under consideration for the prevention and treatment of

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COVID-19. Curcumin has been demonstrated to retard the entrance of SARS-CoV-2 into cells, interfere with its proliferation inside cells, and curb the hyperinflammatory state caused by the virus by modulating immune system regulators, minimizing the cytokine storm effect, and modulating the renin-angiotensin system. This chapter discusses the role of curcumin and its derivatives in the prevention and treatment of COVID-19 infection, considering the molecular mechanisms involved. It will also focus on the molecular and cellular profiling techniques as essential tools in this research, as these can be used in the identification and development of new biomarkers, drug targets, and therapeutic approaches for improved patient care.

Keywords Nutraceutical · Phytochemical · Curcumin · COVID-19

1 Introduction

Coronaviruses are single-stranded ribonucleic acid (RNA) viruses belonging to the family of Coronaviridae. They were first recognized as enzootic infection factors and also as human-contaminating agents [1]. Coronavirus disease 2019 (COVID-19) is a newly emerged disease with a rapid rise in mortality cases after its first detection in December 2019 [2]. The international virus classification committee proposed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as the name of the virus which causes COVID-19 disease [3]. The symptoms of COVID-19 infection can resemble those of the common cold and acute respiratory diseases, with infection of the respiration system (e.g., pneumonia or bronchitis) [4]. Compared to the other members of the coronavirus family, such as SARS and Middle East Respiratory Syndrome Coronavirus (MERS-CoV), SARS-CoV-2 has higher transmissibility. The significance of this fact is that this virus can engage a higher number of people through contact with an infected patient [5].

COVID-19 infection results in acute upper respiration symptoms, such as sneezing, sore throat, fever, dry coughs, fatigue, sputum, dyspnea, and headache [6, 7]. Severe cases of this disease are marked by pneumonia, metabolic acidosis, septic shock, and hemorrhage [8]. The laboratory results of most cases indicate a reduction in the number of white blood cells and lymphocytes [6, 9]. In acute cases, neutrophil counts, urea, and creatinine levels also show a significant rise while the number of lymphocytes is reduced. Inflammatory factors, such as interleukin 6 and 17, and necrosis factors, such as tumor necrosis factor (TNF-α), often increase [10]. The current anti-virus treatments target human cells or the virus itself. Currently, there are

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eight approved treatments for use in the European Union, and this field is rapidly evolving [11]. In addition to investigating the effect of the chemical-pharmaceutical agents on the pathogenicity of the virus, several studies have addressed the influence of phytochemicals on coronavirus due to evidence of the antiviral efficacy of some plant-based compounds. Some phytochemicals have also been found to boost the immune system against various diseases through diverse cellular mechanisms [12, 13].

Curcumin is an important phytochemical compound that is extracted from the rhizome of Curcuma longa, also known as the turmeric plant. Turmeric includes an extensive spectrum of phytochemicals, such as curcumin, demethoxycurcumin, zingiberene, curcumenol, eugenol, triethyl curcumin, and turmerones. However, most of the therapeutic features of turmeric have been ascribed to curcumin [14]. Numerous studies have confirmed curcumin's antioxidant and anti-inflammatory effects, making it a viable candidate for treating diseases marked by disturbances in these pathways [15–28]. The anti-inflammatory effects of curcumin are comparable with those of anti-inflammatory steroids and non-steroid drugs [29] and appear to be mediated by inhibition and suppression of the prostaglandins synthesis and inhibition of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), as well as suppression of the production of cytokines, such as gamma interferon (INFγ) and tumor necrosis factor-alpha (TNFα) and activation of transcription factors such as nuclear factor-κB (NF-κB) [30]. Some studies have confirmed the role of curcumin in the inhibition of the proliferation of some viruses, such as human papillomavirus (HPV) and human immunodeficiency virus (HIV) [31]. Antiviral effects have also been proven against COVID-19 disease [32, 33]. Hence, curcumin appears to be a potential phytochemical for preventing and treating COVID-19 infection.

Recently, phytochemical investigations have been conducted to unveil the various molecular effects and pharmacological properties involved in their mechanisms of action [34]. This is important as some patients do not respond to particular drug therapy or suffer from adverse effects limiting the drug development process [35, 36]. Therefore, validated biomarkers that predict the effects of drugs and establish optimal therapeutic dosages are urgently needed [37]. In addition, the emergence of systems biology techniques has brought dawn to researchers in COVID-19 medication, and there are also many new technologies and strategies for drug design that can promote research in this field [38]. Accordingly, this study was conducted to review the molecular mechanisms and methods used in the study of curcumin and its derivatives in the prevention and treatment of COVID-19.

2 Methods

This review was carried out in a narrative manner by searching the databases PubMed, Web of Science, and Science Direct using coronavirus-19, COVID-19, SARS-CoV-2, curcumin, nanocurcumin, turmeric, nutraceutical, and phytochemical keywords without any limiting search items. The main objective was to report on the clinical trials which have investigated the effects of various forms of curcumin in the treatment of COVID-19 patients (Table 21.1).

Table 21.1 Characteristics and results of the studied articles on clinical trials for protective effects of curcumin on COVID-19 disease

Authors [Ref.]	Objective	Methods	Results
Valizadeh et al. [57]	Investigation of the effect of nanocurcumin on modulation of inflammatory cytokines in COVID-19 patients	Intervention groups: 40 healthy controls and 40 COVID-19 patients Intervention: Group 1 received nanocurcumin (160 mg nano-curcumin for 14 days) Group 2 received a placebo Biomarker techniques used to monitor treatment: Assessment mRNA expression and secretion IL-1β, IL-6, TFN-α, IL-18 by real-time PCR and ELISA	Significant decrease after treatment with nanocurcumin in expression and secretion of IL-6 and IL1β
Hassaniazad et al. [59]	Investigation of the effect of nanocurcumin on clinical variations of cellular immunity subgroups of COVID-19	Intervention groups: 40 patients divided into two groups Intervention: Group 1 received nanocucumin capsules (40 mg) 4/day for 2 weeks Group 2 received placebo over the same schedule Biomarker techniques used to monitor treatment: mRNA expression levels measured by PCR Serum levels of cytokines measured on days 0, 7, and 14	TBX21 and FOXP3 mRNA levels were decreased and increased, respectively, between nanocurcumin and placebo groups on day 7 Reduced serum levels of IFN-γ and IL-17 in the nanocurcumin group Increased serum levels of IL-4 and TGF-β in the nanocurcumin group on day 14 compared to the placebo
Pawar et al. [72]	Determining the effect of curcumin / piperine (to optimize absorption) on symptoms in COVID-19 patients	Intervention groups: 140 patients in two groups of case and control Intervention: The case group received curcumin (525 mg) along with piperine (2.5 mg) in tablet form twice a day Biomarker techniques used to monitor treatment: None	Early symptomatic recovery (fever, cough, sore throat, and breathlessness) and better clinical outcomes in patients who received curcumin/piperine

(continued)

Table 21.1 (continued)

Authors [Ref.]	Objective	Methods	Results
Tahmasebi et al. [67]	Investigation of the therapeutic effects of nanocurcumin on frequency and response of Th17 cells in mild and severe COVID-19 patients	Intervention groups: 40 severe COVID-19 patients (admitted to ICU) 40 mild COVID-19 patients Intervention: Prescription nanocurcumin (80 mg 2/day) or placebo Biomarker techniques used to monitor treatment: Measuring frequency of RNA expression of Th17-relevant factors, and serum levels of inflammatory cytokines Flow cytometry used to measure frequency of the Th17 cell population with monoclonal antibodies against surface and intracellular markers	Decreased number of Th17 cells, Th17-related factors, and Th-17-related cytokines levels in the mild and severe COVID-19 patients treated by nanocurcumin
Askari et al. [73]	Investigation of the efficacy of curcumin/piperine on clinical symptoms, duration, severity, and inflammatory factors of COVID-19 patients	Intervention group: 46 COVID-19 patients (23 in each group of case and control) Intervention: Two curcumin/piperine capsules (500 mg curcumin/5 mg piperine) or placebo for 14 days Biomarker techniques used to monitor treatment: Auto-analyzer used to measure CBC, FBS, serum cholesterol, TG, LDL, HDL, VLDL, ALT, AST, LDH, creatinine, BUN, and CRP using commercial kits	Curcumin/piperine co-supplementation in COVID-19 patients significantly reduced weakness, but not other biochemical and clinical indices

(continued)

Table 21.1 (continued)

Authors [Ref.]	Objective	Methods	Results
Asadirad et al. [68]	Evaluation of the effect of nanocurcumin on inflammatory cytokines of hospitalized mild to moderate COVID-19 patients	Intervention group: 60 COVID-19 patients (two groups receiving nanocurcumin or placebo) Intervention: 240 mg nanocurcumin for 7 days Biomarker techniques used to monitor treatment: Record clinical signs and laboratory parameters on days 0 and 7 Measure serum levels of TNF-α, IL-1β, and IL-6 using ELISA kits	Improved clinical manifestations and laboratory parameters by nanocurcumin treatment Decreased IFN-γ and TNF-α mRNAs by nanocurcumin treatment

A secondary objective was to report on the molecular techniques used in these studies as biomarkers of efficacy or toxicities.

3 Results

Results of the studies on the mechanisms of action showed the effect of curcumin on the prevention and treatment of COVID-19 at four stages of the virus life cycle, including entry into the cell, viral replication, cytokine storm effects, and involvement of the renin-angiotensin system. It should be noted that all the hypotheses mentioned in this study are based on the assumption that the immune response against COVID-19 is similar to that caused by other coronaviruses, which should be confirmed with further studies. This is important to aid preparedness for future coronavirus pandemics. These four stages are discussed in the following sections.

3.1 Cellular Entry of SARS-CoV-2 and Curcumin

Angiotensin-converting enzyme 2 (ACE2) receptor on host cell surface acts as an attachment and entry port for the SARS-CoV-2 virus. The SARS-COV-2 spike glycoprotein has two structural subunits (S1 and S2), which play separate roles in identifying and binding to the receptor and promoting fusion with the host cell membrane [39]. The attachment stage occurs through the binding of the receptor-binding

domain (RBD) in the S1 subunit to ACE2 on the target cells [40]. Studies have shown that curcumin interacts with and can block this stage of SARS-COV-2 infection [41]. Various amino acid residues of ACE2 (e.g., alanine 348, asparagine 394, glutamate 402, histidine 378, and Tyrosine 385) have been identified as being in the active binding site in the interaction with curcumin [41]. Also, curcumin reduces the expression of TMPRSS-2 [42], which is one of the main activating proteases of host cells, permitting entry of the SARS-Cov-2 virus [39]. This enzyme cleaves the spike glycoprotein between the S1 and S2 domains to allow the fusion of the S2 subunit with the host cell membrane. Moreover, curcumin has a high binding affinity to the SARS-CoV-2 nucleocapsid proteins, which regulates replication of the viral RNA, inhibits protein translation, alters the cell cycle, and promotes apoptosis in host cells [43]. Thus, some of the antiviral properties of curcumin might arise by blocking the actions of this protein.

3.2 SARS-CoV-2 Proliferation and Curcumin

A large number of studies have investigated the key factors and enzymes involved in SARS-CoV-2 replication. Most of these have been carried out on the RNA-dependent RNA polymerase (RdRp) and the main protease (MPro; a 3CL-like enzyme). The MPro enzyme is responsible for the proteolytic cleavage of the viral polyprotein into distinct active peptides and the RdRp, which is also known as non-structural protein 12 (NSP12), is responsible for catalyzing the synthesis of the new viral RNA as part of the replication and transcription process [44]. In silico molecular docking studies have suggested that curcumin can bind directly to the MPro enzyme and the RdRp [45–47]. Other molecular docking studies have shown the effects of curcumin in blocking SARS-CoV-2 reproduction by targeting NSP9 of the viral replicase. NSP9 binds to single-stranded RNA and works in concert with the RdRp complex in the replication process [48]. Although a number of drugs have now been identified which can inhibit the activity of the RdRp complex, as well as the MPro enzyme and NSP9 [49], these will require further in vitro and in vivo validation. However, as curcumin has shown inhibitory properties against the viral replication cycle, it is possible that it achieves these effects by targeting some or all of the above SARS-CoV-2 proteins [50, 51]. Thus, curcumin has the potential to interfere with the process of replication of SARS-CoV-2 RNA in the generation of new virus particles.

3.3 COVID-19, the Cytokine Storm, and Curcumin

In any type of viral infection, inflammatory cytokines such as IL-1, IL-6, and TNF-α are actively released by immune cells into the bloodstream [51]. The release of large amounts of cytokines into the systemic circulation is often referred to as a cytokine

storm [52]. Such an increase in cytokine levels in COVID-19 cases is associated with conditions related to acute respiratory distress syndrome (ARDS) and multiple organ damage, which can lead to a poorer prognosis [53, 54]. Curcumin also shows promise as a novel and effective treatment of the cytokine storm effects as the immunomodulatory activities of this molecule have been well established in different studies [32, 55, 56]. The ability of curcumin to suppress the cytokine storm and its potential in treating viral disorders, including those caused by coronaviruses, supports the case that it may be an effective treatment for COVID-19 [32]. The inflammatory factors released in the cytokine storm include IL-1, IL-2, IL-6, IL-10, transforming growth factor-β (TGF-β), interferons (IFNs), and TNF-α. The expression of IL-6 and TNF-α is mainly associated with COVID-19-related ARDS, which may contribute to organ damage in severe cases [53, 54]. Curcumin has a suppressive effect on IFN-α, and its lowering effect on inflammatory cytokines has been attributed to the inhibition of NF-κB signaling [55]. In a study conducted on the effects of nanocurcumin on the modulation of the inflammatory cytokines in COVID-19 patients, curcumin was found to temper the virus-related increase in inflammatory cytokines at both the mRNA and protein levels [57]. In another study of mild and severe nanocurcumin-treated COVID-19 patients, a significant decrease was observed in the number of inflammatory markers, including pro-inflammatory Th17-related cytokines [58]. Hassaniazad et al. also investigated the effect of curcumin nanomicelles in a clinical study on the cellular immune response in COVID-19 patients [59]. This revealed a rise in the levels of the anti-inflammatory cytokines IL-4 and TGF-β in the group receiving nanocurcumin, compared to the placebo group on the day 14.

The effect of curcumin to stimulate the production of anti-inflammatory factors may aid in at least a partial restoration of the cytokine balance by modulating key regulatory elements of immune and inflammatory pathways, thereby reducing the cytokine storm response to viral infection and minimizing the potentially damaging oxidizing effects of excessive reactive oxygen species (ROS) production [32].

3.4 Renin-Angiotensin System, SARS-CoV-2, and Curcumin

ACE2 present on the surface of host cells provides a target for the spike glycoprotein of SARS-CoV-2 as an entry point for viral infection via endocytosis [40]. Simultaneous internalization of ACE2 has been reported during cellular entry of SARS coronaviruses, including SARS-CoV-2 [39, 60]. As ACE2 normally acts to inactivate angiotensin II (AngII), a decrease in cell surface ACE2 levels can lead to the accumulation of this peptide hormone and high levels of AngII have been associated with acute lung injury in COVID-19 patients [54]. The mechanism of this is likely due to the fact that AngII is a peptide hormone that acts as a vasoconstrictor, which can lead to high blood pressure and trigger an inflammatory response. High AngII levels can stimulate the AT1 angiotensin receptor, which can have multiple adverse effects on physiology, including those mentioned above, as well as fibrosis and ARDS [61].

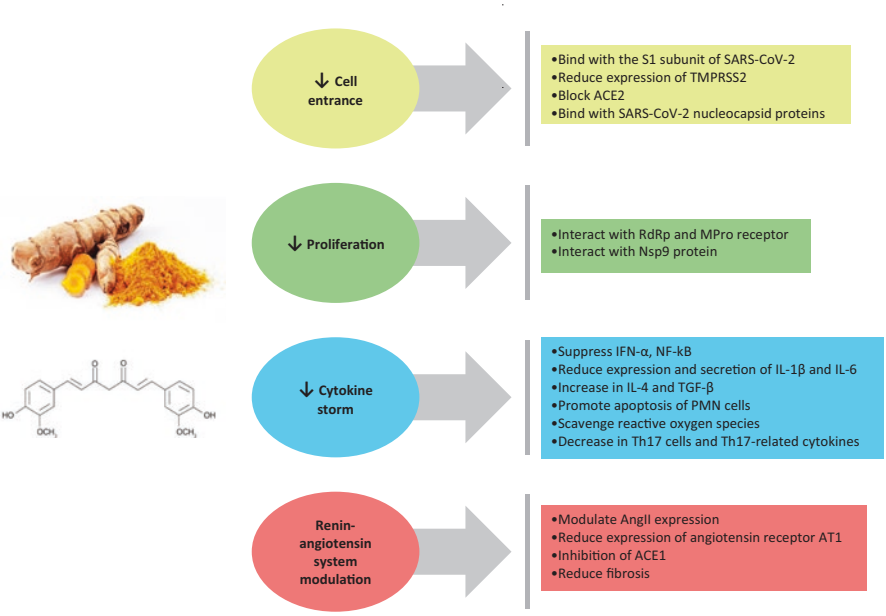

Fig. 21.1 The potential inhibitory mechanisms of curcumin on COVID-19 prevention and treatment

Curcumin has been reported to modulate the level of AngII expression and prevent inflammation-associated fibrosis [62]. Furthermore, the modulation of ACE2 levels by curcumin has also been documented [62, 63]. Such modulation of ACE2 expression by curcumin could lead to reduced AngII cell signaling and the subsequent damage and pathological consequences. Furthermore, curcumin has been found to inhibit high blood pressure by lowering the expression of angiotensin 1 converting enzyme (ACE1) in a rat model of hypertension [64]. Curcumin has also been found to reduce the expression of the angiotensin receptor AT1 in an AngII infusion model of fibrosis in rats [62]. Consistent with this, curcumin treatment was found to reduce AngII-induced hypertension in a mouse model [65].

Taken together, these findings indicate that treatment with curcumin can be used in the treatment of COVID-19 disease effects via multiple complementary pathways (Fig. 21.1).

4 Cellular and Molecular Profiling Techniques Used in the Assessment of Curcumin Efficacy in the Treatment of COVID-19 Disease

Different cellular and molecular profiling techniques were used to evaluate the biomarkers linked with the mechanism of action of curcumin treatment in the above studies. Valizadeh et al. used both real-time PCR and an enzyme-linked

immunosorbent assay (ELISA) approach for evaluating the effects of curcumin on the production of the IL-1β, IL-6, IL-18, and TNF-α in peripheral blood mononuclear cells (PBMCs) and serum from COVID-19 patients [57]. The real-time quantitative PCR method allowed simultaneous multiplex detection of the different cytokines through the use of nucleotide probes linked with different fluorescent tags, as described by Hawkins and Guest [66]. In a similar manner, Hassaniazad et al. used real-time PCR analysis to examine immune response gene expression changes in the transcription factors TBX21, GATA-3, FOXP3, and RAR-related orphan receptor γt (ROR-γT) and ELISA to measure the serum levels of IFN-γ, IL-4, IL-17, and TGF-β cytokines in investigating the effects of curcumin nanomicelles on clinical cellular immune responses in COVID-19 patients [59]. Tahmasbi et al. used a flow cytometry technique to measure the frequency of circulating Th17 cells in patients with COVID-19 and healthy subjects using monoclonal antibodies against surface and intracellular markers [67]. They also evaluated mRNA expression profiles of Th17 cell-related factors RORγt, IL-17, IL-21, IL-23, using real-time PCR and the secreted levels of serum IL-17, IL-21, IL-23, and granulocyte-macrophage colony-stimulating factor (GM-CSF) via ELISA. Similar approaches were employed by Asadirad et al. in the measurement of TNF-α, IL-1β, IL-6, and IFN-γ inflammatory cytokines by the real-time PCR method and analyses of serum levels of TNF-α, IL-1β, and IL-6 using cytokine ELISA kits [68].

Other multiple analyte probing techniques have also been described, which can also be applied in the study of risk factors for COVID-19 and for assessing the effects of this disease in cells and circulation, and also for monitoring the response to various pharmaceutical and phytochemical treatments such as curcumin. These techniques include multiplex immunoassay [69] and biochip arrays [70] for the simultaneous measurement of multiple analytes such as inflammation- and immune-related factors. Given the multi-faceted nature of COVID-19 disease, the multiplex biomarker technologies listed above enable the simultaneous analysis of numerous analytes for interrogation of disease and treatment effects on specific protein pathways such as inflammation and oxidative damage. There are also methods for measuring effects on entire protein pathways, such as kits developed for determining total antioxidant capacity and coagulation status [71] in blood samples. Figure 21.2 indicates some of the main multiplex molecular profiling technologies employed in studying the effects of curcumin on COVID-19 disease.

5 Conclusions and Future Perspectives

In this chapter, we have summarized the disrupted molecular pathways that are potentially targeted by the phytochemical curcumin in COVID-19 disease prevention and treatment. We have also described the main methods which have been used to investigate these effects, with a focus on multiplex molecular profiling techniques such as real-time PCR and multi-analyte immunoassay. Curcumin has protective effects in preventing viruses from entering the cells, reducing virus proliferation,

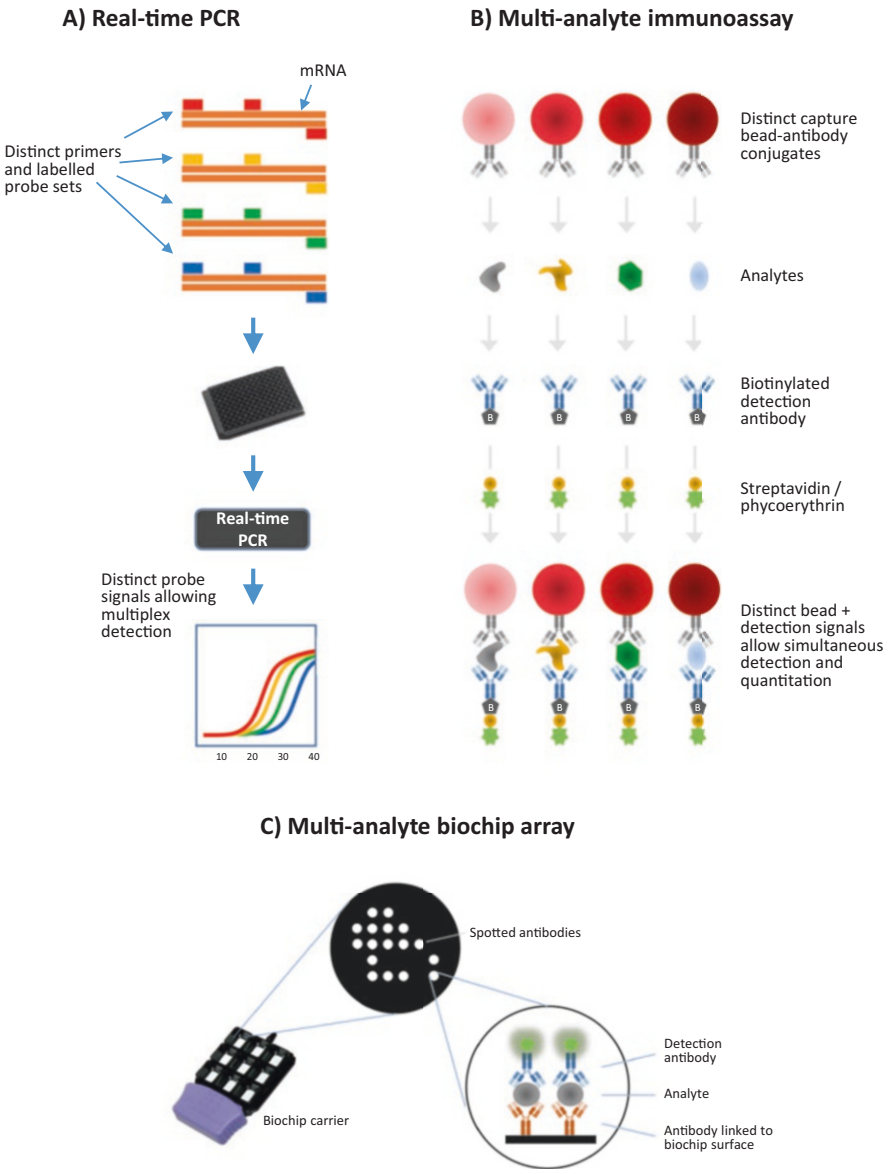

Fig. 21.2 Multiplex molecular profiling technologies employed in studying the effects of curcumin on COVID-19 disease. (**a**) Real-time PCR. (**b**) Multi-analyte immunoassay. (**c**) Multiplex biochip array

decreasing the cytokine storm effects, and modulating the renin-angiotensin pathway. Further studies on the possible use of natural compounds such as curcumin and the application of the appropriate molecular profiling approaches to monitor disease and treatment effects can lead to improved management of coronavirus and other viral infections during the current pandemic and future outbreaks.

Competing Interests MM is the founder of Sami-Sabinsa group of companies.

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Chapter 22
Evaluation of Curcumin-Piperine Supplementation in COVID-19 Patients Admitted to the Intensive Care: A Double-Blind, Randomized Controlled Trial

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Abstract

Background

Curcumin is a traditional remedy for diseases associated with hyper-inflammatory responses and immune system impairment. Piperine, a bioactive compound in black pepper, has the potential to enhance curcumin bioavailability. 0This study aims to examine the effect of the curcumin-piperine co-supplementation in patients infected with SARS-CoV-2 and admitted to the intensive care unit (ICU).

Material and Methods

In this parallel randomized, double-blind, placebo-controlled trial, 40 patients with COVID-19 admitted to ICU were randomized to receive three capsules of curcumin (500 mg)-piperine (5 mg) or placebo for 7 days.

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Results
After 1 week of the intervention, serum aspartate aminotransferase (AST) ($p = 0.02$) and C-reactive protein (CRP) ($p = 0.03$) were significantly decreased, and hemoglobin was increased ($p = 0.03$) in the curcumin-piperine compared to the placebo group. However, compared with the placebo, curcumin-piperine had no significant effects on the other biochemical, hematological, and arterial blood gas and 28-day mortality rate was three patients in each group ($p = 0.99$).

Conclusion
The study results showed that short-term curcumin-piperine supplementation significantly decreased CRP, AST, and increased hemoglobin in COVID-19 patients admitted to the ICU. Based on these promising findings, curcumin appears to be a complementary treatment option for COVID-19 patients, although some parameters were not affected by the intervention.

Keywords Curcumin · Piperine · SARS-CoV-2 · COVID-19 · ICU · CRP

1 Introduction

The COVID-19 outbreak began in Wuhan, China, in December 2019 and spread quickly to other countries [1]. Based on its genome similarity of 79% to Coronaviruses, this new strain was called SARS-CoV-2 [2]. Many new SARS-CoV-2 variants have emerged since the first outbreak, despite isolation, lockdown, and other containment measures [3]. Recently, the WHO reported 600 million cases of confirmed COVID-19 and over 6.5 million deaths [4]. Even with rapid advances in public vaccination, the disease remains a major public health concern [5] and has negatively affected people's lives [6]. Despite early determination of the SARS-CoV-2 structure and the development of some effective treatments and vaccines [7], the virus continued to spread and the pathogenesis is still not entirely clear. However, it appears that a cytokine storm effect caused by alteration of the immune system

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plays a crucial role in disease effects [8]. The cytokine storm effect can lead to inflammatory responses and changes in hematologic parameters, leading to damaging effects such as severe lung damage, liver injury, and death in some cases [9–13].

A number of traditional compounds have shown some promise in curbing some of these effects and prove effective as well-tolerated alternate therapies for COVID-19 infection. Curcumin is a bioactive polyphenol with a multitude of pharmacological effects [14–21] and a number of recent studies have shown that this compound has beneficial effects on diseases associated with hyperinflammatory responses and immune system impairment, such as COVID-19 [8, 22–26]. Many preclinical and clinical studies have indicated the health benefits and safety (tolerated up to 12 g/day) benefits of this nutraceutical [27, 28]. Additionally, a wide range of pharmacological and biological activities have been attributed to its therapeutic mechanism of action, including immunomodulatory, anti-tumor, antimicrobial, antiviral, antioxidant, and anti-inflammatory properties [29–32]. However, the poor solubility in aqueous solutions, extensive metabolism in the liver and intestine, and rapid elimination of curcumin result in low bioavailability. To overcome this issue, compounds, such as piperine, a bioactive compound in black pepper, have been used to enhance curcumin absorption, inhibit metabolic enzymes, and limit curcumin clearance through the P glycoprotein efflux pump [33, 34]. Adding piperine to curcumin can significantly increase its bioavailability in humans [34]. Few studies have shown the benefits of curcumin in COVID-19 infection, but none have investigated the impact of curcumin-piperine supplementation in patients in intensive care units (ICUs). Thus, this study aims to examine the effect of the administration of curcumin-piperine supplementation on ICU patients infected with SARS-CoV-2.

2 Material and Methods

2.1 Study Design and Participants

This parallel randomized, double-blind, placebo-controlled trial assessing the efficacy of co-supplementation of curcumin-piperine on COVID-19 patients admitted to ICUs of Alzahra hospital, an academic hospital affiliated with Isfahan University of Medical Sciences, Isfahan, Iran, between June and September in 2021. The summary of the study protocol was published earlier [35]. The protocol was approved by the ethics committee of the Isfahan University of Medical Sciences (ethic code: IR.MUI.RESEARCH.REC.1400.057) and conducted based on the principles of the Declaration of Helsinki. The trial was also registered in the Iranian Registry of Clinical trials (IRCT) with ID: IRCT20121216011763N52. Before starting the study, the objectives and procedures of the trial were explained to patients or their caregivers, and written informed consent was obtained from all participants. Patients with a definitive diagnosis of COVID-19 confirmed via real-time polymerase chain reaction (RT-PCR), 30–70 years-old, and who were admitted to the ICUs, were

included. Exclusion criteria were as follows: unstable hemodynamic status, renal or liver disease, undergoing dialysis, cancer patients undergoing chemotherapy, and pregnancy. The other exclusion criteria included use of parenteral nutrition, taking anticoagulant drugs such as warfarin and having a history of sensitivity to herbal products such as turmeric and pepper. Patients were withdrawn from the trial if they were unwilling to continue or showed any adverse effects.

2.2 Randomization and Blinding

A total of 40 patients were randomized in a ratio of 1:1 into two groups. An independent statistician conducted the sequencing of the assignment using a table of random numbering and this was kept in opaque, sealed, numbered envelopes until the end of the assessment of the eligibility criteria. Curcumin-piperine and placebo capsules were provided in identical formats with the same shape, size, color, and odor. Participants, investigators, laboratory staff, outcome assessors, and data analyzers were blinded to treatment assignments until the completion of data analyses.

2.3 Intervention

Patients in the intervention group received three curcumin piperine capsules containing 500 mg curcumin and 5 mg piperine per capsule, amounting to a total of 1500 mg curcumin and 15 mg piperine in a day. Capsules were administered orally or with enteral nutrition (gavage) at 9 am, 3 pm, and 9 pm (6 h apart). The duration of the intervention was 7 days. Patients in the control group received three matched placebo capsules a day, each containing 505 mg maltodextrin (1515 mg maltodextrin/day). All capsules were provided by Sami-Sabinsa Group Limited (Bangalore, India). The intervention was started 24–48 h after admission to the ICU when hemodynamic resuscitation and stabilization were carried out and when patients received at least 70% of their energy requirements based on 25 kcal/kg body weight. All patients continued standard treatment as per the physician's prescriptions and were allowed to take their usual medications without any limitations.

2.4 Outcome Measures and Data Collection

Acute physiology and chronic health evaluation II (APACHE II) and NUTRIC score were calculated to assess COVID-19 disease severity and nutritional status of the patients, respectively, at the beginning of the study. Blood samples (5 mL) were obtained early in the morning after approximately 6 h fasting before and after the intervention. These were left for 60 min to allow clotting and centrifuged at room

temperature for 10 min to isolate serum, which was stored at −80 °C until use. The parameters measured were serum calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), chloride (Cl), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin (ALB), C-reactive protein (CRP), complete blood count (CBC) including white blood cells (WBCs), red blood cells (RBCs), hemoglobin (Hb), hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), blood urea nitrogen (BUN), serum creatinine (Cr), prothrombin time (PT), and partial phromboplastin time (PTT). These parameters were assessed at baseline and end of the study at the laboratory center of Alzahra hospital using enzymatic methods and auto-analyzer with commercial kits (Pars Azmun, Karaj, Iran). Furthermore, arterial blood gas (ABG) was taken while the patient was breathing room air.

2.5 Statistical Analysis

The statistical package for the social sciences (SPSS) software version 16 (SPSS Inc., Chicago, IL, USA) was used to analyze data. Paired sample t and chi-squared tests were used to analyze within-group differences. The differences between the groups were assessed using independent student's t-test. Data were reported as mean ± standard deviation (SD) or frequency (percentage). Analysis of covariance (ANCOVA) was used to compare the mean values of continuous outcomes at the end of the study between two groups, considering adjustment for baseline values. Chi-squared or Fisher exact tests were used to compare qualitative outcomes between groups. A p-value less than 0.05 was considered statistically significant.

3 Results

A total of 94 patients were assessed for eligibility, 42 patients were excluded for not meeting inclusion criteria, and 12 persons refused to participate in the study (Fig. 22.1). After this, patients (19 men and 21 women) were randomized to receive the curcumin-piperine (n = 20) or maltodextrin (n = 20) capsules in three divided doses for 7 days. One subject in the curcumin piperine group and one subject in the control group died before the end of the study, and thus analyses were conducted on 38 patients (19 patients in the intervention and 19 samples in the control groups).

The baseline characteristics of patients were comparable between the groups. There was no significant difference between the groups in any of the baseline characteristics, including age, sex, APACHII, or NUTRIC scores (Table 22.1). The effects of curcumin-piperine supplementation on selected metabolic and biochemical parameters are shown in Table 22.2. The intra-group comparison showed a decreasing trend in serum AST in the curcumin-piperine group (p = 0.08) and a

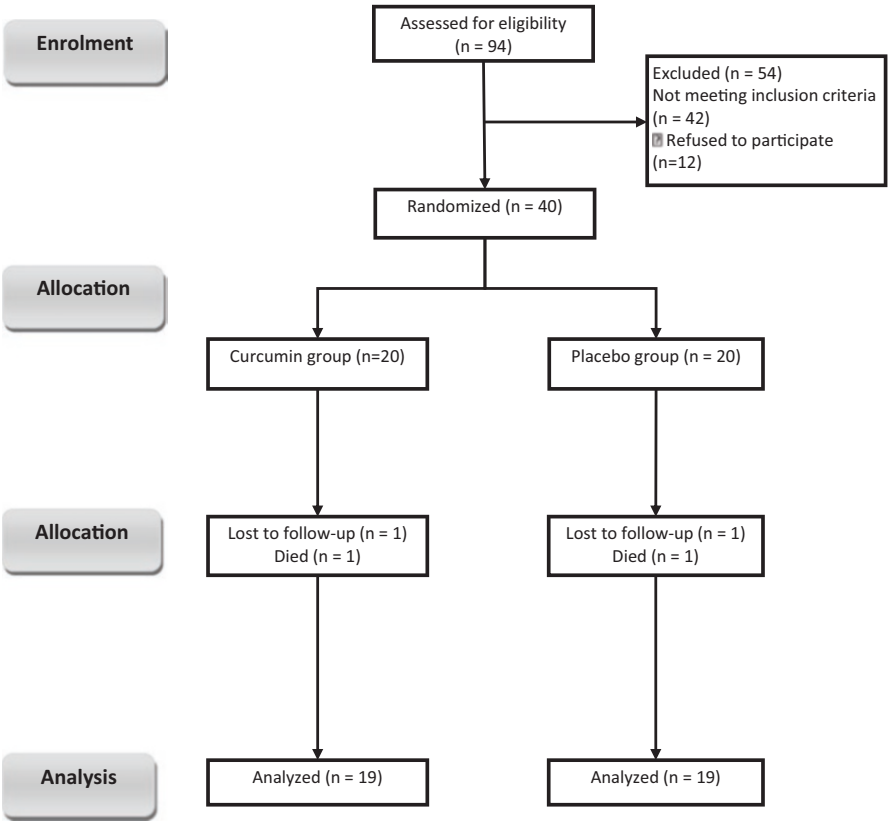

Fig. 22.1 Flowchart showing patient selection

Table 22.1 Summary of baseline characteristics of the patients

Variables	Intervention (20)	Placebo group (n = 20)	P-values
Age, y	50.26 ± 8.83	54.95 ± 12.58	0.513[a]
Sex (%men)	10(50)	9(45)	0.75[b]
APACH II	19.65 ± 5.51	17.30 ± 4.81	0.15[a]
NUTRIC	3.95 ± 1.50	3.70 ± 1.12	0.55[a]

Data are presented as mean ± SD or number (percent)
APACH acute physiology and chronic health evaluation
[a]Based on independent sample t-test
[b]Based on Pearson chi-squared test

significant increase in the level of AST in the placebo group (p = 0.03). Furthermore, compared to the baseline, after 7 days of intervention, a non-significant increase was found in the serum levels of BUN (p = 0.09), Cr (p = 0.07), ALT (p = 0.09) in the placebo and for ALP (p = 0.08) in the curcumin piperine group. Based on the inter-group comparisons, it was found that the AST (p = 0.02) and CRP (p = 0.03)

Table 22.2 Changes in metabolic and biochemical parameters during the study

	Curcumin-piperine group (n = 19)				Placebo group (n = 19)				
Variables	Baseline	Week one	MD ± SE[a]	P-value	Baseline	Week one	MD ± SE[a]	P-value[a]	P-value[b]
BUN (mg/dL)	23.3 ± 7.5	22.9 ± 9.4	−0.4 ± 1.8	0.86	30.0 ± 14.9	36.3 ± 26.5	6.3 ± 3.6	0.09	0.30
Cr (mg/dL)	1.0 ± 0.2	1.3 ± 1.6	0.3 ± 0.4	0.39	1.0 ± 0.3	1.4 ± 1.2	0.4 ± 0.2	0.07	0.77
ALT (IU/L)	58.7 ± 29.1	50.7 ± 43.0	−8.0 ± 10.7	0.46	31.4 ± 12.9	99.0 ± 171.2	67.6 ± 169.4	0.09	0.18
AST (IU/L)	42.7 ± 24.6	35.9 ± 19.3	−6.7 ± 3.6	0.08	29.9 ± 9.6	47.4 ± 33.0	17.5 ± 7.5	**0.03**	**0.02**
ALP (IU/L)	176.0 ± 62.9	206.1 ± 103.8	30.1 ± 16.3	0.08	221.1 ± 79.5	237.8 ± 85.9	16.7 ± 17.5	0.35	0.75
CRP (mg/L)	39.5 ± 35.1	27.8 ± 33.9	−11.6 ± 7.3	0.12	36.6 ± 33.2	49.5 ± 39.5	12.9 ± 8.7	0.15	**0.03**

Significant values ($p < 0.05$) shown in bold text

Data are expressed as means ± SD; p-value <0.05 is significant

MD mean differences, *SE* standard error, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *ALP* alkaline phosphatase, *BUN* blood urea nitrogen, *Cr* creatinine, *CRP* C-reactive protein

[a]Based on paired t-test

[b]Based on analysis of covariance (ANCOVA)

levels significantly decreased in the intervention group in comparison to the placebo group. However, there were no significant differences regarding BUN, Cr, ALT, and ALP between groups.

The effects of curcumin-piperine supplementation on hematological parameters are presented in Table 22.3. Within-group comparisons indicated that one-week supplementation with curcumin piperine led to a significant increase in MCV ($p = 0.009$) and a significant decrease in platelets ($p = 0.02$), while there was no significant change regarding other variables. Also, in the placebo group, the lymphocyte count showed a significant increase ($p = 0.01$), while hemoglobin ($p = 0.07$) and MCHC ($p = 0.08$) showed a non-significant decrease. Between-group analysis showed that in comparison to the placebo, curcumin-piperine supplementation significantly increased the serum level of hemoglobin ($p = 0.03$). Minerals and ABG parameters and their changes are presented in Table 22.4. At the end of the intervention, we observed a significant increase in pCO_2 ($p = 0.02$) and a decrease in pH ($p = 0.01$) in the curcumin-piperine compared to the placebo group. The only significant finding in the placebo group was a decrease in Cl levels ($p = 0.02$). There was no significant difference in minerals and ABG gas parameters between the two groups (p for all > 0.05). Finally, the 28-day mortality rate was 3 (15%) patients in each group, with no statistical difference between the groups ($p = 0.99$).

4 Discussion

The results of this study suggest that curcumin-piperine consumption is efficacious and safe in COVID-19 patients. Recent studies revealed that this polyphenol could positively affect disease symptoms such as sore throat, cough, fever and weakness, O_2 saturation, and length of hospital stay [30, 36, 37]. The main findings of our study are that CRP and AST levels decreased, and hemoglobin concentration increased significantly with curcumin-piperine supplementation for 7 days in COVID-19 ICU patients.

A number of prior studies have obtained similar results regarding anti-inflammatory effects of curcumin in COVID-19. A previous randomized-controlled trial on 60 COVID-19 patients revealed that subjects receiving 160 mg of curcuminoids daily had reduced CRP levels than placebo [38], as we found here. It has also been shown that other inflammatory markers such as IL-6 and IL-1β are also reduced due to curcumin supplementation [22, 39, 40]. A systematic review performed in 2022 indicated that curcumin supplementation reduced not only pro-inflammatory cytokines but also was effective in increasing IL-10, IL-35, and TGF-a as anti-inflammatory cytokines [8]. These effects are most likely driven by the curcumin modulation of inflammatory signaling pathways such as the nuclear factor-κB (NF-kB), mitogen-activated protein kinase (MAPK), activator protein 1 (AP-1), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) transcription factors [24].

Table 22.3 Hematological analysis of blood samples and their changes in the intervention and placebo groups at baseline and at the end of the study

	Curcumin-piperine group (n = 19)				Placebo group (n = 19)				
Variables	Baseline	Week one	MD ± SE[a]	P-value[a]	Baseline	Week one	MD ± SE[a]	P-value[a]	P-value[b]
ALB (g/dL)	2.9 ± 0.3	3.0 ± 0.4	0.1 ± 0.1	0.23	2.9 ± 0.2	2.8 ± 0.4	−0.1 ± 0.1	0.15	0.06
WBC ($\times 10^9$/L)	18.1 ± 26.3	13.0 ± 4.7	−5.2 ± 5.5	0.36	13.0 ± 6.1	11.8 ± 5.6	−1.2 ± 1.2	0.33	0.70
Lym ($\times 10^9$/L)	6.9 ± 2.7	8.4 ± 5.4	1.5 ± 1.1	0.20	5.7 ± 2.6	7.6 ± 4.5	1.9 ± 0.7	**0.01**	0.72
NEUT ($\times 10^9$/L)	87.3 ± 4.3	85.6 ± 6.0	−1.8 ± 1.5	0.26	89.4 ± 5.2	86.9 ± 6.4	−2.5 ± 1.5	0.12	0.80
RBC ($\times 10^{12}$/L)	4.2 ± 0.7	4.2 ± 0.6	−0.02 ± 0.11	0.87	4.3 ± 0.8	4.1 ± 0.8	−0.2 ± 0.1	0.17	0.37
HB (g/dL)	11.9 ± 2.13	12.0 ± 1.8	0.2 ± 0.4	0.65	11.4 ± 1.6	10.9 ± 1.4	−0.5 ± 0.3	0.07	**0.03**
HCT (%)	34.9 ± 5.6	35.3 ± 4.9	0.4 ± 0.9	0.67	33.8 ± 4.4	33.2 ± 4.2	−0.6 ± 0.9	0.49	0.20
MCV (fL/cell)	83.0 ± 5.2	84.2 ± 4.3	1.23 ± 0.4	**0.009**	81.1 ± 8.6	78.6 ± 14.6	−2.5 ± 2.7	0.37	0.19
MCH (pg)	28.0 ± 2.9	28.1 ± 3.5	0.1 ± 0.4	0.83	27.6 ± 3.0	27.5 ± 3.5	−0.1 ± 0.5	0.93	0.84
MCHC (g/dL)	34.3 ± 1.9	34.1 ± 1.7	−0.2 ± 0.4	0.66	34.0 ± 0.9	33.2 ± 2.0	−0.8 ± 0.4	0.08	0.19
Platelets ($\times 10^9$/L)	198.4 ± 80.7	160.1 ± 68.3	38.3 ± 15.3	**0.02**	200.7 ± 90.3	178.2 ± 97.6	−22.5 ± 14.0	0.12	0.39
PT (s)	14.6 ± 7.6	12.1 ± 3.8	−2.5 ± 1.3	0.07	12.6 ± 1.3	12.6 ± 9.7	0.01 ± 2.3	0.99	0.64
PTT (s)	30.6 ± 4.4	34.1 ± 10.2	3.5 ± 2.5	0.18	31.8 ± 11.0	29.3 ± 7.9	−2.5 ± 3.4	0.47	0.14

Significant values ($p < 0.05$) shown in bold text

Data are expressed as means ± SD; p-value <0.05 is significant

MD mean differences, *SE* standard error, *ALB* albumin, *HB* hemoglobin, *Lym* lymphocytes, *HCT* hematocrit, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *NEUT* neutrophils, *PT* prothrombin time, *PTT* partial thromboplastin time, *RBC* red blood cells, *WBC* white blood cells

[a]Based on paired t-test

[b]Based on analysis of covariance (ANCOVA)

Table 22.4 Changes in some minerals and PH during the study

	Curcumin group (n = 19)				Placebo group (n = 19)				
Variable	Baseline	Week one	MD ± SE[a]	P-value[a]	Baseline	Week one	MD ± SE[a]	P-value[a]	P-value[b]
Na (mM)	137.3 ± 3.2	137.8 ± 3.1	0.5 ± 0.7	0.45	141.2 ± 5.6	140.0 ± 5.1	−1.2 ± 1.4	0.39	0.62
K (mM)	4.4 ± 0.4	4.6 ± 0.6	0.2 ± 0.1	0.18	4.6 ± 1.0	4.8 ± 0.7	0.2 ± 0.3	0.51	0.24
P (mg/dL)	3.2 ± 0.7	3.2 ± 0.7	0.02 ± 0.2	0.89	3.1 ± 0.5	3.3 ± 0.8	0.1 ± 0.2	0.48	0.66
Mg (mg/dL)	2.0 ± 0.2	2.0 ± 0.2	0.1 ± 0.1	0.42	2.1 ± 0.2	2.1 ± 0.2	0.01 ± 0.03	0.76	0.68
Cl (mEq/L)	105.4 ± 4.3	104.5 ± 4.6	−0.8 ± 0.9	0.33	107.1 ± 4.8	105.2 ± 3.8	−1.9 ± 0.8	**0.02**	0.66
Ca (mg/dL)	8.0 ± 0.7	8.2 ± 0.5	0.1 ± 0.2	0.42	8.0 ± 0.6	8.2 ± 0.6	0.2 ± 0.1	0.23	0.96
pH	7.5 ± 0.04	7.4 ± 0.1	−0.03 ± 0.01	**0.01**	7.2 ± 0.1	7.4 ± 0.1	−0.1 ± 0.02	0.09	0.23
$_{p}CO_2$ (mmHg)	39.5 ± 8.0	48.2 ± 15.6	8.7 ± 3.5	**0.02**	43.8 ± 10.3	48.8 ± 11.4	5.0 ± 3.1	0.12	0.82
HCO_3 (mEq/L)	26.3 ± 4.6	30.0 ± 9.8	3.7 ± 2.3	0.12	28.3 ± 7.7	28.1 ± 6.1	−0.2 ± 1.7	0.90	0.32

Significant values ($p < 0.05$) shown in bold text

Data are expressed as means ± SD; p-value <0.05 is significant

MD mean differences, *SE* standard error, *Na* sodium, *K* potassium, *Ca* calcium, *Cl* chlorine, *Mg* magnesium, *P* phosphorus

[a]Based on paired t-test

[b]Based on analysis of covariance (ANCOVA)

Higher levels of liver enzymes have been observed in many COVID-19 patients, which is related to the severity of the disease and mortality risk [41, 42]. It has been proposed that elevated levels of ALT and AST indicate a possibility of COVID-19 recurrence [43]. The lowering effects of curcumin on ALT and AST have been shown in some animal and human studies [44–47]. In the present clinical trial, the administration of curcumin-piperine combination reduced the levels of AST in COVID-19 patients compared to the placebo. In the intervention group, both ALT and AST levels decreased at the end of the study compared to the baseline, although these changes did not reach statistical significance. However, AST was significantly increased in the placebo group which may be an indicator of worsening severity. This could have been caused by uncontrolled inflammation, hypoxia, and potential hepatocyte damage caused by the viral infection and replication process in those patients receiving the placebo [48]. Although BUN and creatinine did not change significantly in the curcumin group, these markers increased in the placebo group compared to the baseline as a potential indicator of impaired renal function [49]. Increased BUN and creatinine levels also serve as risk factors for a more severe disease course and increased mortality [49, 50].

We also found that hemoglobin concentrations were significantly increased in individuals who received curcumin-piperine compared to those in the placebo group. Furthermore, hemoglobin concentrations and MCHC values showed non-significant decreases in the placebo group compared with the baseline values. This is consistent with a study by Huang et al. which found that approximately 38% of COVID-19 patients had decreased levels of hemoglobin [51]. In addition, Fouad et al. concluded that hemoglobin concentration is a helpful indicator of disease severity [52]. The effective transport of oxygen in the blood is directly influenced by the hemoglobin concentration and, when an infection occurs, the peripheral tissues require more oxygen, which may result in disease complications like hypoxia and ischemia [53]. This is also consistent with our finding in the curcumin-piperine group of a significant increase in MCV, which is an indicator of red blood cell volume.

Finally, there was no difference in the 28-day mortality rate between the intervention and control groups. This result is not in line with another study which showed that supplementation curcumin-piperine two times per day over 2 weeks reduced the mortality rate in COVID-19 patients [37]. However, it is possible that the larger sample size and longer treatment used in the above mentioned study accounts for this difference.

Our work has some limitations. First, the sample size was relatively small which may have impacted on our ability to detect some significant changes or differences between the groups. Second, the duration of this study was short, although it is common approach in trials of critically ill patients. Finally, the number of biomarkers and physiological parameters that we measured was small and could be expanded to include other inflammation-related analytes, such as cytokine arrays or multiplex immunoassay panels [54–57].

In conclusion, the results of the current randomized controlled trial revealed that short-term curcumin-piperine supplementation is well-tolerated and can significantly decrease CRP, AST, and increase hemoglobin levels in COVID-19 patients admitted to ICU. Based on these findings, further larger studies should be conducted

over both short and longer time periods to investigate the potential use of this compound as a novel therapeutic option for treatment of COVID-19 disease and potentially other respiratory virus infections.

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Competing Interests MM is the founder of Sami-Sabinsa group of companies.

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Chapter 23
Chronobiological Efficacy of Combined Therapy of *Pelargonium Sidoides* and Melatonin in Acute and Persistent Cases of COVID-19: A Hypothetical Approach

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Abstract Since the outbreak of the first SARS-CoV-2 epidemic in China, pharmacists have rapidly engaged and developed strategies for pharmaceutical care and supply. According to the guidelines of the International Pharmaceutical Federation (FIP), clinical pharmacists/hospital pharmacists, as members of care teams, play one of the most important roles in the pharmaceutical care of patients with COVID-19. During this pandemic, many immuno-enhancing adjuvant agents have become critical in addition to antivirals and vaccines in order to overcome the disease more easily. The liquid extract obtained from the *Pelargonium sidoides* plant is used for many indications such as colds, coughs, upper respiratory tract infections,

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sore throat, and acute bronchitis. The extract obtained from the roots of the plant has been observed to have antiviral and immunomodulatory activity. In addition to its anti-inflammatory and antioxidant effects, melatonin plays a role in suppressing the cytokine storm that can develop during COVID-19 infection. Knowing that the severity and duration of COVID-19 symptoms vary within 24 hours and/or in different time periods indicates that COVID-19 requires a chronotherapeutic approach. Our goal in the management of acute and long COVID is to synchronize the medication regimen with the patient's biological rhythm. This chapter provides a comprehensive review of the existing and emerging literature on the chronobiological use of *Pelargonium sidoides* and melatonin during acute and prolonged COVID-19 episodes.

Keywords *Pelargonium sidoides* · Melatonin · Chronotherapy · Clinical pharmacy · Acute COVID-19 · Long COVID-19

1 Introduction

Due to its global spread, the World Health Organization (WHO) classified coronavirus disease (COVID-19) as a pandemic in March 2020 [1]. The virus can be transmitted through the air, surface, or by contact [2]. Entry of SARS-CoV-2 into cells is mediated by angiotensin converting enzyme-2 (ACE2) [3]. ACE2 binds to the receptor-binding domain (RBD) of the SARS-CoV-2 spike (S) protein. In addition, production of the viral S protein for entry into the host cell is necessary for its fusion with the host cell membrane [4]. The S protein is cleaved at the host cell membrane by transmembrane serine protease 2 (TMPRSS2) [5]. Since the pandemic was declared, scientists have conducted several scientific studies to treat and prevent the spread of COVID-19. However, only vaccines, some monoclonal antibodies, and antiviral agents are currently available to treat COVID-19 [6]. One of the most prominent of these is bebtelovimab, which has high neutralizing activity against all subvariants of Omicron [6]. Furthermore, molnupiravir, one of the antiviral agents with proven efficacy against COVID-19, belongs to the therapeutic category [7].

Although many variants of COVID-19 have emerged, the latest and most dominant variant announced by the WHO is the Omicron variant. Omicron was identified as an alarming variant by the WHO in November 2021 [8]. Compared to other known variants, the Omicron variant is more dominant over other variants in terms of mortality and transmission rate [8]. Omicron subvariants, namely, BA.1, BA. 2, BA.3, were found at the same time and reported to be more contagious than the previously dominant Delta variant but have a milder disease course [9]. Subvariant BA.4 was first discovered in January 2022 and subvariant BA.5 in February in South Africa [10]. It is known that the BA.4 and BA.5 subvariants have higher infectivity rates than others [10]. The difference between the new subvariants and their

predecessors is that the symptoms last longer. While the average recovery time in patients with the Omicron variant BA.1/2/3 is 4–5 days, it can be up to 10 days for the BA.4/5 subvariants [11].

Therefore, new treatments against SARS-CoV-2 disease are urgently needed as a therapeutic strategy against COVID-19 to reduce the effectiveness of the virus, prevent transmission, and eliminate severe inflammation as a result of the cytokine storm effect triggered by the virus [12]. Today, as the disease is being caught at an increasingly early stage, many patients try to overcome the infection process by isolating at home unless their condition worsens. The number of patients applying their own treatment in isolation at home is also increasing.

In many cases, infection by the SARS-CoV-2 virus may cause few or no symptoms. However, the absence of severe symptoms does not usually mean that the patient has fully recovered. Coronaviruses can now persist in cells for a long time [13]. According to the terminology of the American Infectious Diseases Association (IDSA), this phenomenon is referred to as "long covid," "post-covid syndrome," or "post-acute covid-19 syndrome" [14]. According to numerous reports, most patients who had COVID-19 are likely to develop a long COVID condition [15]. Some of these patients cannot undergo surgery because of the irreversible damage to their organs [16]. Although there is no visible organ damage in some individuals with other diseases, a condition that mimics the symptoms of the disease still exists. In these cases, COVID-19 can hide under various masks of symptoms. Researchers have identified more than 200 post-COVID symptoms in 10 organ systems [17].

Common pathophysiological syndromes of prolonged COVID-19 are also listed as four clinical pictures. These are systemic inflammation, endothelitis, pulmonitis, and asthenic syndrome [18]. Systemic inflammation is the result of a systemic hyperimmune response. Cytokines continue to be released like a cytokine storm in the lungs. This can also be accompanied by immune inflammation in brainstem structures that trigger the development of neurological complications. Endotheliitis is the general inflammatory damage to vascular endothelial cells. This situation triggers a disturbance of coagulation homeostasis linked to thromboembolisms, decreased energy supply to the myocardium, development of myocardial infarction, stroke, and myocarditis [18]. Pulmonitis is a lung injury that develops due to vascular and alveolocyte damage by viruses and cytokines. The process of fibrosis of lung tissue is activated and lung function (vital capacity) decreases [19]. Asthenic syndrome is a leading clinical syndrome that significantly worsens quality of life and reduces work capacity [19].

After acute COVID-19, mandatory monitoring of respiratory function, cardiac symptoms, nervous system, and mental functions is required, along with a focus primarily on eliminating systemic background inflammation and improving function. In addition, monitoring should be extended to detect the presence of long COVID-19, as evidenced by the persistence of symptoms for more than 1 month after initial diagnosis [20]. Considering their low cost and wide availability, increasing attention is now being paid to the potential use of herbal preparations containing agents as adjunctive treatments against the cytokine storm effects resulting from SARS-CoV-2 infections. Due to the clinical safety of *Pelargonium sidoides* extract

[21], along with its antiviral and/or immunomodulatory activity, the use of this extract may be desirable as an adjuvant in daytime management of COVID-19 [22]. *Pelargonium sidoides* preparations have been used as a herbal supplement to treat viral diseases prior to the COVID-19 pandemic [23]. In addition, melatonin, which is a key component of circadian rhythm, could be used as an adjuvant in treatment due to its immunosuppressive properties against the bedtime cytokine storm caused by COVID-19 infection [24–26]. *Pelargonium sidoides* extracts and melatonin are among the commonly used prescription and over-the-counter drugs in pharmacies for the treatment of COVID-19. Although clinical trials of the antiviral efficacy of both drugs were conducted prior to COVID-19, no clinical trials of their use in combination therapy have been conducted during this pandemic. However, distribution of these prescription and over-the-counter drugs through pharmacies continues. Community pharmacists, as well as clinical pharmacists, are responsible for providing information and educating their patients on this topic. This chapter aims to evaluate the possible mechanisms of action when both drugs are used in combination against acute and long COVID from a chronobiological perspective.

2 The Possible Chronobiological Efficiency of Melatonin in Acute and Long COVID-19 Period

Chronopharmacology is the branch of chronobiology that studies the effects of drugs on the timing and rhythms of biological events and the relationship between biological timing and drug effects [26]. It is used as an important tool in optimizing drugs, maximizing the desired effect and minimizing the undesirable effects of a drug. Processes such as tissue growth, blood pressure, heart rate, and blood glucose level are regulated by a biological clock [27]. The mammalian circadian system is regulated by the suprachiasmatic nucleus (SCN), the central oscillator located in the hypothalamus. The rhythm that occurs in the SCN due to daylight ensures that the peripheral clocks in all cells are synchronized through many neuronal and hormonal rhythms [27]. Peripheral tissue synchronization is related to peripheral clocks. It may also be related to SCN-mediated hormone release and environmental factors.

Melatonin is a hormone secreted in humans under the influence of darkness, suppressed by light, and regulated by the SCN [28]. In mammals, circadian rhythm is influenced by how the SCN center of the hypothalamus is organized in the brain. Pineal gland functions are acutely suppressed when exposed to light [28]. Therefore, the amount of synthesized melatonin changes depending on the day-night rhythm and synthesis peaks at night. Melatonin is known to reduce oxidative stress [29]. Its antioxidant effect comes from scavenging free radicals, reducing metals, and taking part in the secretion of enzymes related to our redox system: catalase, glutathione peroxidase, and superoxide dismutase. It is also effective in the regulation of mitochondrial functions that cause free radical production and related oxidative stress [29]. Melatonin also reduces the production of pro-oxidant nitric oxide synthase

and lipoxygenase enzymes [30]. For these reasons, it has become the target of many research and clinical studies as a therapeutic approach during the COVID-19 pandemic.

Melatonin has recently been proposed as a possible first-line treatment for acute COVID-19 [31]. However, it may also be useful for treating long COVID patients with neuropsychiatric symptoms such as anxiety, insomnia, and depression [32]. The most well-known neurological and psychiatric symptoms of long COVID are impaired smell and taste, sleep problems, memory problems, depression, and anxiety. According to the chronopharmacological approach, taking melatonin before bedtime may positively affect the therapy, especially for people in high-risk groups such as those with type-2 diabetes, asthma, and hypertensive patients, in order to prevent both acute and long COVID-19 symptoms.

3 Melatonin and Its Effect on the Immune System

Melatonin is synthesized from tryptophan and secreted mainly by the pineal gland [33]. Melatonin has strong lipophilic and hydrophilic properties, and it mixes with blood and body fluids without being stored in the body. Most of the melatonin in the blood is bound to albumin [34]. This hormone is primarily metabolized in the liver and secondarily in the kidneys to 6-hydroxymelatonin sulfate and 6-hydroxymelatonin glucuronide [34]. These molecules are excreted from the body in the urine. Due to the rhythmic release of melatonin, the amount of metabolites in the urine is higher at night [33]. Mammals have two distinct melatonin receptors that pharmacologically bind to their cell membranes. These are the MT1 (high affinity) and MT2 (low affinity) receptors [35].

The most important functions of melatonin are the regulation of biological rhythms and sleep patterns. However, many studies have been conducted on its ability to reduce stress and the signs of aging by directly increasing life expectancy [36]. In addition to all of these effects, melatonin has also been shown to directly interact with T lymphocytes in the immune system and increase immunity at the cellular level [37]. Melatonin also has anti-inflammatory effects in addition to having an impact on reactive oxygen species (ROS) [38]. As a result, numerous studies have been conducted on this hormone, and it has been proposed as an adjuvant therapy for several viral diseases that trigger a cascade of immunoinflammatory responses [38]. This is due to its capacity as a potent scavenger of hydroxyl radicals and an inducer of superoxide dismutase and glutathione reductase and several other enzymes. Because of these effects, melatonin elicits an effective immune response against cellular oxidative damage [38]. Melatonin also attenuates negative immunological responses in a number of viral activities, including the COVID-19-induced cytokine storm effect [39]. It has been established that some viruses act to prevent the synthesis of melatonin in order to prevent their own destruction and to allow their replication inside the host cell. The mechanism of this is due to a viral-mediated decrease in gene expression of certain enzymes involved in the formation of the

melatonin-synthesizing amino acid tryptophan. This is consistent with the finding that many viral infections are exacerbated by the decrease in melatonin levels in cells [39].

4 The Antiviral Effects of Melatonin and Its Use Against COVID-19

Both melatonin and *Pelargonium sidoides* root extract have been shown to be curative for their respective therapies in previous studies [40, 41]. The SARS-CoV-2 virus is similar to the respiratory syncytial virus (RSV) that emerged in the pre-pandemic period in terms of the damage it inflicts on the body. RSV infection, like that of SARS-CoV-2, causes degeneration of bronchial epithelial cells. This is achieved by RSV acting through the toll-like receptor 3 (TLR3) to activate the transcription factor nuclear factor kappa B (NF-κB) [42]. This can lead to a massive infiltration of the lung parenchyma by lymphocytes, neutrophils, and macrophages, resulting in damage due to pro-inflammatory and non-specific oxidative stress [43].

Inhibition of NF-κB activation reduces the hyperinflammatory response of the cell to respiratory viruses. In line with this, melatonin administration has been reported to inhibit TLR3-mediated gene expression in RSV-infected macrophages [44]. Melatonin was found to significantly reverse lung injury and suppress tumor necrosis factor (TNF-α) production by CD8 cells in the lungs and spleen of mice infected with influenza A [45]. In addition, treatment with high doses of melatonin has been found to upregulate anti-inflammatory cytokines, such as interleukin IL-10, and this can further reduce the inflammatory response elicited by viruses infecting the lungs [46]. Treatment of RSV-infected mice with melatonin resulted in a normalizing effect on nitric oxide, malondialdehyde, hydroxyl, GSH and SOD levels, which formed the basis of RSV-related acute oxidative lung injury [47].

In other studies, melatonin has been shown to have a protective effect against another viral disease, the Ebola virus, which has numerous similarities with COVID-19 [48]. Melatonin also prevents the severe vascular endothelial damage that leads to multi-organ bleeding caused by the Ebola virus. The harmful effects of the Ebola virus include the induction of inflammatory chemokines and cytokines like monocyte chemoattractant protein-1 (MCP-1), tissue factor, interferon, IL-6, and IL-8. Melatonin‘s ability to counteract Ebola can be attributed to its ability to increase type 2 T helper cytokine production, interferon-gamma response, and natural killer cell activity while decreasing ROS caused by cytokine storms and viral infections [49]. However, melatonin also inhibits Ebola replication by inducing an enzyme called heme-oxygenase-1. Ultimately, melatonin inhibits pro-inflammatory processes, activates endogenous antioxidants, enhances mitochondrial activity, and thus protects endothelial barriers in septic shock, as well as in disseminated intravascular coagulation [49].

Plasma melatonin levels in patients with hemorrhagic fever are significantly low [50]. Therefore, melatonin could possibly act as a protective agent against encephalitis-causing viruses such as West Nile virus and the virus that causes rabbit hemorrhagic disease [50]. Several studies have shown that melatonin significantly reduces viral load in the blood, decreases mortality, and attenuates disease severity [51]. TNF-α increases intercellular adhesion molecules, alters the blood–brain barrier permeability, and promotes lymphocyte entry into the central nervous system (CNS) [52]. Melatonin treatment reduces the damaging hyperinflammatory effects of TNF-α in the CNS while at the same time causing an increase in astrocytic release of nerve growth factor as a protective measure [53].

Melatonin can be used to treat SARS-CoV-2 viral infections, severe inflammatory responses, and the effects of virus-induced oxidative stress [54]. The S1 and S2 subunits of the viral S protein are used by SARS-CoV-2 to enter alveolar epithelial cells via ACE2 [54]. While binding to ACE2 occurs at S1, the S2 mediates cell membrane fusion. At the plasma membrane, calmodulin regulates ACE2 surface area and uptake [55]. Melatonin, on the other hand, inhibits calmodulin and, hence, indirectly prevents ACE2 from binding to SARS-CoV-2 during the infection process [55]. Melatonin also blocks the activity of the primary protease enzyme of SARS-CoV-2 involved in cleavage of the viral polyprotein and the replication process [56]. Binding of SARS-CoV-2 to ACE2 leads to generation of angiotensin II. When angiotensin II is formed, the antioxidant and anti-inflammatory properties of the angiotensin-1-7 peptide are significantly diminished [57]. Left unchecked, angiotensin II overproduction triggers NF-κB signaling, allowing the production of IL-6 and constriction of blood vessels [57]. The cumulative effect of these events leads to lung cell damage which triggers significant inflammatory and adaptive immune responses. However, melatonin is an angiotensin 1–7 agonist and can thereby act as an inhibitor of angiotensin II activation in the above damaging cascade.

5 Anti-Inflammatory Effect of Melatonin in SARS-CoV-2 Infection

SARS-CoV-2 triggers programmed cell death by causing pyroptosis, a highly inflammatory state and consequential severe lung pathologies [58]. A viral protein produced by SARS-CoV-2 interacts with the inflammatory domain of the NLR family pyrin-domain containing 3 (NLRP3) inflammasome at the peak of infection, resulting in an inflammatory release of cellular contents that ruptures the host cell membrane. In addition, activation of NLPR3 stimulates the release of damaging pro-inflammatory cytokines [59].

Melatonin has an anti-inflammatory action in the cell because it prevents pyroptosis and inhibits the activity of NLRP3 [60]. A cytokine storm characterized by elevated levels of some inflammatory interleukins, C-reactive protein, and TNF-α

follows the increased levels of neutrophils brought on by the innate response to SARS-CoV-2 infection [61]. In addition, melatonin inhibits inducible nitric oxide synthase, NF-kB signaling, and cyclooxygenase-2 levels [62].

Exacerbation of COVID-19 disease is due to the accumulation of monocytes and macrophages in the respiratory tract, which causes hyperinflammation during the infection process [63]. The switch to cytosolic anaerobic glycolysis for adenosine triphosphate synthesis results in an increase in the generation of cytokines, killer T cells, and eventual destruction of the alveolar cells [63]. Melatonin induces the transformation of pro-inflammatory glycolytic macrophages into anti-inflammatory macrophages, which allows oxidative phosphorylation to occur [64]. In addition, melatonin inhibits the production of hyperinflammatory macrophages by interacting with multiple signaling pathways such as sirtuin 1 [64].

Upon entry into SARS-CoV-2 cells, deleterious oxidative effects can cause epithelial cell damage with uncontrolled release of mitochondrial ROS [65]. As part of the host immunological response, it also induces macrophages, monocytes, and neutrophils to release ROS [65]. Counter to this, melatonin exerts its antioxidant properties by stimulating antioxidant enzymes to scavenge the damaging oxygen and nitrogen-containing free radicals [66]. It can also achieve this by maintaining mitochondrial homeostasis and suppressing production of pro-oxidative enzymes. Melatonin is also known to regulate autophagy, endoplasmic reticulum stress, and apoptosis through its antioxidant properties [66], and it prevents acute oxidative injury by suppressing ROS and restoring antioxidant enzyme levels [47, 67]. ROS has been identified as a key factor in the hypoxic respiratory failure that occurs in the most severe SARS-CoV-2 infections because of its adverse effects on lung and red blood cell function. High amounts of ROS have an adverse effect on the cytoplasmic and membrane lipids in circulating cells [68]. Red blood cells are a typical example because they show considerable lipid alterations in the capillary bed that influences both red blood cell smoothness and gas transport. These ROS-induced alterations can lead to thrombotic states that adversely impair normal oxygen delivery and vasodilation. Melatonin combats these ROS effects by activating the electron transport chain, preventing ROS damage, and boosting mitochondrial respiration and adenosine triphosphate synthesis [69, 70].

The anti-inflammatory and antioxidant properties of melatonin make it a potentially effective therapy for COVID-19, as it affects all phases of the viral life cycle, including viral entry and deleterious signaling pathways [71]. Theoretically, melatonin should be given at the onset of infection, where it will have the most significant impact on management of COVID-19. It is inexpensive compared with other drugs used for treatment, it is readily available in pharmacies, and has an acceptable safety profile [72]. While it is used to prevent early viral replication, it could also be employed as a preventative measure against viral infection and as remedy in existing COVID-19 patients [73]. In line with this, a single-blind, randomized trial demonstrated that melatonin, when combined with acyclovir, significantly diminished the symptoms of herpes simplex virus [51].

6 *Pelargonium sidoides* Root Extract as an Adjuvant for the Treatment of Acute and Long COVID-19

Pelargonium sidoides commonly known as "African Sardinia" is a perennial plant of the Geraniaceae family that grows in the highlands of South Africa and Lesotho [74]. It has been used therapeutically for diarrhea, colds, skin infections, infections of the upper respiratory tract, and tuberculosis [74]. *Pelargonium sidoides* drew the attention of European scientists in the nineteenth century due to its therapeutic properties against tuberculosis and was brought to Europe for this purpose [74]. The use of *Pelargonium sidoides* root extract gained notice across Europe after it was found out that its root extract could treat severe cases of tuberculosis [75]. The pharmacological activity of *Pelargonium sidoides* has been linked to the biological activity of its constituent flavonoids, coumarins, phenolic, gallic, and hydroxycinnamic acid derivatives [76]. Based on this, *Pelargonium sidoides* extracts are under investigation for antibacterial, antiparasitic, and antiviral properties, especially against *Streptococcus*, *Leishmania amazonensis,* and RSV strains [76]. In their study on the root extract of *Pelargonium sidoides*, Papies et al. found that it has various immunomodulatory and antiviral effects that strengthen host defense mechanisms and reduce inflammation [77].

Another study found reduction of nasally secreted chemokines and epithelial-neutrophil activating peptide (ENA-78) associated with improvement of symptoms in patients with acute bacterial rhinosinusitis treated with *Pelargonium sidoides* [78]. Moreover, an increase in the levels of motif chemokine ligand 10 and 2 suggested that this extract has selective immunomodulatory effects in acute respiratory infections [78]. In particular, Papies et al. investigated the potential effect of *Pelargonium sidoides* against SARS-CoV-2 infection in human lung cells. Their results showed that the extract limited the ability of the virus to spread and differentially controlled the release of immunomodulatory cytokines such as IL-1β [77].

Phytochemical characterization of *Pelargonium sidoides* fractions identified proanthocyanidins as the main active compounds [79]. The immunomodulatory effects of these compounds on pro-inflammatory IL-1 and anti-inflammatory TNF Alpha Induced Protein 3 (TNFAIP3) were found to more potent than those in the extract [79]. *Pelargonium sidoides* suppresses the release of several cytokines and growth factors involved in SARS-CoV-2 infection [80]. The results of these studies are also consistent with the previously studied anti-influenza effects of *Pelargonium sidoides* as well as by inhibitory effects on other viruses [81].

Pelargonium sidoides is a daytime flowering plant [82]. Therefore, from a chronobiological point of view, it is possible that it is more biologically active during the day. In addition, the recommended use of herbal products containing *Pelargonium sidoides* by the European Medicines Agency (EMA) is three times a day: morning, noon, and evening [82]. Therefore, in addition to the use of *Pelargonium sidoides* preparations during the day, nocturnal melatonin supplementation may add a complementary effect by reducing the symptoms commonly observed in the acute and persistent phase of COVID and to facilitate the treatment process.

7 The Role of Clinical Pharmacists in Adjuvant Chronotherapy for Acute and Ongoing COVID-19

Chronotherapy is a way to increase the efficacy and safety of therapy by administering drugs according to rhythmic changes in disease exposure to the drugs and/or the patient's tolerance to the side effects of the drug [83]. In other words, the goal of chronotherapy is to optimize the expected effects of the drug over time while minimizing its side effects. Determining the appropriate time to administer the drug in the appropriate indication, through the appropriate route of administration and ensuring patient compliance are key objectives in this endeavor [83]. As part of the process, clinical pharmacists have important responsibilities related to the timing of drug administration. Answering questions such as at what time of day the patient's symptoms are worse, the duration of the drug's effects, and what times of the day the patient feels most comfortable, will contribute to the prevalence of such time orientation in drug use [83].

7.1 The Role of the Pharmacist

In addition to the traditional role of pharmacists, such as clinical pharmacy and pharmaceutical care, improving the quality of life and achieving positive clinical outcomes, cognitive services are also evolving in this field worldwide [84]. During the pandemic in particular, the role of pharmacists has become patient service-oriented [84]. Because of these changes, pharmacies and pharmacists have assumed new roles during the acute and long COVID-19 periods, and individuals can more easily access medical treatment and supportive care.

In many countries like the United States and the United Kingdom, pharmacies have served as vaccination centers and PCR centers in addition to selling and prescribing medicines during the COVID-19 pandemic [85]. Also, during this pandemic, online pharmacies evolved to minimize physical contact and also served as an escape from some of the bureaucracy that slows down healthcare services. Through this, pharmacists have worked with patients to ensure that they understand their prescriptions correctly and adhere to their therapies. In addition, pharmacists have been able to track updates on long COVID developments through these online networks [86].

Because of their experience with other viruses that have similar effects as SARS-CoV-2, pharmacists can quickly and confidently determine an appropriate treatment plan for the patient. In this regard, pharmacies also offer herbal and hormonal dietary supplements [87] such as *Pelargonium sidoides* and melatonin. Based on the importance of chronotherapy described in this chapter, advice concerning the

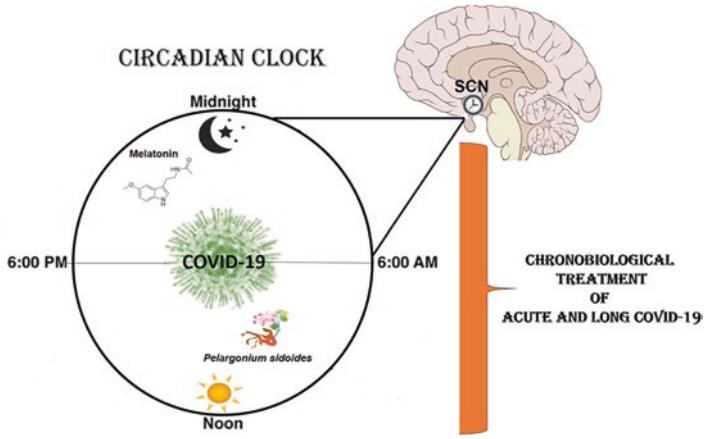

Fig. 23.1 Chronobiological usage of *Pelargonium sidoides* and melatonin combination therapy in acute and long COVID-19

timing of when a patient should take these remedies would be of critical importance. The chronobiological use of *Pelargonium sidoides* in combination with melatonin in long COVID is shown in Fig. 23.1.

8 Conclusions and Future Perspectives

In this chapter, we have described how the use of melatonin along with *Pelargonium* extract as adjuvant therapy may be effective in both the acute and chronic phases of COVID-19 disease. One of the reasons melatonin can be used as an adjunct in the treatment of COVID-19 is its ability to reduce toxicity and increase drug efficacy. In severe cases of acute SARS-CoV-2 infection and in patients at high risk of long COVID illness, melatonin and *Pelargonium sidoides* may be preferred for the treatment of COVID-19 due to their tolerable side effects, low cost, ease of use, and accessibility. However, the chronobiological effects of this combination therapy should be considered to maximize efficacy and reduce potential side effects. Regarding the role of pharmacies and pharmacists in the acute phase, and long COVID, it has been shown that community pharmacies are likely to be part of the front line of health services in future pandemics. Thus, they may play a significant role in the administration of compounds such as *Pelargonium sidoides* and melatonin in acute and persistent viral infections which have chronobiological effects. Taking all of these factors into account, it is clear that further studies are warranted at the laboratory and clinical levels on the use of these compounds as potential viral remedies.

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Chapter 24
The Potential Effect of Royal Jelly on Biomarkers Related to COVID-19 Infection and Severe Progression

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Abstract Royal jelly is a yellowish to white gel-like substance that is known as a "superfood" and consumed by queen bees. There are certain compounds in royal jelly considered to have health-promoting properties, including 10-hydroxy-2-decenoic acid and major royal jelly proteins. Royal jelly has beneficial effects on some disorders such as cardiovascular disease, dyslipidemia, multiple sclerosis, and diabetes. Antiviral, anti-inflammatory, antibacterial, antitumor, and immunomodulatory properties have been ascribed to this substance. This chapter describes the effects of royal jelly on COVID-19 disease.

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1 Introduction

Royal jelly is a yellowish-to-white jelly and creamy-like substance formed from the hypopharyngeal and mandibular glands of worker bees. It is known as a "superfood" and is consumed by queen bees [1–3]. Moreover, it is one of the most fruitful remedies for humans in both modern and traditional medicine. The properties of antiviral, anti-inflammatory, antibacterial, antitumor, and immunomodulatory have been ascribed to this substance. Other beneficial bioactive compounds reported in royal jelly include fatty acids, proteins, adenosine, acetylcholine, polyphenols, and some hormones (such as estradiol, progesterone, and testosterone) [4, 5]. Chemically, royal jelly consists of certain basic components such as water (50–60%), proteins (18%), carbohydrates (15%), lipids (3–6%), mineral salts (1.5%), and vitamins [6]. The main unique fatty acid of royal jelly is trans-10-hydroxy-2-decenoic acid (10-HDA), which has multiple biological properties [7–10]. Moreover, more than half of the proteins in royal jelly are termed the major royal jelly proteins (MRJPs), which also affect several biological pathways [11].

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One of the properties of royal jelly is due to its capability to regulate oxidative stress in the body [12]. The flavonoids and phenolic acids of royal jelly are part of phenolic class of compounds that can have an antioxidant impact [13, 14]. These confer protection of cell membranes from damage caused by over-production of free radicals [15]. Royal jelly collected 24 h after larval transfer showed the most substantial antioxidant activities. Other factors like initial larval age and time of harvest also have an impact on the antioxidant properties in royal jelly [16]. The antioxidants in royal jelly have been shown to block reactive oxygen species (ROS) production and support the antioxidant system in a rat model [17]. Also, in other animal studies, it was observed that royal jelly protected the kidneys from nephrotoxicity caused by cadmium and fluoride, most likely due to its antioxidant and anti-inflammatory effects [18, 19]. Royal jelly suppresses the production of several proinflammatory cytokines such as interleukin-6 (IL-6), IL-1, and tumor necrosis factor α (TNF-α). Additionally, royal jelly reduces capillary permeability in the acute phase of inflammation causing a lower inflammatory response in the human body [20].

Royal jelly has various biological effects on the human body. An intervention with RJ for 3 months significantly decreased total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c) levels by improving the levels of dehydroepiandrosterone sulfate (DHEA-S) [21]. Another study investigated the effect of 6 weeks of selective aerobic exercise and consumption of royal jelly on liver enzymes of multiple sclerosis patients [22]. This showed that royal jelly administration significantly reduced biomarkers of liver damage (aspartate transaminase and alanine transaminase) in these patients. Another study revealed that the administration of royal jelly may be beneficial in weight management of diabetes patients [23]. Also, royal jelly can improve erythropoiesis, glaucous control, and mental health [21]. In another study of multiple sclerosis, royal jelly administration in combination with exercise found a decrease in high-sensitivity C-reactive protein (hs-CRP), TNF- α, and neutrophils [13]. Additionally, 10-HDA can elevate the synthesis of ovulation hormones, maintaining a lower expression of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in young ovarian cells [24]. Royal jelly administration also shortened the cure duration of desquamated skin lesions [25]. A randomized controlled trial recommended that intensive care unit (ICU) patients who are connected to a ventilator inhaled forms of propolis and royal jelly as use of these compounds as adjuvant therapy for COVID-19 treatment helped to reduce disease symptoms [26]. Moreover, many studies have advocated potential antiviral effects of bee products such as royal jelly, honey, propolis, and bee bread, by the direct impact of various bioactive components of these such as peroxides, flavonoids, and phenolics [27].

The key proteins in royal jelly are the MRJPs. MRJP2 and MRJP2 isoform X1 represent two functional dietary proteins present in royal jelly that through their sialidase activity and ability to interact with the angiotensin-converting enzyme 2 (ACE2) binding site of the viral spike receptor-binding domain (RBD) complex are thought to block binding of the SARS-CoV-2 virus to host cells. According to docking analysis, these MRJPs also bind to the active site or cofactor binding site

residues of the SARS-CoV-2 non-structural proteins (NSP) 3, NSP5, NSP9, NSP12, and NSP16 and inhibit their activity. Moreover, these proteins may prevent viral synonyms in the lung, such as hypoxia and related pathogenesis, because of their ability to efficiently bind to most of the oxyhemoglobin and deoxyhemoglobin binding sites on the viral NSPs [28]. In addition, MRJP 3, a glycoprotein isolated from water extract of royal jelly was proposed to have immunosuppressive and anti-inflammatory impacts on T cells and peritoneal macrophages in rat models [6]. Furthermore, the antiviral impact exhibited by royal jelly can also be used as a prophylactic agent because of its favorable effect on immune tone [29]. The alkaline and water obtained from royal jelly have also been shown to be an effective scavenger against ROS [30]. From these properties, it has been proposed that royal jelly administration could be used to diminish the effects of COVID-19 infection [31].

Another effective compound in royal jelly is 3,10-dihydroxy-decanoic acid (3,10-DDA). This molecule has been demonstrated to stimulate maturation of human monocyte-derived dendritic cells (MoDCs) and polarized T cells, contributing to an antiviral immune response [32]. A study in a rat model showed production of antibodies and proliferation of immune-competent cells in animals that received royal jelly supplementation [29, 33].

Other peptides obtained from royal jelly, such as the jelleines (jelleine I–IV), can be effective in controlling co-infections in patients with COVID-19 [1]. The result of a systematic review study showed that 7% of hospitalized patients with COVID-19 had co-infections, which was reported to be twice as high in ICU-admitted patients [34], and such co-infections were found to be reduced in royal jelly–administered patients [33].

In the absence of special antiviral drugs against SARS-CoV-2, apitherapy using royal jelly and related substances may offer hope of relieving some of the risks associated with COVID-19 disease [35–37]. In this review, the effectiveness of royal jelly on biomarkers relevant to the study of COVID-19 disease are reviewed. The effects of royal jelly on various parameters that have been investigated in these different studies are summarized in Table 24.1 and Fig. 24.1.

2 Inflammatory Biomarkers

Mounting evidence during the COVID-19 crisis has shown the detrimental role of the inflammatory response associated with this viral infection, which is responsible for pulmonary complications in these patients, leading to acute respiratory distress syndrome (ARDS) and ultimately septic shock or multi-organ system failure (MOSF) [38–41]. In this inflammatory response, uncontrolled production of inflammatory cytokines is observed [39, 42]. Under these conditions, the clinical manifestations of the disease may be accompanied by a systemic increase in inflammatory mediators and cytokines, known as a “cytokine storm.” This involves massive alterations in the production of interleukin 6 (IL-6), soluble IL-6 receptor, IL-1β, TNF-α, interferon gamma (IFN-γ), IL-10, IL-2, soluble IL-2 receptor, and CRP [39, 43, 44].

Table 24.1 The effects of royal jelly on various biomarkers

Parameters	effect
Oxidative stress, reactive oxygen species (ROS) production, and inflammatory response	↓
Production of proinflammatory cytokines like interleukin-6 (IL-6), IL-1, IL-1β, and IL-8, tumor necrosis factor α (TNF-α), and IL-10	
Liver function tests (AST, ALT, ALP, GGT, and MDA in the liver)	
Autoantibodies against single-stranded deoxyribonucleic acid (ssDNA), and double-stranded deoxyribonucleic acid (dsDNA)	
Pathological damage such as diffuse edema, bleeding, and congestion, capillary permeability	
Level of nitric oxide, and creatine kinase (CK-BM) levels, creatinine	
The curing duration of desquamated skin lesions	
Level of neutrophils, erythrocytes, thrombocyte, thrombosis, and Plasma fibrinogen levels, Hs-CRP, and Neutrophils	
Level of lipids (TC and LDL-c levels) and cholesterol in serum and liver	
Total antioxidant capacity and Immunomodulatory effects	↑
Pro-inflammatory cytokines including TNF-α, IL-1β and IL-8	
Weight management, glaucous control, and delayed formation of atheroma plaque	
Stimulates maturation of human monocyte-derived dendritic cells (MoDCs)	
Levels of DHEA-S, erythropoiesis, level of lymphocytes, platelets, serum uric acid levels and blood urea nitrogen	
Anti-stress and neuroprotective effects, and mental health, and the state of memory and cognitive functions (by improving oxygenation of brain tissue), improves learning processes and spatial memory, and antidepressant activity	

Abbreviations: *ROS* reactive oxygen species, *IL-6* interleukin-6, *TNF-α* tumor necrosis factor α, *ssDNA* single-stranded deoxyribonucleic acid, *dsDNA* double-stranded deoxyribonucleic acid, *CK-BM* creatine kinase, *MoDCs* maturation of human monocyte-derived dendritic cells, *IFN-γ* interferon gamma, *sIL-2R* soluble interleukin 2 receptor, *CRP* C-reactive protein, *DHEA-S* dehydroepiandrosterone sulfate, *ALT* alanine transaminase, *AST* aspartate transaminase, *ALP* alkaline phosphatase, *MDA* malondialdehyde, and *GGT* gamma-glutamyl transferase

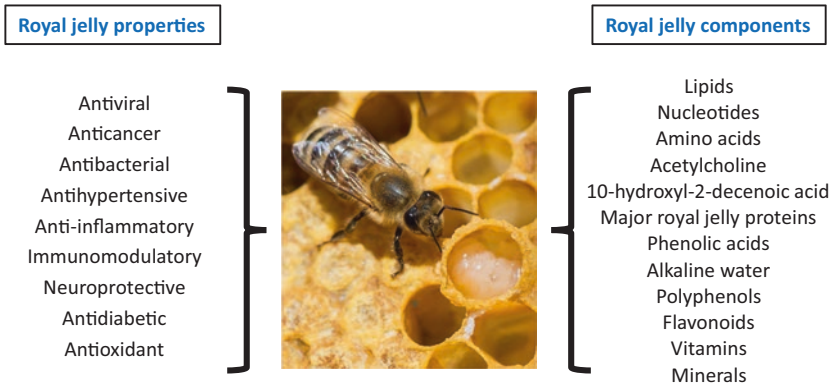

Fig. 24.1 Royal jelly properties and components

In this respect, the presence of 10-HDA in royal jelly can confer an anti-inflammatory effect [45] and inhibit the over-production of pro-inflammatory cytokines by activated macrophages [46]. This has been demonstrated in animal models, which showed that the production of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-8 was inhibited by 10-HAD [47]. In addition, administration of royal jelly to mice has shown a significant decrease in IL-10 serum levels, as well as the circulating levels of autoantibodies against single-stranded deoxyribonucleic acid (ssDNA) and double-stranded deoxyribonucleic acid (dsDNA) [48].

3 Hematological Biomarkers

In the first 2 weeks of contracting COVID-19, the number of leukocytes and lymphocytes in the peripheral blood can be normal or slightly reduced [49]. However, elevated neutrophil/lymphocyte and platelet/lymphocyte ratios can be indicative as biomarkers for risk of a more serious disease course [43, 49]. Complete blood counts (CBCs) are inexpensive and easy to evaluate in this regard, including the composition of white blood cell, lymphocyte, and platelets, as well as mean platelet volume. This routine test provides useful information to the physician and plays an important role in the early diagnosis of diseases such as pneumonia [50, 51]. Neutrophil white blood cells and lymphocytes are among the most indicative parameters that indicate primary inflammation [52]. An increase in the ratio of neutrophils to lymphocytes is an important indicator that inflammation is in progress [50]. In animal models, royal jelly administration has been shown to drive normalization in the neutrophil to lymphocyte ratio, stimulate the production of antibodies, and enhance the immune response [29]. It also reduces the level of erythrocytes and self-reactive B lymphocytes in the spleen [48].

4 Coagulation Biomarkers

Coagulation disorders have been reported relatively frequently in patients with COVID-19, especially in severe cases [53, 54]. Many studies in the field of COVID-19 have shown that the prothrombin time (PT; a measure of clotting time), D-dimer, and fibrinogen are increased in severe cases of COVID-19 [55–57]. The cytokine storm caused by COVID-19 infection appears to lead to development of vascular thrombi [58]. In COVID-19, the number of platelets is usually normal with a small amount of thrombocytopenia [59, 60]. However, high thrombocytopenia has been reported in severe cases of this disease [61]. Khazaei et al. [62] showed that the administration of royal jelly to rats that had thrombocytopenia improved platelet levels. Royal jelly reduces plasma fibrinogen levels in animal samples. Also, the

occurrence of thrombosis in mice treated with royal jelly was less than in untreated control mice [63].

5 Renal Biomarkers

Most COVID-19 patients who experience acute kidney disease (AKI) have proteinuria and hematuria, and in severe cases, they may have acute tubular necrosis and need dialysis [64]. Possible mechanisms in the pathophysiology of AKI related to COVID-19 include direct viral entry into kidney cells, unbalanced activation of the renin-angiotensin system, or damage caused by the cytokine storm, thrombotic status, or non-specific mechanisms, such as heart failure, hypovolemia, hospital sepsis, and nephrotoxicity [65]. Supplementation with royal jelly has been shown to reduce nephrotoxicity, serum uric acid levels, and blood urea nitrogen [66]. A case series on patients with chronic kidney diseases showed royal jelly can also lead to a reduction in the circulating levels of creatinine, a widely used biomarker of kidney function [67].

6 Cardiac Biomarkers

An increased incidence of cardiovascular diseases (CVDs) has been found in patients with COVID-19, especially among those with more severe disease [68–71]. Results from a meta-analysis by Sheth et al. [72] showed that troponin, lactate dehydrogenase (LDH), and brain natriuretic peptide (BNP) levels were higher among patients with COVID-19 who died or were severe ill compared to non-critically ill patients who survived. This study also showed that there was a significant difference in D-dimer levels in patients who were dead or critically ill. Additionally, creatinine kinase (CK) levels were significantly higher only in those who died compared to those who were alive. However, there was no significant difference in CK levels between patients with severe COVID-19 compared to non-severe controls. Another meta-analysis study showed that increased levels of cardiac biomarkers including troponin I, cardiac troponin T, high-sensitivity cardiac troponin, high-sensitivity cardiac troponin I, high-sensitivity cardiac troponin T, creatine kinase-MB, and myoglobin were associated with severity of COVID-19 disease and with an increased risk of mortality [26]. Administration of royal jelly was shown to reduce the levels of malondialdehyde, nitric oxide, and creatine kinase (CK-BM) levels, and this supplementation also ameliorated pathological damage such as diffuse edema, bleeding, and congestion [73]. A meta-analysis by Vittek et al. [74] demonstrated that consumption of royal jelly by experimental animals significantly reduced the levels of lipids and cholesterol in serum and liver and delayed the formation of atheroma plaque in the aorta even in animals that had been fed a high-fat diet.

7 Liver Biomarkers

In addition to respiratory complications, the COVID-19 crisis was also associated with liver dysfunction and damage [75]. In a study in Wuhan, China, it was seen that about half of the examined patients had abnormally increased levels of biomarkers of liver damage [alanine aminotransferase (ALT) or aspartate aminotransferase (AST)] [59]. In a study by Cia et al., [76] more than 70% of patients with COVID-19 showed abnormal levels of these liver enzymes and more than 20% experienced liver damage. In another study, about 40% of patients on admission with COVID-19 had abnormal liver function tests, such as increased ALT, AST, alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), and total bilirubin [77]. In an experimental study, long-term administration of royal jelly significantly reduced the levels of ALT, AST, ALP, GGT, and malondialdehyde (MDA) in the liver, with a protective role against liver lesions. Additionally, royal jelly has been found to enhance total antioxidant capacity, as a mechanism of preventing liver damage [78, 79].

8 Brain Biomarkers

It has been emerging for more than a year now that neurological damage can occur in some COVID-19 patients, consistent with the ability of the SARS-CoV-2 virus to infect the central nervous system (CNS) [80, 81]. In addition, many patients who have recovered from COVID-19 can experience depression, anxiety, and memory loss [82, 83]. Administration of royal jelly was found to improve some of these adverse neurological effects in an albino rat model by reducing oxidative stress levels in brain tissue [84]. Also, results from a randomized clinical trial showed that supplementation with royal jelly had beneficial effects on the level of consciousness in brain trauma injury patients [85]. At least one aspect of the mechanism of these effects appeared to involve enhancement of oxygenation in the brain tissue [86]. Furthermore, royal jelly has shown to have anti-stress and neuroprotective effects under stressful conditions [87]. In addition, 10-HDA has been shown to have anti-depressant activity and improve learning and spatial memory in animal models [88, 89].

9 Conclusion

Royal jelly as a superfood has been shown to have many beneficial effects on COVID-19 disease sequelae, including strengthening of the immune system, as well as antiviral, antibacterial, and antifungal impacts (Fig. 24.1). This can result in protective effects against damage that can occur to organs and tissues as byproducts of viral infection. Thus, further preclinical and clinical studies should be conducted

using relevant molecular and physiological biomarker readouts to investigate the effects of royal jelly and its components during the continuation of the current pandemic and in preparation for the next one. Such treatments may help to alleviate the damaging effects of new viral outbreaks while awaiting development and deployment of effective vaccines.

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Competing Interests The authors declare no competing interests.

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Chapter 25
Statins: Beneficial Effects in Treatment of COVID-19

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Abstract The recent viral disease COVID-19 has attracted much attention. The disease is caused by SARS-CoV-19 virus which has different variants and mutations. The mortality rate of SARS-CoV-19 is high and efforts to establish proper therapeutic solutions are still ongoing. Inflammation plays a substantial part in the pathogenesis of this disease causing mainly lung tissue destruction and eventually death. Therefore, anti-inflammatory drugs or treatments that can inhibit inflammation are important options. Various inflammatory pathways such as nuclear factor Kappa B (NF-κB), signal transducer of activators of transcription (STAT), nod-like receptor family protein 3 (NLRP), toll-like receptors (TLRs), mitogen-activated protein kinase (MAPK), and mammalian target of rapamycin (mTOR) pathways and mediators, such as interleukin (IL)-6, IL-1β, tumor necrosis factor-α (TNF-α),

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and interferon-γ (INF-γ), cause cell apoptosis, reduce respiratory capacity and oxygen supply, eventually inducing respiratory system failure and death. Statins are well known for controlling hypercholesterolemia and may serve to treat COVID-19 due to their pleiotropic effects among which are anti-inflammatory in nature. In this chapter, the anti-inflammatory effects of statins and their possible beneficial effects in COVID-19 treatment are discussed. Data were collected from experimental and clinical studies in English (1998–October 2022) from Google Scholar, PubMed, Scopus, and the Cochrane Library.

Keywords SARS-CoV-2 · COVID-19 · Statins · Cytokine storm · Inflammation

1 Introduction

The global outburst of infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a strain of coronavirus that causes COVID-19 (coronavirus disease 2019), began in China. Global high mortality, constant mutations, lack of knowledge about the nature of the virus, and uncertain treatment options made the disease a worldwide concern. SARS-CoV-2 acts via angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) expressing epithelial cell receptors leading to extensive synthesis and release of inflammatory agents inducing immune cells and acute respiratory distress syndrome (ARDS) (Fig. 25.1) [1]. The increased rate of mortality in COVID-19 patients is attributed to immune dysregulation resulting in a cytokine storm. This results from over-activation of

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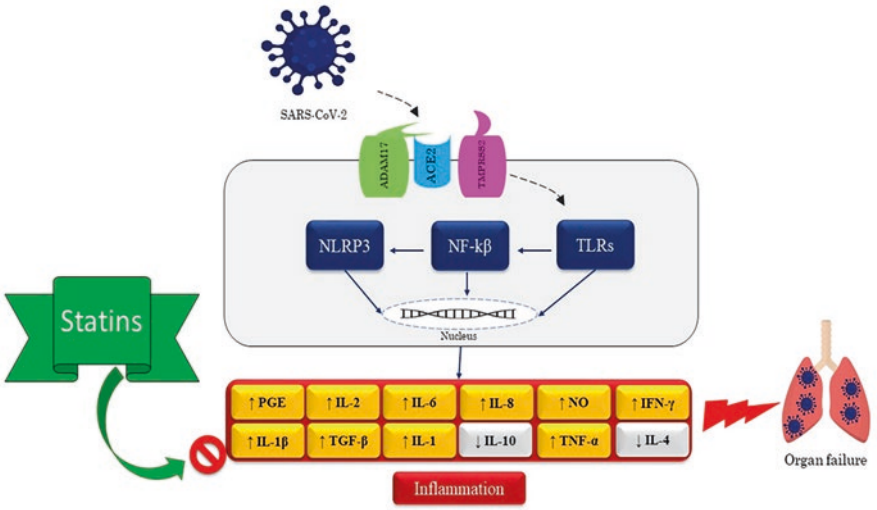

Fig. 25.1 COVID-19 inflammatory signaling pathway; statins could block the inflammatory process caused by COVID-19 infection and might have therapeutic effects

complex inflammatory networks interconnecting different cells, signaling pathways, and cytokines [2]. Activation of nuclear factor kappa light chain enhancer of activated B cells (NF-κB), signal transducer and activator of transcription 3 (STAT3), Janus kinase (JAK), protein kinase B (AKT), mammalian target of rapamycin (mTOR) signaling pathways causes elevated levels of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-18, IL-33, IL-37, IL-1β, tumor necrosis factor-alpha (TNF-α), and interferon-gamma (INF-γ) in COVID-19 patients, while anti-inflammatory cytokines i.e. IL-10 are downregulated by NF-κB [3, 4]. These mediators cause deleterious effects on respiratory, cardiovascular, and digestive system. For COVID-19 prevention and treatment vaccines, immune-based treatments and drugs are used.

For instance, remdesivir, an anti-viral drug, is prescribed for COVID-19 patients with respiratory symptoms leading to a faster recovery. Hydroxychloroquine was found to prevent viral replication in SARS-CoV and was used in Middle East respiratory syndrome coronavirus (MERS-CoV) patients a decade ago [5]. Lopinavir/ritonavir was used as an anti-viral agent, and corticosteroids, such as dexamethasone, methylprednisolone, are recommended for their anti-inflammatory properties. Tocilizumab is used in patients with ARDS, and it reduces elevated levels of IL-6. Besides chemical medicines, herbal medicines such as curcumin and quercetin are also used as a complementary treatment to decrease COVID-19 symptoms by suppressing inflammatory signaling pathways and mediators [6].

Statins have been used for more than three decades as drugs of choice in preventing cardiovascular disease, both in terms of efficiently decreasing plasma low-density lipoproteins cholesterol (LDL-C) and due to their cost-effectiveness. Besides their LDL-C lowering effects, statins have different pleiotropic properties,

such as their anti-inflammatory and immunomodulatory effects, which are beneficial in managing inflammatory conditions [7–17]. Statins are either fungal derivatives (e.g., lovastatin, mevastatin, pravastatin, pitavastatin, and simvastatin) or they are synthetic drugs (e.g., atorvastatin, fluvastatin, and rosuvastatin) [7]. Here, we present a detailed review of the possible use of statins in treating COVID-19 patients. The relevant anti-inflammatory properties of these drugs are discussed in detail.

2 Search Methods

Data were collected from experimental and clinical studies published in English between 1998 and October 2022, from Google Scholar, PubMed, Scopus, and the Cochrane library. Search terms were as follows "SARS-CoV-19" or "COVID-19" and "Statins" and "Cytokine storm" or "Inflammation" and "Novel therapeutic approach."

3 SARS-CoV-2 and COVID-19

3.1 Biology

Coronaviruses are an extremely diverse group of ribonucleic acid (RNA) viruses which cause diseases in mammalian and avian species. They are composed of a positive-sense, single-stranded RNA (+ssRNA) genome varying from 26.4 to 31.7 kilobases. The genome has a 5′ methylated cap and a 3′ polyadenylated tail [18]. The large genome enables this family of viruses to adapt and modify achieving better virulence [19]. Coronaviruses such as SARS-CoV, MERS-CoV, and SARS-CoV-2 can cause several life-threatening infections [20]. SARS-CoV-2 is the coronavirus strain which has caused the ongoing COVID-19 pandemic.

3.2 Structure

Coronavirus virions consist of the RNA genome, helical nucleocapsid, and the viral membrane containing spike protein, membrane protein, and envelope protein [21]. All coronaviruses share a similar structure. The first two-thirds of the genome are open reading frames (ORFs) 1a and 1b encoding 16 nonstructural proteins [18]. The structural proteins, such as spike (S), envelope (E), membrane (M), and nucleocapsid (N), are encoded by the later reading frames [22]. Coronaviruses differ in the number and function of accessory proteins. The reading frames between the nonstructural and structural proteins encode the accessory proteins.

The S protein controls the virus activity and virulence and different accessory proteins that attack the host immune functions [23, 24]. The S protein is composed of S1 and S2 subunits. The S1 subunit has a receptor-binding domain (RBD) that binds with the receptor-binding motif (RBM) to the host surface. S2 subunit mediates receptor attachment and the host membrane fusion [25, 26]. The primary host receptor for SARS-CoV and SARS-CoV-2 is angiotensin-converting enzyme 2 (ACE2), while for MERS-CoV this is dipeptidyl peptidase 4 (DPP4) [27–30].

Coronaviruses are large with an average diameter of 80–120 nm and molecular mass of 40,000 kDa. They are roughly spherical and relatively pleiomorphic viruses with surface spikes [31]. Their RNA genome is situated in the center of the virus and protected by the N and M proteins and lipid bilayer envelope [32, 33]. The S protein is crucial for interaction with the host cell. In addition to S protein, the viral surface also has hemagglutinin-esterase dimer (HE), which is not necessary for replication but is important for viral entry [34, 35]. The E protein is a minor structural protein and is different in different coronaviruses [36]. The M protein is the primary structural protein and shapes the envelope [37]. The N protein is tied to the RNA and enables the virus to take over the host cells [38, 39]. The genome of coronaviruses contains various ORFs. The gene order in all members is 5′-leader-UTR-replicase (ORF1ab)-S-E-M-N-3′UTR-poly (A) tail [40]. Their genomes seem to have a bias against cytosine (C) and guanine (G) nucleotides, with the highest composition of uracil (U) and adenosine (A) [41]. In addition to these components, 16 nonstructural proteins (NSP1 to NSP16) differ between different groups of coronaviruses [18]. These NSPs have important roles in assembling the replication–transcription complex, RNA polymerization, RNA proofreading, mRNA capping, allosteric activation, and repression of the host immune system [42, 43].

To enter the cells, the S protein anchors the virus to ACE2 receptors which are expressed on surface. Transmembrane protease serine 2 (TMPRSS2) and lysosomal proteases also have a significant role in enabling SARS-CoV-2 entry into the cells [44]. After entering into the cytoplasm, the virus induces spatial alteration in the endosome resulting in its uncoating. Finally, the viral genome is released within the cytoplasm and the RTC initiates [45]. A unique characteristic of SARS-CoV-2 among the coronaviruses is the integration of furin-mediated cleavage of the S protein at the polybasic site that amplifies its virulence. It has been proposed that this site in SARS-CoV-2 S protein is necessary to enable the virus to infect humans as well as animals [46].

3.3 Variants

Coronaviruses are members of sub-family of *Orthocoronavirinae* in the family *Coronaviridae* order *Nidovirales* and realm *Riboviria* [47, 48]. Based on the latest International Committee of Taxonomy of Viruses (ICTV) classification, coronaviruses are sorted into four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus. However, the number of species is large

and many coronaviruses are unspecified [47, 49]. The Alphacoronavirus and Betacoronavirus infect only mammalian species, while Gammacoronavirus and Deltacoronavirus infect mammalian and avian species. Coronavirus infection mostly causes respiratory, gastrointestinal, and neurologic disorders [50, 51]. Several variants of concern have been recognized so far [52, 53]. These include: (1) the Alpha (B.1.1.7) variant which was first detected in the United Kingdom in September 2020; (2) Beta (B.1.351) which appeared originally in South Africa in May 2020; (3) Gamma (P.1, B.1.1.28.1) which arose in Brazil in November 2020; (4) Delta (B.1.617.2) which appeared as multiple forms in India in October 2020; and (5) the highly infectious Omicron (B.1.1.529) which arose in Botswana and South Africa in November 2021 and has since given rise to multiple sub-variants (BA.1–BA.5).

4 Pathogenesis of COVID-19: The Role of Inflammation

COVID-19 has often severe respiratory and gastrointestinal manifestations. In addition, extensive hyperinflammatory responses and inflammatory cytokine release have been reported in different organs. COVID-19 disease activates several inflammatory pathways, leading to immune system imbalance and impairment in the renin-angiotensin system (RAS), thus reducing expression of ACE2 and induction of the "cytokine storm." Extensive cytokine (i.e., TNF-α, IL-1β, IL-2R, IL-6, IFN)-γ and chemokine (i.e., C-motif chemokine ligands; CCL-2, CCL-3, CCL-10) release exacerbates the systemic inflammation and worsens patient prognoses. Also, ACE2 downregulation stimulates angiotensin II receptor1 (AT1R), leading to more severe disease [44, 54]. Molecular analyses have demonstrated the involvement of multiple signaling pathways in this inflammatory response, including IL-6-Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, TNF-α-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, and toll-like receptor (TLR)-myeloid differentiation primary response 88 (MYD88)-NF-κB pathway. TNF-α is one of the main pro-inflammatory cytokines that plays a significant role in initiating and propagating the inflammatory signaling transduction. TNF-α activates IL-6 and contributes to activation of the JAK-STAT kinase pathway. TNF-α also stimulates NF-κB signaling. Simultaneously, toll-like receptors (TLRs) and IFN-γ also actively participate in stimulating the inflammatory response. TLRs trigger myeloid differentiation primary response 88 (MYD88) overexpression and activates NF-κB. Furthermore, IFN-γ stimulates JAK-STAT signaling [55, 56]. Activation of these inflammatory pathways can cause acute lung injury, ARDS, thrombosis, organ failure, and an increased morbidity and mortality [55, 57]. Therefore, as mentioned earlier, treatment with medications which have anti-inflammatory effects which suppress these signaling pathways can result in favorable outcomes of COVID-19 and/or decrease mortality.

5 Statins

Statins are potent inhibitors of cholesterol synthesis and the use of these compounds has revolutionized the treatment of hypercholesterolemia [58]. Cholesterol is synthesized from acetyl coenzyme A, in a mechanism that occurs over 30-steps in which the rate-limiting step is modulated by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. This enzyme transforms HMG into mevalonate [59] and statins are competitive, reversible inhibitors of HMG-CoA reductase in the mevalonate pathway [60]. This inhibition results in the lowering of plasma LDL-C concentrations, which is a beneficial effect [61]. The statins family includes atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin. Regarding the origin, simvastatin, lovastatin, and pravastatin are extracted during fungal fermentation, while atorvastatin, fluvastatin, and cerivastatin are chemically synthesized. Lovastatin is produced from *Aspergillus terreus* strains, and simvastatin is a semisynthetic derivative of lovastatin [62]. Rosuvastatin has been synthesized more recently and is more potent than the older statins.

Pravastatin and rosuvastatin are less lipophilic and more hydrophilic in comparison with the other members of the statin family, while atorvastatin, fluvastatin, lovastatin, and simvastatin are more lipophilic. This property is important since lipophilic drugs have greater ability to diffuse into cell membranes, including those of hepatic cells, and water solubility is important for diminishing cytochrome P450 enzyme metabolism. Bioavailability is also an important pharmacological variable. Fluvastatin has a 24% bioavailability, while that of rosuvastatin is 20%, pravastatin 17%, atorvastatin 14%, and simvastatin less than 5% [63]. Regarding elimination half-life, rosuvastatin with 20 h, and atorvastatin with 14 h have a highly prolonged profiles. The elimination half-life of simvastatin, pravastatin, and fluvastatin are 1–2 h. The plasma half-life indicates their first-pass metabolism [64]. Lovastatin, simvastatin, and atorvastatin are metabolized by cytochrome P 450 3A4, while fluvastatin metabolism depends upon CYP2C9. Pravastatin is not significantly metabolized by the CYP family of enzymes [65]. Statins have many pleiotropic effects including modulation of anti-inflammatory responses (Fig. 25.1) [66] and antioxidant pathways [67, 68]. Therefore, statins have additional benefits besides their effects on serum lipoproteins [69].

Statins also change the function of platelets thereby significantly affecting atherosclerosis and thrombosis [70]. Vascular endothelial function is enhanced mostly by the increase of nitric oxide (NO) [71]. Statins can also play a crucial neuroprotective role in neurodegenerative disorders including Parkinson's disease, Alzheimer's disease, multiple sclerosis, and ischemic stroke, due to their anti-inflammatory, anti-oxidative, and anti-excitotoxic properties [72]. The most known adverse effects of statins concern those regarding muscle and liver tissue [73]. Muscle pain, fatigue and weakness, as well as rare rhabdomyolysis, are most common side effects related to statins, particularly if they are applied in high doses [74]. For example, myopathy can occur in 1–2000 patients and abnormalities in liver are seen in 1–2% of patients per year. However, these effects are mostly reversible and cease when the drug is reduced or stopped [75].

5.1 Anti-Inflammatory Effects of Statins

Experimental and clinical trials have showed that statins provide cardiovascular benefits beyond their lipid-lowering effects. These effects include (1) improvement of endothelial function; (2) modulation of inflammation and oxidative stress; (3) increasing plaque stability; and (4) inhibition of the thrombogenesis response [76–80]. These properties of statins are caused by intracellular isoprenoid inhibition and modulation of the reductive-oxidative (REDOX) state and nitric oxide pathway that eventually drive reduced levels of C-reactive protein (CRP) and pro-inflammatory cytokines (Fig. 25.1) [81, 82]. Moreover, statins can intensify ACE2 expression and suppress the TLR-MYD88-NF-κB pathway [83]. On the other hand, statin discontinuation in patients with coronary heart disease can cause adverse cardiovascular events, even without changes of lipid levels [84, 85]. Because of their anti-viral, immunomodulatory, anti-thrombotic, and anti-inflammatory effects, statins may have beneficial roles as adjuvant therapy in COVID-19. MYD88 is one of the host genes stimulated by SARS-CoV-2 infection. Stimulation of MYD88 triggers the NF-κB signaling transduction, reduces IFNs, and amplifies inflammation. Moreover, oxidized LDLs bind to TLR receptors and initiate inflammation via the TLR-MYD88-NF-κB pathway, eventually increasing inflammatory cytokine levels [86, 87]. Statins may exert their protective effects by maintaining the regular activity of the MYD88 pathway and its subsequent downstream products. This effect might be beneficial against COVID-19 by suppressing the beginning of the inflammatory cascade and subsequent release of inflammatory cytokines [88–91].

Administration of lovastatin (20 and 40 mg/day) in 284 intensive care unit (ICU) patients significantly decreased IL-6, IL-8, and CRP levels. The results of this study also showed that the hospitalization duration was reduced in patients who received lovastatin in comparison with control patients [92]. However, studies which evaluated the effect of pravastatin in COVID-19 patients did not find any significant improvements in prognosis. Although decreased mortality rates in patients who received simvastatin and atorvastatin were reported, patients who were treated with pravastatin and rosuvastatin did not show such an improvement [93].

The potential therapeutic effects of fluvastatin against SARS-CoV-2 infection have been studied in vitro and ex vivo. Fluvastatin at a concentration of 5 μM significantly reduced viral proteins, viral replication, and viral protein translation in human lung cells. The outcomes also suggested a slight inhibitory activity of lovastatin, pravastatin, and rosuvastatin in infected human lung cells but this effect was not as potent as that caused by fluvastatin [94]. A retrospective study of 87 COVID-19 patients admitted to ICU showed that atorvastatin treatment caused slower progression of the disease and a slower progression to death but, given the observational nature of this study, these results should be interpreted with caution [95]. Another retrospective cohort study which enrolled 421 confirmed cases of hospitalized COVID-19 patients showed that treatment with atorvastatin was associated with reduced mortality and lower endotracheal intubation rates [96]. A double-blind, randomized clinical trial also showed that adjunct therapy with

atorvastatin was more effective in hospitalized COVID-19 patients compared to the standard antiviral (lopinavir/ritonavir) treatment alone [97]. In contrast, a similar randomized control trial that compared the effect of atorvastatin (20 mg/day) versus placebo in 605 patients failed to confirm any significant beneficial effects of atorvastatin therapy [98], and another randomized clinical trial reported that addition of atorvastatin (20 mg/day) to the standard treatment (hydroxychloroquine + lopinavir/ritonavir) was associated with adverse outcomes in hospitalized COVID-19 patients [99]. This discrepancy in clinical trial results may be due to variations in the standard treatment of COVID-19, duration of treatment, or even the clinical stage of the disease. Another explanation might be that the effects of statins may be restricted to the early phases of inflammatory responses in COVID-19 [98]. Finally, an in silico molecular docking study which evaluated the interactions between statins (lovastatin, fluvastatin, pravastatin, atorvastatin, simvastatin, rosuvastatin, and pitavastatin) and the SARS-CoV-2 main protease (Mpro) suggested that statins may act as inhibitors of this enzyme. However, additional confirmations from experimental studies are needed concerning this issue [100].

6 Statins in COVID-19: New Possibility for COVID-19 Treatment

Several pieces of evidence support the anti-inflammatory effect of statins (Fig. 25.1) [101]. It is also known that statins have anti-viral [102], anti-inflammatory, and antithrombotic characteristics, suggesting their potential use as complementary drugs in COVID-19 therapeutics [90, 91, 103, 104]. Furthermore, statins have effects on reducing viral transmission by effects on cellular membranes [105].

6.1 Clinical Evidence

Retrospective cohort study, including patients who were hospitalized with confirmed diagnosis of severe COVID-19. Baseline characteristics and related clinical data of patients were recorded. Clinical outcomes consist of in-hospital mortality, need for invasive mechanical ventilation, and hospital length of stay. COX regression analysis models were used to assess the association of independent factors to outcomes. Atorvastatin was administered for 421 of 991 patients. The mean age was 61.640 ± 17.003 years. Older age, higher prevalence of hypertension, and coronary artery disease reported in patients who received atorvastatin. These patients have shorter hospital length of stay ($P = .001$). Based on COX proportional hazard model, in-hospital use of atorvastatin was associated with decrease in mortality (HR = 0.679, $P = .005$) and lower need for invasive mechanical ventilation (HR = 0.602, $P = .014$). Atorvastatin add-on therapy in patient with severe COVID-19 was associated with

lower in-hospital mortality and reduced the risk of need for invasive mechanical ventilation which supports to continue the prescription of the medication (Table 25.1) [96].

Atorvastatin is one of the most commonly used statins in treatment of hypercholesterolemia. Many studies have confirmed its pleiotropic effect on inflammation. A double-blind, parallel group, randomized clinical trial by Davoodi et al. analyzing the outcomes of atorvastatin treatment on COVID-19 patients. Forty patients were included in the study, and they were divided into two groups. Half of the patients received lopinavir/ritonavir (400/100 mg twice daily) and were the control group while the rest were treated with lopinavir/ritonavir (400/100 mg twice daily) + atorvastatin (40 mg daily) for 5 days. The hospitalization rate was shorter in the group treated additionally with atorvastatin (9.75 ± 2.29 vs. 7.95 ± 2.04 days; $p = 0.012$) and invasive mechanical ventilation was mandatory only for one patient in the lopinavir/ritonavir group. In addition, the CRP level was decreased, and O_2 saturation (O_2sat) increased significantly on the sixth day in comparison with the first day in the atorvastatin group. In the control group, the O_2 sat was not changed while CRP was increased (Table 25.1) [97].

Another study carried out by Karampoor et al. analyzed the anti-inflammatory effect of lovastatin on COVID-19 patients. The case control study included 284 ICU patients who were randomized into three different groups: (1) 92 patients received no lovastatin; (2) 99 patients were treated with 20 mg lovastatin per day; and (3) 93 patients received 40 mg lovastatin per day for 1 week. The results showed that CRP, IL-6, and IL-8 biomarkers were decreased in patients who received lovastatin in comparison with the control group and the decrease of IL-6 and IL-8 was dose-dependent. Also, IL-6 showed a greater decrease in the group who received 40 mg/day lovastatin than in those who received 20 mg/day. Moreover, IL-8 was higher in the control group than in the two intervention groups ($p < 0.05$). Finally, duration of hospitalization was significantly shorter in lovastatin-treated patients ($p < 0.05$) and the mortality rate was reduced although this effect was not significant (Table 25.1) [92].

Other studies have reported minimal or no effects of statin treatment on COVID-19 outcomes. A randomized controlled trial was done to evaluate the effect of atorvastatin on COVID-19 patients. Out of 587 patients suffering from COVID-19, 290 were assigned to be treated with atorvastatin, and 297 received placebo. Atorvastatin was administered orally (20 mg) or by a naso- or oro-gastric route to those patients who were mechanically ventilated and unable to take the drug orally. The study lasted for 30 days from randomization until the primary efficacy outcome was observed (a composite of venous or arterial thrombosis, treatment with extracorporeal membrane oxygenation, or all-cause mortality within 30 days from randomization). The primary outcome occurred in 33% patients assigned to atorvastatin and 36% assigned to placebo after 30 days follow-up (odds ratio 0.84, 95% confidence interval 0.58–1.21, $p = 0.35$). The median duration of ICU hospitalization was 5 days (interquartile range 3–9 days) in the atorvastatin group and 5 days (2–10) in the control group. No significant difference was found between the two groups concerning atrial fibrillation, venous thromboembolism, and arterial thrombosis. Liver enzyme levels were increased in five atorvastatin-treated patients and in six

Table 25.1 Clinical studies of statins in COVID-19

Study design	Intervention		Number of patients		Treatment duration	Results	Ref.
	Case	Control	Case	Control			
Double-blind, randomized parallel-group, clinical trial	Atorvastatin + lopinavir / ritonavir	Lopinavir / ritonavir	20	20	5 days	CRP level ↓o2sat ↑ Lopinavir/ritonavir + atorvastatin group Duration of hospitalization ↓ Lopinavir/ritonavir + atorvastatin group ($P = 0.012$) On day 6, O_2sat remarkably higher in lopinavir/ritonavir + atorvastatin group	[97]
Case control study	Lovastatin	–	99 patients (20 mg/day lovastatin), 93 patients (40 mg/day lovastatin)	92	1 week	CRP, IL-6, IL-8 levels ↓ Length of hospitalization in ICU and mortality ↓	[92]
Retrospective cohort study	Atorvastatin	Placebo	421	570	March 2020 to July 2020	CRP significantly lower in patients who received atorvastatin ESR higher in patients who did not receive atorvastatin Patients on atorvastatin had lower hospital and lower mechanical ventilation need Hospital mortality ↓	[96]
Randomized controlled trial	Atorvastatin	Placebo	290	297	30 days	Venous thromboembolism 2% in atorvastatin group and 3% in placebo group Liver enzyme increased in five patients who received atorvastatin and six who received placebo	[106]
Pragmatic, open-label randomized trial	Emtricitabine + tenofovir + colchicine + rosuvastatin Colchicine + rosuvastatin	Emtricitabine tenofovir (Standard care arm)	163 161	163 162	28 days	Mortality significantly lower in FTC/TDF + COLCH + ROSUV group vs. SOC group [10.7% (17/159) vs. 17.4% (28/161)] Invasive mechanical ventilation need lower in FTC/TDF + COLCH + ROSUV group vs. SOC group	[107]

placebo-treated patients (odds ratio 0.85, 95% confidence interval 0.25–2.81; $p = 0.79$) while venous thromboembolism occurred in six patients in the atorvastatin group and nine in the placebo group (odds ratio 0.71, 95% confidence interval 0.24–2.06) but myopathy was not clinically diagnosed in either group and the treatment was safe (Table 25.1) [106].

The effect of rosuvastatin plus colchicine, emtricitabine/tenofovir, and combinations of these were evaluated in 633 COVID-19 patients in a randomized, open parallel group multi-center-controlled trial. The patients received either: (1) usual care ($n = 162$; control group); (2) emtricitabine + tenofovir + colchicine + rosuvastatin ($n = 163$); (3) colchicine + rosuvastatin ($n = 161$); or (4) emtricitabine + tenofovir ($n = 163$). The results showed that need for invasive mechanical ventilation and 28-day mortality was significantly lower in the emtricitabine + tenofovir + colchicine + rosuvastatin group than in the standard care group. The results supported the idea that combination therapy with anti-viral and anti-inflammatory drugs can be useful in decreasing the damage in COVID-19 disease and over-activation of the innate immune system (Table 25.1) [107].

It should be stressed again that statin use can have adverse effects on muscle tissues and cause elevations in the levels of creatine kinase, liver enzymes, and serum glucose levels, all of which may already be elevated in severe COVID-19 disease. Some authors have also raised concerns as to whether statins might interfere with response to COVID-19 vaccines, although there has been no evidence shown thus far to confirm this. Also, concomitant administration of statins and some antiviral therapeutics might exacerbate the risk of adverse effects of statins because most statins are metabolized mainly through CYP3A4, and this CYP enzyme is potently inhibited by the antiviral drug Paxlovid [108–111].

6.2 In Vivo/In Vitro Evidence

An in vivo study testing the effects of statin administration were performed on K18-hACE2-transgenic mice infected with either a medium (mock) control or a 10^5 tissue culture infective dose of SARS-CoV-2 gamma strain [112]. In the mice that received 20 mg/kg of simvastatin as pre-treatment and throughout the study, the functional capillary density was higher and adhesion of leukocytes to inflamed endothelium lower than in control mice. In addition, there was a lower number of viral genome copies in the lungs, and less edema, tissue hemorrhage, inflammation, and oxidation in the simvastatin-treated compared to the control animals. In addition, both pre-treatment and post-treatment with 10 μM of simvastatin prevented monocyte death induced by SARS-CoV-2 infection. However, this effect was greater in the pre- compared to the post-protocol suggesting that the simvastatin treatment may be more effective if administered in the early stages of viral infection. At the molecular biomarker level, intracellular adhesion molecule-1 (ICAM-1) and integrin alpha M mRNA levels were decreased, and there were lower levels of inflammatory biomarkers such as TNF-α, IL-6, monocyte chemoattractant protein 1

Table 25.2 In vivo studies of statins in COVID-19

Study design	Intervention		Number of patients		Treatment duration	Results	Ref.
	Case	Control	Case	Control			
In vivo study on K18-hACE2-transgenic mouse model	Simvastatin	Vehicle	10 (simvastatin + SARS-CoV-2)	8 (vehicle+SARS-CoV-2) 5 (mock)	11 days	Simvastatin reduced viral replication, lung damage, and mortality MPO and IL-6 ↓	[112]

(MCP1), IFN-α, chemokine (C-C motif) ligand 5 CCL5, and chemokine (C-X-C motif) ligand 1 (Table 25.2).

In an in vitro study on human lung microvascular endothelial cells, Qian et al. showed that administration of the SARS-CoV-2 N protein led to activation of the NF-kB and MAPK signaling pathways, with increased expression of cellular adhesion and inflammatory molecules [113]. They also found that simvastatin treatment blocked this endothelial activation in a dose-dependent manner, suggesting that this compound might help to ameliorate SARS-CoV-2-induced vasculopathy and coagulopathy in COVID-19 patients (Table 25.3).

Zapatero-Belinchón et al. performed an in vitro study to investigate the effect of statin pre-treatment on lung cells infected with the human coronaviruses, CoV-229E and SARS-CoV-2 [94] (Table 25.3). The statin pre-treatment with 5 mM fluvastatin led to a dose-dependent reduction in the susceptibility of these cells to coronavirus infection. The researchers followed this up by testing the effects of pre-treatment with either 10 or 50 mM fluvastatin on SARS-CoV-2 infected human primary bronchial epithelial cells. This showed that the 10 mM fluvastatin dose decreased viral release moderately and the 50 mM dosage decreased viral release in samples from all donors. In addition, label-free mass spectrometry proteomic profiling showed that the 35 proteins were significantly decreased by the fluvastatin treatment. Many of these proteins were associated with RNA degradation, protein translation, and viral replication processes (Table 25.3).

7 Conclusions and Future Perspectives

COVID-19 is a worldwide pandemic causing often mild symptoms including fatigue, dry cough, dyspnea, myalgia, chills, and fever but also severe symptoms that can cause organ failure. COVID-19 can affect several organs, including the respiratory, digestive, and central nervous system. Inflammation plays a pivotal role in COVID-19 and therefore anti-inflammatory medications might suppress the harmful effects of the virus on organs and tissues. Statins are currently the most

Table 25.3 In vitro studies of statins in COVID-19

Study design	Intervention		Number of patients		Treatment duration	Results	Ref.
	Case	Control	Case	Control			
In vitro study on human lung cells	Statins, particularly fluvastatin	DMSO	–	–	24 h	Fluvastatin induced unique proteins and inhibited some proteins in infected cells SARS-CoV-2 infection in cultured cells↓	[94]
In vitro study	Simvastatin	–	2 x 10^4cell/well	–	24 h	SARS-CoV-2-induced pro-inflammatory response in human neutrophils ↓ TNF, CXCL-8/IL-8, IL-6, and IFN-a in SARS-CoV-2 infected monocytes↓ Both pre- and post-treatment with 10 μM simvastatin hindered monocyte death in SARS-CoV-2-2 infected cells Pretreatment with simvastatin impede IL-6, CXCL8/IL-8 and TNF by SARS-CoV-2-infected Calu-3 cells, reduced viral entry in Calu-3 cells in a dose-dependent manner, diminished SARS-CoV-2-induced cell death Virus entry and adsorption inhibited, ACE2 expression promoted by simvastatin, virus binding and entry was lower	[112]

(continued)

Table 25.3 (continued)

Study design	Intervention		Number of patients		Treatment duration	Results	Ref.
	Case	Control	Case	Control			
In vitro study on HLMECs infected with SARS-CoV-2	155 substances, including simvastatin, lovastatin, atorvastatin, mevastatin, rosuvastatin	–	30 μM, 1 h before induction of N protein (1 μg/mL)	–	8 h	Simvastatin potently and lovastatin mildly inhibited N protein-induced expression of ICAM-1 and VCAM-1, blocked monocyte adhesion to activated endothelial cells Downregulation of NF-κB and MAPK l pathways	[113]

often prescribed and effective LDL-cholesterol lowering drugs that are used to prevent atherosclerotic cardiovascular disease. Statins decrease total and LDL-cholesterol, they slightly reduce triglycerides and slightly increase HDL-cholesterol, therefore decreasing the risk of adverse cardiovascular events. In addition to their effects on cholesterol metabolism, statins reduce the circulating isoprenoid and inactivation of signaling proteins. Statins also have anti-inflammatory, antioxidant, antiproliferative, and immunomodulatory effects. Statins can also stabilize atherosclerotic plaques and prevent platelet aggregation on the plaques. Because of their proven anti-inflammatory effects statins, this review focused on their potential use as an adjuvant therapy in the treatment of COVID-19. Statins are safe drugs without many adverse effects but their musculoskeletal adverse effects should be taken into consideration.

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Part VI
Genomic Surveillance and Future Pandemics

Chapter 26
Multiplex Immunoassay Approaches Using Luminex® xMAP® Technology for the Study of COVID-19 Disease

Shubhagata Das and Sherry Dunbar

Abstract The COVID-19 pandemic, caused by the SARS-CoV-2 virus, has been one of the most severe outbreaks of respiratory illness in history. The clinical symptoms of COVID-19 may be similar to flu, although they can be life-threatening, particularly in the elderly and immunocompromised population. Together with nucleic acid detection, serological testing has been essential for the diagnosis of SARS-CoV-2 infection but has been critically important for studying the epidemiology, serosurveillance, and for vaccine research and development. Multiplexed immunoassay technologies have a particular advantage as they can simultaneously measure multiple analytes from a single sample. xMAP technology is a multiplex analysis platform that can measure up to 500 analytes at the same time from the same sample. It has been shown to be an important tool for studying immune response to the various SARS-CoV-2 antigens, as well as for measuring host protein biomarker levels as prognostic indicators of COVID-19. In this chapter, we describe several key studies where xMAP technology was used for multiplexed analysis of SARS-COV-2 antibody responses and host protein expression in COVID-19 patients.

Keywords MAP technology · COVID-19 · Multiplex · Immunoassay · Serological assay

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1 Introduction

The coronavirus disease-19 or COVID-19 pandemic caused by SARS-CoV-2 has been one of the most severe outbreaks of respiratory illness in recent times. The clinical symptoms of COVID-19 mimic flu, although they can be life-threatening, particularly in the elderly and immunocompromised population. Nucleic acid tests based on viral genome sequences for SARS-CoV-2 are considered the gold standard for detecting current infection and can aid in patient management, infection control, and prevention of transmission [1]. However, nucleic acid tests cannot determine prior exposure to the pathogen, possible immunity, or identify susceptible individuals. Therefore, serology (antibody) testing is essential to provide community-level immune response data to identify exposure, prior infection, potential donors of convalescent plasma, and assist public health officials in implementation of safety policies. Serological testing is also critical for determining the duration of protective immunity against the SARS-CoV-2 virus. From a therapeutic perspective, serological testing is a key component of vaccine and drug development because it assists with determining drug efficacy and immune response to vaccines. Surveillance data obtained from serological testing can provide insights into the rate of community transmission and the efficacy of non-pharmaceutical interventions such as social distancing, quarantine, and travel restrictions [2].

Multiplex testing can be particularly useful in a pandemic, as it can simultaneously analyse large numbers of antigens for large-scale screening and has the potential to replace traditional enzyme-linked immunoadsorbent (ELISA) assays. Compared to ELISA, multiplex assays can shorten the time to results, minimize the volume of sample required, and eliminate excess labour by reducing the amount of testing that is needed. Furthermore, analysis of the proteins expressed in the host during and after COVID-19 may also be key to understanding the pathology of the disease in different patient populations. Several studies have described the role of biomarkers, such as IL-6 and procalcitonin, on the immune response to SARS-CoV-2 which could help determine prognosis and assist with management of COVID-19 patients.

Amongst the various assay platforms that have been developed over the years, xMAP® technology from Luminex® has emerged as one of the most common and well-established platforms for multiplex analysis of proteins, antibodies, and nucleic acids with more than 60,000 peer-reviewed publications. More than 65 Luminex partners offer assay kits for over 1300 analytes and the open architecture of the platform allows custom assay development as well. In this chapter, we describe various applications of xMAP technology in diagnosis, vaccine research, and surveillance of SARS-CoV-2, as well as the study of protein biomarkers relevant to the pathology of COVID-19.

2 xMAP Technology

The xMAP technology is a bead-based multiplexing platform that can rapidly detect and quantify multiple analytes in a single sample. xMAP technology is based on the principles of flow cytometry and uses polystyrene microspheres (beads) that are identical in size, physical properties, and surface composition but have different amounts of internal dyes to allow them to be classified into discrete populations [3]. The beads are dyed with precise amounts of spectrally distinct fluorochromes which are excited at the same wavelength but have unique emission profiles to provide distinct spectral characteristics for each individual microsphere region (bead set) and allow each bead colour to be differentiated from all others in the multiplexed reaction (Fig. 26.1). Each bead set can be covalently coupled with capture molecules that are specific to a target of interest. For a multiplex reaction, a mixture of coupled beads specific to different target molecules are added in a single reaction to simultaneously detect multiple analytes. A reporter fluorochrome, usually R-phycoerythrin (PE), quantifies the binding events on the bead surface, and the fluorescence of the internal dyes allows for differential analysis of the multiplex data (Fig. 26.2).

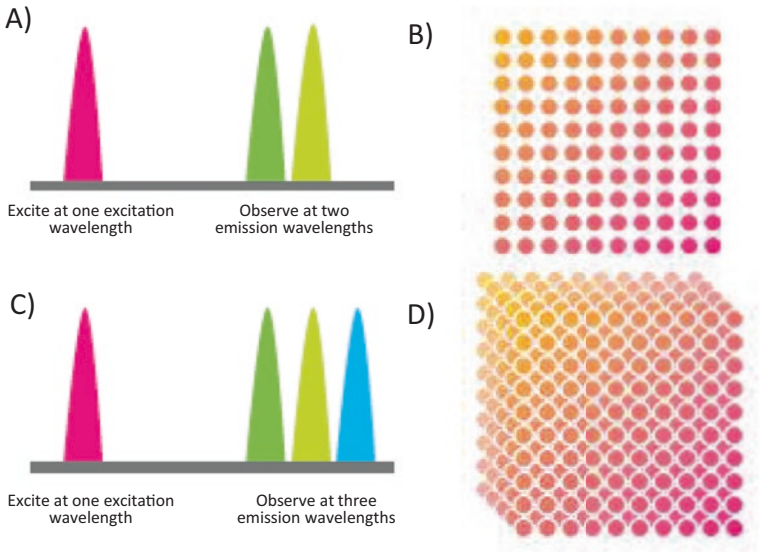

Fig. 26.1 xMAP® microspheres include two dyes, where (**a**) one excitation wavelength allows for the observation of two separate fluorescence emission wavelengths, (**b**) yielding 100 unique microsphere sets (10 × 10 dye matrix), or (**c**) three dyes, where one excitation wavelength allows for observation of three separate fluorescence wavelengths, (**d**) yielding 500 unique microsphere sets (10 × 10 × 5 dye matrix)

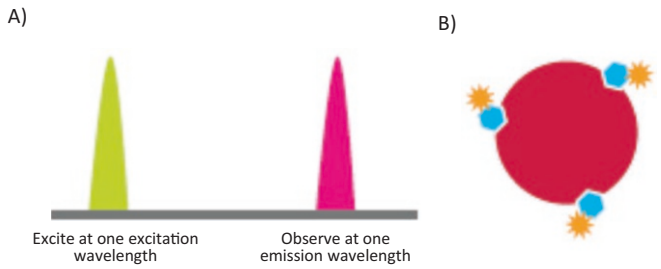

Fig. 26.2 In addition to detection of internal bead dyes (shown in Fig. 26.1), a second excitation wavelength allows for (**a**) observation of a separate fluorescent reporter molecule that (**b**) enables the detection of the analyte captured on the surface of the microsphere

3 Applications of xMAP Technology During the Pandemic

3.1 Diagnosis

Antibody assays are extremely valuable in identifying previous exposure to the pathogen and for detecting asymptomatic infection. It is also essential to identify individuals who have recovered from the disease and do not have an active viral infection. During the COVID-19 pandemic, several laboratories developed xMAP-based diagnostic immunoassays that have been widely used to detect anti-SARS-CoV-2 antibody responses amongst COVID-19 patients for the diagnosis of SARS-CoV-2. Weiss et al. developed a high-throughput xMAP bead-based multiplex immunoassay that can simultaneously measure, qualitatively and quantitatively, the spike (S) protein antibodies and the spike angiotensin-converting enzyme (ACE2) receptor-binding domain (RBD) in serum and plasma samples in less than 2.5 h [4]. The results demonstrated a wide range of serum/plasma antibody levels in the infected patient samples, and also clearly differentiated between specimens that were obtained from COVID-positive and COVID-negative patients. Such an assay is advantageous over traditional ELISA approaches, since the assay used 20-fold less antigen than ELISA and the antigen-coated beads could be stored and prepared in advance. Cameron et al. developed a multiplex xMAP microsphere-based immunoassay that can detect antibodies to three major SARS-CoV-2 antigens: S protein, RBD, and nucleocapsid (N) protein [5]. The assay was able to reveal the overall profile of the IgG serological response and identify the initial and peak responses for each SARS-CoV-2 antigen, timing of the decline in antibody levels, and correlation with decline in the viral load. The assay had 48% sensitivity for samples that were obtained ≤5 days from symptom onset and progressively improved to 92% for samples that were obtained between 16 and 20 days. Additionally, comparable performance was observed between the xMAP-based immunoassay and other commercial immunoassay for samples that were obtained ≥21 days from symptom onset. However, the xMAP-based assay was more sensitive (48.0% vs. 32.0%) for samples obtained at ≤5 days from symptom onset. The results obtained in this study

were comparable with other studies that reported immunoassay sensitivities ranging from 87% to 95.7% between 15 and 21 days of symptom onset and a 100% after 3 weeks of symptom onset [6–8].

In another study, Cameron et al. further modified the previously developed "3Flex" immunoassay to detect both IgM and IgG antibodies to be performed on a new dual reporter instrument (INTELLIFLEX DR-SE) that can measure two fluorescent signals per analyte at the same time [9]. It was observed that the IgM peaked and declined rapidly between weeks 3 and 4 following infection, whereas S- and RBD-specific IgG antibody response plateaued at 80 days from symptom onset. Ndiaye et al. also developed an xMAP-based multiplex immunoassay targeting specific IgM and IgG antibodies against the S1 and S2 domains, RBD, and N antigens [10]. The study reported 100% sensitivity and specificity for S1, RBD, and N for IgG at day 14 after enrolment. The in-house bead-based assay demonstrated a higher sensitivity when compared to two commercially developed ELISA kits, as it detected more true positives than the commercial assays. The results further revealed that COVID-19 symptomatic individuals produce more RBD-specific IgM and confirmed that IgM and IgG responses to SARS-CoV-2 show distinct patterns over time. Dobaño et al. developed a quantitative suspension array (qSAT) assay based on xMAP technology and observed 95.78% sensitivity and 100% specificity for samples obtained at ≥14 days since the onset of symptoms [11]. The researchers further concluded that compared to traditional ELISA, the multiplex immunoassay can capture a wider range of antibody responses, which is critical for diagnosis as some individuals may not respond to one antigen but may respond to other antigens or responses may change over time.

Luminex developed the xMAP® SARS-CoV-2 Multi-Antigen IgG Assay (EUA) which is a multiplex, microsphere-based, highly sensitive, and specific assay that detects the presence or absence of antibodies from serum or plasma samples against three different SARS-CoV-2 antigens: S1, RBD, and N [12]. The assay measures IgG responses against the three antigens, which is consistent with CDC guidelines to assess a multi-target immune response to SARS-CoV-2, particularly in low-prevalence settings [13]. Several studies have validated the assay and evaluated its performance for detection of antibodies against the SARS-CoV-2 antigens. Iriemenam et al. validated the assay using whole blood specimens and reported an overall sensitivity of 75.3% and specificity of 99% [14]. The reported sensitivity was lower than the manufacturer reported sensitivity of 96.3%, however, this could be attributed to the difference in the time point of serum sample collection, which is critical for evaluating the diagnostic performance of serological assays [6]. The sensitivity estimate increased to 83.3% for specimens >14 days post-confirmation of diagnosis. The assay also demonstrated a higher sensitivity (75.3% vs. 73%) when compared to other commercial immunoassays that had been validated previously. The xMAP multi-antigen assay protocol can be further modified to evaluate alternate sample types, antibody isotypes, and potential neutralizing antibody responses [12]. Turgeon et al. validated the modified xMAP multi-antigen assay on dried blood spot specimens and observed a 96.9% concordance of qualitative results with matched sera tested by the reference FDA EUA SARS-CoV-2 serologic assay [15].

3.2 Vaccine Research

Multiplex immunoassays have been deemed essential not only to measure quantitative antibody responses to COVID-19 infection but also responses to the vaccines against SARS-CoV-2. Bartsch et al. evaluated the antibody response elicited by children in response to the adult (100 μg) and paediatric (50 μg) doses of the Moderna mRNA-1273 vaccine [16]. The researchers used an xMAP bead-based assay to analyse antigen-specific antibody isotype, subclass titres, and Fc receptor binding profiles. The study reported that the vaccinated children elicited an IgG-dominant immune response to both doses in a similar manner, but not identical to adults. It was further observed that children generated antibodies with enhanced Fc receptor binding capacity irrespective of the antibody titre recorded. In another study, Benschop et al. investigated the effect of prior prophylactic treatment with bamlanivimab, a SARS-CoV-2 monoclonal antibody, on the response to vaccination with Comirnaty or SpikeVax [17]. The binding inhibition titre for ACE2-RBD was measured using a custom xMAP bead-based assay and it was observed that the effect of prior bamlanivimab treatment on vaccination was minimal, which suggested that the benefits of monoclonal antibody therapy outweigh the costs in terms of reducing vaccine-elicited immune responses. Multiplex immunoassays have also been used to measure the association of symptoms after COVID-19 vaccination with anti–SARS-CoV-2 antibody response. Hermann et al. studied the association between self-reported post-vaccination symptoms with anti–SARS-CoV-2 antibody response [18]. A multiplex xMAP bead-based immunoassay was used to measure IgG antibodies to the SARS-CoV-2 S protein, and it was observed that a greater antibody response was associated with self-reported systemic symptoms after SARS-CoV-2 mRNA vaccination. Gray et al. evaluated the immunogenicity and reactogenicity of the COVID-19 RNA vaccine in pregnant and lactating women [19]. Antibody titres were measured at different time points using a multiplex immunoassay, and it was observed that vaccine-induced antibody titres were equivalent in pregnant and lactating women compared to non-pregnant women.

Multiplex immunoassays have also been used to evaluate the safety and immunogenicity of vaccines during the early development stages and phase 1 trials of the vaccines. Walsh et al. evaluated the dose levels of BNT162b1 and BNT162b2 vaccines in adults [20]. They utilized a bead-based SARS-CoV-2 serum neutralization assay and RBD-binding and S1-binding IgG direct immunoassays to evaluate the immunogenicity before the administration of vaccine or placebo at days 7, 21, 28, and 35. The immunogenicity and safety data were assessed to determine which vaccine candidate should be advanced to phases 2 and 3 of the trial. In a similar study, Frenck et al. assessed the safety, immunogenicity, and efficacy of the BNT162b2 vaccine in a healthy adolescent population between 12 and 15 years old [21]. It was observed that the BNT162b2 vaccine in 12-to-15-year-old recipients was highly effective against COVID-19 and had a favourable safety profile and produced a greater immune response than in young adults.

3.3 Epidemiology and Surveillance

Epidemiological surveillance is key to infection control and monitoring and can assist with disease elimination efforts in low-transmission areas. In addition to pathogen identification, detection of serological markers can provide additional information to estimate recent and past exposure to the pathogen. It is essential to evaluate a set of compatible immunogenic antigens for developing large-scale serological assays for serosurveillance purposes. Mariën et al. designed an xMAP bead-based immunoassay to evaluate the performance of N, RBD, S1, and S2 antigens for the detection of SARS-CoV-2 IgG, IgM, and IgA antibodies using sera from severe and mild cases in the early convalescent phase (<6 weeks) and later after infection (>5 months) [22]. It was observed that neutralizing and binding IgG, IgA, and IgM antibody levels were higher for severe than mild cases in the early convalescent phase (<6 weeks). Additionally, contrary to the hypothesis, both neutralizing and IgG antibodies were detected in >96% of PCR-confirmed cases at least 5 months after infection, although the titre differed between severe and mild/asymptomatic cases. Alternative sample types such as dried blood spots have been used for a long time for serosurveillance in adults and children, particularly in resource-limited countries [23, 24]. This sample type is favoured as it can be self-obtained, uses minimal resources such as a single-use lancet and a filter paper card, and eliminates close contact with phlebotomists in outbreak situations. Schultz et al. developed a bead-based high-throughput multiplex immunoassay for the RBD and the N antigens and validated this using serum and dried blood spot eluates [25]. The multiplex immunoassay could successfully differentiate between SARS-CoV-2 seropositive and seronegative individuals, was more sensitive than ELISA (98% vs. 87%), and could be scalable for rapid and affordable SARS-CoV-2 serosurveillance. Multiplex immunoassays can be also useful to differentiate antibody responses elicited due to natural infection versus vaccine-induced immunity for post-vaccination serosurveys and vaccine effectiveness studies. Laing et al. developed and validated a high throughput multiplex immunoassay to discriminated SARS-CoV-2 natural and vaccine- induced immunity from seasonal human coronavirus humoral responses using dried blood spot specimens [26]. The assay demonstrated 92–99% sensitivity and 94–100% specificity for samples collected as early as 7–10 days from symptom onset. The same group of researchers also developed and characterized a betacoronavirus (β-CoV) multiplex microsphere-based immunoassay to examine differences in SARS-CoV-2 antibody reactivity between widely used antigens [27]. The assay detected seroprevalence of 72% and 98% for HCoV-HKU1 and HCoV-0C43, respectively, and concluded that the assay can be used to investigate the influence of HCoV-induced antibodies on COVID-19 clinical outcomes. Moe et al. used the xMAP multi-antigen assay to study different serological responses between the first and second epidemiological waves of COVID-19 [28]. The study reported that following the first wave, distribution of SARS-CoV-2 positive serology was slightly

higher than expected in the sample cohort, and also observed a scarcity of seropositive cases without COVID-19 diagnosis after the second wave.

3.4 Biomarker Analysis

During the pandemic, identification of reliable biomarkers that can predict the COVID-19 disease progression was essential to categorize high-risk patients following diagnosis to ensure optimal resource management. Several studies have reported elevated levels of various biomarkers such as white blood cells, creatinine, blood urea nitrogen, creatinine, C-reactive protein, and markers of liver and kidney for the severe or fatal cases of COVID-19 compared with milder cases [29–31]. Hyper-inflammatory response and cytokine storm-like syndrome were common during the COVID-19 disease that determined the disease severity and also was responsible for deaths in patients [32–35]. Arsentieva et al. used an xMAP bead-based multiplex immunoassay to study 47 cytokines/chemokines/growth factors for evaluating the significance of specific cytokines in blood plasma as predictive markers of COVID-19 associated mortality [36]. It was observed that four pro-inflammatory cytokines, IL-6, IL-8, IL-15, and IL-18 have the highest significance in determining the disease outcome. The study further concluded that analysing the concentrations of IL-6 and IL-8 prior to treatment might be valuable in terms of clinical outcome. Biró et al. compared the cytokine concentrations between patients who recovered from the disease and patients who died [37]. It was observed that patients who died had higher levels of IL-6, IL-8, IL-10, IL-15, MCP-1, and TNF-α compared to those who recovered. Additionally, the study reported higher levels of IL-8 and IL-10 under plasma therapy compared to tocilizumab administration, thereby concluding although that tocilizumab has some effect on cytokine profile, a higher level of inhibition is needed to effectively reduce the cytokine storm during intensive care unit therapy.

Biomarker analysis is also important to understand the long-term effect of COVID-19 disease including neurological sequelae and symptoms such as headache, fatigue, dizziness, memory loss, confusion, and difficulty focusing. Sun et al. used a multiplex immunoassay to evaluate peripheral biomarkers of inflammation associated with neurological dysfunction to understand the post-COVID-19 neurocognitive symptoms in the early stages of recovery [38]. The study reported an elevated level of plasma cytokine IL-4 in all COVID-19 study participants and observed a positive correlation of IL-6 with the age and severity of the neurological sequelae. Biomarker analysis for vascular transformation blood biomarkers has been studied in COVID-19 survivors to predict if they will encounter long COVID symptoms when patients have diffuse symptoms months after recovering from the COVID-19 infection [39]. The study reported that vascular transformation blood biomarkers were significantly elevated in long COVID patients along with angiogenesis markers (ANG-1/P-SEL).

4 Conclusion

The COVID-19 pandemic has witnessed an unprecedented surge in laboratory developed and commercially available diagnostic assays for the detection of SARS-CoV-2 nucleic acids or antibodies to SARS-CoV-2 antigens. In the last 2 years of the outbreak, several serological assays have been developed and validated for rapid and accurate detection of the viral pathogen, as well as for effective surveillance and monitoring to determine infection rates and status and to allow for the implementation of operational public health policies. Multiplex immunoassays have been particularly advantageous over the traditional ELISA during the outbreak, as they can detect multiple analytes at the same time using less volume of reagent and sample, thereby making it a perfect tool in a resource-limited setting. Although these assays are not suitable for detecting active infection in the first few days of illness, they have been proven useful to determine antibody responses to natural and vaccine-induced immunity, for vaccine development and for epidemiological and serosurveillance purposes. Serological assays are also used to determine the attack rate and immunity in communities and to evaluate the disease progression by detecting appropriate biomarkers. As we emerge from the COVID-19 pandemic, research will focus on a better understanding of the disease and the differences in the pathology in different patient populations. Much work has already been done to look at the cytokine storm in COVID-19 and to identify host protein biomarkers that may aid in determining the prognosis and best management of COVID-19 patients, as well as help predict patients susceptible to long COVID. Multiplexed immunoassay methods, such as the xMAP technology platform, are well-positioned to be an essential tool in this work. As healthcare systems and the diagnostic industry prepare for future pandemics, multiplex immunoassays need to be continuously evaluated and improved, along with nucleic acid based molecular assays to effectively understand the dynamics of the outbreak.

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Chapter 27
Rapid Detection of SARS-CoV-2 Variants of Concern by Genomic Surveillance Techniques

Paul C. Guest, Steve F. C. Hawkins, and Hassan Rahmoune

Abstract This chapter describes the application of genomic, transcriptomic, proteomic, and metabolomic methods in the study of SARS-CoV-2 variants of concern. We also describe the important role of machine learning tools to identify the most significant biomarker signatures and discuss the latest point-of-care devices that can be used to translate these findings to the physician's office or to bedside care. The main emphasis is placed on increasing our diagnostic capacity and predictability of disease outcomes to guide the most appropriate treatment strategies.

Keywords COVID-19 · SARS-COV-2 · Diagnosis · Disease management · Point-of-care · POC · Lab-on-a-chip

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1 Introduction

According to databases such as Worldometer [1] and the Johns Hopkins Institute [2], more than 648 million people have been infected by the SARS-CoV-2 virus which causes COVID-19 disease and more than 6.6 million of these individuals have died (as of December 1, 2022). However, the actual number of infected people is likely to be much higher, with some studies estimating that almost 50% of the world population has been infected [3]. From early on in the pandemic, it was deemed that early, rapid, and accurate detection of COVID-19 disease was critical for better management of the crisis, as well as for facilitating better therapeutic outcomes, and a lower damaging effect on healthcare and financial systems [4–7]. However, at that time, most of the testing for such infectious diseases was performed in centralized laboratories by trained personnel, and it could take up to several days for the results of these tests. Given the urgency evidenced by the COVID-19 pandemic and the threat of future outbreaks, it is clear that there is a need for more user-friendly and diagnostic tests that can be used in a point-of-care (POC) capacity. Advances made in the areas of microfluidics, miniaturization, and integration have now enabled the application of these devices in standard laboratory and clinical environments as well as in emergency use scenarios [8–12].

A major obstacle in the use of POC devices occurs at the sample stage. Importantly, this should involve as little human interaction as possible as this is where errors or biases can be introduced. If there is a rush of infected persons to get to the site of testing, as occurred during the early stages of the COVID-19 pandemic [13], there is also the chance of cross-infections. One solution to this is that the testers visit the prospective patients in their homes or places of work and carry out the testing there. Of course this would require trained professionals and for the testing kit to be portable, with a sample-sealing capability to avoid cross-contaminations. This would help to minimize the number of false positives and false negatives.

If the test is polymerase chain reaction (PCR)-based, the sample acquisition and preparation steps are critical. This is due to the presence of constituents in body fluids such as blood serum/plasma [14] and saliva [15] that can inhibit the amplification step in PCR [16]. However, there have been advances in overcoming these potential effects and making the sample preparation step more PCR-friendly. For example, we recently described the use of a commercially available inhibitor-tolerant PCR mix which circumvents the need for extraction, allowing for a faster and more accurate identification of the infective agent and determination of viral load [17, 18]. Multiplex PCR platforms offer a number of advantages of single assay systems as they can significantly lower test times, conserve samples, lower costs, while allowing for simultaneous analysis of multiple pathogens such as influenza types A and B [17, 19, 20] and different SARS-CoV-2 variants [18, 21, 22]. The correct identification of a pathogen using such systems would also allow patient stratification or triage for the most appropriate treatment and also provide a means of correctly determining across a suspected group of pathogens in the different waves of an outbreak and/or the emergence of a new pandemic.

The COVID-19 pandemic has enhanced the need and drive of researchers around the world to develop POC devices to enable early diagnosis of SARS-CoV-2 infection, variant subtyping, and to lay the groundwork in the advent of future pandemics. In this chapter, we describe some of the major developments which have served to advance these efforts.

2 The Omicron Variant

The B.1.1.529/BA.1 SARS-CoV-2 variant (termed Omicron by the WHO) was first reported on 24 November 2021 [23, 24], with cases appearing in Botswana and then South Africa (Fig. 27.1) [23]. By 10 Jan 2022, it had been reported in 89 countries and reached a peak infection rate of more than four million cases per day on 21 Jan 2022 [1, 2, 25]. After this, several Omicron sub-variants evolved which led to further smaller waves and perpetuation of the pandemic [26]. Because of the increase in diversity and highly infectious nature of this variant, the WHO updated their tracking system with a new arm called 'Omicron sub-variants under monitoring' to help identify the sub-variants which may need to be prioritised in public health warnings (Table 27.1) [27].

With 31 or more mutations, Omicron and its sub-variants have the largest number of spike protein amino acid substitutions compared to the preceding Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Delta (B.1.617.2) variants of concern [28, 29]. Approximately half of these mutations in the spike protein occur within the receptor-binding domain (RBD) which binds to the angiotensin converting enzyme

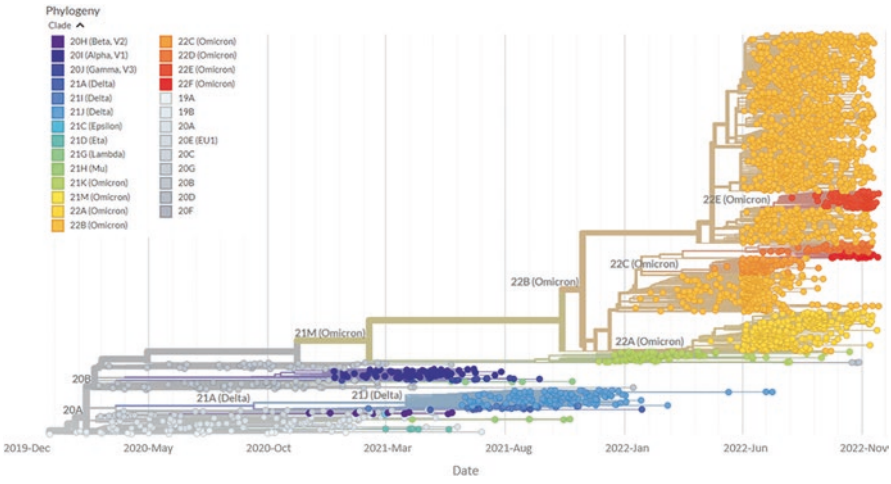

Fig. 27.1 Molecular evolution of SARS-COV-2 with a focus on the omicron strain (phylogeny maintained by Nextstrain, enabled by data from GISAID, Image courtesy: https://nextstrain.org/ncov/open/global/6m)

Table 27.1 Omicron sub-variants under monitoring by the World Health Organization as of October 2022

Sub-variant	Additional possible spike protein mutations
BA.5	R346X or K444X or V445X or N450D or N460X
BA.2.75 (BA.2 +)	K147E, W152R, F157L, I210V, G257S, D339H, G446S, N460K, Q493R (reversion)
BA.2.75.2 (BA.2.75+)	R346T, F486S, D1199N
BJ.1 (BA.2+)	V83A, Y144-, H146Q, Q183E, V213E, G339H, R346T, L368I, V445P, G446S, S:V483A, F490V, G798D, S1003I
BA.4.6 (BA.4+)	R346T, N658S
XBB (BA.2+)	V83A, Y144-, H146Q, Q183E, V213E, G252V, G339H, R346T, L368I, V445P, G446S, N460K, F486S, F490S
BA.2.3.20 (BA.2+)	M153T, N164K, H245N, G257D, K444R, N450D, L452M, N460K, E484R

2 (ACE2) receptor on host cells in the infection process [28–30]. In addition, many of these mutations are known to alter the binding of antibodies produced by the existing vaccines or from previous SARS-CoV-2 infections [31–33].

These changes in properties have led to increasing concerns about the potential emergence of newer variants with increased virulence and capacity to escape the vaccines. However, as the virus adapts to us, we can also adapt to the virus and help to prepare ourselves for a future pandemic like this one or one that is potentially even worse. For example, since the virus has evolved to evade the existing vaccines, we must follow suit and learn to efficiently and effectively update the vaccination programmes to keep pace with these changes. In line with this, Pfizer/BioNTech has released two different bivalent vaccines which target both the original Wuhan spike protein and either the Omicron BA.1 or BA.4–5 spike proteins, and both of these were authorized for use in the European Union in September 2022 [34]. Moderna has also released a bivalent vaccine against the Wuhan and the omicron BA.1 spike proteins which was also approved in September 2022, as well as one that targets the BA.4–5 spike protein, and this is currently under evaluation.

3 Genomic Surveillance

3.1 Next-Generation Sequencing

At the beginning of the pandemic, the SARS-CoV-2 genome was sequenced using a metagenomics approach, which basically allowed determination of the full genome without any prior knowledge of the sequence. After this, it became possible to use more targeted and efficient approaches which involved the design of primers for amplification of multiple overlapping sequences to cover the whole SARS-CoV-2 genome. At this stage, the identification of specific variants required the use

of various bioinformatics pipelines. New lineages are usually assigned using the Nextclade or Pangolin algorithms [35, 36].

Whole genome analysis by next-generation sequencing (NGS) is currently the gold standard technique used for identification and monitoring of new SARS-CoV-2 variants and sub-variants [37]. The method essentially allows parallel sequencing of billions of DNA fragments which are combined afterwards by read assembly [38–40]. This method is typically performed using four basic steps (Fig. 27.2):

1. Library preparation through random fragmentation of the genome and ligation of adapters
2. Generation of clusters by loading the library into a flow cell for capture of the fragments on bound oligonucleotides complementary to the adapters, flowed by bridge amplification of each fragment
3. Reversible terminator sequencing for detection of each nucleotide as it is incorporated into a new strand
4. Data analysis and alignment for detection of single nucleotide polymorphisms, mutations, recombination events, and/or phylogenic tree construction

There are also nanopore sequencing methods that allow maximum coverage of the SARS-CoV-2 RNA genome via PCR tiling [41, 42]. A method called Midnight works through amplification of the genome in overlapping segments of 1000–1200 base pairs which makes it resistant to amplification dropouts due to mutations. The ARTIC method is similar but amplifies the genome in shorter segments of approximately 400 base pairs. This helps to improve coverage of samples that may be partly degraded.

3.2 Real-Time PCR

Once the main viral sequence has been established, there are more rapid and simpler techniques for detecting variants of concern. One of the most useful methods for this is real-time reverse transcription PCR, which has also been the mainstay in COVID-19 screening, diagnostics, and epidemiology [43–45]. This method works through the use of sequence-specific primers and fluorescent reporter probes in repeated cycles of cDNA amplification. The increase in the fluorescent signal with each round of amplification is then related to the amount of viral nucleic acid present in the sample. In addition, different primer/probe sets can be used to detect the presence or absence of specific variant sequences. For example, we described a method which can be used for simultaneous real-time quantitation of the United Kingdom, South Africa, and Brazil SARS-CoV-2 variants, which were prominent during the first year of the pandemic [18]. Figure 27.3a shows the location of the primer and probe sets used to detect these variants in multiplex PCR analyses. In the example shown, target failure by both primer/probe sets 1 and 2 would suggest the presence of the Alpha variant in the sample. Sole failure of primer/probe set 1 indicates potential presence of Beta and Gamma variants in the sample. Finally, target

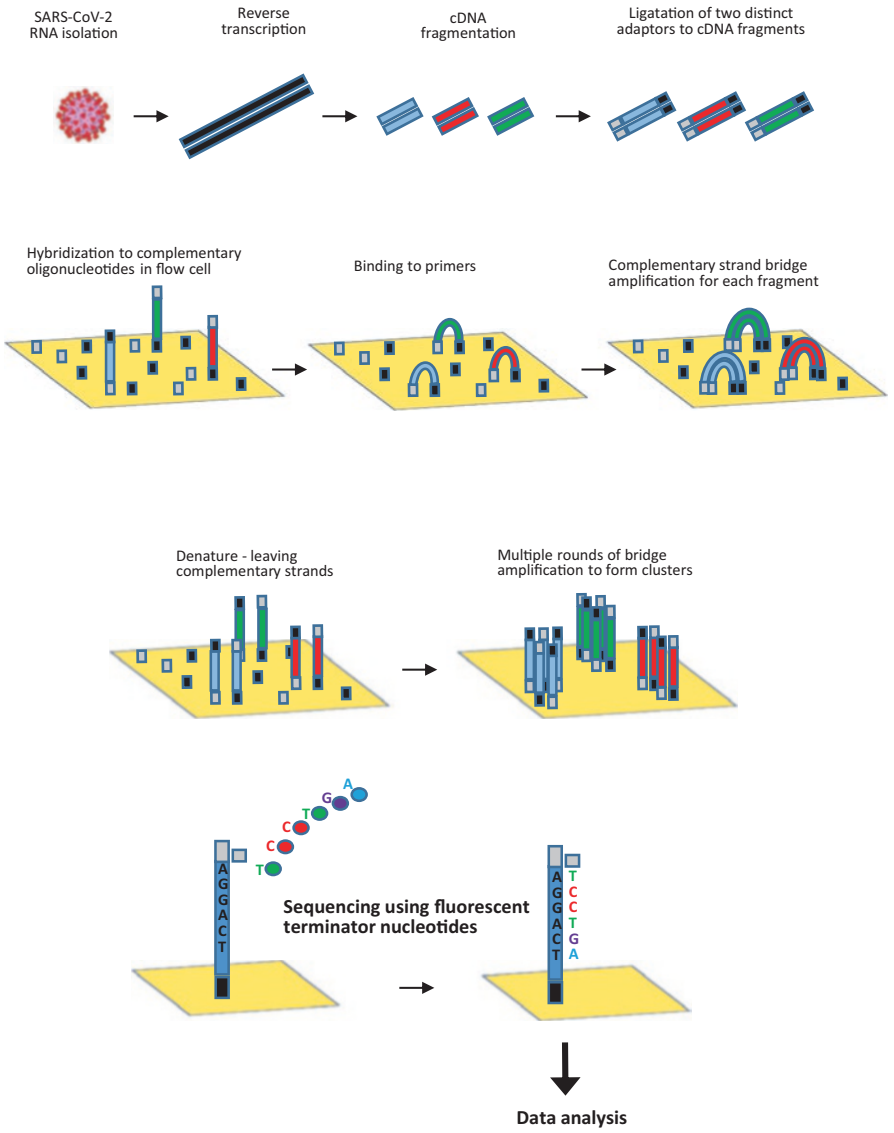

Fig. 27.2 The basic steps of the NGS method. A double-stranded cDNA library is produced by reverse transcription of SARS-CoV-2 single-stranded RNA. Two distinct oligonucleotide adapters are ligated to the cDNA sequences. The adapters allow binding to complementary oligonucleotides linked covalently within the flow cell. Covalently attached cDNA fragments are amplified complementary to the hybridized cDNA templates. Denaturation leaves the new cDNA strands covalently bound to the flow cell and is used to generate multiple copies bridge amplification. This generates DNA clusters reading in forward and reverse directions. Removal of the reverse strands leave only forward DNA strands which are used for sequencing. Primers hybridized to the cDNA strands and fluorescently labelled terminator nucleotides are passed through the cell for sequencing. Finally, all sequencing reads are aligned and mapped to the reference genome

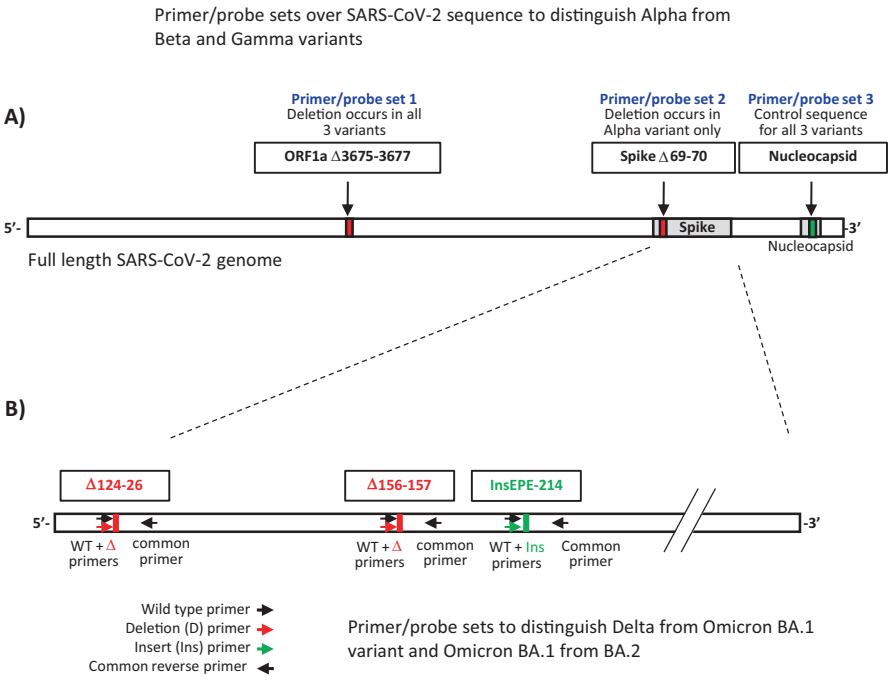

Fig. 27.3 (**a**) Multiplex qPCR to distinguish the Alpha variant from the Beta and Gamma lineages, targeting unique and conserved sites in the full length SARS-CoV-2 genome. (**b**) Multiplex qPCR to distinguish the Delta variant from Omicon BA.1 and BA.1 from BA.2, targeting unique sites in the spike protein

success with primer/probe sets 1–3 indicates that none of these variants are present but cannot rule out the presence of the other SARS-CoV-2 strains.

Similar approaches have been used to detect the Omicron variant. For example, Ayadi et al. described a multiplex PCR screen to distinguish the Delta variant from the Omicron BA.1 and BA.2 sub-variants [46]. This was based on the presence or absence of unique sequences in the spike protein in each of these lineages. Delta has a deletion of the glutamate and phenylalanine residues at amino acid position 156–157 (ΔEF156–157), Omicron BA.1 has a glutamate-phenylalanine-glutamate insert at position 214 (InsEPE-214) and BA.2 has a leucine-proline-proline deletion at amino acids 24–26 (ΔLPP24–26). In the scheme shown in Fig. 27.3b, Delta can be distinguished from Omicron BA.1 using forward wild type and variant primers with a common reverse primer. In a separate reaction, Omicron BA.1 can be distinguished from Omicron BA.2 using a similar strategy.

4 Machine Learning

In contrast with classical statistics, machine learning techniques employ algorithms which can learn from data to enable predictions using pattern recognition and apply this to new datasets. In case of SARS-CoV-2, this could be used to determine how specific features such as molecular biomarker patterns in the host are related to a specific disease status or outcome, as well as response to therapeutics. These relationships can be developed in a training set and then deployed to predict outcomes in new datasets. One big advantage of these approaches is that the algorithms can be retrained in an on-going manner with new input information so that it can be refined and adjusted to enhance predictive accuracy.

Deep learning methods have a complex multi-layered structure and require large datasets as input, but this allows the prediction of outcomes with high accuracy. They are generally constructed of input, hidden, and output layers, with the nodes in each layer representing the conversion of input data into a calculated output weight in connected nodes in the next layer (Fig. 27.4). The data is passed on from layer to layer by an activation function. The hidden layers carry out complex decisions and make changes to the data during this transit, and ultimately relay this information to the output layer. This final layer represents a convergence point for

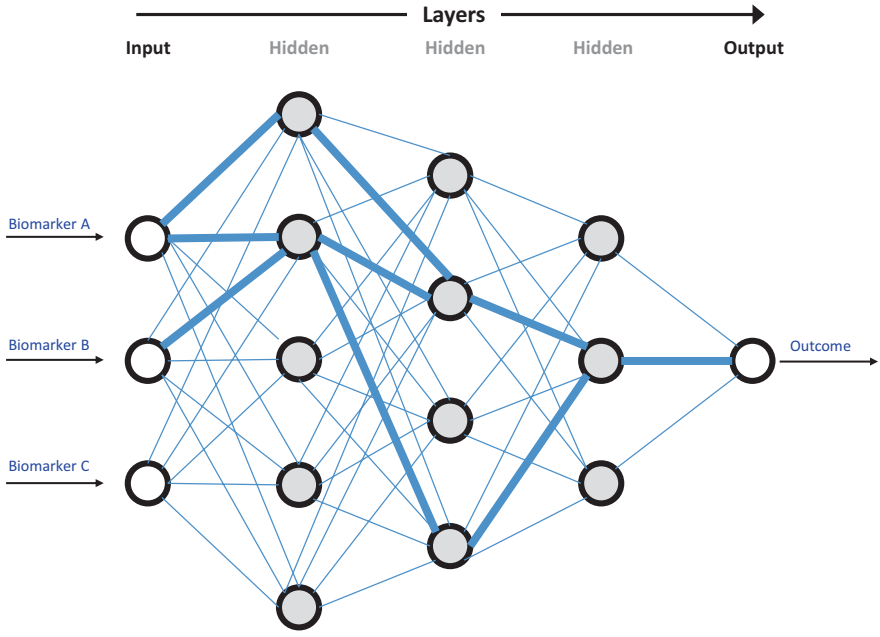

Fig. 27.4 Deep learning showing the input, hidden, and output layers. In the example shown, line thickness between nodes in the various layers indicate biomarker features that have the greatest impact weight on the final output

all data from the previous layers and a final predictive value is made [47]. The learning stage comes from a process called back propagation which involves assigning random weights to the input features and performing several more rounds of training until the most robust combination of input data with the lowest error rate arrives at the correct answer [48]. Following this stage, the model is tested to determine generalizability to new datasets. For this, the study sample can be partitioned into several folds and all but one of these is used in the same iterative way as above to train the model. Next, the model is applied repeatedly to each fold that was not included to assess overall performance. Higher generalizability can be achieved by applying the model to a completely new validation dataset [49, 50].

Machine learning approaches have been used recently to identify robust molecular signatures comprised of transcriptomic [51], proteomic [51, 52], metabolomic [51], and laboratory blood test results [53], for prediction of COVID-19 disease severity and outcomes with excellent sensitivity and specificity scores. Along the same lines, Sardar et al. developed an artificial intelligence algorithm based on a combination of proteomic and clinical biomarkers which had a good overall accuracy for prediction of survival outcomes in COVID-19 patents [54]. Also, another study used machine learning to construct an algorithm from metabolomics data collected from COVID-19 patients at different time points during the disease course, which revealed that a model developed during the earliest phase of the disease was successful in determining disease severity in the later stages [55].

Machine learning algorithms have also been used to identify mutations across the SARS-CoV-2 variants of concern associated with higher infectivity [56, 57] and escape from neutralizing antibodies or the antibodies produced by some of the vaccines [58]. Thus, these approaches could be used to assess current and future variants which would help healthcare workers to manage the disease more effectively.

5 Lab-on-a-Chip Devices

Although miniaturization of the working components is the key to POC devices, this can also cause a number of problems such as issues arising from use outside a designated laboratory and operation by untrained technicians. However, a number of commercialized products have emerged which go some way to overcoming some of these issues. One of these was aimed at detection of the Ebola virus during the 2014–2016 outbreak in West Africa [59, 60]. After the World Health Organization (WHO) declared this outbreak a public health emergency of international concern, an emergency use scheme was put in place to drive research and development of new medical devices for use in public health emergencies [61]. One early success was the GeneXpert Ebola PCR assay which took approximately 5 months to develop and deploy [62]. This was an automated assay which required application of the patient sample into a well on a cartridge, inserting this into a compact reader and retrieving the result within 2 h. Another early example was the FilmArray

BioThreat-E device which was also based on PCR and had a sample application to readout time of 1 h [63].

The standard lateral flow device, as applied by the National Health Service in the United Kingdom [64, 65], consists of a 7 × 2 cm cassette comprising a sample well and an enclosed membrane containing: (1) SARS-CoV-2 nucleocapsid protein antibodies conjugated with colour particles; (2) SARS-CoV-2 nucleocapsid protein antibodies bound on a test (T) line; and (3) secondary antibodies which target the primary antibodies bound on a control (C) line (Fig. 27.5). If virus is present in the sample, this is bound by the detector antibody. The virus-detector antibody complex is carried along the membrane by capillary action to the T line, where it is captured by nucleocapsid protein antibody. Unbound detector antibody also binds to the secondary antibody on the C line. This leads to generation of a colour on both the T and

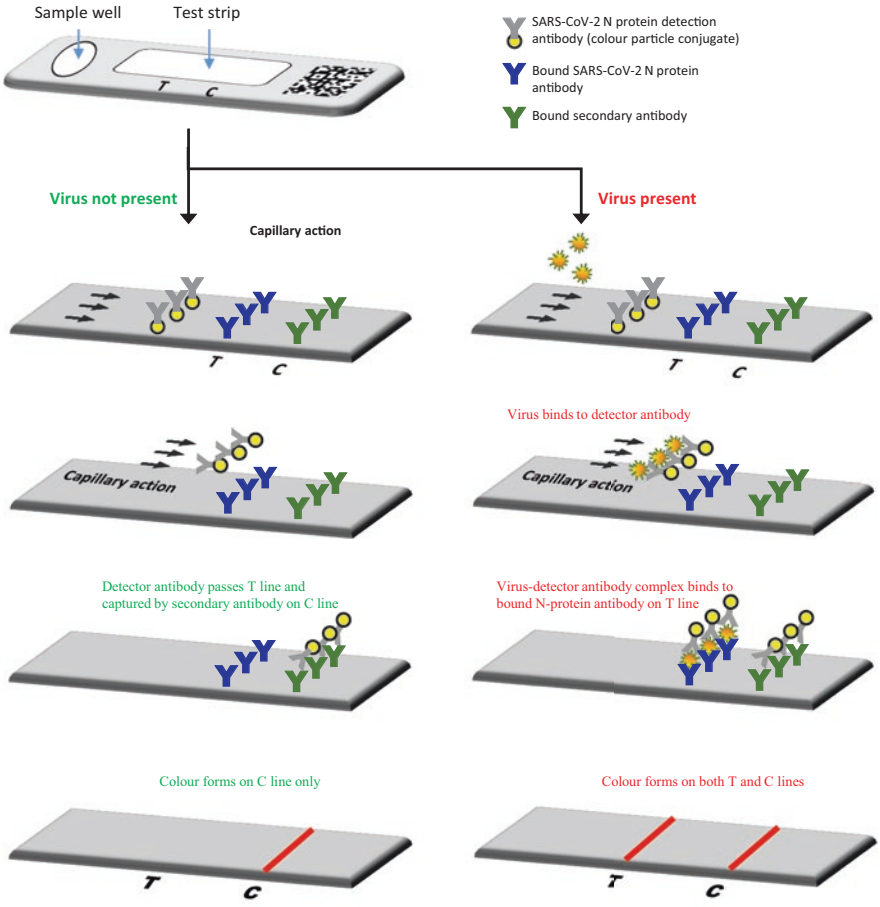

Fig. 27.5 Diagram showing the use of the United Kingdom National Health Service lateral flow device for detection of the SARS-CoV-2 virus

C lines as an indicator of a positive result. If the virus is not present in the sample, the detector antibodies will flow past the T line without binding to be captured by the secondary antibodies on the C line. This results in formation of a coloured line in the C region only as an indication of a negative result.

In 2012, Schumacher et al. reported on the development of a marketable, multi-parameter LOC system that could be used for POC diagnostics [66]. The system consisted of a microfluidic credit card-sized cartridge containing reagent reservoirs, integrated pumping and temperature control mechanisms, and an optical transducer. After the sample(s) are applied to the appropriate wells, the cartridge is inserted into a base unit that contains the essential controlling electronics and an optical system with a touch screen for user-friendly control of the assay and analysis of the results.

5.1 LOC Devices for Diagnosis of COVID-19

Early in the pandemic, Cojocaru reported on the development of microchip real-time PCR assay for detection of SARS-CoV-2 from nasopharyngeal swab samples [67]. This chip contained the primer/probe sets for the SARS-CoV-2 nucleocapsid protein gene in a 1.2-μL reaction volume. They validated the assay using reference and clinical samples and found a detection limit of one RNA copy per reaction. Cui et al. presented a proof-of-concept study of a microfluidic microwave sensing method for diagnosis of COVID-19 [68]. The method employs an immobilized antibody on the sensor to immunoprecipitate the virus which results in a detectable resonance frequency shift. The device showed 4000 copies/mL sensitivity for SARS-CoV-2 virus, and this could be distinguished from the CD4 antigen, MERS-CoV, and CoV-HKU1. Another PCR-based LOC device for COVID-19 detection and quantitation was described by Yin et al. [69]. This was a droplet microfluidic chip capable of multiplex analysis of nine samples with a detection limit of 10 nucleic acid copies per test and a total run time of 15 min. Zai et al. described development of a gravity-driven LOC device for viral nucleic acid diagnosis with extraction-free amplification [70]. They validated this by successful detection of SARS-CoV-2, influenza A/B, and papillomavirus 16/18 viruses. Parker et al. described the use of an optofluidic lab-in-a-fibre device which combines droplet microfluidics with laser-induced fluorescence detection of reverse transcription loop-mediated isothermal amplification (RT-LAMP) products for SARS-CoV-2 diagnostics [71]. The device offers advantages over other LOC systems as fibre technology is ideal for enhanced optical coupling. For monitoring and surveillance purposes, Donia et al. described the use of a LAMP-based LOC device that they used in the detection of SARS-CoV-2 in wastewater samples in COVID-19 hotspots [72].

Another study described a nanoplasmonic LOC device for rapid and quantitative PCR diagnostics [73]. The device consisted of a plasmofluidic chip with glass nanopillar arrays with gold islands, gas-permeable microfluidic channels, reaction arrays, vacuum cell, and a vapour barrier. This allowed sample loading in less than

3 min, and PCR results for the SARS-CoV-2 envelope protein in approximately 5 min. Stambaugh et al. described an LOC device comprising a bead-based solid extraction with sandwich antibody configuration and a fluorescent reporter probe, which they validated in detection of both SARS-CoV-2 and influenza A viruses from nasopharyngeal swab samples [74]. The multiplexing capability was conferred by multispot excitation on a multimode interference waveguide platform, with a sensitivity of 30 ng/mL. Another variation on the LOC concept was described by Kim et al. to enable detection of antigens at low concentrations [75]. This leveraged a rotationally focused flow approach for enhanced sensitivity by wavelength shift of optical sensors upon antigen detection in the module. This worked by addition of a low-density fluid to focus the target fluid into a microchannel and yielded a sensitivity of 0.19 fM, which is more sensitive than single flow methods.

5.2 LOC Devices for Detection of SARS-CoV-2 Antibodies

To aid in determinations of immune protection against new SARS-COV-2 variants, Rajsri et al. described a rapid quantitative POC assay in an injection-moulded polymethyl methacrylate cassette capable of quantifying circulating SARS-CoV-2 antibodies in less than 15 min [76]. Another study reported on the development of a 3D-printed LOC device with multiplexed electrochemical outputs which allows simultaneous detection of SARS-CoV-2 RNA and SARS-CoV-2 immunoglobulins in saliva in less than 2 h [77]. Thus, this could be used for both SARS-CoV-2 diagnosis and for monitoring antibody responses in immunized or infected persons. Along the same lines, Mandal et al. constructed an ultrasonic-guided wave sensor designed in a multi-threaded comb shape with cantilever beams for multiplexing capability [78]. This showed selectivity and sensitivity for detection of SARS-COV-2 antibodies and could be easily adapted for detection or other antibodies or antigens, simultaneously.

5.3 SARS-CoV-2 Disease-associated Effects

We recently described the use of an antibody microarray in combination with an LOC system to automate and increase the speed of multiplex immunoassays for detection of the SARS-CoV-2 cytokine storm effect [79]. For this, we carried out a fully automated LOC immunoassay for detection of C-reactive protein (CRP) in blood samples with pumping of all of the usual assay steps within the cartridge and data analysis using the base unit. The total assay time after application of the sample was 15 min. This is important as most existing multiplex immunoassay protocols are impractical in routine laboratory and clinical tests, as these typically involve long experimental times with the need for sophisticated laboratory equipment and

procedures, as well as trained operators. LOC systems have no such limitations as their user-friendly automated platforms incorporate many of the above steps.

Other LOC devices have also been developed to detect changes in biomarkers-associated COVID-19 disease effects. Recktenwald et al. developed a LOC device called Erysense which can evaluate red blood cell flow properties in samples less than 1 μL [80]. Haghayegh et al. described development of a self-powered automated microfluidic chip which included controls for sample delivery and an electrochemical immune-based biosensor, which allowed detection of the SARS-CoV-2 nucleocapsid protein in phosphate buffer within 15 min [81]. The linear detection range was 10–1000 pg/mL with a limit of detection of 3.1 pg/mL. McRae et al. described the use of 'smart diagnostics' which is powered by the combination of miniaturised electronics, cloud-based computing, and machine learning approaches in the identification and validation of disease signatures [82]. This method also includes deep learning–based inference and clinical decision support, with reporting and integration with healthcare records. In line with this, an Internet of Diseases (IOD) platform has been developed which links an LOC device for SARS-CoV-2 diagnosis using saliva samples to diagnostic data in a cloud-based system for disease control and prevention in a regional manor [83]. Choi et al. demonstrated a similar multiplexed LOC PCR device with a linked smartphone application for automatic processing and cloud storage [84]. Using this, they were able to carry out analysis of nine RNA viruses simultaneously, which included the OC43, 229E, and NL63 human coronaviruses, with high linearity and sensitivity. Also, Heithoff et al. demonstrated a smartphone-based LAMP assay called smaRT-LAMP for detection of SARS-CoV-2 infection, and this showed high concordance with standard RT-PCR tests [85].

5.4 LOC Devices for Detection of SARS-CoV-2 Variants

Based on their capability of identifying specific sequences, PCR-based LOC devices can be used for identification of SARS-CoV-2 variants. Applying this idea in combination with the system described by Schumacher et al. [66], we recently described the development of a microarray LOC device which could be used for diagnosis of COVID-19 infections or for sub-typing of SARS-CoV-2 variants (Fig. 27.6) [86]. We demonstrated this principle through detection of signal nucleotide polymorphisms in methicillin-resistant *Staphylococcus aureus* (MRSA) using the LOC system. Following a PCR stage of 60 min, this resulted in hybridization, washing and readout times of less than 15 min. For analyses of the SARS-CoV-2 virus, we suggest use of inhibitor-tolerant PCR mix such as that developed by Meridian Bioscience to bypass the RNA extraction step. This step is normally rate limiting and may lead to poor recovery and performance of the assay [87]. Kumar et al. described the development of an FnCas9-based CRISPR LOC device for detection of SARS-CoV-2 infections as well as the presence of the N501Y mutation present in multiple SARS-CoV-2 variants [88]. Another report described the development

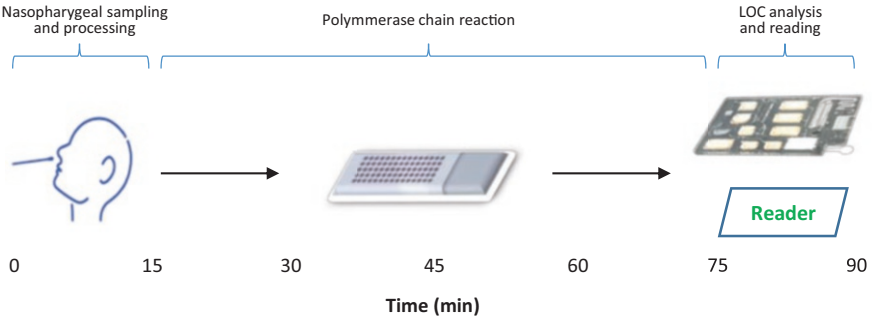

Fig. 27.6 Diagram showing a rapid LOC PCR analysis of a nasopharyngeal sample in less than 90 min

of a microfluidic device capable of discriminating the SARS-CoV-2 Alpha variant from both the SARS-CoV-2 original isolate and negative controls in saliva samples [89]. The assay was based on RT-LAMP PCR in the detection of spike gene target failure as a way of distinguishing the SARS-CoV-2 Alpha variant from the original SARS-CoV-2 strain at least 10 copies/μL within 30 min. They validated the performance of the test by analysis of 38 saliva specimens, which yielded a sensitivity greater than 90% and a specificity of 100%. Another study described the development of a similar device that was used successfully to detect SARS-CoV-2 in clinical samples [90].

6 Conclusions and Future Perspectives

In this chapter, we described attempts to control the COVID-19 pandemic through application of surveillance methods aimed at detection of new SARS-CoV-2 variants of concern and prediction of how specific mutational changes alter the transmissibility, virulence, and immune evasion capabilities of the virus. Other steps that should be taken to prepare us for the next pandemic should include the surveillance and early detection of SARS-CoV-2 and other coronavirus strains and variants in domesticated and wild animals, considering the zoonotic nature of this virus [91]. Although detection of new viral sequences requires whole genome sequencing, once this has been achieved, more targeted methods can be applied for monitoring variants such as real-time PCR. In addition, omic techniques such as multiplex cytokine screening should be used to determine the effects of new viral strains on the host. This would enable development of biomarker testing for prediction of disease severity and outcomes to guide the most appropriate treatment course. Future efforts should also be directed towards translating these methods onto user-friendly platforms, and even handheld devices enabled by smart technologies, for POC testing so that therapeutics could be administered in personalized medicine approach. With

this infrastructure in place, we should be able to curtail any future catastrophic waves caused by emergence of new SARS-CoV-2 variants and other deadly zoonotic viruses.

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